Sources of cholesterol during development of the rat fetus and fetal organs

Helga A. Jurevics, Faisal Z. Kidwai, and Pierre Morell[1]

Neuroscience Center and Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599-7250

Abstract Female rats at various stages of pregnancy were injected intraperitoneally with [^{3}H]water; 4 h later, they were killed, the uterus was removed, and the fetuses were dissected. Lipids were isolated and fractionated by HPLC and the total amount of cholesterol in each organ, as well as radioactivity incorporated into cholesterol and cholesterol precursors, were determined. From the data for cholesterol content at each age we calculated the rate of accumulation of cholesterol during fetal development. As incorporation of label from [^{3}H]water takes place with a stoichiometry defined by a known biosynthetic pathway, we were also able to determine the fraction of cholesterol accumulating in each organ that had been newly synthesized. For the fetus as a whole, more than 93% of the cholesterol accumulating during development was newly synthesized. As the specific radioactivity of cholesterol in the maternal circulation was negligible (because synthesis of cholesterol by maternal liver was suppressed by inclusion of cholesterol in the diet), we conclude that the fetus synthesizes nearly all of its own cholesterol; neither the maternal circulation nor the placenta/yolk sac contribute significant amounts of cholesterol to the fetus. We were also able to quantitate trafficking of cholesterol between fetal organs. Fetal brain is responsible for the synthesis of all of its own cholesterol. In contrast, fetal liver exports cholesterol into the fetal circulation and supplies about half of the cholesterol for development of heart, lung, and kidney.—**Jurevics, H. A., F. Z. Kidwai, and P. Morell.** Sources of cholesterol during development of the rat fetus and fetal organs. *J. Lipid Res.* 1997. **38:** 723–733

Supplementary key words brain • placenta • uterus • cholesterol biosynthesis

The developing fetus depends upon maternal circulation, as mediated by the placenta, for delivery of substrates for energy metabolism as well as precursors for biosynthesis of macromolecules needed during the period of rapid growth and development in utero. However, molecules in the maternal circulation are not all equally available to the developing fetus, as the placenta exercises considerable control over what enters. A question arising is the extent to which cholesterol in the maternal circulation, itself a pool containing cholesterol of dietary origin as well as that synthesized by the liver, can be utilized by the fetus.

The origin of fetal cholesterol has been considered previously. There are a number of lines of evidence which suggest that in several species the dam contributes considerable cholesterol to the fetus (1–3). We have investigated the source of fetal cholesterol in rat and arrive at a contradictory conclusion. Our approach is based on an experimental method exploited by Spady and Dietschy (4, 5). When [^{3}H]water is injected into animals there is rapid equilibration of label with the whole body pool of water. The tritium atoms from this water are incorporated into cholesterol in a stoichiometry defined by known biosynthetic pathways. This allows for determination of the absolute rate of cholesterol biosynthesis in vivo. Belknap and Dietschy (6) extended this methodology to the study of pregnant rats. They showed that the rate of synthesis of cholesterol was several fold higher in the fetus than in the mother, and that dietary cholesterol repressed maternal hepatic cholesterol synthesis, but did not greatly affect synthesis of cholesterol in the fetus. They also found that while the placenta took up ^{125}I-labeled cellobiose-labeled low density lipoprotein, none of the apolipoproteins or cholesterol was transferred to the fetus. This suggested that much of the fetal cholesterol is synthesized within the fetus.

We have further extended these studies by quantitative correlation of the rate of cholesterol synthesis in the fetus with the rate of accumulation of total cholesterol. This allows us to reliably quantitate the fraction of total fetal cholesterol that is synthesized in the fetus. An extension of this methodology has allowed us to study trafficking of cholesterol among different fetal organs as a function of development. Of particular interest is whether the liver of the fetus is a net exporter of cholesterol, and which developing tissues utilize cholesterol produced by the liver. A specific question is

Abbreviations: HPLC, high performance liquid chromatography.

[1]To whom correspondence should be addressed.

whether developing brain relies on the fetal circulation as a significant source of cholesterol. This is relevant in relation to the recent elucidation of the metabolic defect in Smith-Lemli-Opitz syndrome. In this autosomal recessive disorder cholesterol synthesis is impaired at the level of 7-dehydrocholesterol-Δ^7-reductase (7, 8). The nature of this defect has led to the suggestion that elevating circulating cholesterol levels in the mother during pregnancy (or perhaps by surgically mediated delivery of cholesterol to the fetal circulation) might alleviate some of the problems of brain development caused by this disorder (9, 10). Such treatment would only be useful if cholesterol could enter fetal brain from the fetal circulation.

METHODS

Animal model and tissue dissection

To minimize variations in fetal numbers and fetal weights within and among litters, Long-Evans hooded rats in their second pregnancy were purchased from Charles River Breeding Laboratories (Raleigh, NC). Eight dams, timed pregnant to within a 12-h window, were obtained 7 days after mating. Upon arrival at our facility they were placed in individual cages and kept on a 12-h light/dark cycle at constant humidity (50 ± 10%) and temperature (21 ± 2°C). Normal rat chow and water were provided ad libitum. The following day (gestational day 8) dams were placed on a normal rat chow supplemented with 2% cholesterol to suppress cholesterol synthesis by the liver.

Between 10:00 and 11:00 A.M., on gestational days 11, 14, 16, 17, 18, 19, 20, and 22, one of the dams was injected intraperitoneally with 50–75 mCi [^{3}H]water (ARC, St. Louis, MO). After injection, dams were kept in a vented hood without further access to food and water for 4 h, after which they were lightly anesthetized with ether and killed by decapitation. Blood was collected from the aorta to obtain serum for determination of specific radioactivity of body water and for isolation of lipids. The abdomen was opened and both horns of the uterus were excised and rinsed with ice-cold physiological saline. Both horns of the uterus were opened longitudinally and the fetuses were peeled from the uterine lumen with their decidua and placenta intact and protected from evaporation by placement in plastic culture tubes on ice. Dissections were performed in Petri dishes on ice. Fetuses were separated from fetal membranes and weighed individually or as the pooled contents from each horn of the uterus. The decidua was separated from the placenta/yolk sac and the amnion and umbilical cord were included with the latter. Individual tissues were isolated and rinsed with ice-cold physiological saline. Average weights were determined for individual organs either as the pooled contents from each uterine horn in the early gestational ages or as the pooled contents from 3–4 fetuses at later gestational ages. Fetuses before gestational day 14 did not have a decidua and, therefore, each embryo was separated from its placenta/yolk sac membranes. Livers were obtained from fetuses at gestational age 16 and by gestational day 18 other tissues (whole brain, heart, lungs, and kidney) could be dissected intact. Blood was pooled from 4–5 fetuses beginning with gestational age 18. From each gestational age at least two fetuses were left intact for extraction of lipids in the whole fetus.

Preparation and analyses of lipid extracts

Total lipids were extracted from fetal tissues according to Folch, Lees, and Sloane Stanley (11) as modified by Benjamins, Miller, and Morell (12), and sterols were quantitated as described previously (13). Briefly, free cholesterol was separated from its precursors and other lipids by reverse-phase HPLC on a C18 column (LiChosorb RP-18, Hewlett-Packard, Kennett Square, PA). Sterols were eluted isocratically at a flow rate of 2 ml per min within 45 min with acetonitrile–isopropanol 97.5:2.5 and were detected by absorbance at 210 nm. Adjusted (for void volume) retention times for cholesterol and the precursors of interest were as follows: squalene, 10.9 min; desmosterol/lanosterol, 19.2 min; 7-dehydrocholesterol, 22.9 min; cholesterol, 31.5 min. Quantitation of mass of sterols was made by comparing areas under sterol peaks to areas generated by known quantities of that sterol. Eluant fractions of 100 sec duration were collected during the elution period. Fractions were dried and resolubilized in 5 ml of Ecoscint H (Fisher, Raleigh, NC). Radioactivity in each fraction was determined in a Packard 1600TR Liquid Scintillation Analyzer. Specific radioactivity could thus be obtained very accurately as the same eluant fraction was used for assay of both mass and radioactivity.

For some tissue samples a known quantity of the extracted total lipid was subjected to alkaline hydrolysis to determine cholesteryl ester content and radioactivity in cholesterol moiety of the cholesteryl ester. The total lipid sample was dried under nitrogen and 2 ml of water–10% KOH in methanol 1:1 was added. Samples were hydrolyzed for 4 h at 85°C and at room temperature overnight. Non-saponifiable lipids were isolated by the method of Folch et al. (11) and subjected to HPLC analysis as described above. The difference in free cholesterol contents before and after hydrolysis represents the quantity of cholesteryl ester in the total lipid sample. From the difference in radioactivities of free cholesterol before and after hydrolysis, the quantity of newly synthe-

sized cholesterol appearing as ester was calculated as described below.

Experimental design

The initial goal was to establish what fraction of cholesterol accumulating in the fetus is synthesized in the fetus, relative to the amount coming from the maternal circulation. Data needed include the rate of accumulation of total cholesterol in the fetus and rate of accumulation of newly synthesized cholesterol. The rate of cholesterol accumulation was obtained by assay of total cholesterol content of the fetus at various ages followed by calculation of the incremental accumulation of cholesterol. The rate of accumulation of newly synthesized cholesterol in the fetus was determined from the rate of incorporation of label from tritiated water into cholesterol. [^{3}H]water, injected into the dam several hours prior to killing, rapidly equilibrates with body pools of water in the dam and fetus and, subsequently, each molecule of cholesterol synthesized de novo contains tritium with a stoichiometry defined by the biosynthetic pathway. As reviewed by Dietschy and Spady (5), 22 hydrogens, equivalent to that originating from 11 molecules of water, are present in cholesterol. The amount of cholesterol synthesized during the 4 h (1/6th of a day) when [^{3}H]water is present can be calculated from radioactivity present by assuming specific radioactivity of newly synthesized cholesterol is 11 times that of the body pool of water. Thus, the rate of appearance of newly synthesized cholesterol in any organ can be determined by assay of radioactivity in cholesterol.

$$\frac{\text{nmoles cholesterol synthesized}}{\text{day}} = \frac{6 \times \text{dpm in cholesterol in 4 h}}{11 \times \text{dpm per nmole water}}$$

If all the cholesterol present in the fetus is synthesized within the fetus, the rate of accumulation of cholesterol in the fetus should be equal to the rate of accumulation of newly synthesized cholesterol. The other extreme is if all of the cholesterol present in the fetus originates from the maternal circulation. In that case very little newly synthesized (radioactive) cholesterol would be present. This is because synthesis of cholesterol in the liver of the mother is suppressed by inclusion of 2% cholesterol in her diet, so little newly synthesized cholesterol is found in the maternal circulation.

The experimental design, as applied to determination of the source of cholesterol in various organs during postnatal development, is more extensively described in previous publications (13, 14); see also Belknap and Dietschy (6) for consideration of aspects specific to the fetus. It must be verified that the time-course of incorporation of label into cholesterol is linear and that the time needed for equilibration of precursor pools with the specific radioactivity of body water is accounted for. This time lag is, in fact, of the order of 30 to 45 min if synthesis of cholesterol alone is considered (13). In preliminary studies related to the present work we showed that for maternal organs, this lag can be reduced to several minutes if label accumulating in desmosterol, lanosterol, and squalene is also taken into account. This calculation involves expressing label in each of these intermediates as nmoles of intermediate synthesized. For example, synthesis of squalene involves incorporation of 11 hydrogens from water (15) instead of the 22 required for cholesterol synthesis; each molecule of squalene synthesized is presumably committed to becoming cholesterol and was, therefore, assumed to be equivalent to a molecule of cholesterol. We assumed 20 atoms of hydrogen for labeling of desmosterol (present in brain) and 18 atoms of hydrogen incorporated into lanosterol, the primary intermediate present in other tissue. We did not correct for the lag time of equilibration of maternal radioactive body water pools with fetal body water, a value shown to be of the order of 20 min for the rat fetus (6), and to that extent our calculation of locally synthesized cholesterol is a slight underestimate (see Discussion).

The above experimental design is appropriate for evaluating overall cholesterol metabolism in the fetus, as feeding of cholesterol to the mother guarantees negligible specific radioactivity of maternal circulating cholesterol (6, 13). Study of individual fetal tissues adds a second order of complexity in the analysis. Analysis of cholesterol biosynthesis by different fetal organs may be complicated if the specific radioactivity of circulating cholesterol is high. Thus, labeling of cholesterol in the fetal circulation must be determined, and accounted for in the calculations.

RESULTS

Organ weight and cholesterol content

The wet weights of whole fetus and of various dissected organs are plotted as a function of gestational age (**Fig. 1**). As expected, more than three-quarters of the growth of the whole fetus and tissues of fetal origin (placenta/yolk sac) takes place at or after the eighteenth day of gestation. This contrasts with the growth of the uterus and decidua (tissues of maternal origin) which attain full size by gestational age 18. The data for cholesterol accumulation by organ is also available (**Fig. 2**) and track net weight reasonably well. Calculations from the data presented in these figures demonstrate that the organ-specific differences in cholesterol con-

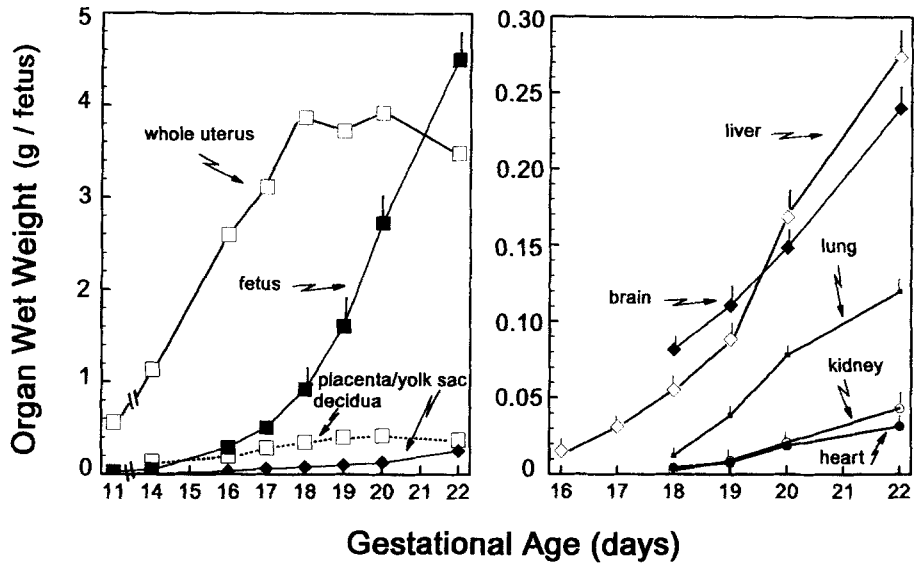

Fig. 1. Wet weight of whole uterus after removal of fetuses and deciduae. Other tissues are on a per fetus basis; these include the decidua (of maternal origin) and the placenta/yolk sac (of fetal origin). The fetus was further dissected to obtain the tissues noted on the right-hand graph. On this and subsequent graphs, experimental points are single observations unless otherwise indicated. Where present, error bars represent a standard deviation for triplicate values.

centration before birth are small; for example, at gestational age 22 days the concentrations of cholesterol in liver, kidney, and brain, respectively, are: 4.05, 5.9, and 7.1 nmol/mg wet weight. In contrast, by postnatal day 35 the cholesterol concentration in these three tissues is 3.2, 8.3, and 36.4 nmol/mg wet weight, respectively (H. Jurevics and P. Morell, unpublished data from our laboratory).

The plateau in weight gain of the uterus before the time of maximum size increase of the fetus is well documented (16), and the actual slight decline in cholesterol after 19 days is not unexpected. Late in gestation there are hormonally mediated structural changes in the uterus leading to a decrease in smooth muscle cell size (16) and an increase in protease activity (17) that could also be accompanied by a loss of tissue cholesterol. Similar results have been reported for decidua; this structure forms from the endometrial cells of the uterine stroma to make a thick capsule around the implanted embryo. As pregnancy approaches term there is cell death in the decidua with a gradual thinning of the tissue (18).

Because conversion of cholesterol to cholesteryl ester is a potential complication in interpretation of our analytical data, we assayed each tissue for cholesteryl ester content as a fraction of total cholesterol at 17, 19, and 22 days of age. Radioactivity in the cholesterol moiety of the cholesteryl esters was also determined. A significant level of cholesteryl ester was found in maternal serum: 55% of total cholesterol was present as the ester but, in these cholesterol-fed animals, there was no significant amount of radioactive labeling of any cholesterol in the maternal circulation. For fetal serum, 20% of the total cholesterol was present as ester at 17 d, increasing to 35% at 22 d; at these ages only about 5% of the label in total cholesterol was present in the cholesterol moiety of the ester. In uterus, about 26% of cholesterol was present as ester but this contained 16% of label at 17 d and less than 1% by 22 d. In fetal liver, 40–15% of cholesterol was present as ester between 17 and 22 days and, correspondingly, 10% and 5% of label was present in the cholesterol moiety of cholesteryl ester. In the placenta/yolk sac about 25% of cholesterol was present as the ester at the three ages and, as cholesterol synthesis in this organ system decreased with development, the fraction of label present in cholesteryl ester decreased from 14% to less than 1%.

Cholesterol labeling in the serum

For label in fetal cholesterol to be equated with fetal synthesis of cholesterol, it must be known that specific radioactivity of maternal circulating cholesterol is negligible. We first determined cholesterol concentration in the serum of dam and fetus (**Fig. 3A**). Note the rise in maternal serum cholesterol later in gestation, similar to that previously reported for humans (19) and rat (20). The levels of cholesterol in fetal serum are at this higher level. However, as indicated by the data below, there is not a precursor product relationship between the two pools of cholesterol.

The plot in Fig. 3B represents the fraction of cholesterol in the serum of the dam and fetus that was synthe-

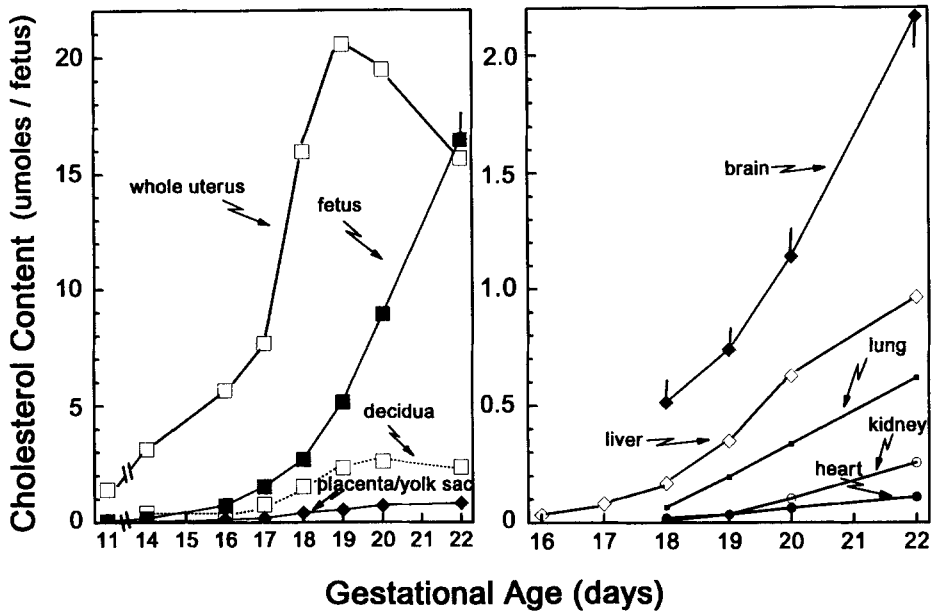

Fig. 2. Cholesterol content of whole uterus and various fetal tissues corresponding to the samples described in Fig. 1. Three to five fetal tissues were pooled for each assay.

sized during the 4-h labeling period. Note that this is actually a plot of corrected specific radioactivity; the value for radioactivity (presented as newly synthesized cholesterol according to the equation given previously) is divided by bulk free cholesterol. For serum in the maternal circulation, much less than 1% of the circulating cholesterol is newly synthesized. This is as expected because synthesis of cholesterol in the maternal liver is suppressed by cholesterol feeding, and any newly synthesized cholesterol is initially diluted by the liver pool of cholesterol and then by cholesterol already in the maternal circulation. Thus, with regard to the data presented in subsequent figures, the specific radioactivity of cholesterol, cholesteryl esters, and cholesterol precursor in the maternal circulation is much too low to account for any significant accumulation of radioactivity in the fetus.

The data for specific radioactivity of serum cholesterol in the fetus (Fig. 3B) contrasts with that for maternal serum cholesterol. After the 4-h labeling period, 16% of the fetal circulating cholesterol is newly synthesized. This is presumably a function of failure of dietary cholesterol to suppress cholesterol synthesis in fetal liver, a smaller pool of liver cholesterol for dilution prior to release into the circulation, and the expected more rapid turnover of circulating cholesterol as it continues to be removed by many of the rapidly growing organs. The specific radioactivity of circulating fetal cholesterol is high enough so that it is a consideration when evaluating cholesterol trafficking within the fetus (see last section of Results). In fetal serum, lanosterol was the only cholesterol precursor found to have significant radiolabel after 4 h. The distribution was 93.9 ± 2.1 (SD)% in cholesterol and 6.1 ± 2.1 (SD)% in lanosterol. This distribution is similar in serum of rats weaned onto a cholesterol-free diet where cholesterol in serum is labeled significantly (13).

Cholesterol balance in whole fetus compared to that of the maternal uterus

The rate of synthesis of fetal cholesterol is similar to the rate of accumulation of fetal cholesterol (**Fig. 4A**). The amount of cholesterol newly synthesized each day was summed between gestational days 11 and 22, and the value was compared to the total amount of cholesterol in the 22-day-old fetus. We found that at least 93% of the cholesterol accumulating in the fetus was newly synthesized (see Discussion). As contribution of radioactive cholesterol from maternal circulation is negligible (shown above) it is apparent that most of the cholesterol in the fetus is synthesized by fetal cells. The possibility that the maternal liver synthesizes a lipoprotein with a high specific radioactivity and that the cholesterol in this lipoprotein travels undiluted by pre-existing pools of this lipoprotein cannot be dismissed entirely but appears highly speculative considering our present knowledge of circulating lipoproteins.

A dramatic contrast to the above results for fetus is offered by the data for the uterus (Fig. 4B) and decidua (Fig. 4D). Most of the cholesterol accumulating in these maternally derived tissues is unlabeled and therefore, as expected, comes from the maternal circulation. The

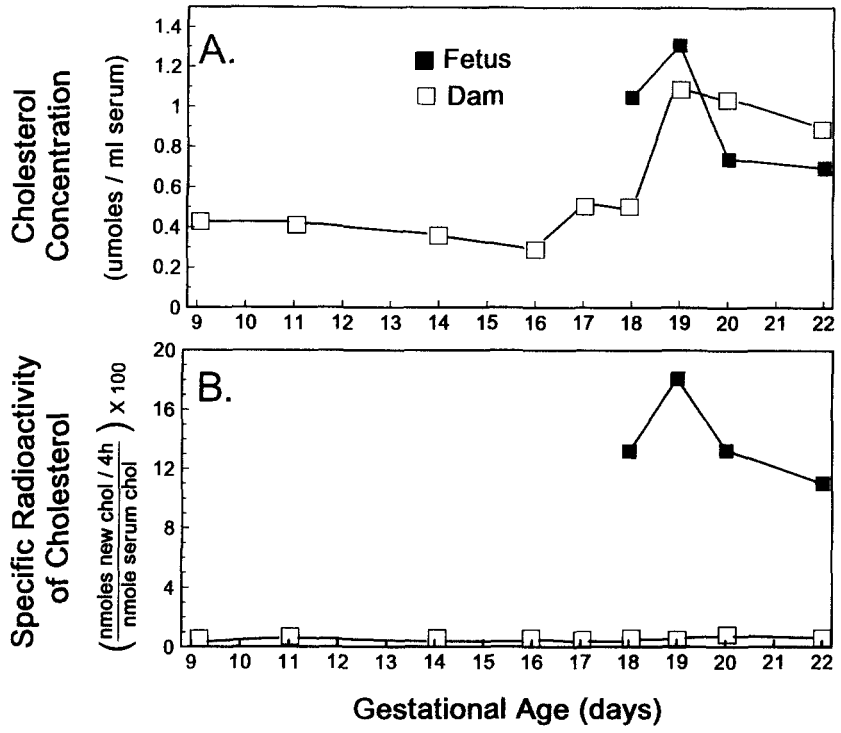

Fig. 3. Cholesterol concentration and appearance of newly synthesized cholesterol in serum of dam and fetus 4 h after injecting the dam with [^{3}H]water. Cholesterol content and radiolabel in cholesterol were determined by HPLC as described in Methods. Cholesterol concentration is plotted in panel A. For panel B the ordinate units correspond to percentage of the total circulating cholesterol that is newly synthesized; this calculation is based on the assumption (see Experimental Design Section) that 22 atoms of H are incorporated per molecule of cholesterol, thus allowing for normalization of differences among animals in specific radioactivity of body water. All values are from a single assay of serum from one dam at each time point and the pooled serum from 4–5 fetuses at each gestational age.

time-course of cholesterol accumulation in uterus and decidua is very different from that of the fetus, with the actual loss of tissue at the end of gestation accounting for the loss of cholesterol at the last age point.

Data for placenta/yolk sac (Fig. 4C) indicate a mass and rate of cholesterol accumulation of the order of 10% of that of the fetus. The slight excess of synthesis of cholesterol over its accumulation occurring after gestational day 18 was less than 0.2 μmol per day suggesting a maximal possible contribution of only a few percent to the fetus, which is accumulating cholesterol at a rate of several μmol a day. The contribution is, in fact, even less as some of this excess newly synthesized cholesterol may be used to account for the synthesis of cholesteryl ester; the cholesteryl ester content of placenta/yolk sac is 25 ± 5% (SD) of total cholesterol. It is also possible that a minor source of error contributing to the overall sterol balance would be production of steroid hormones and/or bile acids by the fetus.

Cholesterol balance for brain and liver

During development of the fetal brain, the rate of accumulation of bulk cholesterol is similar to the rate of accumulation of newly synthesized cholesterol (**Fig. 5A**). Thus, unless the specific radioactivity of fetal serum cholesterol is very high (which is not the case, see Fig. 3), this is evidence that the cholesterol in fetal brain is locally synthesized. The results for the fetal liver (Fig. 5B) stand in marked contrast. At the earliest age at which we could clearly dissect and analyze liver, and proceeding through to birth, the liver synthesizes a great deal more cholesterol than is required for growth of this organ. During early development of the liver, before gestational day 19, 40% of the cholesterol in the liver is in the ester form, but this declines to less than 15% of total cholesterol by gestational day 22. Thus, during early development, we calculated that, of the newly synthesized cholesterol, 30% accumulates as free-cholesterol, 16% is converted to ester, and the remaining 44% is exported from the liver. Over the course of gestational development, the liver synthesizes 1.7 μmol of cholesterol in excess of that accumulating. The assumption that this cholesterol is exported to the fetal circulation and used by other tissues is supported by experiments described below.

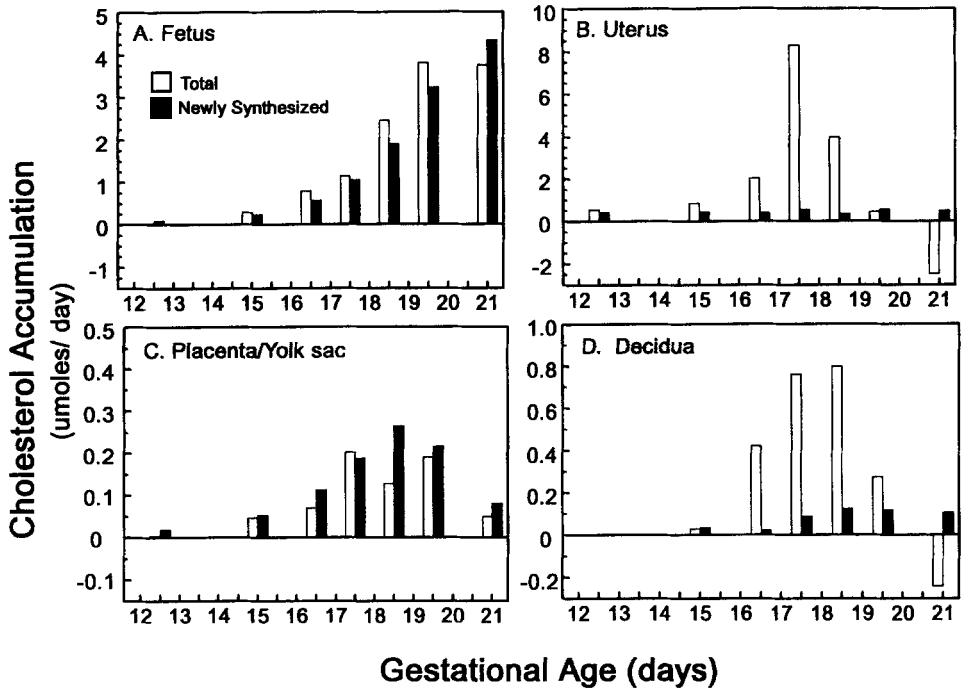

Fig. 4. Accumulation of bulk cholesterol and accumulation of newly synthesized cholesterol in various organs. The accumulation of bulk cholesterol is calculated from the data in Fig. 2. The newly synthesized cholesterol was calculated from incorporated radioactivity as detailed in the Experimental Design Section.

Cholesterol balance during development of kidney, heart, and lung

With respect to data for individual organs of the fetus, in order to equate radioactivity present with locally synthesized cholesterol one would have to assume negligibly low entry of label from the circulation and, therefore, negligibly low specific radioactivity of fetal serum cholesterol. This is not the case (Fig. 3B). Thus, in analysis of these data, a calculation is required to determine how much of the newly synthesized cholesterol was locally synthesized. A first order correction for the accumulation of radioactive cholesterol in each organ was obtained by subtraction of radioactivity corresponding to synthesis of 8% of the accumulation of unlabeled cholesterol. (The fraction of radioactivity in imported cholesterol was set at 8% rather than 16% to account for the linear increase in radioactive cholesterol in the blood during the time period studied.) These corrected values are shown as the third bar in histograms of **Fig. 6.** As is evident, the first order correction is slight (a complete correction is not possible algebraically without further assumptions but would be negligible in view of the relatively low specific radioactivity of serum cholesterol). It is clear that for heart (Fig. 6A), kidney (Fig. 6B), and lung (Fig. 6C), the rate of accumulation of cholesterol is considerably faster than can be accounted for by appearance of newly synthesized cholesterol. Thus, half or more of the cholesterol accumulating is unlabeled and presumably comes from the fetal circulation. For all three organs combined, less than 1 μmol of cholesterol is accumulated by gestational day 22. This amount is considerably less than the excess of cholesterol synthesized by the liver. The proportion of cholesterol for development supplied by the fetal circulation may be quite small in other organs. For example, in other experiments (data not shown) by 22 days the amount of cholesterol accumulated by the urogenital system, stomach, intestines, and pancreas, combined, was 1.7 μmol of which 80% was accounted for by newly synthesized cholesterol.

DISCUSSION

The concept of measuring cholesterol synthesis within the fetus by determination of label incorporated from radioactive water into cholesterol was introduced by Belknap and Dietschy in 1988 (6). They demonstrated that [^{3}H]water administered intravenously to the mother rapidly equilibrates with the body pool of water and that (as that laboratory had previously showed in adults (4)), 22 hydrogen atoms from the body water pool were incorporated into each molecule of newly synthesized cholesterol. When calculated per gram of tissue for various organs, the rate of synthesis

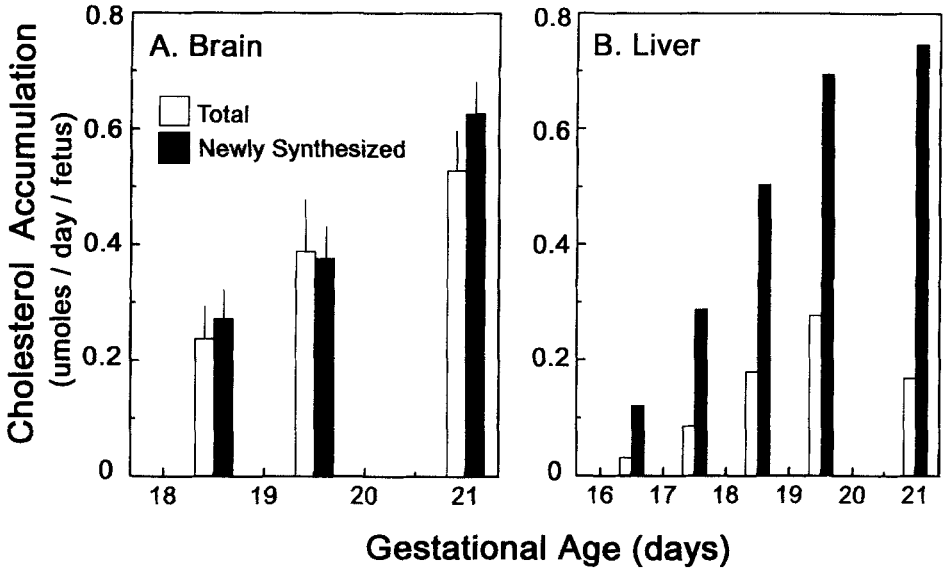

Fig. 5. Accumulation of bulk cholesterol and newly synthesized cholesterol during growth of fetal brain and liver. Accumulation of bulk cholesterol was calculated from the data in Fig. 2 and that in newly synthesized cholesterol from incorporated radioactivity from [^{3}H]water as detailed in Experimental Design Section. The error bars for brain correspond to data from the samples described in Fig. 2.

of cholesterol was several times greater in the rat fetus than in the dam, suggesting that much of the demand for cholesterol for fetal growth was met by the fetus. This conclusion received further support when feeding cholesterol to the mother greatly lowered the specific radioactivity of maternal circulating cholesterol but did not significantly change incorporation of label into fetal cholesterol. Furthermore, there was no transfer of ^{125}I-labeled cellobiose-labeled lipoprotein from maternal circulation to the fetus. These authors concluded "that the rat fetus receives little or no cholesterol from the mother but, rather, satisfies its need for cholesterol during fetal development through local synthesis."

Our experimental design is based on that of Belknap and Dietschy (6), but also included quantitation of the developmental increase in fetal cholesterol, making it possible to calculate a specific radioactivity for newly synthesized cholesterol. Thus, we are able to make a quantitative statement that at least 93% of the cholesterol accumulating in the fetus during development is of fetal origin. This is probably a 4% underestimate, because we did not correct for the approximately 20 min lag in the equilibration of label with fetal body water (6) that would lead to a consequently slight underestimation of the rate of cholesterol synthesis. Thus, inasmuch as our data were interpreted in terms of source of cholesterol in the whole fetus, our results are in accord with the conclusions of Belknap and Dietschy (6). We acknowledge, however, the existence of data from other laboratories interpreted to give conclusions contrary to ours (discussed below).

Transfer of labeled cholesterol from mother to fetus was first reported in rat in 1947 by Goldwater and Stetten (21). Studies on guinea pigs (1) and rhesus monkeys (2) demonstrated that [^{14}C]cholesterol in the dam equilibrated with fetal cholesterol at a rate suggesting significant uptake of maternal cholesterol by the fetus (with the exception of fetal brain). Placental involvement was suggested as this structure is capable of uptake of high density lipoproteins, very low density lipoprotein, and low density lipoprotein cholesterol (6, 22) and contains lipoprotein receptors that bind lipoprotein particles carrying apolipoprotein E (23). There is also evidence for transplacental transport of low density lipoprotein-bound cholesterol by human placenta in vitro (24), and synthesis of apolipoprotein B (presumably for formation of lipoprotein particles) by yolk sac has been reported (25–27). The cholesterol carried by these lipoprotein particles might be for transfer of maternally or locally derived (placenta/yolk sac) cholesterol to the fetus and/or might serve as substrate for placental progesterone synthesis (28). These lipoprotein particles also serve as a source of essential free fatty acids and lipid-soluble vitamins for the developing fetus (for reviews, see 29–31). The yolk sac may play a role in processing of lipoidal material transferred from maternal circulation to the developing fetus, as evidenced by developmental growth retardation of rat fetus upon

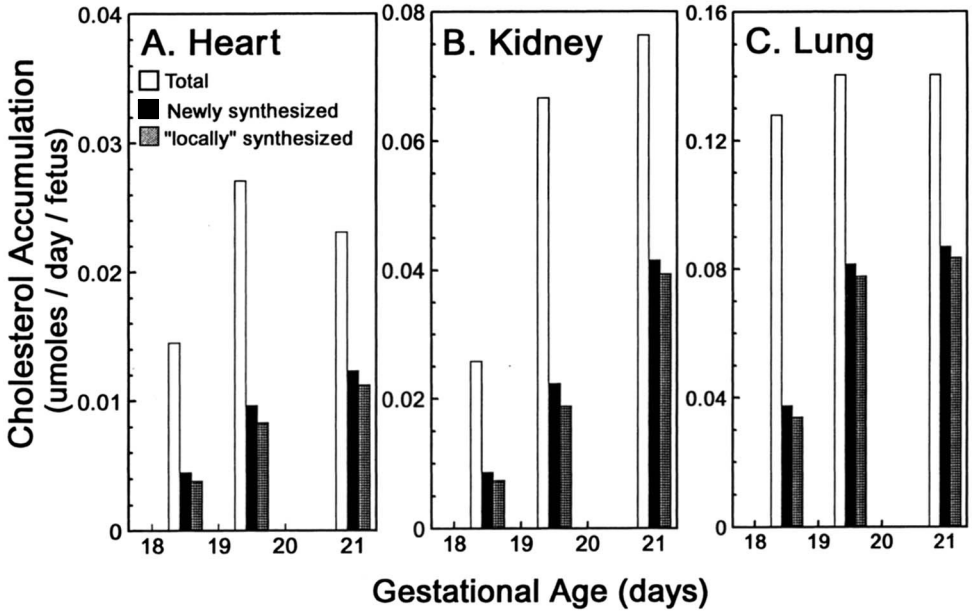

Fig. 6. Accumulation of bulk cholesterol and newly synthesized cholesterol in fetal heart, kidney, and lung. Accumulation was calculated from data in Fig. 2 while newly synthesized cholesterol was determined from incorporation of radioactivity from [^{3}H]water as detailed in Experimental Design Section. A third value, the fraction of newly synthesized cholesterol that was made within the tissue in question (locally synthesized), was calculated as described in the text.

exposure to antibodies to the yolk sac (32). Relevant also is the embryonic lethality of mice homozygous for deletion of the apolipoprotein B gene (33, 34); this apolipoprotein is normally secreted by both the yolk sac (26) and fetal liver (25).

A quantitative study, which concludes that maternal cholesterol may be at least indirectly involved in fetal cholesterol metabolism, is reported by Woollett (3). This author, also studying incorporation of label from [^{3}H]water into cholesterol, concluded that only 40% of cholesterol accumulating during fetal development of the hamster was synthesized by the fetus. She also studied incorporation by fetal tissues of ^{125}I-labeled cellobiose-labeled lipoproteins from the maternal circulation. Her data, similar to that obtained for the rat fetus by Belknap and Dietschy (6), indicated that although the fetus incorporated maternally derived ^{125}I-labeled cellobiose-labeled lipoproteins into placenta and yolk sac, no radioactivity reached the fetus. However, the conclusion reached by Woollett (3) differs from that of Belknap and Dietschy (6); she proposed that the 60% deficit of cholesterol in the fetus could be made up by transfer to the fetus of maternal cholesterol or cholesterol from yolk sac and placenta; the loss of cholesterol from placenta/yolk sac being compensated for by uptake from the maternal circulation. Woollett (3) also recalculated the data of Belknap and Dietschy (6). With certain assumptions (similar to our own) regarding synthesis of cholesterol from [^{3}H]water, she concluded in that study also that locally synthesized cholesterol could only account for about half the cholesterol accumulating in the fetus during development. Thus, it appears that Woollett and Belknap and Dietschy obtained similar results in hamster and rat, respectively, but interpreted the data differently.

The rate of fetal cholesterol synthesis we calculated is greater than those found by the above two groups of investigators; our data show synthesis within the fetus is sufficient to account for almost all the accumulated cholesterol. While we do not have a definitive explanation for the differences in results between laboratories, one explanation is that different times of exposure to [^{3}H]water were used prior to assay of label incorporated into cholesterol. Both Woollett, and Belknap and Dietschy, used a 1-h incubation period while we used a 4-h incubation. In previous studies (13) we had found that a lag period of 30 to 45 min occurred before the incorporation of radiolabel in cholesterol became linear with time; presumably because of a lag in equilibration of label in body water and pools of cholesterol precursors. It is possible that radiolabel in [^{3}H] sterol after an hour of incubation may underestimate cholesterol biosynthesis because all precursor pools may not be radiolabeled at the same rate; such an error would be much less with our 4-h labeling period.

Another possible reason for the discrepancies of our

data in rat, relative to that obtained for hamster (3), is species differences. It has been proposed (Woollett) that, in the hamster, there is considerable contribution to the fetus of cholesterol synthesized by the placenta/yolk sac. This explanation may be relevant to the hamster (3) where the proportional size of the placenta/yolk sac, as well as concentration of cholesterol seem to be greater than the corresponding values we obtain for rat. Thus, at term, the content of cholesterol in hamster placenta/yolk sac is almost half of that in the fetus (3) while our values for rat indicate that placenta/yolk sac has less than 10% of the cholesterol content of fetus.

We also note that although the experimental design we utilized is based on that of Belknap and Dietschy, the analytical techniques used differ greatly. Belknap and Dietschy (6) and Woollett (3) assayed sterol as digitonin-precipitable material. We utilized HPLC techniques that clearly separate from each other the different sterols and their metabolic precursors. This allows for identification and quantitation of each sterol, and for a more exact determination of how much of each sterol is newly synthesized, as incorporation of label from water could be correlated with knowledge about metabolic pathways for synthesis of individual sterols. In any case, our data permit only one simple interpretation, that the rat fetus synthesizes almost all of its own cholesterol.

We were also able to devise techniques to extend [^{3}H]water methodology to study of cholesterol balance among different fetal organs. As when working with the adult animal (13, 14) the assumption is that cholesterol accumulating in any particular organ is either locally synthesized or has come from the circulation. A noteworthy result we obtained is that the developing fetal brain appears almost completely dependent on local synthesis of cholesterol. Whatever barriers to the circulation are involved in preventing entry into brain and utilization of circulating cholesterol in the adult are already present during the period of rapid fetal brain growth (18–22 days of gestation). We have thus extended to the fetal period the observation that, postnatally, most of the cholesterol for brain development is locally synthesized (14, 35 and references contained therein). Our data are compatible with those of Turley et al. (36) who showed that fetal sheep brain does not clear low density lipoprotein from the fetal circulation, again suggesting circulating cholesterol does not enter brain. These results suggest that the failure of the nervous system to develop normally, due to the defect in cholesterol biosynthesis characteristic of Smith-Lemli-Opitz syndrome (7–10), is unlikely to be treatable by cholesterol supplementation of maternal diet or even by direct delivery of cholesterol to the fetal circulation.

It is also interesting that by 16 days gestation, the fetal liver already plays a major role in exporting cholesterol to other organs. This cholesterol is used to meet about half of the demand for cholesterol by heart, lung, and kidney. Although other tissues also take up circulating cholesterol, many of them must do so to a lesser extent. Calculations based on the data in Fig. 5 show that from 16 days upward the overall amount of excess cholesterol synthesized by liver, presumably dedicated to export, is less than 2 μmol. As there is a 15 μmol gain in total fetal cholesterol, we concluded that, even though heart, lung, and kidney obtain significant amounts of cholesterol initially produced by the fetal liver, many other tissues of fetus make most of their own cholesterol.

The above data are compatible with studies of mRNA expression for genes involved in cholesterol biosynthesis; liver, kidney, lung, and brain all express these messages at near-maximal levels during late gestation (37). After birth, as the rat suckles on the high cholesterol content milk, message levels for enzymes in the biosynthetic pathway are rapidly and greatly reduced in liver, not affected in brain, and decline to intermediate levels in other organs. Similar patterns were obtained for enzyme activity by direct biochemical assay of HMG-CoA reductase (38), a major control point in cholesterol biosynthesis.

The methodology we describe allows for quantitative assessment of cholesterol trafficking in the fetus. It gives useful information concerning fetal nutrition and is applicable to studies of other substrates of fetal growth (e.g., origin of fatty acids in fetal lipids). It could also provide a sensitive test as to whether metabolic perturbations, such as administration of a therapeutic drug (for example, for hypercholesterolemia), could affect fetal metabolism of cholesterol.

We thank Dr. Arrel D. Toews for helpful discussions and Ms. Janice Weaver for management of animal care and diets. We thank Mrs. Lou Asmuth for secretarial support. This work was supported by USPHS Grants NS 11615 and ES 01104, in a Center receiving core support from HD 03110.

Manuscript received 23 August 1996 and in revised form 19 December 1996.

REFERENCES

1. Connor, W. E., and D. S. Lin. 1967. Placental transfer of cholesterol-4-^{14}C into rabbit and guinea pig fetus. *J. Lipid Res.* **8:** 558–564.
2. Pitkin, R. M., W. E. Connor, and D. S. Lin. 1972. Cholesterol metabolism and placental transfer in the pregnant rhesus monkey. *J. Clin. Invest.* **51:** 2584–2592.
3. Woollett, L. A. 1996. Origin of cholesterol in the fetal Golden Syrian hamster: contribution of de novo sterol synthesis and maternal-derived lipoprotein cholesterol. *J. Lipid Res.* **37:** 1246–1257.
4. Spady, D. K., and J. M. Dietschy. 1983. Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. *J. Lipid Res.* **24:** 303–315.
5. Dietschy, J. M., and D. K. Spady. 1984. Measurement of

rates of cholesterol synthesis using tritiated water. *J. Lipid Res.* **25:** 1469–1476.
6. Belknap, W. M., and J. M. Dietschy. 1988. Sterol synthesis and low density lipoprotein clearance in vivo in the pregnant rat, placenta, and fetus. Sources for tissue cholesterol during fetal development. *J. Clin. Invest.* **82:** 2077–2085.
7. Irons, M., E. R. Elias, G. Salen, G. S. Tint, and A. K. Batta. 1993. Defective cholesterol biosynthesis in Smith-Lemli-Opitz syndrome. *Lancet.* **341:** 1414.
8. Tint, G. S., M. Irons, E. R. Elias, A. K. Batta, R. Frieden, T. S. Chen, and G. Salen. 1994. Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330:** 107–113.
9. Opitz, J. M., and F. de la Cruz. 1994. Cholesterol metabolism in the RSH/Smith-Lemli-Opitz syndrome: summary of an NICHD Conference. *Am. J. Med. Genet.* **50:** 326–338.
10. Salen, G., S. Shefer, A. K. Batta, G. S. Tint, G. Xu, A. Honda, M. Irons, and E. R. Elias. 1996. Abnormal cholesterol biosynthesis in the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **37:** 1169–1180.
11. Folch, J. M., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
12. Benjamins, J. A., A. L. Miller, and P. Morell. 1976. Metabolic relationships between myelin subfractions: entry of galactolipids and phospholipids. *J. Neurochem.* **27:** 565–570.
13. Jurevics, H. A., and P. Morell. 1994. Sources of cholesterol for kidney and nerve during development. *J. Lipid Res.* **35:** 112–120.
14. Jurevics, H. A., and P. Morell. 1995. Cholesterol for synthesis of myelin is made locally, not imported into brain. *J. Neurochem.* **64:** 895–901.
15. Lakshmanan, M. R., and R. L. Veech. 1977. Measurement of rate of rat liver sterol synthesis in vivo using tritiated water. *J. Biol. Chem.* **252:** 4667–4673.
16. Morton, A. J., and D. F. Goldspink. 1986. Changes in protein turnover in rat uterus during pregnancy. *Am. J. Physiol.* **250:** E114–E120.
17. Afting, E. G., M. L. Becker, and J. S. Elce. 1979. Proteinase and proteinase inhibitor activities of rat uterine myometrium during pregnancy and involution. *J. Biochem.* **177:** 99–106.
18. Scott, J. N., and P. B. Pendergrass. 1981. Scanning electron microscopy of the decidual stalk and decidua basalis in the mouse. *Anat. Embryol.* **162:** 435–441.
19. Neary, R. H., M. D. Kilby, P. Kumpatula, F. L. Game, D. Bhatnagar, P. N. Durrington, and P. M. S. O'Brien. 1995. Fetal and maternal lipoprotein metabolism in human pregnancy. *Clin. Sci.* **88:** 311–318.
20. Innis, S. M., and N. C. Haave. 1988. Effect of chronic modification of diet fat and cholesterol during gestation on plasma hormones and hepatic enzyme activities in rat fetus. *Biol. Neonate.* **53:** 355–361.
21. Goldwater, W. H., and D. Stetten, Jr. 1947. Studies in fetal metabolism. *J. Biol. Chem.* **169:** 723–738.
22. Naoum, H. G., R. C. de-Chazal, B. M. Eaton, and S. F. Contractor. 1987. Characterization and specificity of lipoprotein binding to term human placental membranes. *Biochim. Biophys. Acta.* **902:** 193–199.
23. Whittmaack, F. M., M. E. Gafvels, M. Bronner, H. Matsuo, K. R. McCrae, J. E. Tomaszewski, S. L. Robinson, D. K. Strickland, and J. F. Strauss III. 1995. Localization and regulation of the human very low density lipoprotein/apolipoprotein-E receptor: trophoblast expression predicts a role for the receptor in placental lipid transport. *Endocrinology.* **136:** 340–348.
24. Linn, D. S., R. M. Pitkin, and W. E. Connor. 1977. Placental transfer of cholesterol into the human fetus. *J. Obstet. Gynaecol.* **128:** 735–736.
25. Demmer, L. A., M. S. Levin, J. Elovson, M. A. Reuben, A. J. Lusis, and J. I. Gordon. 1986. Tissue-specific expression and developmental regulation of the rat apolipoprotein B gene. *Proc. Natl. Acad. Sci. USA.* **83:** 8102–8106.
26. Plonne, D., L. Winkler, H. Franke, and R. Dargel. 1992. The visceral yolk sac—an important site of synthesis and secretion of apolipoprotein B-containing lipoproteins in the feto-placental unit of the rat. *Biochim. Biophys. Acta.* **1127:** 174–185.
27. Farese, R. V., Jr., S. Cases, S. L. Ruland, H. J. Kayden, J. S. Wong, S. G. Young, and R. L. Hamilton. 1996. A novel function for apolipoprotein B: lipoprotein synthesis in the yolk sac is critical for maternal–fetal lipid transport in mice. *J. Lipid Res.* **37:** 347–360.
28. Albreght, E. D., and G. J. Pepe. 1990. Placental steroid hormone biosynthesis in primate pregnancy. *Endocr. Rev.* **11:** 124–150.
29. Knopp, R. H., M. R. Warth, D. Charles, M. Childs, J. R. Li, H. Mabuchi, and M. I. VanAllen. 1986. Lipoprotein metabolism in pregnancy, fat transport to the fetus, and the effects of diabetes. *Biol. Neonate.* **50:** 297–317.
30. Hay, W. W., Jr. 1994. Placental transport of nutrients to the fetus. *Horm. Res.* **42:** 215–222.
31. Hay, W. W., Jr. 1995. Current topic: metabolic interrelationships of placenta and fetus. *Placenta.* **16:** 19–30.
32. Brent, R. L., A. J. Johnson, and M. Jensen. 1971. The production of congenital malformations using tissue antisera. *Teratology.* **4:** 255–276.
33. Farese, R. V., Jr., S. L. Ruland, L. M. Flynn, R. P. Stokowski, and S. G. Young. 1995. Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc. Natl. Acad. Sci. USA.* **92:** 1774–1778.
34. Huang, L., E. Voyiaziakis, D. F. Markenson, K. A. Sokol, T. Hayek, and J. L. Breslow. 1995. ApoB gene knockout in mice results in embryonic lethality in homozygotes and neural tube defects, male infertility, and reduced HDL cholesterol ester and apoA-I transport rates in heterozygotes. *J. Clin. Invest.* **96:** 2152–2161.
35. Edmond, J., R. A. Korsak, J. W. Morrow, G. Torok-Both, and D. H. Catlin. 1991. Dietary cholesterol and the origin of cholesterol in the brain of developing rats. *J. Nutr.* **121:** 1323–1330.
36. Turley, S. D., D. K. Burns, C. R. Rosenfeld, and J. M. Dietschy. 1996. Brain does not utilize low density lipoprotein-cholesterol during fetal and neonatal development in the sheep. *J. Lipid Res.* **37:** 1953–1961.
37. Levin, M. S., A. J. A. Pitt, A. L. Schwartz, P. A. Edwards, and J. I. Gordon. 1989. Developmental changes in the expression of genes involved in cholesterol biosynthesis and lipid transport in human and rat fetal and neonatal livers. *Biochim. Biophys. Acta.* **1003:** 293–300.
38. Ness, G. C., J. P. Miller, M. H. Moffler, L. S. Woods, and H. B. Harris. 1979. Perinatal development of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in rat lung, liver and brain. *Lipids.* **14:** 447–450.