# Heterogeneity of Lipoprotein Particles in Hepatic Golgi Fractions

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ABSTRACT Newly synthesized phospholipids, labeled with either [$^{14}$C]choline, [$^{3}$H]myo-inositol, or [$^{33}$P]phosphate, partioned preferentially (>80% of total incorporated radioactivity) in a Golgi membrane subfraction, although the cognate content subfraction contained a relatively large amount of secretory lipoproteins. The labeling pattern was the same for all phospholipids tested in the two subfractions. An active exchange process of polar lipids between Golgi membranes and Golgi secretory lipoproteins is postulated as a plausible explanation for these findings.

Less than half of all Golgi lipoprotein particles have the density of serum VLDLs and a similar, but not identical, biochemical composition. The remaining lipoprotein particles are characterized by a continuous spectrum of sizes, and (to the extent tested) by a lipid and protein composition different from that of serum VLDLs and HDLs. Results obtained in control experiments rule out the possibility that the heterogeneous population of Golgi lipoprotein particles is an artefact caused by our preparation procedures. It is assumed that these heterogeneous particles are immature precursors of both VLDLs and HDLs.

The mammalian blood plasma contains a relatively large amount of lipoprotein particles, resolved at present in the following major density classes: very low density lipoproteins (VLDL), $d \leq 1.006$; —intermediate density lipoproteins (IDL), $1.006 < d < 1.02$; —low density lipoproteins (LDL), $1.02 < d < 1.063$; —and high density lipoproteins (HDL) $1.063 < d < 1.20$. For all these classes the main primary source is the liver. VLDLs and HDLs are produced and secreted directly into the blood plasma by hepatocytes, whereas IDLs and LDLs are the result of modifications incurred by VLDL in the peripheral circulation (16, 17, 40, 48, 51). HDLs are also extensively modified in the circulating plasma by transfer of polar lipids and apoproteins from VLDLs and chylomicrons, and by esterification of their cholesterol either native or subsequently acquired from other plasma lipoproteins or from peripheral tissue sources (40, 48, 51).

In the past, it was assumed that the hepatocytes produce primarily (if not exclusively) VLDLs. Lipoprotein particles, identified as VLDLs by morphological criteria only (size, shape, and density after $OsO_4$ fixation), were detected in the endoplasmic reticulum of these cells, and were found to accumulate in Golgi vesicles before exocytosis (1, 18, 53). Similarly identified VLDLs were described within the vesicles of isolated Golgi fractions for which they were used as morphological markers (8, 30, 37). Moreover, lipoprotein particles of $d \leq 1.006$, isolated from Golgi fractions (14, 32, 35, 39), were characterized biochemically and found to be similar, though not identical, to serum VLDLs.

At present, a more diversified intracellular population of lipoprotein particles is expected, since there is evidence that HDLs are also secreted by hepatocytes (16, 40, 51) and because there may be resolvable steps in the assembly and processing of both VLDLs and HDLs.

We have recently isolated—from a (rat) hepatic Golgi fraction—membrane- and content-subfractions and have identified in the latter all the apoproteins of the lipoproteins of the rat serum (23). The apolipoproteins of the rat serum have been extensively studied by other investigators using a variety of procedures (3, 22, 35, 54, 57), including SDS-gel electrophoresis (56) and isoelectric focusing (13, 55). The relevant information has been recently reviewed (6). In this paper, we present the results obtained in our attempts to resolve and characterize the complex lipoprotein population of the Golgi content subfraction.

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## MATERIALS AND METHODS

### Radiochemicals

[1,2-$^{14}$C]Choline chloride (2–10 mCi/mmol), [9,10-$^{3}$H(N)]palmitic acid (10–30 Ci/mmol), [9,10-$^{3}$H(N)]oleic acid (2–10 Ci/mmol) and carrier-free [$^{33}$P]orthophosphoric acid (50–1,000 Ci/mmol) were purchased from New England Nuclear, Boston, MA. [4,5-$^{3}$H] L-Leucine (62 Ci/mmol) was obtained from Schwartz/Mann, Orangeburg, NY.

### Animals, Ethanol Pretreatment, and In Vivo Labeling

The rats (male Sprague Dawley, 120–150 g) were obtained from the same source and were pretreated with ethanol as in our previous studies (23).

All radioactive precursors were administered intravenously via the saphenous vein. In experiments designed to label and follow in parallel both lipids and proteins destined for export, the animals received simultaneously 0.010 mCi [$^{14}$C] choline and 0.100 mCi [$^{3}$H]leucine. To label the esterified fatty acids of serum VLDLs, free fatty acids were complexed with bovine serum albumin (BSA) and the equivalent of 0.25 mCi [$^{3}$H]palmitic acid and 0.5 mCi [$^{3}$H]oleic acid was administered simultaneously 4 h before sacrifice. [$^{33}$P]phosphate (used to label the phospholipids) was injected twice (2 × 0.250 mCi) at 20 h and 16 h before sacrifice.

Apoproteins were labeled with [$^{3}$H]leucine (0.5 mCi), administered 30 min before sacrificing the animals, when Golgi content lipoproteins were to be studied, and at both 20 h and 4 h before sacrifice, when serum lipoproteins were to be investigated.

### Collection of Serum

Blood was collected (under anesthesia) from the aorta and portal vein and allowed to clot. Then EDTA, adjusted to pH 7.4, was added to a final concentration of 10 mM, and the serum was separated from the clot by low speed centrifugation.

### Cell Fractionation

The isolation of Golgi fractions (both $GF_{1+2}$ and $GF_3$) and their resolution into membrane and content subfractions were carried out as in previous experiments (23). $GF_3$ (1.11 g/cm$^3$ > d < 1.14 g/cm$^3$) consists primarily of cisternal and vesicular elements containing few lipoprotein particles.

### Lipoprotein Isolation

The isolation of lipoprotein particles from either serum or Golgi content subfraction was guided by values already used by other investigators for rat lipoprotein density classes, namely: VLDL < 1.006; IDL 1.006 < d < 1.02; LDL 1.02 < d < 1.063; HDL 1.063 < d < 1.20. All material of d > 1.20 was considered to be plasma proteins. The pertinent literature has been critically reviewed (see reference 6). The loaded samples contained 0.262 M NaCl, and 10 mM EDTA adjusted to pH 7.4; all other solutions were 0.262 M in NaCl and 1 mM in EDTA as used in (20). To all solutions, solid KBr was added to obtain the required density (determined by refractive index measurements).

Two different protocols were used for both preparations, namely sequential flotation (20), and flotation in a single discontinuous gradient modified from (45).

In the first case, the serum and the Golgi content subfraction (already containing NaCl and EDTA as given above) were brought up to a density of 1.08 with solid KBr. 5-ml aliquots of this suspension were loaded in centrifuge tubes and overlaid with a 6-ml layer of a NaCl-EDTA-KBr solution of d = 1.006; the gradients were then centrifuged in a SW-41 rotor at 195,700 $g_{av}$ for 20 h at 4°C in an L5-65 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The same rotor and the same conditions were used for all subsequent centrifugations (see below). Centrifugation time was extended, however, in a number of experiments to check whether equilibrium conditions were reached. After centrifugation, successive 0.25-ml fractions were collected from the top of the gradients, and their density was determined. Fractions of d ≤ 1.006 and d > 1.006 were pooled. For further fractionation, solid KBr was added to the fraction of greater density to make it more dense than the next density class. This fraction became the load of the second step gradient in which the upper layer had a density equal to that of the fraction to be collected. This procedure was repeated in sequence to obtain each of the density classes of interest.

In the alternative fractionation protocol, a single discontinuous gradient was constructed with the sample (serum or Golgi content) brought to a density of 1.26, and loaded below a gradient consisting of 1.5 ml steps of d = 1.20, d = 1.063, d = 1.02, and d = 1.006. The same rotor and centrifugation conditions were used as with the sequential flotation.

Each density class recovered from the KBr gradient was either assayed directly, or first concentrated and washed essentially free of KBr in an Amicon Ultrafiltration Cell with a PM 10 membrane (Amicon, Inc., Lexington, MA).

When a second floatation step was required (for washing particles of a given class, as in the case of serum lipoproteins), the preparation was first concentrated and then its density readjusted with solid KBr to either 1.08 (for final densities of 1.063, 1.02, or 1.006) or 1.26 (for a final density of 1.20). Each sample was loaded in a centrifuge tube under the appropriate gradient. Centrifugation and collection were repeated as above.

### Analytical Procedures

Incorporation of radioactive precursors was measured by counting TCA precipitable material.

Lipids were extracted with chloroform: methanol 2:1 by the method of Folch et al. (12) and separated on HP-TLC plates, silica gel 60 (EM Reagents, Darmstadt, Federal Republic of Germany). For neutral lipids the resolving solvent system was hexane:ether:acetic acid (80:20:1) (50); and for phospholipids, the solvent system for the first dimension was chloroform:methanol:ammonium hydroxide (130:50:10) (27), and for the second dimension chloroform:acetone: methanol:acetic acid:water (100:40:20:20:10) (47). Individual spots were scraped from the plates into vials for counting, or into tubes for digestion (38) and subsequent lipid phosphorus assay (2) or for determining ester linkages by the hydroxamate assay (49).

Protein was assayed by the method of Lowry et al. (31).

### Electron Microscopy

Samples of the isolated density classes from both serum and Golgi content were fixed and processed in parallel for electron microscopy. Fixation was carried out in suspension by mixing equal volumes of the sample with 4% $OsO_4$. After 2 h, the suspension was centrifuged for 30 min at 84,000 $g_{av}$ in an SW 50.1 rotor (Beckman Instruments). The pellets obtained were stained en bloc with 0.5% magnesium uranyl acetate in 0.15 M NaCl, before being dehydrated and embedded in Epon. Thin sections were cut through the entire depth of each pellet and stained with uranyl acetate and lead citrate.

Negative staining was done (after removal of the KBr by Amicon dialysis) by the carbon film technique, using holey grids and 2% phosphotungstic acid adjusted to pH 6.4 with KOH (29). All specimens were examined in a Siemens Elmiskop 102 or a JEM-100 CX electron microscope.

### Gel Electrophoresis

Gel electrophoresis was carried out with the modifications of the Maizel system (34) described in (23), except that the acrylamide gradient was extended from 5 to 16% with a 3% stacking gel to obtain better resolution of the apolipoproteins. Lipoproteins were first concentrated and their lipid extracted with ice cold 90% acetone. The proteins were pelleted and then resuspended in 100 μl of 25 mM Tris-phosphate buffer, pH 6.7, and prepared for electrophoresis as described (23).

After Coomassie Blue staining, the gels were prepared for fluorography with EN$^{3}$HANCE (New England Nuclear, Boston, MA). Their fluorographs were obtained on Kodak Royal X-Omat-XR-5 film (Eastman Kodak Co., Rochester, NY) exposed at −80°C for 14, 30, and 64 d.

## RESULTS

A first series of experiments was designed to explore in further detail the pattern of incorporation of radioactive precursors of phospholipid and protein into the TCA-insoluble components of the GF membrane and content subfractions. Nonparallel incorporation was already apparent from our previously published data (23). The second objective was to investigate the nature of the lipoprotein particles known to be present in the GF content subfraction (8, 14, 23, 32, 35).

### Incorporation of Labeled Lipid Precursors in GF Membrane and Content Subfractions

The experimental protocol involved the concomitant intravenous administration of [$^{3}$H]leucine (0.100 mCi/animal) and [$^{14}$C]choline (0.010 mCi/animal) to ethanol-treated rats, followed 30 min later by the collection and homogenization of their livers. Golgi fractions and subfractions (membrane and

TABLE I

*Distribution of Protein, and Lipid- and Protein-Biosynthetic Label among Golgi Subfractions and Content Density Classes*

| GF subfraction or density class | Lipid biosynthetic label | Protein biosynthetic label | Protein* |
|---|---|---|---|
| | [$^{14}$C]choline† | [$^{3}$H]leucine† | |
| GF-membrane subfraction | 83.0 ± 3.7 | 17.6 ± 1.7 | 53.4 ± 3.0 |
| GF-content subfraction | 17.0 ± 3.7 | 82.4 ± 1.7 | 46.6 ± 3.0 |
| Density classes of GF content | | | |
| $d \leq 1.006$ | 4.4 ± 2.3 | 2.4 ± 0.5 | 1.5 ± 1.0 |
| $1.006 < d < 1.20$ | 9.5 ± 1.4 | 3.9 ± 1.9 | 2.5 ± 2.1 |
| $d > 1.20$ | 3.7 ± 0.8 | 76.1 ± 3.7 | 42.6 ± 1.1 |

* Determined by Lowry assay (31).

† The radioactive precursors were administered i.v. 30 min before sacrifice. The figures give percent distribution ± SD of the corresponding values in total GF. The table shows results based on four experiments.

content) were prepared from the homogenates by procedures described in (23). The GF content subfraction was further resolved by flotation (after density adjustment) into the following density classes: (*a*) $d \leq 1.006$, equivalent to serum VLDLs; (*b*) $1.006 < d < 1.20$, equivalent to serum IDL, LDL and HDL, considered as a group; and (*c*) $d > 1.20$, expected to contain only proteins and glycoproteins destined for secretion into the blood plasma.

The results obtained by analyzing these various subfractions are given in Table I; they compare [$^{14}$C]choline radioactivity (incorporated in phosphatidylcholine) to [$^{3}$H]leucine radioactivity (incorporated in proteins); data on protein distribution are also included. In this new set of experiments, the patterns of $^{3}$H-radioactivity and protein distribution are similar to those previously obtained (23) for unwashed GF membrane subfractions. The distribution of [$^{14}$C]choline radioactivity is, however, strikingly different: 17% is recovered in the content- and 85% in the membrane subfraction. For [$^{3}$H]leucine radioactivity, the corresponding values are practically reversed: 82% in the content- and 17% in the membrane subfraction. A distribution pattern identical to that of [$^{14}$C]choline was obtained in similar experiments with [$^{3}$H]*myo*-inositol (data not shown) and [$^{33}$P]-phosphate (some data shown in [23]). For all phospholipid precursors, the pattern of distribution (between membrane and content subfraction) was generally similar regardless of the time elapsed between the administration of the label and the collection of the livers. It follows that the phospholipid precursors so far tested are incorporated extensively into Golgi membranes; only a relatively small fraction of the radioactivity so imparted (<~20%) appears in the GF content subfraction, presumably in lipoproteins destined for export. Data given in a previous paper (23) indicate that the distribution of $^{33}$P-radioactivity among different phospholipids is similar in the two Golgi subfractions. Taken together, these findings strengthen the assumption that an efficient exchange system operates between Golgi membranes and Golgi lipoprotein particles. This assumption remains to be tested by further work.

## Golgi Lipoprotein Particles

The resolution of the GF content subfraction into three density classes (Table I) showed that a relatively low percentage of its protein and radioactivity (both $^{3}$H and $^{14}$C) separates with the class $d \leq 1.006$, i.e., the VLDL equivalent class. Higher percentages of both proteins and radioactivity appeared in the combined heavier class ($1.006 < d < 1.20$), and most of the $^{3}$H-protein radioactivity was recovered at $d > 1.20$. These findings suggest that the Golgi elements of the hepatocytes contain VLDLs as well as other lipoprotein particles and that the former, i.e., the putative VLDLs, do not necessarily account for the majority of the lipoproteins in Golgi contents.

## Comparison of Golgi- to Serum-VLDL

The validation of this suggestion requires a comparison of serum VLDLs and presumed Golgi VLDLs under conditions designed to eliminate differences or artifacts introduced by nonidentical preparation procedures. Serum VLDLs, for instance, are floated from density modified serum, whereas putative Golgi VLDLs are floated from density modified $Na_2CO_3$ extracts of Golgi fractions. In addition, in our case, Golgi VLDLs were derived from rats to which ethanol had been administered 60 min before the label (and 90 min before sacrifice), whereas serum VLDLs were harvested from animals that received the labeled precursors 20 h and 16 h, and ethanol 90 min before sacrifice. The comparison was intended to cover the morphology as well as the biochemistry of the two different sets of VLDLs, normalized as to preparation procedures.

## Morphology

Serum VLDLs and Golgi VLDLs were prepared and processed for electron microscopy as given under Materials and Methods.

In thin sections of pelleted particles, serum VLDLs appeared as dense, round (although slightly irregular) profiles ranging in diameter from 50 to 95 nm (Fig. 1); only occasionally larger particles were found at the bottom of the pellets. Golgi VLDLs were slightly smaller (30 to 70 nm), as shown by the histogram in Fig. 3, but otherwise similar in shape and density; they were, however, rather often associated with membrane tabs and occasionally contaminated by vesicle remnants (Fig. 2). The tabs were of varied length and polymorphic appearance; all had the usual laminar membrane structure; most were equal in thickness (~8 nm) to usual Golgi membranes, but some were thicker (up to 22 nm) (Fig. 4). The origin of these tabs is uncertain: most of them probably represent fragments of Golgi membranes generated during $Na_2CO_3$ treatment, but some of them may be derived from Golgi contents, since some Golgi vesicles (Fig. 5) contain membrane fragments, as well as lipoprotein particles with tabs. In both preparations, VLDLs appeared in small clusters or chains, presumably as a result of crosslinking incurred during fixation.

In negatively stained specimens, both serum VLDLs (Fig. 6) and Golgi VLDLs appeared more regular in form, most of them being nearly perfect spheres. In both cases, the particles appeared individually dispersed; pairs of fused particles were rare and clusters (of more than two) were absent. Again, the main differences were the presence of membrane tabs on some Golgi VLDLs and the occurrence of occasional vesicular contaminants among the latter (Fig. 7). Tabbed particles were, however, less frequent than in fixed-embedded specimens, a finding which suggested that at least some membrane fragments were crosslinked to particles during fixation.

Special attention was given in our survey to membrane fragments and tabs, because it has been postulated that lipoprotein particles, especially HDLs, begin as bilayer fragments or discs that are converted to globular structures by progressive

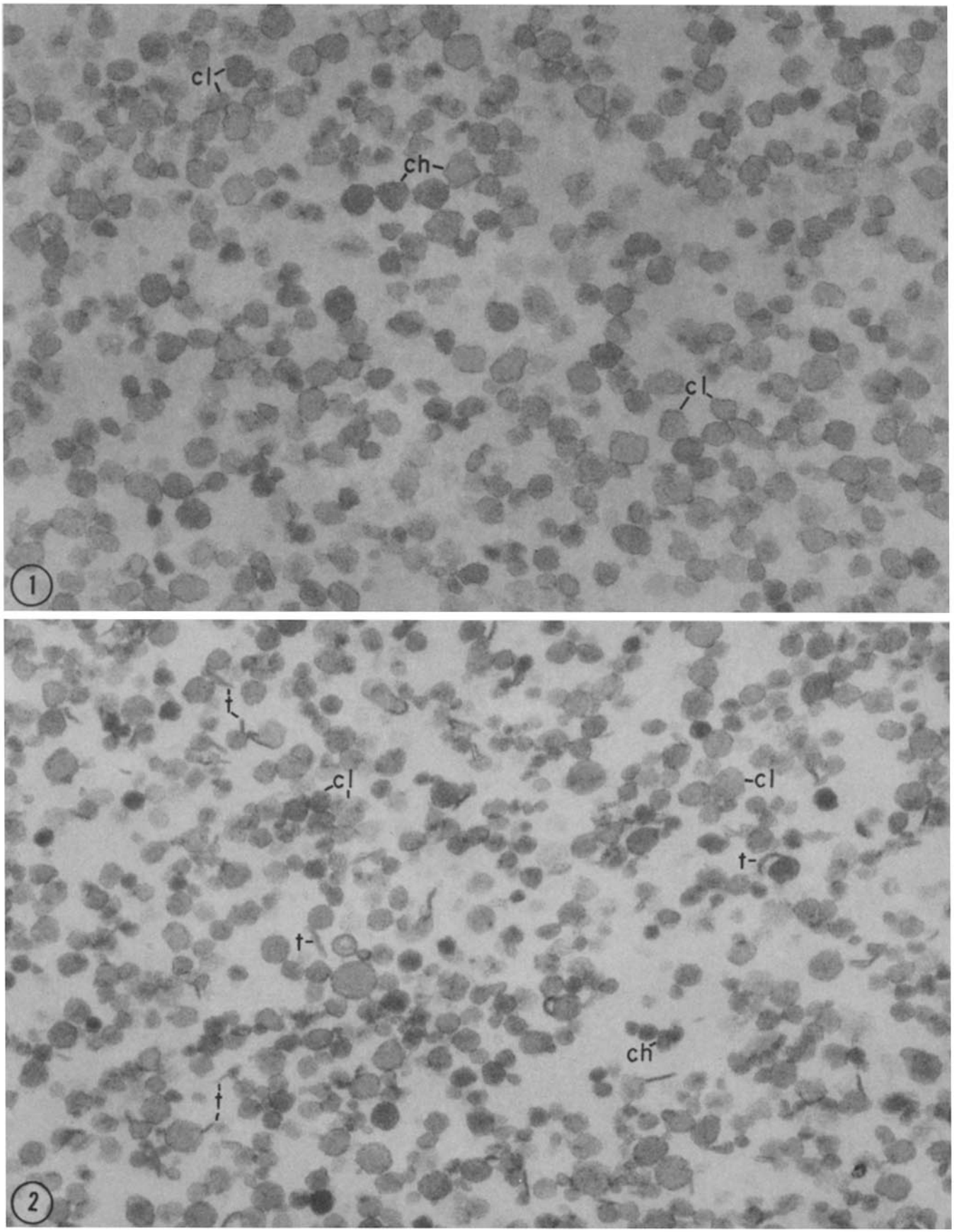



FIGURE 1 Sectioned pellet of serum VLDLs. VLDL particles appear as homogenously dense circular profiles that range in size from 50–95 nm and are deformed to a varied extent by fixation. Chains (*ch*) and clusters (*cl*) of particles are probably generated by crosslinking during fixation. The reason for the noticeable variation in density is unknown; it could be due to differences in unsaturated fatty acyl contents in the triacylglycerols and others lipids of the VLDLs. × 70,000.

FIGURE 2 Sectioned pellet of Golgi VLDLs. The morphology of these particles and their distribution—including the presence of chains (*ch*) and clusters (*cl*)—are similar to those of serum VLDL, except for the presence of frequent membrane tabs (*t*). See Fig. 4 for further details. × 70,000.

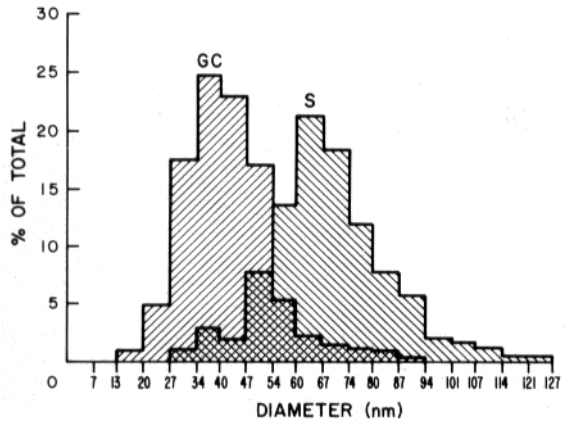



FIGURE 3 Distribution of particle sizes in serum and Golgi VLDL preparations. 250 particles were counted for each group.

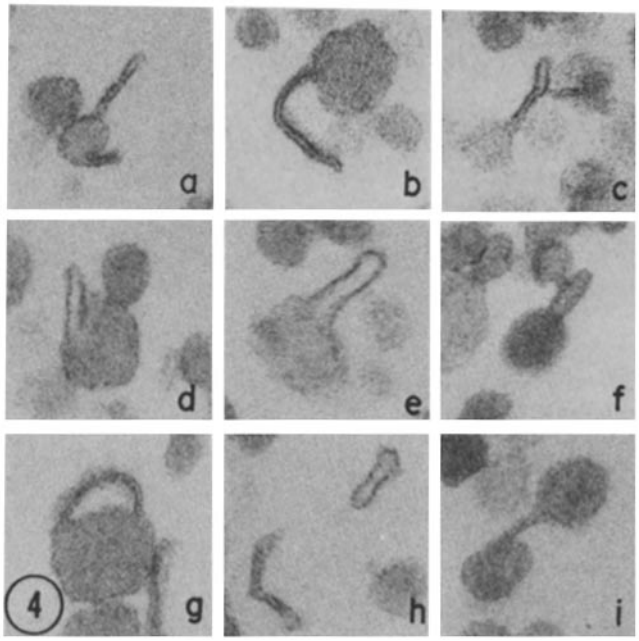



FIGURE 4 Gallery of tabbed Golgi VLDLs. The tabs appear to be continuous with the surface layer of the particles. They vary in shape from short, straight tails (*a*, *d*, *e*, *f*) to long, wavy structures (*b*). All have a typical membrane-like layered appearance, but they vary in thickness from ~8 nm (*a*, *b*) to 12 nm (*d*) or more (*e*), the increase being accounted primarily by a wider light (presumably hydrophobic) band (*d*, *e*, *f*, *h*). In some cases, the thickness changes within the same tab (*c*). All have free margins, and in three dimensions appear to be protruding flat tongues, as suggested by *h*. Occasionally they join two particles (*i*), or form loops anchored at both ends in the same VLDL particle (*g*). × 152,000.

accumulation of nonpolar lipids, primarily cholesteryl esters, within the initial bilayer (15, 17, 19).

## Biochemistry

EFFECTS OF $Na_2CO_3$ TREATMENT OF SERUM VLDLS: Given the difference in preparation procedures already mentioned above, we investigated the effect of $Na_2CO_3$ treatment on the density and chemistry of serum VLDLs.

Serum VLDLs were labeled biosynthetically with [$^3$H]palmitic acid and [$^3$H]oleic acid, and were isolated by a single flotation as given under Materials and Methods. An aliquot of the preparation was treated with 100 mM $Na_2CO_3$, pH 11.3, for 30 min at 4°C (as in the case of Golgi VLDLs), and then refloated as in the original procedure; another aliquot was treated in the same way (incubation at 4°C, reflotation), except for the omission of the $Na_2CO_3$ step. The results, given in Table II, showed that ~85% of the lipid label was recovered at a d ≤ 1.006 in both control and $Na_2CO_3$-treated particles and that differences in chemical composition (to the extent explored) between the two refloated preparations were minimal.

HYDROLYSIS OF LABELED TRIACYLGLYCEROLS UPON $Na_2CO_3$ TREATMENT: The same radiochemical approach can not be used for Golgi VLDLs because, as we have found (and will report later), the ratio of [$^3$H]fatty acid label in phospholipids and triacylglycerols varies with time during intracellular transport. However, a comparison of the amounts of different lipid classes in Golgi and serum VLDLs is possible by assaying for ester linkages in each appropriate spot separated by TLC as given under Materials and Methods. The data in Table III show that the concentration of triacylglycerols and phospholipids in the two types of VLDLs is similar, whereas the concentration of cholesteryl esters in Golgi VLDLs is higher.

The concentrations of different phospholipids in Golgi vs. serum VLDLs was also investigated by assaying for $^{33}$P-radioactivity in each phospholipid spot resolved by two dimensional TLC. The labeling is uniform and, as such, it is assumed to represent actual amounts as shown in (23). The results presented in Table IV show a common composition pattern, with

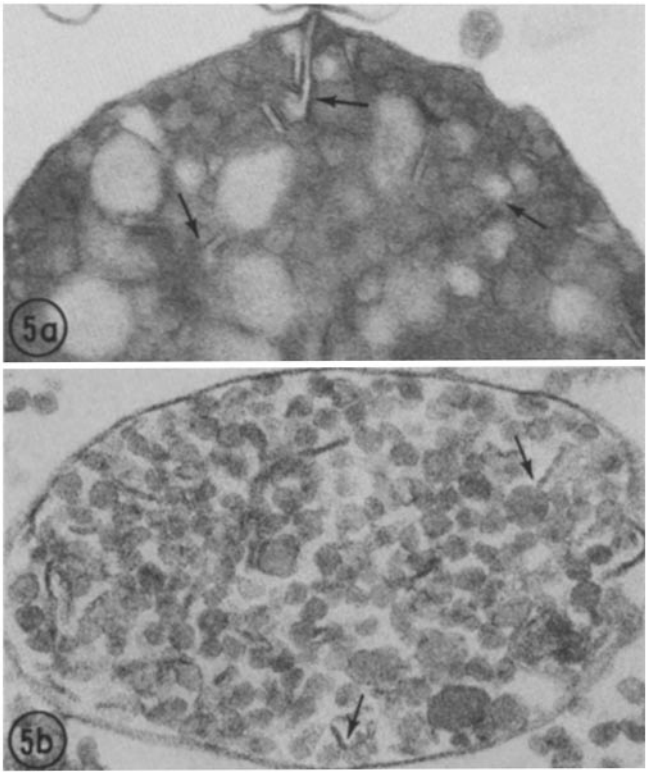
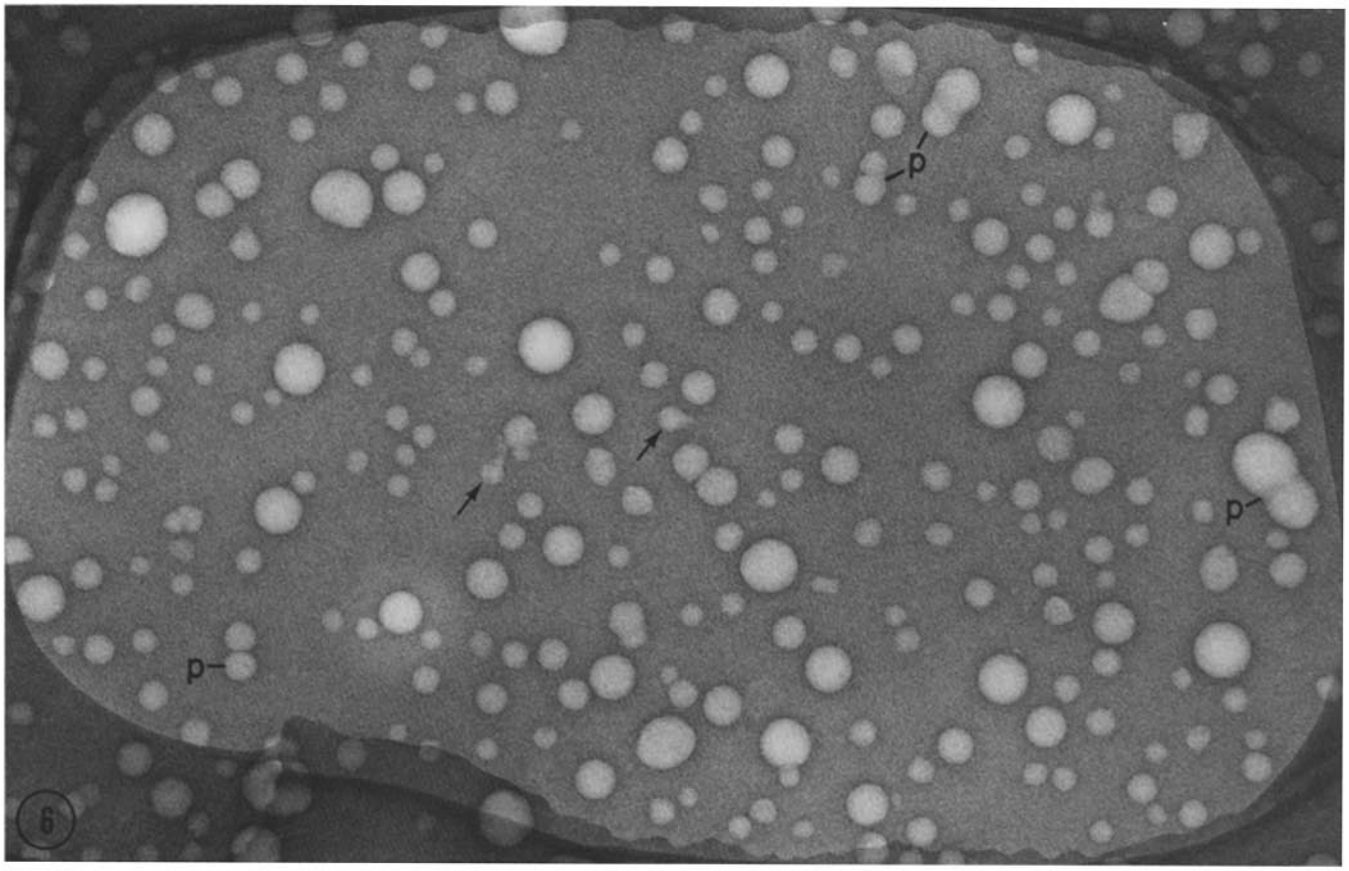



FIGURE 5 Isolated Golgi vesicles before (*a*), and after (*b*) treatment of a Golgi fraction with 0.1 M $Na_2CO_3$. Membrane tabs (arrows) attached to lipoprotein particles are visible in both cases. The Golgi vesicle in *b* is a rare example of a vesicle that has "survived", without extensive membrane disruption, the $Na_2CO_3$-treatment; note in its case, the effective extraction of content elements other than lipoprotein particles, and the size heterogeneity of the latter. *a*, × 120,000; *b*, × 90,000.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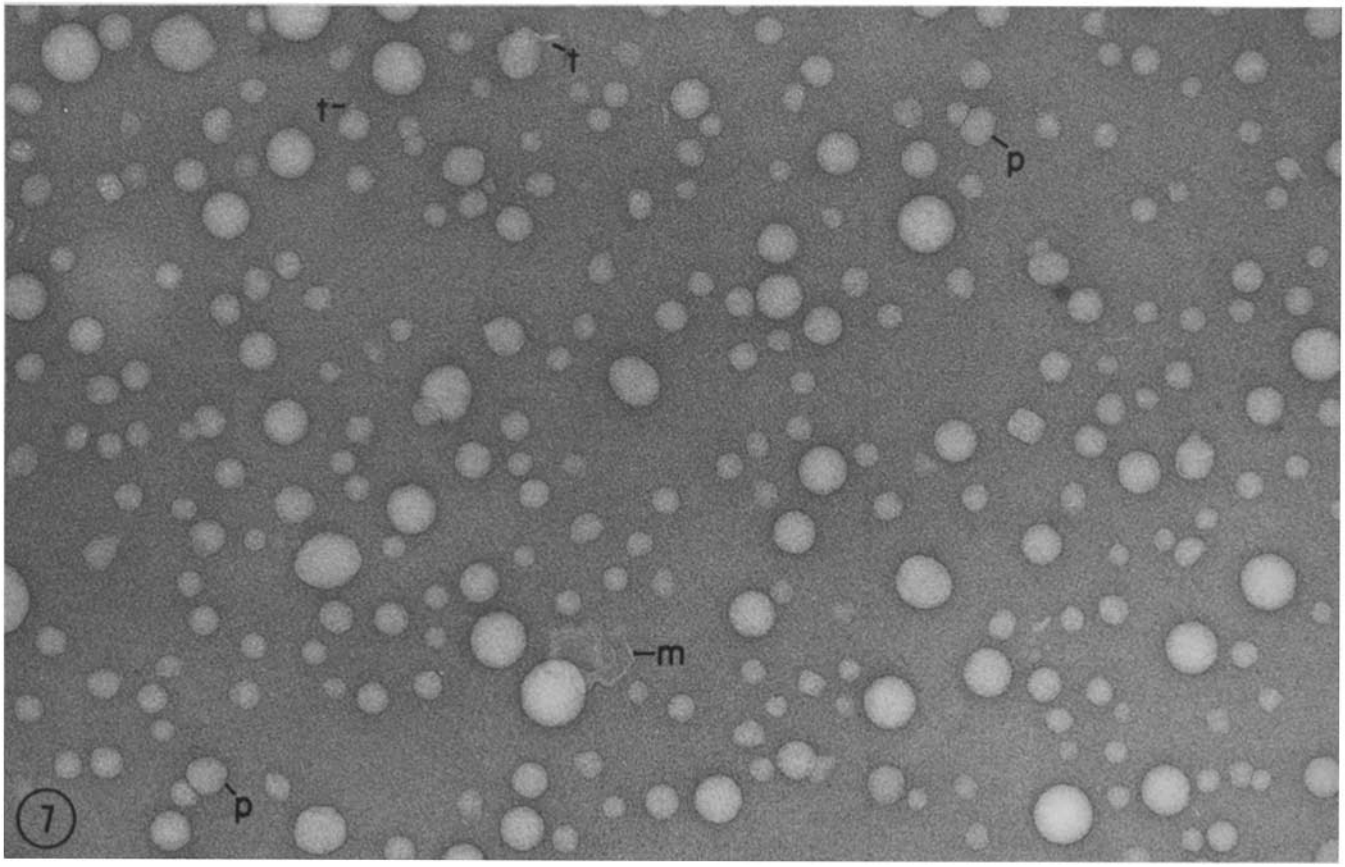
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TABLE II

*Effect of High pH on the Density and Lipid Composition of Serum VLDL and Distribution of Label Introduced as $^3$H-Fatty-Acids Among Different Lipids*

| 1st Flotation | Refloated control | | Refloated after high pH treatment | |
|---|---|---|---|---|
| *Lipids* | VLDL | >VLDL | VLDL | >VLDL |
| ChE | 3 | 2 | 1 | 3.5 | 0 |
| TG | 84 | 83 | 78 | 77 | 75 |
| FFA | 1 | 1 | 2.5 | 2 | 2.5 |
| DG | 3 | 3 | 8.5 | 3.5 | 10 |
| PL | 10 | 10 | 11 | 14 | 13 |
| *Density* * | | | | | |
| | 100 | 85 | 15 | 87 | 13 |

The figures represent percent distribution of total lipid radioactivity assayed in each lipid species resolved (total radioactivity recovered in each preparation being taken as 100). Averages obtained from four samples. Actual recoveries were >95% in each case.

* Density ≤1.006 and >1.006.

TABLE III

*Distribution of Cholesteryl Esters, Triacylglycerols, and Phospholipids among Serum and Golgi Content Lipoprotein Classes*

| | ChE | TG | PL |
|---|---|---|---|
| Serum | | | |
| $d \leq 1.006$ | 5 | 79 | 17 |
| $1.06 < d < 1.20$ | 54 | 15 | 32 |
| Golgi content | | | |
| $d \leq 1.006$ | 10 | 76 | 14 |
| $1.006 < d < 1.02$ | 17 | 22 | 62 |
| $1.06 < d < 1.20$ | 21 | 21 | 59 |
| $d > 1.20$ | 0 | 32 | 68 |

Lipids were extracted from four aliquots of each density class from serum and Golgi content subfractions, and separated by TLC (50). Individual spots were scraped from the TLC plates and assayed for ester linkages by the hydroxamate assay (49). Actual amounts varied from 5 to 1,000 nmol ester. Lauric hydroxamate was used as the standard. Recoveries from the TLC plates were >93%.

The molar content of each esterified component was calculated, averaged among the four samples, multiplied by the average molecular weights, and the results were used to calculate percent distribution for each class. DG is not included; it was <3% in all classes.

small differences affecting primarily the phospholipids present in low amounts.

From the data so far presented, it appears that Golgi VLDLs are similar in morphology, density and lipid composition to serum VLDLs; that only limited differences in lipid composition are detected between the two sets; and, especially, that $Na_2CO_3$ treatment of serum VLDL does not generate new particles of different density or composition that could explain the variety of lipoprotein particles, heavier than VLDLs, found in the Golgi content subfraction.

## Effects of Ethanol Treatment

We also checked the effect of ethanol on the distribution of radioactivity, introduced as [$^{14}$C]choline, among Golgi derived preparations, including lipoprotein particles of $d \leq 1.006$. To this intent, we isolated $GF_{1+2}$ fractions from ethanol-treated and control (no ethanol) rats 30 min after the intravenous administration of [$^{14}$C]choline, 0.010 mCi/rat. The GFs obtained were further subfractionated into membrane and content subfractions and the latter resolved (as given under Materials and Methods) into two density classes ($d \leq 1.006$ and $d > 1.006$). $^{14}$C-radioactivity was assayed in each fraction, subfraction and class and the results are given in Table V. Ethanol treatment correlates with an 80 to 100% increase in incorporated radioactivity in all fractions and subfractions tested, but the pattern of distribution of radioactivity is practically the same for all preparations, including $d \leq 1.006$, i.e., Golgi VLDLs. The comparison was not extended to other chemical components.

## Variations in VLDL Separation Procedures

The fraction of Golgi content phospholipids recovered in the $d \leq 1.02$ remained about the same, irrespective of separation protocol (sequential steps vs. single discontinuous gradient), extension of centrifugation time to 42 h (to check for equilib-

TABLE IV

*Phospholipid Distribution in Serum and Golgi VLDLs*

| Phospholipid species | Serum VLDLs | Golgi VLDLs |
|---|---|---|
| Phosphatidic acid | 0 | 0 |
| Phosphatidylserine | 0.2 | 0.8 |
| Phosphatidylinositol | 3.5 | 3.7 |
| Phosphatidylethanolamine | 2.5 | 6.0 |
| Phosphatidylcholine | 81.5 | 72.5 |
| Lysophosphatidylcholine | 5.9 | 7.5 |
| Sphingomyelin | 6.4 | 9.5 |

The figures are the percent of total $^{33}$P-radioactivity recovered in each phospholipid species resolved by two dimensional TLC. Each figure is the mean of four analyzed samples.

TABLE V

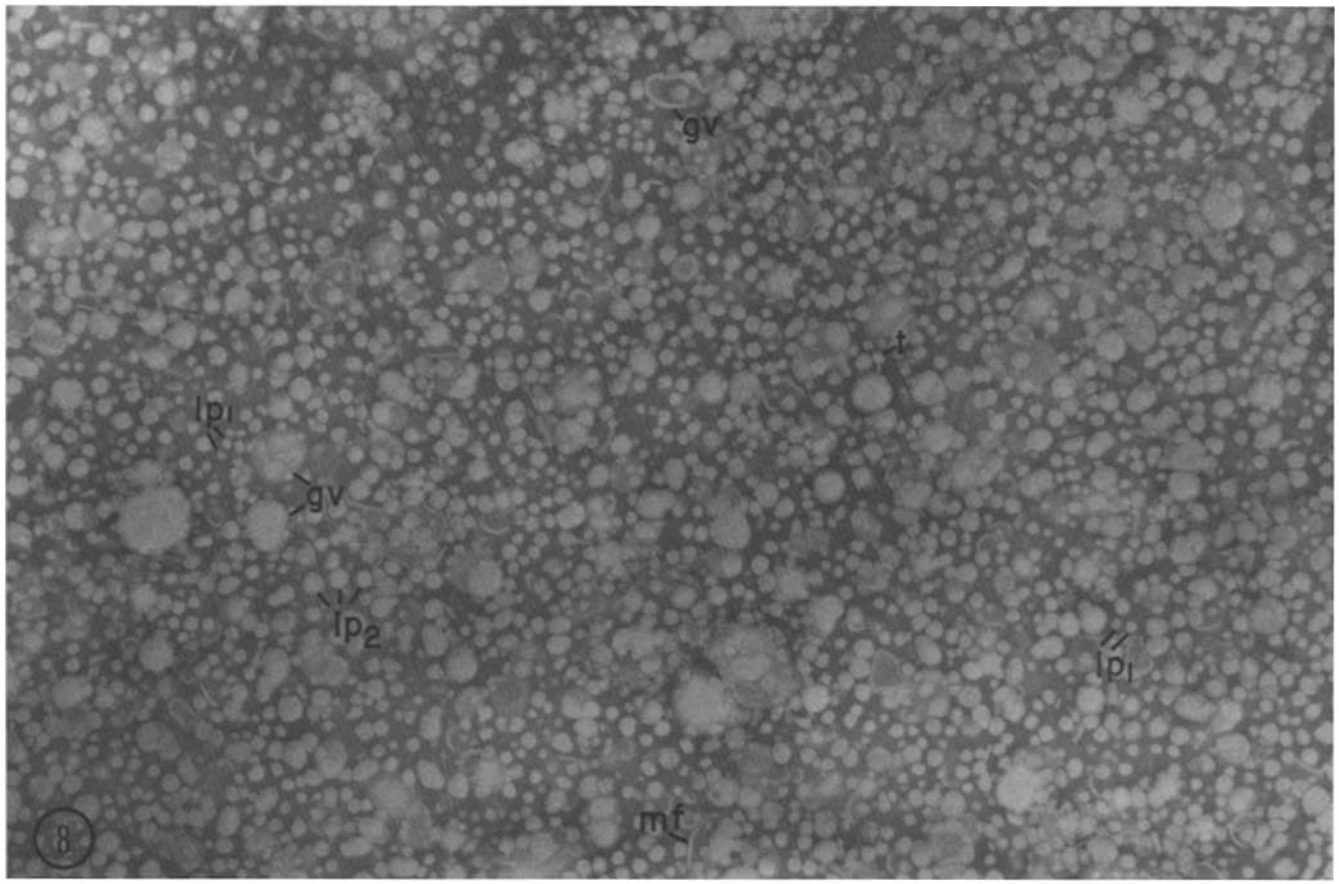
*Distribution of [$^{14}$C]Choline Radioactivity among Golgi Subfractions and Density Classes Isolated from Control and Ethanol Treated Rats*

| | No ethanol | | Ethanol treatment | |
|---|---|---|---|---|
| | *% of total* | | *% of total* | |
| GF Membrane subfraction | 82.9 | | 85.6 | |
| GF Content subfraction | 17.1 | | 14.4 | |
| | | *% of content* | | *% of content* |
| Density classes of GF content | | | | |
| $d \leq 1.006$ | 7.3 | 42.8 | 6.5 | 44.8 |
| $d > 1.006$ | 9.8 | 57.2 | 8.0 | 55.2 |

Total [$^{14}$C]choline incorporated into GF (total) control; 33,530 cpm and GF (total) ethanol-treated; 72,800 cpm. Recoveries were >97%.

FIGURE 6 Negatively stained preparation of isolated unfixed particles. Serum VLDLs range in size from 40–120 nm, and most of them approach the form of perfect spheres; deformed particles (arrows) are extremely rare. Most VLDLs are monodispersed, but coalesced pairs (*p*) are quite frequent. The frame of a window in the holey film used for this type of preparation is visible at the periphery of the field. × 70,000.

FIGURE 7 Golgi VLDLs. Negatively stained preparation of fresh, unfixed VLDLs isolated by flotation from a Golgi content subfraction. The particles range in size from 40–125 nm (in this field) and practically all of them are nearly spherical in shape. A few VLDLs have short membrane tabs (*t*). Most particles are monodisperse; pairing (*p*) is rare. A few membrane fragments contaminate the preparation; an example is marked *m*. × 70,000.


gv
t
lp1
gv
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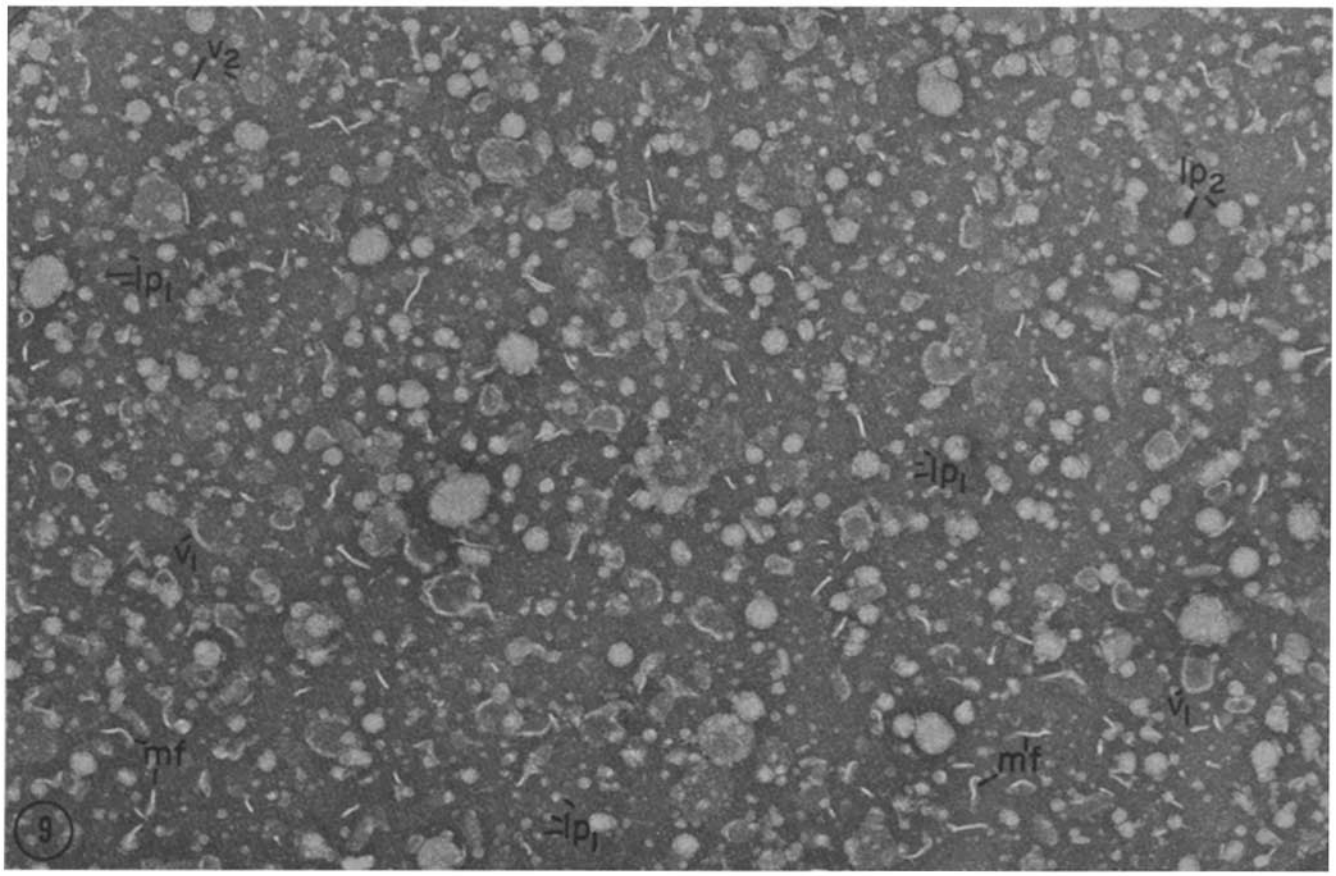
8


v2
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9

rium conditions), or replacement of KBr by sucrose-borate (for density adjustments) (43).

## Morphology of Particles of Density > 1.006

Data concerning the lipid composition of these particles are given in Tables I and III. Complementary morphological information was obtained by electron microscopy of sectioned pellets as well as negatively stained specimens prepared for electron microscopy as given under Materials and Methods. The results obtained with negatively stained preparations proved more reliable, because the particles therein were not deformed and aggregated as in fixed-embedded pellets. Hence, only the findings obtained by the first procedure will be illustrated.

The predominant component of class $1.006 < d < 1.02$ was a regular, spherical particle of ~20–30 nm Diam; minority components were larger lipid droplets (possibly generated by fusion), and membranes present as small vesicles or fragments either free or attached (as tabs) to particles (Fig. 8). Morphologically the most regular component of the combined class $1.02 < d < 1.20$ was a much smaller, dense, spherical particle comparable in diameter (~10–20 nm) to serum HDL; but the class contained in addition larger (up to 40 nm) spherical particles and especially numerous membranes appearing as vesicles, fragments or tabs (Fig. 9). Large populations of discoidal or lamellar particles organized in rouleaux (cf 19) were never seen in these preparations.

The micrographs of the class of $d > 1.20$ showed extensive precipitates presumably proteins, with a few occluded lipid particles (not illustrated).

## Apolipoproteins

The morphological survey of the various lipoprotein classes isolated from the Golgi content subfraction shows that the particles of class $d \leq 1.006$ are generally similar to serum VLDLs, but that heavier classes consist of particles of heterogeneous size, contaminated to a varied, yet significant, extent by membranes. These features rule out a direct correlation with serum lipoproteins of $d > 1.006$. Data on the lipid composition of these classes, given in Tables I and III, lead to the same conclusion: the class of $d \leq 1.006$ is similar to serum VLDLs, whereas the others are quite different.

To gain more insight into the nature of Golgi lipoprotein classes, we decided to compare the apolipoprotein distribution among density classes separated from serum and Golgi content subfractions, and we selected a protocol expected to be insensitive to membrane contaminants and to apolipoproteins contributed by the uptake of chylomicron remnants and recycling serum LDLs or HDLs (5). To this intent, [$^3$H]leucine was administered to the animals and Golgi fractions ($GF_{1+2}$ and $GF_3$) were isolated from homogenates prepared at a time (30 min after [$^3$H]leucine injection) when newly synthesized apolipoproteins were expected to be in transit through the Golgi complex. Lipoprotein classes were separated from the Golgi content subfraction, and their apoproteins were surveyed by fluorography (rather than protein staining) of the corresponding SDS PAGE electrophoretograms. For comparison, serum apolipoproteins were labeled by two i.v. injections of [$^3$H]-leucine at 20 h and 4 h before bleeding; serum lipoprotein classes were isolated (as given under Materials and Methods), and their apoproteins revealed also by fluorography of the corresponding SDS-PAGE electrophoretograms. The results of such an experiment are illustrated in Fig. 10. The apoproteins of the serum density classes were identified on the basis of data already published in the literature (6, 54, 56, 57), whereas those in the Golgi content classes were identified by comparison with their serum counterparts. The recognizable apolipoproteins were: (*a*) apo B, $M_r > 200{,}000$, present as a doublet in serum VLDL, IDL, and LDL with the two bands of the doublet unequally represented in the three classes, the faster moving component predominating in LDL and the slower moving band being particularly prominent in IDL; apo Bs were absent from serum HDL. (*b*) Apo A-IV, $M_r \sim 46{,}000$, a component of HDL (54, 56), but detected also in LDL[1], and, in our case, in small amounts in IDL and VLDL. The presence of apo A-IV has been reported in serum VLDLs of diabetic rats (3). (*c*) Apo E, $M_r$ ~35,000, present in all density classes (13, 44, 54–57). (*d*) Apo A-I, $M_r$ ~27,000, present as the major apoprotein component in HDL (56) and LDL[1], but also detectable in IDL and VLDL. (*e*) Apo C, comprising three peptides ranging in $M_r$ from 11,000 to 7,000 (22), and present in all classes.

Because all these apoproteins appeared radioactively labeled in Golgi lipoprotein classes, we concluded that they are all synthesized by hepatocytes. And since, with three exceptions, they had (within the limits of resolution of our gel system) the same electrophoretic mobility as their serum counterparts, it appeared that in the Golgi complex they had already acquired their mature or secretable form. The exceptions were the two apo Bs that had a faster mobility in the Golgi content than in the serum and apo E which appeared as a doublet in most serum classes, and run as a diffuse band in Golgi VLDLs. The apo E doublet as well as the diffuse band might be accounted for by the existence of isoapoproteins: 4 to 6 forms of apo E ranging in pI from 5.46 to 5.31 have been described in the literature (13). Multiple forms of apo B have been recently reported in rat VLDL (28, 52) and in human chylomicrons and VLDL (26).

All the apoproteins of the lipoprotein classes of the serum were present in the corresponding classes isolated from the

[1] In man apo A-IV and apo A-I are found only in HDL (51).

FIGURE 8 Particles of density class 1.02 g/cm$^3$. ($1.006 < d < 1.02$), isolated by flotation from a Golgi subfraction. Unfixed, negatively stained preparation. Spherical particles ranging in size from 20–30 nm (*lp*$_1$) are the prominent components of this class; some of them have attached membrane tabs (*t*), and most of them appear individually dispersed. Larger (~40 nm) particles (*lp*$_2$) are relatively rare. Additional components of the class are membrane fragments (*mf*) and residual Golgi vesicles (*gv*) some of which still contain lipoprotein particles. × 70,000.

FIGURE 9 Particles of density classes 1.06 and 1.20 g/cm$^3$, ($1.02 < d < 1.20$) isolated by flotation from a Golgi content subfraction. Unfixed, negatively stained preparation. The most characteristic component of this class is a population of small globular particles ranging in size from 10–20 nm (*lp*$_1$). Larger particles (up to 40 nm in size) are relatively frequent (*lp*$_2$). Major contaminants are membrane fragments (*mf*) and residual vesicles ($v_1$); most of the latter appear to be broken open; some ($v_2$) still contain a few small lipoprotein particles. × 70,000.

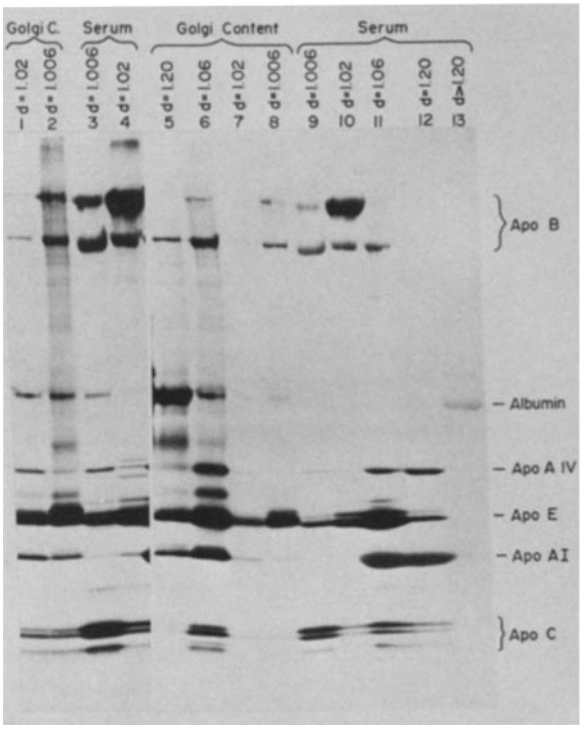


FIGURE 10 Lipoprotein classes isolated from the content of a hepatic light Golgi fraction ($GF_{1+2}$) and from the serum (rat) after in vivo labeling with [$^3$H]leucine (see Materials and Methods for labeling schedule). Composite fluorograph of the same SDS-PA-Gelectrophoretogram. Lanes *1* to *4*: 64-d exposure; lanes *5* to *13*: 30-d exposure. Lane *5* - Golgi HDLs (d = 1.20) contain the fast moving band of the *apo B* doublet, *apo A-IV*, *apo E*, *apo A-I* (doublet), and two bands of the *apo C* triplet; it is heavily contaminated (not refloated preparation) by plasma protein (especially albumin). Lane *12* - Serum HDLs (labeled d = 1.20) lack *apo B*, have *apoA-IV*, *apo E* (doublet), *apo I* (probably doublet), and a complete *apo C* triplet. The band inbetween *apo A-I* and *apoC* is probably apo *A-II*. The preparation (washed) is not contaminated by serum proteins. Lanes *8* and *9* (labeled d = 1.006) compare Golgi VLDLs (lane *8*) to Serum VLDLs (lane *9*); they have the same set of apolipoproteins except that Golgi VLDLs are poorer in *apo A-IV* and richer in apo *A-I* and *apo E* (diffuse doublet); at this exposure, they lack a complete *apo C* triplet, but with longer exposure a complete triplet becomes evident as seen in lane *2*. Lanes *10* and *11* (labeled d = 1.02 and 1.06, respectively) represent Serum IDLs (lane *10*) and LDLs (lane *11*). Note the high concentration of the slow moving band of *apo B* in IDLs and the high content of apo *A-IV*, *apo E* (triplet), apo *A-I*, and *apo C* triplet in LDLs. Lanes *6* and *7* (labeled d = 1.02 and 1.06, respectively) show that the Golgi density equivalents of serum IDLs and LDLs differ from the latter by having quantitatively different spectra of apolipoproteins and by being contaminated by plasma proteins (especially albumin). Lanes *1* to *4* compare *Golgi VLDLs* (lane *2*) and *equivalent IDLs* (lane *1*) to serum VLDLs (lane *3*) and serum IDLs (lane *4*) after longer exposure for fluorography. The differences in *apo B* content between serum IDLs and their Golgi density equivalents are more evident; so are the differences in *apo A-IV*, *apo E*, *apo A-I*, and *apo C* triplet (and in plasma protein contaminants) between Golgi VLDLs and serum VLDLs. Lane *13* (labeled d > 1.20) albumin in the only serum protein clearly labeled.

content subfraction of $GF_{1+2}$; Golgi classes contained, however, additional apoproteins regularly found in serum classes of other densities. For instance, Golgi class d < 1.006 contained (like its serum counterpart), apo B, apo E, and three apo C peptides, but in addition it had the serum HDL specific apo A-IV and apo A–I. It could be argued that the last two apoproteins were present in the light density class on account of HDLs sticking to Golgi VLDLs, but the morphological evidence already presented (Fig. 7) showed practically no particles of HDL size either free or stuck to Golgi VLDLs. The Golgi class of 1.063 < d <1.20 (marked *d = 1.20* on Fig. 10) contained the apoproteins (apo A-IV, apo E, and Apo A-1) of its counterpart, i.e., serum HDL, but it also had the faster band of the apo B doublet. There were a few particles which approached dimensions in this class (Fig. 9) and a few vesicles that could contain mixtures of HDLs and VLDLs. But, the number of such vesicles in the overall particle population was low; moreover, even if present in significant amounts, these contaminants could not explain the presence of only a single apo B in the Golgi HDL density class. "Misplaced" apolipoproteins, like apo B in the example just cited, might be components of lipoprotein particle precursors that have not yet acquired their full complement of lipids and, hence, their final density. This assumption is supported by the apoprotein pattern of Golgi classes d = 1.06 and d = 1.02 which, in principle, correspond to serum LDL and IDL. Because they contain the apoproteins of both HDL and VLDL, and because the liver does not secrete either LDL or IDL, it follows that the classes mentioned may represent mixtures of immature VLDLs and HDLs. In general, similar results were obtained with Golgi VLDLs analyzed with or without refloating. Refloated preparations showed less contamination (than seen in Fig. 10) with plasma proteins, especially albumin, but they regularly lost the apo C peptides. Serum VLDLs have been reported to incur similar apo losses with centrifugation (10, 33).

The probable existence of immature lipoprotein particle in the content subfraction of $GF_{1+2}$ prompted us to examine the apoproteins of lipoprotein classes isolated from a heavy Golgi fraction, $GF_3$, which consists mostly of cisternal elements with a low content of lipoprotein particles. On account of the position of most of its elements in the Golgi complex, $GF_3$ could contain a larger proportion of immature lipoprotein particles. The results obtained (Fig. 11) showed, however, that the correlation of Golgi to serum lipoprotein classes is apparently better for this class than for $GF_{1+2}$. An exception appears to be the higher molecular weight component of the apo C triplet, which is missing from the corresponding Golgi classes.

## DISCUSSION

The first series of experiments reported in this paper gave unexpected results. The phospholipids destined for export were not selectively labeled at the expected time point for transit through the Golgi complex. At all time-points, one minute to 20 h, all the precursors we have tested, i.e., [$^{14}$C] and [$^3$H] choline, [$^3$H]*myo*-inositol, and [$^{33}$P]orthophosphate, were incorporated to an equal specific activity into all phospholipids. Because the Golgi membrane subfraction contains >80% of the total phospholipid, it also contains >80% of the total radioactivity, while the Golgi content subfraction contained <20% of both phospholipid and label. The phospholipid in the content subfraction is in the newly synthesized lipoproteins destined for export. The unexpected character of these findings is brought forward by a comparison with the incorporation pattern of [$^{14}$C]leucine—used as protein precursor. In its case, at 30 min after the administration of a pulse of [$^3$H]leucine, >80% of the total radioactivity was found in the GF content subfraction, presumably in secretory proteins, and <20% separated with GF membranes. Rapid labeling of certain Golgi membrane phospholipids has been reported by Chang et al. (4).

A plausible explanation of these findings is that an efficient phospholipid exchange system operates between membranes and lipoprotein particles within the cisternal spaces of the Golgi complex. This system would function, therefore, in a different compartment than that associated with the phospholipid exchange proteins (43) of the cytoplasmic matrix (58, 59). Exchange of phospholipid between vesicles and lipoprotein particles has been studied by Jonas and Mains (25) and net transport of labeled phospholipids among phospholipid vesicles has been described by Roseman and Thompson (46) and ascribed to phospholipids in monomolecular or micellar dispersion rather than to vesicle collision. Experiments designed to test the assumption that an exchange system operates within the Golgi complex are now in progress.

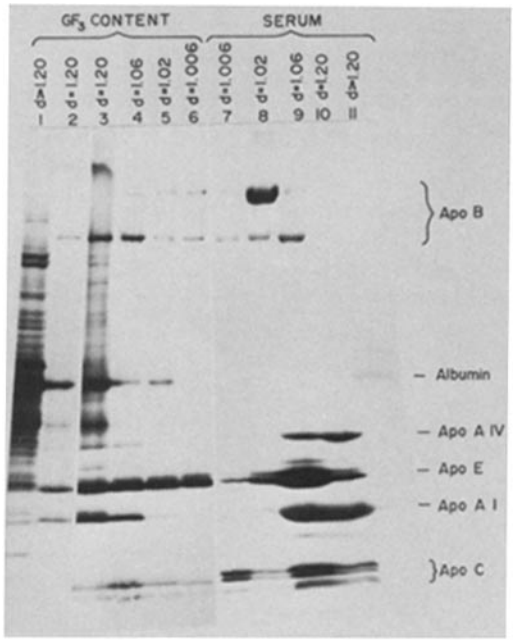


FIGURE 11 Lipoprotein classes isolated from the content of a hepatic heavy Golgi fraction ($GF_3$) and from the serum (rat), after in vivo labeling with [$^3$H]leucine. Composite fluorograph of the same SDS PAGelectrophoretogram. Lanes *1*, *2*, *7–11*: 30-d exposure; lanes *3–6*: 64-d exposure. Lane *1* (d > 1.20) illustrates the multiplicity of hepatic secretory proteins. (Lane *2* and *3*, two exposures) Golgi HDLs (d = 1.20) contain the fast moving band of the *Apo B* doublet; Apo *A–IV*, *Apo E* (doublet), *Apo A–I* (doublet) and the two faster moving bands of the *Apo C* triplet. This preparation (not refloated) is contaminated by various secretory proteins, primarily albumin, the extent of the contamination being more evident in lane *3*. Lane *10* - Serum HDL's (labeled d = 1.20) differ from their Golgi equivalent by lacking any *Apo B*, having larger amounts of Apo *A–IV*, and Apo *A–I*, and having a complete *Apo C* triplet. Contamination of this refloated preparation by plasma proteins is minimal. The band seen in between Apo *A–I* and *Apo C*'s is probably Apo *A–II*. Lane *6* - Golgi VLDL's (labeled d = 1.006) contain the *Apo B* doublet, *Apo E* (doublet) and the two faster components of the *Apo C* triplet. Lane *7* - Serum VLDL's (labeled d = 1.006) have the same set of Apoproteins as Golgi VLDLs, but contain a complete set of *Apo C*'s. In serum VLDLs, Apo *A–IV* and *Apo A–I* can be detected upon longer exposure (see Fig. 10, lane *3*). Lanes *4* and *5*: Golgi density equivalents of serum LDLs and IDLs have a complete set of apoproteins, except for the slower band of the *Apo C* triplet, and are visibly contaminated by plasma proteins (especially albumin). Lanes *8* and *9*: Serum IDLs (labeled d = 1.02) and LDLs (labeled d = 1.06), respectively, have characteristic apoprotein patterns quantitatively different from one another and from their Golgi "equivalents". Note the high concentration of the slow *Apo B* band in IDLs, and the high content of *Apo A–IV*, *Apo E* (triplet), Apo *A–I* and the (probable) presence of *Apo A–II* in LDLs. Lane *11*: Serum proteins (labeled d > 1.20), only albumin is clearly labeled.

The results of the second series of experiments showed that the GF content subfraction contained a variety of lipoprotein particles of which only those of $d \leq 1.006$ were morphologically and (to the extent tested) biochemically similar, but not identical to serum VLDLs. For instance, Golgi VLDL had more cholesteryl esters and different concentrations of apoproteins than their serum counterparts. The other Golgi lipoprotein particles could be separated into density classes equivalent to those of serum lipoproteins; but although similar in density, they were found to be different in size, and lipid and apoprotein content from their serum counterparts. The possibility that these different lipoprotein particles reflect metabolic changes caused by the ethanol treatment of the animals was ruled out, because the distribution of lipoprotein particles was similar in GF content subfractions isolated from animals to which ethanol was not administered. Also ruled out was the production of such particles from Golgi VLDLs during the $Na_2CO_3$ extraction procedure used in our experimental protocol. Serum VLDLs underwent only marginal changes upon $Na_2CO_3$ treatment; they were not converted (in part) to particles comparable to those found in density classes heavier than 1.006 in the GF content subfraction.

Hepatocytes are known to secrete only HDLs and VLDLs. Rat serum HDLs represent a heterogeneous population which has been resolved in at least three different subclasses (6, see also reference 44). The density of one of these subclasses ($HDL_1$) overlaps in part ($1.02 < d < 1.063$) with that of serum LDL. But the particles of none of these subclasses exceeds 20 nm in Diam (6). Assuming that Golgi elements contain *in situ* only mature lipoproteins, the GF content subfraction should comprise only particles of two distinct, well separated size classes: 10–20 nm (for HDLs) and 40–90 nm (for VLDLs) (36, 41). The electron microscopy of the different Golgi density classes reveals, however, a continuous spectrum of sizes with many particles of intermediate diameters (20 to 40 nm). In fact, a similar spectrum of particle exists in Golgi vesicles both *in situ* and in isolated Golgi fractions (Fig. 12). The apparently mixed biochemistry of the different Golgi density classes of $d > 1.006$ could be explained, in principle, by the existence of aggregates comprising HDLs and VLDLs in different proportions. But the electron microscopic survey of the relevant classes detected negligible aggregate formation and the "survival" of few vesicles that may contain mixed particles. The existence of Golgi particles of intermediate sizes and the absence of significant aggregation support the assumption that Golgi elements contain immature lipoprotein particles, which have not yet acquired their final composition and size. Some of those that contain HDL specific apoproteins (apo A–IV, apo A–I) may lose triacylglycerols and phospholipids, while acquiring additional cholesteryl ester during their maturation, whereas some of those having VLDL-specific apoproteins may have to acquire additional lipids, especially additional triacylglycerols. This interpretation assumes that there is no exchange of apolipoproteins among various Golgi lipoproteins, but the assumption may not be valid, since apoprotein exchange has been reported among serum lipoproteins (9, 21, 42).

The interpretation of our results is based on lipoprotein density classes already recorded in the literature. Recent work, however, indicates that the diversity of serum lipoproteins may be considerably larger than assumed in the past (11). The heterogeneity detected among Golgi lipoprotein particles may

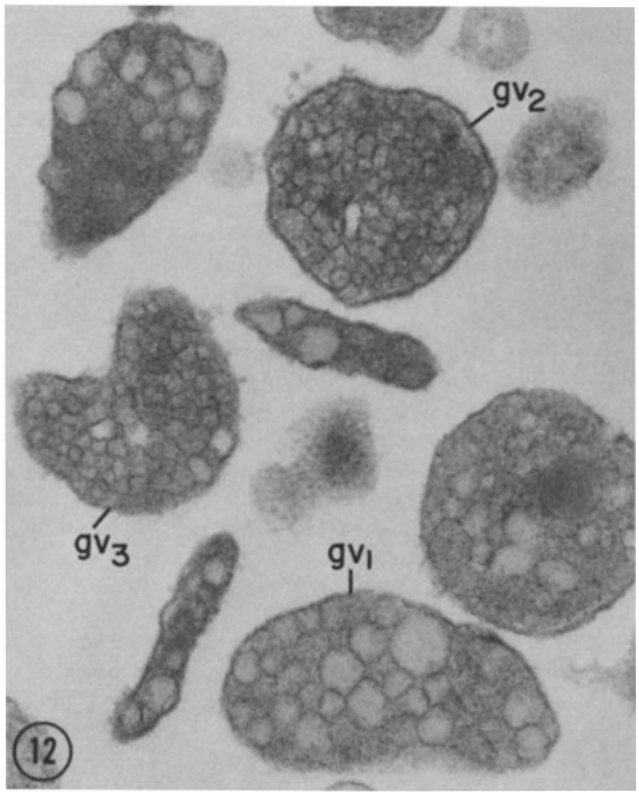


FIGURE 12 Sectioned pellet of a Golgi fraction. The micrograph illustrates the variation in size (from ~20 to ~100 nm) of lipoprotein particles within the same Golgi vesicle ($gv_1$) as well as from one Golgi element to another (compare $gv_1$ and $gv_2$ to $gv_3$). × 76,000.

reflect, at least in part, the increasing complexity of their serum counterparts. Accordingly, our interpretations may require reassessment as the diversity of serum lipoproteins is better understood.

To analyze in further detail the heterogeneity of Golgi lipoprotein particles, we have raised antibodies against specific (rat) apolipoproteins and intend to use them for the separation, by immunoadsorption as in reference 24, and subsequent biochemical characterization of all Golgi apo B - or Apo A–I-containing lipoprotein particles.

The existence of membrane fragments, free or associated as tabs to lipoprotein particles in all Golgi density classes, requires comment. Golgi vesicles *in situ* or in isolated fractions contain membranes as tabs, free fragments or vesicles, but their relative volume density is considerably smaller than that of similar membrane structures found in GF content subfractions. In addition, the membrane fragments found in the latter are quite variable in size and general morphology. Most of them are probably generated by the $Na_2CO_3$ treatment of the Golgi fractions, as indicated by the fact that the GF membrane subfraction contains many membrane fragments of varied sizes. Previous studies have reported that lipoprotein particles originate as bilayer fragments or discs that expand by progressive accumulation of cholesteryl esters in their hydrophobic phase (15, 16, 19). Indeed, such discoidal particles predominate in the lipoprotein output of rat livers perfused with an inhibitor of the enzyme, lecithin:cholesterol acyltransferase (LCAT), that synthesizes cholesteryl esters. But these particles become rapidly spherical when the inhibitor is removed (19). Because the liver produces and secretes LCAT (40), it may be assumed that under normal conditions conversion of lipoprotein particles from discoidal to spherical forms occurs already in the Golgi complex. Discoidal particles may be present, at low concentration, in our GF content subfractions, but they would be difficult to detect among the numerous, polymorphic membrane fragments generated by our extraction procedure. For this reason (among others) it would be desirable to develop in the future subfractionation procedures that avoid membrane fragmentation and improve the separation of membranes from contents beyond the level we have currently achieved in these studies.

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