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Synthesis, Processing, and Secretion of Hepatic Very Low Density Lipoprotein

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Very low density lipoprotein (VLDL) is the major vehicle in the plasma which carries triacylglycerol synthesized in the liver to peripheral tissues for utilization. Estrogen-induced chick parenchymal liver cells (hepatocytes) synthesize and secrete large amounts of VLDL. These cells, in a primary monolayer culture system developed in this laboratory, have been employed to study the operative and regulatory aspects of VLDL synthesis, assembly, and secretion. Some 10 min are required for the translation of the principle VLDL protein constituent, apolipoprotein B, and 30–35 min are required for the two newly translated chick VLDL apolipoproteins, apolipoprotein B and apolipoprotein II, to be secreted. Apolipoprotein B is synthesized on membrane-bound polysomes as a contiguous polypeptide chain of 350K molecular weight (MW) and is not assembled posttranslationally from smaller-peptide precursors. Translocation of puromycin-discharged apolipoprotein B nascent chains into the endoplasmic reticulum lumen and their subsequent secretion are independent of both ongoing protein synthesis and the attachment of the nascent peptides to ribosomes. Apolipoprotein B nascent chains discharged by puromycin assemble with glycerolipid (mainly triacylglycerol) and are secreted as immunoprecipitable VLDL. Core oligosaccharides are added to the apolipoprotein B nascent chain co-translationally in at least two stages, at molecular weights of ~ 120K and ~280K. Inhibition of *N*-linked glycosylation of apolipoprotein B with tunicamycin affects neither the assembly of glycerolipids into VLDL nor the secretion of the VLDL particle, indicating that *aglyco*-apolipoprotein B can serve as a functional component for VLDL assembly and secretion. Active synthesis of the VLDL apolipoproteins is required, however, for glycerolipid assembly into VLDL and secretion from the hepatocyte. The differential kinetics with which newly synthesized apolipoproteins and glycerolipids are secreted as VLDL and the timing of the effects of protein-synthesis inhibitors on their secretion indicate that VLDL constituents are assembled sequentially in the intact liver cell. The bulk of the VLDL triacylglycerol and some VLDL phosphoglyceride is introduced early in the secretory pathway proximal, yet subsequent to apopeptide synthesis, while a significant fraction of VLDL phosphoglyceride associates with the resulting triacylglycerol-rich lipid-protein

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complexes just prior to their secretion as mature VLDL. Within the context of current models for VLDL structure, the late assembly of phosphoglyceride into VLDL is taken to represent a surface maturation of the nascent VLDL particle.

Key words: liver cell (hepatocyte), very low density lipoprotein (VLDL), apolipoproteins, glycerolipids, metabolism, assembly

The major product of lipogenesis by the liver, triacylglycerol, is packaged along with additional lipid and specific proteins ("apolipoproteins") into very low density lipoprotein (VLDL) particles for delivery to extrahepatic tissues via the circulation [1]. The production of VLDL and the maintenance of triacylglycerol supplies to peripheral tissues depend upon at least three functions of the parenchymal liver cell (hepatocyte): *biosynthesis* of protein and lipid, *assembly* of the requisite apolipoprotein and lipid constituents to form a VLDL particle, and *secretion* of the mature VLDL particle into the blood.

The dual character of the VLDL particle both as a supramolecular assembly of protein and lipid and a principle lipid-containing product exported from the liver is reflected in the structural and functional organization of the liver cell. Prior to its discharge into the circulation, the VLDL particle undergoes directed transit within the hepatocyte through a series of membrane-bound intracellular compartments that constitute the "secretory pathway" [1]. Two membranous organelles along the secretory pathway display biosynthetic capabilities particularly important to VLDL biogenesis: The endoplasmic reticulum is the site of VLDL apolipoprotein synthesis [2] and core glycosylation [3] and is also the principle location of hepatic glycerolipid production [4], while the Golgi apparatus is the site of terminal polypeptide glycosylation [5] and some phosphoglyceride synthesis [6].

How the synthesis and assembly of the various VLDL constituents are physiologically, topologically, and temporally regulated and integrated in the liver cell to form the mature, secreted VLDL particle is not understood. Morphological [7,8], radioautographic [9], and immunocytochemical [1] investigations, especially on livers stimulated to produce and secrete VLDL by, for example, chronic ethanol administration [10], indicate that VLDL apolipoproteins are extruded into the endoplasmic reticulum lumen and associate there with lipid to form droplets whose electron density after osmium fixation is reminiscent of serum VLDL. These putative VLDL particles are then transferred to the Golgi complex cisternae, from which they are transported in Golgi-derived, membrane-delimited secretory vesicles to the plasma membrane for release into the blood. Subcellular fractionation studies [11,12] have demonstrated a marked size and compositional heterogeneity of the Golgi lipoprotein population, which has been assumed to indicate the presence of immature precursors of VLDL (and to a lesser extent high-density lipoprotein [12,13]) in the Golgi content that have yet to acquire all their constituents and, hence, to attain the size and buoyant density of serum VLDL. Therefore, it appears possible that extensive introduction of constituents into lipoprotein particles could take place during their transit through the secretory pathway to yield the mature VLDL particles released into the circulation.

The purpose of our work has been to investigate the processing and assembly of the major individual VLDL molecular constituents (apolipoproteins, triacylglycerol, and phosphoglycerides) to form the mature, secreted lipoprotein particle and the role of the apolipoproteins in the control of this assembly. It will be demonstrated that the mature VLDL particle is formed in the intact liver cell by the sequential,

multistep processing and assembly of individual apolipoprotein and glycerolipid constituents in a developmental sequence that does not depend upon ongoing apopeptide production.

LIVER CELL CULTURE SYSTEM FOR THE STUDY OF VLDL BIOGENESIS

In the work to be described, a primary chick liver cell culture system was employed to study in a controlled physiological setting the synthesis and assembly of VLDL and the regulation of these processes. Chick liver cells are ideally suited for studies on VLDL metabolism. The liver accounts for nearly all de novo fatty acid synthesis in the chick [14], as it does in man [15], and provides the major fraction of the fatty acids utilized by other tissues, including adipose tissue, in the form of VLDL triacylglycerol. Moreover, in avian species the liver has the unique role of synthesizing and secreting lipid and protein precursors as VLDL for egg yolk formation in the ovary [16]. Since these processes are under the control of estrogen in avian species [17], it is possible to increase specifically hepatic VLDL output 10–50-fold by administering this hormone to either male or sexually immature chicks [18]. Within a few days after estrogen injection there is an extraordinary rise in the blood VLDL level to 10–15 g/100 ml of serum in the hen [17].

As purified by flotation at serum density in the ultracentrifuge [19] followed by gel filtration on either Sepharose-6B or Biogel A-50m (2% agarose) columns, chick plasma VLDL contains a small amount of protein relative to lipid (Table I). By electrophoresis on 3.3% sodium dodecyl sulfate (SDS)-*N*,*N*′-diallyistartardiamide (DATD)-polyacrylamide gels [19,20], two apolipoproteins can be resolved out of SDS-dissociated chick VLDL: a major, "heavy" 350K glycoprotein, apolipoprotein B; and a "light" 6K polypeptide, apolipoprotein II, which is not glycosylated [19]. In the circulating VLDL particle, apolipoprotein II polypeptides are dimerized through sulfhydryl linkages to form a 12K polypeptide [19]. Glycerolipids account for over

TABLE I. Comparison of Chick and Human Hepatic VLDL*

	Weight-% of total particle mass	
VLDL constituent	Chick[a]	Human[b]
Protein	17	11
Apolipoprotein B (I)	7.1[c]	
Apolipoprotein II	9.9[c]	
Lipid	83	89
Triacylglycerol	54	61
Cholesterol ester	5	7
Cholesterol	4	5
Phosphoglyceride	20	16
PC + PE (PC/PE)	19[d](2.6)	15 (20)
Size (diameter)	300–500 Å	350–750 Å

*PC, phosphatidylcholine; PE, phosphatidylethanolamine.
[a]Plasma VLDL purified from estradiol-treated animals.
[b]Data from Patsch et al [22].
[c]Based on [^{3}H]leucine incorporation into immunoprecipitable VLDL apolipoprotein.
[d]Based on [^{3}H]glycerol and $^{32}P_i$ incorporation into immunoprecipitable VLDL phosphoglycerides.

80% of the mass of the chick VLDL particle, with triacylglycerol predominant along with the phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine [20,21]. The chemical composition of chick VLDL resembles that of human serum VLDL (Table I, and [22]), and chick apolipoprotein B is antigenically related to human apolipoprotein B [23]. Within the context of current models for VLDL structure [24], the particle's phosphoglyceride (along with small amounts of nonesterified cholesterol and triacylglycerol) is located in a surface monolayer that surrounds a neutral lipid (mainly triacylglycerol with some cholesterol ester) core. The apolipoproteins are thought to be disposed mainly within the polar surface film of the VLDL particle.

A technique for this preparation of viable liver cells from estrogenized chicks (1.5 w of age) for monolayer and suspension culture that avoids hepatic perfusion and that is performed under aseptic conditions was developed in our laboratory [19]. Hepatocytes are dissociated from minced liver tissue by external digestion with collagenase. Short-term experiments (1–4 h) can be conducted with hepatocyte suspensions, while longer-term experiments require that the cells be plated and carried in monolayer culture. In both instances, the cells are initially incubated at 37°C in Eagle's basal medium supplemented with glucose (final concentration, 25 mM), 5% rooster serum, amino acids, and insulin under 90% air-10% CO_2 [19]. Subsequently, the hepatocytes can be carried in lipid-free (ie, serum-free) medium [20]. Electron micrographs of cells in monolayer culture indicate polygonal cell boundaries and normal organelle morphology [19]. The secretory capacity of the liver cells is unimpaired after isolation [19]. The cells in monolayer culture carry out fatty acid synthesis, lipogenesis, ketogenesis, and cholesterogenesis at physiological rates for several days and remain responsive to insulin and glucagon [19]. Avian liver cells cultured under these conditions do not proliferate, but like their counterparts in vivo are quiescent.

The capacity of estrognized chick liver cells in monolayer culture to synthesize and secrete VLDL was investigated immunochemically by utilizing antiserum obtained from rabbits immunized with purified chicken serum VLDL [19–21]. The resulting anti-VLDL antibody specifically precipitates only the VLDL component of laying hen serum [19]. As determined from the incorporation of [^{3}H]leucine into [^{3}H]VLDL immunoprecipitable from both cell monolayers and culture medium, it is evident (Fig. 1) that estrogen-induced liver cells in culture actively synthesize and secrete the two apolipoproteins corresponding to the "heavy" and "light" polypeptide constituents of authentic chick serum VLDL [20,21]. Approximately 10% of the total protein synthesized by these cells is VLDL apolipoprotein [19,21]. Furthermore, virtually all of the lipid metabolically labeled during a 2.5-min [^{3}H]palmitic acid or [2-^{3}H]glycerol pulse by hepatocyte monolayers and secreted into the culture medium during a subsequent chase is specifically immunoprecipitated with anti-VLDL using a double-antibody technique [20,21]. The material secreted by the hepatocyte cultures consists of individual, freely dispersed, spherical particles relatively homogenous in appearance [21]. This secretory product closely resembles authentic chicken serum VLDL negatively stained in a similar fashion [23]. Direct, random measurement of individual particles on micrographs of varying magnifications revealed that most are 300–500 Å in diameter, the size range of chicken serum VLDL [23] and chick plasma VLDL as determined by gel-filtration on calibrated 2% agarose [unpublished results]. Thus, it may be concluded that, in monolayer culture, liver cells derived

from estrogenized chicks synthesize, assemble, and secrete large amounts of apparently normal VLDL particles.

SYNTHESIS, PROCESSING, AND SECRETION OF THE VLDL APOLIPOPROTEINS

Electrophoretic Characterization and Quantitation of VLDL Apolipoprotein Synthesis in the Absence and Presence of Cycloheximide or Puromycin

To characterize and quantitate the polypeptide constituents of VLDL secreted by the hepatocyte cultures, liver cell monolayers derived from estradiol-treated chicks were pulse-labeled for 2.5 min with [^{3}H]leucine in lipid-free medium [19] and were

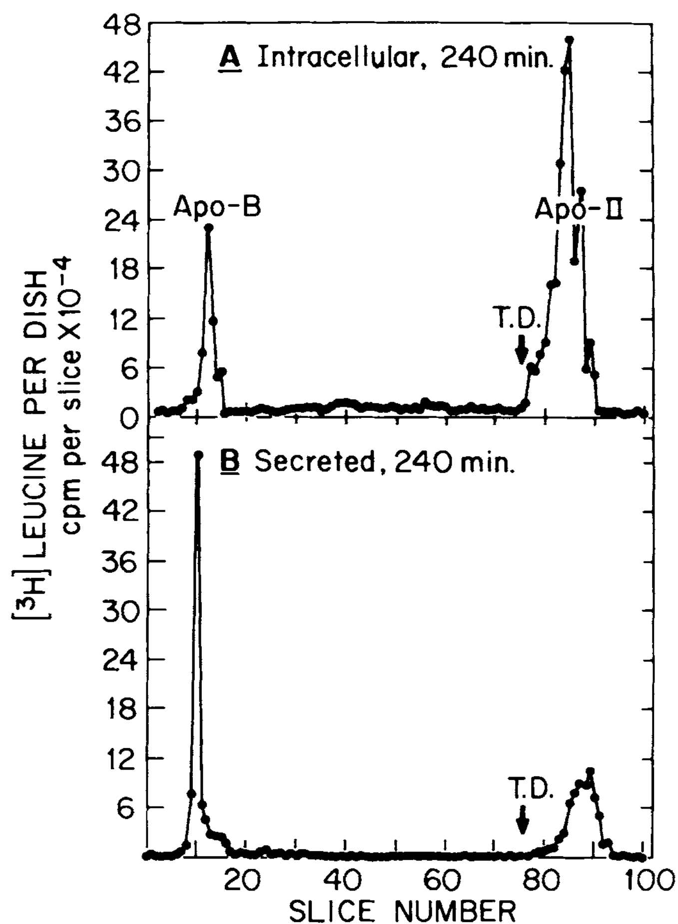

Fig. 1. SDS-DATD-polyacrylamide gel electrophoresis patterns of cellular and secreted immunoprecipitable [^{3}H]VLDL apolipoproteins. Liver cell monolayers, derived from estradiol-treated chicks [19], were labeled with medium [19] containing 2 mCi of L[4,5-^{3}H]leucine for 240 min. Cellular and secreted VLDL [^{3}H]apolipoproteins were then immunoprecipitated and subjected to SDS-DATD-polyacrylamide gel electrophoresis [20,21]. A) Cellular VLDL [^{3}H] apolipoproteins. B) Secreted VLDL [^{3}H]apolipoproteins immunoprecipitated from the culture medium. T.D., tracking dye.

then chased for 240 min with fresh lipid-free medium containing unlabeled leucine. In some cases, the chase medium also contained either 10 μM cycloheximide or 150 μM puromycin. These concentrations of cycloheximide and puromycin inhibit [^{3}H]leucine incorporation into trichloroacetic acid-precipitable cellular protein by 98% within 30 sec (results not shown and [21,25]). Cycloheximide efficiently inhibits polypeptide chain elongation and reinitiation [26], thereby trapping nascent polypeptide chains intracellularly on polysomes in their steady-state pattern [25], whereas puromycin causes premature termination of translation by discharging the nascent chains from the polysomes [27]. The immunoprecipitable VLDL [^{3}H]apolipoproteins labeled by a 2.5-min [^{3}H]leucine pulse and secreted during the subsequent 240-min chase were characterized by SDS-DATD-polyacrylamide gel electrophoresis [19,21] (Fig. 2).

In the absence of protein-synthesis inhibitor (Fig. 2A), two ^{3}H-labeled immunoprecipitable polypeptides corresponding to apolipoproteins B and II, respectively, of authentic chick serum VLDL [19] are secreted. By 240-min of chase time, virtually all (>91%) of the [^{3}H]apolipoprotein B labeled during the preceding 2.5-min pulse has been secreted as VLDL, while only ~70% of the [^{3}H]apolipoprotein II has cleared the cell [21]. The two VLDL [^{3}H]apolipoproteins are also secreted over a 240-min chase in the presence of cycloheximide (Fig. 2B). However, the amount of ^{3}H-labeled apolipoprotein B secreted by the cycloheximide-treated cells is 35% of that secreted by nontreated cultures. The difference in ^{3}H-label associated with apolipoprotein B is recovered quantitatively when puromycin is present throughout the 240-min chase as secretory product immunoprecipitable by anti-VLDL antibody (Fig. 2C). This ^{3}H-label is associated with polypeptide chains of just below 350K to ~20K, which have been demonstrated [22,28] to be VLDL apolipoprotein B nascent chains. The [^{3}H]nascent chains are trapped intracellularly by cycloheximide and can be quantitatively immunoprecipitated from the polysomes (Fig. 3B), whereas puromycin discharges the ^{3}H-labeled nascent chains from the polysomes. The finding that cylcoheximide-arrested nascent chains of VLDL apolipoprotein B are readily discharged by puromycin and are then quantitatively secreted indicates that the translocation of polysome-associated nascent chains into the luminal space of the endoplasmic reticulum requires neither protein synthesis nor the attachment of the polypeptide to the ribosome.

The rather uniform increase in ^{3}H-label from low to high molecular weight in both secreted (Fig. 2C) and polysome-associated intracellular (Fig. 3B) [^{3}H]apolipoprotein B nascent chains suggests that the elongation process occurs at a relatively constant rate of translation, without chain "build-up" at rate-limiting steps. The nascent chain profile shows no evidence of accumulation of a low molecular weight species or of apopeptide subunit(s) of discrete molecular weight. Taken together, these results indicate that the 350K apolipoprotein B polypeptide is synthesized de novo as a contiguous polypeptide chain while attached to the ribosome and is not formed by posttranslational cross-linking or noncovalent aggregation of smaller peptide subunits as proposed by some investigators (see [25]). Further evidence for this conclusion comes from the pattern with which apolipoprotein B is glycosylated (below).

The amount of immunoprecipitable VLDL apolipoprotein II labeled during a 2.5-min [^{3}H]leucine pulse and secreted over a subsequent 240-min chase is the same in the absence or presence of protein synthesis inhibitor (Fig. 2). This is consistent

with the low molecular weight and short translation time of apolipoprotein II and, consequently, its rapid pulse-labeling from [^{3}H]leucine. Since the same amount of VLDL [^{3}H]apolipoprotein II is found in the culture medium whether or not there is ongoing protein synthesis, the secretory capacity of the liver cells is unimpaired by cyclohexmide or puromycin. Significant levels of secreted VLDL [^{3}H]apolipoprotein II nascent chains were not detected when puromycin was present during the 240-min

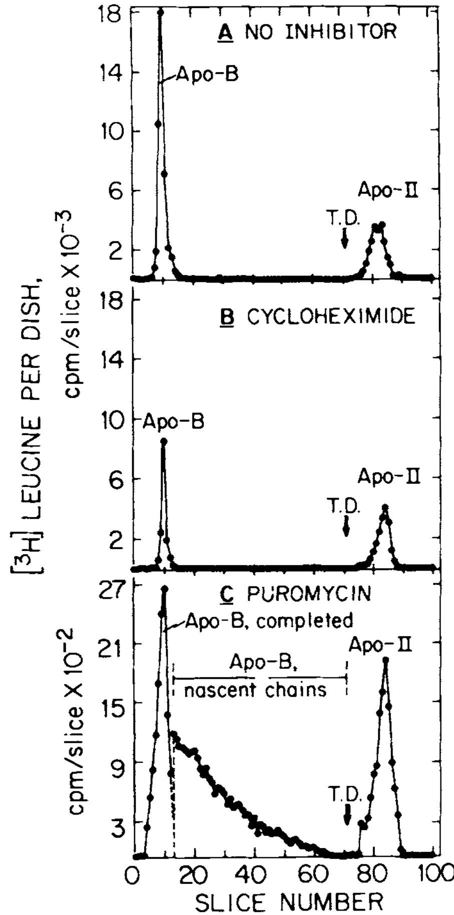

Fig. 2. SDS-DATD-polyacrylamide gel electrophoresis patterns of secreted immunoprecipitable VLDL [^{3}H]apolipoproteins after a [^{3}H]leucine pulse and an unlabeled leucine chase. Liver cell monolayers, prepared from estradiol-treated chicks [19], were pulsed for 2.5 min with lipid-free (serum-free), leucine-free medium [19] containing 2 mCi of L[4,5-^{3}H]leucine and were then chased for 240 min with lipid-free medium containing unlabeled leucine and one of the following: A) no inhibitor of protein synthesis, B) 10 μM cycloheximide, C) 150 μM puromycin. The VLDL apolipoproteins secreted during the chase were immunoprecipitated from the culture media [20,21], and the washed immunoprecipitates were solubilized and subjected to SDS-DATD-polyacrylamide gel electrophoresis [20,21]. Gels were sliced and analyzed by liquid scintillation spectrometry to determine the ^{3}H-activity assimilated into the secreted VLDL apolipoproteins. Apo-B, VLDL apolipoprotein B; Apo-II, VLDL apolipoprotein II; T.D., tracking dye (data from Janero and Lane [21]).

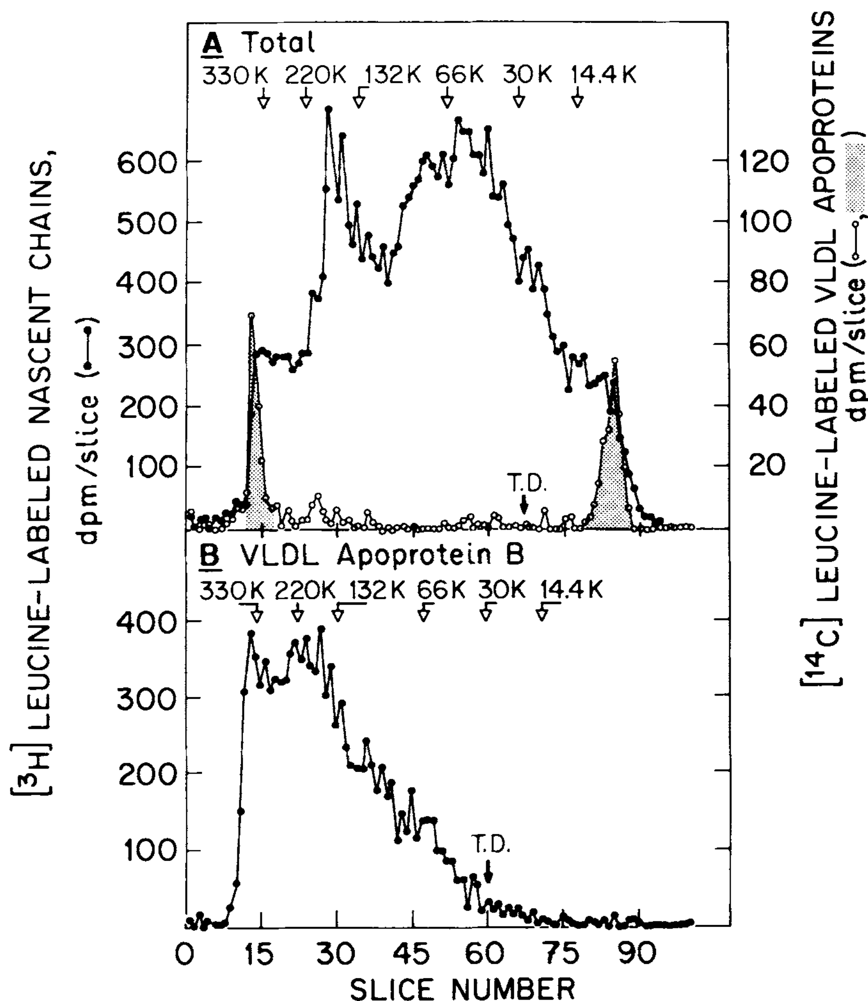

Fig. 3. SDS-DATD-polyacrylamide gel electrophoresis patterns of A) total [^{3}H]leucine-labeled nascent chains coelectrophoresed with secreted [^{14}C]leucine-labeled VLDL apolipoprotein B and VLDL apolipoprotein II markers and B) immunoprecipitable [^{3}H]leucine-labeled VLDL apolipoprotein B nascent chains. Liver cell suspensions, derived from estradiol-treated chicks [19], were subjected to a 10-min L[4,5-^{3}H]leucine pulse (100 μCi/ml), and total polysome-associated nascent chains [25] were used to immunoprecipitate [20,21] VLDL apolipoprotein B nascent chains. Nascent polypeptide chains were separated from contaminating completed chains by anion-exchange chromatography on Celex E [25] and were then subjected to SDS-DATD-polyacrylamide gel electrophoresis [20,25]. The molecular weight standards used were pentamer of bovine serum albumin, 330K; ferritin, 220K; dimer of bovine serum albumin, 132K; bovine serum albumin, 66K; carbonic anhydrase, 30K; and α-lactalbumin, 14.4K. T.D., tracking dye (data from Siuta-Mangano et al [25]).

chase under our conditions of labeling and electrophoretic polypeptide resolution [21,25].

Apolipoprotein B Glycosylation and Its Role in Apolipoprotein Secretion

Both human [29] and avian [19,20] apolipoprotein B are glycoproteins. Human apolipoprotein B is 8–10% carbohydrate by weight and contains both a high-mannose

and a complex oligosaccharide chain [29]. To investigate the mechanism whereby apolipoprotein B is glycosylated, chick liver cells were incubated with either [^{3}H]leucine or [^{3}H]mannose, in glucose-free medium [25]. Deletion of glucose from the medium did not impair the protein-synthesizing capacity of the cells for up to 60 min, as measured by [^{3}H]leucine incorporation into trichloroacetic acid-precipitable protein (results not shown). Total cellular polysomes were isolated by magnesium precipitation [30], and the [^{3}H]leucine- and [^{3}H]mannose-labeled polysomes were subjected to ion-exchange chromatography on Celex E following dissociation and solubilization. Celex E binds peptidyl-tRNA (ie, nascent polypetide chains), which can then be eluted with high salt [31].

Cellular [^{3}H]leucine-labeled nascent chains, recovered quantitatively from the high-salt eluate by ethanol precipitation [25], were characterized by SDS-polyacrylamide gel electrophoresis. Figure 3A shows the electrophoretic profile of cellular [^{3}H]leucine-labeled nascent chains coelectrophoresed with secreted [^{14}C]leucine labeled apolipoprotein B and apolipoprotein II markers. The size distribution of these nascent chains is heterogeneous, ranging in molecular weight from 350K (the size of completed apolipoprotein B) to 6K.

[^{3}H]leucine-labeled nascent chains of VLDL apolipoprotein B were isolated from the total cellular [^{3}H]leucine-labeled nascent chains by indirect immunoprecipitation with rabbit anti-VLDL γ-globulin and goat antirabbit γ-globulin [25]. Figure 3B shows the gel electrophoretic profile of the immunoprecipitated VLDL apolipoprotein B nascent chains. The size distribution of the nascent polypeptides ranged from 350K (the size of completed apolipoprotein B) to ~20 K. The very large nascent chains of VLDL apolipoprotein B detected offer further evidence that this apolipoprotein is synthesized on the polysome as a single high molecular weight polypeptide chain.

Approximately 57% of the [^{3}H]mannose label associated with total cellular polysomes was retained by the Celex E column at low salt concentration and was eluted by high salt [25]. The latter mannose-labeled nascent chain fraction was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4A). The labeling pattern for total polysome-associated nascent peptide chains was heterogeneous, ranging in molecular weight from ~30K to 350K. The mannose-labeled polysomes were subjected to indirect immunoprecipitation [25] using anti-VLDL. The labeling profile of immunoprecipitated polypeptides in the SDS gel shown in Figure 4B reveals that only apolipoprotein B nascent chains of >120K were labeled with [^{3}H]mannose; moreover, there was an abrupt rise in the extent of polypeptide labeling at molecular weight 280K. Labeling experiments with D-[6-^{3}H]glucosamine gave a similar pattern of apolipoprotein B glycosylation (results not shown). If it is assumed that the rate of translation of the mRNA for apolipoprotein B is relatively constant, these results indicate that core (*N*-linked) oligosaccharide attachment occurs cotranslationally in a two- (or multi-) step process, ie, when the growing nascent chain reaches ~120K and 280K. Thus, some 30% of the apolipoprotein B polypeptide (mature MW 350K) is synthesized prior to core oligosaccharide addition. Since core oligosaccharide addition occurs cotranslationally [5], it is difficult to see how mature apolipoprotein B could be derived by oligomerization of small polypeptide precursors without their prior glycosylation. Were this the case, a smaller glycosylated species in the size range of the precursor should have been observed but was not [25]. Rather, the synthesis of the apolipoprotein B chain as a contiguous polypeptide is again indicated.

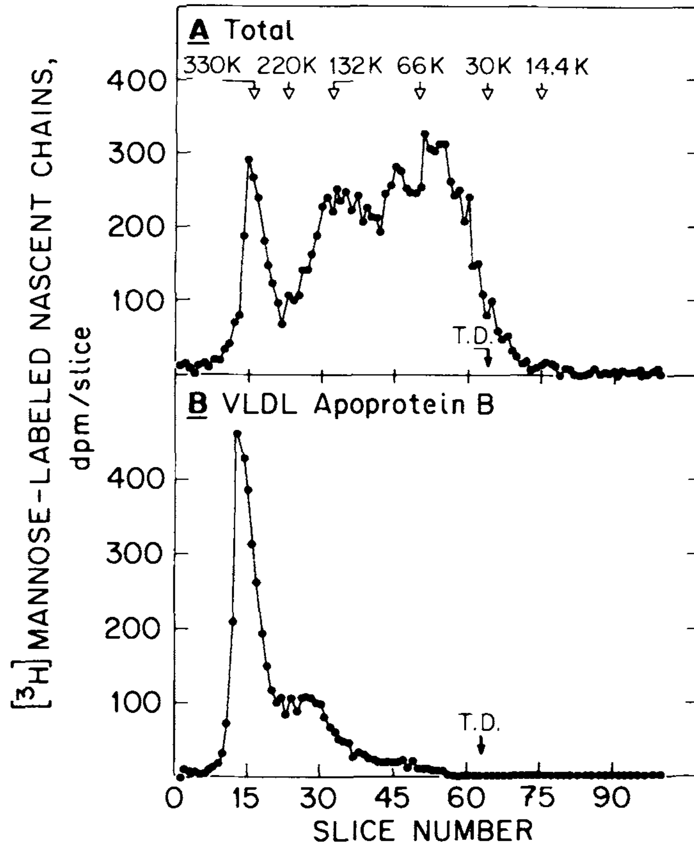

Fig. 4. SDS-DATD-polyacrylamide gel electrophoresis patterns of A) total [^{3}H]mannose-labeled nascent chains and B) immunoprecipitable [^{3}H]mannose-labeled VLDL apolipoprotein B nascent chains. Liver cell suspensions, derived from estradiol-treated chicks [19], were subjected to a 30-min D-[2-^{3}H]mannose pulse (135 μCi/ml), and total polysome-associated nascent chains [25] were used to immunoprecipitate [20,21] glycosylated VLDL apolipoprotein B nascent chains. Nascent polypeptide chains were separated from contaminating completed chains by anion-exchange chromatography on Celex E [25] and then subjected to SDS-DATD-polyacrylamide gel electrophoresis [20,21]. The molecular weight standards used are described in the legend to Figure 3. T.D., tracking dye (data from Siuta-Mangano et al [25]).

Endo-β-N-acetylglucosaminidase *(Endo H)* was used to characterize the oligosaccharide side chains of VLDL apolipoprotein B nascent and completed polypeptides. *Endo H* cleaves asparagine-linked oligosaccharides containing five or more mannose residues ("high mannose") at the di-*N*-acetylchitobiosyl moiety in the inner core region, thereby releasing the intact oligosaccharide from the peptide [32]. "Complex" oligosaccharides and glycopeptides are completely resistant to *endo H* cleavage. Treatment of [^{3}H]mannose-labeled apolipoprotein B nascent chains with *endo H* results in a $>98\%$ release of the [^{3}H]mannose-labeled oligosaccharides [25]; therefore, these nascent chains, like other nascent secretory glycoproteins [5], have acquired a characteristic high-mannose oligosaccharide chain. Incubation of immu-

noprecipitable [^{3}H]mannose-labeled apolipoprotein B secreted by the liver cells (ie, after undergoing intracellular maturation) with *endo H* enzymatically released ~77% of the [^{3}H]mannose-labeled oligosaccharide; however, ~23% of the labeled oligosaccharide was resistant to enzymatic cleavage [25]. Immunoprecipitable [^{3}H]apolipoprotein B secreted from liver cells labeled with [^{3}H]galactose [25] and, therefore, terminally galactosylated in the Golgi complex [25], remains 94% resistant during a 5-hr incubation with *endo H* [25]. Based on the [^{3}H]mannose- and [^{3}H]galactose-labeling results, completed apolipoprotein B secreted by the estradiol-stimulated chick liver cell appears to possess both "high-mannose" and "complex" oligosaccharide side chains. This is in accord with the two peaks of radioactivity observed in the SDS gel patterns for [^{3}H]mannose- (Fig. 4B) and [^{3}H]glucosamine-labeled (results not shown) apolipoprotein B nascent chains. The correspondence between the peaks in Figure 4B and the type of oligosaccharide chain has not been established.

To explore the possible role of the carbohydrate moiety of apolipoprotein B in the secretion of hepatic VLDL, tunicamycin was used to inhibit the formation of *N*-acetylglucosamine-containing lipid intermediates involved in the assembly of the core regions of oligosaccharide chains linked *N*-glycosidically to protein [33]. Liver cell monolayers prepared from estradiol-treated chicks were incubated in serum-free medium [19] either with (0.1 μg/ml final concentration [20]) or without tunicamycin for 4.5 hr, sufficient time for the intracellular VLDL apolipoprotein B synthesized prior to the tunicamycin treatment to clear the cell and be secreted [20]. The tunicamycin-treated and nontreated monolayers were then exposed to either [^{3}H]glucosamine or [^{3}H]leucine for 2.5 hr, after which time the media were recovered to quantitate the incorporation of ^{3}H-label into immunoprecipitable, secreted VLDL apolipoproteins resolved by SDS-DATD-gel electrophoresis [20]. Under these conditions, tunicamycin inhibited [^{3}H]glucosamine incorporation into immunoprecipitable apolipoprotein B by >98% and [^{3}H]leucine incorporation minimally (~40%) [20]. Despite the inhibitory effect of tunicamycin on apolipoprotein B glycosylation, secretion of both the resulting VLDL *aglyco*-apolipoprotein B as well as VLDL apolipoprotein II was unimpaired, and the morphology of the secreted VLDL particles was normal [20,34]. Thus, the carbohydrate moiety of apolipoprotein B does not appear to play an essential role in the secretion of the VLDL apolipoproteins.

Kinetics of Hepatic VLDL Apolipoprotein Synthesis and Secretion

To determine the time required for the synthesis of the VLDL apolipoproteins, cell monolayers derived from estradiol-treated chicks were pulsed with [^{3}H]leucine in lipid-free medium [19] for 2.5 min and then chased with fresh medium containing unlabeled leucine. The appearance of immunoprecipitable "heavy" and "light" ^{3}H-labeled VLDL apopeptides in the cells was then monitored [21,25]. When followed kinetically, maximal incorporation of ^{3}H-label into cellular heavy apolipoprotein B occurs about 10 min after completion of the pulse [21, 25]. Thus, about 10 min are required for the translation of the apolipoprotein B polypeptide. This corresponds to a translation rate of 7.0 amino acid residues/sec, which approximates the values of 5.5–6.9 residues/sec reported [35] for several other proteins in various cell types. The long translation time for apolipoprotein B is consistent with its high (350K) molecular weight. Maximal incorporation of [^{3}H]leucine into cellular apolipoprotein II occurs by the time the pulse is completed (ie, by 0 min of chase) [21,25]. This

finding is consistent with the low molecular weight of the light apolipoprotein (6K) and a calculated translation time of ~0.15 min.

To determine the kinetics with which the newly synthesized VLDL apolipoproteins are secreted, cell monolayers were pulse-labeled for 2.5 min with [^{3}H]leucine in lipid-free medium [19] and were then chased for 240 min with fresh medium containing unlabeled leucine and one of the following: no inhibitor of protein synthesis, 10μM cycloheximide, or 150 μM puromycin [21]. Aliquots of the media removed were immunoprecipitated with anti-VLDL [19], and the solubilized immunoprecipitates were electrophoresed on SDS-DATD-polyacrylamide gels (cf. Fig. 2) to characterize the kinetics with which VLDL [^{3}H]apolipoproteins B and II, labeled during the preceding pulse, are secreted [21].

Immunoprecipitable VLDL apolipoproteins synthesized during the 2.5-min [^{3}H]leucine pulse do not appear in the medium before 30 min into the chase but are present in the medium at significant levels by 45 min (Fig. 5). Therefore, both newly translated VLDL apolipoproteins require at least 30 min to reach the cell surface for secretion as VLDL, do so simultaneously, and continue to appear in the medium concurrently during the 240-min chase. With no protein synthesis inhibitor or with cycloheximide present during the chase, the secreted ^{3}H-label immunoprecipitable by anti-VLDL is associated exclusively with completed VLDL apolipoproteins (Figs. 2A,B, 5A,), whereas in the presence of puromycin the [^{3}H]apolipoprotein B nascent chains are also secreted (Figs. 2C, 5A). ^{3}H-Labeled nascent chains of apolipoprotein B are secreted concurrently with completed VLDL [^{3}H]apolipoprotein B and [^{3}H]apolipoprotein II when puromycin is present (Fig. 5A), and the size distribution of the secreted [^{3}H]apolipoprotein B nascent chains remains heterogeneous, ranging from just under 350K to ~20K (Fig. 2C and [21]).

Quantitatively, the amounts of immunoprecipitable VLDL [^{3}H]apolipoprotein II secreted during the chase and the kinetics of this secretion were unaffected by the presence of either cycloheximide or puromycin in the chase medium (Fig. 5B). In contrast, cycloheximide markedly (by ~65%) depressed the secretion of immunoprecipitable [^{3}H]apolipoprotein B over the 240-min chase, although the kinetic pattern of

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Fig. 5. Kinetics of incorporation of ^{3}H-label into secreted, immunoprecipitable VLDL apolipoproteins after a [^{3}H]leucine pulse and an unlabeled leucine chase. Liver cell monolayers, derived from estradiol-treated chicks [19] were pulsed for 2.5 min with lipid-free (serum-free), leucine-free medium [19] containing 2 mCi of L[4,5-^{3}H]leucine and were then chased for 240 min with lipid-free medium [19] containing unlabeled leucine and one of the following: no inhibitor of protein synthesis (CONTROL), 10 μM cycloheximide (CHX), 150 μM puromycin (PURO). The medium was removed from each dish at various times during the chase, fresh chase medium containing the appropriate inhibitor was immediately added back, and the chase was continued. The secreted A) VLDL [^{3}H]apolipoprotein B (APO-B) and B) VLDL [^{3}H]apolipoprotein II (APO-II) polypeptide chains were immunoprecipitated [20,21] from the chase media at the time intervals indicated and were resolved out of the washed, solubilized immunoprecipitates by SDS-DATD-polyacrylamide gel electrophoresis [20,21]. Incorporation of [^{3}H]leucine into the secreted VLDL apolipoproteins was determined as the sum of the ^{3}H-label (corrected for gel background) in the following regions of SDS-DATD-polyacrylamide gels identical in pattern to those shown in Figure 2 for each respective treatment during the chase: control, total completed VLDL apolipoprotein B (A, □) and total VLDL apolipoprotein II (B, □); cycloheximide, total completed VLDL apolipoprotein B (A, ○) and total VLDL apolipoprotein II (B, ○); puromycin completed VLDL apolipoprotein B chains (A, ◇), nascent VLDL apolipoprotein B chains (A, ▽), total (ie, completed plus nacent) VLDL apolipoprotein B (A, ▲), and total VLDL apolipoprotein II (B, ◇) (data from Janero and Lane [21]).

apolipoprotein B secretion in the presence of cycloheximide was identical to that with which [^{3}H]apolipoprotein B completed chains were secreted in the presence of puromycin (Fig. 5A). At any point in the chase, the difference between the cycloheximide-treated cells and the puromycin-treated cells in the amount of ^{3}H-label associated with apolipoprotein B can be quantitatively accounted for by the secretion of [^{3}H]apolipoprotein B nascent chains arrested intracellularly in the presence of cycloheximide [21,25] but quantitatively released from the puromycin-treated cells [21,28]. The sum of the [^{3}H]apolipoprotein B nascent chains and the [^{3}H]apolipoprotein B completed chains secreted at any time in the chase with puromycin equals the amount of [^{3}H]apolipoprotein B secreted by the control culture (Fig. 5A).

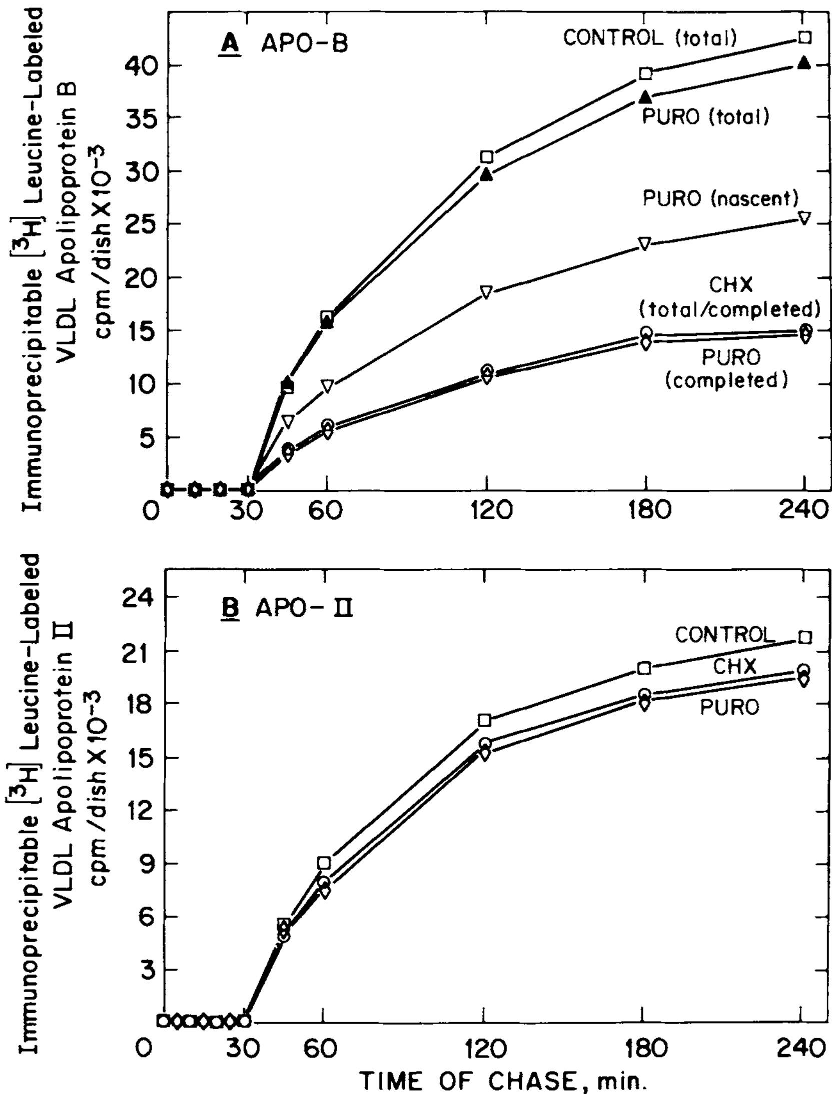

Fig. 5

SYNTHESIS, ASSEMBLY, AND SECRETION OF THE VLDL GLYCEROLIPIDS

Kinetics of Hepatic VLDL Triacylglycerol Secretion and the Influence of Completed and Nascent Apolipoprotein Chains upon VLDL Triacylglycerol Assembly

To determine the kinetics of secretion of VLDL triacylglycerol, liver cell monolayers were pulse-labeled for 2.5 min with [^{3}H]glycerol in lipid-free medium [19] and then were chased for 240 min with fresh lipid-free medium containing unlabeled glycerol and one of the following: no inhibitor of protein synthesis, 10 μM cycloheximide, or 150 μM puromycin [21]. Media were removed during the chase and were subjected to two-step immunoprecipitation [20] with anti-VLDL antibody. Under these conditions, $>85\%$ of the total secreted [^{3}H]glycerolipid is specifically immunoprecipitated as VLDL, while $<3\%$ is immunoprecipitable with nonimmune rabbit serum [20,21]. Lipids extracted from the immunoprecipitates [36] were separated chromatographically [37] and quantitated radiochemically [21] to assess the kinetics of secretion of newly synthesized [^{3}H]triacylglycerol assembled into VLDL [21]. As shown in Figure 6, VLDL [^{3}H]triacylglycerol is detectable in the culture medium by 5-min chase time whether or not protein synthesis is inhibited. Highest rates of immunoprecipitable VLDL [^{3}H]triacylglycerol secretion, though, are not reached until 20 min into the chase, the VLDL [^{3}H]triacylglycerol secreted earlier constituting $<10\%$ of the total secreted during the 240-min chase. The control cells secrete [^{3}H]triacylglycerol as VLDL throughout the chase, with some diminution after 180 min. In the cycloheximide- and puromycin-treated cells, however, VLDL [^{3}H]triacylglycerol secretion is markedly suppressed, and significant amounts are not secreted after 180 min of chase. The halt in VLDL [^{3}H]triacylglycerol secretion when cellular protein synthesis is inhibited indicates that apolipoprotein synthesis is required for VLDL triacylglycerol assembly and secretion [20,21,38]. Since 3 hr are required for $\sim 90\%$ of the VLDL [^{3}H]apolipoprotein B labeled during the 2.5-min [^{3}H]leucine pulse and present at the start of the chase (ie, at the start of protein synthesis inhibition) to clear the cell (Fig. 5A), the immunoprecipitable VLDL [^{3}H]triacylglycerol secreted in the presence of either cycloheximide or puromycin is associated with VLDL apolipoproteins translated prior to inhibitor treatment but not yet secreted [20,21,38].

Although both cycloheximide and puromycin significantly depress VLDL triacylglycerol secretion with respect to the control cells, the inhibition is markedly greater by cycloheximide than by puromycin (Fig. 6). The augmented VLDL [^{3}H]triacylglycerol secretion with puromycin over that with cycloheximide is not accompanied by any alteration in VLDL apolipoprotein II release (Fig. 5B, Table II). However, the coincidence between the greater amounts of VLDL [^{3}H]triacyglycerol secreted (Fig. 6, Table II) and the greater amounts of VLDL [^{3}H]apolipoprotein B (as nascent chains) secreted (Fig. 5A, Table II) by the puromycin-treated cells suggests that the discharged nascent chains load triacylglycerol and undergo secretion as immunoprecipitable lipoprotein throughout the chase. Further evidence for this conclusion comes from the finding that apolipoprotein B nascent chains are degraded when trapped intracellularly for long periods of time on polysomes by cycloheximide, whereas their integrity is preserved when they are discharged (within 30 min after pulse-labeling) by puromycin and secreted along with lipid [mainly triacylglycerol (Table II)] as VLDL [21].

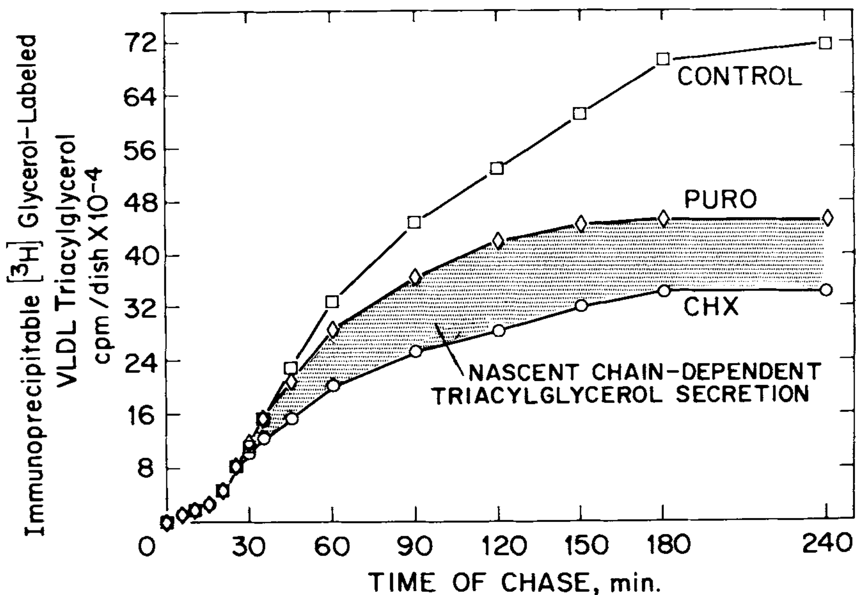

Fig. 6. Kinetics of incorporation of ^{3}H-label into secreted, immunoprecipitable VLDL triacylglycerol after a [^{3}H]glycerol pulse and an unlabeled glycerol chase. Liver cell monolayers, derived from estradiol-treated chicks [19], were pulsed for 2.5 min with lipid-free (serum-free) medium [19] containing 400 μCi of [2-^{3}H]glycerol and then were chased for 240 min with lipid-free medium containing unlabeled glycerol and one of the following: no inhibitor of protein sythesis (CONTROL, □), 150 μM puromycin (PURO, ◇), 10 μM cycloheximide (CHX, ○). The medium was removed from each plate at various times during the chase to 240 min, fresh chase medium containing the appropriate inhibitor was immediately added back, and the chase was continued. Aliquots of the chase media removed at the time intervals indicated were taken for immunoprecipitation in the absence of detergents by a double-antibody technique [20] with the γ-globulin fraction of rabbit-anti-VLDL and goat antirabbit γ-globulin, and the washed immunoprecipitates were subjected to lipid extraction [36]. The triacylglycerol was resolved out of the lipid extracts by thin-layer chromatography [37] and was recovered from the chromatograms and analyzed by liquid scintillation spectrometry to quantitate the incorporation of ^{3}H-label into secreted, immunoprecipitable VLDL [^{3}H]triacylglycerol. The shaded area represents the fraction of VLDL [^{3}H]triacylglycerol secreted by the puromycin-treated cells dependent upon the discharge and secretion of VLDL apolipoprotein B nascent polypeptide chains (data from Janero and Lane [21]).

Kinetics of Hepatic VLDL Phosphoglyceride Secretion and the Influence of Completed and Nascent Apolipoprotein Chains Upon VLDL Phosphoglyceride Assembly

To assess the kinetics of VLDL phosphoglyceride secretion, cell monolayers in lipid-free medium [19] were pulse-labeled for 2.5 min with [^{3}H]glycerol and were then chased for 240 min with lipid-free medium containing an excess of unlabeled glycerol and one of the following: no inhibitor of protein synthesis, 10 μM cyclohex-imide, or 150 μM puromycin. Media were removed at discrete times during the chase, and aliquots were immunoprecipitated [20] with anti-VLDL antibody. Lipids in the washed immunoprecipitates were extracted [36], and the immunoprecipitable

TABLE II. Dependence of VLDL Glycerolipid Assembly/Secretion Upon the Discharge of Nascent Apopeptides of Apolipoprotein B*

	Secreted between 25 and 240 min of chase (cpm per culture dish $\times 10^{-3}$)						
		[^{3}H]Apolipoprotein B[a]		[^{3}H]Triacylglycerol[b]		[^{3}H]Phosphoglyceride[b]	
Treatment during chase	[^{3}H]Apolipoprotein II[a]	Completed apolipoprotein	Nascent apopeptides	Total	Dependent upon nascent apopeptide discharge	Total	Dependent upon nascent apopeptide discharge
No inhibitor	22	43	None	630	—	83	—
Cycloheximide	20	15	None	260	—	34	—
Puromycin	20	15	28	360	100	36	2

*Liver cell monolayers, prepared from estradiol-treated chicks [19], were pulse-labeled for 2.5 min with 2 ml of serum-free medium [19] containing either 2 mCi of L [4,5-^{3}H]leucine or 400 μCi of [2-^{3}H]glycerol and were then chased for 240 min with unlabeled medium containing one of the following: no inhibitor of protein synthesis; 10 μM cycloheximide; 150 μM puromycin. The amounts of VLDL [^{3}H]apolipoproteins and immunoprecipitable VLDL [^{3}H]-glycerolipids secreted between 25 and 240 min of chase were determined by immunoprecipitating [20] samples of the chase media with anti-VLDL antibody, chromatographically separating the VLDL constituents out of the washed immunoprecipitates [20,37,39], and quantitating radiochemically the ^{3}H-label associated with the immunoprecipitated VLDL apolipoproteins, triacylglycerol, and phosphoglyceride (data from Janero and Lane [21]).

[a]Labeled during a 2.5-min [^{3}H]leucine pulse.

[b]Labeled during a 2.5-min [^{3}H]glycerol pulse.

[^{3}H]-phosphoglycerides secreted as VLDL were resolved by thin-layer chromatography [39], recovered, and quantitated by liquid scintillation spectrometry [21]. Four immunoprecipitable phosphoglycerides—phosphatidylethanoamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol—were the sole ^{3}H-labeled polar acylglycerolipids secreted (Fig. 7). All immunoprecipitable VLDL [^{3}H]phosphoglycerides are secreted with biphasic kinetics: A sharp burst of secretion during the first 15 min of chase is followed by a virtual halt in the secretion of additional ^{3}H-labeled VLDL phosphoglyceride over the next 15 min. The resulting plateau, from 15–30 min in the chase, is followed by further VLDL [^{3}H]phosphoglyceride release at 30-min chase time, which then declines after 180 min. The amplitude of the initial burst of VLDL [^{3}H]phosphoglyceride secretion prior to 15 min of chase varies among the individual lipids: For example, the secreted VLDL [^{3}H]phosphatidylethanolamine-to-[^{3}H]phosphatidylcholine ratio changes from >2 at 5 min to <0.5 by 240 min into the chase. A high proportion of VLDL [^{3}H]phosphatidylinositol and [^{3}H]phosphatidylserine (~40% of the total at 240 min) is also secreted before 15 min in the chase.

The early appearance of VLDL [^{3}H]phosphoglyceride in the culture medium and the biphasic nature of VLDL [^{3}H]phosphoglyceride secretion are to be contrasted with the secretion of immunoprecipitable VLDL [^{3}H]triacylglycerol labeled during the 2.5-min [^{3}H]glycerol pulse (Figs. 6, 7). As compared to VLDL [^{3}H]phosphoglyceride secretion, [^{3}H]triacylglycerol secretion as VLDL is negligible (<5% of the total secreted over the chase) during the first 15 min of chase. Maximal rates of VLDL [^{3}H]triacylglycerol secretion are reached only after 20–25 min of chase time, and not before 15 min, as is the case with the VLDL [^{3}H]phosphoglycerides. Cellular phosphoglycerides are not labeled preferentially (as compared to cellular triacylglycerol) from [^{3}H]glycerol during the first 30 min of chase [21], although during this period [^{3}H]phosphoglyceride secretion as VLDL has reached maximal rates, and VLDL [^{3}H]triacylglycerol secretion is minimal (Figs. 6,7). At the end of the 240-min chase period, substantial amounts of [^{3}H]glycerolipid (~80% of the total labeled) remain in the cells [21], although by 180 min the assembly and secretion of pulse-labeled VLDL [^{3}H]glycerolipid is declining (Fig. 7). Thus, the newly synthesized VLDL glycerolipids seem to represent a subpopulation of cellular glycerolipid.

As compared to control cells without protein synthesis inhibitor (Fig. 7), the presence of either cycloheximide or puromycin during the chase did not influence the kinetic pattern with which [^{3}H]phosphoglyceride labeled during a preceding 2.5-min [^{3}H]glycerol pulse was secreted as VLDL [21]. Quantitatively, the same amount of each of the VLDL [^{3}H]phosphoglycerides is secreted prior to 30 min into the chase whether or not protein synthesis is inhibited. Only after 30 min in the chase do cycloheximide and puromycin suppress the secretion of immunoprecipitable [^{3}H]phosphoglycerides, effectively halting VLDL [^{3}H]phosphoglyceride secretion after 180 min as the cells become depleted of VLDL apolipoprotein [10,21,38]. With puromycin present during the chase period, the cells secrete ~6% more immunoprecipitable [^{3}H]phosphoglyceride than they do in the presence of cycloheximide (Table II). The additional VLDL [^{3}H]phosphoglyceride secreted is likely associated with apolipoprotein B nascent chains released from the polysomes by puromycin but trapped intracellularly by cycloheximide (Figs. 2B,C, 5A).

DISCUSSION

The present investigations have employed a chick hepatocyte culture system [19] to identify any discrete steps in the synthesis and processing of VLDL and to evaluate how these events are regulated in order to define the manner in which the major VLDL constituents (apolipoproteins and glycerolipids) are assembled into the mature, secreted VLDL particle by the intact liver cell. Only between 30–35 min of

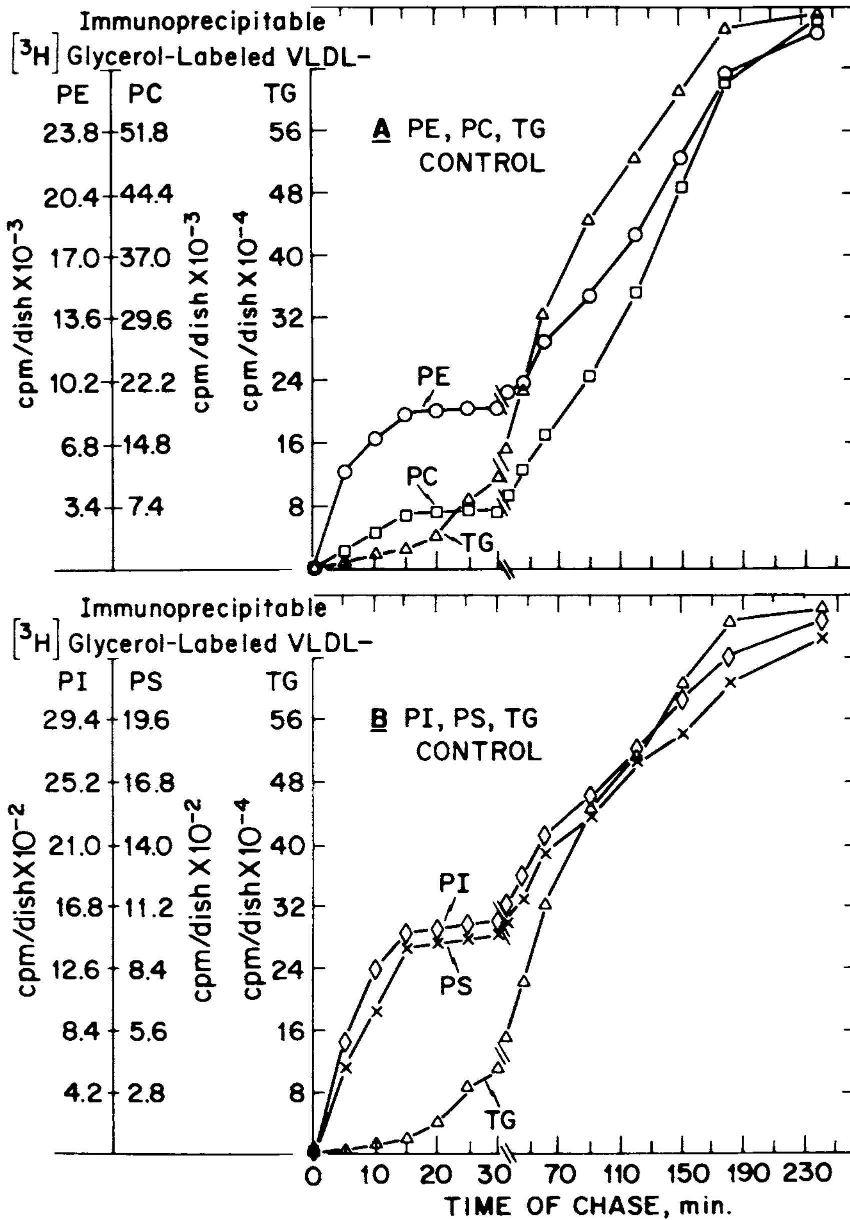

Fig. 7

chase time do both [^{3}H]apolipoproteins and [^{3}H]glycerolipids labeled during a preceding pulse appear in the culture medium as VLDL (Figs. 2, 5–7). Therefore, at least 32.5 min are required for the transit of the nascent VLDL particle along the secretory pathway. Since about 10 min are required for apolipoprotein B translation as a contiguous polypeptide [25], glycosylation in the rough endoplasmic reticulum [25], and extrusion into the endoplasmic reticulum lumen [28], the de novo synthesis and assembly of the VLDL particle would require, minimally, 40 min.

An inverse relationship exists between the time of assembly of a constituent into the nascent VLDL particle along the intracellular secretory pathway and the time of appearance of the constituent in the culture medium. Therefore, in our pulse-chase experiments (Figs. 5–7, [21]), the newly synthesized (ie, ^{3}H-labeled) components first appearing in mature, secreted VLDL are the last to have become assembled into the nascent particle intracellularly. With this principle in mind, the sequential appearance in the culture medium of newly synthesized VLDL apolipoproteins, triacylglycerol, and phosphoglycerides (Figs. 6, 7; [21]) and the mode of synthesis of apolipoprotein B [21,25] suggest the following pathway for the intracellular assembly of VLDL (Fig. 8): Within 15 min after initiation of VLDL apolipoprotein translation at the rough endoplasmic reticulum (ie, within 5 min after the 10 min required [21,25] for apolipoprotein B translation, core glycosylation, and ejection into rough endoplasmic reticulum lumen), the VLDL apolipoproteins associate with some of the phosphoglyceride and the bulk of triacylglycerol they will carry into the medium as VLDL, to be followed some 10 min later by the assembly of virtually all the VLDL triacylglycerol. It would appear that 15 min or less are required for the newly synthesized apolipoproteins to assemble initially with glycerolipid, thereby forming a nascent VLDL particle. Morphological studies [1,8,9] suggest that the initial association between the VLDL apolipoproteins and glycerolipid most likely takes place largely, if not exclusively, within the smooth regions of the endoplasmic reticulum or at the junction of the rough and smooth regions. Some 10–15 min after the initial association of apolipoprotein and glycerolipid (ie, within 5–15 min prior to secretion), additional phosphoglyceride, along with small amounts of triacylglycerol, are introduced into the immature VLDL particles, and apolipoprotein B is terminally glycosylated [21]. Autoradiographic [1,9,10] and subcellular [12,40,41] investigations indicate that the glycerolipid introduced into the nascent VLDL particle 20–25 min after translation of

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Fig. 7. Kinetics of incorporation of ^{3}H-label into secreted, immunoprecipitable VLDL phosphoglycerides after a [^{3}H]glycerol pulse and an unlabeled glycerol chase. Liver cell monolayers, derived from estradiol-treated chicks [19], were pulsed for 2.5 min with lipid-free (serum-free) medium [19] containing 400 μCi of [2-^{3}H]glycerol and were then chased for 240 min with lipid-free medium containing unlabeled glycerol. The medium was removed from each plate at various times during the chase to 240 min, fresh chase medium was immediately added back, and the chase was continued. Aliquots of the chase media removed at the time intervals indicated were immunoprecipitated in the absence of detergents by a double-antibody technique [20] with the γ-globulin fraction of rabbit anti-VLDL and goat antirabbit γ-globulin, and the washed immunoprecipitates were subjected to lipid extraction [36]. Phosphatidylethanol-amine (PE), phosphatidylcholine (PD), phosphatidylinositol (PI), and phosphatidylserine (PS) were resolved out of the lipid extracts by thin-layer chromatography [39] and were recovered from the chromatograms and analyzed by liquid scintillation spectometry to quantitate the incorporation of ^{3}H-label into each secreted, immunoprecipitable VLDL [^{3}H]phosphoglyceride. For comparison, the secretion of newly-synthesized, immunoprecipitable VLDL [^{3}H]triacylglycerol (*TG*) by the hepatocyte monolayers is plotted from the data (CONTROL) in Fig. 6 (data from Janero and Lane [21]).

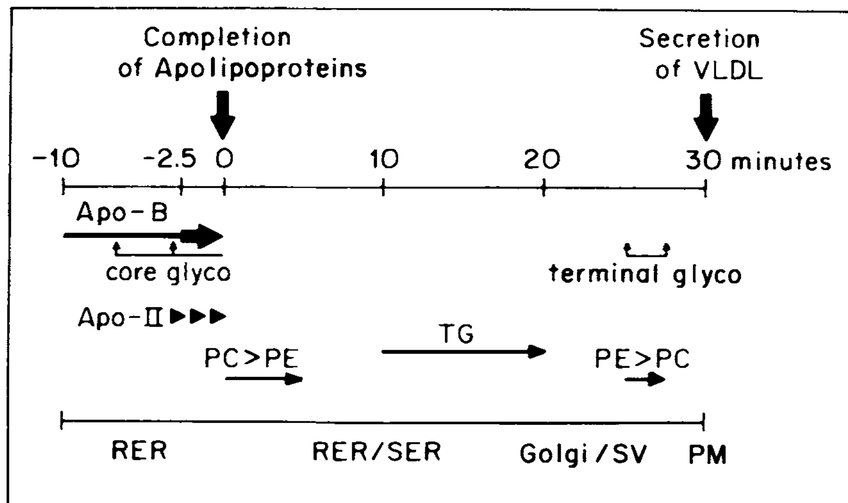

Fig. 8. Proposed sequence of assembly of hepatic VLDL apolipoproteins and glycerolipids. Apo-B represents VLDL apolipoprotein B; Apo-II, VLDL apolipoprotein II; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; SV, secretory vesicle; PM, plasma membrane; glyco, glycosylation. Horizontal arrows represent the progression in time of an immature VLDL particle through the compartments of the liver-cell secretory pathway and highlight some key steps in the assembly of VLDL apolipoproteins and glycerolipids as detailed in the test under Discussion (from Janero and Lane [21]).

apolipoprotein B is assembled within the Golgi complex and perhaps also within the secretory vesicles derived therefrom which shuttle VLDL to the plasma membrane for release. Whether the newly synthesized glycerolipid (mainly triacylglycerol) assembled into VLDL early in the secretory pathway is part of the VLDL "core," while the glycerolipid (mainly phosphoglyceride) introduced just prior to secretion represents a surface maturation of the nascent VLDL particle is an intriguing possibility in light of current structural models of VLDL [24] that envision ~95% of the particle's triacylglycerol in the core phase, the remainder disposed in the surface phase along with all of the phosphoglyceride, most of the apoliporotein, and cholesterol.

Since we observe the same sequential appearance of newly synthesized VLDL apolipoproteins and glycerolipids in the culture medium whether or not either cycloheximide or puromycin is present during the chase (Figs. 5–7 and [21]), cellular protein (and, specifically, VLDL apolipoprotein) synthesis and discharge of completed apolipoprotein chains do not influence the pattern of assembly of VLDL constituents. The inhibitors do halt the assembly of VLDL glycerolipid (Fig. 6 and [20,21]), and hepatic glycerolipid secretion as VLDL requires ongoing apopeptide production [20,38]. Nonetheless, the sequestration of apolipoprotein B polypeptides into the initial compartment of the secretory pathway (ie, the endoplasmic reticulum lumen [28]), the assembly per se of the VLDL constituents, and the transit of the immature VLDL particles so generated through the secretory pathway do not depend upon active protein or apolipoprotein synthesis. Further evidence for this conclusion comes from our finding (Table II) that, in the presence of puromycin, newly-

synthesized nascent chains of apolipoprotein B assemble with apolipoprotein II, triacylglycerol, and phosphoglyceride and are secreted as immunoprecipitable VLDL particles with a kinetic pattern identical with that for completed apolipoprotein B chains (Fig. 5A). The assembly and intracellular transit of VLDL are not influenced by apolipoprotein B glycosylation, since secreted, unglycosylated apolipoprotein B is associated with the usual complement of VLDL constituents [20,34]. Therefore, apolipoprotein synthesis, but not apolipoprotein glycosylation, plays a definite regulatory role in VLDL assembly. Since the integrity of intracellular apolipoprotein B nascent chains at the endoplasmic reticulum is preserved only when they associate with lipid (mainly triacylglycerol) [21], the decisive regulatory event of VLDL biogenesis may be the formation of nascent, triacylglycerol-rich lipoprotein particles, which can then undergo modification along the secretory pathway to yield mature VLDL. The mechanism(s) by which the molecular specificity of constituents assembled into VLDL is maintained remain to be elucidated.

Our investigations indicate that biogenesis of the mature, secreted VLDL particles takes place by the ordered, multistep processing and assembly of molecular constituents (apolipoproteins and lipids) along the secretory pathway (Fig. 8). Such a sequential mode of assembly would imply that the composition of the immature VLDL particle changes while it is in transit to the plasma membrane. It is known [20,42] that the apolipoproteins of the VLDL particle can carry variable amounts of lipid as the physiological situation warrants. That the formation of biological membranes also involves a temporally ordered, sequential association of proteins and lipids [43,44] raises the prospect of a general strategy with which cells form stable supramolecular assemblies of protein and lipid in an aqueous milieu.

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