Biosynthesis of High density Lipoprotein by Chicken Liver: Conjugation of Nascent Lipids with Apoprotein A1

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ABSTRACT To study the assembly of newly synthesized lipids with apoprotein A1, we administered [2-^{3}H]glycerol to young chickens and determined the hepatic intracellular sites of lipid synthesis and association of nascent lipids with apoprotein A1. [2-^{3}H]glycerol was rapidly incorporated into hepatic lipids, reaching maximal levels at 5 min, and this preceded the appearance of lipid radioactivity in the plasma. The liver was fractionated into rough and smooth endoplasmic reticulum and Golgi cell fractions. The isolated cell fractions were further subfractionated into membrane and soluble (content) fractions by treatment with 0.1 M Na_2CO_3, pH 11.3. At various times, the lipid radioactivity was measured in each of the intracellular organelles, in immunoprecipitable apoprotein A1, and in materials that floated at buoyant densities similar to those of plasma lipoproteins. Maximal incorporation occurred at 1 min in the rough endoplasmic reticulum, at 3–5 min in the smooth endoplasmic reticulum, and at 5 min in the Golgi cell fractions. The majority (66–93%) of radioactive glycerol was incorporated into triglycerides with smaller (4–27%) amounts into phospholipids. About 80% of the lipid radioactivity in the endoplasmic reticulum and 70% of that in the Golgi cell fractions was in the membranes. The radioactive lipids in the content subfraction were distributed in various density classes with most nascent lipids floating at a density ≦1.063 g/ml. Apoprotein A1 from the Golgi apparatus, obtained by immunoprecipitation, contained sixfold more nascent lipids than did that from the endoplasmic reticulum. These data indicate that [2-^{3}H]glycerol is quickly incorporated into lipids of the endoplasmic reticulum and the Golgi cell fractions, that most of the nascent lipids are conjugated with apoproteins A1 in the Golgi apparatus, and that very little association of nascent lipid to apoprotein A1 occurs in the endoplasmic reticulum.

High density lipoprotein (HDL)[1] represents one of the major classes of plasma lipoproteins. Plasma HDLs are small, spherical particles, ~8–10 nm diam, which float at a density of between 1.063 and 1.21 g/ml and consist of a layer of phospholipids, free cholesterol, and apoproteins surrounding a membrane lipid core composed mainly of cholesterol esters and triglycerides (8, 16, 20, 28, 46).

In mammals the study of HDL synthesis and secretion is complicated by the fact that there is more than one site of apoprotein and lipid synthesis. In addition, there are several apoproteins that are shared by both very low density lipoprotein (VLDL) and HDL (8, 16, 19, 20, 46). In recent years, the avian species has attracted considerable attention as an animal model for the study of the biosynthesis and secretion of HDL. It has been reported that in young chickens, plasma HDL is 92% of the total serum lipoproteins (33), that 90% of the protein content of HDL is composed of one apoprotein (27, 32), and that liver is the main site of apoprotein and lipid biosynthesis (3, 5, 20, 39, 42).

The various steps of biosynthesis, intracellular transport, and secretion of lipoproteins have been studied extensively (1, 6, 17, 18, 22, 38, 47, 48) and the intracellular site of synthesis of proteins (1, 3, 6), lipids (17, 18, 38, 44, 47, 48), and carbohydrate moieties (43) have been identified. How-

[1] *Abbreviations used in this paper:* HDL, high density lipoprotein; RER and SER, rough and smooth endoplasmic reticulum, respectively; VLDL, very low density lipoprotein.

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ever, none of these studies has clarified the exact cellular sites at which the attachment of the various lipid components to apoproteins occurs or the mechanism governing this assembly process. Recently, we have shown that the newly synthesized apoprotein A1, though present within the rough (RER) and smooth (SER) endoplasmic reticulum fractions, failed to float between densities of 1.063 and 1.21 g/ml, whereas the apoprotein A1 which is present within the Golgi complex is capable of floating at the HDL buoyant density (3). These results indicate that apoprotein A1 has not acquired its full complement of lipids in the endoplasmic reticulum fraction and that most of the lipids are assembled with apoprotein A1 within the Golgi apparatus. Pulse-chase studies of estrogen-induced chick parenchymal liver cells with [^{3}H]leucine and [^{3}H]glycerol suggest that VLDL particles are formed by a multistep assembly of apoproteins and the lipid components of VLDL (29). However, the sequential association of lipids and apoproteins in isolated hepatic cellular fractions has not been demonstrated. In this study we have examined the hepatic sites of incorporation of [2-^{3}H]glycerol into phospholipids and acylglycerides and the time and cellular location at which these nascent lipids combine with apoprotein A1 to form nascent HDL particles.

MATERIALS AND METHODS

Materials

Sucrose, ultrapure grade, was obtained from Schwarz/Mann (Div. of Becton Dickinson, Orangeburg, NY), [2-^{3}H]glycerol, 500 mCi/mmol, from Amersham Corp. (Arlington Heights, IL), and all other chemicals from Fisher Scientific Co. (Pittsburgh, PA).

Antiserum against rooster HDL apoprotein was prepared in female albino rabbits and the specificity was checked by immunoelectrophoresis and by immunodiffusion on agar plates as described in our previous study (3). 1 ml of anti-rooster HDL serum precipitated ~0.15 mg of HDL-apoprotein.

Injection of Animals

Young Leghorn chickens, 5-d old, supplied by Spafas Poultry Farms (Norwich, CT), were maintained in a light- and temperature-controlled room for 24 h. They had free access to water and were fed Purina Chick Starter Chow. Young birds were placed under light ether anesthesia and [2-^{3}H]glycerol (200 μCi/100 g, body wt), evaporated and resuspended in 0.15 M NaCl, was injected into the jugular vein. The birds, at specified times, were killed by decapitation. For each time point two or three birds were used. Blood, when necessary, was collected by heart puncture.

Cell Fractionation

All centrifugations were performed in a Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) and all operations were carried out at 4°C. Livers (8–10 g) were homogenized in 0.25 M sucrose filtered through a layer of cheese cloth, and a postmitochondrial supernatant fraction was obtained by centrifuging the homogenate at 16,000 *g* for 10 min. A total microsomal pellet consisting of a mixture of RER and SER and Golgi elements was obtained by centrifuging the postmitochondrial supernatant at 105,000 *g* for 90 min. Individual organelles were separated from the microsomal pellet by the following procedure. The microsomal pellet was suspended in a small volume of 0.25 M sucrose and adjusted to 1.38 M sucrose by addition of sufficient 2.1 M sucrose. The RER, SER, and Golgi cell fractions were isolated from this suspension by a combination of the procedures of Ehrenreich et al. (12) and Ragland et al. (40). 10-ml aliquots of the adjusted microsomal suspension were layered over a 10-ml cushion of 2.0 M sucrose and overlaid with a discontinuous sucrose gradient composed of 10 ml of 1.1 M sucrose followed by 5 ml of 0.6 M sucrose and 3 ml of 0.25 M sucrose. The gradient was centrifuged for 18 h at 82,500 *g* in a Beckman SW-27 rotor. The Golgi-rich fraction floated at the interface between 0.6 and 1.1 M sucrose, the SER-enriched fraction concentrated at the interface between 1.1 and 1.38 M sucrose, and the RER fraction remained at the boundary between 2.0 and 1.38 M sucrose. The membrane layers were collected individually, diluted with water to obtain a final sucrose concentration of 0.25 M, and harvested as pellets by sedimenting in a Beckman 60 Ti rotor at 105,000 *g* for 60 min. Unless otherwise stated, all sucrose solutions discussed in this paper were in water.

Lipid Extraction and Determination of Radioactivity

Aliquots from the liver homogenate, the RER, SER, and Golgi cell fractions, and from plasma were treated with equal volumes of cold 10% trichloroacetic acid and the resulting precipitates were washed three times with cold 5% trichloroacetic acid and once with ice-cold water. Lipids were obtained from the washed pellet by two extractions with ethanol-ether (3:2 [vol/vol]) at room temperature (32). The ethanol-ether extract was used for the determination of radioactivity and for further separation of lipids into triglycerides and phospholipids. We measured the radioactivity incorporated into the ethanol-ether extract by drying the samples in glass vials, adding 10 ml of Hydrofluor (National Diagnostic, Inc., Somerville, NJ), and determining radioactivity in a liquid scintillation counter.

For thin-layer chromatography, aliquots of the ethanol-ether extracts were dried under nitrogen atmosphere and the dried lipids were dissolved in a small volume of chloroform and applied on Uniplates (Analtech, Inc., Newark, DE). We separated the phospholipids and neutral lipids by developing duplicate plates in hexane-ether-acetic acid (80:20:1 [vol/vol]) (45). The chromatographed lipids were visualized by treatment with iodine vapor. From one plate, the stained lipids were scraped into scintillation vials and radioactivity was measured as described above. From the other plate, the lipids were similarly removed and used for triglyceride and lipid phosphorus determination. Triglycerides were measured by the method of Carlson (7), using tripalmitate as standard, and phosphorus was determined as previously described (3).

Isolation of Nascent Lipids from Intracellular Organelles

The membranous organelles were disrupted by treatment with 0.1 M Na_2CO_3, pH 11.3, as described by Fujiki et al. (15) and by Howell and Palade (24), and the membranes were separated from the content subfractions by centrifugations. Radioactive lipids that were either associated with apoprotein A1 or that could float were then isolated from the various organelle contents either by sequential flotation following the precedure of Havel et al. (23), as previously described (3), or by immunoprecipitations using rabbit antiserum to rooster serum apoprotein A1.

Immunoprecipitation of [2-^{3}H]Glycerol-labeled Lipids

The content subfractions were dialyzed extensively with buffered saline (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) and four equal aliquots, each containing 1 g tissue equivalent, were taken. They were treated as follows: (*a*) with 100 μl of rabbit antiserum to rooster HDL; (*b*) with 100 μl of rabbit preimmune serum; (*c*) with 100 μl rabbit antiserum to chicken albumin and 100 μl of 10-times diluted chicken serum; and (*d*) with 100 μl of rabbit antiserum to rat plasma and 100 μl of 100-times diluted rat plasma. They were all incubated at 37°C for 60 min followed by 18 h at 4°C. The antigen-antibody precipitates were collected by sedimentation through a discontinuous buffered sucrose gradient as detailed by Janero and Lane (29) and washed several times with buffered saline. The sample treated with preimmune rabbit serum did not contain precipitated material. The radioactive lipids were extracted from the washed immunocomplex by two treatments with ethanol-ether (3:2 [vol/vol]) and the extract was used for the determination of radioactivity. The mean radioactivity present in the control samples was subtracted from the experimental value and expressed as counts per minute per gram of liver. In a typical experiment, at 10 min after the administration of [2-^{3}H]glycerol, the actual counts were 3,941 in the experimental samples, 140 in the preimmune rabbit serum, 230 in rabbit anti-chicken albumin, and 240 in rabbit anti-rat plasma controls.

Bio-Gel A-5m Chromatography

The samples obtained by either immunoprecipitation or flotation were mixed with 4 mg of carrier rooster serum HDL and applied to 6% beaded agarose gel (Bio-Gel A-5m, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA) columns (0.5 × 52 cm). Lipoprotein particles were eluted with 200 mM NaCl, 1 mM EDTA, and 0.02% NaN_3 as described by Kruski and Scanu (32). The elution profile of rooster serum HDL was monitored by the absorbance at 280 nm and aliquots were taken for the determination of lipid radioactivity.

Nondenaturing Polyacrylamide Gradient Slab Gel Electrophoresis

Gradient gel electrophoresis was carried out following the procedure of Anderson et al. (2). Radioactive lipids, obtained by immunoprecipitation or by sequential flotation either from the Golgi cell fractions or from serum, were equilibrated in Tris-borate buffer (0.09 M Tris, 0.08 M boric acid, 0.1 g/liter EDTA, and 0.5 g/liter NaN_3, pH 8.35) and applied to a 4–30% polyacrylamide gradient slab gel (Pharmacia Inc., Piscataway, NJ). Electrophoresis was carried out on a Pharmacia GE-4 electrophoresis apparatus at 150 V for 24 h at 10°C in the equilibrating buffer. For molecular size determinations the following proteins of diameter 13.3–7.4 nm (13), supplied by Pharmacia Inc., were used: thyroglobulin, apoferritin, catalase, lactic dehydrogenase, and BSA. In addition, rooster serum HDL was used as a marker protein. The standard proteins were stained with Coomassie Blue and the lipids were visualized by staining overnight with 0.04% Oil red O in 60% ethanol. We detected the radioactive proteins by cutting the gel into 0.5-mm slices and determining radioactivity in the excised pieces. The slices were digested at 70–75°C in 0.1 ml of hydrogen peroxide and 0.2 ml of perchloric acid followed by counting in 10 ml of Scintiverse II (Fisher Scientific Co.).

Characterization of Subcellular Fractions

BIOCHEMICAL: To determine the purity of the isolated organelles and their recovery from the homogenate, we assayed the cell fractions for glucose-6-phosphatase and galactosyl transferase as described by Howell and Palade (26). RNA was determined by the method of Mejbaum (37), and protein by the method of Lowry et al. (34) using BSA as standard.

MORPHOLOGICAL: The isolated fractions were fixed in suspension at 4°C immediately after preparation by the addition of an equal volume of ice-cold 4% paraformaldehyde-glutaraldehyde mixture in 0.2 M cacodylate buffer, pH 7.4. After 30 min, the suspensions were centrifuged in a Beckman SW 50.1 rotor for 20 min at 37,500 *g*, and the pellets were stained, dehydrated, embedded, and sectioned as described earlier (3).

Lipid particles, collected at different density classes from the contents of RER, Golgi cell fractions, and plasma, were dialyzed extensively against 0.14 M NaCl and 0.001 M EDTA and negatively stained by using 2% phosphotungstic acid (pH 6.4) as described earlier (3). The stained samples were examined and photographed on the same day. Electron microscopy was performed with a Philips 410 electron microscope.

RESULTS

Incorporation of [2-^{3}H]Glycerol into Hepatic and Plasma Lipids

Preliminary experiments were performed to establish the time required for the intracellular synthesis of hepatic lipids and to determine the temporal relationship of radioactive intracellular lipids to that of plasma lipids. The results of such an experiment are shown in Fig. 1. The incorporation of radioactive glycerol into hepatic lipid was rapid and reached a peak within 5 min and dropped to 80% of its maximal level by 15 min. During the first 3 min there was only a small amount of radioactive lipid in the plasma, but after 3 min it rose linearly, reaching the maximal amount at 15 min.

Characterization of Cell Fractions

In this study the procedures previously established for the isolations of RER, SER, and Golgi cell fractions from chicken liver (3) were modified to simplify the method. The organelles prepared in this manner were characterized by morphological and biochemical methods.

Examination of the isolated cell fractions by electron microscopy showed that the RER fraction was composed of polysomes bound to the outer surface of membrane vesicles. In contrast, the SER cell fraction consisted mainly of smooth vesicles with some Golgi cisternal elements. The Golgi cell fractions consisted of a mixture of Golgi cisternal elements and Golgi vesicles, some filled with dense material and some broken and empty. A few, scattered, intact or broken smooth membranes, some of which were also studded with ribosomes, were also present (Fig. 2).

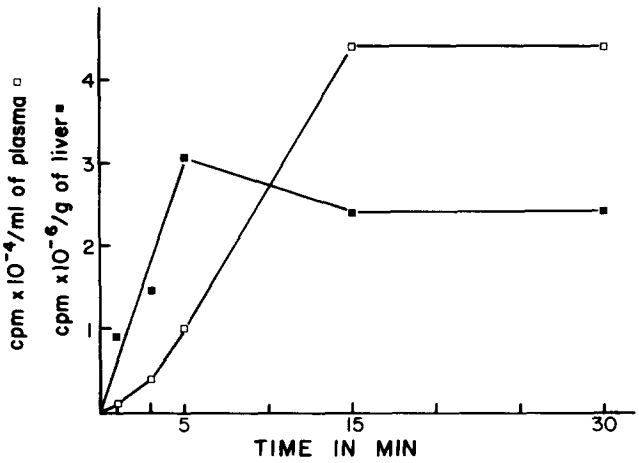

FIGURE 1 Time course of incorporation of [2-^{3}H]glycerol into hepatic and plasma lipids. Young chickens were injected intravenously with [2-^{3}H]glycerol (200 μCi/g body wt) and the blood and livers were collected at specified times. We isolated radioactive lipids from the liver homogenate and from plasma. Details are given in Materials and Methods. ■, liver; □, plasma.

Glucose-6-phosphatase analyses indicated that most of this enzyme activity was localized in the RER and SER cell fractions whereas galactosyl transferase was preferentially localized in the Golgi cell fractions (Table I). RNA and protein determinations indicated that the RER had an RNA to protein ratio of 0.34, which is eightfold higher than that of the SER and Golgi cell fractions. On the basis of a previously published report (4) that 60% of the RNA in the homogenate is present in membrane-bound polysomes, we estimated that the recovery of RER cell fractions was 50–55%. Although most of the galactosyl transferase was recovered in the Golgi cell fraction (32.8%), significant amounts were present in the SER (20.1%) and the RER fractions (8.8%) (Table I).

Time Course of Incorporation of [2-^{3}H]Glycerol into Lipids of Different Subcellular Organelles

To determine the intracellular sites of attachment of the newly synthesized lipids to apoprotein A1 and the subsequent formation of nascent lipoproteins, it was first necessary to examine the kinetics of incorporation of [2-^{3}H]glycerol into the lipids of organelles that are thought to participate in plasma protein secretion. To do this, young chickens were administered [2-^{3}H]glycerol, at specified times the liver was removed and fractionated into RER, SER, and Golgi cell fractions, and the incorporation of radioactive glycerol into the lipids of these fractions was measured. The incorporation was found to be very rapid. Within 1 min after the intravenous administration of radioactive glycerol, lipid radioactivity reached its peak in the RER. Maximal incorporation into lipids of the SER occurred within 3 min and in the Golgi cell fractions at 5 min. In all three fractions the lipid radioactivity dropped to 50% of its maximal level by 15 min (Fig. 3).

Distribution of Nascent Lipid in Membrane and Cisternal Content

Newly synthesized plasma proteins are generally seques-

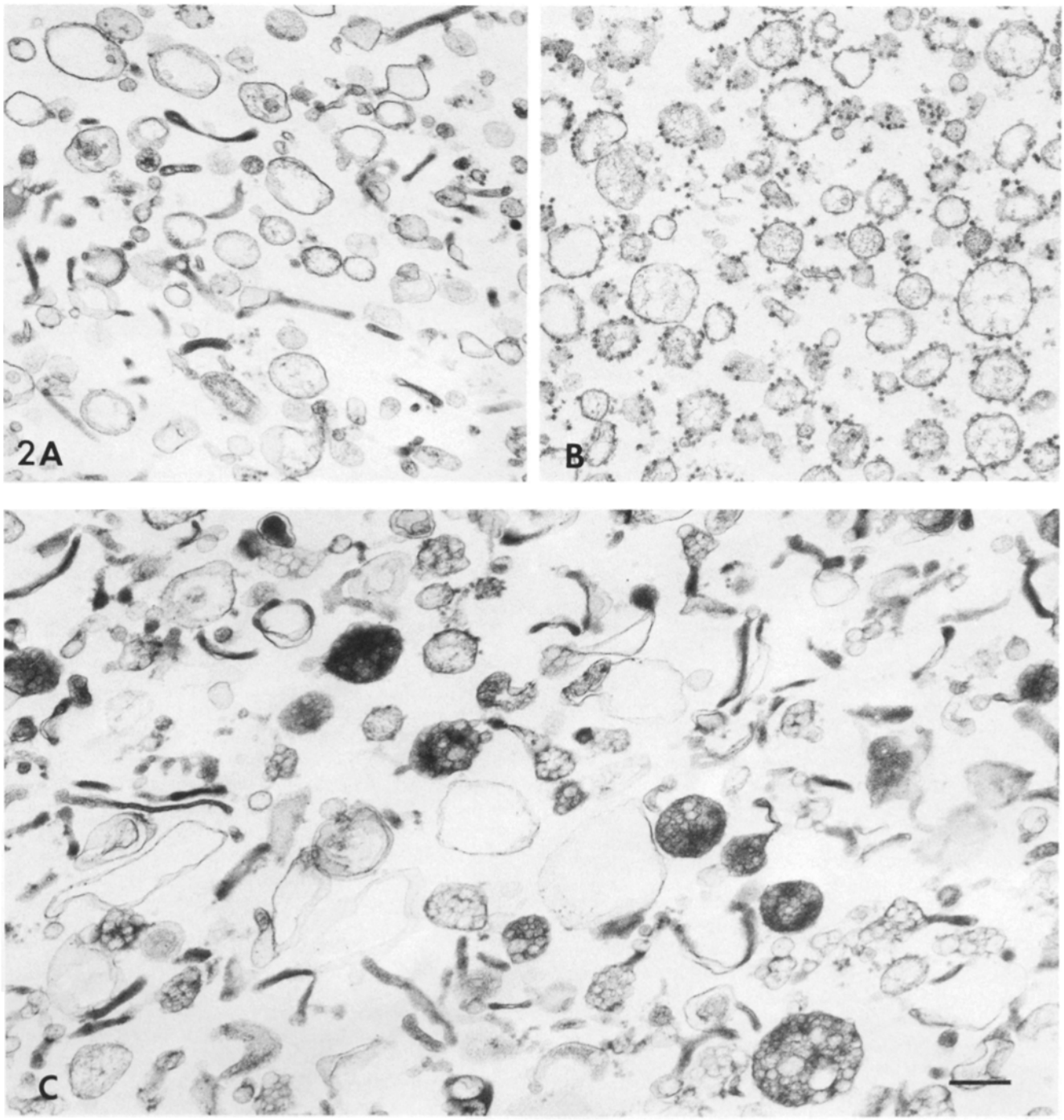

FIGURE 2 Electron micrographs of RER, SER, and Golgi cell fractions. We prepared the cell fractions from livers of young chickens and fixed as described in Materials and Methods. (*A*) SER consisting of smooth membrane vesicles and some Golgi elements. (*B*) RER fraction consisting mostly of membrane vesicles bound with ribososomes. (*C*) Golgi cell fraction; the predominant elements are secretion vacuoles, some of which are filled with lipoprotein particles. Many flattened Golgi cisternae are also present. Bar, 250 nm. × 40,000.

tered within the cisternal spaces of the RER, SER, and Golgi cell fractions during transport from their site of synthesis to the site of discharge. Conventionally, the nascent proteins are recovered from the cisternal space by membrane rupture with detergents. However, since the use of detergent would release lipids from the membrane, the membranous organelles were disrupted by treatment with 0.1 M Na_2CO_3, pH 11.3 (15, 24), and the membranes were separated from the cisternal content by centrifugation.

Lipid radioactivity was measured 10 min after the administration of [2-^{3}H]glycerol in the total cell fractions, in the membrane and content subfractions, and in material that floated at buoyant densities similar to those of plasma lipoproteins. In the RER cell fraction, ~17% of the lipid radioactivity was recovered in the content subfraction and 81% was incorporated into the membrane subfraction. In the Golgi cell fractions, 29% of the radioactive lipids were present in the content subfraction and 70% were in the membrane

TABLE I

Recoveries of RNA, Glucose-6-Phosphatase, and N-acetylglucosamine Galactosyl Transferase in Cell Fractions

Fractions	Protein*	RNA*	Ratio RNA/protein	Glucose-6-phosphatase Sp act‡	Glucose-6-phosphatase Rel act§	Galactosyl transferase Sp act‖	Galactosyl transferase Rel act¶
	mg/g liver						
Homo	152	8.52 (100)	0.05	0.54	82.2 (100)	6.3	957 (100)
Microsome (105,000 g pellet)	16.2	3.04 (35.6)	0.18	0.75	12.2 (14.8)	49	686 (71.6)
RER	6.78	2.36 (27.6)	0.34	1.5	10.17 (12.3)	12.4	84.3 (8.8)
SER	3.84	0.16 (1.9)	0.04	1.2	4.60 (5.5)	50.3	193 (20.1)
GF	0.46	0.02 (0.2)	0.04	0.4	0.18 (0.21)	393	314 (32.8)

Values in parentheses are percent recoveries.
* Wet weight.
‡ Micromoles of P_i released per 30 min/mg of protein.
§ Micromoles of P_i released per 30 min/mg of protein.
‖ Nanomoles galactose transferred per 30 min/mg of protein.
¶ Nanomoles galactose transferred per 30 min/g of liver.

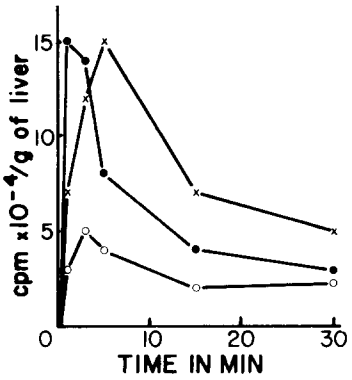

FIGURE 3 Incorporation of [2-^{3}H]glycerol into glycerolipids of RER, SER, and Golgi cell fractions. [2-^{3}H]-glycerol (specific activity, 500 mCi/mmol) was injected into the jugular vein (200 μCi/100 g body weight) of young chickens. At various times the livers were removed and fractionated into RER, SER, and Golgi cell fractions as described in Materials and Methods. All time points given in this and the following figures represent the entire period after injection until the livers were placed on ice cold sucrose and minced. The incorporation of radioactivity into lipids is expressed per unit weight of liver. ●, RER; O, SER; ×, Golgi cell fractions.

TABLE II

Distribution of [2-^{3}H]glycerol-labeled Lipids

Subfractions	RER	Golgi
	% of radioactive lipids	
Membrane	81.2 ± 5.57	70.2 ± 1.63
Content	17.3 ± 6.71	28.4 ± 4.31
$d \leqq 1.063$	9.2 ± 2.3	19.1 ± 1.73 (1.2)
$1.063 < d < 1.21$	2.2 ± 0.9	6.5 ± 1.25 (12.0)
$d > 1.21$	5.0 ± 0.8	2.1 ± 0.75 (3.0)

Radioactive glycerol was administered intravenously 10 min before the chickens were sacrificed. The values given are a mean percent of total lipid radioactivity in the organelles from three experiments. Numbers within parenthesis represent percent radioactivity immunoprecipitated with rabbit antibody to rooster HDL.

TABLE III

Incorporation of [2-^{3}H]glycerol into Phospholipids and Triglycerides

Cell fractions	Membrane Phospholipids	Membrane Triglycerides	Content Phospholipids	Content Triglycerides	Apoprotein A1 Phospholipids	Apoprotein A1 Triglycerides
			% radioactivity			
RER	27	66	6	84	5	90
SER	26	64	12	84	8	84
Golgi	4	93	3	92	13	82

[2-^{3}H]glycerol was administered in vivo for 15 min. The RER, SER, and Golgi cell fractions were isolated and subfractionated into membrane and content subfractions. Apoprotein A1 was obtained from the content subfraction by immunoprecipitation. The values are the mean determinations from three experiments. They represent the percent of total lipid radioactivity recovered by thin-layer chromatography in the phospholipid and triglyceride areas.

subfraction (Table II).

To determine the types of lipids into which [2-^{3}H]glycerol is incorporated at different cellular locations, the radioactive lipids from various organelles were separated by thin-layer chromatography into phospholipids, monoglycerides, diglycerides, triglycerides, cholesterol, and cholesterol esters. Significant radioactivity was only detected in the phospholipids and triglycerides, with most of it found in the triglycerides (Table III). In the membrane subfraction of the RER and SER, 26–27% of the radioactivity was in the phospholipids and 64–66% was in triglycerides. In the membranes of the Golgi cell fraction only 4% of the radioactivity was incorporated into phospholipids and 93% in triglycerides. In the cisternal contents of all cell fractions analyzed, most of the radioactivity (84–92%) was incorporated into triglycerides with little radioactivity (3–12%) in phospholipids (Table III).

The content subfractions of the RER and Golgi complex contained radioactive lipids that floated at a density (d) ≦ 1.063, at $1.063 < d < 1.21$ g/ml, and at $d > 1.21$ g/ml. In both the RER and the Golgi cell fractions most of the radioactivity occurred at $d \leqq 1.063$ g/ml. However, all floated subfractions contained significant lipid radioactivity (Table II). Similar results were obtained from the SER cell fraction, but since morphologically this appears to be a mixture of

endoplasmic reticulum and Golgi cisternal elements, the results are not presented.

The radioactive floated materials obtained from the Golgi cell fractions were treated with antiserum to rooster serum HDL. Only 1.2% of the radioactive lipids that floated at $d \leqq 1.063$, and 3% of those at $d > 1.21$, were immunoprecipitated. By contrast, a larger amount, 12%, of the radioactivity that floated between 1.063 and 1.21 g/ml was immunoprecipitated by the antibody (Table II).

Specific Radioactivity of Nascent Lipids in Organelle Membranes and Contents

At various times, from 1 to 30 min, after the administration of [2-^{3}H]glycerol to the chickens, the specific radioactivity (counts per milligram of phospholipid and counts per milligram of triglycerides) of the lipids from the membrane and content subfractions, separated by thin-layer chromatography, was determined. Maximal specific radioactivity of triglycerides occurred at 1 min in the membranes of the RER and at 2 to 3 min in the Golgi membranes. In the contents of these organelles, maximal triglyceride specific radioactivity occurred at 3 min in the RER and 5 min in the Golgi apparatus. These data suggest that triglycerides are rapidly transferred from the RER to the Golgi complex, Moreover, the specific radioactivity of the membrane triglycerides was higher than that of the content triglycerides (Fig. 4, *A* and *B*).

By contrast, the nascent phospholipids reached maximal specific radioactivity at the same time in both the RER and the Golgi apparatus. In the membranes of the RER and the Golgi apparatus, the phospholipids reached maximal specific radioactivity at 3 min, and in the content subfraction at 5 min. The specific radioactivity of the content phospholipids was about twice that of the membrane phospholipids (Fig. 4, *C* and *D*).

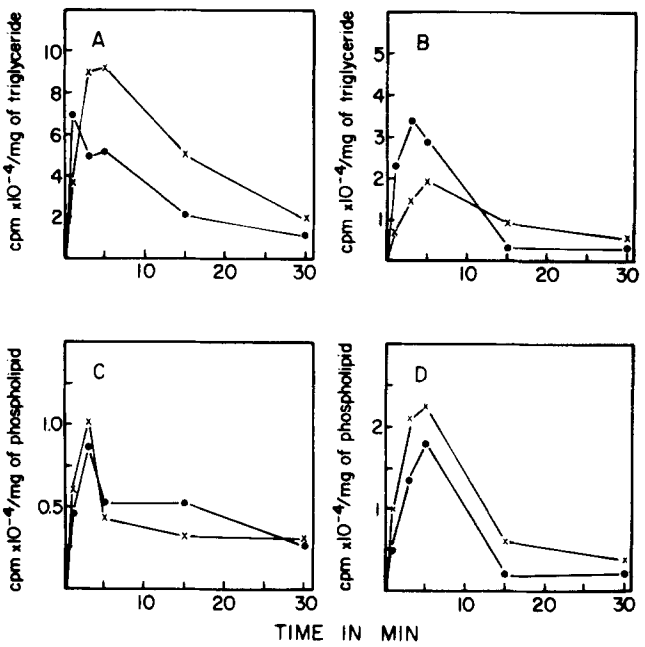

FIGURE 4 Incorporation of [2-^{3}H]glycerol into lipids of the membrane and content subfractions obtained from the RER and the Golgi cell fractions. We administered radioactive glycerol to chickens and isolated the RER and Golgi cell fractions as described in the legend to Fig. 3. We further fractionated the RER and the Golgi cell fractions into membrane and content subfractions by treatment with 100 mM Na_2CO_3 (pH 11.3). *A* and *B* show the triglyceride radioactivity; *C* and *D* show the phospholipid radioactivity. *A* and *C* show the membrane subfractions; *B* and *D* show the radioactivity in the content subfractions. ●, RER; ×, Golgi cell fractions.

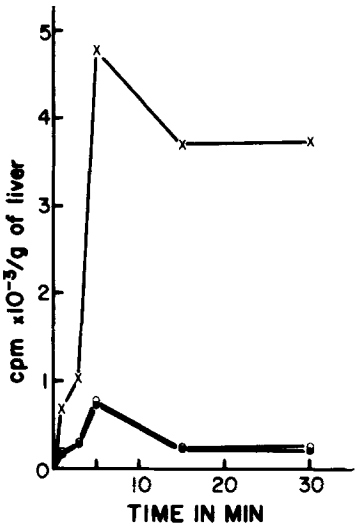

FIGURE 5 Intracellular site of conjugation of nascent lipids to apoprotein A1. We injected [2-^{3}H]glycerol (specific activity, 500 mCi/mmol) intravenously (200 μCi/100 g body wt) into the jugular vein of young chickens. We killed the birds at different time intervals, removed the livers, and prepared and subfractionated the RER, SER, and Golgi cell fractions into membrane and contents as described in Materials and Methods. Apoprotein A1 was isolated from the content subfractions by immunoprecipitation, and the lipid radioactivity in the washed antigen-antibody complex was determined. The lipid radioactivity associated with apoproteins A1 is expressed per unit weight of liver. ●, RER; O, SER; ×, Golgi.

Cellular Sites at which Nascent Lipids Interact with HDL Apoproteins

Radioactive glycerol is readily incorporated into lipids and a significant portion of the radioactive lipids appeared in the cisternal contents of the endoplasmic reticulum and the Golgi complex (Fig. 4). To determine the amount of radioactive lipids associated with apoprotein A1, this protein was isolated from the RER, SER, and Golgi cell fractions by immunoprecipitation and the amount of radioactive lipids associated with the antigen-antibody complex was measured at various times.

Only small amounts of radioactive lipids were recovered from the antigen-antibody complex obtained from the contents of the RER and SER cell fractions whereas substantially more (sixfold) lipid radioactivity was recovered from the Golgi content subfraction. In the Golgi cell fraction the conjugation of radioactive lipid with apoprotein A1 reached a peak within 5 min after the administration of [2-^{3}H]glycerol and dropped to 80% of its maximal level by 30 min, which was the duration of the experiment (Fig. 5).

The small amount of nascent lipids that was complexed to apoprotein A1 in the RER and SER cell fractions was mostly (84–90%) in the form of triglycerides and only 5–8% in phospholipids. In contrast, the apoprotein A1 in the Golgi apparatus had a greater percent of its radioactivity (13%) in phospholipids (Table III), but the triglycerides still contained most (82%) of the radioactivity.

Nondenaturing PAGE and Agarose Chromatography of Immunoprecipitated and Floated Radioactive Lipids

To further establish that the radiolabeled lipids were in association with apoprotein A1, the radiolabeled lipids were isolated either by immunoprecipitation or by flotation from serum and from the Golgi fraction and subjected to both Bio-Gel A-5m column chromatography and nondenaturing PAGE. Radioactive lipids that were floated from the serum at d = 1.063–1.21 g/ml eluted as a single peak, in a manner similar to rooster serum HDL, on Bio-Gel A-5m column chromatography. However the majority of radioactive lipids obtained at a density of 1.063–1.21 g/ml from the Golgi cell fractions eluted as larger particles than rooster serum HDL. The Golgi samples appeared not to be homogeneous. Most of the radioactivity was eluted following the void volume of the column but some radioactivity trailed the major peak and appeared as a broad area which ran into the position at which serum HDL was eluted (Fig. 6).

In nondenaturing polyacrylamide gradient gel electrophoresis, rooster serum HDL appeared to be heterogeneous. When stained for protein (Coomassie Blue) there was a broad area from 9.5 to 13.3 nm and a more discreet band at 7.5 nm. When stained for lipids (Oil Red O) the particles extended from the top of the gel to the 9.5-nm size marker. The radioactive lipids obtained from chicken serum HDL were also distributed as a broad band with sizes ranging from 8 to 13.3 nm. Most of the lipid radioactivity was at 8.6–10.2 nm (Fig. 7). But the radioactive lipid particles, floated from the Golgi cell fraction at a density similar to that of serum HDL, failed to penetrate the 4–30% gel, again suggesting that Golgi HDL is much larger in size than newly secreted serum HDL.

The radioactive lipids obtained by antibody precipitation, either from serum HDL or from Golgi fractions, failed to penetrate either the 4–30% polyacrylamide gel or the Agarose Bio-Gel column, indicating again that radioactive lipids remained complexed to the immunoprecipitates. Attempts to first disrupt the immunoprecipitate by treatment with 6 M urea or by lowering the pH to 2.5 stripped the radioactive lipids from proteins.

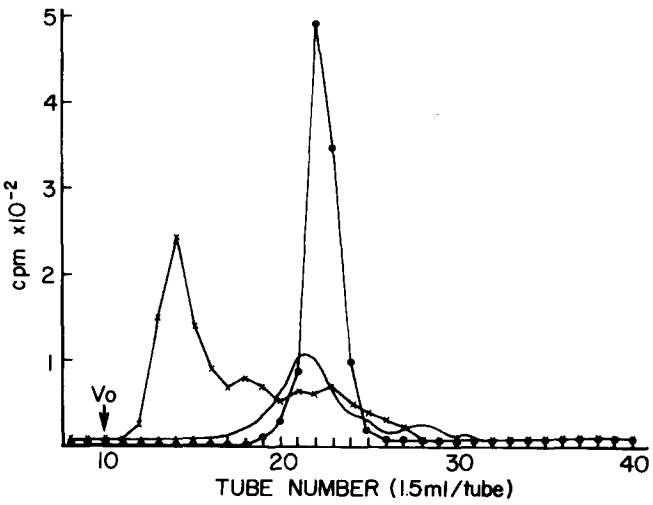

FIGURE 6 Agarose gel chromatography of Golgi and serum HDL. We analyzed the following samples by column chromatography on Agarose (Bio-Gel A-5m) gels as described in Materials and Methods: (*a*) tracing of the protein absorbance of rooster serum HDL (——); (*b*) Golgi-derived radioactive lipid particles that floated at d = 1.063–1.21 g/ml, 1,270 cpm (×); and (c) radioactive chicken serum HDL, obtained 30 min after the administration of [2-^{3}H]glycerol, 1,500 cpm (●).

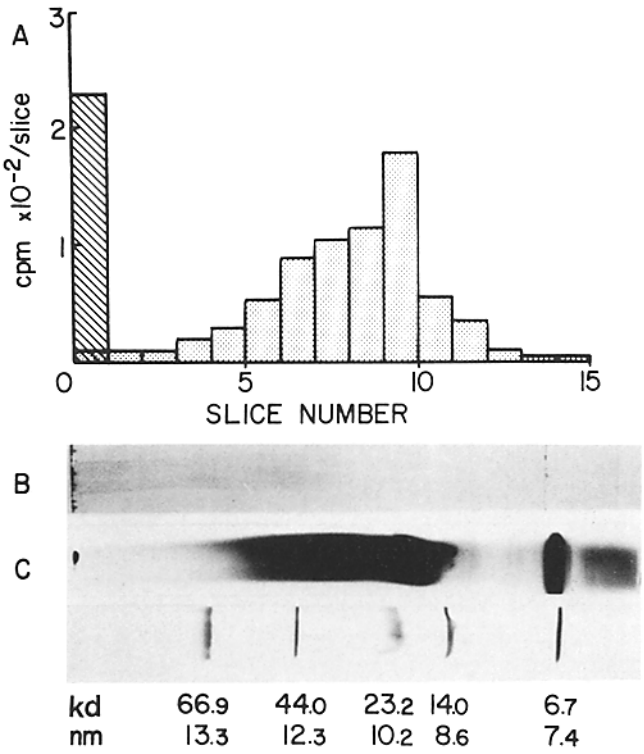

FIGURE 7 PAGE patterns of radioactive lipids isolated from Golgi fractions and from serum. We injected young chickens (95 g body wt) with 200 μCi/100 g body wt of [2-^{3}H]glycerol. One half hour after injection, the bird were decapitated, blood was collected, and the liver was removed and then fractionated into various cell fractions. The radioactive lipids from Golgi cell fractions and from serum were collected by sequential flotation at d = 1.063–1.21 g/ml and analyzed by electrophoresis in nondenaturing conditions in 4–30% polyacrylamide gradient gels as described in Materials and Methods. We stained the gel lanes that contained molecular size markers by Oil Red O or Coomassie Blue, cut and digested those that contained radioactive lipids into 0.5-mm slices, and determined the radioactivity as described in Materials and Methods. *A* shows the radioactive pattern of Golgi-derived HDL (diagonal lines) and serum HDL (dotted bars); *B* shows the lipid pattern of rooster HDL, stained with Oil Red O; *C* shows the Coomassie Blue-stained protein pattern of rooster HDL. The locations in the gels of protein markers are indicated in terms of molecular weight (kilodaltons) and estimated diameters are in nanometers (*nm*).

Morphology of Lipids Contained in the RER and Golgi Cell Fraction

The content subfractions of the RER and Golgi cell fractions contained nascent lipids (Table II) which float at a buoyant density equivalent to that of plasma VLDL–low-density lipoproteins ($d \leqq 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml). To study the nature of these radioactive lipids, we negatively stained the material that floated at $d \leqq 1.063$ and at $1.063 < d < 1.21$ g/ml with phosphotungstic acid and examined it by electron microscopy. The lipids obtained at $d \leqq 1.063$ g/ml from the RER appeared as spherical particles of widely different sizes, ranging from 10–50 nm diam (Fig. 8*A*). Those obtained from the Golgi apparatus were for the

most part larger (20–60 nm) and slightly irregular in shape and contained some larger lipid droplets and a few flattened disk-like structures (Fig. 8*B*).

The particles obtained at $1.063 < d < 1.21$ g/ml, from the RER, were slightly smaller in diameter (6–20 nm) than those obtained at $d \leqq 1.063$ g/ml (Fig. 8*C*). The particles that floated at $1.063 < d < 1.21$ g/ml, from the Golgi apparatus, appeared to be different from those obtained from the RER

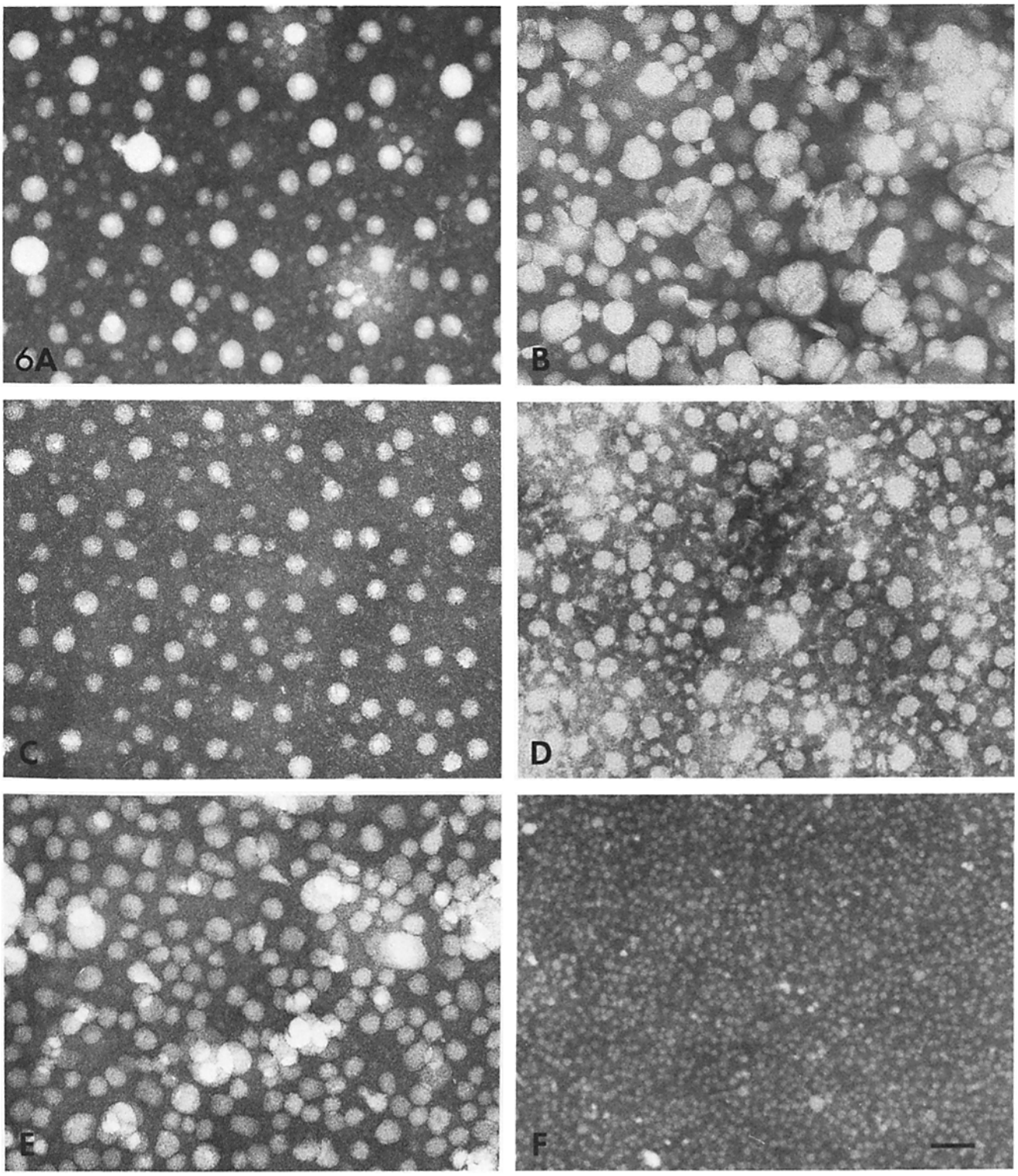

FIGURE 8 Negatively stained preparations of lipid particles obtained from the contents of the RER and Golgi apparatus and from chicken serum. We obtained lipid particles from the contents of the RER and Golgi cell fractions and from serum by buoyant density centrifugation. Particles that floated at $d \leqq 1.063$ and at $1.063 < d < 1.21$ g/ml were collected, negatively stained, and examined by electron microscopy as described in Materials and Methods. (*A*) Particles isolated from the RER at $d \leqq 1.063$ g/ml; (*B*) particles obtained from the Golgi apparatus at $d \leqq 1.063$ g/ml; (C) particles isolated from the RER at $1.063 < d < 1.21$ g/ml; (*D*) particles obtained from the Golgi apparatus at $1.063 < d < 1.21$ g/ml; (*E*) serum VLDL–low-density lipoproteins particles; (*F*) serum HDL particles. Bar, 50 nm. × 150,000.

cell fractions. They were more heterogeneous in size, ranging from 6 to 25 nm diam, and seemed to contain three groups of particles. One group was observed to be spherical while another was irregular in shape. The spherical particles were smaller than the irregular-shaped particles and were comparable in diameter to serum HDL. In addition, this class also contained numerous membrane fragments, some of which were found attached to lipid particles (Fig. 8*D*). The morphology of this latter group of particles appears to be very similar to those described in our earlier report (3). The membrane fragments may be a part of the Golgi membrane which are associated as a contaminant with the floated lipid particles or may represent an intermediate stage in particle formation.

For comparison, lipid particles were also obtained from the serum after similar treatment with 0.1 M Na_2CO_3, pH 11.3, and flotation. The serum lipids that floated at $d \leqq 1.063$ were a heterogeneous mixture of spherical particles, from 14 to 40 nm-diam, which are typical of VLDL and low-density lipoproteins (Fig. 8*E*). Those that floated at $1.063 < d < 1.21$ g/ml were more homogeneous and smaller in size, 7–12-nm diam, and appear to be typical serum HDL particles (Fig. 8*F*).

DISCUSSION

In contrast to most other animals, the circulating lipoproteins of young chickens is mostly (92%) composed of HDL (33), and the appropriate content of the HDL is similar to that of humans in that 90% of its apoprotein is a single protein, apoprotein A1 (27, 32). In mammalians, the major sites of both apoprotein and lipid synthesis are the liver, small intestine, and adipose tissues (19, 20, 47, 48), but in avians apoprotein A1 is also synthesized in such tissues as kidney, aorta, and skeletal muscle (5, 42), although the liver is thought to be the major site of lipid synthesis (39).

It is well established that the endoplasmic reticulum is the principal site of lipid synthesis in hepatocytes (9, 10, 31, 41) and that the enzymes responsible for lipid synthesis are located on the cytosolic side of the endoplasmic reticulum membranes (10). In recent years, the distribution and mobility of the microsomal lipids have been widely studied and the results are often contradictory (49) because newly synthesized lipids are required for both the biogenesis of intracellular membranes (11, 52) and the formation of secretory lipoproteins (4, 17, 47). Our present study shows that all three intracellular organelles, RER, SER, and Golgi apparatus, which are involved in plasma protein secretion, obtain radioactivity into triglycerides and phospholipids soon after the administration of [2-^{3}H]glycerol. Although the in vivo incorporation of [2-^{3}H]glycerol into these lipids is very rapid, and it is difficult to measure kinetics, the results of these experiments suggest that nascent triglycerides are rapidly transferred from the endoplasmic reticulum to the Golgi apparatus. RER membrane triglycerides reach peak specific radioactivity at 1 min and Golgi membrane triglyceride at 2–3 min. Also, the triglycerides in the cisternal spaces of the RER are at a maximum specific radioactivity at 2 min and those in the Golgi fractions 1 min later, at 3 min (Fig. 4). That nascent triglycerides are transferred from the endoplasmic reticulum to the Golgi apparatus is also supported by the findings that the synthesis of triglycerides occurs mainly in the microsomal fractions of liver and fat cells (9, 51) and that diacylglycerol acyltransferase is not present in the Golgi apparatus (50). This implies that radioactive triglycerides present in the Golgi cell fractions are derived from the endoplasmic reticulum.

By contrast, the membrane and content phospholipids of both the RER and the Golgi fraction reach maximal specific radioactivity at the same time—in the membrane at 3 min and in the cisternal contents at 5 min. Since, in addition, the specific radioactivities of both the RER and Golgi phospholipids are similar, in vivo phospholipids are probably synthesized at the same time in both the RER and the Golgi cell fractions. These studies are in accordance with the view that the endoplasmic reticulum is the principal site of phospholipid synthesis in the liver but that the Golgi apparatus also contains enzymes that can synthesize phospholipids (30).

The occurrence of nascent radioactive glycerolipids within the cisternal contents of the endoplasmic reticulum and the Golgi cell fractions is of interest since phospholipids and triglycerides are thought to be synthesized on the cytoplasmic side of the organelle membranes (10). In both phospholipids and triglycerides, incorporation of [2-^{3}H]glycerol occurs first in the membrane lipids and then shortly afterwards, within 1 min, in the cisternal contents (Fig. 4). This suggests that many of the nascent [2-^{3}H]glycerol-labeled lipids are quickly compartmentalized within the luminal space of the endoplasmic reticulum and the Golgi apparatus.

In the RER 53% of the radiolabeled lipids in the cisternal contents floated at a density ≤1.063 g/ml. A smaller percent of the nascent lipids (13%) floated at a density equivalent to that of plasma HDL. There was also a significant amount (29%) of lipid radioactivity at a density >1.21 g/ml but this material when examined by electron microscopy failed to reveal any clearly defined particles such as those that floated at $d \leqq 1.063$ and $1.063 < d < 1.21$ g/ml. Apoprotein A1, present in the RER, appears not to be complexed to nascent lipids even though some of these lipids have a buoyant density similar to that of serum HDL. As shown in Fig. 5, only small amounts of the lipid radioactivity present in the RER was recovered together with the immunoprecipitated apoprotein A1, and previously we showed that RER lipid particles that float at a density equivalent to that of HDL do not contain apoprotein A1 (3). Thus the RER appears to contain both apoprotein A1 and lipid particles within its cisternal space but they are separate. The manner in which apoprotein A1 is segregated within the RER is not presently known.

A different condition exists in the Golgi cell fractions. A greater percent of the nascent lipids in the cisternal fraction floats at a density ≤1.063, but nascent lipids are also present at a buoyant density between 1.063 and 1.21 and >1.21 g/ml. Apoprotein A1 is complexed to nascent lipids, as measured by immunoprecipitation (Fig. 5), and in addition, nascent apoprotein A1 present in the Golgi cell fraction floats at a buoyant density similar to that of plasma HDL (3, 25). However, the electrophoretic mobility, the gel permeation chromatographic profile, and the morphological appearance and size of the apoprotein A1 containing lipid particles obtained from the Golgi cell fractions are different from that of plasma HDL (Figs. 6–8), suggesting that in the Golgi apparatus the conjugation of lipid and protein is not yet complete.

On the basis of morphological and autoradiographic studies, it has been suggested that triglyceride and phospholipid assembly into VLDL takes place in the SER (17, 18, 47, 48). Similar studies have not been performed on the assembly on HDL. Nascent VLDL and HDL have been isolated, by flotation, from the contents of the Golgi cell fractions from rat liver. When these materials were analyzed biochemically and

morphologically, they were found to be similar to serum VLDL and HDL (21, 25, 35, 36). Glaumann et al. (17) have also been able to float VLDL particles from the contents, released by sonication, of the RER, SER, and Golgi fraction of rat liver and have shown that there is an increase in the lipid content of intracellular VLDL as it travels from the endoplasmic reticulum to the Golgi apparatus. Our studies on HDL biosynthesis indicate that very little of the newly formed lipid particle present in RER are associated with apoprotein A1 and that the Golgi cell fractions are the major sites at which nascent lipids are associated with apoprotein A1 (Fig. 5 and Table II). It is difficult from these studies to determine what role the SER plays in HDL biosynthesis since the SER fraction isolated from chicken liver is a mixture of smooth vesicles and Golgi-derived elements.

The intracellular events that lead to the formation of a core of triglycerides and cholesterol ester surrounded by a layer of apoproteins, phospholipids, and cholesterol are still not understood. The results of our experiments suggest that lipid particles rich in nascent triglycerides are formed very early in the secretory process, in the RER, but at that stage they do not contain much apoprotein A1. In the Golgi apparatus, the major association with apoprotein A1 occurs, and presumably phospholipids and free cholesterol are added since material obtained from the Golgi complex, which floats like serum HDL, contains these lipid moieties (3). However, we do not know whether there are progressive and increasing levels of lipid-apoprotein A1 interaction as the nascent lipids and proteins migrate in parallel fashion from RER to SER to Golgi apparatus, with apoprotein A1 finally being part of a lipid particle which attains the same buoyant density at plasma HDL when it reaches the Golgi apparatus or whether apoprotein A1 is mostly free of lipids in the endoplasmic reticulum and is then inserted into preformed lipid particles in the Golgi apparatus.

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