Cholesterol Synthesis and Accretion Within Various Tissues of the Fetal and Neonatal Rat

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The rate of cholesterol synthesis is reported to be higher in fetal relative to adult rats. Along with the observation that maternal diets high in fat and cholesterol are unable to alter the rate of cholesterol synthesis in the fetus, this has been taken as indirect evidence that the fetal rat meets its cholesterol needs through de novo synthesis. This study quantified the rates of cholesterol synthesis and accumulation in the liver, brain, intestine, and carcass of the fetal and neonatal rat and the placenta to determine whether these developing tissues are able to support their own cholesterol needs without the uptake of plasma lipoprotein cholesterol. The rate of cholesterol synthesis was determined in vivo using [^{3}H]water. The rate of cholesterol accumulation was determined by calculating the difference in tissue cholesterol content between 2 subsequent days of development. Total fetal body cholesterol synthesis was sufficient to support the rate of cholesterol accumulation. Fetal and neonatal liver synthesized cholesterol at a rate in excess of cholesterol accumulation, suggesting hepatic secretion of cholesterol into the plasma. Before the onset of suckling, the rates of de novo cholesterol synthesis in the intestine, brain, and carcass were also sufficient but not higher than the need for cholesterol accretion. After the establishment of suckling, the rate of cholesterol accumulation in the intestine and carcass was in excess of synthesis, suggesting that neonatal tissues derive some of their cholesterol from dietary milk or liver. These studies suggest that the perinatal rat does not require exogenous cholesterol to support tissue cholesterol accretion. However, the fetal liver may support cholesterol accretion in other tissues through rates of synthesis in excess of accumulation and secretion into plasma. The placenta may derive some cholesterol from the maternal and/or fetal plasma.

CHOLESTEROL is required by mammals for the maintenance and synthesis of cell membranes. In addition, cholesterol is an obligate precursor for vitamin D, steroid hormones, and bile acids.[1] However, all mammals have the inherent ability to synthesize cholesterol within their tissues despite the availability of cholesterol from the diet. Hence, there has been much discussion about the relative significance of the contribution made to the body cholesterol pool by exogenous sources versus de novo synthesis. This is of particular interest with regard to the role development may play in setting the stage for diseases such as atherosclerosis[2] and Smith-Lemli-Opitz syndrome[3,4] and the role diet may play in development.[5]

Cholesterol in fetal tissues and plasma may originate from fetal de novo synthesis or placental transport from the maternal compartment. Information from studies of radiolabeled cholesterol transfer have indicated that placental transfer of cholesterol is low, probably accounting for only 10% to 20% of cholesterol in the late-gestation fetus.[6-8] However, the changing pool size and substrate specific activity caused by the lack of steady-state conditions in the rapidly growing fetus and mother[9] require cautious interpretation of these studies.

Low placental transfer of cholesterol to the fetus has also been inferred indirectly by studies of fetal cholesterol biosynthesis in pregnant rats fed cholesterol[8,10,11] and cholestyramine.[12] These studies found that cholesterol biosynthesis in fetal rats was not altered by maternal cholesterol or cholestyramine feeding, and the rate of fetal cholesterol biosynthesis was higher versus maternal or nonpregnant rat liver. This finding implies that fetal tissue cholesterol originates mainly from endogenous fetal de novo synthesis rather than placental transfer.[8,10,11] Alternatively, placental cholesterol transfer may be regulated at a set level independent of the maternal plasma cholesterol concentration.

Some studies have investigated the ability of individual tissues to supply their own cholesterol during development. The neonatal rat brain has been investigated by assessing the uptake of D_7-cholesterol, fed in milk, into the brain and other organs. Results of these studies have indicated that neonatal rat brain synthesizes all of the cholesterol it requires from postnatal day 5.[13] Similar conclusions were reported in studies using the incorporation of [^{3}H]water into cholesterol to evaluate whether the rate of de novo cholesterol synthesis in the brain is sufficient to support the rate of cholesterol accumulation.[14] In contrast, some previous studies reported that exogenous cholesterol contributes to brain cholesterol accumulation during postnatal development of the rat,[15,16] but others report that the exogenous contribution is insignificant.[17,18]

Studies using [^{3}H]water incorporation into cholesterol have shown that suckling rat kidney does not rely solely on de novo cholesterol synthesis,[19] and the rate of cholesterol synthesis in the skin decreases shortly before birth.[20] Measures of hepatic cholesterol synthesis using [^{3}H] incorporation have similarly found high rates before birth followed by low rates during suckling.[21] However, the relative contribution of de novo cho-

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lesterol synthesis to total cholesterol accretion in the skin and liver was not determined in the latter studies.

The use of [^{3}H]water to label cholesterol synthesized in vivo bypasses many of the problems inherent to many other methods used to assess the rate of cholesterol synthesis. Hepatic hydroxymethyl glutaryl coenzyme A (HMG CoA) reductase is the rate-limiting enzyme of cholesterol synthesis in the adult rat, but the activity of this enzyme may be uncoupled from cholesterol synthesis in fetal rat liver.[21] Potential differences in the recovery of microsomes and in enzyme kinetics among different tissues also limit the use of HMG CoA reductase activity in comparative studies of cholesterol synthesis in different organs.[22] Radiolabeled substrates such as [^{14}C]acetate or [^{14}C]octanoate have different patterns of distribution in the metabolic pools of different tissues, making a determination of specific radioactivity necessary but problematic.[22] [^{3}H]water, on the other hand, distributes equally within the entire body water pool, allowing determination of the specific radioactivity of body water through sampling of the plasma once the equilibration kinetics are determined.[22]

Recently, Jurevics et al[23] quantified the incorporation of [^{3}H]water into fetal rat cholesterol and concluded that the fetal rat synthesizes nearly all of its own cholesterol. In contrast, Woollett[24] found that only 40% of fetal hamster cholesterol could be accounted for by fetal tissue synthesis, and suggested that delivery of cholesterol from the placenta and yolk sac contributed the remainder. The present report presents the results of studies using [^{3}H]water incorporation with a 1-hour incubation period confirming that the fetal rat does not appear to derive significant cholesterol from its dam, and extends these findings to show the changes that occur in cholesterol synthesis during the first 2 postnatal days. We also report for the first time the rates of cholesterol synthesis in comparison to accretion in the intestine and placenta (without the yolk sac) of the perinatal rat.

MATERIALS AND METHODS

Chemicals and Radioisotopes

[^{3}H]OH 1 Ci/g was purchased from Dupont Canada (Mississauga, Ontario, Canada). Enzymatic assay kits for the determination of cholesterol were obtained from Biopacific Diagnostic (West Vancouver, British Columbia, Canada). All other chemicals were reagent-grade and acquired from BDH Chemicals Canada (Vancouver, British Columbia, Canada) or Sigma Chemical (St Louis, MO).

Animals and Treatment

Female Wistar rats (250 g) were used in all experiments. They were housed and bred as previously described[25] and fed a standard laboratory rat chow (Purina Mills, St Louis, MO) ad libitum. Pregnant rats were anesthetized after a gestation of 18, 19, 20, or 21 days (normal term, 22 days), and the fetuses were delivered by rapid hysterectomy. Neonatal rats were killed by decapitation immediately after birth before having suckled, or after normal nursing for up to 48 hours after birth, as indicated in the Results. Fetal and newborn tissue within each litter were pooled prior to analyses with an n value of 3, 2, 6, 6, 3, and 3 litters for gestation days 18, 19, 20, and 21 and postnatal days 0 (presuckling) and 2 (postsuckling), respectively. Adult males (n = 7) were killed at 162 ± 38 days of age (mean ± SEM) and 530 ± 19 g body weight. All procedures were reviewed and approved by the University of British Columbia Animal Care Committee and conformed to the guidelines of the Canadian Council on Animal Care.

In Vivo Determination of Cholesterol and Fatty Acid Synthesis Rates

[^{3}H]water administration and tissue collection 1 hour after administration were performed as previously described.[21] Briefly, pregnant rats were lightly anesthetized with ether and received an injection of [^{3}H]water through the tail vein and were left unrestrained in a fume hood. One hour later, the rats were again anesthetized with ether and the blood, liver, intestine, brain, carcass, or whole body from newborn and fetal rats were collected and immediately frozen in liquid nitrogen. Solid tissues were first rinsed in ice-cold saline before freezing. The intestines were additionally flushed of their contents prior to freezing. Separated tissues were pooled for each litter. Plasma was prepared as described previously.[26] The placentas were collected on postconception days 18 to 21 and prepared similarly.

Tissue lipids were extracted[27] and analyzed for [^{3}H][21] and for cholesterol[28] content by precipitating cholesterol as the digitonide followed by splitting the digitonide and isolating cholesterol as previously reported.[21] The mean specific activity of plasma water 1 hour after injection[21] and the rate of cholesterol synthesis as nanomoles of [^{3}H]cholesterol synthesized per hour per gram of tissue[19] were calculated.

Data Analysis

Tissue cholesterol accumulation for a given 24-hour period was calculated as the difference between the mean content on a given day and the mean cholesterol content on the preceding day. The standard error (SE) of the cholesterol accumulation for a given day (S_{x-y}) was calculated as

$$S_{x-y} = \sqrt{S_x^2 + S_y^2},$$

where S_x is the SE of the tissue cholesterol content for a given day and S_y is the SE of the tissue cholesterol content for the preceding day.

A paired-sample, 2-tailed *t* test was used to determine significant differences between the amount of cholesterol synthesized and the amount accumulated in a given tissue for each 24-hour period studied. These results were interpreted to indicate whether a particular tissue is likely to synthesize sufficient cholesterol, or if the uptake of cholesterol from plasma is important for cholesterol accretion in that tissue. The *t* statistic was calculated from the mean difference between cholesterol synthesis and accumulation,

$$t = \frac{X_d - 0}{S_d},$$

where X_d is the mean of the difference between cholesterol synthesis and accumulation for a given 24-hour period and S_d is the SE of the difference.

The SE of the mean difference for a given 24-hour period was calculated as

$$S_d = \sqrt{S_A^2 + S_S^2 - 2rS_AS_s},$$

where S_d is the SE of the mean difference between cholesterol synthesis and accumulation, S_A is the SE of the mean of cholesterol accumulation, S_s is the SE of the mean of cholesterol synthesis, and *r* is the correlation between tissue cholesterol synthesis and total cholesterol content.

Note that this method of determining in vivo cholesterol synthetic rates minimizes the problems of newly synthesized cholesterol exchange between tissues by virtue of the short 1-hour incubation period. However, it does ignore any diurnal variation in cholesterol synthesis.

In contrast, the method of determining the rate of tissue cholesterol accumulation is performed over a 24-hour period, thereby accounting for any diurnal variation. However, the cholesterol content of a given tissue may not necessarily derive from de novo synthesis within that same tissue, and instead may derive from the uptake of plasma lipoprotein cholesterol even if the results with this method indicate no difference between the rates of cholesterol synthesis and accretion. This method seems to be the best compromise between these limitations for determining whether perinatal tissues have the cholesterol synthetic capability to support cholesterol accretion during perinatal development.

RESULTS

The rates of cholesterol synthesis were not corrected for radiolabeled cholesterol cleared from plasma by the tissues, as the short 1-hour incubation time should minimize tissue uptake of plasma cholesterol. In addition, corrections taking this into account[23] made little (0.1% to 1.2%) difference in the calculated rate of cholesterol synthesis.

The rate of cholesterol synthesis in the fetal liver was consistently significantly higher than the rate of cholesterol accumulation (Fig 1). In contrast, there was no apparent statistical difference between the rates of cholesterol synthesis and accumulation in the liver on the second day after birth. Further, the period from gestation day 21 to the first day after birth indicates a possible net loss of cholesterol from the liver, which could be explained by the cessation of nutrient delivery from the placenta and the time required to establish enteral nutrition by suckling, or by the beginning of bile acid synthesis which occurs during this same period.[29]

The fetal intestine appeared to synthesize its own cholesterol at a rate sufficient to meet its needs, without producing excess, for example, for intestinal lipoprotein synthesis. However, on postnatal day 2 significantly more cholesterol accumulated in the intestine than was synthesized (Fig 2).

There was no statistical difference between the rates of cholesterol synthesis and accumulation in the brain at any age studied (Fig 3).

There was also no statistically significant difference between the rates of cholesterol synthesis and accumulation in the carcass, except on postnatal day 2, when significantly more cholesterol was accumulated than synthesized. However, the actual mean daily accumulation of cholesterol in the carcass was consistently about 2-fold higher than the amount synthesized (Fig 4).

Similarly, the whole fetus was able to meet its own cholesterol needs through de novo synthesis (Fig 4). There was no statistically significant difference between the rates of fetal whole-body cholesterol synthesis and accumulation.

In contrast, there was a downward trend in the amount of cholesterol accumulation in the placenta over the last 4 days of gestation. On day 19 of gestation, more cholesterol accumulated in the placenta than was synthesized (Fig 5).

DISCUSSION

These studies confirm a previous study showing that the fetal rat is able to synthesize sufficient cholesterol to support tissue cholesterol accumulation, at least during the latter stages of gestation. Further, these studies show that fetal liver cholesterol

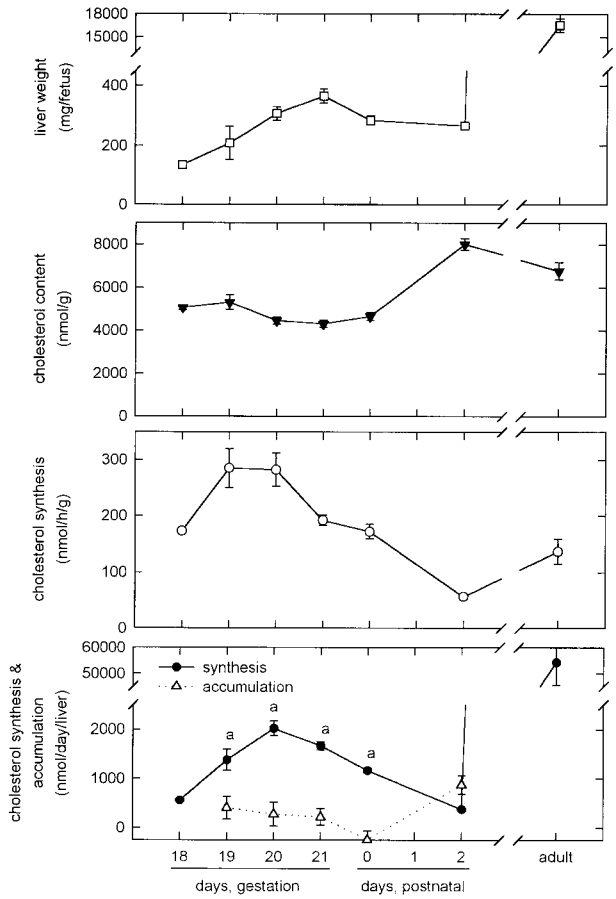

Fig 1. Liver weight (□), cholesterol content (▼), cholesterol synthesis/h/g liver (○), cholesterol synthesis/d/liver (●), and cholesterol accumulation (△) during perinatal development. Perinatal livers were pooled for each litter. Neonates < 2 hours old had not suckled. Adult samples were from nonlittermate rats. Each point is the mean ± SEM unless too small to signify. Statistical differences between cholesterol synthesis and accumulation per day per liver for a given day were determined using a paired-sample, 2-tailed *t* test ([a]*P* < .05).

synthesis proceeds at a rate in excess of the need for cholesterol accretion. In addition, they show that hepatic cholesterol synthesis in excess of the need for growth continues in the period immediately following birth, but then declines as suckling becomes established. Thus, cholesterol synthesis in fetal rats in excess of the need for liver growth may be secreted into the plasma for uptake by other developing organs. This interpretation agrees with previous studies showing relatively high levels of apolipoprotein B (apoB) mRNA in fetal rat liver,[30] indicating that the fetal liver is an important site for the synthesis and secretion of low-density lipoprotein (LDL), the major cholesterol-carrying lipoprotein in fetal rat plasma.[31-34] The intestine, on the other hand, appears to be a relatively insignificant source of apoB-containing lipoprotein until at least the day before birth.[29] This again is consistent with the results of studies herein showing that the intestine does not synthesize cholesterol in excess of need in the prenatal period. Other studies have shown low expression of LDL receptor protein[35] and lower ability to bind apoB/E-containing lipoproteins in fetal versus

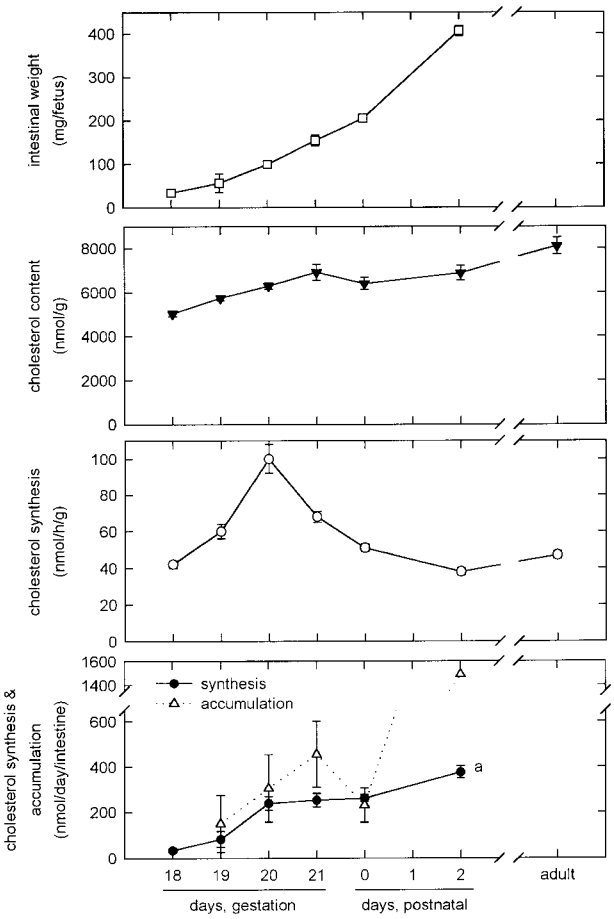

Fig 2. Intestinal weight (□), cholesterol content (▼), cholesterol synthesis/h/g intestine (○), cholesterol synthesis/d/intestine (●), and cholesterol accumulation (△) during perinatal development. Perinatal intestines were pooled for each litter. Neonates < 2 hours old had not suckled. Adult samples were from nonlittermate rats. Each point is the mean ± SEM unless too small to signify. Statistical differences between cholesterol synthesis and accumulation per day per intestine for a given day were determined using a paired-sample, 2-tailed *t* test ($^aP < .05$).

adult liver,[34] with the suggestion that the resulting limited delivery of lipoprotein cholesterol to the liver may allow greater availability of plasma cholesterol for other tissues.[35]

Similar to the fetal liver, the fetal intestine synthesized sufficient cholesterol to meet its own accretion needs. However, unlike the fetal liver, the fetal intestine did not synthesize cholesterol in excess of that required to sustain tissue accretion. Hence, it is unlikely that the fetal intestine secretes cholesterol into the plasma for uptake by other tissues. In contrast to the fetal period, the increased rate of cholesterol accumulation in the rat intestine on postnatal day 2 may reasonably be explained by the contribution of cholesterol from the milk diet.

Consistent with previous studies,[23] the results of the study reported here add further support to the belief that the fetal brain does not rely on plasma cholesterol for cholesterol accretion. In addition, the present study also suggests that the neonatal brain likewise does not require plasma cholesterol to sustain its rate of cholesterol accretion. Similarly, rates of deuterium (from deuterated water) incorporation into cholesterol appear to be higher in developing rat brain than in any other tissue,[18] and recent studies by Edmond et al[13] found that D_7-cholesterol fed in milk did not contribute to brain cholesterol from postnatal day 5 onward. In contrast, Jurevics and Morell[14] reported that cholesterol accumulation in the rat cerebral hemispheres and brainstem, but not the cerebellum, was more rapid than the rate of cholesterol synthesis during the first postnatal week. Others have also suggested that exogenous cholesterol can contribute to developing postnatal brain cholesterol. For example, Dobbing[16] found significant amounts of radiolabeled cholesterol following a single injection of [4-^{14}C]cholesterol to postnatal rats. Also, there are reported estimates that as much as 50% of cholesterol accumulation between postnatal days 10 and 20 is derived from exogenous sources.[15] Possibly, some of the discrepancies among the findings of different studies are explained by developmental

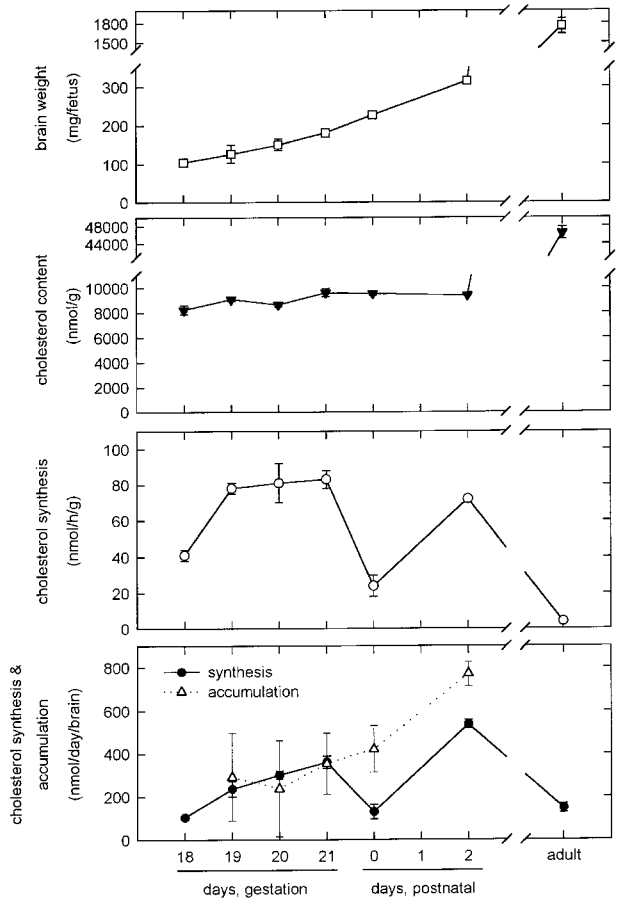

Fig 3. Brain weight (□), cholesterol content (▼), cholesterol synthesis/h/g brain (○), cholesterol synthesis/d/brain (●), and cholesterol accumulation (△) during perinatal development. Perinatal brains were pooled for each litter. Neonates < 2 hours old had not suckled. Adult samples were from nonlittermate rats. Each point is the mean ± SEM unless too small to signify. There were no statistical differences between cholesterol synthesis and accumulation per day per brain for any given day.

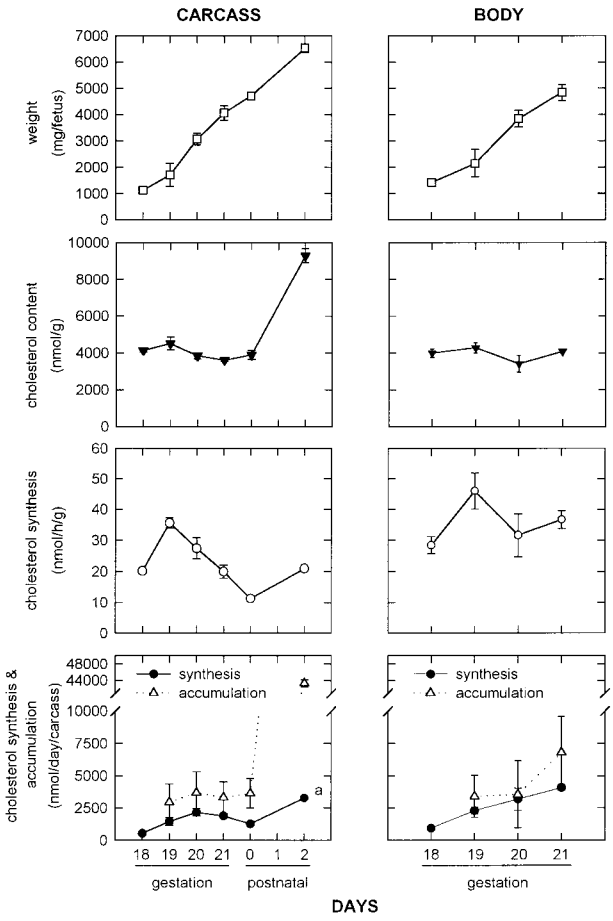

Fig 4. Carcass and fetal body weight (□), cholesterol content (▼), cholesterol synthesis/h/g carcass or fetus (○), cholesterol synthesis/d/carcass or fetus (●), and cholesterol accumulation (△) during perinatal development. Perinatal carcasses were pooled for each litter. Neonates < 2 hours old had not suckled. Each point is the mean ± SEM unless too small to signify. Statistical differences between cholesterol synthesis and accumulation per day per carcass for a given day were determined using a paired-sample, 2-tailed *t* test ([a]*P* < .05). There were no statistical differences between cholesterol synthesis and accumulation per day per fetus for any given day.

changes in the ability of cholesterol to enter the brain, for example, with development of the blood-brain barrier in rats,[36] or by methodologic differences, for example, perfusion of the brain to remove contaminating plasma prior to analysis. Interestingly, similar results for the neonatal lamb have also suggested that the principle source of brain cholesterol is de novo synthesis.[37]

Compared with the prenatal period, the rate of cholesterol synthesis in the carcass, as in the liver, decreased while the cholesterol content continued to increase after birth. This may be explained by a decrease in de novo tissue cholesterol synthesis in the presence of an increase in exogenous cholesterol supply with the progression of suckling. This interpretation of the results in this study is consistent with data from Jurevics and Morell[19] showing that most of the cholesterol for the kidney (included in the analysis of the carcass in the present study) is derived from the plasma after birth.

The results of the present study show that the accumulation of cholesterol in the placenta proceeded at a rate higher than that likely to be supported by de novo synthesis in the organ itself. Previous studies have shown that the rat and hamster placenta can take up maternal plasma LDL.[11,24] Although it has recently been reported that high-density lipoprotein cholesterol in the maternal circulation of mice can influence placental cholesterol synthesis,[38] it seems likely that the placenta is not a net contributor of de novo cholesterol to the fetus during normal pregnancy.

The method used in this study compares synthesis and accumulation rates of tissue cholesterol over a 24-hour period. The synthetic rate has been extrapolated from a 1-hour incubation period with tritiated water. Hence, the method assumes that either there is no diurnal variation in the rate of cholesterol synthesis or the extrapolation closely approximates the 24-hour mean rate of cholesterol synthesis. Although a diurnal rhythm

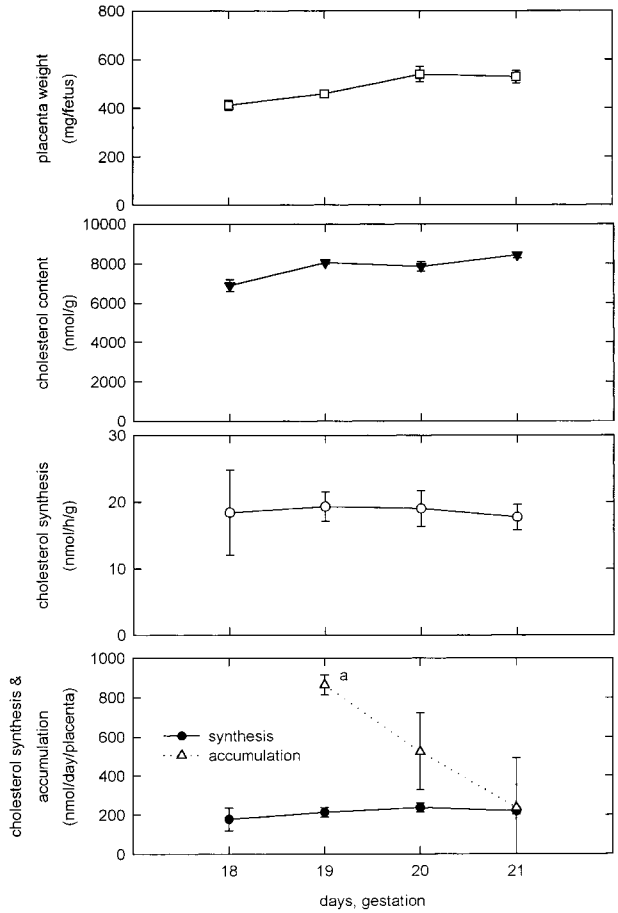

Fig 5. Placental weight (□), cholesterol content (▼), cholesterol synthesis/h/g placenta (○), cholesterol synthesis/d/placenta (●), and cholesterol accumulation (△) during fetal development. Placentas were pooled for each litter. Each point is the mean ± SEM unless too small to signify. Statistical differences between cholesterol synthesis and accumulation per day per placenta for a given day were determined using a paired-sample, 2-tailed *t* test ([a]*P* < .10).

for cholesterol synthesis has been demonstrated in adult rat liver,[39] it has not been demonstrated in fetal or neonatal rats. A diurnal rhythm for hepatic HMG CoA reductase activity has been shown in late fetal liver,[40] but other studies have shown that the activity of this enzyme is not representative of cholesterol synthesis at this time.[21]

Lengthening the period of radioactive water incubation would solve the problem of any diurnal rhythm that might exist in perinatal rats. However, this leads to the problem of radioactive cholesterol potentially being exchanged between tissues through the plasma, thereby yielding inaccurate results for specific tissue rates of cholesterol synthesis. Given the two alternatives, in the present study we opted to minimize the amount of time available for net movement of radiolabeled cholesterol from the sites of synthesis to other tissue compartments, as in similar studies.[39]

In contrast, the rate of cholesterol accumulation in specific tissues was calculated from the difference in tissue cholesterol content between subsequent days of development. Any changes in cholesterol content were assumed to be due to changes in endogenous cholesterol synthesis or export or import to or from the plasma. The data cannot definitively identify the source or destination of any cholesterol possibly imported or exported to or from the plasma. In addition, it is possible that some changes in cholesterol content are due to the utilization of cholesterol for steroid or bile acid synthesis, which cannot be determined with the methodology of this study.

In summary, the results of the studies reported here indicate that the fetal liver, in addition to synthesizing cholesterol for its own needs, is the only tissue able to supply extrahepatic developing organs other than the brain with cholesterol. In contrast to the carcass and possibly the placenta, the fetal brain and intestine both appear to be fully capable of synthesizing cholesterol at rates sufficient to meet their needs for new tissue membrane lipid synthesis.

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