The use of the Dhcr7 knockout mouse to accurately determine the origin of fetal sterols

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Abstract **Mice with a targeted mutation of 3β-hydroxysterol Δ^7-reductase (*Dhcr7*) that cannot convert 7-dehydrocholesterol to cholesterol were used to identify the origin of fetal sterols. Because their heterozygous mothers synthesize cholesterol normally, virtually all sterols found in a *Dhcr7* knockout fetus having a Δ^7 or a Δ^8 double bond must have been synthesized by the fetus itself but any cholesterol had to have come from the mother. Early in gestation, most fetal sterols were of maternal origin, but at approximately E13–14, in situ synthesis became increasingly important, and by birth, 55–60% of liver and lung sterols had been made by the fetus. In contrast, at E10–11, upon formation of the blood-brain barrier, the brain rapidly became the source of almost all of its own sterols (90% at birth). New, rapid, de novo sterol synthesis in brain was confirmed by the observation that concentrations of C24,25-unsaturated sterols were low in the brains of all very young fetuses but increased rapidly beginning at approximately E11–12. Reduced activity of sterol C24,25-reductase (Dhcr24) in brain, suggested by the abundance of C24,25-unsaturated compounds, seems to be the result of suppressed *Dhcr24* expression. The early fetal brain also appears to conserve cholesterol by keeping cholesterol 24-hydroxylase expression low until approximately E18.**—Tint, G. S., H. Yu, Q. Shang, G. Xu, and S. B. Patel. **The use of the Dhcr7 knockout mouse to accurately determine the origin of fetal sterols. *J. Lipid Res.* 2006.** 47: **1535–1541.**

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Fetal mice carrying mutations that completely disrupt the transport of cholesterol across the yolk sac and placenta (1–4) or that prevent the synthesis of any sterol whatsoever die early in gestation (5, 6). In contrast, mutations in genes coding for enzymes in the postsqualene portion of the cholesterol biosynthetic pathway that inhibit the conversion of a sterol precursor to cholesterol but allow the fetus to synthesize a sterol other than cholesterol (7–9) or that materially reduce but do not abolish the synthesis of apolipoprotein B (apoB) by placenta and yolk sac (10) often permit survival to term. In humans, six heritable syndromes have been identified, each caused by a block at a different step of cholesterol biosynthesis, in which an abundance of steroidal cholesterol precursors but limited quantities of cholesterol are synthesized. These diseases are generally characterized by reduced, but nonzero, prenatal and postnatal survival, multiple severe structural abnormalities, developmental delay, and mental retardation (11).

Although the fetal mouse cannot develop without endogenously synthesized cholesterol, proof that humans or mice cannot survive without the delivery of maternal cholesterol has yet to be established. The demonstration of prenatal lethality in mice that cannot synthesize apoB means that the fetus, to survive, needs cholesterol supplied by the yolk sac and placenta (3). However, the inability of the relatively large quantities of cholesterol normally made by the fetal tissues that constitute these organs (12) to reach the fetus, rather than a lack of maternal cholesterol, might be the cause of the early demise. Arguing against the need for a great deal of maternal/fetal cholesterol transfer in humans, perhaps, is the observation that, unlike mice that exhibit severe developmental abnormalities and early death (1, 4, 10), humans with abetalipoproteinemia or hypobetalipoproteinemia, who secrete either no apoB or a truncated form of this lipoprotein, appear to be anatomically normal at birth, and women with these diseases give birth to normal-appearing offspring (13). Although there is little doubt that some cholesterol from the maternal circulation becomes incorporated into all mammalian fetuses (14–22), exactly how much is transferred, when it arrives, and how essential it is for proper development and survival are unanswered questions.

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Many strategies have been used to determine the balance between cholesterol synthesized by the fetus and by the mother (reviewed in 21), but we chose to use the 3β-hydroxysterol Δ^7-reductase (*Dhcr7*) knockout mouse (*Dhcr7*$^{-/-}$), a model class not used previously for this purpose. The biosynthesis of cholesterol requires that, at some point, the C7,8 double bond of a precursor sterol must be reduced by the enzyme DHCR7 (23, 24). Mutations in *DHCR7* lead to the accumulation of the cholesterol precursor 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol; 7DHC) and, in humans, cause Smith-Lemli-Opitz syndrome (SLOS) (24–26). For the current study, we took advantage of the demonstrated inability of our *Dhcr7*$^{-/-}$ (SLOS) mice to synthesize cholesterol (8). This means that all of the cholesterol that one finds in a *Dhcr7*$^{-/-}$ fetus must have come from its mother. Conversely, because cholesterol and 7DHC concentrations in *Dhcr7*$^{+/-}$ mice are essentially the same as those in *Dhcr7*$^{+/+}$ (wild-type) mice (8), almost no maternal 7DHC is available for transport to the fetus and fetal 7DHC concentrations reflect only endogenous (i.e., fetal) synthesis. Therefore, *Dhcr7*$^{-/-}$ mice allow us to easily and accurately determine the relative amounts of sterols synthesized by mother and fetus in any fetal organ at any time during gestation by comparing the levels of cholesterol with those of the appropriate di- and tri-unsaturated sterols. Furthermore, if maternal/fetal transport is not greatly perturbed by the accumulation of the precursor sterols, and if total sterol synthesis in the knockouts is not too different from that in the wild-type fetus, one can also use this model to predict the maternal contribution of cholesterol to a normal mouse fetus.

Our results indicate that until approximately E10–11 in brain and E12–14 in liver and lung, the dam provides a great deal, if not most, of the cholesterol necessary for her fetus. After that, endogenous sterol synthesis increases abruptly, and at birth the fetus has synthesized 55–60% of the sterols incorporated into its liver and lungs. This process is accentuated in the central nervous system (CNS), so that the brain of the newborn mouse has been forced to make 90% of its own sterols. We were also able to demonstrate that the well-known accumulation of Δ^{24}-unsaturated sterols in the fetal CNS is probably the result of the reduced expression of sterol C24,25-reductase (*DHCR24*) in the brain and that cholesterol efflux from the brain via 24*S*-hydroxylation is likely to be limited until just before birth.

MATERIALS AND METHODS

Animals

Collection of embryonic tissues was performed as described previously (27). Briefly, mice fed a commercial chow were housed in an animal facility at 22°C with a 12 h light/dark cycle, and all protocols were approved by the Institutional Animal Welfare Committee. Mice heterozygous for a null mutation in *Dhcr7* (8) were backcrossed onto a C57BL/6J background for 10 successive generations, and their offspring were used in this study. Timed pregnant females were euthanized, and the embryos were dissected from the uteri and placed in 1× PBS or in RNAlater (Ambion, Austin, TX) for RNA analysis. Brains (with the exception that in E9.5 mice the whole head was used), lungs, and livers were dissected intact from E9.5, E11.5, E13.5, E15.5, E17.5, and E19.5 mice and neonates, blotted free of excess fluid and weighed. *Dhcr7* genotyping was performed by PCR, as described previously (8).

Quantitative RT-PCR

The following primers were used to measure the expression of *Dhcr24*, *Dhcr7*, and cholesterol 24-hydroxylase (*Cyp46A1*): Dhcr24-F (5′-GGTGTTCGTGTGCCTCTTCTTG-3′) and Dhcr24-R (5′-GCCCTGTTCCTTCCATTCCC-3′); Dhcr7-F (5′-GCCAAGACACCACCTGTGACAG-3′) and Dhcr7-R (5′-TGGACGCCTCCCACATAACC-3′); and Cyp46A1-F (5′-CAGCCGCTATGAGCACATCC-3′) and Cyp46A1-R (5′-AGAAACACATCTTGGAGCACACG-3′). Cyclophilin was used as the control. Samples of RNA from mouse liver and brain were reverse-transcribed according to the SuperScript First-Stand synthesis system (Invitrogen, Carlsbad, CA) using random hexamers in a final total reaction volume of 20 μl. Quantitative RT-PCR was performed on an Applied Biosystems (Foster City, CA) model 7300 real-time PCR system. The reaction volume was 10 μl containing 1× QuantiTect SYBR Green PCR master mix (Qiagen, Valencia, CA), 2 units of heat-labile uracil-DNA-glycosylase (Roche, Indianapolis, IN), 0.7 μl of the cDNA template from the reverse transcription reaction, and 100–500 nM oligonucleotide primers. The initial steps of RT-PCR were 2 min at 50°C for glycosylase activation followed by 15 min at 95°C to hydrolyze any contaminant DNA that contained uracil. Amplification consisted of 40 cycles of a 15 s melt at 95°C followed by a 1 min annealing/extension at 60°C and finally by a 60°C incubation for 1 min. After amplification, the samples were subjected to a 20 min ramp to 95°C to construct dissolution curves that ensured that single PCR products had been obtained. Relative quantities of *Dhcr24* and *Cyp46A1* message from liver and brain were normalized to cyclophilin. Relative mRNA expression levels were calculated by the threshold cycle ($\Delta\Delta C_T$) method and expressed as the fold increase $2^{-\Delta\Delta CT}$ according to the manufacturer's recommendations.

Tissue sterol measurements

We had reported previously that Dhcr7 activity in wild-type animals was >200 pmol/mg protein/min but was undetectable in the knockouts (8), confirming that these SLOS mice cannot reduce 7DHC to cholesterol. However, some of the accumulated 7DHC is isomerized to 8-dehydrocholesterol (cholesta-5,8-dien-3β-ol; 8DHC) (28, 29). 8-DHC was identified and quantified as a precursor sterol.

Tissue sterol concentrations from *Dhcr7*$^{+/+}$ and *Dhcr7*$^{-/-}$ fetal mice at E9.5 to P0 (n = 4 for each time point) were measured by capillary column gas chromatography (GC) as described previously (8). Compound identifications were confirmed by both GC-mass spectrometry (GC-MS) and the comparison of GC retention times with those of available standard sterols. Sterols not represented by a visible GC peak were considered to be of negligible importance (i.e., ≤1% of total sterols) and are not reported.

Except for trace quantities of lathosterol (cholest-7-en-3β-ol) averaging no more than 1% of the total, no other sterols with double bonds at C7,8 or C8,9 were detected by the GC assay in any tissue from wild-type mice. Therefore, for the purposes of this study, we define the term "precursor sterol" to mean the sum of all compounds with a Δ^7 or Δ^8 double bond. Because *Dhcr7*$^{-/-}$ mice cannot synthesize cholesterol, and because the pregnant mothers do not supply the fetus with appreciable quantities of 7DHC or of any sterol precursors that the affected fetus can convert to cholesterol, the fraction of sterol synthesized in each

organ by the fetus is, to a very good approximation, equal to P/(P + C), where P and C are the precursor sterol and cholesterol concentrations, respectively. We could not differentiate between sterols made in the fetus itself and those synthesized in the yolk sac or in tissues of fetal origin in the placenta, so we refer to any sterol synthesized by any fetal tissue as an endogenous sterol.

Data are expressed as means ± SD, except where indicated, and significance was assessed by the unpaired Student's *t*-test using GraphPad InStat (GraphPad Software, San Diego, CA).

RESULTS

Plasma cholesterol, 7DHC, 8DHC, and lathosterol levels in four heterozygous pregnant female mice measured by GC-MS were found to be 59 ± 12, 0.030 ± 0.018, 0.026 ± 0.019, and 0.44 ± 0.12 mg/dl, respectively, demonstrating that only trace quantities of Δ^7 or Δ^8 sterols are available for transport to the fetus. Therefore, it is highly likely that all of the 7DHC found in the fetus was made by the fetus. Levels of cholesterol, 7DHC, 8DHC, and lathosterol in pregnant wild-type mice were very similar at 52 ± 12, 0.022 ± 0.012, 0.014 ± 0.013, and 0.48 ± 0.30 mg/dl, respectively (n = 4, *P* = NS vs. *Dhcr7*$^{+/-}$ mice).

Only cholesterol was detected by GC in lung and liver of wild-type fetuses, whereas desmosterol (cholesta-5,24-dien-3β-ol) and, occasionally, small amounts of lathosterol were found in brain. GC peaks arising from 7DHC were not seen. Cholesterol, 7DHC, and 8DHC were identified in liver, lung, and brain from *Dhcr7*$^{-/-}$ fetuses of all ages, and we identified and estimated the concentrations of lathosterol, cholesta-5,7,24-trien-3β-ol (7-dehydrodesmosterol), and cholesta-7,24-dien-3β-ol in brains from SLOS mice.

There were no differences between total sterol concentrations in *Dhcr7*$^{-/-}$ and wild-type fetal brain during gestation. In contrast, total sterol levels in *Dhcr7*$^{-/-}$ fetal livers were 20–50% less than the concentrations measured in livers from wild-type animals, whereas total sterol levels tended to be somewhat reduced in the *Dhcr7*$^{-/-}$ fetal lung (**Fig. 1**).

The time course of the cumulative fraction of brain, liver, and lung sterols in the *Dhcr7*$^{-/-}$ mouse fetus that were synthesized by the fetus is shown in **Fig. 2**. It is evident that very early in gestation most of the cholesterol in these three organs is being supplied by the mother. However, as development continues, the fetus begins to take over its own synthesis, as reflected by the steady accumulation of 7DHC and other Δ^7 and Δ^8 precursor sterols. This process was especially evident in the CNS, so that after E15 close to 85% and, at birth, 90% of the sterols in the rapidly growing brain had been made by the fetus itself. In contrast, by the time of its birth, the fetus needed to make only 55–60% of the sterols incorporated into its liver and lung, with the remainder being supplied by its mother.

Increased levels of the Δ^{24}-unsaturated sterol desmosterol are common in the brains of normal fetal and newborn mammals. We estimated the concentrations of all Δ^{24}-unsaturated sterols in brains from all mice, specifically, desmosterol in wild-type mice and 7-dehydrodesmosterol and cholesta-7,24-dien-3β-ol in *Dhcr7*$^{-/-}$ animals (**Fig. 3**).

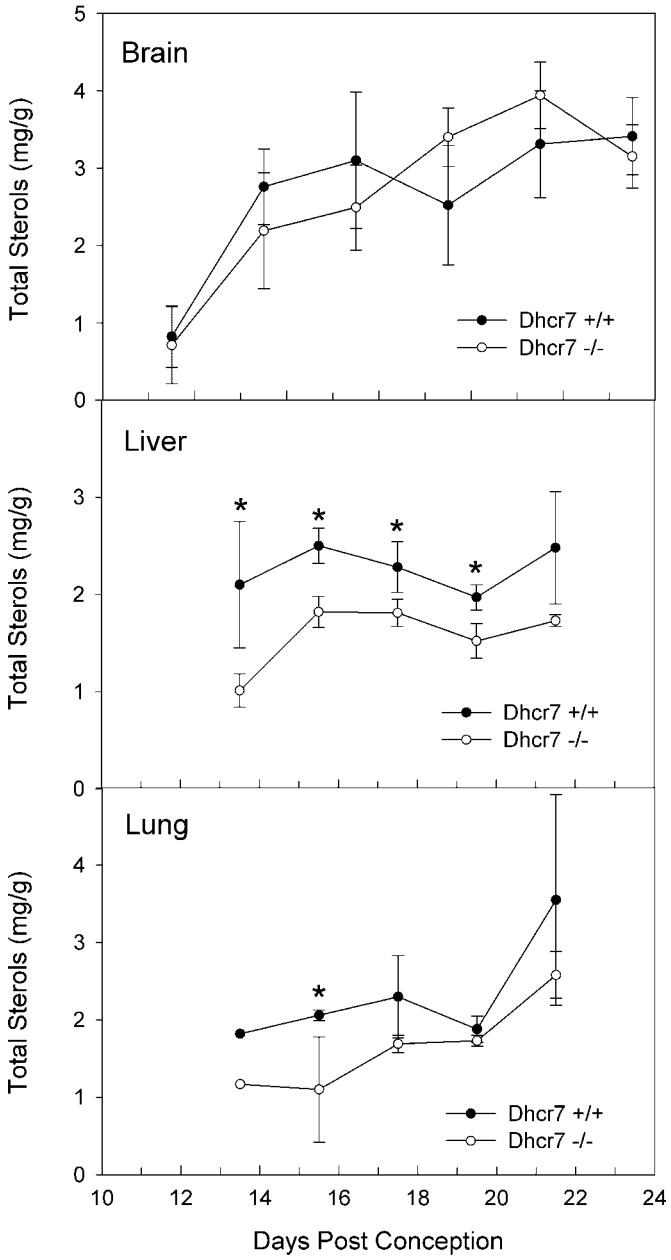

Fig. 1. Total sterol concentrations in brain, liver, and lung of fetal 3β-hydroxysterol Δ^7-reductase knockout (*Dhcr7*$^{-/-}$) and *Dhcr7*$^{+/+}$ (wild-type) mice (means ± SD, n = 4) as measured by gas chromatography at various times during gestation. * $P < 0.05$, *Dhcr7*$^{-/-}$ versus *Dhcr7*$^{+/+}$.

The percentage of sterols with a C24,25 double bond was rather low in both wild-type and knockout brain at E11.5, reflecting the maternal origin of its sterols. Soon after, because formation of the blood-brain barrier prevented cholesterol from the peripheral circulation from entering this organ and the brain was required to make most of its own sterol, the fraction of Δ^{24}-unsaturated sterols increased markedly. There was also a tendency for the *Dhcr7*$^{-/-}$ mice to have a lower proportion of Δ^{24}-unsaturated sterols, but this difference achieved statistical significance only at E13.5 and E17.5. Because increased levels of Δ^{24} sterols suggested that 3β-hydroxysterol Δ^{24}-

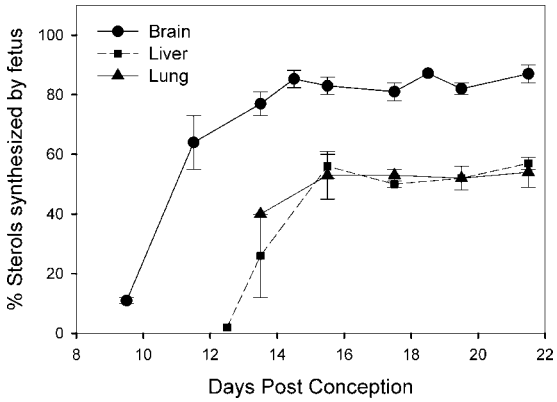

Fig. 2. Cumulative percentage of total sterols in brain, liver, and lung of fetal *Dhcr7*$^{-/-}$ mice that have been synthesized by the fetus during its gestation, calculated from the expression 100 × precursor sterols/total sterols (means ± SD, n = 4).

reductase (Dhcr24) activity ought to be reduced in brain compared with other organs, we compared *Dhcr24* mRNA expression in wild-type brain and liver at various gestational ages by quantitative RT-PCR (**Fig. 4A**) and found the levels to be consistently lower in brain.

Much of the cholesterol that is removed from brain is first converted to 24(*S*)-hydroxycholesterol by the enzyme CYP46 (30, 31). So we asked whether, as the cholesterol concentration in brain increases during gestation, the transcription of *Cyp46A1* keeps pace or if the brain attempts to conserve its cholesterol. We observed the latter: *Cyp46A1* mRNA expression levels remained relatively low until ~3 days before birth, when there was an abrupt increase (Fig. 4B).

DISCUSSION

Because the *Dhcr7*$^{-/-}$ mouse cannot reduce the sterol C7,8 double bond, all of the cholesterol in their liver, lung,

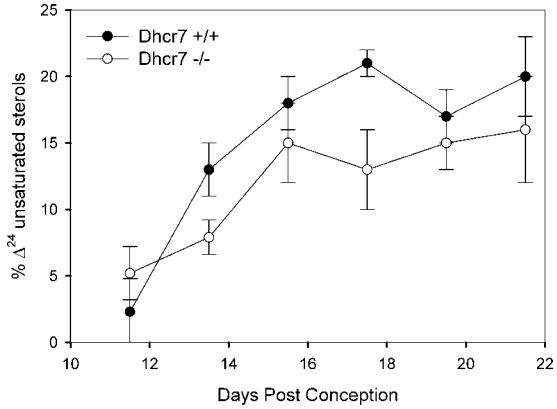

Fig. 3. C24,25-unsaturated sterols in brains of fetal *Dhcr7*$^{-/-}$ and wild-type mice (means ± SD, n = 4), expressed as the percentage of total sterols at various times during gestation. These compounds are cholesterol precursors found in abundance only in the central nervous system and are markers for in situ sterol biosynthesis.

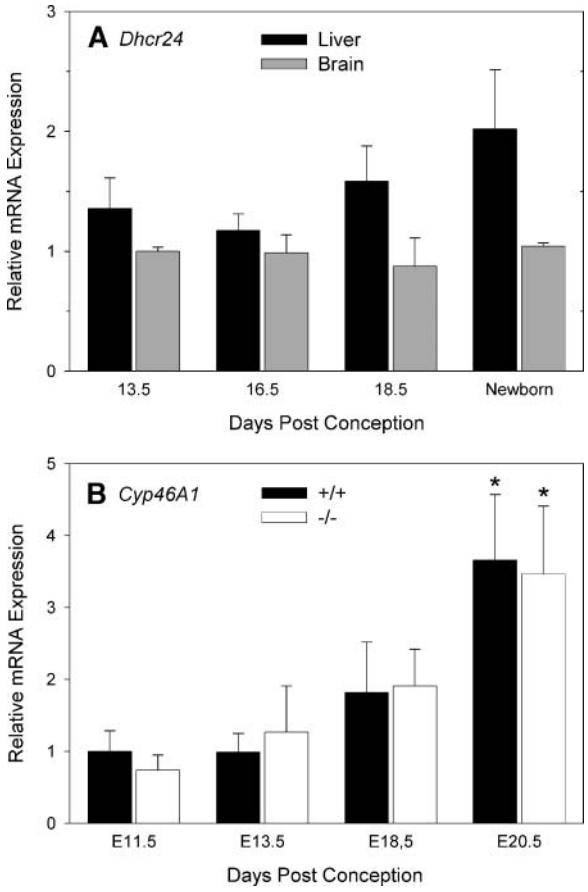

Fig. 4. A: Relative levels of sterol C24,25-reductase (*Dhcr24*) mRNA in wild-type mice measured by quantitative RT-PCR, demonstrating reduced expression in brain (black bars) compared with liver (gray bars) throughout gestation. Normalized to E13.5 brain, threshold cycle = 19.18 ± 0.09 (means ± SEM, n = 2). B: Relative levels of cholesterol 24-hydroxylase (*Cyp46A1*) mRNA measured by quantitative RT-PCR in brains of *Dhcr7*$^{-/-}$ and wild-type mice (white and black bars, respectively) during gestation, demonstrating that expression is suppressed until approximately E18. Normalized to E11.5 wild-type mice, threshold cycle = 21.12 ± 0.32 (means ± SEM, n = 3). * $P < 0.05$ versus expression in wild-type mice at E11.5.

and brain must have been of maternal origin. Thus, this mouse model enables us to make robust estimates of the relative amounts of cholesterol transferred to the fetus by the mother during gestation. One possible source of error, other than attempting to account for the rather small quantities of precursor sterols present in the maternal plasma, might be the existence of a small residual sterol Δ^7-reductase activity in the fetus; that is, this process might, during the 3 weeks of gestation, convert measurable amounts of accumulated 7DHC to cholesterol and skew the results in favor of maternally synthesized sterols. If this did happen, it must have been inconsequential, because we detected only small concentrations of cholesterol in the brains of the newborn knockouts.

During the first 10–12 days of the pregnancy, the pregnant dam supplies most of the cholesterol needed by her fetus (Fig. 2), a result first reported in the rat by Chevallier (14) >40 years ago. Then, at approximately E10–11 in

brain and E13–14 in liver and lung, the major source of cholesterol becomes the fetus itself, so that at birth 55% of lung and 60% of liver sterols have been synthesized endogenously. However, because the concentration of cholesterol in these organs becomes essentially constant by E16–17, while the fetus is still growing, the mother must be able to continue to supply some cholesterol to her fetus until it is born. The very steep increase in the curve of the percentage of endogenous brain sterols beginning at approximately E10 (Fig. 2) and the low percentage of cholesterol in the newborn brain suggest that an effective blood-brain barrier begins to form at this time, requiring the brain to make essentially all of its own sterols (14, 22, 32, 33).

Removal of cholesterol from the brain is facilitated by its conversion to 24(*S*)-hydroxycholesterol by CYP46 (30, 31). The time course of *Cyp46A1* expression in the brains of our mice (Fig. 4B) suggests that the brain attempts to limit cholesterol egress by restricting the transcription of this gene for most of its prenatal development. The observation in newborn mice of a steady increase in *Cyp46A1* expression in the perinatal period (34) appears to be a continuation of the trend that begins at E18–19.

Although our conclusion that only 55–60% of liver and lung sterols in the newborn SLOS mouse are synthesized endogenously is consistent with previous studies in rats (14), mice (22), and hamsters (17), it is in sharp disagreement with results from studies that used ^3H_2O to measure fetal cholesterol synthesis; these have reported that fetal rats make essentially all of their own cholesterol (33, 35). The source of this discrepancy may be that those studies measured fetal cholesterol synthesis over relatively short time intervals late in gestation, when the flux of maternal cholesterol, most prominent soon after conception, has probably diminished. Woollett (17), in contrast, using the same ^3H_2O technique in the hamster, reported results more consistent with ours. There seems little doubt, however, that a fully developed and functioning maternal/fetal transport apparatus exists in all mammals studied to date (3, 21, 22).

We conclude that, for the first third of its life, the newly conceived mouse receives most of its cholesterol from its mother, but that after E10–11 its brain is required to make essentially all of its own sterols. This scenario explains why genetic manipulations that block maternal/fetal cholesterol transfer or that prevent fetal sterol synthesis in the mouse necessarily lead to early fetal death. The demonstrated need for maternal cholesterol early in gestation also helps to explain why we were able to induce severe developmental abnormalities in Wistar rats but not in ICR mice after the administration of a chemical inhibitor of DHCR7 to pregnant dams (36, 37): treating rats caused maternal plasma sterols to decline from 50 mg/dl before to 20 mg/dl after treatment, but pretreatment and post-treatment sterol levels in the mice remained 2- to 3-fold higher at 110 and 70 mg/dl, respectively. It further explains why the rat fetus can be partially rescued in such experiments by diets that markedly increase maternal plasma cholesterol levels (18, 37, 38). In humans, absence of a maternal apoE2 allele is thought to allow for more rapid maternal/fetal cholesterol transport and tends to result in a less severe SLOS phenotype (39). In addition to early labeling studies (15, 16), proof of a quantitatively important flow of cholesterol from a human mother to her fetus comes from the detection of significant quantities of cholesterol in two fetuses and a newborn with the most severe form of SLOS (40–42), who, like our SLOS mice, are probably unable to convert any 7DHC to cholesterol (43, 44). This flux appears to be sufficient to provide as much as 30% of non-CNS tissue sterols (but only 2–3% of brain sterols).

Cholesterol biosynthesis requires that the Δ^{24} double bond of lanosterol, the first true sterol in the pathway, be reduced. Because concentrations of these compounds are vanishingly small in maternal or fetal plasma, all Δ^{24} sterols in the fetal brain must be synthesized in situ. Our observation that most of the cholesterol in mouse brain comes from its mother early in gestation, implying reduced de novo sterol synthesis, is consistent with our finding of low levels of Δ^{24} sterols in the brains of both wild-type and SLOS mice before E11.5 (Fig. 3). Increased concentrations of desmosterol in normal fetal and newborn mammalian brain also suggest that the activity of sterol 3β-hydroxy-Δ^{24}-reductase (DHCR24) must be low (8, 45), and measurements in 3 day old rats have confirmed that Dhcr24 activity is reduced 7-fold compared with its activity in liver (46). These results are in agreement with our finding that *Dhcr24* expression in the fetal brain is suppressed (Fig. 4A).

Although the results described above fairly represent what takes place in the *Dhcr7*$^{-/-}$ fetus, is it a reasonable approximation to a normal mouse? That is, are rates of endogenous sterol synthesis and transfer of cholesterol from mother to fetus similar in wild-type and *Dhcr7*$^{-/-}$ animals? Total sterol concentrations in brain were similar in both mice and were only slightly reduced in lungs from the knockouts (Fig. 1). The sterol values in brain are especially informative, because this organ needs to make almost all of its own sterols beginning at approximately E11.5. Although we have no explanation for why sterol concentrations were significantly lower in the livers of knockout mice (Fig. 1), our results do suggest that, for the most part, the rates at which the nearly pure C57BL/6J *Dhcr7*$^{-/-}$ and wild-type mice make sterols are similar. There is little available information, however, that would allow us to predict whether the ability of a pregnant dam to transfer cholesterol to a fetus might be affected by mutations in *Dhcr7* and the subsequent accumulation of 7DHC. The best that can be said, at present, is that in humans with SLOS, the synthesis and transport of apoB (47) and LDL receptor activity (48, 49) seem not to be impaired, suggesting that cholesterol transport to an affected fetus might not be too greatly perturbed.

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