Hepatic Golgi Fractions Resolved into Membrane and Content Subfractions

KATHRYN E. HOWELL and GEORGE E. PALADE
Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510. Dr. Howell's present address is the European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany.

ABSTRACT Golgi fractions isolated from rat liver homogenates have been resolved into membrane and content subfractions by treatment with 100 mM Na$_2$CO$_3$ 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, 50-100 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, 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 $\mu$Ci [3H]leucine by intraperitoneal injection, or 50 $\mu$Ci [3H]palmitic acid and 100 $\mu$Ci [3H]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 x 250 $\mu$Ci) in the saphenous vein at 20 h and 16 h before sacrifice.
Gel Electrophoresis was carried out at room temperature at a constant current. 100 µl of sample, containing 165 µg protein, was applied to each slot of solution, and 1% bromphenol blue (10 µl/ml) was introduced as electrophoretic disulfide bonds. 2 M sucrose was added (200 µl/ml) to increase the density of the 0.8 M 1.15 M interface of the sucrose gradient, in a fraction designated GFs.

**ISOZYMES:**

GF contains 12–15% of the total galactosyltransferase activity (the most commonly used marker enzyme for Golgi-derived vesicles) of the whole homogenate. 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 GFp.

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 centrifuged for 60 min at 105,000 g in a 60 Ti rotor (Beckman Instruments). The resulting pellet was resuspended in a protein concentration of <2 mg/ml in 100 mM Na2CO3–NaHCO3 buffer 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 centrifuged for 60 min at 106,500 g 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 Na2CO3 supernatant is defined as the GF content subfraction and the Na2CO3 pellet, resuspended in 0.25 M sucrose, as the GF membrane subfraction.

**Electron Microscopy**

Samples of the fractions and subfractions of interest were fixed in suspension by mixing equal volumes of the sample and 4% O2O2. After 2 h, the suspensions were centrifuged for 20 min at 37,500 g, 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.

Neutral lipids were separated with the solvent system hexane:ether:acetic acid (80:20:1) (50). Spots, detected by exposure of the TLC plates to iodine vapor, were identified by comparison with known standards (obtained from Supelco, Bellanfonza, PA), scraped directly into vials for counting or into tubes for digestion (39) and subsequent 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:acetonemethanol: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, Bellanfonza, PA), scraped directly 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 MgCl2, 0.1% Triton-X-100, 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 0.5 M 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 1 (paraphosphoric diester phosphohydrolase; EC 3.1.4.1): Alkaline p-nitrophenyl phosphatase and phosphodiesterase 1 activities were estimated by measuring the absorbances 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 Na2CO3-NaHCO3 buffer, pH 10.0, 2.5 mM MgCl2, 0.1% Triton-X-100, and 5 mM substrate. For alkaline phosphodiesterase 1, the incubation mixture contained 10 mM Na2CO3-NaHCO3 buffer, pH 10.0, 2.0 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

---

1 Density units will not be restated in the rest of the text.
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 Na₂CO₃, 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 [³H]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 [³H]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 subtraction). Release can be increased slightly if the pellet (i.e., membrane subtraction) 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 Na₂CO₃, 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 subtraction. These figures represent 5% of the radioactivity and 3 to 5% of the protein content of the original GF. If the resuspended membrane subtraction 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 MgCl₂) 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.
The membrane subfraction washed as under (a) contains ~13% of the [\textsuperscript{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 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 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 [\textsuperscript{3}H]leucine label, the major protein components by CB staining are in the 50,000 M\textsubscript{s} 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\textsubscript{s} 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\textsubscript{s} from 200,000 to 100,000 are revealed by fluorography in the membrane subfraction, in addition to another labeled band of apparent M\textsubscript{s} ~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 [\textsuperscript{32}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 \textsuperscript{32}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 above.

3 At higher load or after longer exposure, apoA IV (M\textsubscript{s} 46,000) and apoA I (M\textsubscript{s} 27,000) are also detected in the content subfraction.

\textsuperscript{2} 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. X 32,000.

The inset illustrates, at higher magnification, the stratified structure (m) of the membranes, and the existence of free edges (fm). X 133,000.
between the GF membrane and the GF content subfraction: 84% of both \( ^3P \)-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 \( ^3P \)-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 \( ^3P \)-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. Phosphatidyserine 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 phosphatidyserine 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. For phosphatidylinositol, a convenient radioactive precursor is available ([\( ^3H \)]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 [\( ^3H \)]palmitate and [\( ^3H \)]oleate. The neutral lipids of each extract were resolved by thin layer chromatography,

- Ethanolamine radioactivity is expected to appear in phosphatidyl ethanolamine, phosphatidylcholine, and phosphatidyserine.
secretory proteins of higher molecular weight. Synthesized apo E and other unidentified, radioactive, (presumably) albumin, apo B, and apo C, but is still contaminated by newly
companion paper [25]. Bands found only in the GF membranes are
content subfractions. Electrophoretograms of membrane (M) and
content subfractions with each other’s components. The membrane
limit is -rl% of the phospholipid of the total membrane
content subfraction; expressed as percent phospholipid, the
subfraction (see Fig. 2). Assuming that all the triacylglycerol
represents contamination by content VLDLs, the data set an
subfraction, and 12 to 16% with the 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, [33P]-Radioactivity, and Phospholipids in Golgi Subfractions |
|-----------------------------------|----------------------------------|--------|
|                                  | Protein | [33P] | Phospholipid | mg PL |
|                                  | 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% |

5'-nucleotidase, alkaline phosphodiesterase I, and alkaline
phosphatase have an alkaline pH optimum and are not in-
hibited, or only partially inhibited, by the high pH procedure.
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 find-
ings 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 activ-
ities are recovered in the content subfraction, which suggests
that 5 to 10% of the total amount of Golgi membranes contam-
inate the content. The distribution of alkaline phosphatase
activity is quite different. The enzyme is partially (~30%) inactiv-
ated 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 signifi-
cantly stabilized if 0.2 mM ZnCl₂ is added to the 100 mM
Na₂CO₃. In this case, 91% recovery is obtained and the enzy-
matic 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 triacylglycerol in the membrane subfraction is contributed by VLDL-like particles with a ratio TG:PL of 5:1 (see reference 43).
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 microsomal 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

### TABLE IV
Phospholipid Distribution in Golgi Fraction and Derived Membrane and Content Subfractions

| Phospholipid species | Golgi fraction | Membrane subfraction | Content subfraction |
|----------------------|---------------|-----------------------|---------------------|
|                      | % PL | % SPL | % SF PL | % SPL | % SF PL | % PL | % SPL | % SF PL | % SPL | % SF PL |
| Origin               | 0.66 | 0.65 | 0.67 | 0.01 | 0.03 | 100.00 | 74.66 | 25.39 |
| Phosphatidic acid    | 0.11 | 0.11 | 0.14 | 0.00 | 0.00 | 100.00 | 74.66 | 25.39 |
| Phosphatidylserine   | 2.74 | 2.30 | 3.08 | 0.17 | 0.66 | 100.00 | 74.66 | 25.39 |
| Phosphatidylinositol | 4.59 | 3.27 | 4.35 | 1.32 | 5.20 | 100.00 | 74.66 | 25.39 |
| Phosphatidylethanolamine | 16.97 | 15.73 | 20.92 | 1.34 | 5.35 | 100.00 | 74.66 | 25.39 |
| Phosphatidylcholine  | 58.98 | 43.94 | 58.87 | 16.58 | 65.32 | 100.00 | 74.66 | 25.39 |
| Lyso phosphatidylcholine | 6.90 | 4.08 | 9.46 | 2.81 | 11.07 | 100.00 | 74.66 | 25.39 |
| Sphingomyelin        | 9.34 | 6.18 | 0.28 | 3.16 | 12.45 | 100.00 | 74.66 | 25.39 |
| % of total GF phospholipid | 100.00 | 74.66 | 25.39 | 100.00 | 74.66 | 25.39 |

The figures are percent ^32P-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 subfrations | 5'-Nucleotidase | Phosphodiesterase I | Alkaline phosphatase |
|-------------------|-----------------|---------------------|----------------------|
|                   | % Distribution  | % Distribution | % Distribution |
| Membrane subfraction | 95.3 ± 2.07 | 89.7 ± 6.25 | 59.7 ± 10.5 |
| Content subfraction | 4.7 ± 2.07 | 10.3 ± 3.65 | 40.3 ± 10.5 |
| Recovery           | 100% | 109% | 70% |

The figures give percent distribution ± SD. Specific activities were: 89 ± 16.6 nmol P₄ liberated from 5'-AMP/min/mg protein for 5'-nucleotidase; 655 ± 58 nmol p-nitrophenolphosphate from p-nitrophenyl thymidine 5'-phosphate/min/mg protein; 2.28 ± 0.25 µmol p-nitrophenolphosphate from p-nitrophenyl phosphate/min/mg protein for alkaline phosphatase. The table shows the results of three experiments.

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 microsomal 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

### 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.
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, cytochrome b₅, and nucleoside diphosphate 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 salt (0.4 M NaCl, basic conditions (0.14 M NaHCO₃), and disruption in a Parr bomb. This technique has the advantage that the galactosyltransferase activity is maintained (7% is released to the supernatant) (17). 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

---

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

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 [32P]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.

We acknowledge with gratitude the assistance of Stephen Massaro. We also thank Hans Stuckenbrock for microtomy, Pamela Ossorno for photography, and Rick Andrews and Cynthia Davis for typing the manuscript.

This work was supported by National Institutes of Health grants GM 27303 and GM 21714.

Received for publication 28 May 1981, and in revised form 15 October 1981.

REFERENCES

1. Ames, B. N., and D. T. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235:769-775.
2. Anderson, L. E., and W. O. McClure. 1973. An improved vitellinification cocktail of high-solubilizing power. Anal. Biochem. 51:173-179.
3. Bergeron, J. J. M., J. H. Ehrenreich, P. Sieverkez, and G. E. Palade. 1973. Golgi fractions prepared from rat liver homogenate. II. Biochemical characterization. J. Cell Biol. 59:73-88.
4. Bergeron, J. J. M., W. H. Evans, and I. I. Geschwind. 1973. Insulin binding to rat liver Golgi fractions. J. Cell Biol. 59:771-775.
5. Bergman, A., and G. Dallner. 1976. Distribution of protein-bounded sugar residues in microsomal subfractions and Golgi membranes. Biochim. Biophys. Acta. 433:496-508.
6. Berta, R., H. Bretz, and G. E. Palade. 1980. Distribution of terminal glycosyltransferases in hepatic Golgi fraction. J. Cell Biol. 84:87-101.
7. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.
8. Castle, J. D., J. D. Jamieson, and G. E. Palade. 1975. Secretion granules of the rabbit parotid gland. Isolation, subfractionation, and characterization of the membrane and content subfractions. J. Cell Biol. 64:182-210.
9. Castle, J. D., and G. E. Palade. 1978. Secretion granules of the rabbit parotid. Selective removal of secretory contaminates from granule membranes. J. Cell Biol. 76:323-340.
10. Cheng, H., and M. G. Farquhar. 1976. Presence of adeylate cyclase activity in Golgi and other fractions from rat liver. I. Biochemical determination. J. Cell Biol. 70:670.
11. Cheng, H., and M. G. Farquhar. 1976. Presence of adeylate cyclase activity in Golgi and other fractions from rat liver. II. Cytochemical localization within Golgi and ER membranes. J. Cell Biol. 70:671-684.
12. Ehrenreich, J. H., J. J. M. Bergeron, P. Sieverkez, and G. E. Palade. 1973. Golgi fractions prepared from rat liver homogenate I. Isolation procedure and morphological characterization. J. Cell Biol. 59:45-72.
13. Elder, J. E., and D. J. Moore. 1975. Synthesis in vitro of intrinsic membrane proteins by free, membrane-bound, and Golgi apparatus-associated polyribosomes from rat liver. J. Cell Biol. 70:554-568.
14. Farquhar, M. G., J. J. M. Bergeron, and G. E. Palade. 1974. Cytochemistry of Golgi fractions prepared from rat liver. J. Cell Biol. 60:25.
15. Farquhar, M. G. 1978. Traffic of products and membranes through the Golgi complex. In: Transport of Macromolecules in Cellular Systems. S. Silverstein, editor. Dahlem Conference, Berlin. 341-362.
16. Fleischer, B. 1974. Isolation and characterization of Golgi apparatus membranes from rat liver. In: Methods in Enzymology. S. P. Colowick and N. D. Kaplan, editors. Academic Press, New York. 31:180-191.
17. Fleischer, B., and M. Snigul. 1978. Solubilization and properties of galactose transferase and mannosidase activities of Golgi membranes in Triton X-100. J. Cell Biol. 25:1632-1638.

H OWELL AND PALADE Hepatic Golgi Fractions 831
19. Fleischer, S. and B. Fleischer. 1977. Membrane diversity in the rat hepatocyte. In: Membrane Alteration as Basis of Liver Injury. H. Popper, editor. MTP Press Ltd., Lancaster, England. 31-47.

20. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

21. Glassman, H. and G. Dallner. 1968. Lipid composition and turnover of rough and smooth microsomal membranes in rat liver. J. Lipid Res. 9:720-728.

22. Hamilton, R. L., D. M. Regen, M. E. Gray, and V. S. LeQuire. 1967. Lipid transport in liver. I. Electron microscopic identification of very low density lipoproteins in perfused rat liver. Lab. Invest. 16:305-319.

23. Haimont, R., L. A. Keyden, 1974. The liver and the formation of normal and abnormal plasma lipoproteins. In: The Liver: Normal and Abnormal Functions. Part A. F. F. Decker, editor. Marcel Dekker, Inc., New York. 531-572.

24. Howell, K., E. A. Ito, and G. E. Palade. 1978. Endoplasmic reticulum marker enzymes in Golgi fractions. What does this mean? J. Cell Biol. 79:581-589.

25. Howell, K. E., and G. E. Palade. 1982. Heterogeneity of lipoprotein particles in hepatic Golgi fractions. J. Cell Biol. 92:833-843.

26. Ito, A., and G. E. Palade. 1978. Presence of NADPH cytochrome P-450 reductase in rat liver Golgi membranes: evidence obtained by immunoadsorption method. J. Cell Biol. 79:590-597.

27. Jamieson, J. D., and G. E. Palade. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. J. Cell Biol. 34:597-615.

28. Keenan, T. W., and D. J. Morre. 1970. Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions. Biochemistry 9:13-25.

29. Kreibich, G., P. Debey, and D. D. Sabatini. 1973. Selective release of content from microsomal vesicles without membrane disassembly. I. Permeability changes induced by low detergent concentrations. J. Cell Biol. 58:436-462.

30. Kreibich, G., and D. D. Sabatini. 1974. Selective release of content from microsomal vesicles without membrane disassembly. II. Electrophoretic and immunological characterization of microsomal subfractions. J. Cell Biol. 61:789-807.

31. Krenn, T., M. H. Washler, and W. H. Evans. 1976. The lipid composition of plasma membrane subfractions originating from the three major functional domains of the rat hepatocyte cell surface. Biochim. Biophys. Acta. 453:651-664.

32. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of 3H and 14C in polyacrylamide gels by fluorography. Eur. J. Biochem. 50:333-341.

33. Little, J. S., and C. C. Wunderlich. 1977. Evidence for the translocation of 5'-nucleotidase across hepatic membranes by protein perturbants. J. Supramol. Struct. 1:220-232.

34. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

35. Mahley, R. W. and V. S. LeQuire. 1969. Characterization of lipoprotein particles isolated from Golgi apparatus of rat liver. J. Lipid Res. 10:433-439.

36. Mahley, R. W., T. P. Berner, V. S. LeQuire, R. I. Levy, H. G. Windmueller, and W. V. Brown. 1970. Identity of very low density lipoprotein apoproteins of plasma and liver Golgi apparatus. Science (Wash. D. C.) 168:380-382.

37. Mazel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. In: Methods of Virology. K. Maramorosh and H. Koprowski, editors. Academic Press, Inc., New York. 5:179-246.

38. Mendelsohn, J. 1974. Dynamics of cytoplasmic membranes in the guinea pig pancreatic acinar cells. I. Synthesis and turnover of membrane proteins. J. Cell Biol. 61:1-13.

39. Morrison, W. R. 1964. A fast, simple, and reliable method for the microdetermination of phosphorus in biological materials. Anal. Biochem. 7:218-224.

40. Neville, D. M., Jr. 1975. Isolation of cell surface membrane fractions from mammalian cells and organs. In: Methods in Membrane Biology. E. D. Korn, editor. Plenum Press, New York. 5:1-49.

41. Novikoff, A. B. 1976. The endoplasmic reticulum: a cytochemist's view. (A Review). Proc. Natl. Acad. Sci. U. S. A. 73:2791-2797.

42. Palade, G. E. 1975. Intracellular aspects of the process of protein secretion. Science (Wash. D. C.) 189:347-358.

43. Pasquale-Ronzoni, J., S. Calandra, M. Razzani-Contri, and M. Mazzaghi. 1975. The ultrastructure of rat plasma lipoproteins. J. Ultrastruct. Res. 53:180-192.

44. Peters, T., B. Fleischer, and S. Fleischer. 1971. The biosynthesis of rat serum albumin. IV. Apparent passage of albumin through the Golgi apparatus during secretion. J. Biol. Chem. 246:340-344.

45. Redman, C. M., D. Banerjee, K. Howell, and G. E. Palade. 1975. Co爪chicine inhibition of plasma protein release from rat hepatocytes. J. Cell Biol. 66:42-59.

46. Rouzer, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids. 5:494-496.

47. Ruderman, N. B., D. C. Richards, V. Valles de Bourges, and A. L. Jones. 1968. Regulation of production and release of lipoprotein by the perfused rat liver. J. Lipid Res. 9:613-619.

48. Scheele, G. A., G. E. Palade, and A. M. Tartakoff. 1978. Cell fractionation studies on the guinea pig pancreas. Redistribution of exocrine proteins during tissue homogenization. J. Cell Biol. 78:110-130.

49. Shkolnikov, W. D., and C. Entenman. 1962. The determination of esterified fatty acids in glycerides, cholesterol esters, and phospholipids. J. Lipid Res. 3:356-360.

50. Skipski, V., and M. Barclay. 1969. Thin-layer chromatography of lipids. In: Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press, Inc., New York. 14:350-359.

51. Steck, T. L., and J. Yu. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. J. Supramol. Struct. 1:220-232.

52. Stein, O., and Y. Stein. 1967. Lipid synthesis, intracellular transport, storage, and secretion. 1. Electron microscopic radioautographic study of liver after injection of tritiated palmitate or glycerol in fasted and ethanol-treated rats. J. Cell Biol. 33:319-339.

53. Zambrano, F., S. Fleischer, and B. Fleischer. 1975. Lipid composition of the Golgi apparatus of rat kidney and liver in comparison with other subcellular organelles. Biochim. Biophys. Acta. 380:377-369.