Hepatic Golgi Fractions Resolved into Membrane and Content Subfractions

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ABSTRACT Golgi fractions isolated from rat liver homogenates have been resolved into membrane and content subfractions by treatment with 100 mM Na_2CO_3 pH 11.3. This procedure permitted extensive extraction of content proteins and lipoproteins, presumably because it caused an alteration of Golgi membranes that minimized the reformation of closed vesicles. The type and degree of contamination of the fractions was assessed by electron microscopy and biochemical assays. The membrane subfraction retained 15% of content proteins and lipids and these could not be removed by various washing procedures. The content subfraction was contaminated by both membrane fragments and vesicles and accounted for 5 to 10% of the membrane enzyme activities of the original Golgi fraction. The lipid composition of the subfractions was determined, and the phospholipids of both membrane and content were found to be uniformly labeled with [^{33}P]phosphate administered in vivo.

Golgi fractions isolated from rat liver homogenates represent membrane-bound compartments (vesicles, cisternae, and vacuoles) containing plasma proteins and lipoproteins, all destined for export (13, 35, 36, 44, 45). The ratio of exportable protein to Golgi membrane protein is expected to be smaller in cisternal and small vesicular Golgi elements and larger in large Golgi vesicles or vacuoles, the equivalent of secretory granules in other cell types.

It was our aim to isolate and study Golgi membranes and Golgi content free from cross contamination. To this end, we separated membrane- and content subfractions from a Golgi fraction (GF) that had been isolated by our modifications (24) of the procedure of Ehrenreich et al. (13). In this paper, we describe the subfractions obtained and provide data for assessing the degree to which they are intercontaminated.

To define content proteins, we have used a simple and convenient criterion, namely, their rapid in vivo labeling by radioactive amino acids. In hepatocytes, the most rapidly labeled group of proteins consists of secretory products which (as a result of segregation and intracellular transport) become the predominant content components of the endoplasmic reticulum (ER) as well as the Golgi complex (42). In the few other glandular cells so far investigated, membrane proteins are labeled at a much slower rate than secretory proteins (10, 38). Rapidly labeled proteins have been used successfully as content markers for hepatic microsomes by Kreibich et al. (29) and Kreibich and Sabatini (30). The secretory nature of these proteins can be further ascertained by comparative polyacrylamide gel electrophoresis (PAGE) of Golgi content and blood plasma (or serum), because the latter can be considered—to a large extent—a physiological standard (cf. 9) of hepatocyte protein secretion. Short term protein labeling combined with PAGE provides a convenient method for identifying content proteins and monitoring their selective release from their membrane containers.

MATERIALS AND METHODS

Materials

Enzyme substrates and specific biochemical compounds were purchased from Sigma Chemical Company, St. Louis, MO; [4,5-^{3}H]L-Leucine, 62 Ci/mMole, was obtained from Schwarz-Mann, Orangeburg, NY, and [^{33}P] carrier-free orthophosphoric acid, 50–1000 Ci/mMole, [9,10-^{3}H(N)]palmitic acid, 10–30 Ci/mMole, and [9,10-^{3}H(N)]oleic acid, 2–10 Ci/mMole, were purchased from New England Nuclear, Boston, MA.

Animals, Ethanol Pretreatment, and Labeling

110 to 160-g male, Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were starved overnight and then given 0.6 g ethanol/100 g body weight in a 50% solution by stomach intubation; 60 min later, they received—under light ether anesthesia—either 250 μCi [^{3}H]leucine by intraportal injection, or 50 μCi [^{3}H]palmitic acid and 100 μCi [^{3}H]oleic acid (bound to bovine serum albumin) by injection into the saphenous vein; 30 min later, the animals were sacrificed by decapitation. [^{33}P]phosphate was injected twice (2 $\times$ 250 μCi) in the saphenous vein at 20 h and 16 h before sacrifice.

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Cell Fractionation

ISOLATION OF A COMBINED GOLGI FRACTION: A combined light and intermediate Golgi fraction (GF_{1+2}), isolated by our modifications (24) of the original method of Ehrenreich et al. (3, 13), was used in these investigations. GF_{1+2} (for convenience designated GF in this paper) was selected because it is reasonably well-characterized and has a high content of lipoproteins. Details concerning the isolation procedure and the characterization of the fraction are given in references 3, 13, and 24.

The entire fractionation procedure was carried out at 4°C. The rat livers were excised, minced, forced through a tissue press of 1 mm mesh, and homogenized in 0.25 M sucrose. A Beckman L5-65 centrifuge (Beckman Instruments, Fullerton, CA) was used for all separations. Centrifugation of the homogenate for 10 min at 10,000 g_{av} in a 60 Ti rotor produced a common pellet of cell debris, nuclei, and mitochondria. The resulting supernatant was centrifuged for 90 min at 105,000 g_{av} to give microsomal pellets, which were resuspended in 0.25 M sucrose and then mixed with enough 2.0 M sucrose to give a refractive index of 1.3920, equivalent to that of 1.22 M sucrose. 10-ml aliquots of this total microsomal suspension were loaded under a discontinuous sucrose gradient with 8.5-ml steps of 1.15 M, 0.86 M, and 0.25 M sucrose, and centrifuged for 180 min at 82,500 g_{av} in an SW 27 rotor (Beckman Instruments). GF was defined as the material that floated to the 0.25 M/0.86 M interface and therefore had a density of <1.11 g/cm^3.[1]

GF contains 12–15% of the total galactosyltransferase activity (the most commonly used marker enzyme for Golgi-derived vesicles) of the whole homogenate (6, 24). From morphological characterization (13, 24) it is evident that GF does not represent the entire spectrum of Golgi elements: Golgi cisternae are under-represented in the fraction; such elements isolate mostly at the 0.86 M/1.15 M interface of the sucrose gradient, in a fraction designated GF_3.

SUBFRACTIONATION OF THE GOLGI FRACTION: GF was removed from the gradient and immediately diluted with cold distilled water to a refractive index of 1.3450, equivalent to that of 0.25 M sucrose. The fraction was then pelleted by centrifugation for 60 min at 105,000 g_{av} in a 60 Ti rotor (Beckman Instruments). The resulting pellet was resuspended to a protein concentration of <2 mg/ml in 100 mM Na_2CO_3 with 6–8 strokes of a teflon pestle in an AA glass homogenizer (A. H. Thomas Co., Philadelphia, PA). This suspension was mixed (with a vortex mixer) three times while being kept for 30 min at ~4°C, and then pelleted by centrifugation for 60 min at 106,500 g_{av} in a 50 Ti rotor (Beckman Instruments). As will be shown, this procedure results in extensive separation of the content of Golgi elements from the corresponding membranes; hence, the Na_2CO_3 supernatant is defined as the GF content subfraction and the Na_2CO_3 pellet, resuspended in 0.25 M sucrose, as the GF membrane subfraction. For washing, membrane subfractions were resuspended in an appropriate medium and repelleted as described for the Na_2CO_3-release step.

Electron Microscopy

Samples of the fractions and subfractions of interest were fixed in suspension by mixing equal volumes of the sample and 4% OsO_4. After 2 h, the suspensions were centrifuged for 20 min at 37,500 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, stained with uranyl acetate and lead citrate, and examined in a JEM 100 CX.

In some cases, the preparations of interest were fixed for 2 h in suspension in 2.5% glutaraldehyde in 0.15 M Na cacodylate-HCl buffer, pH 7.4, then pelleted and postfixed for 2 h in 2% OsO_4 in the same buffer. The rest of the preparation procedure was as given above.

Gel Electrophoresis

SDS PAGE (37) was carried out on a 1-mm thick (20 × 24 cm) slab gel apparatus with a linear 7.5 to 13% acrylamide gradient stabilized with sucrose and prepared from dilutions of a stock solution (acrylamide:bisacrylamide = 30: 0.8). The stacking gel contained 3% acrylamide. Samples for electrophoresis were prepared at a concentration of 2 mg protein/ml in a medium containing 25 mM Tris-phosphate buffer, pH 6.7, (stacking gel buffer [37]), 5 mM EDTA, 10 mM dithiothreitol, and 2.5% SDS. They were boiled for 1 min, then cooled and reacted with iodoacetic acid (final concentration 50 mM) to alkylate the reduced disulfide bonds. 2 M sucrose was added (200 μl/ml) to increase the density of the solution, and 1% bromphenol blue (10 μl/ml) was introduced as electrophoretic marker. 100 μl of sample, containing 165 μg protein, was applied to each slot of the gel. Electrophoresis was carried out at room temperature at a constant current of 12.5 mA for ~15 h. After electrophoresis the gels were stained in 0.02% Coomassie Brilliant Blue R in 50% methanol—7% acetic acid; destained in 30% methanol—7% acetic acid; photographed with Kodak Ektapan film (Eastman Kodak Company, Rochester, NY); and prepared for fluorography by the methods of Bonner and Laskey (7) and Laskey and Mills (32).

[1] Density units will not be restated in the rest of the text.

Analytical Procedures

RADIOACTIVITY ASSAYS: To measure the incorporation of radioactive precursors into trichloroacetic acid (TCA) precipitable materials, an equal volume of cold 20% TCA was added to each sample of cell fraction or subfraction of interest. The resulting precipitate was washed three times with cold 10% TCA; the final pellet was resuspended in 1% SDS (0.5 ml), and counted in a Beckman LS 200 liquid scintillation spectrometer, using the Triton-X-114/xylene scintillation fluid of Anderson et al. (2).

LIPIDS: EXTRACTION AND SEPARATION: Lipids were extracted from each preparation of interest with 20 vol of chloroform:methanol, 2:1, according to Folch et al. (20). The organic phase was dried under nitrogen, and the residue redissolved in a known volume of chloroform. Aliquots of the chloroform solution were taken for radioactivity assays and for digestion (39) before lipid phosphorus determinations (1). The data thus obtained were used to calculate specific radioactivity of phospholipids.

For thin layer chromatography (TLC), aliquots were spotted on HP-TLC plates (Silica Gel 60, EM Reagents, Darmstadt, FRG).

PHOSPHOLIPIDS: Phospholipids were resolved in a two dimensional system, using chloroform:methanol:ammonium hydroxide (130:50:10 ml) for the first dimension (46), and chloroform:acetone:methanol:acetic acid:water (100:40:20: 20:10) for the second dimension (31). Spots, detected by exposure of the TLC plates to iodine vapor, were identified by comparison with known standards (obtained from Supelco, Bellanfonte, PA), scraped directly into vials for counting or into tubes for digestion (39) and subsequent lipid phosphorus assay (1).

NEUTRAL LIPIDS: Neutral lipids were separated with the solvent system hexane:ether:acetic acid (80:20:1) (50). Spots were detected by exposing the TLC plates to iodine vapors, and standards (obtained from Supelco) were run in parallel for their identification. Individual spots were scraped from the plates into vials for scintillation counting, or into tubes for determining ester linkages by the hydroxamate assay (49).

PROTEIN ASSAY: Protein was assayed by the method of Lowry et al. (34) using bovine serum albumin as the standard.

Enzyme Assays

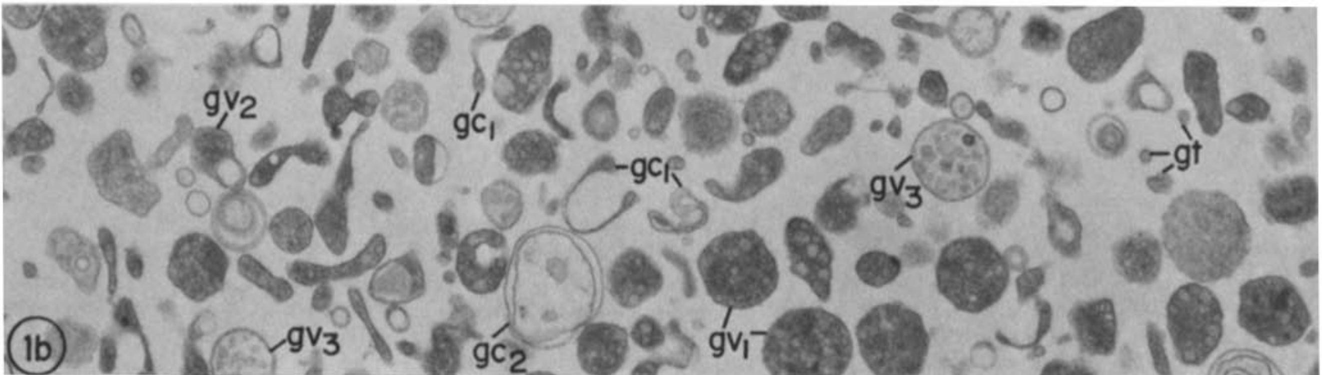
5′-NUCLEOTIDASE (RIBONUCLEOTIDE PHOSPHOHYDROLASE; EC 3.1.3.5): The assay measured the release of inorganic phosphate from adenosine 5′-monophosphate and was carried out in 0.5 ml vol containing 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1% Triton-X-100, 5 mM substrate and 20–100 μg fraction protein. Activity was measured at 37°C at successive intervals ranging from 0 to 30 min and stopped by the addition of 0.2 ml of 30% TCA. The sample was cleared by centrifugation and an aliquot of the supernatant was assayed for inorganic phosphate as in reference 1.

ALKALINE PHOSPHATASE (*P*-NITROPHENYL PHOSPHATASE; EC 3.1.3.1) AND ALKALINE PHOSPHODIESTERASE I (PARAPHOSPHORIC DIESTER PHOSPHOHYDROLASE; EC 3.1.4.1): Alkaline *p*-nitrophenyl phosphatase and phosphodiesterase I activities were estimated by measuring the absorbance at 410 nm of *p*-nitrophenol liberated at 37°C from *p*-nitrophenylphosphate and *p*-nitrophenyl thymidine 5′-phosphate, respectively, using 20–100 μg fraction protein in a 0.5 ml final vol. The reactions were stopped at intervals ranging from 0 to 30 min by adding 1 ml of glycine buffer, pH 10.7. For alkaline phosphatase, the incubation mixture contained 100 mM Na_2CO_3-$NaHCO_3$ buffer, pH 10.0, 2.5 mM $MgCl_2$, 0.1% Triton-X-100, and 5 mM substrate. For alkaline phosphodiesterase I, the incubation mixture contained 10 mM Na_2CO_3-$NaHCO_3$ buffer, pH 10.0, 2 mM Zn acetate, 0.1% Triton-X-100, and 1.5 mM substrate.

RESULTS

Morphological Characterization of the Golgi Fraction

A micrograph of GF is presented in Fig. 1 in which (*a*) and (*b*) represent fields from the top and mid-portion of the pellet, respectively. The major component of the fraction is large (0.3 to 0.5 μm Diam) Golgi vesicles, the equivalent of secretion granules and condensing vacuoles in other secretory cells. These vesicles are filled with particles identified as very low density lipoprotein (VLDL) primarily on the basis of their


gv2
gc1
-gc1
gv3
-gt
1b
gv3
gc2
gv1

morphological similarity (size, shape, density) to VLDLs released in the plasma or in liver perfusates (22, 23, 47, 52). Specifically, they fall within the size range, 25–70 nm, of VLDLs isolated from rat serum (43). However, besides VLDLs of expected size, the vesicles contain both smaller particles (which become more visible when the vesicular content is partially extracted), particles of larger diameter (which may represent coalesced VLDLs), and occasionally membranes present as fragments or small vesicles. All these particles are embedded in a dense homogenous matrix provided by other secretory proteins (primarily albumin). At the top of the pellet, there are many Golgi cisternae with minimal lipoprotein content, some small vesicles containing only one lipoprotein, and a few small, apparently empty, vesicles of unidentified origin. Recognizable contaminants, present in very small amounts only, include lysosomes and rough microsomes.

Subfractionation of the Golgi Fraction

We have tested a variety of procedures to release the content of Golgi vesicles (and thereby permit content separation from membrane), including disruption by sonication or a Paar Bomb (17), shearing in a French Press (13), Tris/Water/Tris wash (5, 21), hydrophobic washing (14) and saponin treatment (9). The most (though not completely) satisfactory results have been obtained by treating GF with 100 mM Na_2CO_3, pH 11.3, as described in Materials and Methods. For convenience, this method will be referred to hereafter as "the high pH procedure".

Content proteins were labeled in vivo by a single injection of [^{3}H]leucine into the portal vein of anesthetized rats 30 min before the sacrifice of the animals. GF was prepared and the content of the fraction was released by the high pH procedure. The distribution of the [^{3}H]leucine label, marking content proteins, and of total protein is shown in Table I; 82% of the label and 47% of the protein are released to the supernate (i.e., content subfraction). Release can be increased slightly if the pellet (i.e., membrane subfraction) is resuspended in a variety of media and either repelleted or sedimented to equilibrium in a continuous sucrose gradient as shown in Table II. Resuspending the pellet in different media: (*a*) 5 mM Tris-HCl buffer, pH 7.4, (*b*) 0.25 M sucrose, (*c*) 100 mM Na_2CO_3 with sonication, followed by repelleting, released in all cases only 25 to 28% of the radioactivity and 6 to 9% of the protein remaining in the corresponding membrane subfraction. These figures represent 5% of the radioactivity and 3 to 5% of the protein content of the original GF. If the resuspended membrane subfraction were placed on a continuous sucrose gradient (density = 1.05 to 1.14) and centrifuged to equilibrium (instead of simply pelleting the preparation), the same percent of release was obtained (*e*). In this gradient, the membranes sedimented to a density of 1.089. Resuspension of the first pellet in a concentrated salt solution (*d*) (0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 0.5 M KCl, and 5 mM $MgCl_2$) followed by repelleting released a comparable amount of radioactivity but considerably more protein: ~41%. High ionic strength solutions are known to release extrinsic proteins (51) as well as adsorbed proteins (48) from other membrane preparations.

TABLE I

Distribution of [^{3}H]Leucine-labeled Proteins between Golgi Membrane and Content Subfractions

	Protein distribution	Leucine counts distribution
	%	%
Golgi fraction	100	100
Content subfraction	47.0 ± 4.6	81.9 ± 2.5
Membrane subfraction	53.0 ± 4.6	18.1 ± 2.5
Washed membrane subfraction	48.9 ± 0.7	13.4 ± 0.2

Cell fractionation was started 30 min after an intraportal injection of 250 μCi [^{3}H]leucine. Over this interval (30 min), the liver incorporated 6.8 to 7.9% of the radioactivity injected. The figures give percent distribution ± SD. Example of actual figures: Golgi fraction = 347,008 counts/min/mg protein; Membrane subfraction = 108,568 counts/min/mg protein; Content subfraction = 615,889 counts/min/mg protein The table shows results of eight experiments.

TABLE II

Effect of Washing the Golgi Membrane Subfraction with Various Media and Procedures

	(a) Buffer washed	(b) Sucrose washed	(c) Na_2CO_3 washed + sonication	(d) STKM *washed	(e) Sucrose washed + gradient isolation
	%	%	%	%	%
Label					
Extract (wash)	26 (4.7)	26	26	25 (4.5)	28
Pellet	74 (13.3)	74	74	75 (13.5)	72
Protein					
Extract (wash)	8 (4.2)	8	6	41 (21.7)	9
Pellet	92 (48.7)	92	94	59 (31.2)	91

Data obtained in a representative experiment and given as percent of the radioactivity or protein of the cognate membrane subfraction. Corresponding values, calculated for the radioactivity and protein of the original GF, are given in parentheses. Similar results were obtained in five other experiments for (*a*) and (*b*), two other experiments for (*d*), and three other experiments for (*e*).

Radioactivity recovery ranged from 91 to 102%, for total radioactivities ranging from 171 × 10^3 to 191 × 10^3 cpm. For protein, the corresponding figures were 91 to 103% and 1.090 to 1.239 mg.

* STKM = 0.25M Sucrose, 50 mM Tris-HCl, pH 7.4, 0.5 M KCl, and 5 mM $MgCl_2$.

FIGURE 1 *Golgi Fraction.* (*a*) Representative field of the bulk of a GF pellet (from the bottom to immediately under top layer). Golgi vacuoles (gv_1), whose content is marked by clustered lipoprotein particles of varied sizes, are the predominant component of the fraction. Some vacuoles (gv_2) still retain their cisternal connections. Golgi cisternae (*gc*) are rare, and so are small, circular profiles (of vesicles or tubules) (*gt*) containing single lipoprotein particles. The only rare, particulate contaminants of the entire fraction are rough microsomes (*m*) and lysosomes (*ly*); the latter amount to ~4% of the total particle population in this field in which they appear to be in higher concentration than in the rest of the preparation.

(*b*) Representative field in the top layer of the same pellet. The volume density of cisternal elements is increased. They appear in normal (gc_1) or parallel (gc_2) sections to their bottom. The latter have a characteristic double ring appearance. Also increased is the volume density of small vesicular profiles (*gt*) marked by single lipoprotein particles, and that of damaged Golgi vacuoles (gv_3) recognizable on account of the partial loss of their content. × 30,000.

The membrane subfraction washed as under (*a*) contains ~13% of the [^{3}H]leucine label and ~49% of the total protein of the parent Golgi fraction.

Both the morphology and the electrophoretograms of the two main subfractions, GF membrane and GF content, will be presented and discussed.

MORPHOLOGY

MEMBRANE SUBFRACTION: The membrane subfraction (Fig. 2) is a reasonably homogeneous preparation in which membranes are the major component. Most of them have retained their usual layered structure, but appear to have undergone extensive reorganization, since in addition to membrane vesicles of varied sizes, the preparation contains small membrane fragments and large membrane sheets with free edges. These sheets must be the result of fusion of vesicles followed by their rupture, because in many cases their dimensions exceed the circumference of the largest vesicles present in the original GF. Recognizable contaminants are a minor component of the membrane subfraction; they include: (*a*) a few VLDLs occluded among, or fused to, the membranes; (*b*) some lipid droplets, larger than VLDLs (up to 500 nm Diam) which are probably fused VLDLs; and (*c*) some membrane-associated precipitates which probably represent residual vesicular content (proteins destined for export).

CONTENT SUBFRACTION: Homogeneously dense, spherical particles (deformed to a varied extent by fixation) are the predominant component of the content subfraction. As in the original Golgi fraction, they have the general appearance of serum lipoprotein particles, but they range in diameter from ~450 nm to ~10 nm. In addition to these presumptive lipoprotein particles, the subfraction contains small membrane fragments, a few small vesicles and fibrillar precipitates. We assume that the latter represent secretory proteins precipitated around and in between lipoprotein particles during fixation (Fig. 3).

PROTEINS OF GOLGI SUBFRACTIONS (SDS PAGE)

The two GF content and membrane subfractions were further characterized by processing them through SDS-PAGE and comparing their electrophoretograms and corresponding fluorographs. Rapidly labeled proteins, defined as content proteins in both subfractions, were easily identified in these fluorographs. Plasma proteins and the apoproteins of serum lipoproteins were run in parallel as standards.

The results of such an experiment are illustrated in Fig. 4 in which electrophoretograms stained with Coomassie Brilliant Blue R are shown under *A*, and their fluorographs under *B*. In the content subfraction, which accounts for ~80% of the incorporated [^{3}H]leucine radioactivity, albumin is the major Coomassie Blue-stained band and also the most heavily labeled. It accounts for 45% of the total protein radioactivity of the subfraction, as estimated by counting gel slices. Other proteins, identifiable in the content subfraction by their electrophoretic mobilities and rapid labeling, are the VLDL apoproteins: i.e., the high M_r > ~210,000 apoprotein B which appears regularly as a doublet, the ~35,000 M_r arginine-rich protein (apoE), and the 7,000–10,000 M_r apoC proteins.[2] Examples of the SDS-PAGE electrophoretograms of the apoproteins of isolated serum lipoproteins used as standards are shown in Figs. 10 and 11 of the companion paper (25). From the fluorograph it is evident that each of these proteins is heavily labeled. So are additional content proteins found between 200,000 and 100,000 daltons, around 80,000 daltons, and between 60,000 and 55,000 daltons.[3] None of the latter is identified at present.

In the membrane subfraction, which contains 15% of the short term [^{3}H]leucine label, the major protein components by CB staining are in the 50,000 M_r region. The corresponding bands are unlabeled and so are three other bands in the low molecular weight region that have slightly different mobilities than the apoC proteins.

From Fig. 4, part *B*, it is evident that bands of identical mobilities to content bands are retained in the membrane subfraction, and that their retention is not proportional to their concentration in the content subfraction. Albumin, which is the major content protein, is almost entirely released (only trace amounts are detected) while the M_r 35,000 apoE is selectively retained. In contrast, the apoC proteins and apparently apoB are effectively released.

Two other unidentified content contaminants ranging in M_r from 200,000 to 100,000 are revealed by fluorography in the membrane subfraction, in addition to another labeled band of apparent M_r ~300,000. The latter does not appear in the content subfraction; accordingly it may represent a polymer of some of the polypeptides of the faster bands.

LIPIDS OF GOLGI SUBFRACTIONS

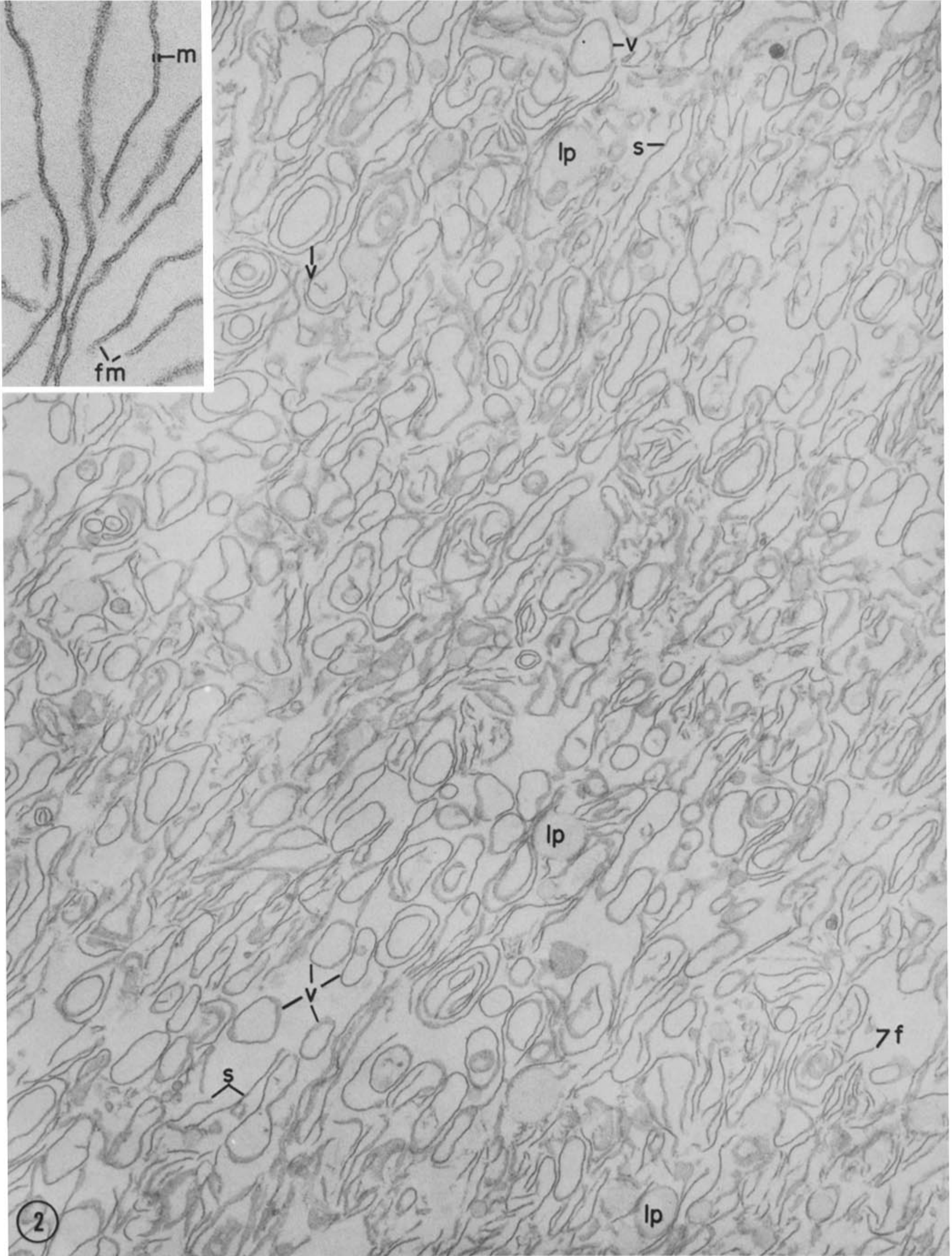
PHOSPHOLIPIDS: Total phospholipids were labeled in vivo with [^{33}P]phosphate given in two intravenous injections 20-h and 16-h before sacrificing each animal. Golgi fractions and subfractions were prepared, and their lipids extracted as described under Materials and Methods. The distribution of ^{33}P-radioactivity and of chemically assayed lipid phosphorus was determined and the results are given in Table III. They show that the two sets of values distribute in strict parallelism

[2] At higher load or after longer exposure, apoA IV (M_r 46,000) and apoA I (M_r 27,000) are also detected in the content subfraction.

[3] Fluorograms of plasma protein standards (after long-term labeling) are also included in these figures. A more comprehensive record of secretory (mostly plasma) proteins can be seen in Fig. 11, lane *l*, of the companion paper which illustrates results obtained by short-term labeling.

FIGURE 2 Representative field in the pellet of a Golgi membrane subfraction. The preparation consists primarily of membranes that appear as vesicles (*v*), or large sheets (*s*), or small fragments (*f*) with free edges. The dense content of Golgi vesicles has been extensively or completely removed, but a few lipoprotein particles (*lp*) or lipid droplets remain occluded amidst the membranes. Many of these particles are larger than Golgi content lipoproteins; such particles may be the result of partial fusion during the extraction procedure. × 32,000.

The inset illustrates, at higher magnification, the stratified structure (*m*) of the membranes, and the existence of free edges (*fm*). × 133,000.


m
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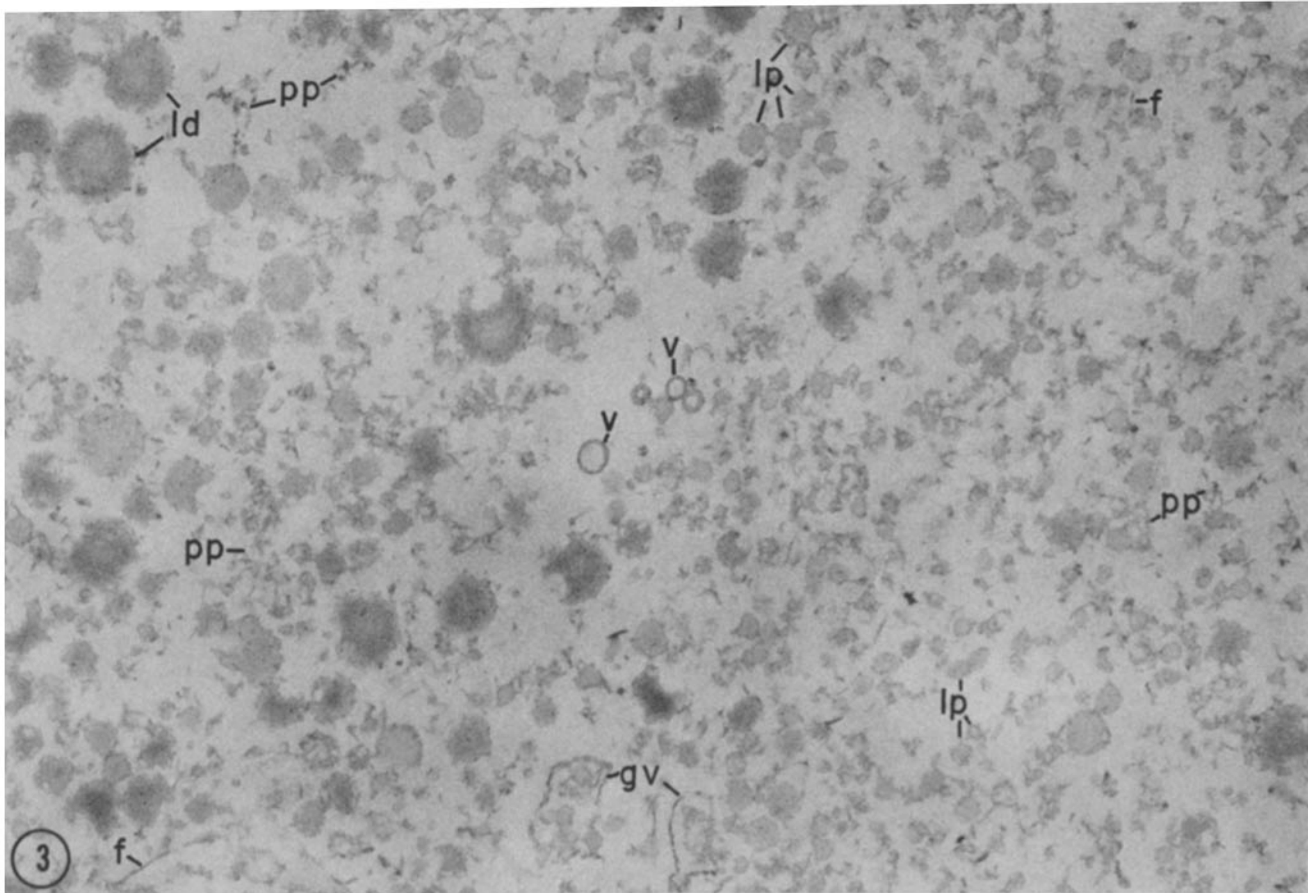

FIGURE 3 Pellet of a Golgi content subfraction. The bottom layer of the preparation contains primarily lipoprotein particles (*lp*) or lipid droplets (*ld*) (partly deformed by fixation), precipitated proteins (*pp*), and membrane remnants that range from numerous fragments (*f*) to a few vesicles (*v*), some (*gv*) still containing lipoprotein particles.

The right half of the field is representative of the bulk of the pellet. In the bottom ⅕ of the pellet, the size range of lipoprotein particles is larger than in Golgi elements; in the rest of the pellet, it is comparable to that of particles seen in intact Golgi elements (see Fig. 1). × 32,000.

between the GF membrane and the GF content subfraction: 84% of both ^{33}P-radioactivity and lipid phosphorus content are recovered in the membrane subfraction, with 16% of each in the content subfraction. Table III also gives protein distribution and the phospholipid:protein ratios for the two Golgi subfractions. The ratio for the membrane subfraction is remarkably high (0.91); the low value for the content subfraction reflects the high concentration of secretory proteins and glycoproteins (in addition to lipoproteins) within GF vesicles.

Aliquots of the two lipid extracts were resolved into individual phospholipid species by a two dimensional TLC system, the ensuing spots were identified by comparison with known standards, and the lipid phosphorus content and ^{33}P-radioactivity of each spot determined as indicated under Materials and Methods. The results are given in Table IV which includes (for comparison) data for the parental Golgi fraction. For each Golgi subfraction, the values obtained are expressed as percent of the total phospholipid in the parental Golgi fraction (% T PL), and as percent of the phospholipid in each subfraction (% SF PL). As before (see Table III), there was strict parallelism in the distribution of ^{33}P-radioactivity and lipid phosphorus content, this time at the level of individual phospholipids, in all preparations examined (data not shown). In this experiment a slightly lower percentage than usual of the total phospholipid of the parental GF (~75%, instead of 82–84%) distributed with the membrane subfraction, and the percent found in the content subfraction was commensurately higher (~25%). In each subfraction, phosphatidylcholine is the major phospholipid: ~62% in the membrane - and ~76% in the content subfraction. The relative concentration of different phospholipids varies from one subfraction to the other, but neither one nor the other contains unique phospholipids. Phosphatidylserine and phosphatidylethanolamine have the most unequal distribution: the first is 4.4 times, and the second 3.9 times, more concentrated in the membrane than in the content subfraction. As a first approximation, both could serve as lipid markers for Golgi membranes, because ~92% of the phosphatidylserine and ~93% of the phosphatidylethanolamine of the parental Golgi fraction are recovered in the membrane subfraction, but there is no convenient, reasonably stable, radioactive precursor for either one.[4] For phosphatidylinositol, a convenient radioactive precursor is available ([^{3}H]*myo*-inositol), but the percent recovered in the membrane subfraction is generally lower, ranging from 71% (in this experiment) to 80% in others.

NEUTRAL LIPIDS: The distribution of neutral lipids was followed on lipid extracts prepared from Golgi fractions and subfractions isolated from animals labeled in vivo with a combination of [^{3}H]palmitate and [^{3}H]oleate. The neutral lipids of each extract were resolved by thin layer chromatography,

[4] Ethanolamine radioactivity is expected to appear in phosphatidyl ethanolamine, phosphatidylcholine, and phosphatidylserine.

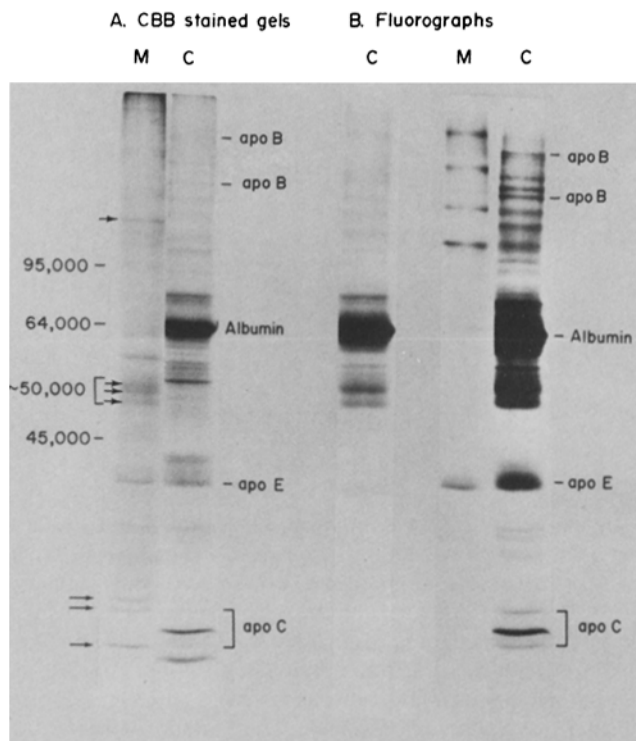

FIGURE 4 SDS PAG Electrophoretograms of Golgi membrane and content subfractions. Electrophoretograms of membrane (*M*) and content (*C*) subfractions stained with Coomassie Brilliant Blue are shown under *A*. The figures to the left of *M* indicate the position of molecular markers. Identified content bands are indicated on the right side of lane C. (Examples are shown in Figure 10 and 11 of the companion paper [*25*]). Bands found only in the GF membranes are marked by arrows.

Fluorographs of the same gels are shown in the last two lanes (*M* and *C*) under *B*. The main apolipoproteins (the *apo B* doublet, *apo E*, and three *apo C's*), albumin, and other unidentified secretory proteins are heavily labeled. The membrane subfraction is free of albumin, *apo B*, and *apo C*, but is still contaminated by newly synthesized apo E and other unidentified, radioactive, (presumably) secretory proteins of higher molecular weight.

A shorter exposure of the fluorograph in lane *B3* is shown in lane *B1* to demonstrate additional secretory proteins in the 80–50 Kdal region, obscured in lane *B3* as a result of a longer exposure.

and identified and quantitated as given under Materials and Methods.

The data obtained indicate that 84 to 92% of the labeled triacylglycerol of the parental GF separates with the content subfraction, and 12 to 16% with the membrane subfraction. This finding is in agreement with the presence of morphologically recognizable lipoprotein-like particles in the membrane subfraction (see Fig. 2). Assuming that all the triacylglycerol represents contamination by content VLDLs, the data set an upper limit to the contamination of membrane subfraction by content subfraction; expressed as percent phospholipid, the limit is ~1% of the phospholipid[5] of the total membrane subfraction.

ASSESSMENT OF GF SUBFRACTIONATION: From both biochemical and morphological studies, it is clear that there is detectable cross contamination of both membrane and content subfractions with each other's components. The membrane

TABLE III

Distribution of Protein, [^{33}P]-Radioactivity, and Phospholipids in Golgi Subfractions

	Protein		[^{33}P]		Phospholipid		mg PL / mg protein
	mg	*%*	*cpm*	*%*	*mg*	*%*	
Total GF	2.32		136,552		1.308		
Membrane subfraction	1.18	57	106,050	84	1.076	84	0.91
Content	0.88	43	19,725	16	0.202	16	0.23
Recovery	89%		92%		98%		

subfraction accounts for ~15% of the short term [^{3}H]leucine label, and retains specific content bands identified by PAGE and fluorography. In addition, it accounts for ~14% of the label introduced in triacylglycerol by [^{3}H]palmitate and [^{3}H]-oleate, and ascribable to contamination by content lipoproteins. The morphological survey also indicates that the membrane subfraction retains some lipid particles the size of VLDLs or larger. Conversely, it is difficult to get from electrophoretic analyses an accurate estimate of the contamination of the content subfraction by membrane components, especially membrane proteins, but the morphological survey shows convincingly that the preparation contains membrane fragments. Since it is not easy to assess the extent of this contamination by morphometry, we have tried to estimate it by assays for a few membrane enzymes.

DISTRIBUTION OF PHOSPHATASE ACTIVITIES IN GOLGI SUBFRACTIONS

5′-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphatase have an alkaline pH optimum and are not inhibited, or only partially inhibited, by the high pH procedure[6]. All these enzymes are membrane proteins often considered to be plasmalemmal markers (40). But, as in the case of other plasmalemmal enzymes (11, 12) and receptors (4), our findings indicate that they are present in Golgi membranes in addition to the plasmalemma. The data in Table V show that ~5% of the 5′-nucleotidase and ~10% of the phosphodiesterase I activities are recovered in the content subfraction, which suggests that 5 to 10% of the total amount of Golgi membranes contaminate the content. The distribution of alkaline phosphatase activity is quite different. The enzyme is partially (~30%) inactivated during the high pH procedure and the remaining activity (~70%) partitions during the GF subfractionation so that ~42% is recovered in the membrane - and ~28% in the content subfraction. Recalculated for 100% recovery, the figures become 60% for the membrane - and 40% for the content subfraction. The alkaline phosphatase activity can be significantly stabilized if 0.2 mM $ZnCl_2$ is added to the 100 mM Na_2CO_3. In this case, 91% recovery is obtained and the enzymatic activity distributes 60% with the membrane - and 40% with the content subfraction. Some of the released activity may represent true content, because alkaline phosphatase activity is present in rat serum. Two other possible interpretations are that the alkaline phosphatase is a membrane protein (as gen-

[5] Calculated on the basis of the following assumptions: all triaclyglycerol in the membrane subfraction is contributed by VLDL-like particles with a ratio TG:PL of 5:1 (see reference 43).

[6] Galactosyltransferase, the most widely accepted marker enzyme for Golgi membranes, was inactivated by the high pH procedure as used in these experiments.

TABLE IV

Phospholipid Distribution in Golgi Fraction and Derived Membrane and Content Subfractions

Phospholipid species	Golgi fraction	Membrane subfraction		Content subfraction	
	% PL	*% TPL*	*% SF PL*	*% TPL*	*% SF PL*
Origin	0.66	0.65	0.87	0.01	0.03
Phosphatidic acid	0.11	0.11	0.14	0.00	0.00
Phosphatidylserine	2.74	2.30	3.08	0.17	0.66
Phosphatidylinositol	4.59	3.27	4.35	1.32	5.20
Phosphatidylethanolamine	16.97	15.73	20.92	1.34	5.35
Phosphatidylcholine	58.98	43.94	58.87	16.58	65.32
Lysophosphatidylcholine	6.90	4.08	5.46	2.81	11.07
Sphingomyelin	9.34	6.18	8.28	3.16	12.45
% of total GF phospholipid	100.00	74.66	—	25.39	—

The figures are percent ^{33}P-radioactivity recovered in each phospholipid species resolved by two dimensional TLC. The distribution of lipid phosphorus (chemically assayed) among the same phospholipid species is similar. For further details see Materials and Methods.

TABLE V

Distribution of Enzymatic Activities in Golgi Membrane and Content Subfractions

Golgi subfractions	5'-Nucleotidase	Phosphodiesterase I	Alkaline phosphatase
		% Distribution	
Membrane subfraction	95.3 ± 2.07	89.7 ± 6.25	59.7 ± 10.5
Content subfraction	4.7 ± 2.07	10.3 ± 6.35	40.3 ± 10.5
Recovery	100%	109%	70%

The figures give percent distribution ± SD. Specific activities were: 89 ± 16.6 nmol P_i liberated from 5'-AMP/min/mg protein for 5'-nucleotidase; 855 ± 58 nmol *p*-nitrophenol liberated from *p*-nitrophenyl thymidine 5'-phosphate/min/mg protein, for phosphodiesterase I; and 3175.5 nmol *p*-nitrophenol liberated from *p*-nitrophenyl phosphate/min/mg protein for alkaline phosphatase. The table shows the results of three experiments.

erally assumed), but is partially released by the high pH treatment; or that the membrane fragments and vesicles found in the content subfraction are specifically enriched in this enzyme.

DISCUSSION

We have characterized the Golgi fraction used as starting preparation in these experiments primarily by morphological criteria, and - by this account - it appears to be reasonably homogeneous. The characterization of the same fraction by assays for marker enzymes has been carried out recently (24, 26) and closely related fractions (3, 6) have been the object of more extensive enzymic studies in the past. We felt that a more detailed investigation along such lines was not warranted at present, since recent findings indicate that the degree of enzymic overlap between the ER and the Golgi complex, on the one hand (24, 26), and between the Golgi complex and the plasmalemma, on the other hand (4, 11, 12, 40), is more extensive than assumed in the past, at the time when enzyme markers for the ER and plasmalemma were defined. A redefinition of the marker enzyme concept for the successive compartments of the secretory pathway (ER, Golgi complex, plasmalemma) is needed to establish to what extent we can rely on absolute markers, and to what extent we must depend on quantitative differences in the concentration of enzymes or receptors when characterizing different subcellular components and their corresponding cell fractions.

An additional problem is the extensive heterogeneity of the elements of the Golgi complex (cf 16) suggested or indicated by histochemical (15, 33) and biochemical (24, 26) findings. The histochemical heterogeneity, especially that connected with the acid phosphatase positive elements defined by Novikoff as GERL (41), has not yet been corroborated by biochemical data; and to obtain such data, more refined fractionation procedures than currently available would be required.

The resolution of Golgi fractions into Golgi content and Golgi membrane subfractions achieved by our high pH procedure is definitely not complete. The evidence indicates that the upper limit for the contamination of the membrane subfraction by content proteins is ~15% of the total content - identified as radioactive proteins in a short term labeling experiment. The contamination by content lipoproteins, identified via radioactive triacylglycerol, is of the same order. Conversely, the upper limit of content contamination by membrane protein is ~10% on the basis of enzymatic activities. Notwithstanding its limitations, this separation is the best thus far obtained, and it is the only example in which the type and extent of contamination of membranes by content proteins has been reasonably well assessed.

Complete separation of content from membranes has been difficult to achieve for most cell fractions. In the case of hepatic rough microsomes, for instance, Kreibich et al. (29) and Kreibich and Sabatini (30) have achieved the release of 45% of the content (defined by short term [5 min] radioactive labeling) and 20% of the total protein by using 0.049 to 0.098% deoxycholate (DOC) at a fraction protein concentration of 3–6 mg/ml. The recovered membranes retained their phospholipids and the activities of their electron transport systems. Greater release of content, i.e., 64% of the label and 26% of the protein, was achieved with 0.15% Triton X-100 in the case of smooth microsomes, but the recovered membranes were not further characterized. Other investigators have isolated microsomal membrane preparations, but have characterized them less extensively. Elder and Morré (14) have prepared intrinsic membrane proteins by treating microsomal fractions with 1.5 M KCl, 0.1% DOC, 0.01 M Tris-HCl, pH 7.6, and extensive sonication, followed by a water wash and a sonication step. For rough microsomes, this intrinsic membrane protein fraction was 22% of the total protein. An antibody against it was reported to form no precipitation lines with the protein fraction extractable by KCl and DOC. The same separation procedure was used to prepare intrinsic proteins from Golgi fractions, but the efficiency of the separation was not defined.

Glaumann and Dallner (21) and Bergman and Dallner (5) prepared both microsomal and Golgi membranes with a Tris-water-thermal washing procedure, but data on the efficiency

of the technique were given only for microsomes. Their final microsomal membrane preparation comprised 45% of the original protein and retained most of the phospholipid, NADPH cytochrome *c* reductase, cytocheom b_5, and nucleoside diphosphatase activities of the original microsomal fraction.

To date the most effective separation of content from membranes was achieved with parotid secretion granules (9, 10): 98% of the content removed by saponin-high salt treatment at the price of extensive membrane degradation.

The only characterized procedure for the release of content from Golgi vesicles is that of Flesicher (17); it involves high slat (0.4 M NaCl), basic conditions (0.14 M $NaHCO_3$), and disruption in a Parr bomb. This technique has the advantage that the galactosyltransferase activity is maintained (7% is released to the supernatant) (17).[7] The extent of content release approaches that achieved by our procedure: protein - 35%, phosphate - 30%, and albumin - 70% (measured by precipitation with specific antibody). The Golgi subfractions were not characterized morphologically (17).

This survey of the literature substantiates what we have already stated: removal of content is a difficult operation for all cell fractions so far tested, and the results of our procedure for separating Golgi content from Golgi membranes compare favorably with those already published in the literature except for the inactivation of galactosyltransferase activity.

From our data it is possible to estimate how much of the protein of the Golgi vesicles is in membranes and how much is protein destined for export (content). A reliable estimate would be useful in normalizing enzymic data, and critical in making comparisons between different membranes. Assuming that our content marker (rapidly labeled proteins) is valid, and assuming its complete release from the membrane, 40% of the protein of GF would be membrane protein. This is intermediate between microsomes (ER) in which membrane protein would account for 56% of the total protein by the same calculation (29, 30), and parotid secretion granules in which the corresponding figure is estimated as 1.5% (9, 10). The relatively high percentage of Golgi protein recovered in Golgi membranes is consistent with a number of observations: by microscopy, the proteins destined for export appear to be less extensively concentrated in hepatic Golgi elements than in most secretion granules, and in pulse-chase experiments, autoradiographic grains are not as concentrated over hepatic Golgi elements (52) as they are over secretion granules in other cell types (8, 27). In addition, in hepatocytes, a large fraction of the Golgi content is accounted for by lipoproteins which contain proteins in low concentration: VLDLs are ~10% protein and ~90% lipid (43). Yet the estimate mentioned above may well be excessive, because a high salt wash (see Table II) removes a large fraction of nonradioactive protein (presumably peripheral membrane proteins or proteins relocated by adsorption) from the GF membrane subfraction.

Data on the phospholipid composition of hepatic Golgi fractions (19, 28, 53) and membranes prepared from Golgi fractions (19, 53) are available in the literature. Only the membrane preparations analyzed by Zambrano et al. (53) and Fleischer and Fleischer (19) are comparable to our GF membrane subfractions (although they appear to include more Golgi content). The relative concentration of the various phospholipids in the two preparations is generally similar, but our subfractions contain less phosphatidylinositol and phosphatidylserine and more phosphatidylcholine than their Golgi membranes.

One of the most interesting findings in relation to Golgi phospholipids is the extensive, uniform, long-term labeling by [^{33}P]phosphate of all phospholipids in both membrane and content subfractions. This finding definitely requires further investigation, because it suggests effective exchange mechanisms between membrane and content phospholipids within the Golgi complex.

In the future, the GF membrane - and GF content subfractions, isolated and partially characterized in the experiments reported in this paper, can be used to investigate the biogenesis of serum lipoproteins and the Golgi phase of their intracellular processing, as well as the metabolism of Golgi membrane and Golgi lipoprotein lipids. We realize that a definitive analysis of these subfractions will become possible only when a better separation than the one here described will be achieved. However, even at the current level of resolution of Golgi subfractions, useful information can be obtained on certain topics. An example is given in the companion paper (25) in which we present the results of a study on the lipoproteins of the Golgi content subfraction.

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[7] The enzyme can be solubilized to >90% by treating Golgi membranes with 2 mg of Triton X-100/ml (18).

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