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THE GOLGI COMPLEX OF THE HEPATOCYTE: ISOLATION  
AND PARTIAL ENZYMOLOGICAL CHARACTERIZATION

A thesis submitted to the Faculty of The Rockefeller University  
in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy

by  
John H. Ehrenreich, B. A.

*Approved for publication,*

*George E. Palady*

*Professor, Cell Biology.*

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The Rockefeller University  
New York, New York





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## SUMMARY

The elements of the Golgi complex of the hepatocyte are characterized by their content of 30-80m osmiophilic particles, which have been identified by others as very low density lipoprotein. The vast majority of such particles in the cell are in the elements of the Golgi complex. Moreover, the number in the complex can be increased still further by acute ethanol intoxication. This light lipoprotein content afforded the opportunity to isolate a purified fraction of Golgi-derived vesicles from the liver cell, since the content itself would make these vesicles lighter than vesicles derived from other intracellular sources (which do not contain lipoprotein particles) and since the clusters of particles could serve as a unique marker for Golgi-derived material.

Accordingly, a fraction of Golgi-derived vesicles has been isolated from microsomes obtained from the livers of ethanol-intoxicated rats, by centrifugation on a discontinuous sucrose gradient under conditions designed to favor purity at the expense of yield. The fraction has been examined in the electron microscope and found to consist almost exclusively of vesicles containing clusters of electron opaque 30-80m particles. There are no recognizable mitochondria or rough surfaced microsomes in the fraction and only a small number of smooth microsomes not containing dense particles. About 1% of the vesicles in the fraction have an appearance suggestive of lysosomes. On purely morphological grounds, we estimate that at least 90-95% of the vesicles in the fraction are derived from the Golgi complex.

The vesicles have been broken open by suspension in alkaline hypotonic medium followed by shearing in a steep pressure gradient (French press). After this treatment, a fraction of membranes largely free of lipoprotein particles could be isolated by centrifugation. This fraction is assumed to represent the membranes which bounded the Golgi vesicles in the cell.



The total Golgi fraction (membrane and contents) and the isolated Golgi membranes have been examined with respect to gross chemistry and to a series of enzymatic activities, and compared to a total microsomal fraction (and in some cases to lysosomal and mitochondrial fractions). The Golgi membranes have the same phospholipid-to-protein ratio as microsomal membranes, and contain no detectable RNA. They have no detectable glucose-6-phosphatase, NADPH-cytochrome c reductase, or cytochrome P-450 activity, all enzymes associated with microsomal fractions, and their specific activity for NADH-cytochrome c reductase and cytochrome b<sub>5</sub>, enzymes associated with both microsomal and plasma membranes, is only about 15% of that of microsomal fractions and about equal to that reported for plasma membranes. The Golgi fractions have an AMPase activity about equal to that of microsomal fractions and about 10% that of plasma membrane fractions. We believe this represents, in part at least, activity in the Golgi membranes themselves, not merely plasma membrane contamination of the fraction. The Golgi fractions also have high thiamine pyrophosphatase activity.

The Golgi fraction contains less than 1% of the cytochrome oxidase specific activity of purified mitochondrial fractions, consistent with our failure to observe mitochondrial contaminants morphologically. The acid phosphatase activity of the Golgi fractions is about 3% that of purified lysosomal fractions. This may reflect the lysosomal contamination seen in the fraction with the electron microscope, but it may also come from acid phosphatase activity in the Golgi vesicles themselves.

The biochemical evidence supports the conclusion drawn from morphology that the fraction consists at least 90-95% of Golgi-derived material. The enzyme activities are discussed with respect to the interrelationships which they imply among the Golgi complex, the rest of the endoplasmic reticulum, and the plasma membrane.



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## CHAPTER ONE

### GENERAL INTRODUCTION

The nature, function, and very existence of the Golgi complex were subjects of controversy for a long period after the discovery of the organelle in nerve cells by Camillo Golgi in 1898. The controversy stemmed from several sources: First, the Golgi complex could not be demonstrated in the living cell by conventional light microscopic techniques, except in the special case of the spermatocytes and spermatids of certain species. Second, the metal impregnation techniques classically used to demonstrate the Golgi complex in fixed material were difficult, and in the hands of some investigators, at least, led to failure to observe the complex in a number of cell types. Finally, the metal impregnation techniques were empirical and had no known specificity as histochemical reactions; hence, there was no assurance that the structures demonstrated with them in various cell types were chemically homologous. For these reasons, many investigators came to believe that in many cell types the structures described as the Golgi apparatus were artifacts of preparation techniques, or, in any event, were a heterogeneous set of subcellular structures that could be impregnated with metal in only some types, and which accordingly should not be lumped together under the common rubric "Golgi apparatus". Comprehensive reviews of the early Golgi controversy can be found in Bowen (1929) and Hibbard (1945). The controversy continued with considerable bitterness well into the 1950's; see Gatenby (1955), Lacy and Challice (1957) and Palay (1958) for later reviews supporting the reality of the Golgi complex, and Baker (1957) for a review supporting the artifact theory.

The controversy over the existence and structural homology of the Golgi complex in various cell types was not laid to rest until better methods for demonstrating the complex in both fresh and fixed



tissue became available. The phase contrast microscope permitted the ready demonstration in certain unfixed cells of a "Golgi zone" or "Golgi region" which had a refractive index differing from that of the surrounding cytoplasm, and which corresponded in location to the Golgi network observed in fixed, metal impregnated tissue. Finally, Dalton and Felix (1954) showed that metal impregnation techniques revealed a classical Golgi network in the Golgi region in the epithelial cells of the mouse epididymis, and electron microscopic examination of the same region demonstrated that the sites of metal deposition were a characteristic complex of large vacuoles, flattened saccules, and small vesicles. More extensive electron microscopic surveys showed that a structurally similar complex of vesicles could be demonstrated in the Golgi zone in a wide variety of vertebrate cell types, including secretory and absorptive cells, nerve cells, germ cells, and tumor cells (Sjostrand and Hanzon, 1954; Dalton and Felix, 1956, 1957) and in various cells of invertebrates and protozoa (Grasse and Carasso, 1957).

In light microscopy, however, there remained difficulties in convincingly demonstrating a homologous Golgi complex in a number of cell types, partly on account of the capriciousness of the classical methods of impregnating the complex in these cells. It was not until 1961 that a simpler and more reliable staining procedure for demonstrating the Golgi network was found--the histochemical reaction for the enzyme thiamine pyrophosphatase (Novikoff and Goldfischer, 1961). The localization of the reaction product was the same as that of the metal deposits of the old impregnation procedures in virtually all animal cell types examined. Using this reaction as a way of identifying the Golgi region and examining the structures revealed therein by electron microscopy led the last major proponent of the "artifact" school, J.R. Baker, to concede the reality of the Golgi complex: "The author accepts, after long hesitation, the view that the 'Golgi apparatus' in the neurons of vertebrates corresponds with the organelle





of the same name in other cells" (Baker, 1963).

The same difficulties which attended efforts to demonstrate the existence and universality of the Golgi complex made many early discussions of the function of the complex little more than speculation. Despite these difficulties, however, the observations of Nassonov (1923, 1924), Bowen (1929) and others on the topographical relations between the Golgi complex and newly forming secretion granules in various secretory cells, and on changes in the Golgi complex correlated with the secretory cycles (summarized in Bowen, 1929), strongly suggested that the Golgi complex played an "immediate role in the process of accumulation and final synthesis of the secretion produce" (Bowen, 1929). More precise evidence on the nature of this role did not become available until the electron microscope made it possible to obtain detailed information on the morphological relationship between the Golgi complex and other subcellular constituents (notably the rough endoplasmic reticulum and the secretion granules) in normal and experimentally altered states of the cell (cf. reviews of Palay, 1958, and Beams and Kessel, 1968). Since this information is based on static images of cells, however, the conclusions drawn from it are, at best, suggestive.

Direct evidence on the functions of the Golgi complex is even more recent. Specific staining methods and histochemical reactions applicable at the electron microscopic level (e.g. for certain carbohydrates (Rambourg et al., 1969); for phosphatases (Sheldon et al., 1954); for specific antibodies (Leduc et al., 1968)) have permitted a more detailed exploration of the chemical and enzymatic nature of the Golgi complex and its content than was possible with the light microscopic versions of these procedures. Moreover, the development of high resolution autoradiography (Caro, 1962; Caro and von Tubergen, 1962) has enabled sites of incorporation of precursors to be identified in various classes of subcellular constituents and has permitted sequential



localizations of cellular products during their transport from point of synthesis to later destinations in the cell.

Methods such as these have implicated the Golgi complex of various cell types in a variety of functions. These have been recently reviewed by Beams and Kessel (1968) and Whaley (1969) and so will not be elaborated on here. Rather, since the liver cell is the subject of the experimental work in this thesis, we will illustrate the knowledge of the structure and function of the Golgi complex for the specific case of the hepatic parenchymal cell.

#### A. The Golgi Apparatus of the Hepatic Parenchymal Cell

The fine structure of the Golgi complex in the hepatocyte of the rat has been studied by numerous investigators (Fawcett, 1955; Novikoff and Shin, 1964; Bruni and Porter, 1965, inter alia). Our own observations, described below, are in accord with these previous reports. Further details on topographical relations between the Golgi complex, the rest of the endoplasmic reticulum, and lysosomes are discussed by Novikoff and Shin (1964) and Bruni and Porter (1965). The nature and possible function of the "coated vesicles" are discussed by Bruni and Porter (1965).

In thin sections of liver, several Golgi complexes are usually seen in each cell. They are most frequently found in the region between the nucleus and the bile capillaries, although other locations are also noted. Frequently, but not always, one or more stacks of cisternae of the rough endoplasmic reticulum are found closely associated with the Golgi complex.

The complex itself is made up of several kinds of vesicles, distinguished by size, content, and nature of their limiting membrane. Generally, (although not evident in every section) the Golgi complex has a distinct polarity: two "faces" can be recognized.

1. At one face of the Golgi complex (generally the outer,



convex face) there are numerous, small (60-150m $\mu$ ) smooth surfaced "vesicles", which frequently contain one or more rarely two electron opaque, 30-80m $\mu$  diameter particles. Identical particles are often seen in vesicles of similar size located near the cisternae of the rough endoplasmic reticulum. Moreover, smooth surfaced tubules are frequently seen in continuity with rough surfaced cisternae, generally at the ends of the latter. Such "transitional" elements between the rough and the smooth, reticular form of the endoplasmic reticulum often contain dense particles. Finally, similar images are seen in transitional elements (part rough and part smooth) at the periphery of the Golgi complex\* suggesting that the free, smooth surfaced vesicles of the complex may form by pinching off from these regions of the endoplasmic reticulum.

2. Continuing across the Golgi complex, we find 3 or 4 large, flattened, smooth surfaced vesicles or "saccules", in three dimensions probably disc shaped, often dilated along one or both rims, and lying more or less parallel to each other. Such a stack of flattened vesicles is called a "dictyosome". The saccules in the normal liver cell often contain a few electron opaque particles similar to those seen in the small vesicles at the periphery of the complex, and seldom, some amorphous dense material. Images are occasionally seen suggesting that the dilated ends of the saccules may pinch off to form large vacuoles.

3. At the other face of the Golgi complex (generally the inner, concave face) are several large (up to 600m $\mu$ ) smooth surfaced "vacuoles" containing clusters of electron opaque particles and occasionally some

---

\*Note: The transitional elements at the periphery of the Golgi complex are less prominent in the hepatocyte than in many other cell types, in contradistinction to the transitional elements between the rough and the smooth, reticular part of the system which are particularly frequent and easily demonstrable.



amorphous material. These vacuoles are often about the same size as the dilated ends of the Golgi saccules. Identical particle-filled vacuoles are found in the cytoplasm close to the plasma membrane facing on the sinusoid, and occasional images are seen suggesting that such vacuoles fuse with the plasma membrane to discharge their contents into the space of Disse.

4. Additionally, small vesicles (about 50m $\mu$  diameter), whose membrane appears to have a fuzzy outer coat are seen in the Golgi region. Similar vesicles are seen just under the plasma membrane. The same kind of fuzzy coat is occasionally seen on small evaginations from transitional elements and Golgi saccules and on invaginations from the plasma membrane.

5. Other subcellular components seen associated with the Golgi complex are lysosomes, usually of secondary type.

6. In other cell types, the two centrioles occupy the center of the Golgi region, but in the liver cell they are rarely encountered in this location or anywhere else in the cell.

The functions of the Golgi complex in the liver cell are still largely obscure. In addition to the difficulties inherent in studying the Golgi complex in general, the hepatocyte poses the problem of being a multi-functional cell, involved in the synthesis, transport, and metabolism of a wide variety of proteins, carbohydrates, and lipids. The products may be used in the cell or secreted in either endocrine or exocrine fashion. There is evidence of Golgi involvement in a number of these functions.

1. Proteins and lipoproteins for use outside the cell: The liver produces serum albumin and other serum proteins, including lipoproteins. Peters (1962a, 1962b) has shown by cell fractionation studies that serum albumin is synthesized in rough microsomes and is then transferred to smooth microsomes (which include vesicles derived from the





Golgi complex) before release into the blood. Droz (1966a) and Peters and Ashley (1967) have shown by autoradiography, using  $^3\text{H}$ -leucine as a precursor for proteins, that label is first incorporated in the rough endoplasmic reticulum. Subsequently, a very large proportion of the label appears in the Golgi region, prior to its appearance in the blood. But this finding does not necessarily prove that newly synthesized albumin passes out of the cell by way of the Golgi complex. First, there is no assurance that the labelled protein thus observed is albumin rather than some other protein, although Peters (1962b) has shown that at the time when label is over the Golgi region, a large proportion of the labelled protein is albumin. Second, the resolution of autoradiography is not sufficient to prove that the labelled protein is within Golgi vesicles rather than in the cell sap in the Golgi region. Assuming that the protein observed is albumin, however, Peters' (1962a) observation that there is very little free albumin in the cell sap in liver cells supports the assumption that the newly synthesized protein is in the elements of the Golgi complex.

Finally, if the Golgi complex of the liver cell takes part in the secretion of albumin into the blood, it is surprising that the complex is located between the nucleus and the bile capillaries rather than between the nucleus and the sinusoid. In secretory cells, the Golgi complex is usually (but not always) positioned between the nucleus and the surface where the cell product is extruded (cf. Bowen, 1929). The "unusual" location of the complex in liver cells suggests that other routes of discharge of albumin--perhaps directly from the rough endoplasmic reticulum, or by way of vesicles of the smooth endoplasmic reticulum, which are abundant near the sinusoidal surface--may be involved for at least part of the albumin secreted.

The involvement of the Golgi complex in the production and transport of plasma lipoprotein is somewhat clearer, since intracellular very low density lipoprotein can be identified with the



electron microscope as the 30-80m $\mu$  particles described above in elements of the Golgi complex (Hamilton et al., 1967; Jones et al., 1967); this subject is discussed in more detail below, Chapter 2). When perfused livers are stimulated to produce large amounts of lipoprotein by inclusion of fatty acid in the perfusion medium, these particles appear in greatly increased numbers first in the transitional elements of the endoplasmic reticulum (the junctions between the rough and smooth surfaced regions), in smooth surfaced vesicles, and in the elements of the Golgi complex. Subsequently they are found in vacuoles near the cell surface and in the space of Disse (Hamilton et al., 1967; Jones et al., 1967). Treatment of animals with intoxicating doses of ethanol also causes a dramatic accumulation of similar particles in the Golgi complex (Stein and Stein, 1965), and under these conditions, autoradiography using <sup>3</sup>H-palmitate as a precursor for triglycerides (the major lipid component of the very low density lipoprotein of the plasma) shows a pathway identical to that shown by the dense, 30-80m $\mu$  particles after fatty acid perfusion.

In this case, since the secretory product--the lipoprotein particles--is uniquely identifiable, the involvement of the Golgi complex in the secretion into the blood is clearly demonstrated, despite the peri-canalicular location of the complex. It should be noted, however, that in this case, too, a secondary pathway of secretion, directly from the smooth endoplasmic reticulum vesicles into the blood remains possible, since such vesicles, containing one or two dense particles, are seen far from any Golgi complex, near the sinusoidal surface.

2. Lysosomal and microbody enzymes: Novikoff (1963) has reported that the characteristic lysosomal enzyme acid phosphatase is found occasionally, but not always in a few of the vesicles of the Golgi region of the liver cell. Clear evidence that Golgi vesicles do, in fact, transport acid phosphatase or other enzymes to lysosomes in the liver is, however, lacking (Novikoff, 1963; Novikoff and Shin,



1964).

Bruni and Porter (1965) have proposed, on the basis of morphological observations, that microbodies are formed by the aggregation of the small coated vesicles seen in the Golgi region. However Novikoff and Shin (1964) disagree on the interpretation of the morphological evidence, and Higashi and Peters (1963) found the microbody enzyme catalase to be restricted almost entirely to the rough microsomes; the smooth microsomal fraction, presumably including Golgi-derived vesicles, contained virtually no enzyme.

3. Glycoproteins: Cell fractionation experiments by Molnar et al. (1965), Lawford and Schachter (1966) and Molnar and Sy (1967) indicate that the sialic acid moieties of liver glycoproteins are probably attached to the protein primarily in smooth microsomes, while glucosamine residues are apparently attached while the protein chain is still bound to the ribosomes. Galactose is also known to be added to the carbohydrate chain after the peptide is separated from the ribosomes, although the exact location (e.g., rough vs. smooth endoplasmic reticulum) is not known (Sarcione, 1964). Rambourg et al. (1969) have developed an electron microscopic version of the periodic acid - Schiff test for certain carbohydrates and have demonstrated that reactive carbohydrates occur in the Golgi complex of a wide variety of cell types, including hepatocytes. Droz (1966b) using autoradiography with  $^3\text{H}$ -galactose as a label for newly synthesized glycoproteins, reports that five minutes after injection of the labelled precursor, by far the highest concentration of grains appears over the Golgi complex in rat liver cells. Thus, there is evidence suggesting that the Golgi complex of the liver cell may be involved in the elongation of the carbohydrate chains of glycoproteins.

4. Membranes: Various authors have reported morphological evidence suggesting that pieces of membrane are transferred from the endoplasmic reticulum to the Golgi complex and from the Golgi complex



to the plasma membrane, by successive processes of vesicles pinching off from one membrane system, migrating to the next, and fusing with the membranes at the new site (Bruni and Porter, 1965; Jones et al., 1967; Hamilton et al., 1967, inter alia). No more direct evidence is available, however. (See below, Chapter 5, for an extended discussion of the relation of Golgi membranes to other intracellular membranes).

The paucity and uncertainty of information on the role of the Golgi complex is particularly striking when compared to the abundance of information available on the activities of the rest of the endoplasmic reticulum in liver cells. The difference is largely accountable for by the availability of microsomal fractions (Claude, 1946; Hogeboom, Schneider, and Palade, 1948), derived from the endoplasmic reticulum (Palade and Siekevitz, 1956). Such fractions, which can be further subfractionated into rough and smooth microsomal fractions, derived primarily from the rough and smooth parts of the endoplasmic reticulum respectively (Moule et al., 1960; Rothschild, 1963; Dallner, 1963), permit direct chemical analysis of the membrane and contents of the microsomal vesicles, allow the direct detection and assay of enzymes other than those few for which histochemical reactions have been developed, render possible quantitative analysis of changes under various physiological or experimental conditions, and permit direct study of precursor-product relationships. The tools to which we have been limited in studying the Golgi complex--morphology, staining, and autoradiography--are indirect and crude by comparison. Without the ability to prepare purified Golgi fractions, it remains difficult to study the nature and function of the Golgi complex as well as its relation to other subcellular components.

In this thesis we will describe a scheme for obtaining purified fractions of Golgi-derived vesicles from rat liver cells. In Chapter 2 we will discuss the obstacles in the way of obtaining such a fraction and the approach we took to overcome these difficulties. In Chapters 3 and 4 we will present the details of how the fraction is obtained.





In Chapter 4 we will also present morphological and biochemical evidence attesting to the purity of the fraction and some preliminary biochemical information bearing on the relationships between the Golgi complex membranes and the membranes of the rest of the endoplasmic reticulum and the plasma membrane. In Chapter 5 we will further discuss these relationships.



## CHAPTER TWO

### INTRODUCTION TO THE METHOD

#### A. The Problem of Isolating Golgi Fractions

In attempting to isolate purified fractions of membranes derived from the Golgi complex, two sets of difficulties arise.

First, when the tissue is homogenized, the Golgi complex presumably fragments into small vesicles or microsomes, just as the rest of the endoplasmic reticulum and, in part, the plasma membrane do (Palade and Siekevitz, 1956). These vesicles are likely to overlap in size and density the vesicles formed from other cellular membranes. Since differences in size and density are the basis for the isolation of subcellular fractions by centrifugation procedures, such an overlap would prohibit purification of Golgi-derived fractions by these techniques. We can hope to overcome this difficulty only if (1) the Golgi-derived vesicles have, or can be caused to have, a size or density different from vesicles derived from other sources, or (2) the particular cell type contains, to begin with, few other membranes which could give rise to vesicles of size and density similar to those of the Golgi-derived vesicles.

Second, there is no generally accepted way of identifying Golgi-derived vesicles in the course of isolation, or of assaying the purity of supposed Golgi fractions. Morphologically, vesicles derived from the Golgi complex presumably look more-or-less like smooth surfaced vesicles derived from any other smooth membranes in the cell. Enzymologically, little is known that would distinguish Golgi membranes from other intracellular membranes. There is histochemical evidence that the enzyme thiamine pyrophosphatase is primarily localized in the Golgi complex (Novikoff and Goldfischer, 1961), but its presence at lower concentrations in other membrane systems is not excluded. Similarly, the failure to demonstrate by histochemical methods that the Golgi complex



contains enzymes such as glucose-6-phosphatase, which are known to occur in other membranes, cannot be considered as adequate evidence that the Golgi complex is totally lacking in these enzymes.

The absence of a unique intrinsic marker for Golgi-derived vesicles has led previous workers to shift their attention from isolation of a complete Golgi fraction to isolation of dictyosomes. Under suitably gentle conditions of homogenization, the dictyosome does not fragment. If the characteristic stacking of its saccules can be maintained during purification, the morphology of the entire dictyosome serves as a marker for Golgi-derived material. Difficulties remain, however, since the density of the dictyosome may not be sharply differentiated from the density of other components of the homogenate. Moreover, the price for such a procedure is that only a part of the Golgi complex is isolated. Any Golgi vesicles or vacuoles not firmly attached to the dictyosome are lost in the course of purification.

These difficulties can be illustrated from the history of attempts to isolate Golgi fractions.

(1) Schneider and Kuff (1954) and Kuff and Dalton (1959) attempted to isolate Golgi fractions from rat epididymis. In these cells, the Golgi complex is extensively developed, making up a large portion of total intracellular membranes, and the dictyosome portion of the complex is quite prominent (Kuff and Dalton, 1959; Fawcett, 1966). The basis of the procedure for isolating the Golgi complex was the observation that if cells were gently homogenized in sucrose solutions containing high salt concentrations (0.34M NaCl), then in the phase contrast microscope the Golgi apparatus retained the configuration and optical properties seen in situ. The high salt concentration apparently stabilized the structure of the dictyosome, maintaining it as a set of stacked saccules rather than allowing it to break down into separate vesicles. Furthermore, since the saccules held together, whatever material was involved in binding them to one



another may have contributed to the density of the dictyosomes.

The homogenate was then layered on top of a discontinuous sucrose gradient (Schneider and Kuff, 1954) or placed at the bottom of a similar gradient (Kuff and Dalton, 1959) and centrifuged. The Golgi-rich fraction accumulated at the interface between sucrose of densities 1.09 and 1.13. If the salt stabilization was omitted, few intact dictyosomes were found in the homogenate and few could be isolated (Kuff and Dalton, 1959).

Thus the difficulties discussed above were, in part at least, overcome: that part of the Golgi complex that was maintained as an entire dictyosome had a unique size and possibly a unique density and could be reliably identified by its morphology. Despite these precautions, the best preparations obtained by Kuff and Dalton contained considerable quantities of impurities. From the NADH-cytochrome c reductase activity and the RNA content (Kuff and Dalton, 1959) we might estimate that the Golgi fraction may have contained as much as 25-35% contamination by microsomes and possibly mitochondria. The fraction also had considerable acid phosphatase activity. Histochemical evidence indicates that the Golgi cisternae of the epididymal epithelium do not contain acid phosphatase (Novikoff et al., 1962). However, the cells of the epididymis may also contain lysosomes (Novikoff et al., 1962; Fawcett, 1966), which might be another contaminant in the fraction. The morphological evidence presented by Kuff and Dalton is too sparse to make any judgement about the type and extent of contamination of their fractions.

(2) Morré, Mollenhauer, and collaborators have applied a similar approach to the isolation of Golgi-rich fractions from plant cells having relatively numerous Golgi complexes. As with animal cells, the Golgi complex of onion, maize, and radish stems tends to fragment into vesicles after normal homogenization procedures. Morré and Mollenhauer (1964) reported conditions under which intact





dictyosomes could be found in homogenates. These conditions included very gentle homogenization (in some experiments merely chopping the tissue with a razor blade), careful control of pH and sucrose concentration, addition of monovalent and divalent cations (e.g., 0.1M NaCl and 0.001M  $\text{CaCl}_2$ ), and, in some experiments, the addition of 1% dextran to the homogenate. With material homogenized under these conditions, they were able to prepare a cityosome rich but heavily contaminated fraction by differential centrifugation. In later experiments Morré et al. (1965) and Mollenhauer (1966) used very low concentrations of glutaraldehyde to get better stabilization of the Golgi structure, and then purified the dictyosomes by centrifugation on a sucrose gradient. As in the case of the epididymis fractionation, they thus obtained a structure in the homogenate with a more-or-less unique size and density. Moreover, by preserving the dictyosomes intact, they had a way of identifying Golgi material in their fractions. However, as was the case with Kuff and Dalton, they were not able to determine the source of other membraneous material seen in the fractions.

(3) Jamieson and Palade (1966) isolated fractions derived from the Golgi or Golgi related elements from guinea pig pancreas. In their case, it was evident from previous electron microscope studies of intact acinar cells that almost all of the smooth membranes in the cell were located in the Golgi region, the main exceptions being the plasma and zymogen granule membranes. Hence there was no need to depend on maintaining the topography of the dictyosome to identify Golgi-derived material. The combined zymogen granules and condensing vacuoles could be isolated by a low speed spin and the total microsomes could be separated into rough and smooth microsomes on a sucrose density gradient. The smooth microsomes were, presumably, largely Golgi-derived vesicles.

Although no true marker for Golgi vesicles was available, other experimental evidence helped confirm that this fraction was, in fact, enriched in Golgi material. Autoradiographic evidence showed that about



20 minutes after pancreatic slices were pulse-labelled with radioactive leucine, most of the label was found in the Golgi region. We would predict that if the smooth microsomal fraction was enriched in Golgi-derived elements, then if isolated from slices at this time, it would show a high specific radioactivity. In fact, this is what was found.

The absence of any direct Golgi marker, however, makes it impossible to assess just how pure the fraction is, especially since no adequate markers are available for the most likely major source of contamination, the plasma membrane, either.

(4) Finally, Morré et al. (1968a, 1968b) have attempted to isolate the Golgi complex of rat liver, again relying on preservation of the dictyosome throughout the procedure. After homogenization of the liver and some preliminary spins, the material was loaded onto a discontinuous sucrose gradient. The final Golgi-rich fraction was obtained at the interface between sucrose of density 1.12 to 1.14. Apparently in this case, gentle homogenization alone, without chemical stabilization, was enough to retain the dictyosomal structure; the morphology of the isolated material was "similar to that observed in the living cell" (Morré et al., 1968a). Among other morphological features, the dense granules seen in the interior of Golgi elements in situ were retained in the fractionated material. These investigators claim that their fractions consist of about 80% Golgi-derived material, based on morphological, chemical, and enzymatic evidence.

#### B. A New Approach to the Problem of Isolating Golgi Fractions

As we have seen, the several earlier attempts to isolate Golgi membranes have been only partially successful. The principle difficulties encountered were the lack of unique size or density of the Golgi-derived components present in cell homogenates, and the lack of adequate morphological or enzymological markers to distinguish Golgi-derived membranes from membranes derived from other subcellular sources.



In the case of the liver, we assumed that both general obstacles to isolation of highly purified Golgi fractions could be overcome: there was evidence that the Golgi-derived vesicles might acquire a unique density and a characteristic morphological marker under appropriate experimental conditions.

First, the 30-80m $\mu$  dense particles observed in the Golgi complex in liver have been identified as very low density lipoprotein. This very light content might serve as a buoy, making the Golgi-derived vesicles lighter than the vesicles derived from the rest of the endoplasmic reticulum, which do not contain such lipoprotein. Various experimental procedures cause the Golgi complex to become unusually heavily loaded with lipoprotein, which might increase the differential in density still more. Moreover, the characteristic clusters of dense particles could serve as a unique morphological marker for Golgi-derived vesicles, enabling us to monitor our isolation procedures.

In the concluding part of this chapter, we will summarize the evidence that the Golgi complex of rat hepatocytes contains very low density lipoprotein which is morphologically identifiable. Then we will discuss the evidence that under suitable conditions, the Golgi complex becomes "overloaded" with this lipoprotein. Finally, we will discuss the method we used to induce Golgi "overloading" in the experimental work of this thesis and its general effects on the cell.

#### 1. Golgi involvement in lipoprotein production in the liver

The Golgi complex of the liver cell of the rat and numerous other species contains clusters of 30-80m $\mu$  diameter osmiophilic particles (Fawcett, 1955; Parks, 1962; Chandra, 1963; Biava, 1964; Smuckler et al., 1965; Toro and Viragh, 1966, inter alia). Similar particles are also occasionally seen in transitional elements of the endoplasmic reticulum and in vesicles and tubules of the reticular form of the smooth endoplasmic reticulum. In the two latter locations they almost always



occur as single units. Clusters of particles are also seen in vacuoles in the cytoplasmic matrix near the sinusoidal surface of the cell, and free particles are seen in the space of Disse (Fawcett, 1955; Chandra, 1963; Bruni and Porter, 1965; Stein and Stein, 1966; Hamilton et al., 1967; Jones et al., 1967, inter alia).

The identification of these particles has been a subject of some controversy. They have been variously considered to be protein (Trotter, 1964; Bruni and Porter, 1965), chylomicrons taken up from the blood (Ashworth, 1961 and 1965; Parks, 1962; Trotter, 1964; Jordan, 1964), newly forming glycogen (Karrer, 1960), and newly synthesized plasma lipoprotein (Chandra, 1963; Trotter, 1964; Stein and Stein, 1965; Baglio and Farber, 1965; Novikoff et al., 1966; Hamilton et al., 1966). The bulk of the evidence suggested that the particles were lipoprotein.

First, the density of the particles seen in the liver cell after osmium fixation is consistent with their containing unsaturated fatty acids (cf. Stoeckenius, 1959) like the triglycerides of serum lipoproteins. Second, the size of the particles is similar to that of very low density lipoprotein particles isolated from plasma, from chyle, or from liver perfusate, as determined by physical chemical methods (Oncley, 1964) and by electron microscopic examination (Hayes and Hewitt, 1957; Casley-Smith, 1962; Hamilton et al., 1967; Jones et al., 1967).

Further evidence for the lipid nature of the electron opaque granules was provided by the experiments of Parks (1967) and of Stein and Stein (1966, 1967). Parks showed that the resistance of the particles to extraction in the course of fixation, dehydration, and embedding procedures used in electron microscopy is related to the diet fed to the animals before killing. If the animals were fed a diet rich in unsaturated fatty acids, which bind osmium (Stoeckenius, 1959), the particles were well preserved. If the animals were fed diets rich in saturated fatty acids, however, many of the particles





were extracted in the course of the processing. Since hepatic lipids reflect the recent dietary history of the animal (cf. Robinson, 1964), Parks concluded that the particles contained lipid: such a content would explain the variable osmium binding efficiency of the particles under various dietary conditions and their correlated resistance to extraction by organic solvents.

Stein and Stein (1966, 1967) studied by autoradiography the fate of injected radioactively labelled fatty acid and glycerol in the livers of fasted rats. They showed that esterified label appears first in the rough and smooth endoplasmic reticulum, then over the Golgi complex and over vesicles and vacuoles containing dense particles, both near the Golgi complex and in the vicinity of the sinusoidal cell surface, and finally over the space of Disse. Although the resolution of autoradiography permits concluding only that the lipid label was associated with the structures containing the electron opaque particles, it supports the contention that the particles themselves contain the lipid visualized.

More direct evidence that the particles are newly synthesized lipoprotein in the process of being transported out of the cell into the blood comes from experiments with perfused liver preparations. Hamilton et al. (1967) and Jones et al. (1967) showed that when rat livers were perfused with fatty-acid rich medium, the number of electron opaque particles in the liver increased dramatically over control levels. The particles appeared within two to five minutes after the start of perfusion in the transitional elements of the endoplasmic reticulum, in vesicles of the smooth endoplasmic reticulum, and in very great numbers in the Golgi complex. After longer perfusion (30-60 minutes), increased numbers of particles were seen in large vacuoles near the space of Disse and in the space of Disse itself, and identical particles could be observed in the perfusate, from which increased amounts of very low density lipoprotein could be isolated with a similar lag time after the



beginning of the perfusion. Moreover, Jones et al. (1967) showed that puromycin, an agent which depressed the incorporation of leucine and oleate into lipoprotein, also reduced fatty acid stimulation of particle formation in the tissue. Thus the electron opaque particles appear to be newly formed very low density lipoprotein.

Finally, direct proof that the particles in the Golgi complex of the hepatocyte are lipoprotein has been provided by Mahley et al. (1968) who broke open the vesicles in dictyosome-enriched fractions from rat liver (prepared by the procedure of Morré et al., 1968) to obtain their contents. By physical chemical and immunological methods, they directly identified the contents as very low density lipoprotein.

There is thus a convincing body of evidence that the 30-80m $\mu$  electron opaque particles seen in rat liver Golgi complexes are very low density lipoprotein. For our purposes, this identification has two significant aspects: First, the lipoprotein involved is very light. The average density of the very low density lipoprotein fraction of the plasma has been reported to be 0.98 (Oncley et al., 1957). Second, the lipoprotein particles are primarily localized in the vesicles, saccules, and vacuoles of the Golgi complex, both in normal animals and in animals which have been experimentally induced to speed up the production of lipoprotein. Although some isolated particles appear elsewhere in the cell, it is virtually only in the elements of the Golgi complex that clusters of particles are found. The only exception is the large vacuoles seen near the space of Disse; even these, however, can be considered to be the equivalent of the zymogen granules of the pancreatic exocrine cell, in the sense that they are Golgi-derived vacuoles in which the secretory products are sequestered and transported to their ultimate destination (Jones et al., 1967; Hamilton et al., 1967).

These observations suggested that the lipoprotein content of the Golgi vesicles of the liver cells could be the basis for their isolation. If the Golgi vesicles are filled with very light material, they should



become lighter than the other vesicles formed from endoplasmic reticulum or plasma membrane, which do not contain such material. And since essentially only the Golgi elements contain clusters of the easily identifiable electron opaque particles, a morphological marker became available for Golgi-derived vesicles. Any vesicle which contained clusters of 30-80m $\mu$  particles could be presumed to be derived, almost certainly, from the Golgi complex.

## 2. Inducing "Overloading" of the Golgi Complex with Lipoprotein

The observations of Jones et al. (1967) and of Hamilton et al. (1967) also emphasize the fact that under appropriate experimental conditions, the number of particles seen in the Golgi complex increases sharply. Such an induced loading of the elements of the Golgi complex with lipoprotein promised to facilitate the isolation of a purified Golgi fraction. First, Golgi vesicles which normally held no lipoprotein particles might be induced to contain such particles. Thus the population of vesicles which we could hope to isolate due to their light contents would be expanded. Second, the number of particles in any given Golgi vesicle might increase, thus further differentiating its density from that of the general population of microsomal vesicles.

A variety of procedures other than perfusion of the liver with fatty acid rich medium are known to cause an increased in the number of electron opaque particles in the Golgi complex of the hepatocyte. These include partial hepatectomy (Trotter, 1964), feeding corn oil (Ashworth et al., 1960; Parks, 1962), and acute ethanol intoxication (Ashworth et al., 1965; Stein and Stein, 1965). Opaque particles, often somewhat larger than 80 m $\mu$ , are also seen in the Golgi complex in the hepatocytes of animals treated with orotic acid (Novikoff et al., 1966), ethionine (Baglio and Farber, 1965) and cortisone (Hill, 1965). We chose to use ethanol intoxication, which we will discuss in greater detail below.

We will start by describing the morphological results of acute ethanol intoxication, and then proceed to a discussion of the metabolic



effects involved in their production. Stein and Stein (1965) have investigated the fine structural alterations in rat liver cells after administration of a single large dose of ethanol. One to three hours after the ethanol is administered, large numbers of electron opaque particles, 30-80m $\mu$  in diameter, were observed in elements of the Golgi complex. Control animals showed fewer such particles. Generally a single Golgi saccule or vacuole contained many such particles. Occasional particles were also observed in vesicles of the smooth endoplasmic reticulum, usually as single units. Many particles were also seen in the space of Disse. The appearance is thus identical to that seen by Jones et al. (1967) and Hamilton et al. (1967) after fatty acid perfusion. No other abnormalities of cell fine structure were reported. Later, at sixteen hours after ethanol administration, there was an extensive accumulation of larger lipid droplets in the cytoplasmic matrix and some mitochondria appeared to be budding, but otherwise the cells appeared normal.

The effects of ethanol on lipid metabolism in the liver cell have been extensively studied. It is well known that both acute and chronic administration of ethanol lead to accumulation of triglyceride in the hepatocytes (see reviews by Isselbacher and Greenberger, 1964, and by Lieber, 1967). This may stem from decreased release of triglyceride-containing lipoprotein from the cell, or from increased production of triglyceride without a correspondingly increased release. Increased production, in turn, could stem from several causes: increased uptake of fatty acids derived from adipose tissue, increased hepatic fatty acid synthesis, or decreased hepatic catabolism of lipids.

The evidence on all of these possibilities is voluminous and conflicting. Thus, ethanol administration may lead to an increased mobilization of fatty acids from adipose tissue. Lieber et al. (1966) and Scheig and Isselbacher (1965) have shown that after a single large dose of ethanol, the types of fatty acid which accumulate in the liver





are similar to those found in adipose tissue. However, other (Elko et al., 1961; Poggi and DiLuzio, 1964; Jones et al., 1963) have argued that other mechanisms than increased mobilization could account for this; it could, for example, merely reflect increased uptake of fatty acid already present in the blood. The same investigators have also presented direct evidence that there is no increased mobilization of fatty acid from adipose tissue.

With respect to possible increases in hepatic lipid synthesis, Lieber and Schmid (1961) have demonstrated an increase in the incorporation of acetate into fatty acids in ethanol treated rats. Horning et al. (1963) and Scheig and Isselbacher (1965) have shown an enhancement of the esterification of fatty acids under similar conditions. Nikkila and Ojala (1963) have confirmed this finding and have linked it to an increase in  $\alpha$ -glycerophosphate levels in the liver consequent upon ethanol administration.

With respect to decreased catabolism of fatty acid in the liver, Reboucas and Isselbacher (1961) have demonstrated that ethanol intoxication reduces the rate of oxidation of palmitate, and Lieber and Schmid (1961) have shown the same for acetate oxidation.

Finally, the hypothesized blockage of lipid transport out of the cell is argued for by Schapiro et al. (1964), who have shown that in perfused livers, when ethanol is added to the perfusate the rate of appearance of newly esterified triglyceride in the perfusate is decreased, although such triglyceride accumulates in the liver. But Jones et al. (1963) and Lieber et al. (1965) have argued in opposition to this interpretation that, in vivo, ethanol consumption leads to increased plasma triglyceride levels. The many variables involved in in vivo experiments have clouded this issue.

In addition to these effects on lipid metabolism, other effects of ethanol in the liver cell have been demonstrated. Various investigators have shown that the metabolism of ethanol leads directly to a considerable



increase in the ratio of NADH to NAD in the liver (Forsander et al., 1958; Smith and Newman, 1959; Cherrick and Leevy, 1965; Lieber, 1966, inter alia). Lieber and Davidson (1962) have proposed that this increased hepatic NADH level is the primary biochemical lesion produced by ethanol, accounting for the effects on lipid metabolism described above. They have further pointed out that at least two enzymes whose activities are known to decrease after ethanol administration--UDP-galactose-4-epimerase and glutamic dehydrogenase--are both inhibited directly by NADH (Isselbacher and Krane, 1962; Isselbacher and Greenberger, 1964). These latter enzyme inhibitions, in turn, may be related to other general metabolic effects of ethanol on the liver, which include interference with gluconeogenesis (Freinkel et al., 1962, 1963; Field et al., 1963) and with amino acid oxidation (Isselbacher and Greenberger, 1964).

Ethanol administration does not, however, interfere with many other enzyme activities. Ashworth et al. (1965) has reported that there is no gross loss of succinic dehydrogenase, NAD diaphorase, NADP diaphorase, or ATPase activity in liver after ethanol intoxication, as indicated by histochemistry, and Isselbacher and Greenberger (1964) report that glucose-6-phosphatase, nucleoside diphosphatase, and glucoronyl transferase activities are not affected.

Finally, it should be noted that there may be indirect ethanol effects in in vivo experiments. The central nervous system, the pituitary, and the adrenal are affected by ethanol, and through them, the liver may be indirectly involved (Frederickson and Gordon, 1958; Perman, 1958; Maickel and Brodie, 1963).

### 3. Isolation of a Golgi Fraction by Means of Loading with Lipoprotein

This evidence indicated to us that we could cause the Golgi complex of the hepatic parenchymal cell to become loaded with lipoprotein by giving the animal a single intoxicating dose of ethanol one to three hours before removing the liver. This should not cause



any serious derangements of cellular function. The Golgi vesicles, after treatment, should become much lighter than the rest of the microsomal vesicles, as a result of their lipoprotein load; hence, we should be able to isolate them on a suitable density gradient. Finally, since intracellular clusters of 30-80m $\mu$  electron opaque particles are found essentially only in the elements of the Golgi complex and not in other membrane bound structures of the liver cell, the particles themselves could serve as a unique marker for Golgi derived vesicles. The two general obstacles to the isolation of Golgi fractions--lack of unique density and lack of a unique marker--might thus be overcome. This, then, was the approach we took in our attempt to obtain a purified Golgi fraction.



### CHAPTER THREE

#### MATERIALS AND METHODS

##### A. Preparation of the Golgi Fraction

The general procedure for obtaining a Golgi fraction is outlined in the simplified flow sheet shown in Figure 1. Rats are given an intoxicating dose of ethanol. Their livers are then excised and homogenized in sucrose, and the homogenate is centrifuged at low speed to sediment cell debris, nuclei and mitochondria. The resulting supernatant is spun again to yield a microsomal pellet. The microsomes are resuspended and loaded onto a discontinuous sucrose density gradient. After spinning, the Golgi vesicles are collected from the appropriate interface of the gradient, broken up by a combination of chemical and mechanical means, and their membranes separated from their contents by a centrifugation. The purity of the Golgi fraction is assessed morphologically, with the electron opaque lipoprotein particles serving as a marker for Golgi-derived material.

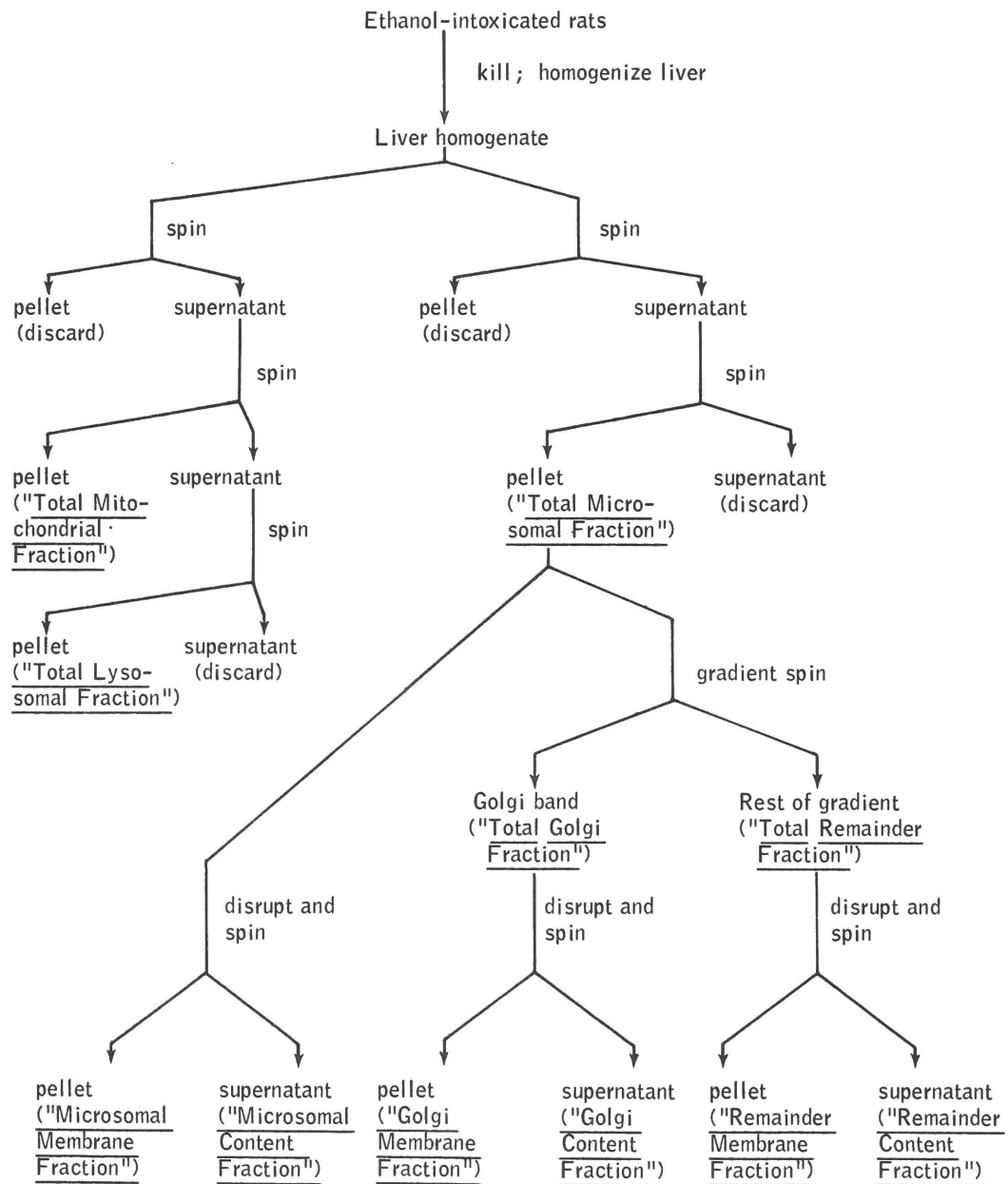
The method thus falls into four parts: (1) preparation of animals; (2) obtaining a microsomal fraction by standard methods; (3) obtaining a Golgi fraction by sub-fractionation of the microsomes; (4) obtaining the membranes of the Golgi vesicles free from the contents. Since the purpose of this project was the development of a procedure for isolating the elements of the Golgi complex, the details of the subfractionation procedure will be discussed in the Results and Discussion section (Chapter 4). They are summarized in the detailed flow sheet on page 76 (Figure 4). In this section we will discuss the preparation of the animals and the obtaining of the microsomal fraction. We will also discuss the procedures used for obtaining mitochondrial, lysosomal, and microsomal fractions used for comparisons in chemical and enzymatic assays.

##### 1. Preparation of animals

All experiments were performed on male Sprague-Dawley rats





**FIGURE 1**Outline of Procedure for Preparing Golgi and Related Fractions



weighing 150-250 grams. The animals were starved overnight and then given 0.6 grams of ethanol per hundred grams body weight in 50% (w/v) solution through a stomach tube. Ninety minutes after ethanol administration, the animals were killed by decapitation. In some experiments it was desirable to have an easily assayable marker for the intra-Golgi lipoprotein. Stein and Stein (1966) have shown by autoradiography that intravenously injected fatty acid appears in the Golgi complex (presumably in the lipoprotein granules) within ten minutes of injection. At this time it is also associated with the endoplasmic reticulum and with large intracellular fat droplets. It is thus not a unique marker for the intra-Golgi lipoprotein. However it could give us some indication of the fate of the lipoprotein particles when we try to remove these particles from isolated Golgi vesicles. Hence, as a marker, 100  $\mu$ C of sodium <sup>3</sup>H-palmitate, bound to bovine serum albumin was injected intravenously ten minutes prior to killing.

## 2. Obtaining microsomes and related fractions

### a. Homogenization

After killing the animals, the livers were excised and immediately placed in ice-cold 0.25M sucrose. All further procedures were carried out at approximately 4<sup>o</sup>. The liver was finely minced with scissors and then forced through a tissue press. 25 to 35 grams of tissue were then homogenized in 150 ml of 0.25M sucrose. Homogenization was effected with six strokes of a motor driven Teflon pestle in a Potter-Elvehjem homogenizer.

### b. Preparation of microsomal fraction used to obtain Golgi vesicles

The homogenate was centrifuged in a Spinco #40 rotor in a Spinco model L or model L2 ultracentrifuge for 10 minutes at 12,000 rpm (average field 10,000 x g). The pellets were discarded. The supernatants were pooled and diluted back to 150 ml with 0.25M sucrose. They were then spun for 90 minutes at 40,000 rpm (average field 105,000 x g) in a Spinco #40 rotor. The resulting pellets, consisting primarily of microsomes,



were resuspended in 1.5 ml of 1.15M sucrose per gram wet weight of liver originally processed, by means of three strokes of a loosely fitting motor-driven Teflon pestle. This material was loaded onto a discontinuous sucrose gradient to obtain the final Golgi fraction (see below, section 3).

c. Preparation of mitochondrial and lysosomal fractions

For purposes of comparison of certain enzymatic activities, it was desirable to isolate from the same homogenate mitochondrial and lysosomal fractions. Aliquots of the total liver homogenate were spun for 15 minutes at 1700 rpm (average field 485 x g) in an International Centrifuge, model SBV, in order to sediment unbroken cells, cell debris, and nuclei. The supernatant was decanted into tubes for a Spinco #40 rotor and centrifuged for 15 minutes at 7000 rpm (average field 3300 x g). The supernatant was carefully drawn off with a pipette, avoiding the loosely sedimented material at the top of the pellet, and used to obtain the lysosomal fraction; the pellet was used to obtain the mitochondrial fraction.

(1) Mitochondrial fraction: The pellet from the above spin was gently resuspended in 0.25M sucrose with a loosely fitting Teflon pestle and spun for 10 minutes at 7000 rpm in a Spinco #40 rotor (average field 3300 x g). The pellet was resuspended and spun again for the same time at the same speed. The final pellet was resuspended in water or buffer. It is referred to as the "total mitochondrial fraction."

(2) Lysosomal fraction: The supernatant from the first 7000 rpm spin was respun at 13,500 rpm for 20 minutes in a Spinco #40 rotor (average field 12,500 x g). The supernatant from this spin was decanted and discarded and the surface of the pellet was rinsed with 0.30M sucrose. The pellet was then resuspended in 0.30M sucrose and spun at 11,700 rpm for 10 minutes (average field 9500 x g). The resulting pellet was again rinsed with 0.30M sucrose, resuspended in 0.30M sucrose, and spun at 11,700 rpm for 10 minutes. The final pellet was resuspended in 0.30M sucrose, and is referred to as the "total lysosomal fraction."



This procedure is a modification of the method of Sawant et al., (1964).

d. Preparation of total microsomal fraction for assays

Since it was also desirable to have a companion microsomal fraction for comparisons of enzymatic activities, an aliquot of the preparation obtained by resuspending in 1.15M sucrose the pellet resulting from the second, 40,000 rpm spin (section A.2.b. above) was diluted with water to a sucrose concentration of 0.25M, and spun for 60 minutes at 40,000 rpm in a Spinco #40 rotor. The resulting pellet was resuspended in water or buffer. It is referred to as the "total microsomal fraction."

3. Subfractionation of microsomes to obtain the Golgi fraction

Our primary aim was to obtain, from the microsomal fraction described in section A.2.b., a fraction of Golgi-derived vesicles free of contaminating non-Golgi derived microsomes. A discontinuous gradient centrifugation was used to effect this separation on the basis of the following considerations:

The Golgi vesicles are a heterogeneous population. Since the density of any individual vesicle depends on the ratio of light lipoprotein content to heavy limiting membrane (a ratio which varies considerably from vesicle to vesicle), the Golgi-derived vesicles could be expected to overlap in density the general population of microsomal vesicles, especially since some of the latter may also contain a few lipoprotein particles. The size distribution of both Golgi and other microsomal vesicles could also be presumed to be heterogeneous and overlapping. These considerations precluded differential centrifugation as a means of separating Golgi vesicles from microsomal vesicles, since rate of sedimentation on a centrifugal field depends on size and density.

However, the overlap in density distributions of Golgi-derived vesicles and "true" microsomal vesicles (i.e., microsomal vesicles derived from intracellular membrane systems other than the Golgi complex)





should be only partial, since smooth microsomes contain few very low density lipoprotein particles, while generally, Golgi-derived vesicles contain many (Jones et al., 1967; Hamilton et al., 1967). Ethanol intoxication should reduce the overlap by inducing the overloading of the Golgi vesicles with very low density lipoprotein particles (Stein and Stein, 1965). Therefore, if the Golgi-plus-microsomal vesicles were spread out on a density gradient, regions of lower density should be progressively enriched in Golgi derived vesicles. So we decided to try to separate Golgi-derived vesicles from total microsomal vesicles on a sucrose density gradient.

One possibility was to layer the microsomes on top of a sucrose density gradient. Upon centrifugation, then, the relatively heavy microsomes would sediment away from the relatively light Golgi vesicles. The microsomes would descend through the gradient, leaving the Golgi vesicles at or near the top of the gradient. However, the Golgi vesicles are only a small fraction of the total microsomal material. We would be in the position of removing a large amount of "true" microsomes from a small amount of Golgi vesicles. It seemed likely that we would get a cleaner fraction by reversing the procedure--by plucking the Golgi vesicles away from the "true" microsomes. This could be done by placing the total microsomal fraction, suspended in dense sucrose, at the bottom of the centrifuge tube and building the gradient, of lighter sucrose, on top of them. Then, in spinning, we would float the Golgi vesicles free of contaminating "true" microsomal vesicles. This procedure would have the additional advantage of continuously "washing" the fraction as the vesicles float upwards through the sucrose. Although some of the lighter smooth microsomes might also float up, we would expect that at sucrose densities below the limits of the range of density of "true" microsomes, we would find relatively pure Golgi fractions. This was the procedure adopted.

Since the Golgi vesicles were assumed to be heterogeneous in



density, however, we did not expect them to move as a sharp peak in the gradient. Rather, we expected that the Golgi material would be spread out throughout the entire gradient above the microsomes. So we decided to pool the material having certain ranges of density by using a discontinuous gradient. The actual densities of the layers used in the final gradient were determined empirically, using the purity of the Golgi fractions, as determined morphologically, as the criterion: Various possibilities were tried. The interfaces between layers of differing density were collected and were examined in the electron microscope. Osmiophilic lipoprotein particles in the interior of vesicles were taken as a marker for Golgi-derived vesicles.

The details of the preliminary and final gradients used will be found in Results and Discussion (Chapter 4).

#### 4. Rupture of Golgi vesicles--obtaining a Golgi membrane fraction

The fraction obtained by the gradient centrifugation outlined above consisted of Golgi-derived vesicles loaded with lipoprotein particles. For biochemical and chemical analyses of the fraction, it was desirable to obtain the membranes alone, uncontaminated by the lipoprotein content. This was done by using appropriate combinations of alkaline treatment, hypotonic shock, and mechanical disruption to break open the vesicles and free the contents. Then, contents and membranes were separated by a single centrifugation. Details are given in Results and Discussion (Chapter 4).

#### B. Electron Microscopy

0.5 to 1.0 ml aliquots of all the fractions of interest were mixed with an equal volume of 2% osmium tetroxide in water and placed in the bottom of a centrifuge tube. The remainder of the tube was filled with water and the tube spun in a Spinco #40 rotor for 1 hour at 40,000 rpm. The supernatant was discarded and the pellet was dehydrated through increasing concentrations of ethanol and propylene oxide. After dehydration



it was cut into strips which were embedded in Epon in such an orientation that sections could be cut through the entire depth of the pellet (Luck, 1961).

Samples of liver tissue from normal starved rats and from rats which had been given ethanol 90 minutes previously were fixed in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.4, for 2 hours. The tissue was dehydrated as above and embedded in Epon.

Thin sections were cut on a Porter-Blum Servall MT2 automatic microtome with a Dupont diamond knife, and mounted on Formvar coated copper grids. The sections were stained for 1 minute in ethanolic uranyl acetate (Watson, 1958) and then for five minutes in alkaline lead citrate (Venable and Coggeshall, 1965). They were examined with a Siemens Elmiskop I electron microscope operated at 80 kilovolts with a double condenser, a 400  $\mu$  condenser aperture, a 50  $\mu$  objective aperture, and a pointed filament, or with a Hitachi 11C electron microscope operated at 75 kilovolts with a double condenser, a 500  $\mu$  condenser aperture, a 50  $\mu$  objective aperture, and a pointed filament.

### C. Enzyme Assays

The procedures used for quantitative determination of the various enzymes were standard procedures or slightly modified forms thereof. Hence, only summaries of the assays are given. In all cases, it was determined that conditions were such as to give a linear relation between measured activity and enzyme concentration in the range of activities examined.

#### 1. Glucose-6-phosphatase

The method was a modification of that of Swanson (1950). In this, as in the other phosphatase assays, inorganic phosphate liberated from the substrate is measured. The assay mixture consisted of 4mM glucose-6-phosphate, 5mM magnesium chloride, 30mM Tris-maleate buffer, pH 6.5, and enzyme in a total volume of 1.0 ml. The reaction



was carried out for 20 minutes at  $37^{\circ}$  and was stopped by the addition of 1.0 ml of cold 5% trichloroacetic acid. Inorganic phosphate was determined by the method of Ames and Dubin (1960).

## 2. Thiamine pyrophosphatase

The method was a slight modification of that of Novikoff and Heus (1963). There are several precautions which must be observed. First, thiamine pyrophosphate precipitates the ammonium molybdate used in colorimetric assays for inorganic phosphate. Hence, low concentrations of thiamine pyrophosphate must be used. Second, the terminal phosphates on thiamine pyrophosphate are apparently acid labile (Ord and Stocken, 1959). The Ames assay for inorganic phosphate is carried out in 1.0 N sulfuric acid. This results in the partial hydrolysis of the substrate, which then registers as having a high inorganic phosphate content. Hence, multiple blanks must be run, and the reaction must be carried out for a long enough time to give a reading well above the blank.

The assay mixture consisted of 0.56mM thiamine pyrophosphate, 3mM magnesium chloride, 50mM Tris-maleate buffer, pH 7.4, and enzyme in 0.7 ml total volume. The reaction was carried out for 40 minutes at  $37^{\circ}$  and was stopped by the addition of 0.3 ml of cold 30% trichloroacetic acid. Inorganic phosphate was determined by the method of Ames and Dubin (1960).

## 3. 5'-Nucleotidase (AMPase)

The method is a modification of that of Heppel and Hilmoe (1955). The assay mixture contained 3.5mM AMP, 3mM magnesium chloride, 28mM Tris - HCl buffer, pH 8.6, and enzyme in 0.7 ml final volume. The reaction was carried out for 20 minutes at  $37^{\circ}$  and was stopped by the addition of 0.3 ml of cold 30% trichloroacetic acid. Inorganic phosphate was determined by the method of Ames and Dubin (1960).

## 4. Acid Phosphatase

The method is that of Giannetto and de Duve (1955) as modified





by Wattiaux and de Duve (1956). The reaction mixture contained 5 mM sodium  $\alpha$ -glycerophosphate, 50 mM sodium acetate-HCl buffer, pH 5.0, 0.1% Triton X-100, and enzyme, in a final volume of 1.0 ml. The reaction was carried out for 10 minutes at 37° and was stopped by the addition of 1.0 ml cold 20% trichloroacetic acid. Inorganic phosphate was determined by the method of Ames and Dubin (1960).

#### 5. NADH-Cytochrome c Reductase and NADPH-Cytochrome c Reductase

In these assays, the reduction of cytochrome c by NADH or NADPH is followed spectrophotometrically (Strittmatter and Ball, 1954). The assay mixture contained 0.825 mg/ml cytochrome c, 82 mM nicotinamide, 0.33 mM potassium cyanide, 10 mM Tris-maleate buffer, pH 7.5, enzyme, and either 0.1 mM NADH or 0.1 mM NADPH in 3.0 ml total volume. The reaction was followed at 550 m $\mu$  in a Gilford recording spectrophotometer.

#### 6. Cytochrome oxidase

In this assay, the oxidation of reduced cytochrome c is followed spectrophotometrically (Cooperstein and Lazarow, 1951). Reduced cytochrome c was prepared by adding a few milligrams of sodium borohydride to cytochrome c, 6.1 mg/ml in 0.02M phosphate buffer, pH 7.4, plus 0.01 mM EDTA. The reduced cytochrome was then dialyzed against the same buffer for at least 6 hours. 0.3 ml of the reduced cytochrome solution was added to 2.7 ml of the same buffer plus EDTA, and enzyme was added. The oxidation of the cytochrome c was followed at 550 m $\mu$  in a Gilford recording spectrophotometer.

#### 7. Cytochrome b<sub>5</sub> and Cytochrome P-450

These were determined by difference spectra. Two identical cuvettes of the sample were prepared. The material in one cuvette was reduced by the addition of about 2 milligrams sodium dithionite. The absorption spectrum of the reduced sample was read against the oxidized blank over the range 400 m $\mu$  to 650 m $\mu$  in a Cary scanning spectrophotometer. The difference in optical density between 424 m $\mu$  and 410 m $\mu$  was taken



as an arbitrary absolute measure of cytochrome  $b_5$  concentration (Garfinkel, 1957).

The oxidized blank was then similarly reduced with dithionate and carbon monoxide was bubbled through the test sample for 1 minute. The carbon monoxide saturated sample was then measured against the dithionite reduced but aerobic blank, over the range 400 m $\mu$  to 550 m $\mu$ . The difference in optical density between 450 m $\mu$  and 490 m $\mu$  was taken as an arbitrary absolute measure of cytochrome P-450 concentration (Klingenberg, 1958).

#### D. Gross Chemistry

Aliquots of fractions of interest were taken for measurement of protein, phospholipid, and ribonucleic acid.

##### 1. Protein

Protein was assayed by the method of Lowry et al. (1951). Bovine plasma albumin was used as a standard.

##### 2. Phospholipid

Lipids were extracted by the method of Folch et al. (1957) and washed with 0.73% NaCl. The extracts were washed and then hydrolyzed in acid to convert phosphate esters into inorganic phosphate, and the inorganic phosphate determined (Ames and Dubin, 1960).

##### 3. Ribonucleic Acid

Aliquots of the fractions were precipitated and washed several times with cold 10% trichloroacetic acid. The precipitates were then extracted for 20 minutes with hot (90 $^{\circ}$ ) 5% trichloroacetic acid. RNA in the extract was determined by the orcinol method of Mejbaum (1939).

#### E. Sources of Materials

Palmitic-9,10- $^3\text{H}$  Acid was obtained from New England Nuclear Corporation.



Glucose-6-phosphate (disodium salt), cytochrome c (type III, from horse heart), and dihydronicotinamide adenine dinucleotide phosphate (reduced form) were obtained from Sigma Chemical Co.

Adenosine monophosphate dihydrate (yeast), thiamine pyrophosphate, and dihydronicotinamide adenine dinucleotide were obtained from Mann Research Laboratories.

Bovine plasma albumin was obtained from Armour Pharmaceutical Co.

All other reagents used were analytical or biological grade.



## CHAPTER FOUR

### RESULTS AND DISCUSSION

#### A. Fractionation Method

##### 1. Preparation of animals: lipoprotein loading of the Golgi elements

The method we proposed to use for subfractionating the microsomes to obtain Golgi elements is dependent on the hypothesis that a unique density range can be imparted to the Golgi-derived vesicles by causing them to become loaded with very low density lipoprotein. The use of acute ethanol intoxication to induce such loading was suggested by the observations of Stein and Stein (1965). They showed by electron microscopic examination that one to three hours after giving rats large doses of ethanol, large numbers of osmiophilic particles are found in the elements of the Golgi apparatus of hepatocytes (for details, see Chapter 2, page 21). Control animals also showed similar particles in the Golgi vesicles, but the accumulations were not as sizeable as in the ethanol treated animals.

Our observations confirm those of Stein and Stein. Plates 1 and 2 show liver cells from a normal, fasted rat. The Golgi apparatus contains a few electron opaque particles, mainly in the large vacuoles. Plates 3 and 4 show liver cells from an animal which had been fasted and then given an intoxicating dose of ethanol  $1\frac{1}{2}$  hours prior to killing. The Golgi saccules, the nearby large vacuoles, and the small vesicles are filled with 30-80m $\mu$  particles. Plate 5 shows, for comparison, several other regions from liver cells from the same animal. Occasional particles are seen in tubules of the smooth endoplasmic reticulum and in vesicles and vacuoles throughout the cytoplasm, but nothing like the Golgi accumulation is found elsewhere. The only exception is some large vacuoles, filled with numerous particles, at some distance from the Golgi complex. Similar vacuoles are often seen in close association with the Golgi complex. We may speculate that they





PLATES 1-5Liver cells from normal and ethanol-intoxicated rats

Plate 1: hepatocytes of normal, starved rat. The large Golgi vacuoles (V) contain numerous electron opaque particles, but only a few particles are seen in the Golgi saccules (S) and vesicles. Occasional single particles are also observed in transitional elements of the endoplasmic reticulum (arrows). Magnification: 31,500.

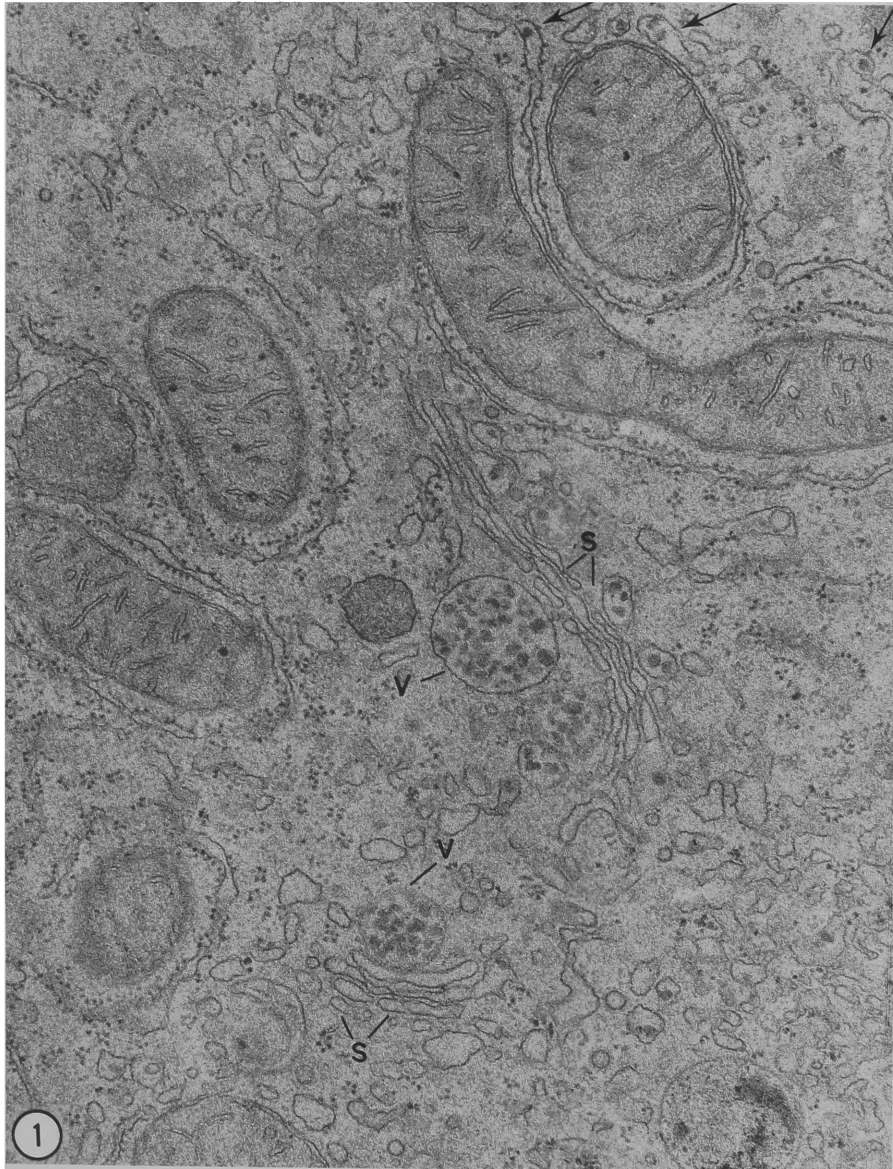
Plate 2: hepatocyte of normal starved rat. The Golgi elements (G) contain only a few electron opaque particles. Occasional single particles are observed in smooth surfaced vesicles away from the Golgi region (arrows). Magnification: 47,500.

Plate 3: hepatocytes of ethanol-intoxicated rat. Virtually all of the elements of the several Golgi complexes (G) in the field contain clusters of electron opaque particles. Magnification: 24,000.

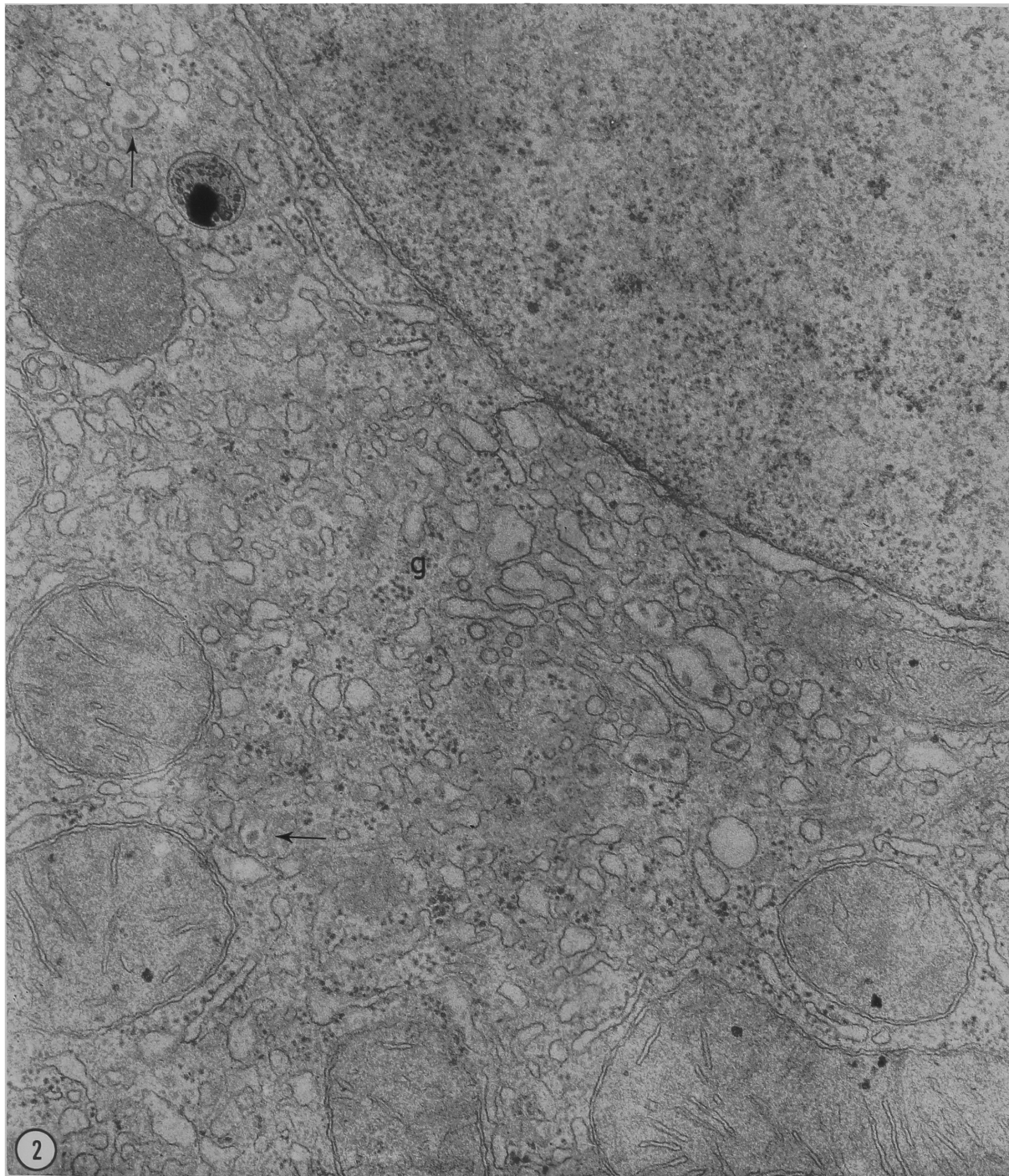
Plate 4: hepatocytes of ethanol-intoxicated rat. Virtually all of the elements of the Golgi complex contain clusters of electron opaque particles. Magnification: 47,500.

Plate 5: hepatocyte of ethanol-intoxicated rat. A large vacuole (V) near the surface, much like those seen in the Golgi region, contains electron opaque particles. Single particles are seen in several locations in tubules and vesicles of the smooth endoplasmic reticulum (arrows). Particles are also seen in the space of Disse (at D). Magnification: 57,000.

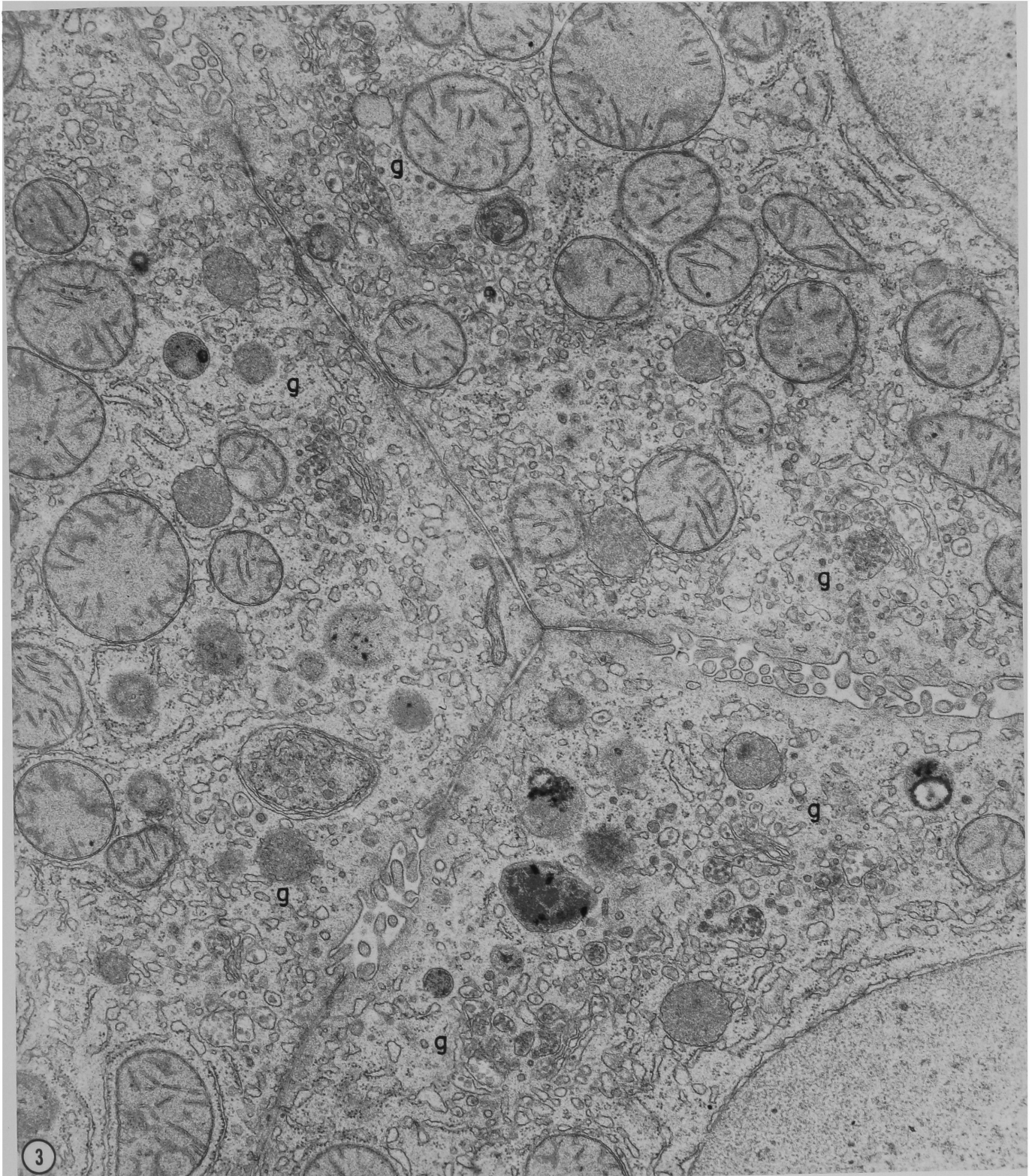






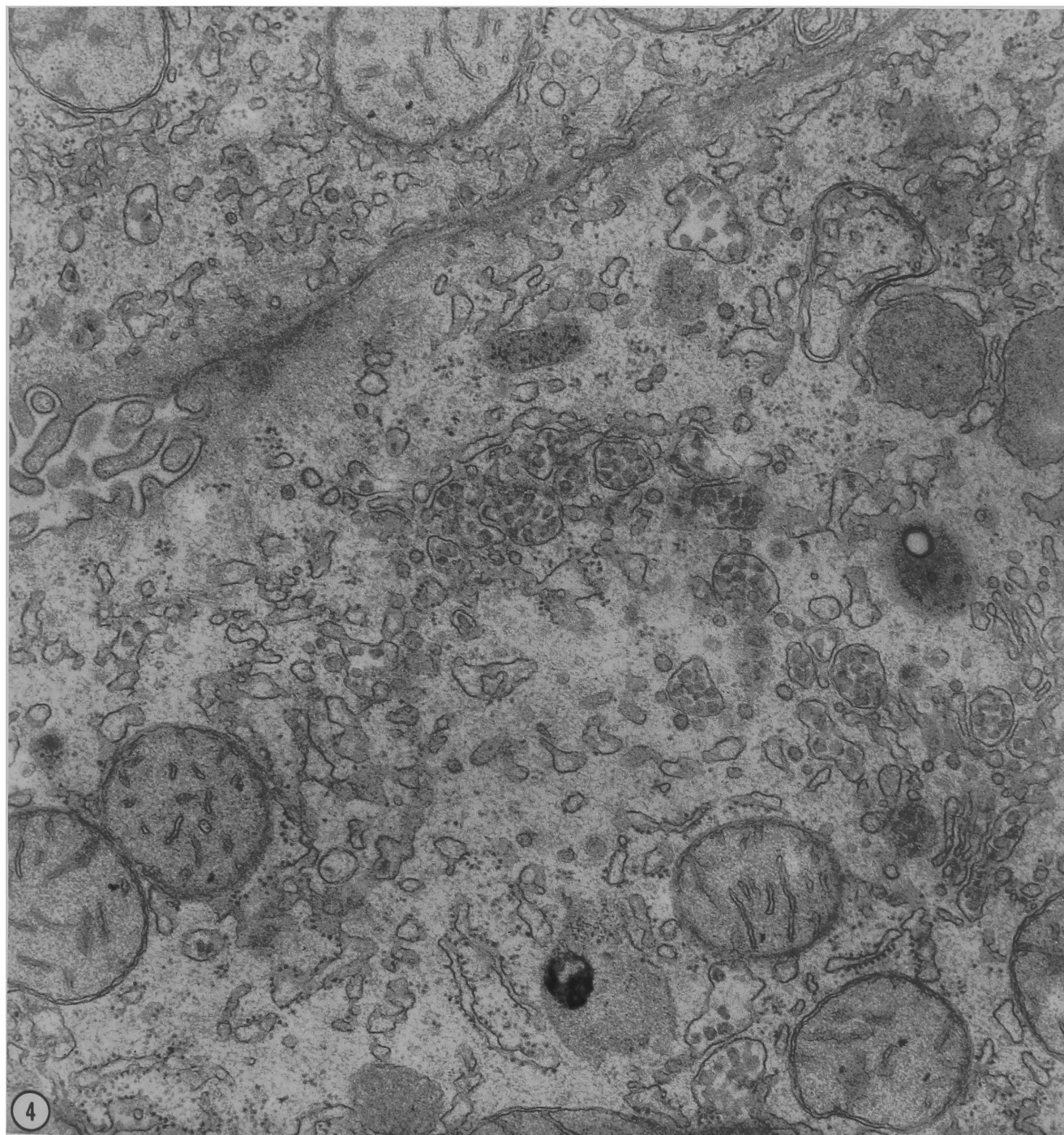




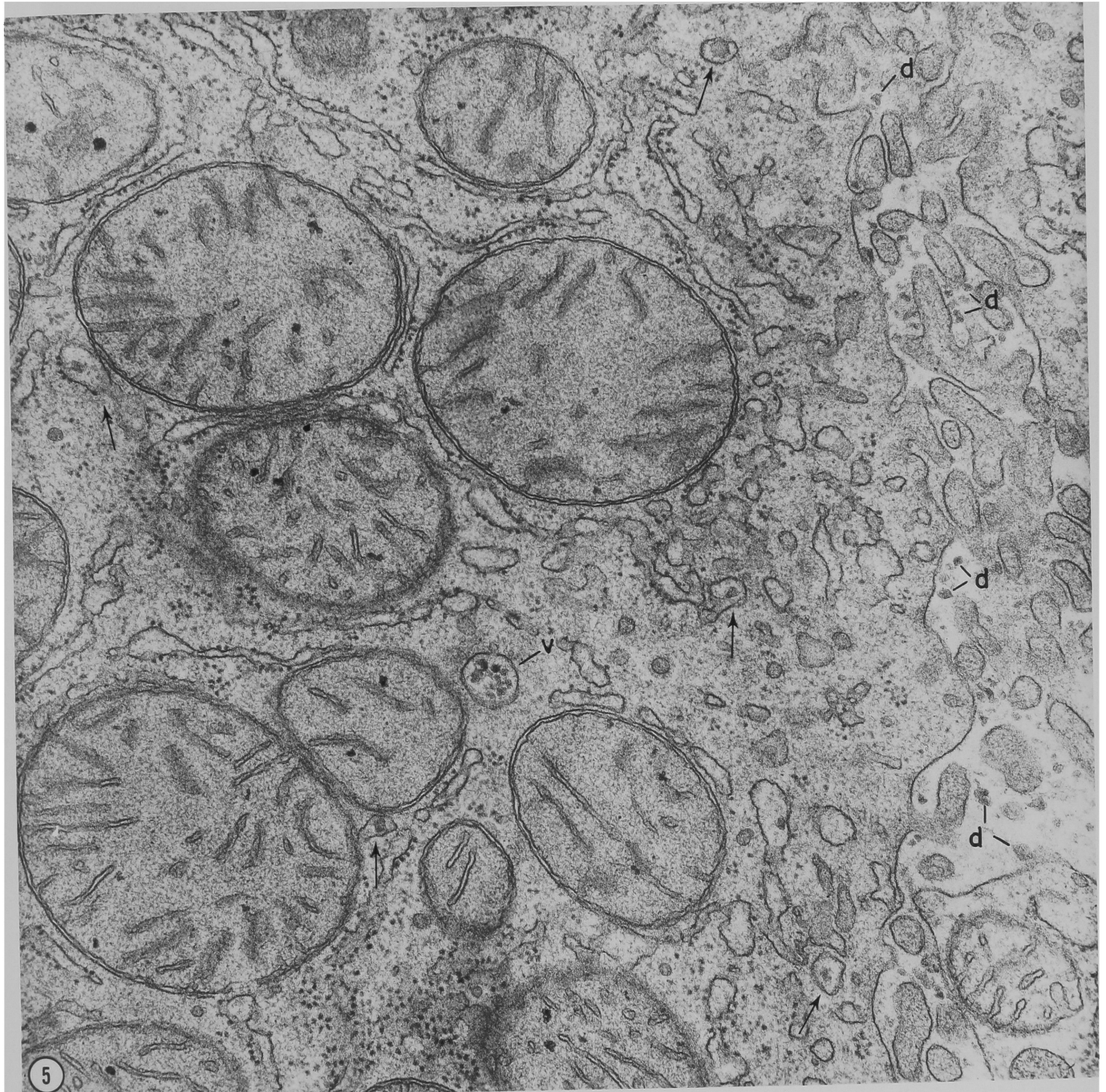














represent the equivalent of a zymogen granule: a post-Golgi, membrane-bound compartment in which completed lipoprotein particles are sequestered until such time as they are released into the blood (Jones et al., 1967; Hamilton et al., 1967).

These results suggested that: (1) we can cause the Golgi vesicles and saccules to become loaded with very low density lipoprotein by intoxicating the animals with ethanol, as Stein and Stein reported. This treatment both increases the percentage of Golgi vesicles which contain lipoprotein particles, and decreases the density of the average Golgi vesicles by enlarging their load of lipoprotein. The overall effect is thus to shift the density distribution of the total population of Golgi-derived vesicles toward lower densities. Such a shift should make it possible for us to obtain a clean fraction of Golgi-derived vesicles from the total microsomal population. (Table 1 shows the shift toward lower densities of hypothetical mixtures of very low density lipoprotein and density 1.18 membrane, as the lipoprotein content is increased). (2) Clusters of 30-80m $\mu$  particles can serve as a characteristic marker for vesicles derived from the Golgi complex, since there is no other quantitatively significant source of vesicles which contain such clusters of particles in the liver cell. We can thus attempt to purify the elements of the Golgi complex using some of their characteristic morphological features (such as type of membrane and especially type of content) as a necessary and sufficient means of identification after isolation. The biochemical characterization of the fraction is based therefore on the morphological identification of its components.

## 2. Obtaining microsomes as a source of Golgi vesicles

The loading of the Golgi complex with very low density lipoprotein could, conceivably, introduce a complication into the standard procedure for isolating microsomes. In the standard procedure, the post-mitochondrial supernatant is centrifuged for one to three hours



at approximately 100,000 x g. This is normally sufficient to bring down the microsomes (presumably including the vesicles derived from the Golgi complex) into the pellet. But, if we make the Golgi vesicles light, it is possible that they would no longer sediment in this field over this time.

We therefore checked the microsomal pellet and the post-microsomal supernatant obtained after  $1\frac{1}{2}$  hours of centrifugation by electron microscopy to see if they contained vesicles loaded with particles. The pellet was fixed in the centrifuge tube. The supernatant was mixed with osmium tetroxide and recentrifuged. The resulting pellet was then processed for electron microscopy along with the microsomal pellet. The microsomal pellet did contain particle-loaded vesicles among other kinds of vesicles. The supernatant consisted of free ribosomes, a few empty vesicles, a number of vesicles containing dense particles, and some large fat droplets. We concluded that, although there were vesicles derived from the Golgi complex in the supernatant, the number was relatively small.

We therefore felt safe in using the total microsomal pellet, obtained by conventional procedures, as the source material for a Golgi fraction. A procedure of centrifuging the post-mitochondrial supernatant for 90 minutes at 40,000 rpm in a Spinco #40 rotor was adopted as standard. The resulting pellet was resuspended and used as the starting material for isolating the Golgi fraction.

### 3. Isolating a Golgi fraction from microsomes

#### a. Preliminary subfractions of microsomes: densities greater than 0.75M sucrose

A preliminary subfractionation of the microsomes was carried out to determine whether we could obtain a Golgi-enriched microsomal fraction and to get an idea of the range of densities into which Golgi-derived vesicles would fall. The microsomes were resuspended in 1.15M





TABLE 1Densities of Sucrose Solutions and of Lipoprotein-Membrane Mixtures

<u>Sucrose molarity</u>	<u>Density at 0°</u>	<u>ratio of density 1.18 membrane to density 1.006 lipoprotein giving equivalent overall density</u>
0.00	1.000	--
0.25	1.034	0.16
0.30	1.041	0.20
0.40	1.054	0.28
0.50	1.068	0.36
0.60	1.081	0.43
0.70	1.095	0.51
0.75	1.101	0.55
0.88	1.118	0.64
1.00	1.134	0.73
1.15	1.154	0.85

Sources: Sucrose density figures are interpolated from the table of de Duve et al., 1958. Equivalent membrane-lipoprotein mixtures were calculated from the formula  $1.18 \times (\text{membrane fraction}) + 1.006 \times (1 - \text{membrane fraction}) = \text{overall density}$ . 1.18 is the density of isolated rat liver plasma membranes (Emmelot et al., 1964); 1.006 is the upper limit of density of very low density lipoprotein (Oncley, 1964).



sucrose and loaded into the bottom of a cellulose nitrate centrifuge tube. Discontinuous sucrose gradients were constructed above them by carefully pipetting several layers of successively lighter sucrose solutions one above another. The lightest sucrose used was 0.75 M. (Table 1, page 47, shows the densities of various sucrose solutions). At the top of the tubes was a layer of water. The gradients were spun for 6 hours at 40,000 rpm in a Spinco #40 rotor. Then the material at the interfaces between sucrose of various concentrations and between the top water layer and the underlying sucrose was collected with a syringe, mixed with osmium tetroxide, and centrifuged. The pellets obtained were processed for electron microscopy.

The fraction at the interface between water and 0.75 M sucrose was of particular interest. It contained large numbers of vesicles filled with 30-80 m $\mu$  electron opaque particles. Using the particles as markers, these vesicles were identified as being derived from the Golgi complex. However, the fraction also contained a variety of empty vesicles as well as some very large empty vacuoles. Some of the latter were collapsed, so that they appeared as a sheet made up of two closely opposed membranes. The origin of these vesicles and vacuoles is unclear. Without the electron opaque particles we could not assign them to the Golgi complex. It thus appeared that we could obtain a fraction greatly enriched in Golgi-derived vesicles, but that the fraction was seriously contaminated with significant amounts of membranes possible derived from non-Golgi sources. For a pure Golgi fraction it would be necessary to obtain only material of a density somewhat less than that of 0.75M sucrose.

b. Preliminary subfractions of microsomes: densities less than 0.75M sucrose

To this intent a series of gradients was constructed with sucrose solutions of concentrations below 0.75M layered above the load, which, as in the earlier series of experiments, was in 1.15M



sucrose. The gradients were spun for three hours at 25,000 rpm in a Spinco SW25.1 swinging bucket rotor (average field 63,500 x g). The material which accumulated at the various interfaces was collected, mixed with osmium, centrifuged, and the pellets processed for electron microscopy. The various fractions appeared as follows:

(1) water/0.30M sucrose interface: this fraction contained a few vesicles filled with electron opaque particles, but most of the pellet consisted of large, free fat droplets.

(2) water/0.40M sucrose interface: this fraction was composed primarily of vesicles loaded with electron opaque particles. There were a few large free fat droplets at one end of the pellet.

(3) water/0.50M sucrose interface: this fraction was virtually identical to the water/0.40M material. There was considerably more material at this interface, however, than at the water/0.40M interface.

(4) 0.30M/0.50M and 0.40M/0.60M sucrose interfaces: these fractions were virtually identical. Almost all of the vesicles in them contained opaque particles. There were almost no empty vesicles and no free fat droplets.

(5) 0.50M/0.70M and 0.50M/0.75M sucrose interfaces: these fractions were made up primarily of vesicles filled with electron opaque particles. However, about  $\frac{1}{4}$  to  $\frac{1}{3}$  of the pellet was made up of large vacuoles, often twisted or collapsed, and small empty vesicles. The origin of these latter materials was unclear. In the absence of the particles, they could not be ascribed to the Golgi complex.

Thus, the density range from the density of water to that of 0.75M sucrose could be divided into three parts. At densities less than 0.30M sucrose, there were a few Golgi vesicles, but free fat droplets predominated. At densities between that of 0.30M sucrose and that of 0.60M sucrose, almost all the vesicles derived from the Golgi



complex, according to the criterion of the electron opaque particles. And at densities greater than 0.60M sucrose, although there were many Golgi-derived vesicles, there was also much membrane derived from an unknown source.

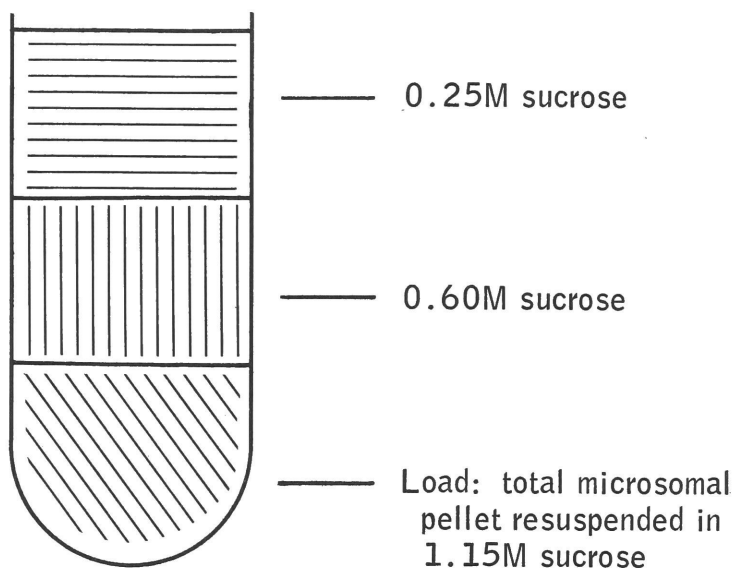
c. The final gradient

Since we wanted to obtain the purest fraction possible, even at the price of a low yield of Golgi membranes, we designed a final gradient which would (1) eliminate the fat droplets of the light range and the extended membrane and empty vesicles of the heavy range, and at the same time (2) give us an acceptable yield of vesicles containing electron opaque particles. The final density range we chose to try was from the density of 0.25M sucrose to that of 0.60M sucrose (densities 1.034 to 1.081; see Table 1, page 47).

The final gradient, then, was constructed by layering 10-15 ml of 0.60M sucrose over the load and filling the remainder of the centrifuge tube with 0.25M sucrose above the 0.60M sucrose (see Figure 2, page 51, for details of the gradient construction). The gradient was spun for 3½ hours at 25,000 rpm in a Spinco SW25.1 or for 3 hours at 25,000 rpm in an SW25.2 rotor ( 13,000,000 g-minutes at the 0.60M/0.25M interface in either case). The fluid above the 0.25M/0.60M interface was drawn off with a syringe and discarded. The cloudy band at the interface was collected, mixed with 2% osmium tetroxide in water, spun down, and the pellet processed for electron microscopy. The pellet was cut into longitudinal strips which were oriented in the Epon block in such a way that a single cut could provide a cross section of the entire depth of the pellet. Thus we could distinguish and comparatively examine the slower sedimenting top regions of the pellet and the more rapidly sedimenting bottom regions. Since the material was fixed in suspension, the position in the pellet reflects size and density after osmication. It does not reflect the sedimentation characteristics of the native material.





FIGURE 2Final Density Gradient Used in Isolation of Golgi FractionIn SW25.1 rotor:

volumes: load: 8-12 ml.  
 0.60M sucrose: 10 ml.  
 0.25M sucrose: 10-14 ml.

radii: axis to top of tube: 5.3 cm.  
 " " 0.60M/0.25M interface: 7.9-8.6 cm.  
 " " load/0.60M interface: 10.0-10.7 cm.  
 " " bottom of tube: 12.9 cm.

field: at 25000 rpm,  $G_{\max}$  is 90,137 x g;  $G_{\min}$  is 37,031 x g.

In SW25.2 rotor:

volumes: load: 15-20 ml.  
 0.60M sucrose: 15 ml.  
 0.25M sucrose: 25-30 ml.

radii: axis to top of tube: 6.4 cm.  
 " " 0.60M/0.25M interface: 10.0-10.7 cm.  
 " " load/0.60M interface: 12.0-12.7 cm.  
 " " bottom of tube: 15.3 cm.

field: at 25000 rpm,  $G_{\max}$  is 106,000 x g;  $G_{\min}$  is 44,700 x g.



d. Morphology of the 0.25M/0.60M interface material

The pellet obtained from the material which collected at the 0.25M/0.60M sucrose interface is shown in Plates 6 to 9.

The bottom of the pellet (Plate 6) is composed largely of roundish vesicles of diameter 150-500 $\mu$ , packed with 30-80 $\mu$  electron opaque particles. The vesicles are bounded by a unit membrane 75-90 A thick, which is the thickness typical of the membranes of the Golgi complex in situ. The vesicles are similar in size range to many of the Golgi vacuoles and saccules seen in tissue sections, and the particles inside the vesicles are similar in size to the particles seen in the Golgi elements in situ (see Plates 3 and 4). There are no vesicles in the pellet quite as large as the largest saccules seen in situ, however. Moreover, in tissue sections, the matrix of the Golgi elements is light and the particles dark, while in the pelleted Golgi fraction, the particles appear relatively light against a dark matrix (although they are still, absolutely speaking, "dark"). There is relatively little matrix space visible in the vesicles in the pellet as compared to the situation seen in tissue sections. In the former case, the matrix appears to have been concentrated and the membrane is applied closely to the tightly packed particles. Further, many of the vesicles in the pellet appear to contain a larger number of particles than do vesicles of similar overall diameter seen in situ. Finally, both in this part of the pellet and its remainder, the proportion of vesicles that have a more or less circular cross section is larger than in situ. All of these observations can be accounted for if we assume that many of the Golgi saccules lose water and take on a more compact, rounded up form upon isolation, possible as a result of prolonged exposure to hypertonic sucrose in the isolation procedure.

There are also in the pellet occasional vesicles, of similar size to those discussed above, which do not contain discrete opaque particles, but rather a non-homogeneous dense material. These are



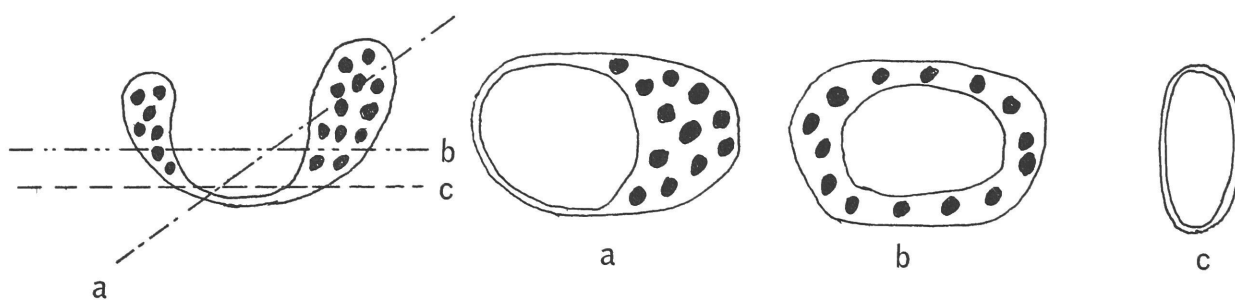
probably lysosomes. They comprise less than five per cent of the vesicles of the bottom layers of the pellet and are confined to the bottom fifth of the pellet. Thus they comprise only a very small proportion of the vesicles of the entire pellet--probably less than 1%. There are also a few empty vesicles in this part of the pellet. Some of these may be cross sections of empty portions of vesicles which would be seen to contain dense particles in sequential sections (see below). Occasional, particle-filled vesicles are elongated or even dumbbell or barbell shaped, reminiscent of the shapes of some Golgi saccules seen in tissue sections.

The middle region of the pellet (Plate 7) shows a greater variety of sizes and shapes of vesicles, but they are virtually without exception loaded with particles. There are large (500m $\mu$ ) roundish vesicles, dumbbells, and many smaller elongated profiles. The latter measure 50-100m $\mu$  wide and 200-300m $\mu$  long. There are also more or less circular profiles, 50-100m $\mu$  in diameter, which suggest that the small elongated or circular profiles may be a population of small tubular elements.

Many of the vesicles in this part of the pellet show particle-free loops of membrane extending from them. These may be cross sections of flattened, curved Golgi saccules. In sectioned tissue, one often sees Golgi saccules distended with dense particles at one or both ends, but more or less flattened or collapsed in their central regions. Similar images are seen in the top two-thirds of the Golgi pellet (Plates 7 and 8). Higher magnification micrographs (Plate 9) of this portion of the pellet reveal that the membrane loops in many cases consist, in fact, of two closely opposed membranes. As is shown in Figure 3, this is what we would expect if these vesicles are appropriate cross sections through curved cisternae having cup-shaped, collapsed regions.

The top of the pellet (Plate 8) consists primarily of small,



FIGURE 3Schematic Views of "Looped" Vesicles

Side section  
of vesicle

Top sections of same vesicle,  
cut as indicated at left





elongated vesicles. Some of these are narrow in their central region, with particles in enlargements at one or both ends. There are a few roundish vesicles, approximately 150-200m $\mu$  in diameter, too. As in the remainder of the pellet, virtually all of the vesicles, large or small, contain several electron dense particles.

Using the 30-80m $\mu$  dense particles in the interior of vesicles as a criterion for considering a vesicle to be of Golgi origin, we conclude that the 0.25M/0.60M interface is a highly purified Golgi fraction. From the morphological evidence presented, it contains an extremely small fraction of lysosomes (probably less than 1-2%) and occasional empty vesicles which may originate from cellular membrane systems other than the Golgi complex.

As discussed earlier, there are some elements of the smooth endoplasmic reticulum other than the Golgi vesicles which contain dense granules (see Plate 5). There is no way in which we could unambiguously distinguish on structural grounds such vesicles formed from smooth endoplasmic reticulum from Golgi-derived vesicles. It is clear, however, from examination of sectioned tissue that the vast bulk of those vesicles containing more than one electron opaque particle are associated with the Golgi complex. In the fraction obtained above, virtually all of the vesicles contain several particles. This suggests that most of the vesicles in the fraction are in fact derived from the Golgi complex. Later in this thesis we shall present evidence that the Golgi fraction lacks various enzymes associated with the rest of the endoplasmic reticulum. This will provide further support for the conclusion that virtually all of the vesicles in the fraction are of Golgi origin.

The question remains whether the fraction is representative of all of the components of the Golgi complex or only of certain parts of the Golgi vesicle population. The Golgi complex is composed of a variety of saccules, vesicles, and vacuoles of various sizes, which



PLATES 6-9The Total Golgi Fraction

Plate 6: Bottom region of pellet from material obtained at 0.25M/0.60M interface. Most of the vesicles (GV) are roundish and filled with particles. Some of the vesicles, however, are elongated (GV<sub>1</sub>). There are a few small empty vesicles (v) and a few large vesicles which do not contain particles and which may be lysosomes (L). Magnification: 39,500.

Plate 7: Middle region of pellet from material obtained at 0.25M/0.60M interface. A variety of shapes of vesicles are seen: roundish (GV<sub>1</sub>), dumbbell shaped (GV<sub>2</sub>), small and elongated (GV<sub>3</sub>), small and round (GV<sub>4</sub>) and large, with membrane loops extending from them (GV<sub>5</sub>). Almost all of these various vesicles are filled with particles. Magnification: 42,500.

Plate 8: Top region of pellet from material obtained at 0.25M/0.60M interface. Most of the vesicles are small and often elongated (GV<sub>1</sub>), but a few larger, roundish vesicles also occur (GV<sub>2</sub>). Some of the small elongated vesicles contain particles only at one or both ends (GV<sub>3</sub>). Magnification: 37,500.

Plate 9: Higher magnification micrographs of selected vesicles from the middle region of the pellet from material obtained at 0.25M/0.60M interface, showing how various images could result from different planes of sectioning similar vesicles (cf. Figure 3, page ).

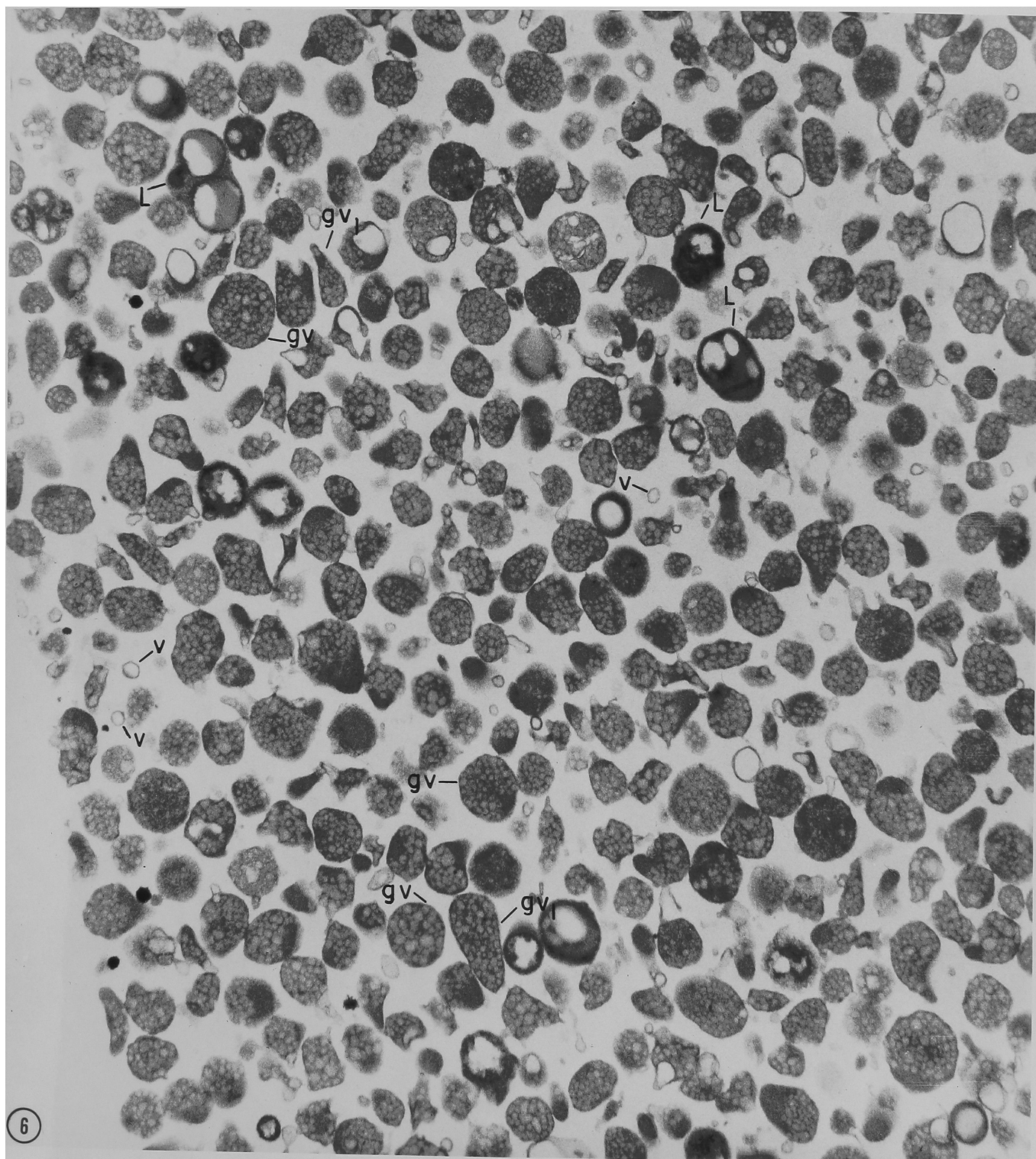
Plate 9a: side view of elongated, curved vesicle (cf. Figure 3, left).

Plate 9b and 9c: ring shaped vesicles (cf. Figure 3b).

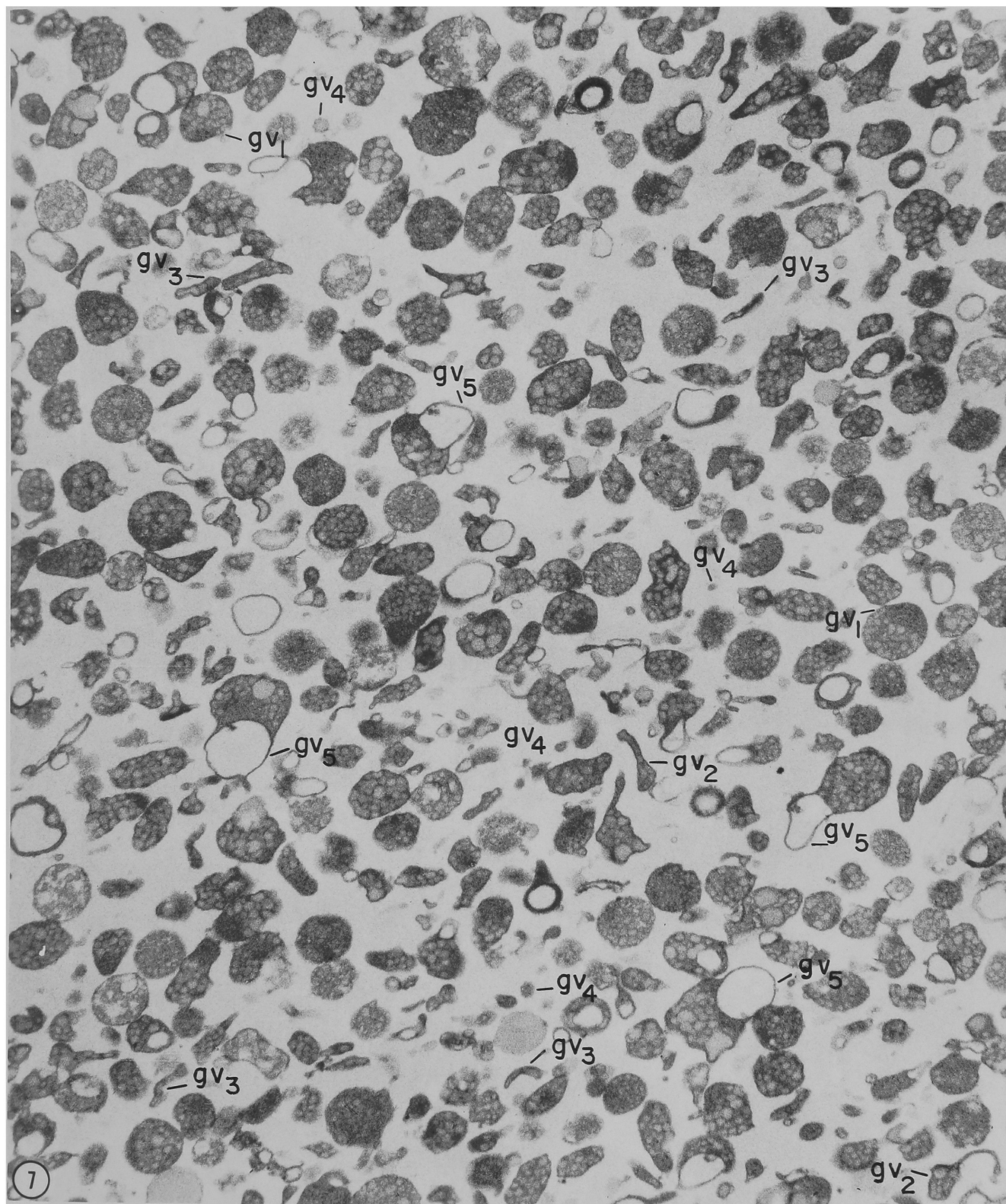
Plate 9d, 9e, 9f: vesicles with "loops" extending from them. At the single arrows it can be seen that the "loops" consist of two membranes, which may in some regions be fused (cf. Figure 3a). At the double arrow in Plate 9f is an image which might be a section through such a loop or through an empty region of a vesicle as in Fig. 3c.

Magnification: 86,000.



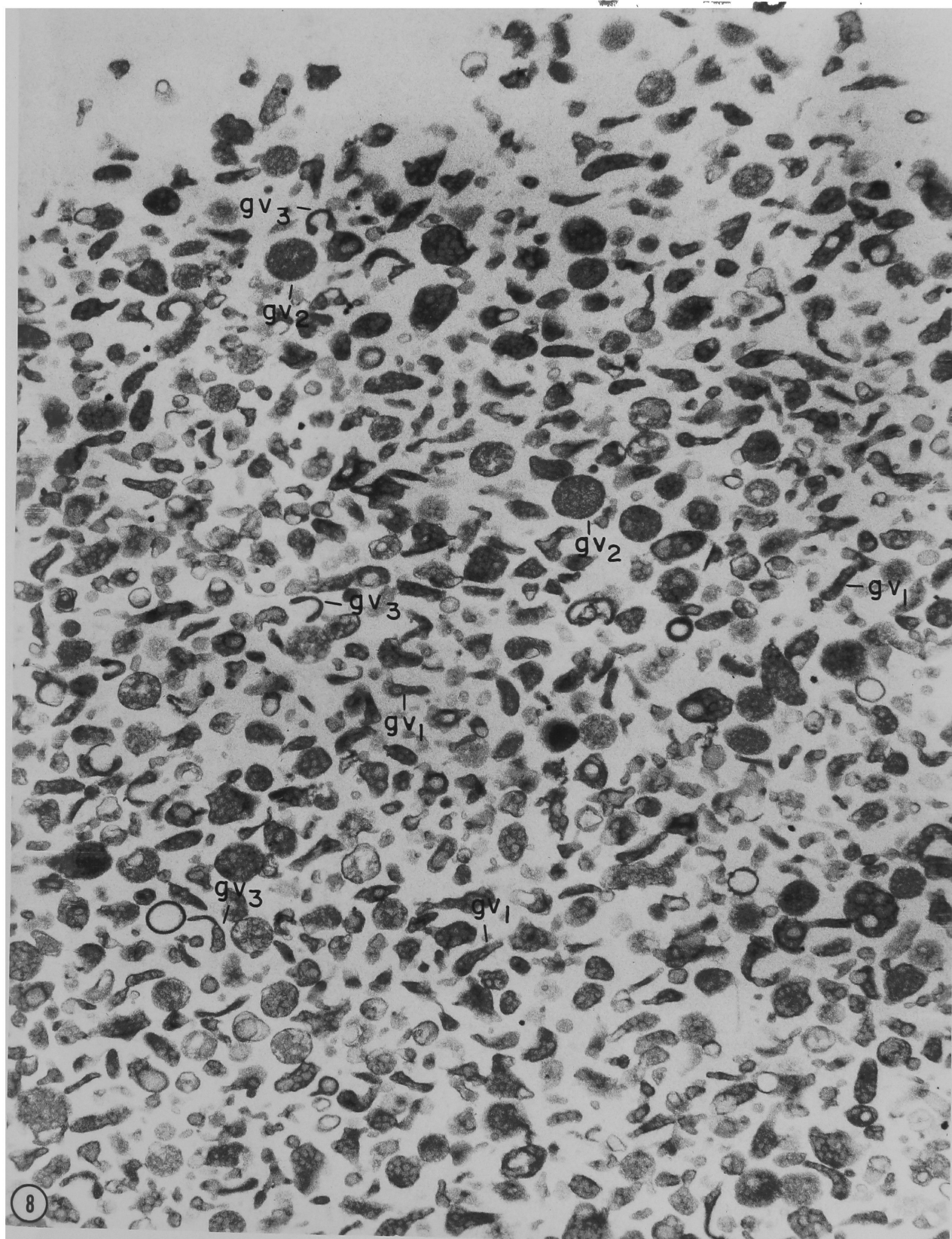




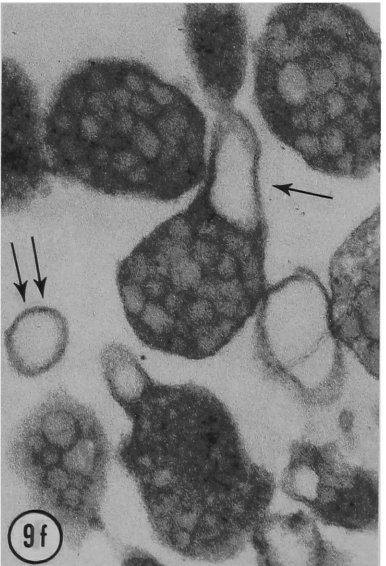
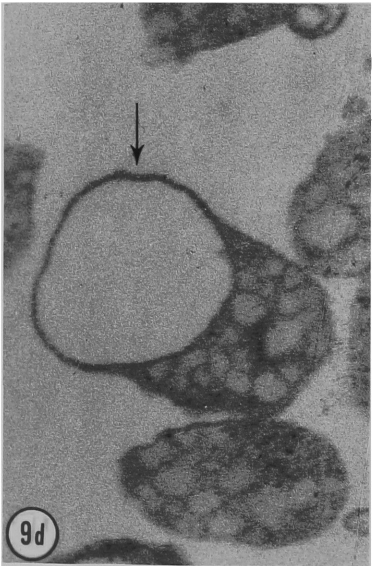
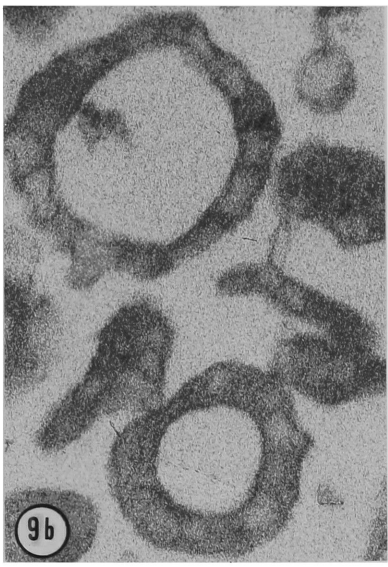
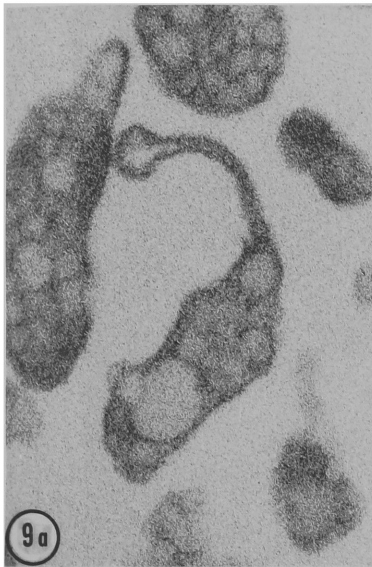














may be differentiated considerably in content, as well. The method of isolation we are using selects out those vesicles in which the ratio of light lipoprotein content to heavy limiting membrane is high. In situ, virtually all of the elements of the Golgi complex of ethanol-intoxicated rats appear to contain particles. However, a small vesicle has a greater ratio of surface area to volume than a large one. Since the membrane surface is heavier than the contents (lipoprotein), a small vesicle needs a greater concentration of lipoprotein in its interior than a large vesicle if they are to have the same density. There is no way of telling whether the degree of lipoprotein loading of the various elements of the Golgi complex is such that, for example, the population of large Golgi vacuoles and the population of small Golgi vesicles have similar density distributions. When we collect a specific part of the density distribution of the entire Golgi-derived vesicle population, we may be preferentially isolating only certain sub-populations of such vesicles.

The only reliable morphological criterion we have for Golgi origin is the presence of lipoprotein particles. The vesicles have been subjected to strong shearing forces and centrifugal fields during homogenization and centrifugation. They have been passed through a series of chemically and osmotically varying media. Finally, they have been fixed with osmium tetroxide, dehydrated, and embedded in epoxy resin. As a result, they are unlikely to reflect accurately the morphology of the vesicles seen in tissue sections, which have been subjected to a different ensemble of procedures. For example, as we have pointed out, in tissue sections the dense particles are not packed as tightly as they are in the vesicles in the fractionated material.

It is nevertheless clear from the morphology of the fraction that a considerable variety of Golgi-derived elements are present. There are roundish vesicles ranging in size from 100 to 500m $\mu$  in diameter.



There are large and small elongated vesicles and tubules and dumbbell and barbell shaped vesicles. These are all reminiscent, in size range and in variations in shape, of sizes and shapes of vesicles seen in the Golgi complex in tissue sections. The only Golgi elements seen in situ which may be lost appears to be the small, spherical vesicles seen at the periphery of the Golgi complex. In sections of tissue, these small vesicles generally contain at most one dense particle; hence, they may be too heavy to be isolated by our procedure.

It seems safe to conclude that virtually the full range of vesicles, saccules, and vacuoles found in the intact Golgi apparatus is represented in our fraction (with the possible exception noted). There is no way of determining, however, whether the quantitative mix of different kinds of vesicles is similar to the mix in the intact Golgi apparatus.

On the basis of the morphology of the fraction obtained at the interface between 0.25M and 0.60M sucrose, then, we conclude that we have obtained a highly purified and reasonably representative fraction of vesicles derived from the Golgi apparatus of the hepatic parenchymal cell.

e. Final procedure for isolating a Golgi fraction

The final procedure for isolating the Golgi fraction is then as follows:

The total microsomal fraction is obtained from the livers of ethanol intoxicated rats by differential centrifugation (details in Materials and Methods, Chapter 3). The microsomes are resuspended in 1.15M sucrose and loaded into a cellulose nitrate centrifuge tube. A discontinuous gradient consisting of one layer of 0.60M sucrose and one layer of 0.25M sucrose (see Figure 2, page 51) is constructed above the load, and the gradient is spun for  $3\frac{1}{2}$  hours at 25,000 rpm in a Spinco SW25.1 or for 3 hours in a Spinco SW25.2 swinging bucket rotor.





The cloudy band at and below the interface between 0.25M and 0.60M sucrose is the Golgi fraction. The liquid above this is drawn off and discarded. The Golgi band is collected with a syringe, diluted with an equal volume of water to bring the sucrose concentration down to approximately 0.25M, and recentrifuged for 1 hour at 40,000 rpm in a Spinco #40 rotor to wash away any remaining low molecular weight solutes and to concentrate the membranes. The resulting whitish-tan pellet is resuspended in a small volume of water or buffer. This resuspended fraction is referred to as the "total Golgi fraction."

In order to be able to establish recovery factors for enzymes, it was necessary to collect the remainder of the gradient. To do this, the entire gradient below the Golgi band, including the pellet, was mixed, diluted 3:1 with water to bring down the sucrose concentration, and spun 1 hour at 40,000 rpm in a Spinco #40 rotor. The pellet is resuspended in water or buffer, and is referred to as the "total remainder fraction."

#### 4. Rupture of Golgi vesicles: obtaining a pure membrane preparation

The fraction obtained above consists of membrane bound vesicles loaded with lipoprotein particles. It was desirable to obtain the Golgi membranes alone, free of the lipoprotein content, because the content (a) might interfere with enzyme activities; (b) would in any event give the fraction an artificially high protein content for use in calculating specific activities of the membranes; and (c) make it impossible to do any meaningful chemistry on the membranes for comparison with other types of cell membranes, due to the high background of non-membrane lipid and protein.

The procedure we developed to eliminate the contents consisted of breaking open the vesicles by chemical and mechanical means, allowing the contents to escape, and then separating the membranes from the contents by centrifugation.



a. Disrupting the membranes and obtaining their membrane

A variety of methods have been used by other investigators to break open cells and subcellular vesicles. We investigated the use of several of these procedures alone and in combination. They included suspending the Golgi vesicles in hypotonic medium, hoping to swell and perhaps rupture them; exposure of the vesicles to dilute alkali (known to cause the lysis of pancreatic zymogen granules, presumably by loosening the structure of their membranes (Greene *et al.*, 1963)), thus rendering them more susceptible to rupture; sonication; and shearing in a steep pressure gradient (French press; Milner *et al.*, 1950).

Now the original vesicles, consisting of membrane plus content, had earlier been sedimented in 0.25M sucrose. We reasoned that the heavier membrane without its lipoprotein buoy could certainly be sedimented in 0.15-0.25M sucrose. The lipoprotein content, on the other hand, would be light (very low density lipoprotein is the fraction having a density less than 1.006 (Oncley, 1964)) and would not sediment under these conditions. So to separate the membrane from the released content, 1.0M sucrose was added to the suspension to bring the final sucrose concentration to 0.15M or 0.25M. This suspension was then centrifuged for 1 hour at 40,000 rpm in a Spinco #40 rotor.

The effect of the attempted release of lipoprotein particles and subsequent centrifugation was followed in two ways. First, the experiment was performed on animals which had been injected prior to killing with  $^3\text{H}$ -palmitate. The label is expected primarily in lipoprotein particles, although a fraction of it may be incorporated into the membrane itself or into materials not dislodged from the membrane when the granules are freed. Thus the distribution of label between pellet and supernatant after centrifugation was one means of determining how much of the content had been released by the procedure.

Table 2 shows the distribution of radioactive label between the



TABLE 2Effectiveness of procedures for disrupting Golgi vesicles

<u>Series<sup>a</sup></u>	<u>Procedure</u>	<u>Per cent of label recovered in pellet</u>
I.	Freezing and thawing, 10 times	90
	hypotonic shock <sup>b</sup>	95
	freezing and thawing, 6 times in hypotonic medium	94
	sonication <sup>c</sup> , 20 sec, in hypotonic medium	59
	sonication, 50 sec, in hypotonic medium	45
	Freezing and thawing, 6 times in hypotonic medium, followed by sonication, 20 seconds in hypotonic medium	47
II.	Alkaline, hypotonic shock <sup>d</sup> (Tris, pH 8.0)	62
	Alkaline, hypotonic shock (Tris, pH 8.0) followed by sonication, 20 sec	34
	Alkaline hypotonic shock (Tris, pH 8.0) followed by sonication, 20 sec, followed by passage through French press <sup>e</sup>	21
III.	Alkaline hypotonic shock <sup>f</sup> (Veronal, pH 7.0) followed by passage through French press one time	49
	same, pH 7.5	34
	same, pH 8.0	26
	same, pH 8.5	16
	Alkaline hypotonic shock, (Veronal, pH 8.0) followed by passage through French press two times	21
	same, pH 8.5	16

Notes: on following page



Notes to TABLE 2

General note: the total Golgi fraction from animals which had been injected with  $^3\text{H}$ -palmitate 10 min prior to killing was treated according to the procedures listed. The sucrose was added to the suspension, and the whole centrifuged. The distribution of label between pellet and supernatant was assayed.

- a. Series: Series I - the Golgi fraction used was the impure material from the 0.25/0.75M sucrose interface. The final sucrose concentration for the differential centrifugation was 0.15M. Centrifugation was 1 hr at 40,000 rpm in a Spinco 40.3 rotor.

Series II - The same Golgi fraction was used as in Series I. The final sucrose concentration for the differential centrifugation was 0.25M. Centrifugation was 1 hr at 40,000 rpm in a Spinco 40.3 rotor.

Series III - The Golgi fraction used was the pure material from the 0.25/0.60M sucrose interface. The final sucrose concentration for the differential centrifugation was 0.25M. Centrifugation was 1 hr at 40,000 rpm in a Spinco 40 rotor.

- b. Hypotonic shock: in Series I, the total Golgi fraction was originally suspended in 0.25M sucrose. It was diluted to 0.08M sucrose with water for hypotonic treatment.
- c. Sonication: sonication was performed with a Branson Ultrasonic Corp. probe sonicator Model LS75 at maximum power output.
- d. Alkaline hypotonic shock: in Series II, the total Golgi fraction was resuspended in water, and Tris buffer added to make the final concentration 0.04M Tris of the pH noted.
- e. French press: the material in the hypotonic buffer was passed through the French press under a pressure of 4,000 pounds per square inch.
- f. Alkaline hypotonic shock: in Series III, the total Golgi fraction was resuspended in water, and veronal-HCl buffer added to make the final concentration 0.04, sodium veronal of the pH noted.





pellet and the supernatant after various disruptive treatments followed by a single centrifugation. As a result of the evidence from the labelling experiments, the more promising procedures were repeated and the pellets and the supernatants fixed with osmium tetroxide and processed for electron microscopy. The relative patterns were identical to those predicted by the labelling: in those procedures in which the pellet retained little label, the vesicles appeared relatively free of contents. In those procedures in which the pellet retained a large proportion of the radioactivity, the vesicles in the pellet retained a considerable part of their electron opaque particle content.

As a result of these experiments, the final procedure was as follows: The total Golgi fraction was resuspended in 0.04M sodium veronal-HCl buffer, pH 8.5. The suspension was passed through the French press twice under a pressure of 4,000 pounds per square inch. 3 ml of 1.0M sucrose was added to 9 ml of the disrupted suspension and the whole centrifuged for 1 hour at 40,000 rpm in a Spinco #40 rotor. The pellet was resuspended in buffer or water. It is called the "Golgi membrane fraction." The supernatant is called the "Golgi content fraction". Membranes obtained in this way contain 15-25% of the lipid label of the total Golgi fraction from which they were derived.

For purposes of comparisons of enzymatic activity, the other fractions obtained earlier were treated identically--suspended in hypotonic veronal buffer, passed through the French press, brought up to 0.25M sucrose, and centrifuged. The resulting pellets are called "mitochondrial membrane", "microsomal membrane", and "remainder membrane" fractions respectively. The resulting supernatants are called "mitochondrial content", "microsomal content", and "remainder content" fractions respectively. Presumably the treatment released much of the contents of these other vesicle suspensions, just as it did in the case of the total Golgi fraction. This was not investigated in greater detail, however, so the use of the terms "membranes" and



"contents" is largely arbitrary; it implied only that they are fractions which have been treated in a way corresponding to the Golgi fractions.

It should be noted that treatment with alkali may remove some protein from the membranes and thus alter the distribution of enzymes in the "Golgi membrane fraction" relative to that in the native Golgi membranes. As will be seen later, this presents difficulties in interpretation only for the case of cytochrome  $b_5$ .

b. Morphology of the Golgi membrane fraction

Plates 10-13 show the membrane pellet obtained from the total Golgi fraction as described above. It consists almost entirely of empty vesicles and broken open vesicles. Only at one end of the pellet are there some vesicles having a few granules in them. In some cases it appears that there is moderately dense content in an occasional vesicle, but the structure of a granule 30-80m $\mu$  has been lost. The morphology of the preparation appears consistent with the labelling evidence in which 75-85% of the label is lost to the supernatant. Since some of the remaining label may not be associated with the lipoprotein content of the vesicles, the membrane fraction appears to be sufficiently freed of content for chemical and enzymological study.

c. Morphology of the Golgi content fraction

Plate 14 shows the pellet obtained when the supernatant from the centrifugation of the ruptured Golgi suspension is mixed with osmium tetroxide and centrifuged. It consists entirely of electron opaque particles, of diameters 30-80m $\mu$  primarily, although there are a few larger particles.

5. Summary of isolation

We have shown that a fraction of Golgi-derived vesicles could be obtained by centrifugation of microsomes on a discontinuous gradient. Morphological examination, using the characteristic Golgi content of 30-80m $\mu$  electron opaque particles as a marker, has established that



PLATES 10-14The Golgi Membrane and Golgi Content Fractions

Plates 10-13: Golgi membrane fraction.

Plate 10: Top of pellet obtained by spinning the disrupted total Golgi fraction. Most of the vesicles are empty or still broken open

Plate 11: Upper middle region of pellet. Most of the vesicles are empty or still broken open.

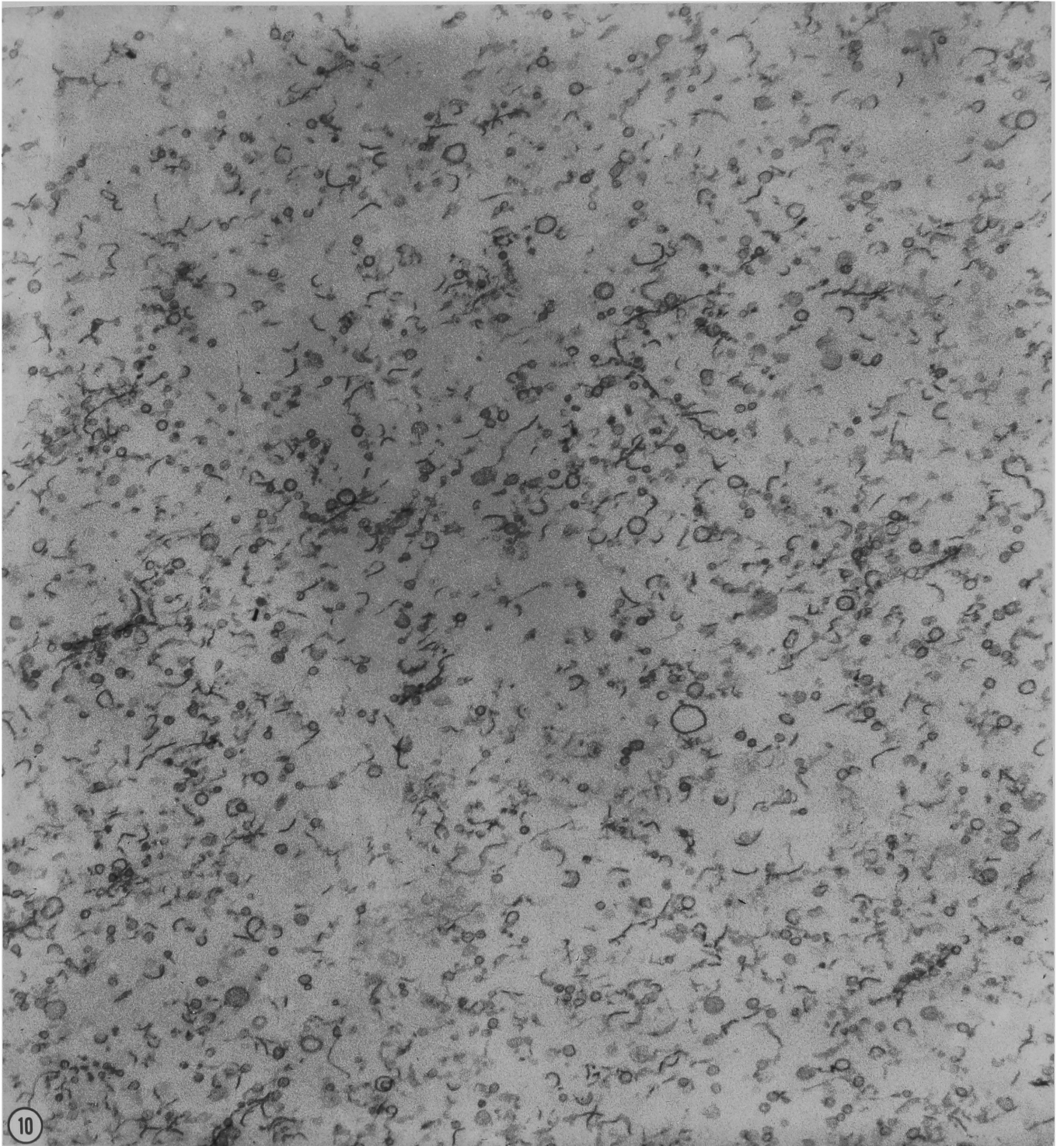
Plate 12: Lower middle region of pellet. A few vesicles (arrows) contain dense particles.

Plate 13: Bottom of pellet. A few vesicles (arrows) contain dense particles.

Magnification: 37,500.

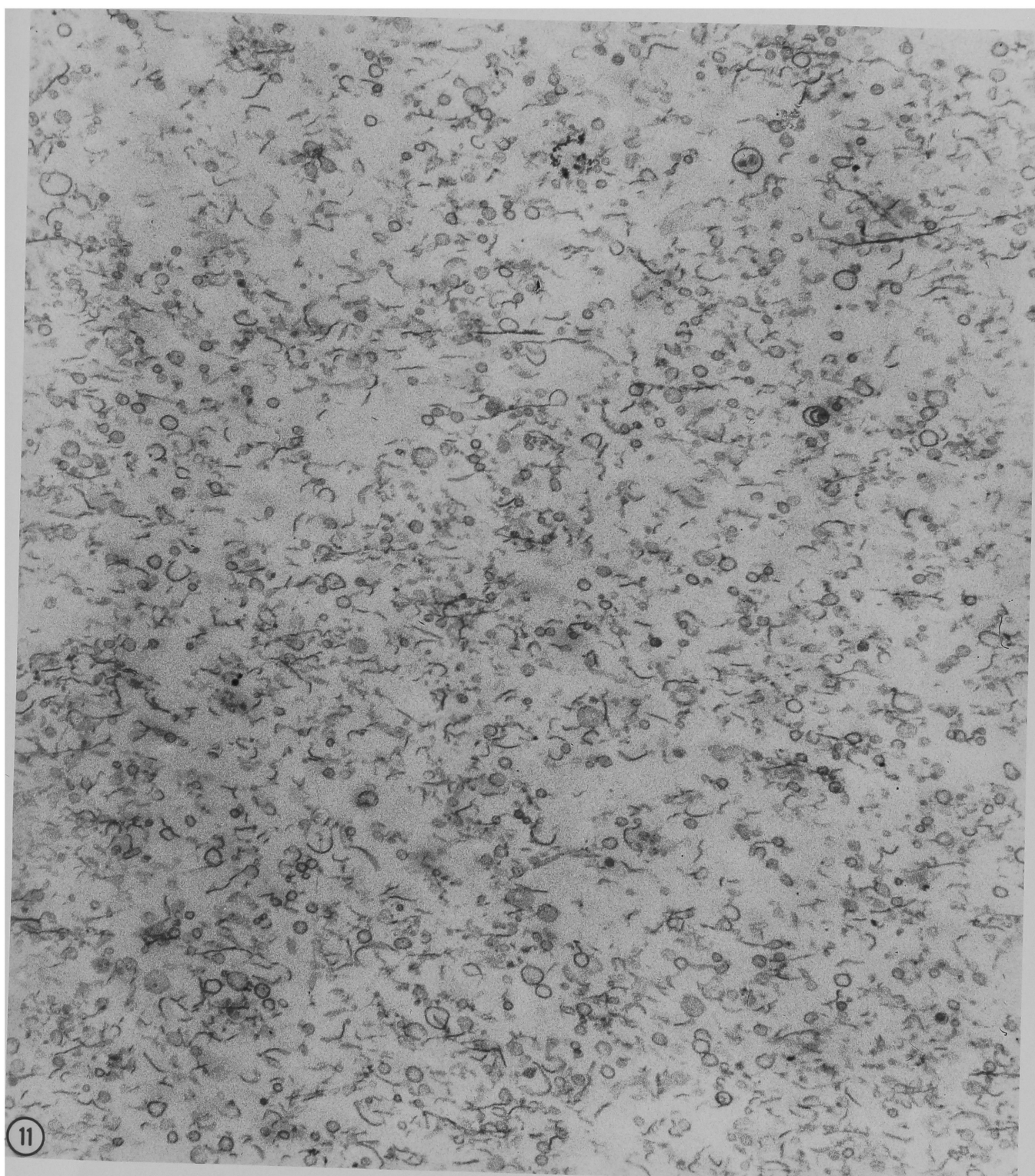
Plate 14: Golgi content fraction: various regions of the pellet obtained by mixing the supernatant which resulted from spinning the disrupted total Golgi fraction with osmium tetroxide and re-spinning. Plate 14a is near the top of the pellet, Plate 14b is in the central region, and Plate 14c is near the bottom. The entire pellet consists of dense particles. Magnification: 45,000.



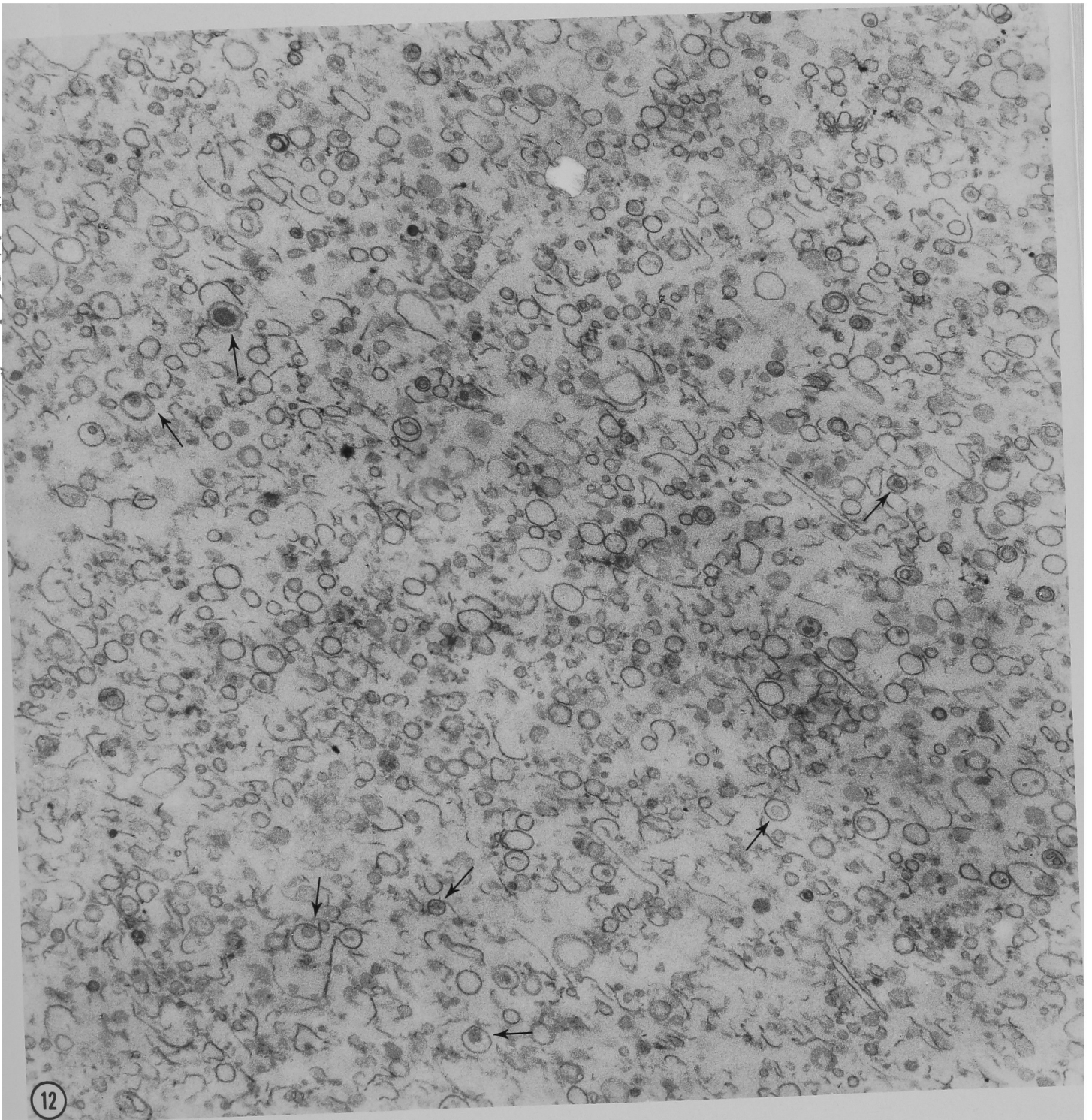






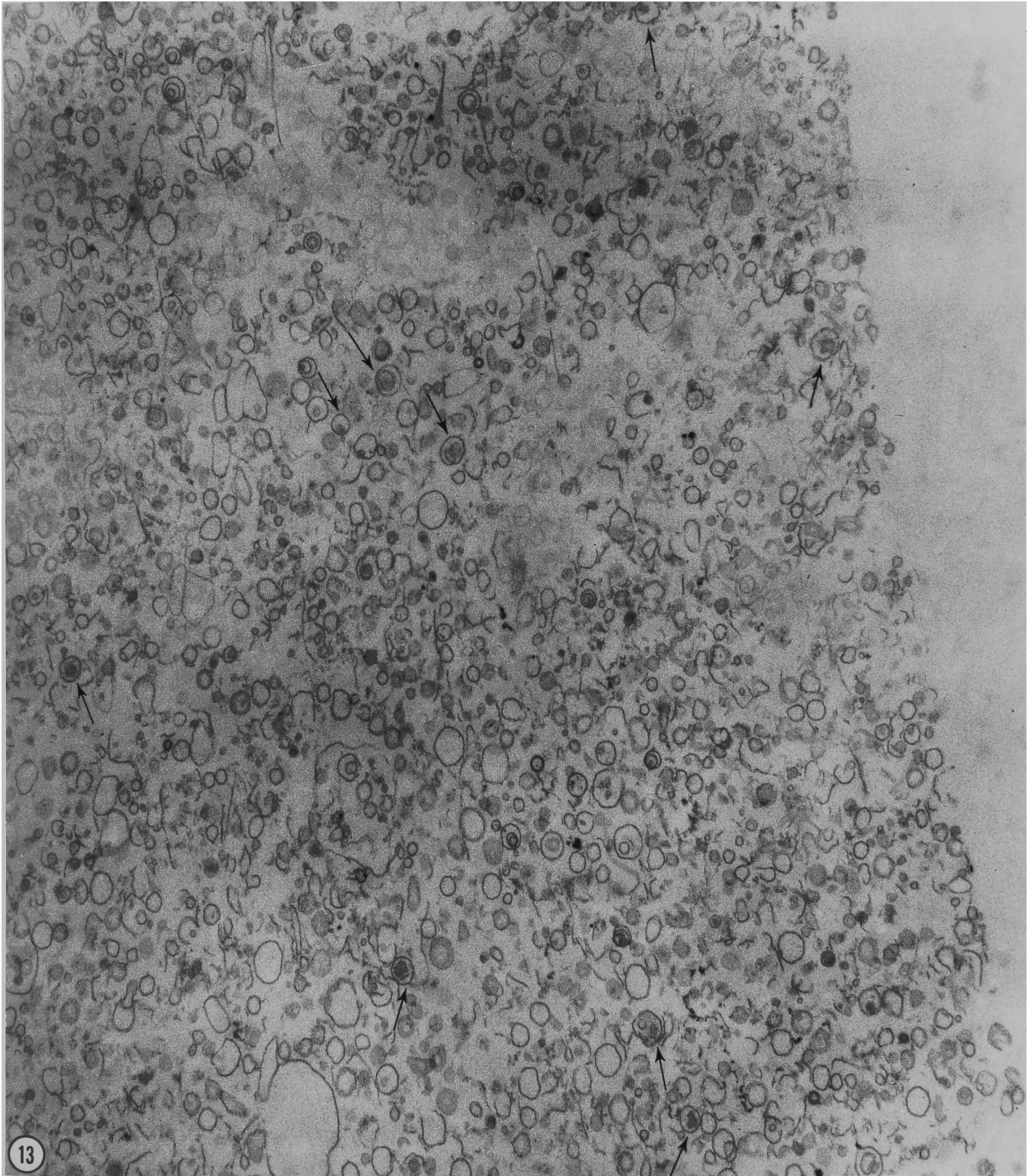




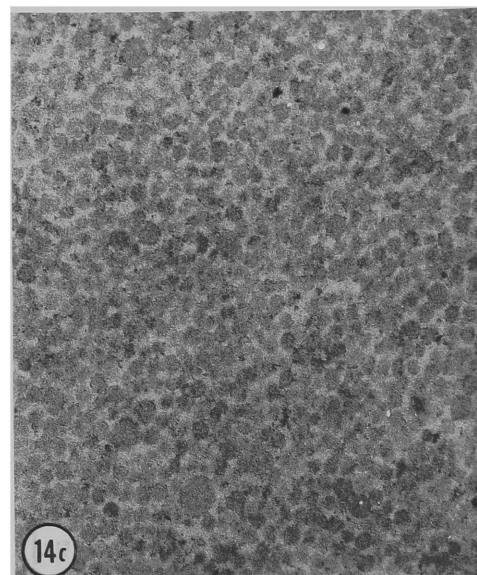
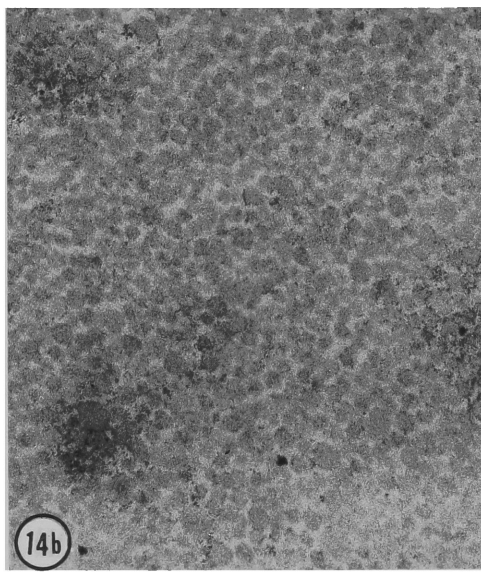
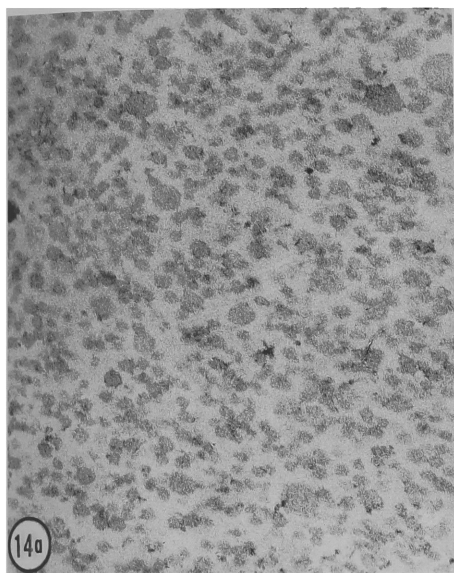
















the fraction is highly purified. The vesicles can be broken open, allowing the lipoprotein content to escape, and the membranes can then be recovered, free of the content. The entire procedure is summarized in detail in the flow sheet in Figure 4 (page 76).

We turn now to the chemical and enzymological characterization of this fraction.

## B. Gross Chemistry of Fractions

### 1. Protein

The total Golgi fraction, isolated as described in section A, generally contained 50-60  $\mu\text{g}$  protein per gram liver (Table 3).<sup>\*</sup> In occasional experiments, the figure was as low as 25  $\mu\text{g}$  and as high as 80  $\mu\text{g}$ . The usual figures represent approximately 0.3% of the protein contained in the total microsomal fraction from which the total Golgi fraction was derived.

The total Golgi fraction consists of membrane bound vesicles heavily loaded with lipoprotein particles. Thus the membranes themselves constitute only a fraction of the 50-60  $\mu\text{g}$  protein per gram liver. When the contents are released and the membranes isolated, we find that about 60% of the protein is attributable to the contents. The final Golgi membrane fraction averages 19  $\mu\text{g}$  protein per gram liver.

These yields undoubtedly represent only a small fraction of the total Golgi membranes of the cell. A very rough guess, from examination of sectioned material, is that the Golgi complex membranes make up of the order of 5 to 10% of the total intracellular membranes (endoplasmic reticulum) of the liver cell. If we assume that our total microsomal fraction is more or less representative of the membrane populations of the cell, then we are obtaining a Golgi fraction

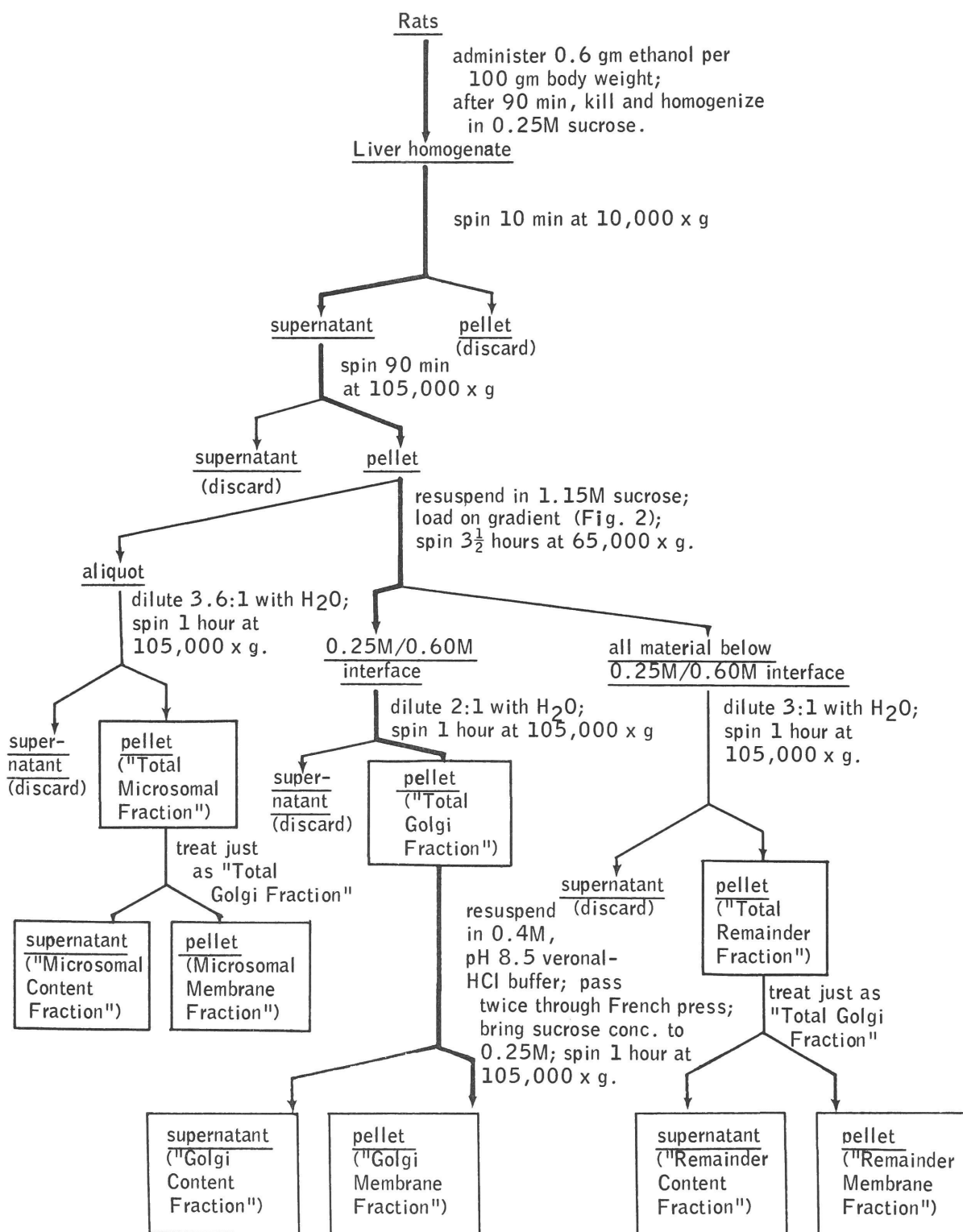
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<sup>\*</sup>Note: The layout of the tables in this and the following section is explained in Figure 5.



FIGURE 4

# Procedure for Isolating Golgi and Other Fractions





comprising, at most, about 5% of the total Golgi membranes of the cell. This low yield stems, of course, from the decision to sacrifice yield in favor of purity.

The total microsomal fraction and the total remainder fraction (which is essentially the total microsomal fraction with the total Golgi fraction removed) show a less dramatic loss of contents. About 65% of the protein of these total fractions are recovered in the corresponding membrane fractions. This difference in behavior from the total Golgi fraction undoubtedly reflects the very heavy loading of the Golgi vesicles.

When an attempt was made to isolate Golgi-derived vesicles without previous treatment of the animals with ethanol, a band of cloudy material is obtained at the 0.25M/0.60M interface. It contains, however, only  $\sim 5$   $\mu$ g protein per gram liver--about 1/10 the yield after ethanol intoxication. Both the success of the isolation procedure after ethanol treatment and the much lower yield when this treatment is omitted testify to the correctness of our hypothesis that loading the Golgi complex with lipoprotein by ethanol intoxication would make it possible to obtain a pure and reasonably abundant Golgi fraction.

## 2. Phospholipid

The total Golgi fraction contains 0.50 mg phospholipid per mg protein (Table 4). By contrast, the total microsomal fraction contains only 0.26 mg phospholipid per mg protein. That this difference reflects the differing contents of the two populations of vesicles and not a major difference in the membranes themselves can be seen by examining the membrane and content fractions derived from the total Golgi and total microsomal fractions:

The Golgi content fraction is very rich in phospholipid. In our experiments it contains 0.60 mg phospholipid per mg protein. This



raises the ratio of phospholipid to protein in the total fraction relative to that in the membrane fraction. The Golgi membrane fraction itself has only 0.36 mg phospholipid per mg protein.

By contrast, the microsomal content fraction is very poor in phospholipid--0.075 mg per mg protein. The content presumably includes albumin and proteins for "internal" cell use (e.g., lysosomal and micro-body enzymes) as well as some lipoprotein (Peters, 1962a). Thus, the phospholipid-to-protein ratio of the total microsomal fraction is lower than that of the microsomal membranes alone. The microsomal membrane fraction has 0.35 mg phospholipid per mg protein. This is virtually the same ratio as the Golgi membrane fraction.

Not surprisingly, the microsomal and the Golgi membrane fractions are thus grossly similar in phospholipid and protein content. For yet another comparison, plasma membranes from rat liver have been reported to contain 0.27 to 0.37 mg phospholipid per mg protein--again, grossly similar (recalculated from Emmelot et al., 1964; Pflieger et al., 1968; and Widnell and Unkeles, 1968). It is only when the lipids of plasma membrane are examined in more detail (with respect to sphingomyelin and cholesterol content, for instance) that significant differences with the microsomal membranes appear.

### 3. Ribonucleic Acid

Due to difficulties encountered in determining RNA, we can only present upper limits on the amount of RNA in the Golgi fractions. The total Golgi fraction contained less than 0.015 mg RNA per mg protein. The Golgi membrane fraction contained less than 0.01 mg RNA per mg protein. The total microsomal fractions, by comparison, contained 0.125 to 0.17 mg RNA per mg protein (Table 5).

The difficulties stemmed from contaminants, presumably carbohydrates, present in the TCA extracts from the total Golgi and Golgi membrane fractions. Various substances, mainly sugars, react





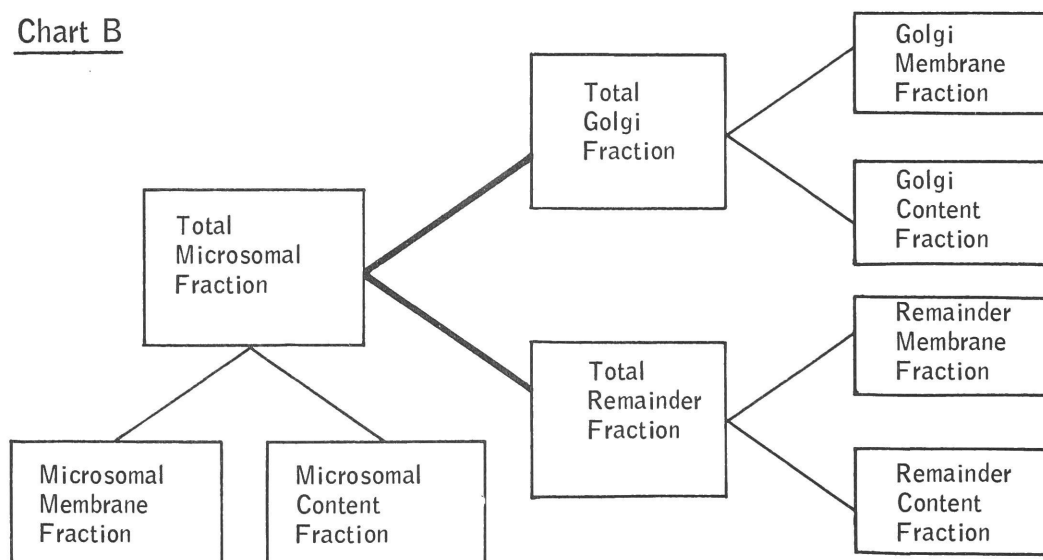
FIGURE 5  
Layout of Tables

The tables in this section and in section C (Comparative Enzymology of Fractions) are presented in a standardized way to facilitate comparisons of fractions which are derived from one another. (A few charts are modified slightly). Chart A below is a replica of the standard pattern in which the tables appear. Chart B is a redrawing of the various categories so that the relations between the various fractions can be recalled.

Chart A

	Total Microsomal Fraction	Total Golgi Fraction	Total Remainder Fraction
	Microsomal Membrane Fraction	Golgi Membrane Fraction	Remainder Membrane Fraction
	Microsomal Content Fraction	Golgi Content Fraction	Remainder Content Fraction

Chart B



(Heavy lines indicate discontinuous gradient centrifugation; light lines indicate disruption by alkaline hypotonic shock and passage through the French press followed by differential centrifugation.)



TABLE 3Protein Content of Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fractions"	15.0 (14.6)	0.055 (0.047)	15.2 (13.9)
"Membrane Fractions"	10.7 (9.4)	0.019 (0.016)	10.2 (9.4)
"Contents Fractions"	2.9 ( - )	0.031 ( - )	3.3 ( - )

All figures are mg protein/gram liver. Upper figures are averages of 4 experiments in which protein in "total", "membrane", and "content" fractions were all assayed. Lower, bracketed figures are averages of 15 experiments for which complete figures are available only for the "total" and "membrane" fractions.

Ranges: total microsomal fraction: 11.6-18.7 mg/gram liver  
microsomal membrane fraction: 6.3-12.1  
total Golgi fraction: 0.025-0.080  
Golgi membrane fraction: 0.007-0.025



TABLE 4  
Phospholipid Content of Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fractions"	0.26 (3.80)	0.50 (0.042)	0.27 (3.71)
"Membrane Fractions"	0.35 (3.26)	0.36 (0.0085)	0.35 (3.28)
"Contents Fractions"	0.075 (0.21)	0.60 (0.026)	0.085 (0.36)

Upper figures are mg phospholipid/mg protein; lower, bracketed figures are mg phospholipid/gram liver.

Figures are averages of 4 experiments.

Ranges: total microsomal fraction: 0.22-0.31 mg phospholipid/mg protein  
microsomal membrane fraction: 0.30-0.40  
total Golgi fraction: 0.43-0.56  
Golgi membrane fraction: 0.31-0.41

Figures from literature for comparison: (mg phospholipid/mg protein)

total microsomal fraction: 0.24 (Palade and Siekevitz, 1956)  
0.20-0.27 (Dallner, 1963)  
0.26-0.29 (Orrenius et al., 1965)  
0.31 (Dallner et al., 1966b)

plasma membrane fractions: 0.27-0.35 (Benedetti and Emmelot, 1968)  
0.27 (Pfleger et al., 1968)  
0.37 (Widnell and Unkeles, 1968)



TABLE 5  
Ribonucleic Acid Content of Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fractions"	0.15	$\leq 0.015^*$	0.15
	(2.28)	( $\leq 0.0008$ )	(2.38)
"Membrane Fractions"	0.135	$\leq 0.009^*$	0.14
	(1.54)	( $\leq 0.00035$ )	(1.52)
"Contents Fractions"	0.099	$\leq 0.026^*$	0.089
	(0.32)	( $\leq 0.0008$ )	(0.28)

\*Note: see text, page 78, for explanation of upper limits.

Upper figures are mg RNA/mg protein; lower, bracketed figures are  
 mg RNA/gram liver.

Figures are averages of 4 experiments.

Ranges: total microsomal fraction: 0.125-0.17 mg RNA/mg protein  
 microsomal membrane fraction: 0.127-0.15  
 total Golgi fraction:  $\leq 0.007$ - $\leq 0.030$   
 Golgi membrane fraction:  $\leq 0.006$ - $\leq 0.04$

Figures from literature for comparison: (mg RNA/mg protein)  
 total microsomal fraction: 0.145 (Palade and Siekevitz, 1956)  
 0.16-0.26 (Dallner, 1963)  
 0.10-0.20 (Ernster et al., 1962)  
 0.18-0.20 (Orrenius et al., 1965)  
 0.22 (Glaumann and Dallner, 1968)





with orcinol under the conditions of the RNA assay, to give a product which absorbs light at 665 m $\mu$ , which is the absorption maximum for orcinol reacted with RNA (Hutchinson and Munro, 1961). Brown (1945) has proposed that absorption at 565 m $\mu$  of orcinol reacted extracts be taken as a test for such contamination. The extracts from the Golgi fractions, after reaction with orcinol, all had absorptions at 565 m $\mu$  at least 2-3 times greater than their absorptions at 665 m $\mu$ . Repeated washings of the fractions with cold TCA before extracting the RNA with hot TCA decreased, but did not eliminate this contaminant absorption. (In contrast to this behavior, a purified RNA sample, dissolved in hot TCA and reacted with orcinol, had an absorption at 665 m $\mu$  four times as great as that at 565 m $\mu$ , and the extract from the total microsomal fraction absorbed three times as much at 665 m $\mu$  as at 565 m $\mu$ ).

The figures in Table 5 for all of the fractions are calculated directly from the absorption at 665 m $\mu$ , ignoring the probability that all or part of the absorption of the Golgi fractions came from contaminants. Thus, these figures must be regarded as upper limits on the RNA content, not as accurate representations of it.

### C. Comparative Enzymology of Fractions

The various fractions which we obtained were assayed for a series of enzyme activities.

The first group in this series is represented by glucose-6-phosphatase, NADPH-cytochrome c reductase, cytochrome P-450, NADH-cytochrome c reductase, cytochrome b<sub>5</sub>, and 5'-nucleotidase, i.e., enzymes which are all found in either microsomal fractions or plasma membrane fractions or both. They were of interest because of what they might reveal about the relations between the Golgi-derived membranes and the endoplasmic reticulum membranes (the main source of microsomal membranes in the cell) on the one hand, and between the Golgi-derived membranes and the plasma membrane on the other hand.



The next enzyme discussed--thiamine pyrophosphatase--was of interest because histochemical evidence has indicated that it is associated with the Golgi complex in a wide variety of cell types, and in some of them, at least, it appears to be exclusively found in this complex.

A final pair of enzymes--cytochrome oxidase and acid phosphatase--gave us information on the level of contamination of the Golgi fraction by other subcellular components, the mitochondria and the lysosomes.

In the discussion of the enzyme activities associated with the various fractions, we will frequently have cause to distinguish between the Golgi elements and the remainder of the endoplasmic reticulum. In the liver cell, the endoplasmic reticulum, the main source of microsomes, consists of a rough surfaced part and a smooth surfaced part (Palade, 1956). The elements of the latter are typically organized in a tightly meshed three dimensional network associated with glycogen deposits, and in a series of Golgi complexes generally located near bile capillaries (Fawcett, 1955; Palade and Siekevitz, 1956; Bruni and Porter, 1965). All of these parts fragment upon tissue homogenization and contribute to the population of microsomal vesicles (Palade and Siekevitz, 1956) from which we are now trying to isolate the vesicles of Golgi origin. For convenience, and without implying any conclusions as to the functional unity of the system, we will refer to the rough part and to the smooth reticular part of the endoplasmic reticulum, as well as to the microsomes derived therefrom as "true." This designation will facilitate the presentation and the discussion of a comparison of these elements with the Golgi complex and vesicles or microsomes of Golgi origin. Moreover, it is well supported by the final results which show that typical (or "true") microsomal enzymatic activities are absent from Golgi-derived vesicles (see below).



1. Microsomal and plasma membrane enzymes

- a. The Golgi membranes and microsomal enzymes

Direct structural continuity between rough and reticular smooth endoplasmic reticulum is well established (Palade, 1956) and is frequently seen in electron micrographs of liver and other cells in which such a smooth reticulum is present (Palade, 1956; Palade and Siekevitz, 1956; Bruni and Porter, 1965; Jones et al., 1967; Hamilton et al., 1967). Material in the cisternae of the rough endoplasmic reticulum thus has access to the lumen of at least some smooth membrane bounded compartments. The membranes of the rough and smooth reticular endoplasmic reticulum also appear to be qualitatively, if not strictly quantitatively, similar in phospholipid compositions, in amino acid compositions of their protein components, and in concentrations of various enzymes, including glucose-6-phosphatase, ATPase, cytochrome  $b_5$ , cytochrome P-450, NADH-cytochrome c reductase, and NADPH-cytochrome c reductase, and 5'-nucleotidase (Dallner, 1963; Remmer and Merker, 1963; Mangianello and Phillips, 1965; Widnell and Unkeles, 1968).

The relation between the rough endoplasmic reticulum and the other form of smooth endoplasmic reticulum, the Golgi complex, is less clearly understood. Early reported observations of direct structural continuity (e.g., Palade, 1955, 1956) are rare and questionable. However, regions of the rough endoplasmic reticulum and the Golgi complex are, in many cell types, closely related topographically, and the appearances encountered within the corresponding areas are compatible with the assumption that vesicles formed from the rough endoplasmic reticulum migrate to the Golgi regions and become or fuse with Golgi-associated vesicles: The rough endoplasmic reticulum cisternae in the region near the Golgi complex often are smooth along the surface or at their ends facing the complex and hence have been referred to as "transitional" elements. Electron microscopic images are often seen which suggest that the smooth membrane may bleb out and pinch off from



such transitional elements, forming smooth surfaced vesicles. Similar vesicles are seen nearby at the periphery of the Golgi complex. This set of appearances is observed in a variety of cell types, including cells of rat liver (Novikoff and Shin, 1964), rat hepatomas (Essner and Novikoff, 1962), guinea pig exocrine pancreas (Palade, 1959, 1962), goblet cells of rat intestinal epithelium, mouse plasma cells, and albumen secreting cells of the hen oviduct (Zeigel and Dalton, 1962), Brunner's gland in mouse (Friend, 1965), thyroid cells (Wissig, 1964), salivary gland cells in *Sciara* (Phillips and Swift, 1965), cells of the fat body of the butterfly (Locke and Colin, 1965), dragonfly spermatids (Kessel, 1966), and follicle cells of some tunicate oocytes (Kessel, 1967).

As a result of such morphological observations, many authors have postulated that the contents of the endoplasmic reticulum cisternae are transported to the Golgi complex by small vesicles: pieces of endoplasmic reticulum membrane which pinch off, trapping some of the contents of the cisternae inside them, and migrate to the Golgi region where they fuse with or become Golgi vesicles (Palade, 1959). This interpretation is strengthened in some cases by the observation of morphologically identifiable cellular products in the cisternae of the rough endoplasmic reticulum, in the smooth surfaced vesicles close to the rough endoplasmic reticulum, in the smooth surfaced vesicles at the periphery of the Golgi complex, and in other elements of the Golgi complex (for example, the dense particles seen in the liver; for other examples, see Leduc, 1968; Bainton and Farquhar, 1968; Kessel, 1967 inter alia). More direct evidence for transfer of cellular products from rough endoplasmic reticulum to Golgi complex has been derived from autoradiographic studies of pathways of transport of newly synthesized protein in guinea pig pancreas (Caro and Palade, 1964), guinea pig fibroblasts (Ross and Benditt, 1965), rabbit heterophil myelocytes (Fedorko and Hirsch, 1966), and mouse peritoneal macrophages (Cohn et al., 1966). In rat liver, both newly synthesized





protein (Droz, 1966a; Peters and Ashley, 1967) and newly synthesized triglyceride (Stein and Stein, 1966, 1967) have been shown by autoradiography to pass from rough endoplasmic reticulum to the Golgi region.

Although the passage of materials from rough endoplasmic reticulum to Golgi complex within membrane bound compartments has been proved in the pancreatic exocrine cell for secretory protein in general (Jamieson and Palade, 1966) and in the liver cell (for very low density lipoprotein only (Jones et al., 1967; Hamilton et al., 1967), and although similar operations are suggested in many other cases, the fate of the membrane involved in this transport is unknown. One possibility is that when vesicles derived from the rough endoplasmic reticulum move to the Golgi region and become or fuse with Golgi vesicles, their membranes are permanently transferred so that they become an integral part of the membranes of the Golgi complex. Then, unless enzymes in the membranes are activated or inactivated as a result of such transfers, we would expect the membranes of the rough endoplasmic reticulum and the Golgi complex to be more or less similar with respect to their constitutive activities.

Alternatively, the "true" endoplasmic reticulum membranes and the Golgi membranes may remain distinct. Pieces of "true" endoplasmic reticulum membrane or of Golgi membrane might pinch off to form vesicles, migrate to the other system, and fuse with its membranes. Then the same patch of membrane might pinch off again and return to its original station. In this situation, cellular products could be transported from one compartment to the other, but without permanent mixing of the membranes of "true" endoplasmic reticulum and Golgi complex, and without equilibration of their constitutive enzyme activities.

It was thus of interest to determine whether Golgi membranes and "true" endoplasmic reticulum membranes are similar or distinctive with respect to their enzyme activities. This information will impose



restraints upon our interpretation of what actually takes place at the interface between rough endoplasmic reticulum and the Golgi complex. We therefore looked at enzymes which are entirely or primarily associated with microsomal fractions from rat liver cells and hence presumably represent markers of the "true" endoplasmic reticulum (the major component of the total microsomes): glucose-6-phosphatase, which has been classically considered as a "marker" enzyme for microsomal membrane (deDuve et al., 1955); NADPH-cytochrome c reductase and cytochrome P-450, for which there is also evidence for an exclusively microsomal localization (Phillips and Langdon, 1962); and NADH-cytochrome c reductase and cytochrome  $b_5$ , which are present in large amounts in microsomes (Hogeboom and Schneider, 1950b), but which are also present in smaller quantities in the plasma membrane (Emmelot et al., 1964) and in mitochondrial membranes (Ernster et al., 1963; Sottacasa et al., 1967).

#### (1) Glucose-6-phosphatase

Glucose-6-phosphatase has been widely used as a marker for microsomal enzymes (deDuve et al., 1955). Microsomal fractions, however, contain vesicles derived from the Golgi apparatus, as well as vesicles derived from the "true" endoplasmic reticulum (Palade and Siekevitz, 1956). Thus there was no biochemical evidence which would indicate whether the Golgi-derived vesicles could be expected to share in this enzyme activity or not. The electron microscopic histochemistry of Goldfischer et al. (1964), Ericsson (1966) and Saito (1968) indicates that there is no glucose-6-phosphatase activity in the Golgi complex of hepatocytes; reaction product is confined to the "true" endoplasmic reticulum (both rough and smooth). Such histochemical evidence must be accepted cautiously, however, since there are many factors such as penetration of substrate into membrane-enclosed compartments and absolute concentration of enzyme at the time of assay, usually after fixation, which can determine whether an enzyme is demonstrable by histochemical methods or not. Nevertheless, the



histochemical results suggested that Golgi membranes might not resemble the "true" microsomal membranes with respect to glucose-6-phosphatase activity.

As is shown in Table 6, neither the total Golgi fraction nor the Golgi membrane fraction show any detectable glucose-6-phosphatase activity. The figures listed under the Golgi categories in Table 6 represent the minimum specific activity in the fraction which would be detectable, given the sensitivity of the assay and the protein content of the aliquots assayed. The histochemical evidence is thus confirmed.

This evidence demonstrates not only that the Golgi membranes differ from those of the true endoplasmic reticulum with respect to glucose-6-phosphatase activity, but also that the level of contamination of the Golgi fractions by "true" microsomes is extremely low. It should be stressed that, had there been activity in the Golgi fractions, we would have had no way of deciding whether the activity was associated with the vesicles derived from the Golgi apparatus or with "true" microsomal contaminants in the fraction. But in the absence of detectable activity we can conclude, first, that the "true" microsomal membranes and the Golgi membranes are different: that glucose-6-phosphatase is exclusively found in the "true" microsomes; and second, that the total Golgi fraction and the Golgi membrane fraction contain no detectable contamination from "true" microsomes. If the Golgi fractions contained as much as 4% non-Golgi derived microsomal vesicles, we could detect glucose-6-phosphatase activity in them. We cannot; hence the contamination is less than this. The finding is consistent with the conclusions reached from the electron microscopic examination of the total Golgi fraction. In particular, it supports our earlier contention that those non-Golgi derived vesicles which contained lipoprotein granules must represent a very small part of the total Golgi fraction (see page 55).

## (2) NADPH-cytochrome c reductase and cytochrome P-450

Liver microsomes contain two electron transport chains, one



TABLE 6  
Glucose-6-Phosphatase Activity of Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fractions"	1.69	0.07*	1.63
	(34.5)**	( 0.03)	(34.9)
"Membrane Fractions"	3.53	0.13*	3.46
	(37.2)	( 0.025)	(37.3)
"Contents Fractions"	0.20	0.03*	0.22
	(0.63)	( 0.01)	(0.33)

\*Note: see text, page 89, for explanation of upper limits.

\*\*Note: activity of total homogenate is 66.3  $\mu$ moles  $P_i$ /20 min/gram liver

Upper figures are  $\mu$ moles  $P_i$ /20 min/mg protein; lower, bracketed figures are  $\mu$ moles  $P_i$ /20 min/gram liver.

Figures are averages of 3 experiments.

Ranges: total microsomal fraction: 1.53-1.75  $\mu$ moles  $P_i$ /20 min/mg protein  
microsomal membrane fraction: 3.08-4.13  
total Golgi fraction: 0.03- 0.09  
Golgi membrane fraction: 0.03- 0.23

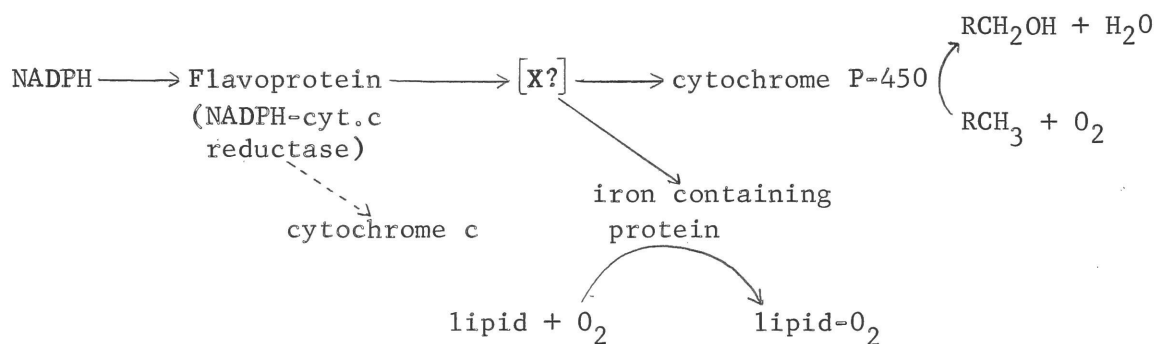
Figures from literature for comparison: ( $\mu$ moles  $P_i$ /20 min/mg protein)  
total microsomal fraction: 0.88 (Hogeboom and Schneider, 1950b)  
2.5-4.5 (Ernster et al., 1962)  
2.17-3.72 (Dallner, 1963)  
3.00-3.40 (Orrenius et al., 1965)

plasma membrane fractions: 0.48 (Emmelot et al., 1964)  
0.24-0.27 (Dod and Gray, 1968)





utilizing NADH as electron donor and the other utilizing NADPH. A cytochrome electron acceptor has been identified in each chain-- cytochrome  $b_5$  in the first and cytochrome P-450 in the second. The NADPH-chain, which we will discuss in this section, is involved in the hydroxylation of steroids (Mueller and Rumney, 1957; King, 1961; Conney and Klutch, 1963 *inter alia*), fatty acid, fatty alcohol, and hydrocarbon oxidations (Imai and Sato, 1959; Marsh and James, 1962; Hochstein and Ernster, 1963; Tietz *et al.*, 1963, *inter alia*), and drug detoxification (Conney *et al.*, 1957; Pettit and Zeigler, 1963, *inter alia*). (reviewed in Mason *et al.*, 1965). The proposed electron transport pathway is



(Omura *et al.*, 1965; Mason *et al.*, 1965; Ernster and Orrenius, 1965). The enzyme system is exclusively microsomal. There is no equivalent mitochondrial system (Phillips and Langdon, 1962). Plasma membrane fractions also contain no cytochrome P-450 (Benedetti and Emmelot, 1968).

We can make further inferences about the localization of NADPH-cytochrome c reductase and cytochrome P-450 from the response of animals to a variety of drugs (Ernster and Orrenius, 1965; Conney and Burns, 1959). If a rat is given daily doses of phenobarbital, for instance, the NADPH-cytochrome c reductase and cytochrome P-450 levels in the liver rise dramatically. The increase appears first in rough microsomes and then in smooth (Orrenius, 1965a). The enzymes apparently accumulate in the smooth, which eventually shows a considerably greater increase in activity than does the rough (Orrenius *et al.*, 1965). The



amounts of rough and smooth endoplasmic reticulum also increase, as shown by electron microscopy and by protein and lipid yields in the rough and smooth microsomal fractions. As with the enzymes, after the first day of treatment, the greater increase is in the smooth fraction (Remmer and Merker, 1962; Orrénus et al., 1965). The increase in the amount of enzymes is thus associated with proliferation of the membrane systems with which the enzyme is associated.

The Golgi complex in hepatocytes of phenobarbital treated rats does not increase in size, unlike the rest of the smooth endoplasmic reticulum (Jones and Fawcett, 1966). Indeed, it appears to become smaller absolutely as well as relatively (Palade, unpublished). This suggests that the Golgi complex does not participate in the NADPH-cytochrome c reductase electron transport system in which phenobarbital induces and increase.

As Table 7 shows, there is no detectable NADPH-cytochrome c reductase activity in the Golgi fractions. The figures listed in Table 7 under the Golgi categories represent the lower limits of detectability of the enzyme for the amounts of protein assayed.

Table 8 shows the content of cytochrome P-450 of the various fractions. The assay for cytochrome P-450 is relatively insensitive, as the specific extinction coefficient of the cytochrome is quite low. Within the limits of detectability, however, the total Golgi fraction and the Golgi membrane fraction have no cytochrome P-450. Although the limits of detectability in this case create a serious limitation on the meaningfulness of the data, the results are nevertheless consistent with the NADPH-cytochrome c reductase results, and consistent with the conclusion that the Golgi fraction does not share in this electron transport system with the "true" microsomes.

The results of the assays for components of the NADPH-cytochrome c reductase - cytochrome P-450 electron transport system thus agree with the glucose-6-phosphatase results. Since the enzymes are present in the



TABLE 7

NADPH - Cytochrome c Reductase Activity of Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fraction"	0.39 (6.3)	< 0.02* ( < 0.0008)	0.40 (6.1)
"Membrane Fraction"	0.48 (4.8)	< 0.10* ( < 0.0010)	0.48 (5.4)

\*Note: See text, page 92 for explanation of upper limits.

Upper figures are  $\Delta OD_{550m\mu}$  /min/mg protein; lower, bracketed figures are  $\Delta OD_{550m\mu}$  /min/gram liver.

Figures are averages of 4 experiments.

Ranges: total microsomal fraction: 0.33-0.45 ( $\Delta OD$ /min/mg protein)  
microsomal membrane fraction: 0.38-0.51  
total Golgi fraction: < 0.01- < 0.05  
Golgi membrane fraction: < 0.04- < 0.17

Figures from literature for comparison:

total microsomal fraction: 0.28-0.51 ( $\Delta OD$ /min/mg protein) (Ernster et al., 1962)  
0.073  $\mu$  moles cyt.c reduced/min/mg protein (Phillips and Langdon, 1962)  
0.02-0.03 " (Dallner, 1963)  
0.019-0.026 " (Orrenius et al., 1965)  
(Our figure in chart above is equivalent to 0.021  $\mu$  mole/min/mg protein)



TABLE 8  
Cytochrome P-450 in Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fraction"	0.031 (0.37)	< 0.005* ( < 0.0001)	0.014 (0.24)
"Membrane Fraction"	0.051 (0.41)	< 0.010* ( < 0.0002)	-

\*Note: See text, page 92, for explanation of upper limits.

Upper figures are  $OD_{450m\mu} - OD_{490m\mu}$  /mg protein; lower, bracketed figures are  $OD_{450m\mu} - OD_{490m\mu}$  /gram liver.

Figures are averages of 3 experiments.

Ranges: total microsomal fraction: 0.014-0.045 ( $OD_{450m\mu} - OD_{490m\mu}$  /mg protein)  
microsomal membrane fraction: 0.031-0.068  
total Golgi fraction: <0.003- <0.006  
Golgi membrane fraction: <0.010- <0.026

Figures from literature for comparison: ( $OD_{450m\mu} - OD_{490m\mu}$  /mg protein)  
total microsomal fraction: 0.024-0.033 (Dallner, 1963)  
0.023 (Mason et al., 1965)  
0.019-0.025 (Orrenius et al., 1965)  
0.023 (Smuckler et al., 1967)

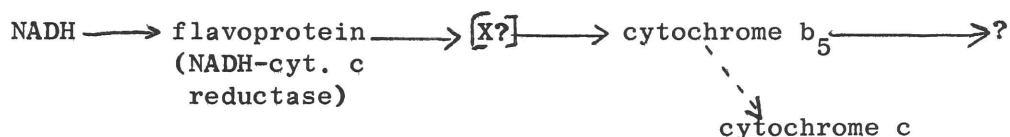




total microsomal membranes but are not present in the Golgi membranes, Golgi membranes differ from "true" microsomal membranes. Moreover, there are few if any "true" microsomal membranes present as contaminants in the Golgi fraction.

### (3) NADH-cytochrome c reductase and cytochrome b<sub>5</sub>

Liver microsomes contain another electron transport system, in which NADH rather than NADPH is the electron donor (Hogeboom, 1949). Cytochrome b<sub>5</sub> serves as an electron acceptor in this system. The proposed complete electron pathway is



(Strittmater, 1965).

This enzyme is a less satisfactory marker for microsomes than glucose-6-phosphatase and NADPH-cytochrome c reductase. Mitochondria contain at least two NADH-cytochrome c reductases, one in the inner and one in the outer membrane. These can be distinguished from the microsomal on the basis of sensitivity of the inner membrane enzyme to inhibitors such as rotenone (Ernster et al., 1963; Sottacasa et al., 1967) and sensitivity of the outer membrane enzyme to dicoumarol and sulfhydryl reagents (Sottacasa et al., 1967).

More recently, a NADH-cytochrome c reductase has been reported to be present in rat liver plasma membrane preparations with a specific activity about one-fifth that of the microsomes (Emmelot et al., 1964). It is not clear whether this activity should be considered as associated with the plasma membrane since the same preparations have about one-fifth the glucose-6-phosphatase activity of microsomes. The reductase activity thus may be simply microsomal contamination, although Emmelot et al. presents arguments against this possibility.

Despite the non-exclusivity of the microsomal localization, since



the enzyme does occur in high concentration in microsomes, it was of interest to see whether the Golgi fraction contained this activity. As is shown in Table 9, the total Golgi fraction has one-fifth the activity of the total microsomal fraction. The Golgi membrane fraction has one-eighth the activity of the microsomal membrane fraction. Thus, the Golgi fractions appear to resemble plasma membrane rather than microsomes in their NADH-cytochrome c reductase activity.

Caution is needed in accepting these results, however. The actual results in individual experiments varied considerably, with the specific activity of the total Golgi fraction ranging from one one-hundredth to one-third of the specific activity of the total microsomal fraction. NADH-cytochrome c reductase is well known to be an extremely labile enzyme (Ernster et al., 1962), whose measured activity in microsomal fractions varies with concentration, age, and probably other factors. Since the fractions we have obtained have showed such variable activities, it seemed advisable to obtain a check on their validity by examining the second component of the electron transport chain of which NADH-cytochrome c reductase is a part.

We therefore assayed cytochrome  $b_5$ . As is shown in Table 10, the total Golgi fraction contains about one-eighth as much cytochrome  $b_5$  per unit protein as does the total microsomal fraction, while the Golgi membrane fraction contains one-sixth as much as the microsomal membrane fraction. These results were quite consistent from experiment to experiment, and they are quite consistent with the averages of the NADH-cytochrome c reductase results.

Again, however, a word of caution is needed. Cytochrome  $b_5$  is relatively loosely bound to the membranes of microsomes (Dallner, 1963), and it is readily removed from them by chemical and mechanical means. As the recovery figures in Table 10 reveal, a large part of the cytochrome is lost from the membrane during the disruption used to free the membrane of its contents. Hence, the figures for the total fractions



TABLE 9

NADH - Cytochrome c Reductase Activity of Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fraction"	2.67 (44.8)	0.53 (0.04)	2.53 (42.3)
"Membrane Fraction"	3.55 (43.8)	0.45 (0.01)	2.58 (39.7)

Upper figures are  $\Delta OD_{550m\mu}$  /min/mg protein. Lower, bracketed figures are  $\Delta OD_{550m\mu}$  /min/gram liver.

Figures are averages of 4 experiments.

Ranges: total microsomal fraction: 2.26-3.08 ( $\Delta OD_{550m\mu}$  /min/mg protein)  
microsomal membrane fraction: 3.36-4.31  
total Golgi fraction: 0.16-1.16  
Golgi membrane fraction: 0.00-1.36

Figures from literature for comparison:

total microsomal fraction: 3.8-13.5 ( $\Delta OD_{550m\mu}$  /min/mg protein) (Ernster  
et al., 1962)

0.41  $\mu$ moles cyt.c reduced/min/mg protein  
(deDuve et al., 1955)

0.91-1.47 " (Dallner, 1963)

0.62-0.80 " (Orrenius et al., 1965)

(Our figure in chart above is equivalent  
to 0.15  $\mu$ mole cyt c. reduced/min/mg protein)



TABLE 10

Cytochrome B5 in Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fractions"	0.197 (2.76)	0.026 (0.0007)	0.157 (2.16)
"Membrane Fractions"	0.099 (0.79)	0.017 (0.0002)	0.089 (0.70)
"Contents Fractions"	0.650 (2.08)	--	--

Upper figures are  $OD_{424m\mu} - OD_{410m\mu}/mg$  protein; lower, bracketed figures are  $OD_{424m\mu} - OD_{410m\mu}/gram$  liver.

Figures are averages of 3 experiments.

Ranges: total microsomal fraction: 0.180-0.207 ( $OD_{424m\mu} - OD_{410m\mu}/mg$  protein)  
microsomal membrane fraction: 0.084-0.109  
total Golgi fraction: 0.022-0.029  
Golgi membrane fraction: 0.012-0.026

Figures from literature for comparison: ( $OD_{424m\mu} - OD_{410m\mu}/mg$  protein)  
total microsomal fraction: 0.03-0.07 (Dallner, 1963)  
0.08-0.11 (Orrenius et al., 1965)  
0.038 (Smuckler et al., 1967)





are probably more reliable than those for the membrane fractions, although the former may be quantitatively distorted by the unequal contents of the different vesicle populations.

The NADH-cytochrome c reductase activity and the cytochrome  $b_5$  amount cited here cannot be ascribed to mitochondrial contamination of the Golgi fraction. As will be shown later, the level of mitochondrial contamination is extremely low.

Both the NADH-cytochrome c reductase results and the cytochrome  $b_5$  results are thus open to challenge in so far as any rigorous quantitative conclusions are concerned. But they give a similar and reproducible qualitative picture, which suggests that the Golgi membranes resemble the plasma membrane rather than the "true" microsomal membranes with respect to the electron transport pathway in which NADH-cytochrome c reductase and cytochrome  $b_5$  participate.

We have examined three different enzyme systems present in high activities in microsomal fractions of rat liver. Two of these--glucose-6-phosphatase and NADPH-cytochrome c reductase -cytochrome P-450 electron transport chain--prove to be absent from the Golgi membranes, to the limits of detection of the assay procedures. The third--the NADH-cytochrome c reductase - cytochrome  $b_5$  electron transport chain, is present in the Golgi membranes, but at a concentration much lower than in the "true" microsomes.

We shall return to a discussion of these comparative activities in Chapter 5.

#### b. The Golgi membranes and plasma membrane enzymes

Just as the Golgi membranes appear to be related to the membranes of the rest of the endoplasmic reticulum, so they appear to interact with the plasma membrane in a wide variety of cell types.

In the cells of the exocrine pancreas, newly synthesized enzymes are transferred to the condensing vacuoles of the Golgi complex (Caro and



Palade, 1964). These mature into zymogen granules which move to the apical region of the cell. The membrane of the zymogen granule then fuses with the plasma membrane on the luminal aspect, thus discharging the contents of the zymogen granule into the acinar lumen (Palade, 1959). Thus membrane which was originally located in the Golgi complex becomes a part of the plasma membrane. A similar process has been proposed, on the basis of morphology, cell fractionation experiments, or autoradiography in various other secretory cells, including those of the mammary gland (Wellings and Deome, 1961), the submucosal gland of Brunner in the mouse (Friend, 1965), the anterior pituitary (Farquhar, 1961) and the parotid (Amsterdam et al., 1969). In the liver, images suggesting such a process for secretion of lipoprotein have been recorded (Hamilton et al., 1967; Jones et al., 1967).

In dividing root tip cells of maize, the Golgi complex appears to be the source of small vesicles which fuse to form the cell plate. The membranes themselves become the new cell membranes; the contents of the vesicles may be incorporated into the new cell wall (Porter and Caulfield, 1958; Whaley and Mollenhauer, 1963). In later stages of root tip development, large vesicles form from the Golgi complex, move to the plasma membrane, and discharge their contents by fusing with it (Mollenhauer et al., 1961; Northcote and Pickett-Heaps, 1966).

Various investigators have interpreted electron micrographs of various cells as showing that conversely, the plasma membrane may invaginate and pinch off, forming vesicles which then move to the Golgi region and become part of the Golgi complex (e.g., Daniels, 1964, for amoeba). There is little experimental evidence for this. Loni et al. (1967) have shown that ferritin placed in the medium of ATP-stimulated HeLa cells appears after some time in Golgi vesicles. Amsterdam et al. (1969) have shown that when rat parotid glands are caused to discharge rapidly all of their zymogen granules by injection of isoprenaline, the acinar lumen enlarges greatly. The return of the lumen to normal size is correlated with the appearance of small, smooth surfaced vesicles in



the apical region of the cell. Such small vesicles may possibly be incorporated into new condensing vacuoles, but there is no direct evidence for this assumption. In liver cells, various authors have interpreted the direction of movement of the dense-particle filled vesicles as being from cell membrane inward to Golgi complex, but, as we have discussed above (Chapter 2), the weight of evidence is against this interpretation.

There is thus evidence of possible transfers of membrane between the Golgi complex and the plasma membrane in a number of cells, including those of the liver. We have already noted that the Golgi membranes appear to be similar to the plasma membrane with respect to their content of NADH-cytochrome c reductase and cytochrome  $b_5$ , each membrane system having about one-fifth to one-tenth the specific activity of the total microsomal fraction. The plasma membrane and the Golgi membranes also share the negative characteristic of not containing detectable NADPH-cytochrome c reductase or cytochrome P-450, which are markers of "true" microsomal membranes. It was of interest to examine an enzyme which is known to be primarily localized in the plasma membrane to give us more information on the relationship between Golgi membranes and plasma membranes.

#### (1) 5'-Nucleotidase (AMPase)

Preparations of plasma membranes from rat liver contain a highly active AMPase (Emmelot et al., 1964). The plasma membrane specific activity is 5 to 10 times higher than that found in microsomes (Emmelot et al., 1964; Widnell and Unkeles, 1968; Stein et al., 1968). Histochemical evidence suggests that the plasma membrane is the only site of the enzyme in the liver cell (Wachstein and Meisel, 1957; Essner et al., 1958; El Aaser et al., 1966). Novikoff and Goldfischer (1961) report that there is no AMPase activity demonstrable in the Golgi complex of the hepatocyte.

The hypothesis that AMPase is exclusively a plasma membrane



enzyme has won widespread acceptance in spite of the fact that rough and smooth microsomal fractions invariably contain a significant amount (more than half) of the enzyme activity (Widnell and Unkeles, 1968). It has been argued that the activity found in the microsomal fractions represents plasma membrane contamination, and the argument is hard to disprove. Indeed, the specific activity of the plasma membrane is much higher than that of the microsomal fractions, so that only a small amount of contamination of the latter by the former would suffice to account for the findings. Plasma membrane can presumably pinch off to form vesicles which would sediment with the microsomes and which would be morphologically indistinguishable from smooth microsomal vesicles. But the inconclusiveness of the dispute remains, and given the imprecision of the histochemical evidence, the exclusiveness of the plasma membrane localization must be considered unproven.

As is shown in Table 11, the specific activities in the total Golgi fraction and in the Golgi membrane fraction are very similar to those in the corresponding microsomal fractions. In all experiments done, the Golgi fraction values were 15-30% less than the microsomal fraction values.

The most reasonable explanation of these results is that the greater part of the Golgi fraction and microsomal fraction activities are real (that is, not accountable for by plasma membrane contamination). First, from the ratio of the activity in our Golgi fractions to those reported for plasma membranes, if all of the activity in our fractions were due to contamination, then almost 10% of the Golgi fractions would have to be plasma membranes. This much contamination should be morphologically detectable. But there are neither extended sheets of membrane nor smooth vesicles not containing lipoprotein particles in the total Golgi fraction in anywhere near this concentration.

It is possible that vesicles derived from the plasma membrane move to the Golgi complex and fuse with the Golgi membranes (see below,





TABLE 11  
5'-Nucleotidase Activity of Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fractions"	2.15 (31.6)*	1.59 (0.097)	2.17 (32.8)
"Membrane Fractions"	2.23 (23.9)	1.93 (0.043)	2.17 (22.6)
"Contents Fractions"	0.97 (2.9)	1.70 (0.050)	0.90 (2.4)

\*Note: Activity of the total homogenate is 194  $\mu$ mole  $P_i$ /20 min/gram liver.  
178  $\mu$ moles  $P_i$ /20 min/gram liver is recovered in the combined  
nuclear-mitochondrial pellet.

Upper figures are  $\mu$ moles  $P_i$ /20 min/mg protein; lower, bracketed figures  
are  $\mu$ moles  $P_i$ /20 min/gram liver.

Figures are averages of 4 experiments.

Ranges: total microsomal fraction: 1.75-2.49  $\mu$ moles  $P_i$ /20 min/mg protein  
microsomal membrane fraction: 1.73-2.60  
total Golgi fraction: 1.23-2.07  
Golgi membrane fraction: 1.54-2.27

Figures from literature for comparison: ( $\mu$ moles  $P_i$ /20 min/mg protein)  
total microsomal fraction: 1.84 (Widnell and Unkeles, 1968)  
2.8 (Stein et al., 1968)

plasma membrane fractions: 9.2 (Widnell and Unkeles, 1968)  
20.4 (Stein et al., 1968)  
10.7 (Emmelot et al., 1964)  
4.5-14.9 (Dod and Gray, 1968)



page 128). If this occurs, no plasma membrane "contamination" would be observed morphologically, since the plasma membrane fragments would be at least temporarily integrated into the Golgi membranes. It would be, then, however, a matter of semantics whether we call the resulting AMPase activity "Golgi activity" or "plasma membrane contamination"-- in either case, the enzyme activity would be found in Golgi associated membranes.

Second, if the entire microsomal activity were accounted for by plasma membrane contamination, it would be rather surprising if, upon dividing the microsomes into Golgi and remainder fractions, the plasma membrane contaminants were to partition so nearly proportionally between the two fractions. As already shown, the Golgi fraction consists of 0.3% of the total microsomal fraction and has a density between 1.034 (the density of 0.25M sucrose) and 1.081 (the density of 0.60M sucrose) (see Table 1). To explain the results obtained, it would be necessary to postulate that almost exactly 0.3% of the plasma membrane contaminants of the total microsomal fraction also have densities between 1.034 and 1.081. In fact, the membranes of the plasma membrane fractions isolated from rat liver have densities of 1.16 to 1.18 in sucrose solutions (Emmelot et al., 1964). Hence, when we float the Golgi vesicles free of the microsomes, we would expect to decrease the plasma membrane contamination relative to that of the microsomes.

Just such a phenomenon may account for the difference in the AMPase activity between the Golgi fractions and the microsomal fractions. A part, at least, of the microsomal activity may be plasma membrane contamination. In isolating the Golgi fraction, on the basis of density, we may be eliminating much of this contamination.

Although the easiest explanation of the observed activities is that the Golgi fractions and the microsomal fractions have AMPase associated with them, the possibility of at least some amount of plasma membrane contamination makes it hard to draw rigorous quantitative



conclusions. In any case, however, it is clear that the Golgi membranes seem to resemble the microsomal membranes rather than the plasma membrane with respect to AMPase activity.

The Golgi membranes thus contain three of the enzymes which appear in the plasma membrane--AMPase, NADH-cytochrome c reductase, and cytochrome  $b_5$ . Their low level of AMPase, the one of the enzymes which is primarily associated with the plasma membrane, does not suggest a very close relationship with the plasma membrane, however.

## 2. Thiamine pyrophosphatase

Thiamine pyrophosphatase, an enzyme which inactivates the coenzyme thiamine pyrophosphate (cocarboxylase) has been identified histochemically as occurring in the Golgi apparatus in a wide variety of cell types (Novikoff and Goldfischer, 1961). The function of the enzyme in the living cell is unknown (but see the discussion by Novikoff et al., 1962). The enzyme is so universally observed in the Golgi complex, however, that its histochemical reaction has been widely used as a marker for the Golgi area and a means of demonstrating the Golgi complex, replacing the classical metal impregnation methods (Novikoff et al., 1962; Baker, 1963; Goldfischer et al., 1964).

In many types of cell, thiamine pyrophosphatase appears, by histochemical procedures, to be exclusively a Golgi enzyme; no reaction product is found in other membranes. The rat liver enzyme is less restricted in localization by these methods, however. Goldfischer et al., (1964) and Saito (1968a) have demonstrated the usual Golgi localization in hepatocytes, but have noted a less dense reaction product in the endoplasmic reticulum as well. Thus, we expected to find thiamine pyrophosphatase activity concentrated in the Golgi fraction, but not necessarily restricted to it.

Table 12 indicates that the total Golgi fraction shows a seven-fold increase in thiamine pyrophosphatase activity over the total micro-



TABLE 12Thiamine Pyrophosphatase in Fractions

	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fraction"	0.041	0.28	0.042
	(0.55)*	(0.010)	(0.61)
"Membrane Fraction"	0.067	0.62	0.064
	(0.63)	(0.009)	(0.68)

\*Note: Activity of total homogenate is 2.17  $\mu\text{moles P}_i/40 \text{ min/gram liver}$  or  
0.013  $\mu\text{moles P}_i/40 \text{ min/mg protein}$ .

Upper figures are  $\mu\text{moles P}_i/40 \text{ min/mg protein}$ ; lower, bracketed figures  
are  $\mu\text{moles P}_i/40 \text{ min/gram liver}$ .

Figures are averages of 3 experiments.

Ranges: total microsomal fraction: 0.035-0.042  $\mu\text{moles P}_i/40 \text{ min/mg protein}$   
microsomal membrane fraction: 0.054-0.076  
total Golgi fraction: 0.260-0.291  
Golgi membrane fraction: 0.514-0.764





somal fraction, and a twenty-five fold increase over the total homogenate. Removing the contents from the Golgi fraction results in a further concentration of activity, to ten times that of the microsomal membrane fraction and almost sixty times that of the homogenate.

It is impossible to determine conclusively from the data whether the thiamine pyrophosphatase is exclusively a Golgi enzyme or whether there is activity in the microsomes as well. The total Golgi fraction contains only 2% of the total thiamine pyrophosphatase activity of the microsomal starting material. We have estimated that our total Golgi fraction comprises of the order of 5% of the Golgi-derived vesicles contained in the total microsomes (see page 75). This very crude estimate would imply that about 40% of the thiamine pyrophosphatase of the microsomal fraction is associated with Golgi membranes. The rest would then be associated with microsomal membranes derived from sources other than the Golgi complex.

The histochemical evidence in the liver cell is consistent with this conclusion, but due to the uncertainty of the estimate of the proportion of the Golgi material we are recovering, the only firm conclusion we can make is that thiamine pyrophosphatase is indeed concentrated in the Golgi fraction.

### 3. Contamination of Golgi fractions by other subcellular particles

On the basis of the results obtained above for glucose-6-phosphatase and NADPH-cytochrome c reductase, it is clear that there is very little contamination of the Golgi fractions by "true" microsomes. The morphology of the fraction together with its enzymatic activities indicate that there is also little contamination by plasma membrane fragments. It remains to be shown that there is little contamination by the remaining major candidates, mitochondria and lysosomes.

#### a. Mitochondria: Cytochrome Oxidase

From the morphology of the Golgi fractions, there was no reason



to think there was any significant mitochondrial contamination. No mitochondria and no images suggestive of modified mitochondria were seen. A good marker for mitochondria exists, so we could check this point biochemically.

Cytochrome oxidase is believed to be exclusively a mitochondrial enzyme (Hogeboom et al., 1948). (Strictly it is a marker of the mitochondrial inner membrane; Sottacasa, 1967). Its presence in non-mitochondrial fractions has been used as an indicator of contamination of the fractions by mitochondria (e.g., Applemans et al., 1955). It remains theoretically possible, however, that at least part of the low cytochrome oxidase activity commonly found in microsomal fractions is associated with one or another sub-category of microsomal membranes.

As Table 13 shows, the cytochrome oxidase activity of the total Golgi fraction is less than 0.5% of that of our total purified mitochondrial fraction. (Mitochondrial fractions have been prepared with 4 times the specific activity of our fraction; 0.5% contamination is thus a high estimate (Sottacasa et al., 1967; Schnaitman et al., 1967)). Using the same reasoning as for glucose-6-phosphatase, we can conclude that the mitochondrial contamination of the Golgi fractions is less than 0.5%. This is consistent with the morphological evidence.

b. Lysosomes: Acid Phosphatase

The total Golgi fraction, when examined in the electron microscope, contains a number of vesicles with a dense, somewhat heterogeneous content which is quite unlike the electron opaque lipoprotein particles (see Plate 6). On morphological grounds we believe these vesicles to be lysosomes: they resemble in size and contents the vesicles of purified lysosomal fractions and those identified as lysosomes in tissue sections of liver (Novikoff et al., 1956). To test the degree of lysosomal contamination, we assayed the Golgi fractions for acid phosphatase.



TABLE 13Cytochrome Oxidase Activity of Fractions

	Mitochondrial Fractions	Microsomal Fractions	Golgi Fractions	Remainder Fractions
"Total Fraction"	5.45	0.08	0.025	0.06
	( - )	(1.42)	(0.003)	(1.64)
"Membrane Fraction"	--	0.17	0.095	0.13
		(1.39)	(0.005)	(1.42)

Upper figures are  $OD_{550m\mu}/\text{min}/\text{mg}$  protein; lower, bracketed figures are  $OD_{550m\mu}/\text{min}/\text{gram}$  liver.

Figures are averages of 5 experiments, except mitochondrial figure is average of 2 experiments.

Ranges: mitochondrial fraction: 5.0-5.9 (  $OD_{550m\mu}/\text{min}/\text{mg}$  protein)  
total microsomal fraction: 0.06-0.12  
microsomal membrane fraction: 0.12-0.27  
total Golgi fraction: 0.003-0.05  
Golgi membrane fraction: 0.03-0.20

Figures from literature for comparison: 0.742  $\mu\text{moles}$  cyt. c oxidized/min/mg protein  
(Sottacasa et al., 1967)  
1.23 " (Schnaitman et al., 1967)  
0.47 " (Stein et al., 1968)  
(Our figure is equivalent to 0.30  $\mu\text{moles}/\text{min}/\text{mg}$  protein.)



There is a problem in using acid phosphatase activity as a marker to distinguish lysosomes from Golgi vesicles, however, since there is reason to assume that the Golgi complex itself, in many cell types, may contain some acid phosphatase (Novikoff, 1963). Novikoff has shown that in liver and in several other cell types, some Golgi vesicles, though not all, contain histochemically demonstrable acid phosphatase activity. He has hypothesized that one route for packaging of acid hydrolases as lysosomes may involve the Golgi complex, in a manner quite analagous to the Golgi involvement in processing of proteins for export in glandular cells such as those of the exocrine pancreas. Hydrolytic enzymes synthesized in the rough endoplasmic reticulum may, according to this hypothesis, pass into the Golgi complex where they are further processed and packaged to form primary lysosomes or their immediate precursors. If this hypothesis applies, then at any given time a certain amount of acid phosphatase would be found in the Golgi vesicles themselves, although their specific activity for acid phosphatase might be far less than that characteristic of mature lysosomes. Thus, acid phosphatase activity in the Golgi fractions indicates only an upper limit on lysosomal contamination. At least part of any activity we find may be Golgi-associated rather than associated with contaminant mature lysosomes.

As is shown in Table 14, the total Golgi fraction releases  $0.53 \mu\text{moles P}_i/10 \text{ min/mg protein}$  from sodium  $\beta$ -glycerophosphate at pH 5.0. In order to calculate the amount of lysosomal contamination which would result in this much activity, we need to know the activity of purified lysosomes. Rather than repeat the elaborate purification schemes others have developed, we decided to compare the activity of the Golgi fraction with published values for purified lysosomal fractions, after first establishing the validity of such a comparison.

We prepared a partially purified lysosomal fraction by a slight modification of the procedure of Sawant et al. (1964). (Our





TABLE 14Acid Phosphatase Activity of Fractions

Lysosomal Fraction	Total Microsomal Fraction	Total Golgi Fraction	Total Remainder Fraction
16.50*	0.33	0.53	0.35
	(4.58)	(0.024)	(5.35)

\*Note: Lysosomal fraction figure is recalculated from Leighton et al. (1968).  
See text, page 110, for justification of this comparison).

Upper figure is  $\mu\text{moles } P_i / 10 \text{ min/mg protein}$ ; lower, bracketed figure is  
 $\mu\text{moles } P_i / 10 \text{ min/gram liver}$ .

Figures are averages of 3 experiments, except microsomal figure is average  
of 6 experiments.

Ranges: total microsomal fraction:  $0.27\text{--}0.43 \mu\text{moles } P_i / 10 \text{ min/mg protein}$   
total Golgi fraction:  $0.45\text{--}0.61$



final fraction was approximately equivalent to Sawant's "F II pellet", which is essentially de Duve et al.'s (1955) light mitochondrial fraction). The specific activity of our preparation was  $3.45 \mu\text{mole } P_i / 10 \text{ min/mg}$  protein. This agrees well with the activity reported by Sawant et al. for their F II fraction, and is about seven times the activity of our Golgi fraction.

Sawant et al. (1964) reported on a further purification of the lysosomal fraction, resulting in a fourfold increase in specific activity. Leighton et al. (1968) have also reported on fractions which were purified beyond the level of the original light mitochondrial fraction and which have even higher activities. Since we are using the same assay system as Sawant et al. and Leighton et al., and since we can readily reproduce the acid phosphatase activities in fractions comparable to their relatively impure fractions, we feel justified in comparing the acid phosphatase activity of our Golgi fraction to the value these authors have reported for highly purified lysosomal preparations.

We can calculate from the data in Leighton et al. (1968) that their best fraction has an acid phosphatase activity of  $16.5 \mu\text{moles } P_i / 10 \text{ min/mg}$  protein. Sawant et al.'s (1964) most highly purified fraction has a specific activity of  $55 \mu\text{moles } P_i / 10 \text{ min per mg nitrogen}$  at pH 6.8, which is equivalent to  $8.8 \mu\text{moles } P_i / 10 \text{ min/mg}$  protein. Sawant reports that at pH 5.0, the activity is twice as high as at pH 6.8, so these figures agree well with Leighton's.

Comparing these activities with that of our purified Golgi fraction, we estimate that the lysosomal contamination of the Golgi fraction must be less than about 3%. The electron microscopic examination of the fraction suggested an even lower level of contamination--less than 1% of the vesicles appear morphologically to be lysosomes. The discrepancy can probably be ascribed to acid phosphatase activity associated with the Golgi fraction itself.



## CHAPTER 5

### RELATIONS BETWEEN CELLULAR MEMBRANE SYSTEMS

In the preceding chapter, we have compared the Golgi membranes to "true" microsomal and plasma membranes with respect to several enzymes. In this chapter we will consider what these comparative activities, along with other information from the literature, imply for the functional relationships among the various membrane systems.

#### A. Membrane Transfer Associated With Transport of Cell Products

We have argued above that there is persuasive evidence that the "true" endoplasmic reticulum membranes, the Golgi complex, and the plasma membrane are functionally related in many, and possibly all, cell types. The contents of the "true" endoplasmic reticulum are transferred to the Golgi complex and thence to the cell surface in an operation which is probably accomplished through a system of vesicular shuttles: vesicles pinch off from the endoplasmic reticulum, trapping within some of the contents of its cisternae, and move to and become part of the Golgi complex. Then Golgi-derived vesicles or vacuoles, loaded with the same cellular products (possibly further processed while in the Golgi complex), move to the plasma membrane and fuse with it, discharging their load. Thus the movement of cell products from "true" endoplasmic reticulum to Golgi complex to plasma membrane is accompanied by transfers of membrane from one sub-cellular compartment to another. Electron micrographic images, strongly suggestive of just such membrane transfers, are seen in a variety of cell types including liver. Static images suggesting vesicles shuttling from "true" endoplasmic reticulum to Golgi complex are, of course, identical to the images we would see if the vesicles were moving in the opposite direction, and such reverse transfers of membrane may very well occur. Speculations on other interchanges of membrane (between rough endoplasmic reticulum and plasma membrane, for example) are less firmly based on evidence.



It is apparent that in the living cell, something approaching a steady state is maintained under normal conditions with respect to these presumed membrane exchanges. Membrane does not pile up in the Golgi complex or at the plasma membrane, nor do any of the membrane systems continually diminish in size. Membranes must, then, be removed from each of the three membrane systems at a rate more or less equal to the rate at which it is added to them. The same should apply to the addition of membranes to the sites that initially lose them. Any model of membrane interrelations in the living cell must meet the constraint of such a dynamic equilibrium. This equilibrium need only hold over the long run, of course. For example, Palade (unpublished) has shown that if cells of the exocrine pancreas are stimulated to discharge suddenly and massively the contents of the zymogen granules into the acinar lumen, the surface area of the membrane lining the lumen enlarges greatly. This is, of course, the result of the fusion of large amounts of zymogen granule membrane with the plasma membrane facing on the lumen. The cell's complement of zymogen granules is correspondingly diminished. Within two hours, however, the lumen has returned to normal size: the excess plasma membrane of the luminal region has been eliminated. By a few hours later, the population of condensing vacuoles and zymogen granules is reestablished. Amsterdam et al. (1969) have demonstrated a similar sequence in rat parotid gland after discharge of amylase into the acinar lumen.

The time period involved in transfer of membrane in association with transport of products is quite short. Autoradiographic and cell fractionation experiments studying the transfer of labelled content from one subcellular compartment to another indicate that the time needed for transfer of proteins from rough endoplasmic reticulum through the Golgi complex to the cell surface is less than one hour (Peters and Ashley, 1967; Droz, 1966a; Peters, 1962b for the case of the liver; Jamieson and Palade, 1966, for the exocrine pancreas). Since the liver secretes a large amount of protein into the blood, a significant amount





of membrane flow, associated with transport of secretory products, must be considered to occur over a matter of minutes or hours.

#### B. Membrane Flow Associated With Membrane Biogenesis

In addition to the relations implied by their participation in the transport of cell products, the cellular membrane systems considered ("true" endoplasmic reticulum, Golgi complex, plasma membrane) may be related biogenetically. Dallner et al. (1966a, 1966b) have studied the development of endoplasmic reticulum membranes in pre- and neo-natal rat livers. During the first few days after birth, large amounts of the reticular variety of smooth endoplasmic reticulum membranes are synthesized, and there is a corresponding increase in microsomal enzymes. (Note that unlike the transport case discussed above, this case involves no steady state; there is a net increase in the amount of smooth membrane present). Dallner et al. showed that during this period the amounts of the microsomal membrane enzymes glucose-6-phosphatase and NADPH-cytochrome c reductase increase first in the rough microsomes and only subsequently in the smooth microsomes. Since administration of actinomycin D or puromycin inhibited the appearance or increase of enzyme activity, they concluded that they were observing the production and transfer of newly synthesized enzyme. Although a classical precursor-product relationship could not be demonstrated (probably due to interconversion of the two types of membrane at a relatively slow rate), the results suggested that new membrane components were synthesized in the rough endoplasmic reticulum and were subsequently transferred to the smooth endoplasmic reticulum (presumably to the reticular form of smooth endoplasmic reticulum, which is the source of the bulk of the smooth microsomes).

The hypothesis can also be advanced that new plasma membrane is likewise synthesized elsewhere in the cell and is subsequently transferred to the cell surface. The pathway of membrane movement may pass through the Golgi complex, but so far the evidence for this view



is entirely based on static images seen in the electron microscope. The plasma membrane, in many cell types including liver cells, is thicker than the rough endoplasmic reticulum membranes (Yamamoto, 1963; Sjostrand, 1963; Benedetti and Emmelot, 1968). In some cell types, the Golgi membranes are intermediate between the two in thickness (Sjostrand, 1963). Moreover, in others the membranes of the piled cisternae show a characteristic differentiation. At one side of the stack, the membrane of the cisternae resembles that of the rough endoplasmic reticulum in thickness and staining intensity, while at the opposite side it is morphologically similar to the plasma membrane (Yamamoto, 1963; Daniels, 1964; Hicks, 1966; Grove et al., 1968).

There are other evidences suggestive of progressive changes in the membrane in different parts of the Golgi complex as well. Novikoff et al. (1964) have shown by histochemistry that thiamine pyrophosphatase activity (which appears to be membrane bound, from our own results in which no enzyme is released when the contents of the Golgi vesicles is released) is restricted to the saccules at the "outer" face of the Golgi complex in rat spinal ganglion cells. The remaining saccules and vesicles contain no histochemically demonstrable thiamine pyrophosphatase activity. In various other tissues, including rat liver, only a few of the Golgi saccules stain for thiamine pyrophosphatase, although the polarity is not as evident (Goldfischer et al., 1964; Saito, 1968a). In addition, there are other staining reactions showing polarization across the Golgi complex, including osmium deposition (Friend and Murray, 1965), colloidal thorium staining, indicative of acid mucopolysaccharide (Berlin, 1967), and an electron microscopic version of the periodic acid-Schiff test for certain carbohydrates (Rambourg et al., 1969), but there are difficulties in assessing the results obtained with these procedures. First, it is difficult to determine whether these staining methods are showing differences in the membranes themselves across the dictyosome or only differences in the contents of the various elements of the Golgi complex. Second, all of these observations are based on static images;



there is no way of determining what the time course of such transitions in the membranes might be.

The second point above is especially significant since the time scale of membrane transfer associated with transport of product may be quite different from that associated with biogenesis. Omura et al. (1967), Widnell and Siekevitz (1967), and Schimke et al. (1968) have studied the turnover of membranes in adult rat liver by labelling the cellular membranes with radioactive protein or lipid precursors ( $^3\text{H}$ -leucine,  $^3\text{H}$ -acetate,  $^3\text{H}$ -glycerol) and then watching the loss of radioactivity from these membranes over time. These investigators have shown that total proteins from nuclear membrane, rough microsomes, smooth microsomes, and plasma membrane all turn over with virtually identical average half lives of 2-3 days, while total phospholipids turn over 10-30% more rapidly. Determination of turnover times for more specific membrane components revealed that membrane turnover occurs asynchronously. Thus the microsomal enzymes cytochrome  $b_5$  and NADPH-cytochrome c reductase have half lives of 120 and 80 hours respectively (using  $^3\text{H}$ -leucine as label). The fatty acid portion of microsomal phospholipids have a half life of 60-80 hours (using  $^3\text{H}$ -acetate as label), while the glycerol backbone has a half life of about 30 hours (using  $^3\text{H}$ -glycerol as label) (Omura et al., 1967). Moreover, Schimke et al. (1968) have shown that there is extensive reutilization of the amino acids produced by membrane degradation: guanidino labelled arginine, which is rapidly hydrolyzed in liver and hence is poorly reutilized or not reutilized, reveals a half life for membrane proteins shorter by 30% than does uniformly labelled leucine.

So far the data indicate that transport of secretory protein is rapid (from synthesis to release it takes less than 60 minutes) while the turnover of membrane is slow (several days) and asynchronous. Most probably the images of vesicles pinching off from, or fusing with the membranes of the Golgi complex and of the "true" endoplasmic reticulum are primarily linked to transport, not to biogenesis, as indicated by the following considerations:



First, turnover of rough endoplasmic reticulum, smooth endoplasmic reticulum, and plasma membrane proceeds at comparable rates (Widnell and Siekevitz, 1967; Schinke et al., 1968) although the masses of membrane in the three systems are quite different. Suppose, for example, a piece of membrane pinches off from the endoplasmic reticulum and fuses with the plasma membrane, and suppose by some unknown process an equal amount of membrane is restored to the endoplasmic reticulum and an equal amount of membrane is removed from the plasma membrane. Overall, the total mass of membrane in each system remains constant, while in each system, an equal absolute amount of membrane has "turned over." But in liver cells, for instance, the total mass of the endoplasmic reticulum is considerably greater than that of the plasma membrane. After the number of transfers required for one half of the plasma membrane to be replaced (which requires a time of one "half life") only a much smaller fraction of the endoplasmic reticulum membrane will have been renewed. Thus the half life of the plasma membrane is not equal to that of the endoplasmic reticulum membrane. In general, if turnover is mediated by bulk transfers of membrane from one membrane system to another, then the half lives of membrane systems of unequal mass will differ. The data indicate that this is not the case.

Second, results so far obtained indicate that the turnover of the various constituents of the endoplasmic reticulum and plasma membranes is asynchronous. If the mode of replacement of degraded membrane were the transfer of pieces of complete membrane, we would expect synchronous loss and replacement of all membrane components. Hence it is unlikely that the bulk transfer of membrane implied by vesicular fission-fusion can be part of the turnover process.

### C. Enzyme Relationships of Membrane Systems

Further information on the relations between these membrane systems is provided by the data presented in this thesis. Our comparative studies of enzyme activities of the "true" endoplasmic reticulum, Golgi complex,





and plasma membrane show that with respect to NADH-cytochrome c reductase and NADPH-cytochrome c reductase, the Golgi fractions resemble the plasma membrane rather than the "true" microsomal membrane; conversely, with respect to AMPase, they resemble the microsomes rather than the plasma membrane. The Golgi membranes also contain a high level of thiamine pyrophosphatase, an enzyme which is present only at low levels in the "true" microsomes and probably (from the histochemical evidence) in the plasma membrane. The most striking thing about this list of enzyme activities is that with the exception of thiamine pyrophosphatase, the Golgi membranes do not have high levels of activity of any of the enzymes assayed. As between the plasma membrane and the "true" microsomal membranes, the Golgi membranes resemble whichever of the two has the lower activity with respect to the enzymes assayed.

#### D. Models of Relationships Between Membrane Systems

We have, then, three sorts of information about the relationships between "true" endoplasmic reticulum, Golgi complex, and plasma membrane: first that patches\* of membrane are physically transferred between "true" endoplasmic reticulum and Golgi complex and between Golgi complex and plasma membrane over fairly short times, with the mass of the system being preserved; second, that each of the membrane systems has differing, though partially overlapping patterns of enzyme activities; and third, that all of the membranes considered (with the exception of the Golgi membranes, for which there are no data) are renewed according to an asynchronous pattern at rates considerably slower than transport of secretory proteins. On account of the difference in rates between the transport process and the turnover process, it will simplify matters to consider first models describing relationships among the three membrane systems which will be consistent with the first two lines of evidence only.

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\*Note: A "patch" of membrane, as used here, means the membrane bounding a vesicle involved in shuttling contents from one membrane system to another. It is, then, a piece of membrane having a surface area of upwards of  $250,000 \text{ \AA}^2$ , if the smallest vesicles involved have diameters of about  $500 \text{ \AA}$ .

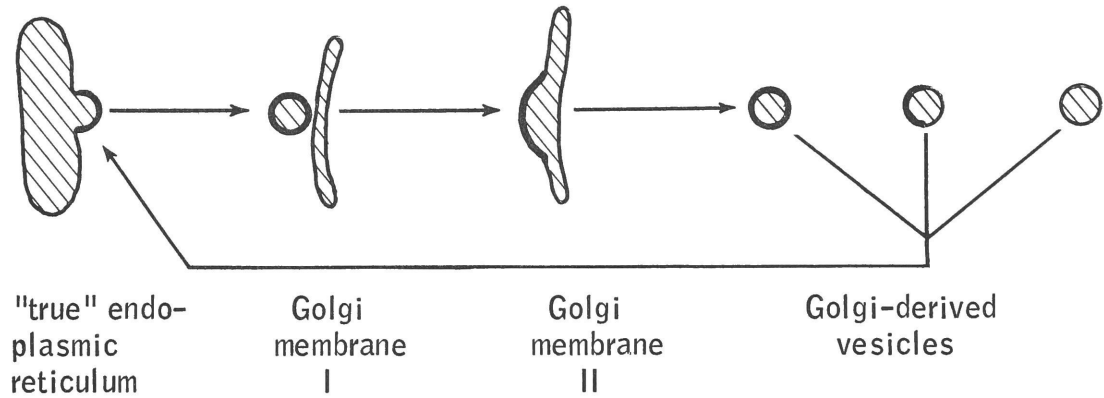
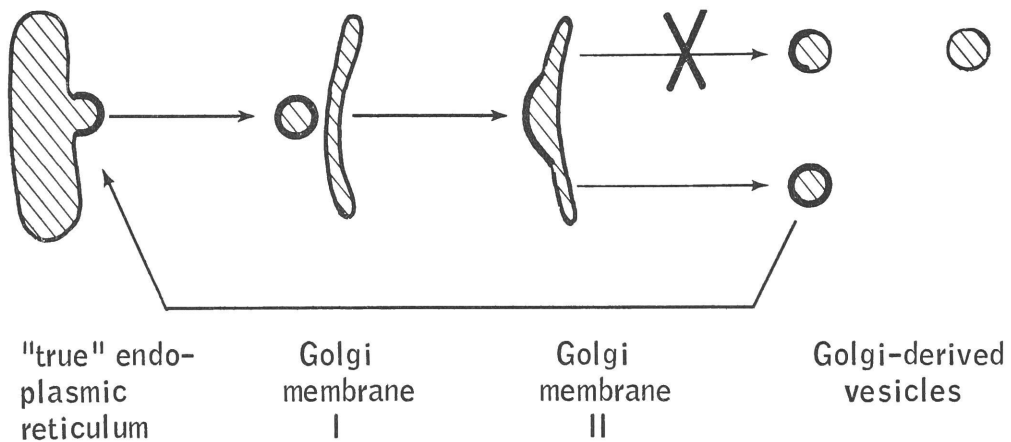


There are several variables we can manipulate in relation to these two lines. First, transport by means of vesicular shuttles may or may not imply mixing of the membranes of the two terminals of the shuttle system. Consider here only the shuttle "true" endoplasmic reticulum-Golgi complex. Membrane transfers can be a random or a non-random process. By "random" we mean that once a piece of membrane from the "true" endoplasmic reticulum fuses with or becomes a Golgi membrane, it is indistinguishable from the rest of the membrane of the Golgi (or of that part of the Golgi complex, at least). If, at a later time, a patch of membrane pinches off from the Golgi element to form a vesicle which moves back to the endoplasmic reticulum or is otherwise removed, the second patch of membrane may be the identical physical piece of membrane which came from the "true" endoplasmic reticulum or a different physical piece of membrane or a mixture of the two. By "non-random" transfer, we mean that, on the contrary, the identity of the piece of Golgi membrane derived from the "true" endoplasmic reticulum is preserved. The piece of Golgi membrane which later is brought back to the endoplasmic reticulum or is otherwise eliminated from the Golgi complex is, in this form of transfer, the same piece which was earlier transferred from the "true" endoplasmic reticulum (see Figure 6). In the same way, we could distinguish between random and non-random membrane interchange between the Golgi complex and the plasma membrane.

Second, enzyme activities can be preserved or transformed. By "preserved" we mean that once a patch of membrane is synthesized, its complement of enzymes is fixed. It can be transferred as a patch of membrane from "true" endoplasmic reticulum to Golgi complex, or from Golgi complex to plasma membrane, in whole or in part, and the enzyme activity of any stretch of the original patch will remain constant. "Transformed" implies, on the contrary, that enzyme activities may be lost or gained as a patch of membrane moves from one membrane system to another, or, in the course of time, after it has been transferred.

Short term variations in concentration are known for a number of



FIGURE 6Random and Non-Random Membrane TransferExample of a "random" membrane transfer sequenceExample of a "non-random" membrane transfer sequence



enzymes of the cell sap (e.g., tryptophan pyrrolase; Feigelson et al., 1959) but for membrane bound enzymes, the information so far available indicates that the time periods involved in induction of activity (by drugs, for instance: Remmer and Merker, 1963; Orrenius et al., 1965; see above, page 91) and in turnover (Omura et al., 1967; Widnell and Siekevitz, 1967; Schimke et al., 1968; see above, page 117) are of the order of hours or days--much longer than the time spans associated with the transport of secretory products.

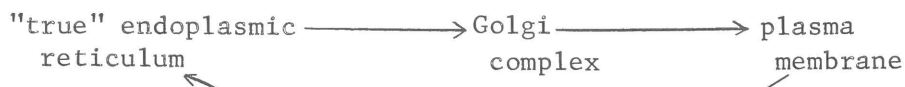
Third, in considering patterns of membrane flow, we must recall that the enzyme activities, staining properties, and thickness differ from one type of cellular membrane to another and that the relative volumes of the membrane systems differ as well. Ignoring for the moment the possibility of short term alterations in these properties, all of these properties must be taken into consideration in any attempt to distinguish the source of membrane involved in a transfer: If a significant amount of "true" endoplasmic reticulum-derived membrane shuttles to the Golgi complex from which it is later removed, at any given time the relatively small Golgi complex will have a relatively large proportion of its membranes made up of "true" endoplasmic reticulum membranes. If a significant amount of Golgi-derived membrane shuttles to the "true" endoplasmic reticulum to pick up a load of cellular products and then returns to the Golgi complex, at any given time the relatively large "true" endoplasmic reticulum will have a relatively small proportion of its membranes made up of Golgi membranes. Since Golgi and "true" endoplasmic reticulum membranes have different patterns of enzyme activities, the two situations are clearly not symmetrical and will have different consequences with respect to the partial or complete mixing of membranes implied by membrane transfers. This will be true, moreover, whether transfers are random or non-random.

Finally, regardless of the original source of membranes, type of transfer (random or non-random), or concomitant transformations of enzymatic activity and membrane structure, we could postulate any of





several patterns of membrane flow. For example, the quantitatively significant forms of membrane flow over a given time span might be:



All systems would stay the same size, so the steady state constraint is met, and the overall transfer of endoplasmic reticulum contents to Golgi complex to plasma membrane is accomplished. Alternatively, the pathway could be:



and the same constraints are met. Other, more complex patterns could also apply.

With these several variables to manipulate, we can now construct several models describing relationships between the various membrane systems and compare these models to the data available. The first three models will examine the difficulties in reconciling the rapid membrane mixing--implied by membrane transfer associated with product transport--with the maintainance of great differences in enzymatic activities. We will show that the only way the difficulties can be overcome, without transformation of enzyme activities, is to have Golgi membranes shuttling back and forth to the "true" endoplasmic reticulum and to the plasma membrane, picking up and discharging their loads alternately, while retaining their identity as Golgi membranes (model 3). Other models, in which, for example, "true" endoplasmic reticulum membranes form the shuttle system, are unsatisfactory (models 1 and 2). Then we will examine the consequences of permitting enzyme activities to be transformed (model 4) and we will indicate why enzyme transformation is more likely to be of significance for models of membrane flow associated with biogenesis rather than with transport of secretory products.

Model 1: Enzyme activities are preserved. Patches of "true" endoplasmic reticulum membrane are transferred from the

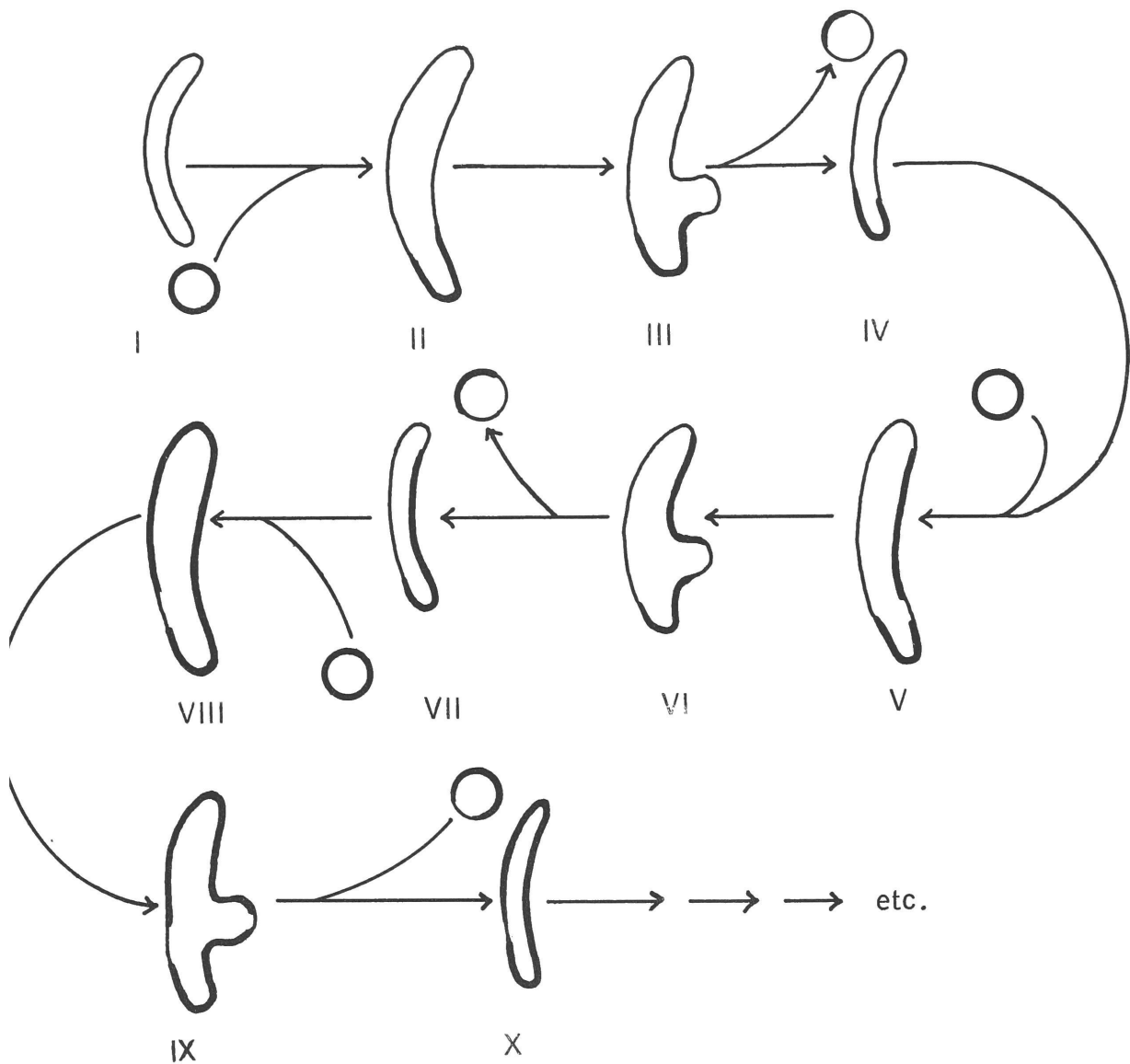


"true" endoplasmic reticulum to the Golgi complex, and then Golgi membrane is randomly transferred back to the endoplasmic reticulum, on to the plasma membrane, or is degraded.

In this model, patches of membrane from the "true" endoplasmic reticulum, containing "true" endoplasmic reticulum enzymes, are transported to the Golgi complex, from which an equal mass of membrane is subsequently removed. The outgoing patch may or may not overlap in whole or in part the incoming patch of the previous transfer. Thus, in the first transfer, "true" endoplasmic reticulum enzymes are added to the Golgi complex; in the second transfer, all, part of, or no part of these enzymes are removed. Such a transfer process implies equilibration, with the enzyme content of the Golgi membranes approaching that of the "true" endoplasmic reticulum (see Figure 7). If membranes from other sources also feed into the Golgi complex (transfer of membrane back from the plasma membrane, for example), the equilibrium levels of "true" endoplasmic reticulum enzymes in the Golgi complex may be lower than in the "true" endoplasmic reticulum itself. But in any case, if transfer from the "true" endoplasmic reticulum to the Golgi complex occurs at any significant rate, the Golgi complex must contain at least some amount of all enzymes found in the "true" endoplasmic reticulum.

In fact, as we have shown, the Golgi membranes contain very little NADH-cytochrome c reductase and cytochrome b<sub>5</sub> and neither glucose-6-phosphatase, NADPH-cytochrome c reductase, nor cytochrome P-450, so this model is unlikely. It could be argued that the endoplasmic reticulum membranes are heterogeneous, and that the part of the "true" endoplasmic reticulum which gives rise to the membrane which transfers to the Golgi complex is atypical, not containing glucose-6-phosphatase, NADPH-cytochrome c reductase, or cytochrome P-450. Counter to this argument, however, is the observation that by histochemical methods, glucose-6-phosphatase at least appears to be present in all membranes of the "true" endoplasmic reticulum, including those in close topographical relationship to the Golgi complex (Ericsson, 1966; Saito, 1966b; Leskes, unpublished).



Schematic Representation of Model 1

"True" endoplasmic reticulum membrane, with "true" endoplasmic reticulum enzymes: **——**

Golgi membranes, with Golgi enzymes: **—**

With time, after many sequential transfers of "true" endoplasmic reticulum membrane to the Golgi complex and with random removal of membrane from the complex, the complex itself is increasingly made up of "true" endoplasmic reticulum membrane with "true" endoplasmic reticulum enzymes.



Model 2: Enzyme activities are preserved. Patches of membrane are transferred from the "true" endoplasmic reticulum to the Golgi complex, and then the identical patches of membrane are non-randomly removed and transferred back to the endoplasmic reticulum, on to the plasma membrane, or are degraded.

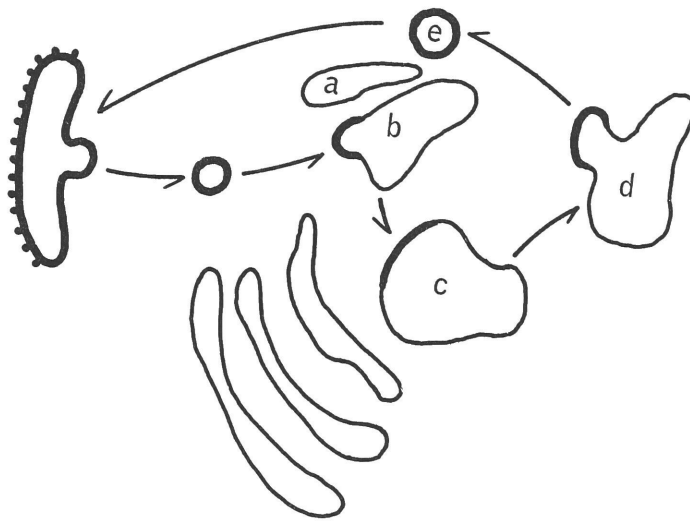
In this model, membrane from the "true" endoplasmic reticulum, with its "true" endoplasmic reticulum enzyme content, is transferred to the Golgi complex. The constant size of the complex is maintained by eliminating from it the identical pieces of membrane. "True" endoplasmic reticulum enzymes are thus added to the Golgi complex, and then the same amount of enzyme is removed. There is no tendency for enzyme activities of the "true" endoplasmic reticulum and the Golgi complex to equilibrate. At any given time, however, some fraction of the membranes of the Golgi complex are "true" endoplasmic reticulum membranes bearing "true" endoplasmic reticulum enzymes. Thus, the Golgi complex will have all of the enzyme activities of the "true" endoplasmic reticulum membranes, although at lower concentrations (see Figure 8).

The absence of measurable glucose-6-phosphatase and NADPH-cytochrome c reductase activities lead to two possibilities: If, in fact, there is none of these enzymes at all in the Golgi membranes, then the model is ruled out. If, on the other hand, there is some activity (there can be up to about 5% of the specific activity found in the microsomes, due to the limits on the sensitivity of the assay), the model is possible. In the latter case, however, the amount of "true" endoplasmic reticulum membrane in the Golgi system at any time must be extremely small--less than about 5% of the total Golgi membrane.

Model 3: Enzyme activities are preserved. Golgi membranes shuttle to the "true" endoplasmic reticulum where they fuse with the endoplasmic reticulum membrane. The identical membrane then non-randomly pinches off and migrates, with its contents, back to the Golgi complex.





FIGURE 8Schematic Representation of Model 2

"True" endoplasmic reticulum membrane with "true" endoplasmic reticulum enzymes (—) is transferred to elements of the Golgi complex and then non-randomly removed therefrom. At any given time, some Golgi elements are in states a, b, c, d, or e. The average vesicle thus contains some "true" endoplasmic reticulum membrane.



The significant feature of this model is that the membrane involved in the shuttle system is Golgi membrane. To preserve this identity, the transfers must be non-random. Otherwise mixing and consequent loss of identity as Golgi membranes would occur. This model is consistent with entirely different enzyme activities in the various systems. At any given time, the only membrane mixing consists of a small amount of Golgi membrane fused with a relatively large amount of "true" endoplasmic reticulum. The Golgi complex has, as far as studied, low enzyme activities. Thus, the "true" endoplasmic reticulum glucose-6-phosphatase activity would be slightly lowered due to the admixture of non-glucose-6-phosphatase containing Golgi membrane. This lowering would be undetectable, since there is no way of separating the Golgi component from the native component in preparations of endoplasmic reticulum membranes. The only Golgi enzyme which we might expect to find in the endoplasmic reticulum as a result of this mixing is thiamine pyrophosphatase, since this enzyme is present in relatively high concentration in the Golgi membranes. (Of course, at any given time, if relatively little Golgi membrane is mixed in with the other membranes, we might detect no thiamine pyrophosphatase activity in the latter location). Our very uncertain thiamine pyrophosphatase recover figures (see above, page 107) are at least consistent with this suggestion.

It should be noted that while the "true" endoplasmic reticulum origin of vesicles involved in the Golgi complex-endoplasmic reticulum shuttle appears unlikely from the enzyme data available (see models 1 and 2), the data do not rule out the possible plasma membrane origin of the vesicles involved in the Golgi complex--plasma membrane shuttle. Additional information on the activity of the Golgi enzyme, thiamine pyrophosphatase, in the plasma membrane and on the activity of other plasma membrane enzymes (e.g., leucyl aminopeptidase) in the Golgi membranes might indicate the likely source of such vesicles.

Several different patterns of membrane transfer can be imagined, consistent with the maintenance of constant volumes for the various



membrane systems. For example, only a part of the Golgi complex may be involved in the shuttle system. Any processing or concentrating of the secretory products then would occur only in this set of Golgi elements. (For instance, the Golgi saccules at the face of the Golgi proximal to the "true" endoplasmic reticulum generally contain few or no lipoprotein particles in normal animals. These saccules, then, possibly may not be involved in the shuttle system to any significant extent. Other explanations of the observations are possible, however.) Alternatively, there may be a transfer of secretory products within the Golgi complex, as well as between "true" endoplasmic reticulum and Golgi complex and between Golgi complex and plasma membrane (see Figure 9).

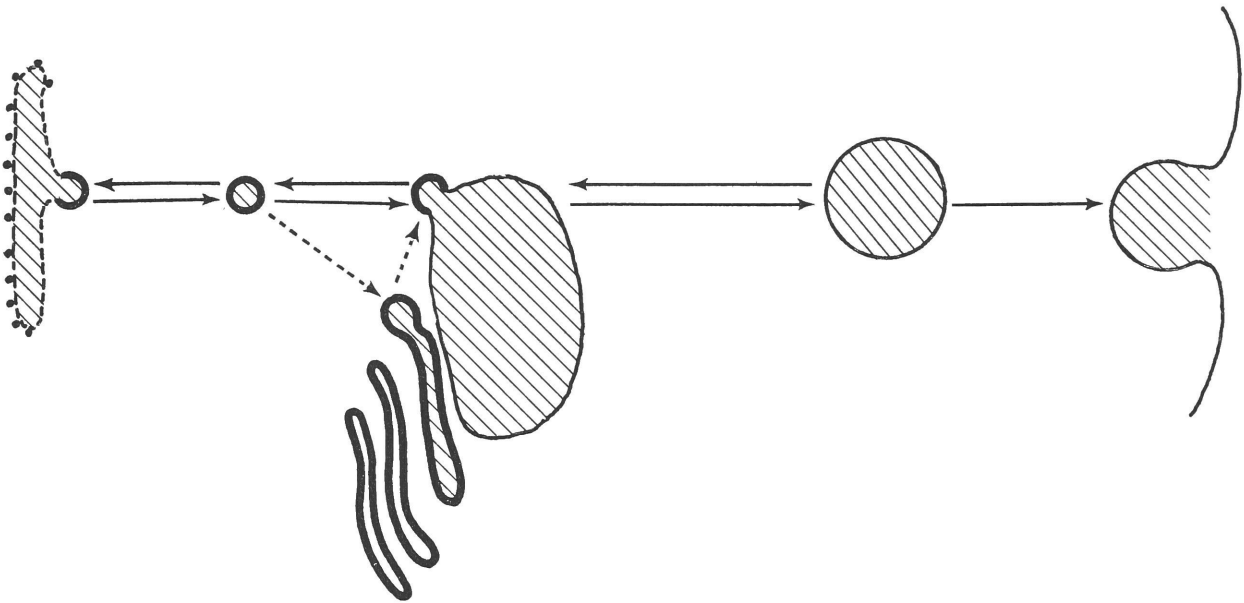
This model takes the differences in enzymatic content of the various membrane systems simply as given. It does not try to explain them. It separates the long term biogenetic relations from short term interrelationships connected with transport of secretory products. It is thus not a complete model--it explains only one part of the data. It tells us that a vesicular shuttle transport system can operate between membrane systems differing in enzymatic content without significantly disturbing their different enzyme systems.

Model 4: Membranes are transferred from "true" endoplasmic reticulum to Golgi complex, where the enzyme activities are transformed. Golgi membranes are then randomly or non-randomly transferred to the plasma membrane (see Figure 10).

The criticism which this model must face is the time scale involved. Short term alterations in membrane structure or chemistry may conceivably occur in some situations, but so far there is no convincing evidence for changes of this kind in membrane enzymes. In accounting in the framework of this model for the differences between the membranes of the "true" endoplasmic reticulum, the Golgi complex,



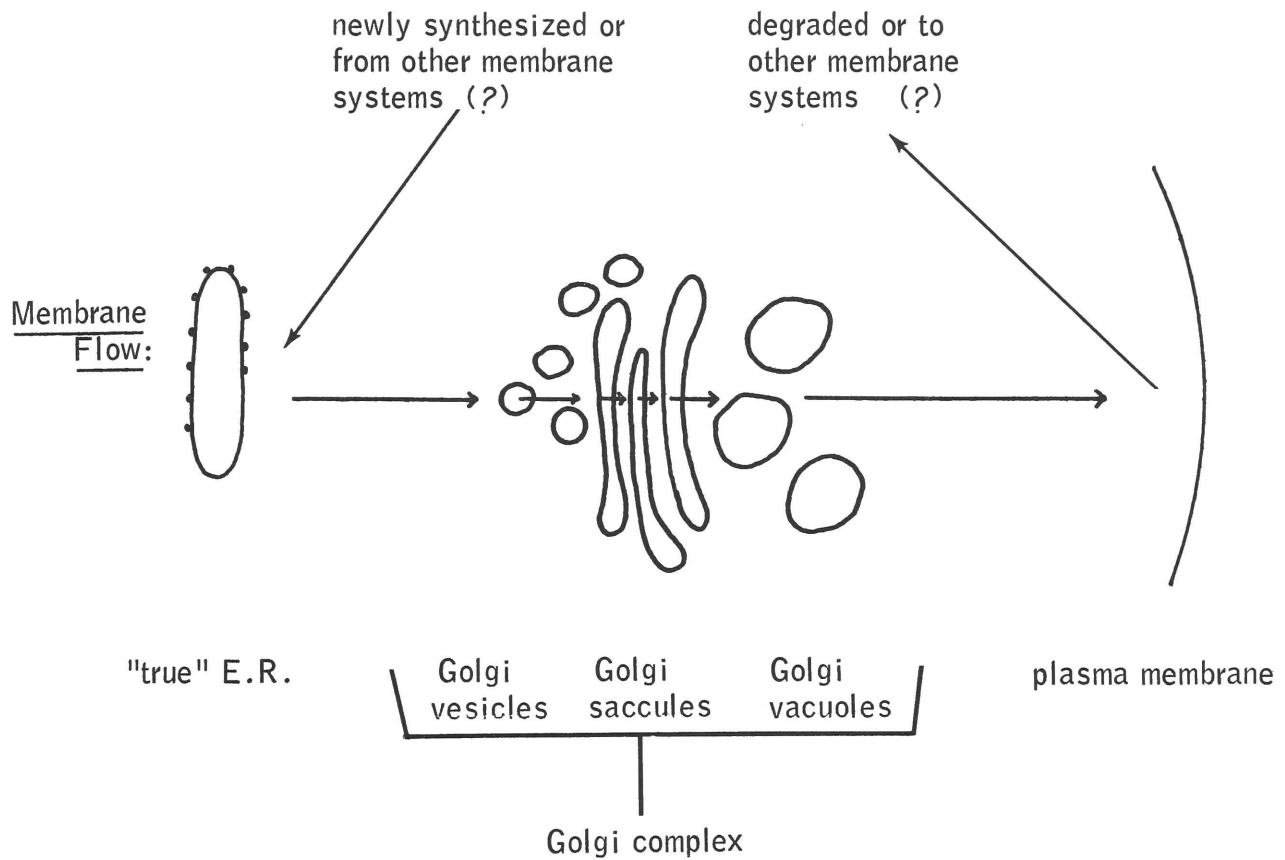
Figure 9

Schematic Representation of Model 3

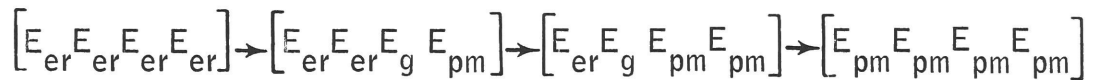
Vesicles derived from the Golgi complex shuttle back and forth from the Golgi complex to the "true" endoplasmic reticulum, picking up "true" endoplasmic reticulum products and depositing them in the elements of the Golgi complex. A similar shuttle system transfers products from the complex to the cell surface, although the membrane of the vesicles of the latter system may be derived from the Golgi complex or the plasma membrane (the figure illustrates the latter case). Possibly not all of the proximal Golgi sacculi are involved in the transfers, as indicated in the figure (see text). "true" endoplasmic reticulum membrane: (-----); Golgi membrane: (—); plasma membrane: (—); content: \\\\'





FIGURE 10Schematic Representation of Model 4

Relative  
Concentration  
of Enzymes:



( $E_{er}$ : "true" E.R. enzymes;  $E_g$ : Golgi complex enzymes;  $E_{pm}$ : plasma membrane enzymes)



and the plasma membrane, we are dealing with a whole complex of changes. A wide variety of enzymatic activities differ greatly. The lipid compositions of the endoplasmic reticulum and cell membrane are also different (Beneddetti and Emmelot, 1968). There may also be additional chemical, and there are obvious structural changes, as evidenced by differences in thickness and staining properties. It is difficult to see how all of these latter changes could occur rapidly, and it is easier to assume that such major changes could be part of the more general process of membrane biogenesis. The latter, as we have emphasized, is a slow process. We are probably, though not certainly, justified in excluding enzyme transformations as a way out of the difficulties encountered in accounting for patterns of enzyme activities imposed by membrane flow in models 1 and 2 above.

A transformation model could still apply within the much slower process of membrane biogenesis, although it should be clear that many other possibilities remain open, and that for the moment there is no evidence to allow any meaningful restrictions. Hence further consideration of the model should be postponed until further evidence, of the type obtained on the rough endoplasmic reticulum-smooth endoplasmic reticulum system, becomes available for Golgi and plasma membranes.



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