# Developmental coordinate expression of triacylglycerol and small molecular weight apoB synthesis and secretion by rat hepatocytes

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**Abstract** Glycerolipid and apoB synthesis and secretion were examined in hepatocytes obtained from fetal, suckling (day 6), and adult rats in order to examine the developmental regulation of lipoprotein production. The capacity to synthesize [$^3$H]triacylglycerol (from [$^3$H]glycerol) followed the order: adult > day 6 > fetal. Addition of 1 mM oleic acid to the incubation media stimulated the incorporation of [$^3$H]glycerol into triacylglycerol 6.7- and 3.6-fold by fetal and adult hepatocytes, respectively. After maximal stimulation by 1 mM oleic acid, triacylglycerol secretion by fetal cells was still only 39% of the amount secreted by adult cells that had been treated similarly. Fetal cells stimulated with 1 mM oleic acid synthesized the same amount of triacylglycerol as adult cells that had been treated with 0.1 mM oleic acid. However, the fetal cells secreted only one-third as much triacylglycerol, further demonstrating relatively impaired secretion of triacylglycerol. In order to determine whether low triacylglycerol secretion was associated with differences in apoB metabolism, cells were incubated with [$^{35}$S]methionine and apoB was quantified after immunoprecipitation. Fetal cells synthesized and secreted nearly equal amounts of large molecular weight and small molecular weight apoB. In contrast, adult cells synthesized and secreted nearly twice as much small molecular weight apoB as large molecular weight apoB. Moreover, although fetal and adult cells secreted large molecular weight apoB at similar rates, adult cells synthesized and secreted small molecular weight apoB at rates that were nearly two times higher than fetal cells. These data suggest that the ability to assemble and secrete VLDL varies in parallel with the developmental expression of small molecular weight apoB. These studies also show the usefulness of the cultured rat hepatocyte model for examining the ontogeny and regulation of lipoprotein assembly/secretion. —**Coleman, R. A., E. B. Haynes, T. M. Sand, and R. A. Davis.** Developmental coordinate expression of triacylglycerol and small molecular weight apoB synthesis and secretion by rat hepatocytes. *J. Lipid Res.* 1988. **29:** 33–42.

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VLDL serves as the vehicle with which endogenously synthesized triacylglycerol is transported from the liver to peripheral cells where it is rapidly utilized in the form of free fatty acids for energy and anabolic purposes. VLDL consists of a monolayer shell of phospholipids, free cholesterol, and specific apolipoproteins (principally apoB and apoE) that surround a hydrophobic core of triacylglycerol and small amounts of cholesteryl ester (1). ApoB is an unusually large and hydrophobic polypeptide having two major molecular weight forms (2). Genetic alterations leading to a specific defect in the ability to secrete both molecular weight forms of apoB leads to a complete inability to assemble/secrete VLDL triacylglycerol (3). In the rat, the liver secretes both the large molecular weight form ($apoB_L$) and the small molecular weight form ($apoB_S$) associated with VLDL (4–8).

Studies using cultured hepatocytes show that VLDL assembly/secretion is responsive to the metabolic state of the donor animal: fasting decreases (9), whereas carbohydrate feeding increases (10) the rate of VLDL assembly/secretion. Changes in metabolic state are associated with a coordinate repression (fasting) or induction (carbohydrate feeding) of regulatory lipogenic enzymes and apoB (but not apoE) (9, 10). Since substrate stimulation of lipogenesis (oleic acid to glycerolipid (5), glucose to glycerolipid and sterols (9, 10), and mevalonate to cholesterol and cholesteryl ester (11)) has no effect on apolipoprotein synthesis or secretion, the coordinate regulation of lipogenesis and VLDL assembly/secretion by metabolic

Abbreviations: VLDL, very low density lipoprotein; BSA, bovine serum albumin; ORN-media, ornithine-supplemented, arginine-deficient media; FBS, fetal bovine serum; $apoB_L$, large molecular weight apolipoprotein B; $apoB_S$, small molecular weight apolipoprotein B; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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state cannot be explained solely by changes in the availability of VLDL lipids. The identity of the effectors for this apparent coordinate regulation remains elusive.

The ability of the hepatocyte to synthesize triacylglycerol increases perinatally (12), concomitant with a change at birth to a milk diet that provides 70% of the neonate's calories as triacylglycerol (13). The increased ability of the perinatal liver to synthesize glycerolipids is reflected by 4- to 74-fold increases in activities of microsomal enzymes involved in phosphatidylcholine and triacylglycerol synthesis (14, 15). Furthermore, the activity of a hepatic-specific 2-monoacylglycerol acyltransferase is 700-fold greater than that found in adult rat liver (16, 17). Whether a coordinate induction in VLDL assembly/secretion occurs with the developmental induction of lipogenic enzymes has not been reported. However, the increased capacity for triacylglycerol synthesis that occurs during the suckling period is accompanied by a 200-fold increase in the hepatic content of triacylglycerol (12). The cause of the hepatic steatosis has not been elucidated with certainty, but it has been suggested that livers from suckling rats secrete little, if any, triacylglycerol (18). The finding that hepatic-derived apoB is absent from the plasma of 16-day-old-suckling rats (19) is consistent with lack of VLDL secretion, but might also result from rapid peripheral metabolism of VLDL.

To gain a better understanding of the developmental regulation of VLDL assembly/secretion, we studied the ability of hepatocytes obtained from fetal, suckling, and adult rats to synthesize and secrete triacylglycerol and apoB. We also studied the role of apoB synthesis and secretion in determining the capacity of the developing hepatocyte to assemble/secrete VLDL triacylglycerol.

## EXPERIMENTAL PROCEDURES

### Materials

Bovine serum albumin, essentially fatty acid-free (BSA), hyaluronidase type I-S, and DNAase I were purchased from Sigma Chemical Company. Ornithine-supplemented media (ORN-media) was prepared from a Gibco MEM Selectamine Kit, by replacing arginine with 0.1 mM ornithine. Fetal bovine serum (FBS) and penicillin–streptomycin solution were purchased from Gibco. Vitrogen 100 collagen solution was obtained from Flow Laboratories. [2-$^{3}$H]Glycerol, [1-$^{14}$C]acetate, and L-[$^{35}$S]methionine were obtained from New England Nuclear. Thin-layer chromatography plates (250 $\mu$ silica gel G) were from Analtech. Bond Elut aminopropyl columns with stainless-steel frits were purchased from Analytichem. Collagenase was purchased from Cooper Biomedical.

### Cell isolation and culture

Timed-pregnant Sprague-Dawley rats (Zivic-Miller) were housed in individual cages in a 12-hr light cycle (0600–1800 hr) and fed rat chow and water ad libitum. Livers were obtained between 0900 and 1000 hr from 21-day fetuses after decapitation of the dam and from suckling rats after decapitation. Approximately $4 \times 10^{6}$ cells/liver were routinely obtained from fetal and suckling rats. Hepatocytes from fetal and suckling rats were isolated by a modification of the method of Acosta, Anuforo, and Smith (20). Briefly, livers were placed in flasks with 1–2 ml of disassociation medium (0.5 g albumin, 0.05 g collagenase, 0.1 g hyaluronidase in 100 ml $Ca^{2+}$-free Hank's balanced salts) per liver and shaken for 10 min at 125 rpm, 23°C. The tissue pieces were filtered through 150 micron Nitex mesh and the filtrate was discarded. The tissue was then placed in fresh disassociation medium and shaken for 12 min. Tissue chunks were gently disrupted by pipeting up and down several times in a wide-bore pipet and filtered again through Nitex. The remaining tissue was returned to fresh disassociation medium, incubated until only connective tissue remained, and refiltered. Usually, three incubations were required for fetal livers and four for neonatal livers. The hepatocyte filtrates were combined and pelleted by a brief centrifugation with culture medium and 10% FBS. When the cells clumped, a drop of 10 mg/ml DNAase was added to the cell suspension. Adult hepatocytes were prepared by collagenase perfusion (21–23). Hepatocytes from 7-day-old rats were also isolated by retrograde perfusion via the inferior vena cava using the same procedure as for adult hepatocytes. Ten ml each of the same perfusion solutions (EDTA-media followed by collagenase-media (22, 23)) used to isolate adult hepatocytes was pushed by hand at 2 ml/min. More than 92% of the cells isolated by each of these methods excluded trypan blue.

Cells were plated at a density of $4 \times 10^{6}$ cells on 60 mm collagen-coated culture dishes in 2 ml of an ornithine-supplemented, arginine-deficient minimal essential media (ORN-media) (24) plus 10% FBS and incubated at 37°C with 5% $CO_2$, 95% air. After 1 to 2 hr, unattached cells were removed and fresh medium was added. Glycerolipid labeling experiments were begun after an overnight incubation. The medium was replaced with ORN-media + 10 mg/ml BSA. Glycerol was added at a concentration of 1.3 or 6.5 mM, with [$^{3}$H]glycerol, 1 to 4 $\mu$Ci/ml, or [$^{14}$C]acetate at 0.1 mM, 0.5 $\mu$Ci/ml. As previously shown by incubating adult hepatocytes with $^{35}$S-labeled, secreted VLDL (9), fetal hepatocytes did not take up label when they were incubated with media containing secreted triacyl[$^{3}$H]glycerol. Thus little, if any, secreted triacylglycerol appears to be further metabolized. Other conditions are given in the appropriate figure legends.

### Protein isolation

Cells were plated in ORN-media + 10% FBS. After 4 hr the medium was changed to serum-free ORN-media. After an overnight incubation, the media was replaced with 2 ml of 50 $\mu$Ci/ml [$^{35}$S]methionine (1097 Ci/mmol) in methionine-free ORN-media + 10 mg/ml BSA. After a 4 hr incubation, the medium was removed, centrifuged at 1000 rpm to pellet cellular debris, and transferred to a clean tube. To the cells and to the medium was added 0.5 ml of boiling buffer (50 mM Tris-HCl, pH 7.4, 2% sodium dodecyl sulfate, 50 $\mu$M leupeptin, 1 mM *p*-chloromercuriphenyl sulfonic acid, 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) followed by enough second buffer (10 mM sodium phosphate, 0.5% Zwittergent 3-14, 50 mM NaCl, 10 mM dithiothreitol, 20 $\mu$M leupeptin, 5 mg/ml BSA, pH 7.4) to give a final volume of 5 ml. The samples were boiled for 5 min, cooled and stored at −70°C until processed. An aliquot was precipitated with trichloroacetic acid to determine total incorporation of [$^{35}$S]methionine into protein (5). A second aliquot was used to immunoprecipitate apoB, separate large and small molecular weight apoB by SDS-PAGE, and quantify radioactivity as described (9).

### Lipid extractions

At various timepoints after incubation with radiolabeled acetate or glycerol, the incubation medium was removed. The cells were rinsed with 1 ml of Medium I (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA); the rinse was added to the removed incubation medium, and centrifuged at 1000 rpm for 5 min to pellet cellular debris. A 1.5-ml aliquot was extracted according to the method of Bligh and Dyer (25). The cells were scraped from the culture dishes in Medium I using a rubber policeman, rinsed once with the same medium, and centrifuged as above. The cell pellet was mixed with 0.5 ml of deionized water and extracted, and residues of both cells and media were mixed with 0.5 ml of deionized water and reextracted (25). The chloroform extract was dried under vacuum and the lipid classes were separated by thin-layer chromatography on silica gel G plates developed in heptane–isopropyl ether–glacial acetic acid 60:40:4 (v/v). Cholesterol and diacylglycerols were separated by applying an aliquot of the chloroform extract to Bond Elut aminopropyl columns and eluting with the solvent sequence of Kaluzny et al. (26).

### Other methods

Protein was measured by the method of Lowry et al. using BSA as the standard (27) after removing collagen (28). Fatty acid CoA ligase (15), diacylglycerol acyltransferase (15), monoacylglycerol acyltransferase (16), and total and microsomal (*N*-ethylmaleimide-sensitive) glycerol-P acyltransferase (15) activities were determined by radiochemical methods.

## RESULTS

Technical difficulties preclude obtaining large quantities of hepatocytes from young rats via in situ hepatic perfusion. In order to determine whether the shake method of Acosta et al. (20) produces hepatocytes whose metabolism of glycerolipids is similar to that of cells isolated by collagenase perfusion, hepatocytes from 7-day-old rats were isolated by both methods. Selected microsomal glycerolipid synthetic enzyme activities were determined in total particulate preparations of hepatocytes before plating. For the perfusion and shake methods, respectively, fatty acid CoA ligase activities were 31.4 and 22.2 nmol/min per mg, total glycerol-P acyltransferase activities were 2.04 and 1.69 nmol/min per mg, microsomal glycerol-P acyltransferase (*N*-ethylmaleimide-sensitive) activities were 1.68 and 1.32 nmol/min per mg, monoacylglycerol acyltransferase activities were 88.4 and 58.9 nmol/min per mg, and diacylglycerol acyltransferase activities were 5.8 and 5.5 nmol/min per mg. After maximal stimulation by oleate, hepatocytes isolated by perfusion synthesized half as much triacylglycerol as those isolated by shaking; secretion of triacylglycerol was virtually identical for both methods (**Table 1**). These data suggest that although activities of selected enzymes of glycerolipid synthesis are 10 to 30% lower in the nonperfused hepatocytes, the cells' basal and oleate-stimulated ability to synthesize triacylglycerol is not impaired and their rate of triacylglycerol secretion is similar to perfusion-isolated hepatocytes. Since the shake method was technically easier and yielded reasonable numbers of metabolically active hepatocytes, it was used for subsequent studies.

In order to determine whether hepatocytes obtained from fetal and suckling rats retain the lipogenic capacity of livers from the same age rats, synthesis of fatty acid-containing glycerolipids and cholesterol was determined using [$^{14}$C]acetate (**Fig. 1** and **Fig. 2**). The rates of endogenous synthesis of glycerolipids by the different groups of hepatocytes followed the order: fetal > adult >> day 6 (suckling). These relative rates of synthesis were found for all lipids examined: triacylglycerol, phospholipid, cholesterol, and cholesteryl esters, and are consistent with results obtained using liver slices (29, 30). These results reflect marked postnatal decreases in fatty acid synthase, ATP citrate lyase, and malic enzyme (29, 31) and of 3-hydroxy-3-methylglutaryl CoA reductase (32). The concordance in results obtained with the cultured hepatocytes and those obtained using fresh tissue suggest that the hepatocytes retain the lipogenic properties of the livers from which they were obtained.

In contrast to the relative rates of lipogenesis displayed by the different groups of hepatocytes, the rates of secretion of de novo synthesized [$^{14}$C]triacylglycerol followed the order: adult > fetal > day 6 (suckling). The strik-

TABLE 1. Triacylglycerol synthesis and secretion by hepatocytes obtained by different methods from 7-day-old rats

| Method[a] | Oleate (1 mM) | Cell Triacylglycerol | Media Triacylglycerol |
|---|---|---|---|
| | | *nmol [$^3$H]glycerol incorporated/$10^6$ cells per 4 hr* | |
| Perfusion | – | 3.2 ± 0.6 | 0.2 ± 0.02 |
| Perfusion | + | 25.5 ± 2.0 | 2.0 ± 0.1 |
| Shake | – | 8.6 ± 0.9 | 0.2 ± 0.01 |
| Shake | + | 50.5 ± 1.5 | 2.1 ± 0.5 |

[a] Hepatocytes from six 7-day-old rats were isolated by retrograde perfusion of the inferior vena cava as described under Experimental Procedures. After perfusion, the liver was kept in the collagenase solution on ice until all six livers had been prepared. The livers were then teased apart, filtered through 150 μm Nitex, rinsed in culture media, and plated. The shake method hepatocytes were isolated from seven littermates as described in Experimental Procedures. For both methods, >92% of the cells excluded trypan blue. Total time for the shake method, from killing the rats to cell plating, was 2 hr; total time for the perfusion method was 5 hr. Cell recovery was $7.4 \times 10^6$ cells and $3.3 \times 10^7$ cells per rat for the shake and perfusion methods, respectively. After 18 hr, cells were incubated with [$^3$H]glycerol as described and lipids were extracted and quantified. Each value is the mean ± SEM for three plates.

ingly reduced ability of cells from fetal rats to secrete [$^{14}$C]triacylglycerol is expressed as a dramatically decreased percentage of the amount of de novo synthesized triacylglycerol that was secreted: 3% by fetal cells, compared to 20% by adult cells. However, since cells from fetal and suckling rats secreted the same (or greater) amounts of de novo synthesized [$^{14}$C]cholesterol, [$^{14}$C]cholesteryl ester (Fig. 2), and [$^{14}$C]phospholipid (Fig. 1), the attenuated secretory capacity of cells from fetal and suckling rats was apparently confined to VLDL triacylglycerol and was not the result of a general inability to secrete lipid (i.e., HDL and biliary lipids).

Whereas [$^{14}$C]acetate incorporation affords a measure of triacylglycerol synthesis from de novo synthesized fatty acids, [$^3$H]glycerol incorporation more accurately reflects total glycerolipid synthesis (from both de novo synthesized and from exogenous sources of fatty acids). Cultured cells were incubated with [$^3$H]glycerol for 2 to 8 hr in order to examine the ontogeny of hepatocyte synthesis and secretion of triacylglycerol (**Fig. 3**). The synthesis and secretion of triacylglycerol was linear with respect to time. Cells in culture for 24 or for 72 hr synthesized virtually identical amounts of [$^3$H]triacylglycerol; however, secretion of triacylglycerol by hepatocytes from fetal and 6-day-old rats almost doubled after cells were in culture for 72 hr, suggesting that the reduced secretion of triacylglycerol displayed by these cells was reversed, in part, by adaptation to the culture conditions. The ability of the different groups of cells to synthesize [$^3$H]triacylglycerol followed an order different from that obtained using [$^{14}$C]acetate: adult > day 6 (suckling) > fetal (**Fig. 4**), consistent with the increasing activities of microsomal enzymes of triacylglycerol synthesis expressed by livers during these different stages of development (14, 15). However, the ability of the different groups of cells to secrete [$^3$H]triacylglycerol followed the same order as that observed using [$^{14}$C]acetate: adult > fetal > day 6 (suckling) (Figs. 1 and 4). Thus, irrespective of the source of fatty acids used for triacylglycerol synthesis, hepatocytes from suckling and fetal rats displayed a markedly diminished capacity to secrete triacylglycerol.

The reduced ability of fetal hepatocytes to secrete triacylglycerol might be caused, in part, by decreased triacylglycerol synthesis and, thus, decreased availability of triacylglycerol. In order to determine whether cells from fetal rats have a reduced capacity to secrete triacylglycerol

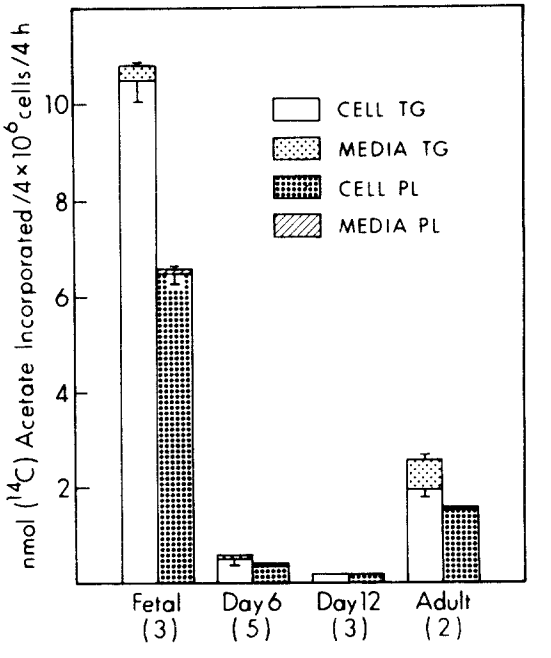


**Fig. 1.** Incorporation of [$^{14}$C]acetate into glycerolipids by hepatocytes from fetal, suckling, and adult rats. After incubation overnight in ORN-media, the medium was replaced with 0.1 mM [$^{14}$C]acetate in ORN-media. After 4 hr, chloroform extracts of media and cells were obtained as described in Experimental Procedures. Data are expressed as means ± SEM for the indicated number of experiments in parentheses; TG, triacylglycerol; PL, phospholipid.

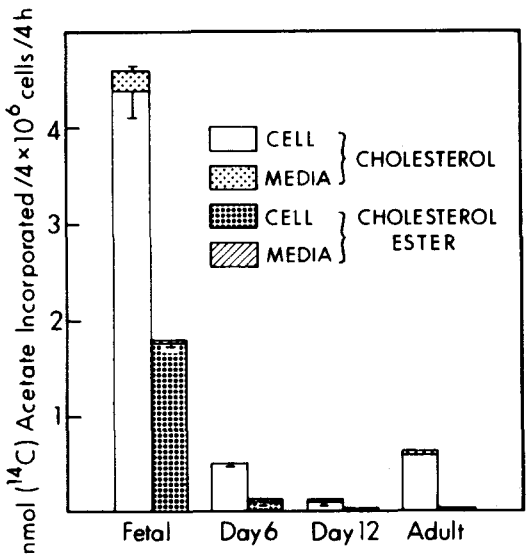


**Fig. 2.** Incorporation of [$^{14}$C]acetate into cholesterol and cholesteryl esters by hepatocytes from fetal, suckling, and adult rats. Conditions were the same as for Fig. 1. Data are expressed as the mean ± SD for three determinations.

irrespective of triacylglycerol availability, fetal and adult cells were incubated with increasing amounts of oleic acid (**Fig. 5**). Fetal cells were more responsive than adult cells to 1 mM oleic acid. Triacylglycerol synthesis increased 6.7-fold by cells from fetal rats, compared to a 3.6-fold increase by cells from adult rats. Furthermore, oleate increased triacylglycerol secretion by cells from fetal rats by 7.6-fold compared with only a 2-fold increase by cells from adult rats. However, even at the highest concentration of oleic acid (1 mM was found to maximally stimulate triacylglycerol synthesis and secretion by hepatocytes from adult rats and fetal rats [data not shown]), fetal cells secreted only 39% as much triacylglycerol as adult cells. In the presence of 1 mM oleic acid, fetal cells incorporated as much [$^3$H]glycerol into cell triacylglycerol as did adult cells exposed to 0.1 mM oleic acid. Despite the equal amounts of cellular [$^3$H]triacylglycerol available for secretion, fetal cells still secreted only 39% as much [$^3$H]triacylglycerol as adult cells. These data suggest that, regardless of differences in triacylglycerol synthesis, fetal cells have a reduced ability to secrete triacylglycerol compared to adult cells.

The capacity of adult hepatocytes to secrete triacylglycerol depends on the synthesis of apoB (2, 3, 9, 10). In order to determine whether the inability of fetal cells to secrete triacylglycerol was accompanied by decreased synthesis of apoB, hepatocytes from the different groups were incubated with and without 1 mM oleic acid. Protein synthesis was determined by labeling with [$^{35}$S]methionine.

The relative amount of TCA-precipitable protein synthesized was fetal > adult > day 6 (suckling) (**Table 2**). Despite the fact that adult cells synthesized twice as much protein as day-6 cells, the proportion of protein that was secreted was identical. Cells from fetal rats, however, secreted proportionately less de novo synthesized protein than did day-6 or adult cells. Consistent with previous results obtained using adult rat hepatocytes, oleic acid did not significantly ($P > 0.05$) increase the synthesis or secretion of [$^{35}$S]methionine-labeled apoB (**Table 3, Fig. 6**). Because the day-6 and adult hepatocytes contained 0.8- and 3.2-fold more protein, respectively, than did the fetal hepatocytes (Table 3), the amount of apoB$_S$ secreted by adult hepatocytes increases substantially when calculated per cell.

To minimize potential differences in labeling the methionine pools of the different groups of cells, rates of protein synthesis were normalized to the rate of total [$^{35}$S]methionine incorporation into TCA-precipitable protein (Fig. 6). Although the amount of [$^{35}$S]methionine-labeled apoB$_L$ in the cells and secreted into the medium was similar for all groups, compared to adult hepatocytes,

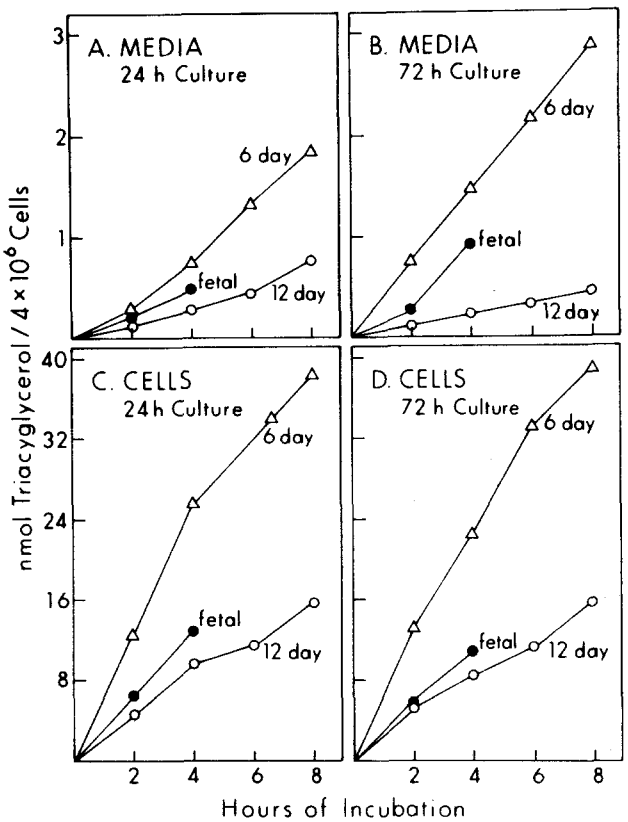


**Fig. 3.** Time course of [$^3$H]glycerol incorporation into cellular and secreted triacylglycerol by hepatocytes from fetal and suckling rats after 24 and 72 hr in culture. Hepatocytes were isolated and plated in ORN-media with 10% FBS. After 24 hr (A, C) or 72 hr (B, D) the medium was changed to ORN-media + 1.3 mM [$^3$H]glycerol. At the indicated intervals, the cells and media were removed and the lipids were extracted and identified. Values are reported as the average of duplicates that varied by less than 10%.

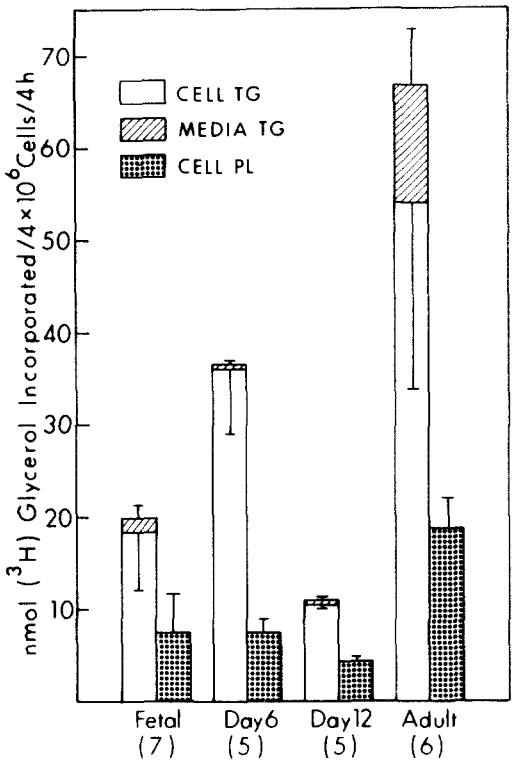


**Fig. 4.** Incorporation of [$^3$H]glycerol into glycerolipids by hepatocytes from fetal, suckling, and adult rats. After incubation overnight in ORN-media, the medium was replaced with 6.5 mM [$^3$H]glycerol in ORN-media. After 4 hr, lipids were extracted from cells and media and identified as described in Experimental Procedures. Media phospholipid was not quantified. Data are presented as means ± SEM for the indicated number of experiments in parentheses; TG, triacylglycerol; PL, phospholipid.

cells from fetal and day-6 (suckling) rats displayed a 30% lower cellular content of [$^{35}$S]methionine-labeled apoB$_S$. Moreover, cells from fetal and suckling rats secreted apoB$_S$ at rates that were also only 30% of adult rates. These data suggest that the decreased synthesis of apoB$_S$ contributes, at least in part, to the decreased ability of cells from fetal and day-6 (suckling) rats to secrete triacylglycerol. Since the proportion of de novo synthesized [$^{35}$S]methionine-labeled protein that was secreted was similar for both the suckling and adult cells (Table 2), the inability of cells from suckling rats to secrete apoB cannot be explained by a generalized defect in protein secretion.

## DISCUSSION

Monolayer cultures of hepatocytes have served as a model system with which to study lipoprotein synthesis and secretion (4, 5, 9–11). Hepatocytes retain for up to 48 hr the lipogenic properties and alterations in lipoprotein secretion rates of the in vivo liver, as demonstrated by studies of cholesterol and fat feeding (11), sucrose feeding (10), and fasting (9). The VLDL secreted by hepatocyte cultures is structurally similar to VLDL secreted by perfused livers and to VLDL found in plasma (21). Furthermore, cultured hepatocytes rapidly augment VLDL triacylglycerol secretion in response to fatty acids (5, 33–35) showing that this model maintains an adaptive process similar to that found in perfused livers (36–38). The adaptive response to fatty acids is likely to reflect the stimulation of diacylglycerol acyltransferase, the microsomal activity unique to triacylglycerol biosynthesis, observed in vivo after fatty acid supplementation (39).

The results obtained in this study support the use of the hepatocyte culture model to examine the ontogeny of VLDL assembly/secretion. Cells from fetal and day-6 (suckling) rats accurately reflect changes in glycerolipid and cholesterol synthesis (Figs. 1–5) that have been documented using fresh liver tissue (29, 30).

The marked changes in energy metabolism that occur during the transition from fetal to postnatal life and through the weaning period are accompanied by changes in the activities of lipogenic enzymes. Glycerolipid biosynthesis from glycerol-3-phosphate in liver homogenates increases 185% by 1 day after birth (12). During the perinatal period, microsomal enzyme activities of triacylglycerol and phospholipid biosynthesis increase 4- to 74-fold, most reaching adult levels by the end of the first postnatal week (15). Additionally, during this time the monoacylglycerol pathway of diacylglycerol synthesis reaches a potential level of activity 700-fold higher than is observed in the adult rat (16). As a result of these

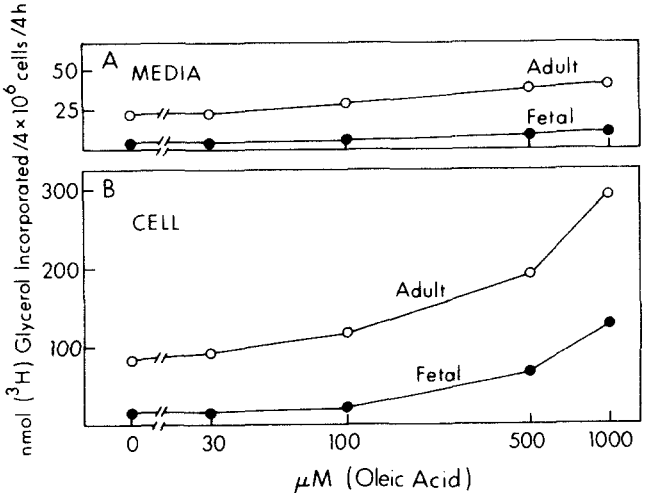


**Fig. 5.** Effect of oleic acid on [$^3$H]triacylglycerol synthesis and secretion by hepatocytes from fetal and adult rats. Hepatocytes were isolated and plated in ORN-media with 10% FBS. After 24 hr the medium was changed to ORN-media plus 6.5 mM [$^3$H]glycerol. Zero to 1 mM oleic acid in 10 mg/ml BSA was added. After 4 hr incubation, the cells and media were removed. Lipids were extracted and identified as described in Experimental Procedures. Values are reported as the mean of two determinations.

TABLE 2. Incorporation of [$^{35}$S]methionine into cellular and secreted protein

| Cells | Oleate (1 mM) | Total | Cell | Media | Percent Secreted |
|---|---|---|---|---|---|
| | | *cpm × $10^{-3}$/mg cell protein* | | | % |
| Fetal | – | 632 ± 78 | 580 ± 20 | 87 ± 3 | 13.8 |
| Fetal | + | 655 ± 41 | 552 ± 23 | 103 ± 9 | 15.7 |
| 6-Day | – | 179 ± 18 | 143 ± 12 | 36 ± 2 | 20.1 |
| 6-Day | + | 140 ± 15 | 107 ± 18 | 33 ± 3 | 23.6 |
| Adult | – | 355 ± 51 | 273 ± 14 | 82 ± 2 | 23.1 |
| Adult | + | 354 ± 16 | 275 ± 13 | 79 ± 2 | 22.3 |

Dishes of cells were incubated with [$^{35}$S]methionine as described under Experimental Procedures and TCA-precipitable protein was quantified. Results are expressed as the mean ± SD of samples done in triplicate.

changes in lipogenic enzyme activities, the liver of the suckling rat is capable of synthesizing increased amounts of glycerolipids from both exogenously derived long-chain fatty acids and from 2-monoacylglycerols. Whereas carbohydrate makes up 70–80% of dietary calories during the fetal and adult periods, during the suckling period the majority of dietary calories are provided as triacylglycerol (13). Carbohydrate initially enters the VLDL assembly/secretion pathway via the production of de novo fatty acids (endogenous pathway), whereas dietary fat enters the hepatic VLDL pathway as free fatty acids that are esterified to form glycerolipids (exogenous pathway). In hepatocytes obtained from adult rats, carbohydrate coordinately induces both lipogenesis and the VLDL assembly/secretion pathway (10). The induction by dietary carbohydrate of the hepatocyte's ability to assemble and secrete VLDL triacylglycerol is probably responsible for the prevention of steatosis despite the marked elevation in lipogenesis (10). On the other hand, dietary fat stimulates hepatic lipogenesis by providing fatty acid substrate for glycerolipid synthesis. But, in contrast to carbohydrate, free fatty acids (5) do not induce the hepatocyte's maximum capacity to assemble/secrete VLDL triacylglycerol. Thus, although both carbohydrate and fat feeding augment VLDL triacylglycerol secretion, only dietary carbohydrate induces the maximal capacity to secrete VLDL. As a result, dietary fat can cause an accumulation of fat in the liver (11).

During the change in diet from carbohydrate prenatally to fat postnatally, the triacylglycerol content of the suckling rat liver increases 200-fold (12). Studies by Frost, Clark, and Wells (18) suggest that the liver secretes little triacyglycerol during this period; the inability of the hepatocyte to secrete triacylglycerol may account for its massive intracellular accumulation. Our results support this proposal and further suggest that the defect in VLDL assembly/secretion displayed by the rat during the early phases of development may be intimately linked to impaired synthesis and secretion of apoB$_S$. The reduction of absolute apoB synthesis by cells from 6-day-old rats mirrors the marked decrease in apoB mRNA in livers from 8- and 14-day old rats compared to hepatic apoB mRNA in fetal and adult rats (40). It is clear from our studies that, although cells from both fetal and suckling rats do not synthesize as much triacylglycerol as do adult cells (Figs. 1–4), decreased synthesis cannot account for the decreased capacity to secrete triacylglycerol in response to oleic acid stimulation (Fig. 5). The ability of oleic acid to stimulate glycerolipid synthesis without affecting apoB synthesis afforded us the opportunity to determine the

TABLE 3. Incorporation of [$^{35}$S]methionine into apoB

| Cells | Oleate (1 mM) | Cell $B_L$ | Cell $B_S$ | Media $B_L$ | Media $B_S$ |
|---|---|---|---|---|---|
| | | *cpm/mg cell protein* | | | |
| Fetal | – | 512 ± 49 | 553 ± 64 | 283 ± 22 | 106 ± 20 |
| Fetal | + | 623 ± 89 | 595 ± 80 | 324 ± 31 | 136 ± 20 |
| 6-Day | – | 131 ± 44 | 182 ± 65 | 62 ± 11 | 43 ± 3 |
| 6-Day | + | 86 ± 4 | 127 ± 16 | 47 ± 3 | 43 ± 7 |
| Adult | – | 240 ± 31 | 540 ± 39 | 156 ± 29 | 271 ± 41 |
| Adult | + | 178 ± 25 | 430 ± 72 | 142 ± 4 | 207 ± 40 |

Dishes of cells were incubated with [$^{35}$S]methionine as described under Experimental Procedures. Apo$B_L$ and apo$B_S$ were quantified as described in Experimental Procedures. Results are expressed as the mean ± SD of samples done in triplicate. Qualitatively similar results were obtained from three additional independent experiments, each performed in triplicate. Protein in fetal, day-6, and adult cells was 0.44, 0.79, and 1.39 mg/$10^6$ cells, respectively.

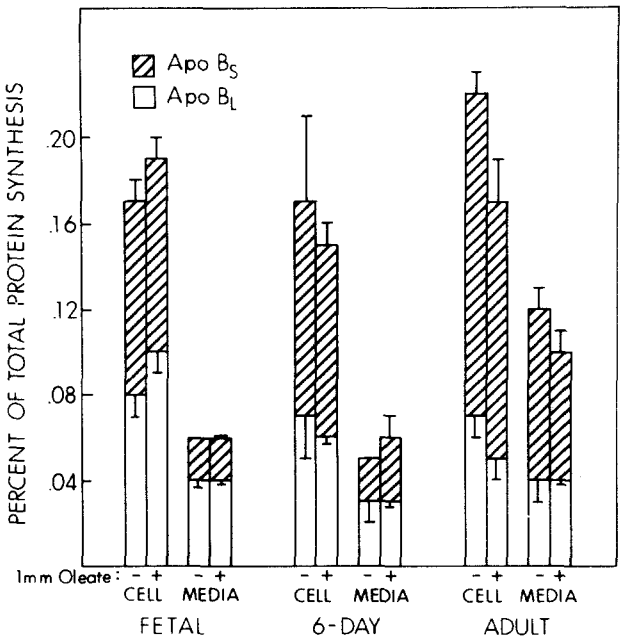


**Fig. 6.** ApoB synthesis and secretion by hepatocytes from fetal, 6-day-old, and adult rats. Hepatocytes were incubated with [$^{35}$S]methionine for 4 hr and total TCA-precipitable cpm (Table 2), apo$B_L$ cpm, and apo$B_S$ cpm were quantified as described in Experimental Procedures. ApoB results are expressed as mean percents of total protein synthesized. Error bars indicate SD > 0.0004%. These data are qualitatively similar to results obtained in three additional experiments performed similarly.

capacity of the different developmental stages of cells to secrete triacylglycerol. Thus, while cells from fetal rats were stimulated to synthesize triacylglycerol at rates similar to those displayed by adult cells, the rate of triacylglycerol secretion was still only 39% of the adult rate (Fig. 5: compare 1 mM oleic acid in fetal cells to 0.1 mM oleic acid in adult cells). Moreover, examination of the apoB synthesized by the different groups of hepatocytes, revealed a developmental change in the molecular weight form. Fetal hepatocytes synthesized more apo$B_L$ than apo$B_S$ (Fig. 6). Cells obtained from suckling rats synthesized similar amounts of apo$B_L$ and apo$B_S$, whereas adult cells synthesized twice as much apo$B_S$ as apo$B_L$. Similar developmental changes in the relative amounts of apo$B_L$ and apo$B_S$ in fetal and adult rat liver have been reported (39). The relative reduction in apo$B_S$ synthesis and the decreased capacity to secrete triacylglycerol displayed by cells from fetal and suckling rats is similar to changes expressed by hepatocytes obtained from fasted rats (9). The combined data of both studies support the hypothesis that the relative rate of apoB synthesis may determine, at least in part, the capacity to assemble/secrete VLDL triacylglycerol.

Despite extensive investigative efforts, the processes responsible for the synthesis of apo$B_S$ remain unknown. Recent studies indicate that a single gene codes for both apo$B_L$ and apo$B_S$ (41–44). In humans, the liver secretes only apo$B_L$, whereas the intestine secretes only apo$B_S$ (1), and the synthesis of apo$B_S$ by the human intestine is developmentally regulated (45). Furthermore, the size of apoB mRNA is similar in both liver and intestine, yet liver apo$B_L$ has a molecular weight that is nearly 250,000 daltons larger than intestinal apo$B_S$. The lack of an intron at the junction of overlap between apo$B_L$ and apo$B_S$ can be inferred from the sequence, and supports the view that both forms (46, 47) are derived from a common mRNA. It has not been established whether apo$B_S$ is derived from apo$B_L$ via proteolytic cleavage. This study shows that the formation of apo$B_S$ by rat liver is developmentally regulated and is expressed more actively as the rat ages and as the liver's capacity to assemble/secrete VLDL increases.

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