Analytical Study of Microsomes and Isolated Subcellular Membranes from Rat Liver VIII. Subfractionation of Preparations Enriched with Plasma Membranes, Outer Mitochondrial Membranes, or Golgi Complex Membranes

MAURICE WIBO, DENISE THINÈS-SEMPOUX, ALAIN AMAR-COSTESEC, HENRI BEAUFAY, and DANIÈLE GODELAINE
Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, B-1200 Brussels, Belgium. Dr. Wibo's present address is Laboratoire de Pharmacodynamie Générale et de Pharmacologie, Université de Louvain, B-1200 Brussels, Belgium. Dr. Thinès-Sempoux's present address is Laboratoire de Biologie Cellulaire, Université de Louvain, B-1348 Louvain-la-Neuve, Belgium.

ABSTRACT   Preparations enriched with plasmalemmal, outer mitochondrial, or Golgi complex membranes from rat liver were subfractionated by isopycnic centrifugation, without or after treatment with digitonin, to establish the subcellular distribution of a variety of enzymes. The typical plasmalemmal enzymes 5'-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphatase were markedly shifted by digitonin toward higher densities in all three preparations. Three glycosyltransferases, highly purified in the Golgi fraction, were moderately shifted by digitonin in both this Golgi complex preparation and the microsomal fraction. The outer mitochondrial membrane marker, monoamine oxidase, was not affected by digitonin in the outer mitochondrial membrane preparation, in agreement with its behavior in microsomes. With the exception of NADH cytochrome c reductase (which was concentrated in the outer mitochondrial membrane preparation), typical microsomal enzymes (glucose-6-phosphatase, esterase, and NADPH cytochrome c reductase) displayed low specific activities in the three preparations; except for part of the glucose-6-phosphatase activity in the plasma membrane preparation, their density distributions were insensitive to digitonin, as they were in microsomes. The influence of digitonin on equilibrium densities was correlated with its morphological effects. Digitonin induced pseudofenestrations in plasma membranes. In Golgi and outer mitochondrial membrane preparations, a few similarly altered membranes were detected in subfractions enriched with 5'-nucleotidase and alkaline phosphodiesterase I. The alterations of Golgi membranes were less obvious and seemingly restricted to some elements in the Golgi preparation. No morphological modification was detected in digitonin-treated outer mitochondrial membranes. These results indicate that each enzyme is associated with the same membrane entity in all membrane preparations and support the view that there is little overlap in the enzymatic equipment of the various types of cytomembranes.

The precise distribution of enzymes through the various membrane systems of rat liver cells is still a matter of debate. Indeed, the development of methods to isolate plasma membranes (50), outer membranes of mitochondria (54), and Golgi complex elements (27, 31, 47) has brought direct evidence that these membranes differ from one another and from the endo-

plasmic reticulum in their biochemical characters. High relative specific activities are currently attained for such enzymes as 5'-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphatase in plasma membrane-rich fractions (for a review, see reference 26), for galactosyltransferase and some other glycosyltransferases in Golgi-rich preparations (14, 31, 49, 56), and for monoamine oxidase in outer mitochondrial membranes (58). In addition, many enzymes, including glucose-6-phosphatase, esterase, and NADPH cytochrome c reductase, reach their highest specific activity in microsomes, a subcellular fraction particularly enriched in vesicles that derive from the endoplasmic reticulum. However, none of the membrane fractions is entirely free of the enzymes predominant in other subcellular preparations.

This partial intermixing of enzyme activities within subcellular preparations has been variously interpreted during the last few years. As pointed out earlier (23, 24), it may be that membrane entities are highly specific in their enzyme characters but are still cross-contaminated with other membranes in the best preparations available so far. The opposite view, that many membrane-bound enzymes are not exclusively associated with one particular type of membrane, has also been favored recently (19, 30, 38, 39, 41, 42, 48). The question of whether the various membranes of the cell generally differ from one another on a qualitative or a quantitative basis is crucial as far as the functional and organizational aspects of membranes are concerned. A nonexclusive association of enzymes with their major host membrane might reflect the biogenetic route of membrane proteins, from the site of synthesis of the polypeptide chain to the membrane domain where the protein exerts its specific function; or it could be the consequence of lateral diffusion along either transient or stable connections between the subcellular compartments through which secretory products are channeled (23, 48, 53).

To gain more information on this matter, we subjected various membrane preparations to density gradient analysis, using procedures similar to those we had used in previous studies on the microsome fraction from rat liver (4, 10). These earlier studies led us to classify the microsomal enzymes into several groups, each group being identified by a characteristic behavior under a variety of conditions. We proposed that these groups are associated with distinct subcellular entities present in the microsome fraction and corresponding to, or related with, the outer mitochondrial membrane (group a1), the plasma membrane (group a2), and the Golgi apparatus (group a3), whereas groups b and c include authentic constituents of endoplasmic reticulum. We investigate here the centrifugal behavior of typical enzymes of these groups in membrane preparations enriched with plasma membranes, outer mitochondrial membranes, or Golgi elements to demonstrate either that each enzyme shows identical characteristics in the various membrane preparations, supporting a specific association with one particular membrane entity, or that each enzyme behaves in a variable manner as a function of the particular membrane preparation used, supporting a widespread distribution through the various subcellular membranes. The results have been reported in part, in condensed form (62, 67).

MATERIALS AND METHODS

Materials

Uridine diphospho-N-acetyl-D-[U-^{14}C]glucosamine (UDP-[^{14}C]GlcNAc, >200 mCi/mmol) and [2-^{3}H]AMP (15–22 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, England), and cytidine 5'-monophosphate

[4,5,6,7,8,9-^{14}C]sialic acid (CMP-[^{14}C]sialic acid, 150–250 mCi/mmol) from New England Nuclear (Dreieich, Federal Republic of Germany). Sialidase from Clostridium perfringens, ovalbumin grade V, and UDP-GlcNAc were obtained from Sigma Chemical Co., St. Louis, Mo. Dextran T-500 and Sephadex were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. α_1-Acid glycoprotein, isolated either from pooled human plasma (65) or from Cohn fraction VI (Calbiochem-Behring Corp., American Hoescht Corp., San Diego, Calif.) (15), was treated with 0.05 mg sialidase/mg glycoprotein for 11 h at 37°C (18). The sialidase was then inactivated by incubation at 60°C for 10 min, and free sialic acid was removed by chromatography on Sephadex G-25. The source of other chemicals was given in a previous article (9).

Animals

Female rats of the Wistar strain, weighing 160–200 g, were given only water for 18 h before being killed by decapitation.

Preparation of Subcellular Membranes

All fractions obtained in the course of the fractionation procedures described below were saved for biochemical assays to establish yields and balance-sheets. When not otherwise stated, sucrose solutions contained 3 mM imidazole-HCl buffer at pH 7.4. Concentrations of the sucrose solutions used for density gradient centrifugation are given in gram of sucrose per 100 g of solution.

PLASMA MEMBRANES: Plasma membranes were prepared from livers homogenized in 1 mM NaHCO$_3$, pH 7.5, as described by Song et al. (59). The large plasma membrane sheets were first concentrated from the homogenate by low-speed centrifugation and then purified by flotation through layers of sucrose containing 1 mM NaHCO$_3$. This step was usually performed in a Beckman SW25.2 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 30-ml portions of sample, brought to density 1.22 by the addition of sucrose up to 46.8% final concentration, were overlaid successively with 12 ml of 39.3% and 10 ml of 35% sucrose solutions in 1 mM NaHCO$_3$ (d = 1.18 and 1.16, respectively). After centrifugation for 90 min at 25,000 rpm, the two upper layers were recovered by suction without removing the material equilibrated at the 1.18–1.22 interface, but including a white band located above this interface. The suspension was diluted fourfold with 1 mM NaHCO$_3$ and centrifuged for 60 min at 25,000 rpm in a Beckman no. 30 rotor. The pellet was resuspended in 1 mM NaHCO$_3$ or in 0.25 M sucrose–1 mM NaHCO$_3$, and the suspension was designated plasma membrane preparation. For large-scale preparation, the flotation was carried out in a Beckman Ti-14 zonal rotor according to a similar procedure.

GOLGI FRACTION: All sucrose solutions contained 37.5 mM Tris-maleate, pH 6.5, 1% Dextran T-500, and 5 mM MgCl$_2$, as in the procedure described by Morré et al. (47). Minced livers were ground with 2–3 ml of 0.5 M sucrose/g of tissue in a Dounce-type homogenizer (Kontes Co., Vineland, N. J.) fitted with a loose pestle. After 20 strokes, the homogenate was centrifuged for 10 min at 1,700 rpm in a no. 253 rotor (International Equipment Co., Needham Heights, Mass.). The supernate was decanted and the loose pellet (about one-third of the volume) was washed twice by homogenization (six strokes) with 0.5 M sucrose and centrifugation for 10 min at 1,500 rpm. The pooled supernates were centrifuged at 25,000 rpm (no. 30 rotor) by a time integral of the squared angular velocity W = $\int_0^t \omega^2 dt$ = 3.1 rad^2 ns^{-1} (equivalent to 7.5 min at top speed). The pellets were suspended in 0.5 M sucrose for another run under the same conditions. The washed material was suspended in 37.4% sucrose, by means of a Dounce grinder fitted with a tight pestle, to a final volume of 1 ml/g of tissue processed. Tubes of a Beckman SW25.2 rotor were loaded successively with 10 ml 50.4% sucrose (d = 1.24), 25 ml of sample (d = 1.16), 12 ml 30.4% sucrose (d = 1.14), and 9 ml 16% sucrose (d = 1.07). The rotor was spun at 25,000 rpm for 2.5 h. The whitish material at the 1.07–1.14 interface was sucked up, gently resuspended, and designated Golgi preparation.

Before subfractionation by isopycnic centrifugation, this Golgi preparation was gently rehomogenized in a salt-free medium to dissociate the aggregates present (see Results). After concentration by centrifugation, the material was resuspended in 0.25 M sucrose—3 mM imidazole, pH 7.4, by a Dounce homogenizer (loose-fitting pestle). The suspension was spun as such or after addition of digitonin (see below) for 45 min at 40,000 rpm (no. 40 rotor), and the sediment was finally suspended in 0.25 M sucrose. Less than 10% of the amount of constituents in the Golgi preparation was lost in the supernatant fluids.

OUTER MITOCHONDRIAL MEMBRANES: A mitochondrial fraction was first obtained according to the method of de Duve et al. (25), except that the homogenization medium was the one described by Parsons et al. (54). We pelleted mitochondria by centrifugation at 12,500 rpm in a no. 30 rotor (W = 0.45 rad^2 ns^{-1}). Washed mitochondria were allowed to swell for 30 min in 0.02% bovine serum albumin–20 mM phosphate buffer, pH 7.2 (54), and afterward were subjected to shearing in a Dounce homogenizer by six strokes of the tight pestle. This treatment improved the yield of free outer mitochondrial membranes, which were then partially separated from the mitochondrial ghosts (mitoplasts) by

differential centrifugation (54). The final pellet was resuspended in 20 mM phosphate buffer, pH 7.2. This suspension is designated outer mitochondrial membrane preparation.

MICROSOMES: They were prepared as previously described (3).

Treatment of Membrane Preparations with Digitonin

In most experiments, the density distribution of enzymes was determined on the original preparation and on a sample treated with digitonin. The digitonin solution was added dropwise to the membrane preparation kept in ice. Except in the experiment of Fig. 9, the amount of digitonin added was adjusted to match the endogenous cholesterol, estimated quickly by the Liebermann-Burchard method (see reference 9), or by protein assay when the cholesterol:protein ratio was approximately known. The actual digitonin:cholesterol molar ratio ranged from 0.7 to 1.2.

Digitonin-treated Golgi preparations were spun and resuspended in digitonin-free 0.25 M sucrose, as described above. Microsomes were treated with digitonin and washed as previously described (4). The other digitonin-treated membrane preparations were analyzed by density equilibration without further washing.

Subfractionation of Membrane Preparations by Isopycnic Centrifugation

Density equilibration was achieved, as previously described (10), in sucrose-H_2O gradients extending linearly from a density of 1.09 or 1.10 to 1.25 or 1.28. The E-40 rotor was spun at 40,000 rpm for 140 min (microsome, plasma membrane, and Golgi preparations) or 30 min (outer mitochondrial membrane preparations). In some experiments, several gradients were run simultaneously in a Beckman swinging-bucket rotor, as indicated in the figure legends. The methods described by Beaufay et al. (12) were then used to make the gradients and to

recover 10-15 subfractions. Results were calculated and presented as described in paragraph 8.2 of reference 7.

Biochemical Determinations

Most constituents were assayed according to previously described procedures (9), except that protein, acid phosphatase, and 5'-nucleotidase were determined manually. Sialyltransferase was assayed by measuring the transfer of radioactivity from CMP-[^{14}C]sialic acid to sialidase-treated α_1-acid glycoprotein (56). Leucine-β-naphthylamidase was determined by the procedure of Goldberg and Rutenburg (34).

N-Acetylglucosaminyltransferase was assayed by measuring the transfer of N-acetylglucosamine from radiolabeled UDP-GlcNAc to ovalbumin. The incubation mixture contained, in a final volume of 60 μl, 4% (wt/vol) ovalbumin, 0.11 mM UDP-[^{14}C]GlcNAc (40,000 cpm), 100 mM cacodylate-HCl buffer, pH 6.4, 5 mM ADP, 20 mM $MnCl_2$, 0.4% (wt/vol) Triton X-100, and 20 μl of tissue fraction or 0.25 M sucrose (blank). After 50 min at 37°C, 40 μl of the incubated mixtures was pipetted onto strips (3 × 2 cm) of Whatman 3 MM chromatography paper, which were immediately dipped into ice-cold 10% (wt/vol) TCA for 30 min. The strips were washed three times for 5 min in 5% (wt/vol) TCA at room temperature, with occasional shaking, and twice for 5 min in ethanol. They were dried and transferred to vials that contained 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole [PPO], 120 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene [Dimethyl POPOP], and 1 liter toluol). Most (>90%) of the protein-bound radioactivity was found to behave like ovalbumin when incubated mixtures were analyzed by gel chromatography (not shown). The radioactivity transferred to endogenous acceptors was usually not measured separately.

In some experiments, glucose-6-phosphatase activity was assayed before and after incubation for 30 min at 37°C and pH 5 (10 mM acetate buffer) in the absence of substrate. Under these conditions, the authentic glucose-6-phosphatase is inactivated, and the residual activity is that of other, stable phosphohydrolases (11). The distribution patterns shown in Figs. 8 and 14 are those of the pH 5-labile glucose-6-phosphatase.
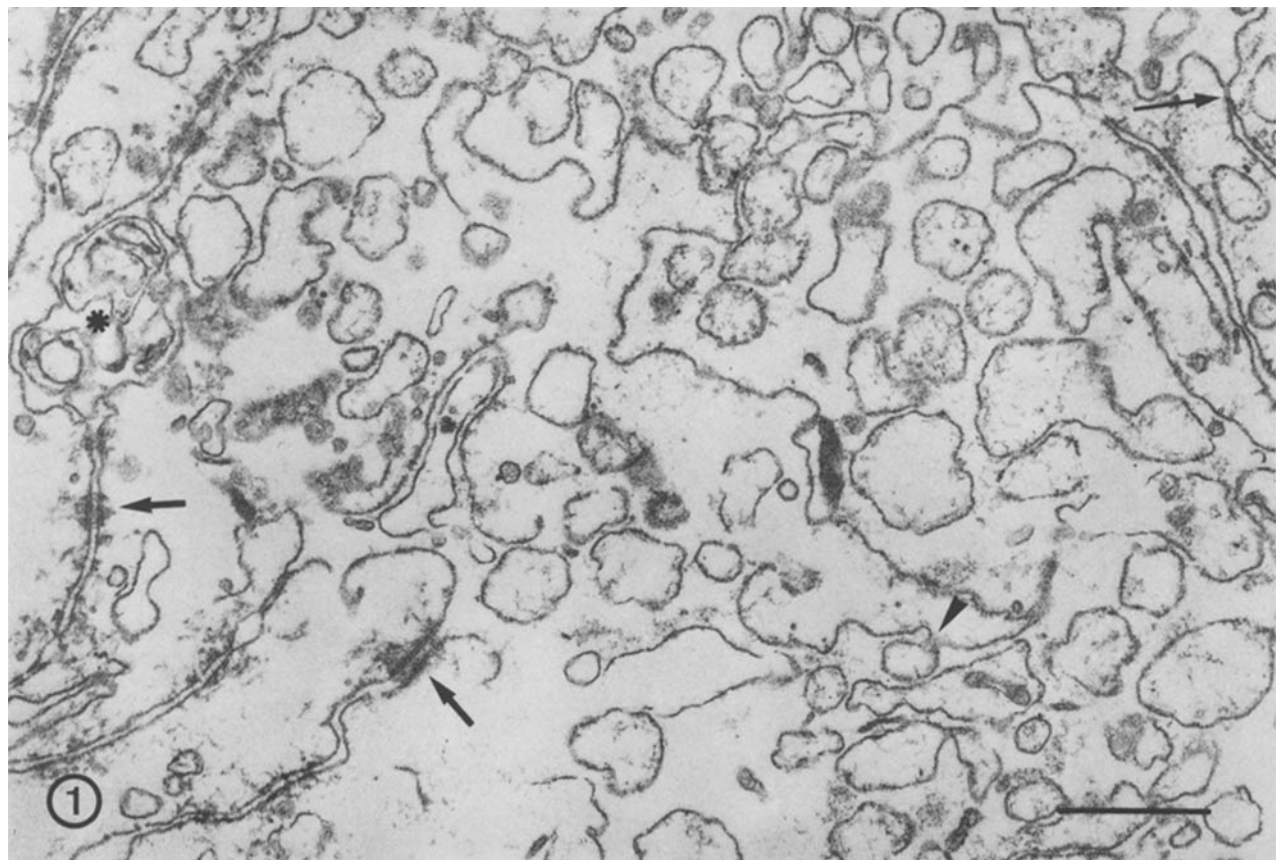

FIGURE 1 Micrograph of a plasma membrane preparation. Vesicular profiles of variable size and large membrane sheets dominate the picture. Usually these sheets are arranged in pairs, held together by junctional complexes. Desmosomes (short arrows), a segment of gap junction (long arrow), and a bile canaliculus (asterisk) are unambiguously identifiable. Some small vesicles are likely to be connected to large sheets, as evidenced by images of pediculated elements (arrowhead). A network of fibrillar material is associated with many membranes, usually on one side only. Bar, 0.5 μm. × 40,500.

Electron Microscopy

Representative samples of membrane fractions were obtained by the filtration technique developed by Baudhuin et al. (6). They were processed for electron microscopy as previously described (66).

RESULTS

Morphology of Membrane Preparations and Alterations Induced by Digitonin

The membrane preparations were examined by thin-section and negative-staining electron microscopy, to assess their composition and to look for alterations after treatment with digitonin. Plasma membrane preparations consist mainly of smooth vesicles of variable size and, more characteristically, of large membrane sheets frequently held in pairs by junctional complexes (Fig. 1). Although these features largely dominate, some obvious contaminants (in particular mitochondrial ghosts and rough microsomes) are also conspicuous in most preparations. After treatment with digitonin, the morphology of the preparation is dramatically modified (Figs. 2 and 3). Usually, digitonin induces a broken or fenestrated appearance of the membranes (with the exception of gap junctions, of some small vesicles that sometimes bear a few ribosomes and of contaminating mitochondria). In spite of discontinuities, the outlines of vesicles and membrane sheets are still clearly detected.

Grazing sections show unstained patches. These alterations are identical to those described previously for the microsomal vesicles that contain 5'-nucleotidase and the other enzymes of group $a2$ (4). More rarely, the membranes show stiff segments, with a sharp triple-layered aspect (Fig. 2 b) previously described by Colbeau et al. (21) in rat liver microsomes incubated with digitonin. The reason for this variability in the morphological response to digitonin is unclear. As reported earlier (22), in negatively stained preparations the digitonin-treated membranes appear undisrupted and show straight portions and sharp bends. This rigid appearance, contrasting with the smoothly rounded contours seen in control preparations, is reminiscent of that shown in Fig. 2 b.

The Golgi preparations consist mainly of structural assemblies showing the essential components of the Golgi complex in situ, i.e., stacked cisternae, interconnected tubules, and vacuoles loaded with very-low-density lipoprotein (VLDL) particles (Fig. 4). The dictyosomal arrangement is fairly well preserved, as is to be expected for preparations obtained in the medium originally proposed by Morré et al. (47). Aggregates of rough vesicles are often seen in close contact with authentic Golgi components; recognizable mitochondrial contaminants are scarce. Due to their aggregated condition, such preparations were unsuitable for subfractionation by isopycnic centrifugation. After washing and resuspension in 0.25 M sucrose, aggregation is no longer apparent, and dictyosomes are dismantled
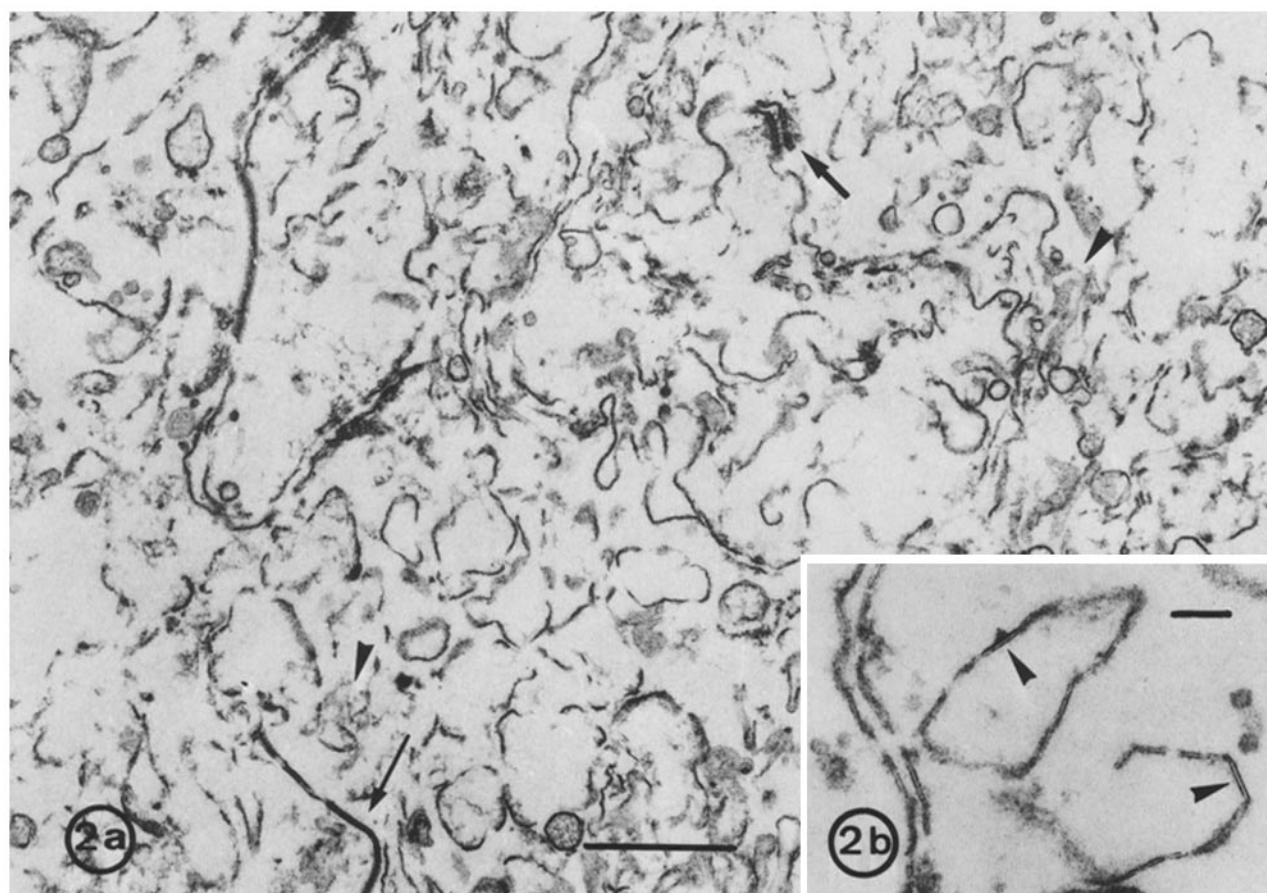

FIGURE 2   Morphology of plasma membranes after treatment with digitonin. (a) The untreated sample from the same preparation is shown in Fig. 1. The major morphological characters of the untreated sample, in particular desmosomes (short arrow) and gap junctions (long arrow), are still recognizable, but, with the exception of some small vesicles, most membranes present discontinuous contours. Unstained patches are visible in face view (arrowheads). Bar, 0.5 μm. × 44,000. (b) Instead of the usual fenestrated aspect, the membrane profiles of this digitonin-treated preparation show small straight segments with a highly contrasted trilaminar appearance (arrowheads). Bar, 0.1 μm. × 80,000.

into individual cisternae, tubules, and vesicles containing VLDL particles (Fig. 5). When the washing medium is supplemented with digitonin, the only obvious modification is the occurrence of poorly contrasted tubular profiles (Fig. 6). Similar profiles have been observed in cholesterol-containing liposomes exposed to digitonin (29). The alterations induced by digitonin are more prominent after negative staining (Fig. 7). This method reveals rigid tubules permeated by the contrasting agent, which coexist with apparently intact elements. Broken-looking vesicles of the type observed with plasma membranes are seen occasionally in digitonin-treated Golgi preparations (see Fig. 16b). In some cases, a decrease in the number and electron density of intravesicular VLDL particles was also noted.

Outer mitochondrial membrane preparations (not shown) consist mainly of empty membranous bags and swollen mitoplasts. Digitonin induces no morphological change in these major components (see Fig. 18), but a few vesicles acquire the broken-looking aspect characteristic of plasma membranes.

These electron microscope examinations thus indicate that each preparation is distinctly enriched in one particular species of subcellular membranes. Some contamination was, however, consistently observed; it was not quantified because we were unable to unambiguously identify too many membrane elements. In addition, we observed that plasma membranes are
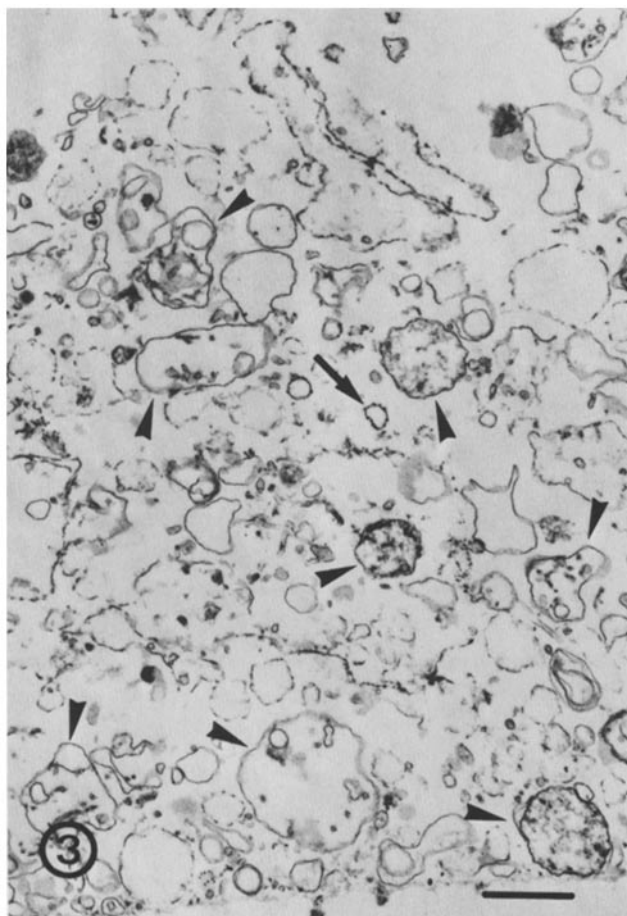

FIGURE 3 Micrograph of a digitonin-treated plasma membrane preparation. In addition to the typical broken-looking membranes, this preparation contains a number of mitochondrial profiles (arrowheads) that do not show the fenestrated aspect. Apparently unaltered small vesicles, some of them with attached ribosomes (arrow), are also present. Bar, 1 μm. × 12,200.

characteristically altered by digitonin, whereas the morphology of the Golgi elements is changed in a different, less uniform manner.

Biochemical Properties of Membrane Preparations

The biochemical data referring to the various membrane preparations are presented in Tables I and II, which give, respectively, the yields and the specific activities of enzymes and chemical constituents. In agreement with many other studies (see the Introduction for references), each membrane preparation is characterized by several enzymes that are recovered with a fair yield and attain a specific activity (italics in Table II) that is distinctly higher than in the whole homogenate. In addition, every preparation shows the enzyme activities typical of other membrane preparations, but these occur more variably and with much lesser yields and do not reach specific activities significantly greater than those in the starting homogenate.

Alkaline phosphodiesterase I and 5'-nucleotidase are typical of plasma membrane preparations in the sense defined above. They are also detected in Golgi and outer mitochondrial membrane preparations, but with specific activities 8- to 20-fold less. Alkaline phosphatase is another typical constituent of plasma membrane preparations, although its yield and relative specific activity are comparatively low, partly because one-third of the alkaline phosphatase activity is unsedimentable in liver homogenates (3). Golgi preparations are characterized by galactosyltransferase, N-acetylglucosaminyltransferase, and sialyltransferase, the specific activities of which are increased almost two orders of magnitude above the values in homogenates. In contrast, galactosyltransferase is barely detectable in the two other membrane preparations, and its relative specific activity varies noticeably. Monoamine oxidase and, to some extent, NADH cytochrome c reductase are typical of outer mitochondrial membrane preparations. The mean relative specific activity of monoamine oxidase is 24, a value 10- and 80-fold higher than in plasma membrane and Golgi preparations, respectively. The level of NADH cytochrome c reductase (rotenone-insensitive) activity is distinctly less because, in liver, 75–80% belongs to the endoplasmic reticulum (3, 25).

Enzymes associated with the endoplasmic reticulum, particularly NADPH cytochrome c reductase, glucose-6-phosphatase, and esterase, are also present in the various membrane preparations, but their relative specific activities are only on the order of 1. The stability of NADPH cytochrome c reductase and glucose-6-phosphatase in the Golgi preparation (resuspended in 0.25 M sucrose–3 mM imidazole, pH 7.4) was examined by measuring these enzyme activities soon after isolation of the membranes (11 h after killing the rat), or after up to 40 h of storage at 0°C at protein concentrations varying from 0.4 to 6 mg/ml. In contrast with the results reported by Howell et al. (38), but in agreement with those of Borgese and Meldolesi (16), in our preparations, glucose-6-phosphatase and NADPH cytochrome c reductase activities did not decrease over this time period (not shown). Acid phosphatase occurs in the Golgi preparations with a relative specific activity of ~4. Cytochrome c oxidase is nearly absent from the Golgi preparations, but is found with a relative specific activity of 1.6 and 1.3 in plasma membrane and outer mitochondrial membrane preparations, respectively.
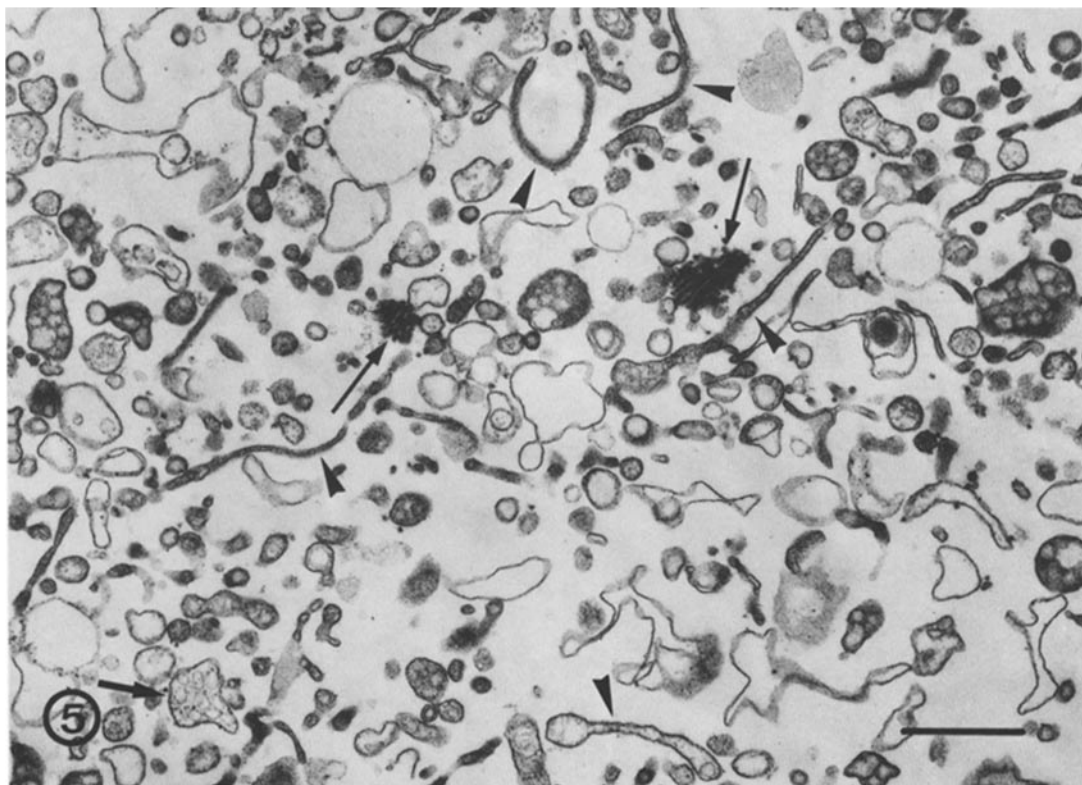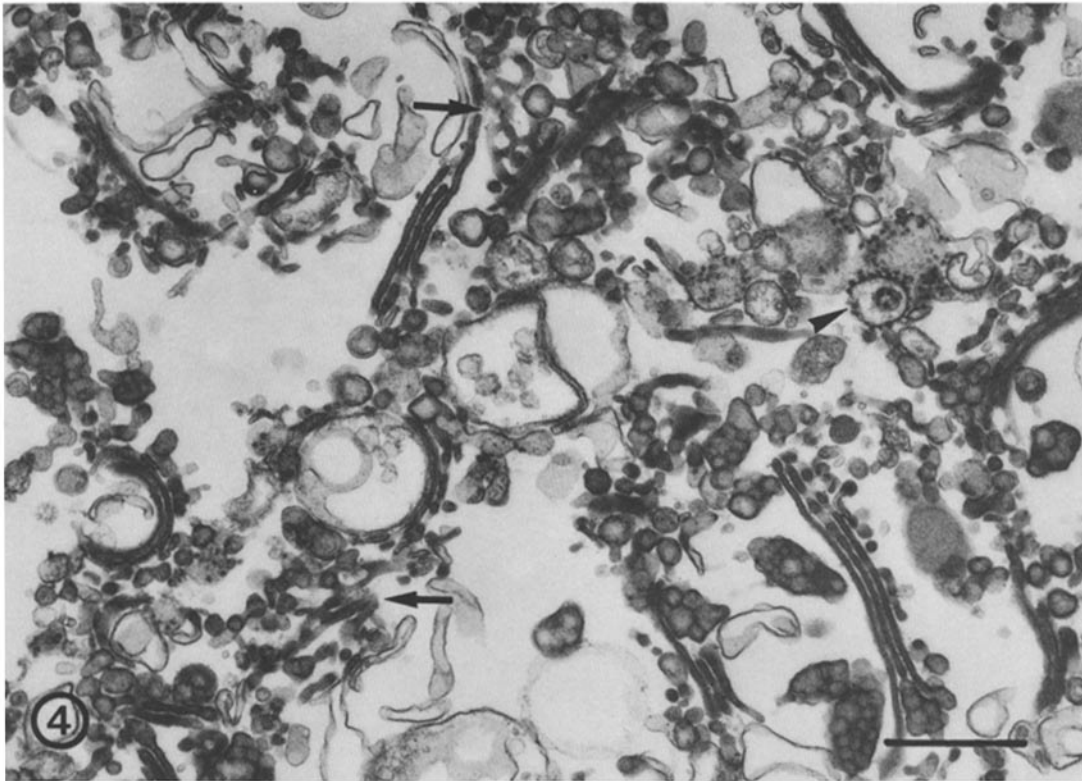
FIGURE 4 Micrograph of a purified Golgi complex preparation. The predominant structures are stacks of three to four cisternae, vacuoles filled with VLDL particles, and networks of tubules (arrows). Note a clump of rough vesicles (arrowhead) in close contact with other membranous elements. Bar, 0.5 μm. × 35,000.

FIGURE 5 Micrograph of a sucrose-washed Golgi complex preparation. Numerous isolated cisternal profiles (arrowheads) are present. Other large and apparently empty membrane profiles could arise from dilated cisternae. Several elements contain VLDL particles, but sometimes the dense matrix that surrounds these particles is absent (short arrow). Two peroxisomal cores are indicated by long arrows. Bar, 0.5 μm. × 31,000.
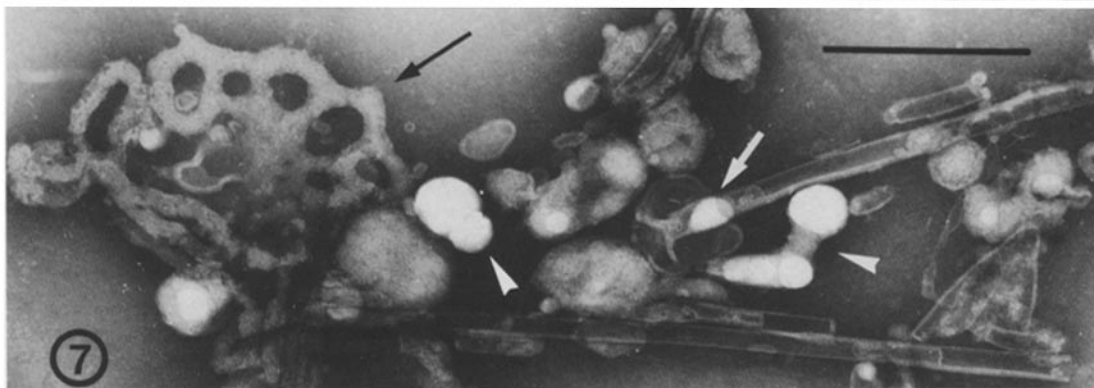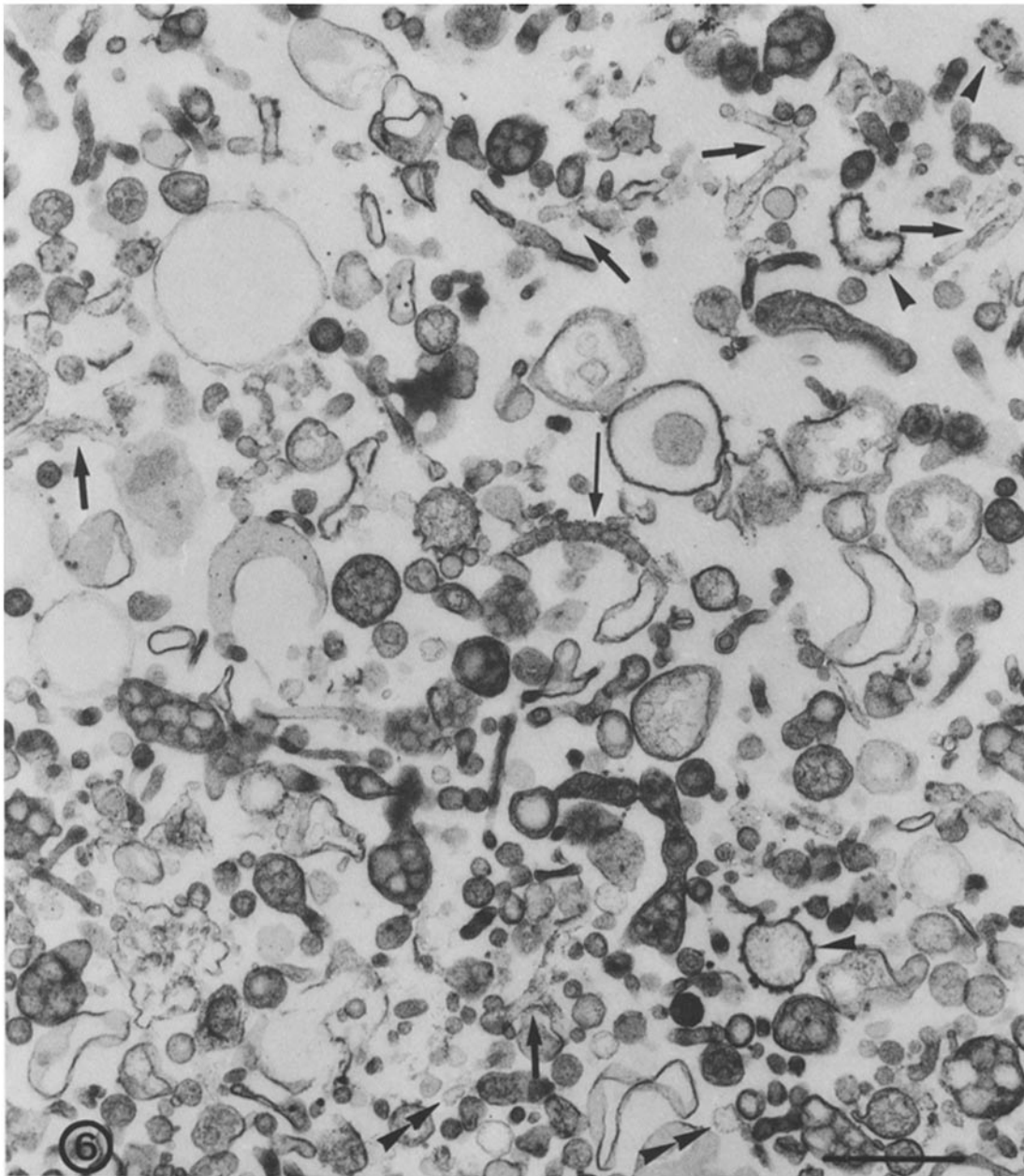
FIGURE 6 Micrograph of a digitonin-treated Golgi complex preparation. This preparation is characterized by the presence of tubular profiles delineated by weakly contrasted edges (short arrows). One probably contains VLDL particles (long arrow). Several small, faint profiles could be cross sections through these elongated elements (double arrowheads). The preparation also contains numerous tubular and vesicular profiles limited by an apparently unaltered membrane, as well as VLDL-loaded elements. A few rough vesicles are visible (arrowheads). Bar, 0.5 μm. × 40,000.

FIGURE 7 Appearance of a digitonin-treated preparation after negative staining. This preparation was fixed with 1.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, and examined after negative staining with 1% potassium-phosphotungstate, pH 7.0. Several elements appear unaltered: a fenestrated cisterna (long arrow), tubules, and elements that probably contain VLDL particles (arrowheads). In addition, several elongated, rigid-looking profiles are present. One of them seems to contain a VLDL particle and to be continuous with an unaltered vesicle (short arrow). Bar, 0.5 μm. × 54,400.

Analysis of Membrane Preparations by Density Equilibration

PLASMA MEMBRANE PREPARATIONS: The density distributions found for the control and digitonin-treated samples from a typical preparation are compared in Fig. 8. The enzyme activities remaining in the sample layer (d <1.1, not shown) were low, particularly in the digitonin-treated preparation (<2% of the activities in the sample); thus, treatment with digitonin did not solubilize the enzymes assayed. Consistent with our previous results (4), neither did this treatment cause any significant loss of these enzyme activities.

With the untreated sample, all distribution profiles are asymmetrical, with a peak at 1.175 (except for NADH cytochrome c reductase) and little or no material above 1.19. This truncated shape is expected because denser elements were removed during purification. The spreading along the low-density portion of the gradient varies markedly; it is minimal for alkaline phosphatase and maximal for glucose-6-phosphatase and NADH cytochrome c reductase.

TABLE I

Yields of Constituents in Membrane Preparations *

Constituent	Homogenate	Plasma membrane	Golgi	Outer mitochondrial membrane
	Absolute value	Yield	Yield	Yield
	mg/g liver	%	%	%
Protein	214.1 ± 33.8 (42)‡	1.08 ± 0.36 (21)	0.41 ± 0.15 (15)	0.74 ± 0.24 (6)
Phospholipid§	26.7 ± 4.8 (11)	2.02 ± 0.24 (5)	1.83 ± 0.43 (3)	2.00 ± 0.90 (3)
Cholesterol	2.49 ± 0.52 (19)	6.65 ± 2.15 (8)	2.31 ± 1.28 (7)	1.03 ± 0.52 (4)
	unit/g liver‖			
5'-Nucleotidase	13.4 ± 2.3 (31)	17.2 ± 7.0 (19)	0.70 ± 0.31 (12)	0.83 (1)
Alkaline phosphodiesterase I	15.6 ± 4.2 (27)	18.9 ± 8.2 (15)	1.14 ± 0.60 (11)	1.14 ± 0.30 (3)
Alkaline phosphatase	1.35 ± 0.30 (16)	8.90 ± 4.90 (16)		
Galactosyltransferase	0.0151 ± 0.0058 (19)	0.41 ± 0.31 (5)	25.0 ± 8.4 (15)	0.23 ± 0.30 (3)
N-Acetylglucosaminyltransferase	0.0017 ± 0.0002 (3)		26.3 ± 14.1 (3)	
Sialyltransferase	0.0013 ± 0.0003 (2)		36.2 ± 18.2 (2)	
Monoamine oxidase	0.482 ± 0.082 (14)	2.10 ± 0.52 (4)	0.13 ± 0.10 (4)	16.9 ± 9.1 (6)
NADH cytochrome c reductase	99.7 ± 29.4 (18)	1.25 ± 0.48 (4)	0.60 ± 0.34 (9)	4.80 ± 2.20 (5)
NADPH cytochrome c reductase	3.47 ± 0.72 (3)		0.33 ± 0.10 (3)	
Glucose-6-phosphatase	21.6 ± 4.8 (23)	0.92 ± 0.43 (7)	0.42 ± 0.22 (15)	0.38 ± 0.06 (2)
Esterase	266.3 ± 65.0 (7)		0.70 ± 0.45 (3)	0.79 ± 0.44 (4)
Cytochrome c oxidase	30.5 ± 11.9 (17)	1.91 ± 1.35 (5)	0.05 ± 0.04 (10)	1.03 ± 1.04 (5)
Acid phosphatase	6.06 ± 0.91 (13)	0.62 ± 0.24 (5)	1.45 ± 0.45 (8)	

* Values are given as means ± SD. Yields in membrane preparations are the percent of the sums of the amounts or activities recovered in all fractions. These sums comprised between 90 and 99% of values in the homogenate for plasma membrane preparations, 83 and 104% for Golgi preparations, and 88 and 111% for outer mitochondrial membrane preparations.
‡ Number of experiments is given in parentheses.
§ Weight amount of phospholipid is taken to be 0.775 mg/μmol of lipid phosphorus.
‖ Units are micromoles of product formed per minute, except for cytochrome c oxidase, which obeys first-order kinetics (see reference 9).

TABLE II

Specific Activities of Constituents in Membrane Preparations *

	Plasma membrane	Golgi	Outer mitochondrial membrane
		mg/g liver	
Protein	2.15 ± 0.78	0.91 ± 0.28	1.44 ± 0.54
		mg/mg protein	
Phospholipid	0.35 ± 0.04	0.45 ± 0.09	0.55 ± 0.20
Cholesterol	0.088 ± 0.016	0.055 ± 0.011	0.018 ± 0.012
		relative specific activity‡	
5'-Nucleotidase	16.0 ± 3.5	1.7 ± 0.4	0.8
Alkaline phosphodiesterase I	18.4 ± 3.3	2.5 ± 0.9	1.3 ± 0.05
Alkaline phosphatase	8.0 ± 2.8		
Galactosyltransferase	0.4 ± 0.3	63.3 ± 11.6	0.25 ± 0.32
N-Acetylglucosaminyltransferase		76.1 ± 13.0	
Sialyltransferase		105.0 ± 2.0	
Monoamine oxidase	1.9 ± 0.8	0.3 ± 0.2	23.7 ± 13.9
NADH cytochrome c reductase	1.4 ± 0.5	1.2 ± 0.4	6.6 ± 2.4
NADPH cytochrome c reductase		1.0 ± 0.3	
Glucose-6-phosphatase	0.9 ± 0.4	1.0 ± 0.3	0.8 ± 0.6
Esterase		1.3 ± 0.5	1.1 ± 0.8
Cytochrome c oxidase	1.6 ± 0.5	0.1 ± 0.1	1.3 ± 1.1
Acid phosphatase	0.6 ± 0.1	3.9 ± 1.2	

* Values are given as means ± SD and correspond to the experiments reported in Table I.
‡ Relative specific activity is the ratio of the yield (percent value, as defined in Table I) of enzyme to that of protein; mean values greater than 5 are printed in italics.

Digitonin has a clear-cut effect on alkaline phosphatase, 5'-nucleotidase, alkaline phosphodiesterase I, and cholesterol. These constituents are shifted to markedly higher equilibrium densities and recovered above 1.19 for the most part. NADH cytochrome c reductase and esterase (not shown) are little affected. Protein and glucose-6-phosphatase behave in a more complex manner, which results in nearly bimodal distributions. Apparently, some 50–60% of the protein and some 30–40% of the pH 5–labile glucose-6-phosphatase activity are shifted as much as alkaline phosphatase and its companion enzymes, whereas the remainder is not displaced by digitonin. Density shifts estimated on the median equilibrium densities are given in Table III for protein, phospholipid, and the major constituents of the plasma membrane preparations. The lower value for phospholipid and protein results from the bimodal shape of their density distributions in digitonin-treated preparations and reflects the presence of membrane components insensitive to digitonin. The relationship between the amount of digitonin added to the preparation and the median equilibrium density of alkaline phosphatase is shown in Fig. 9. This enzyme was selected because its density distribution is the least polydisperse under all conditions, which makes comparison of median

values meaningful. The density increases in an essentially linear fashion, up to a digitonin:cholesterol molar ratio of unity, and then levels off abruptly, as expected for an effect produced by reaction of digitonin with cholesterol. A similar relationship was obtained for 5'-nucleotidase and cholesterol.

MECHANICALLY DISRUPTED PLASAMA MEMBRANE PREPARATIONS: Because the density distributions of enzymes typical of plasma membrane preparations differed moderately but reproducibly (Fig. 8, control), our preparations were subjected to shearing in an Ultra-Turrax homogenizer (Janke and Kunkel KG., Staufen i. Br., W. Germany) in an attempt to more clearly resolve enzymically distinct zones of the membrane. The sheared membranes were subsequently equilibrated

TABLE III

Median Densities of Constituents in Untreated and Digitonin-treated Plasma Membrane Preparations *

Constituent	Untreated	Digitonin-treated	Difference
Protein	1.170 ± 0.005 (12)	1.198 ± 0.012 (5)	0.028‡
Phospholipid	1.173 ± 0.006 (3)	1.195 ± 0.005 (2)	0.022§
Cholesterol	1.169 ± 0.007 (6)	1.207 ± 0.008 (5)	0.038‡
5'-Nucleotidase	1.165 ± 0.003 (11)	1.203 ± 0.003 (5)	0.038‡
Alkaline phosphodiesterase I	1.169 ± 0.005 (8)	1.205 ± 0.002 (4)	0.036‡
Alkaline phosphatase	1.173 ± 0.003 (11)	1.208 ± 0.002 (4)	0.035‡

* Density values are means ± SD. The number of experiments is given in parentheses.
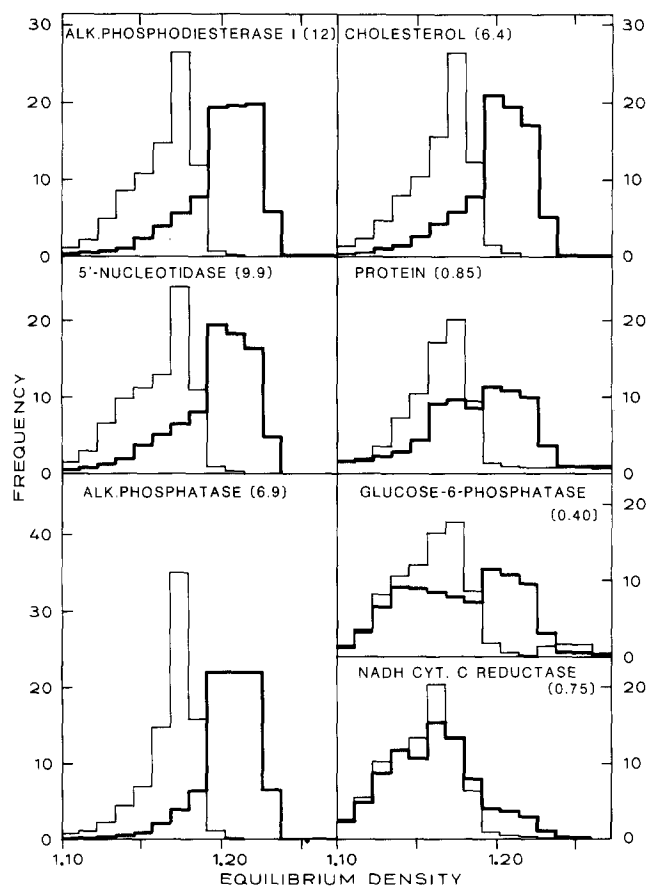‡ Significant at $P < 0.001$.
§ Significant at $P < 0.05$.

FIGURE 8 Density distributions of constituents in control and digitonin-treated samples from a plasma membrane preparation. Yields of constituents, expressed in percent of the liver content, are given in parentheses. Digitonin was added to a portion of the preparation (digitonin:cholesterol molar ratio was 0.7) and the digitonin-treated (thick line) and the untreated (thin line) samples were subfractionated in the E-40 rotor (see Materials and Methods). Distribution profiles of the constituents were superimposed after normalization. The pH 5–labile glucose-6-phosphatase activity (see Materials and Methods) shown here amounted to 83% of the total activity in the plasma membrane preparation.
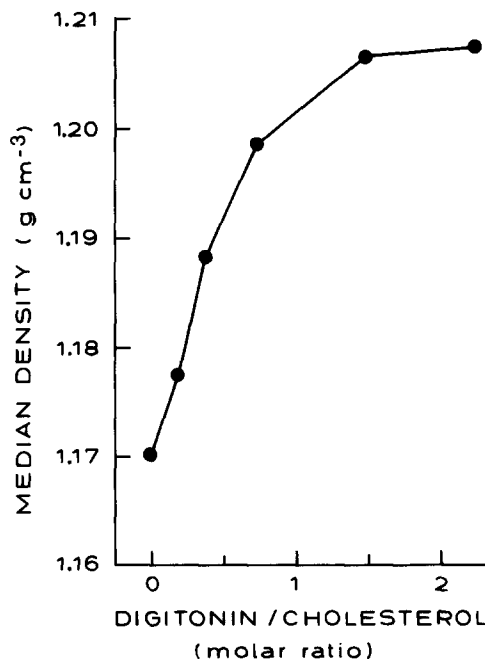

FIGURE 9 Dependence of the median equilibrium density of alkaline phosphatase upon digitonin concentration. The plasma membrane preparation contained 5.7% of the alkaline phosphatase and 0.8% of the protein of the homogenate. Six samples were suspended in 1 mM NaHCO₃ at a final protein concentration of 4.3 mg/ml and at digitonin concentrations ranging from 0 to 2.1 mg/ml. Isopycnic centrifugation was carried out in a SW50 rotor (50,000 rpm, 210 min).

in sucrose gradient as such or after treatment with digitonin.

In the experiment reported in Fig. 10, care was taken to minimize breakage of the membranes during the purification procedure. After density equilibration (Fig. 10 a), alkaline phosphatase is largely recovered in a single subfraction, with an average density of 1.175. That subfraction contains ~60% of the 5'-nucleotidase activity, which shows a second peak at 1.14. Protein behaves like alkaline phosphatase, except for a small excess in the low-density part of the gradient. As a consequence of gradual mechanical disruption (Fig. 10 b and c), all distributions become broader. More strikingly, 5'-nucleotidase separates from alkaline phosphatase and protein. Although the bulk of these two constituents remains centered around the density of 1.18, the peak of 5'-nucleotidase in this part of the gradient progressively disappears, with concomitant increase of the light peak. This sharp dissociation between 5'-nucleotidase and alkaline phosphatase after mechanical disruption of membranes was found consistently. Alkaline phosphodiesterase I and leucine-β-naphthylamidase behave in an in-

termediate fashion, and protein follows alkaline phosphatase more closely than any other enzyme (not shown). Like us, House et al. (37) obtained a heavy subfraction enriched in alkaline phosphatase with respect to other plasma membrane enzymes; others (64, 68), however, have found alkaline phosphatase and 5'-nucleotidase concentrated in light subfractions.

Gradient subfractions from an Ultra-Turrax-treated preparation were examined under the electron microscope. The light subfractions, enriched in 5'-nucleotidase, appear quite heterogeneous and contain a number of very small membranous elements (Fig. 11 a). Heavy subfractions, enriched in alkaline phosphatase, comprise larger elements, in the form of vesicles or membrane fragments with many junctional structures (Fig. 11 b). Transition between these aspects is gradual with increasing density.

Although 5'-nucleotidase dissociates from alkaline phosphatase in sheared membranes, Fig. 12 shows that both enzymes remain associated with digitonin-sensitive membrane fragments. No activity to speak of is recovered below the density
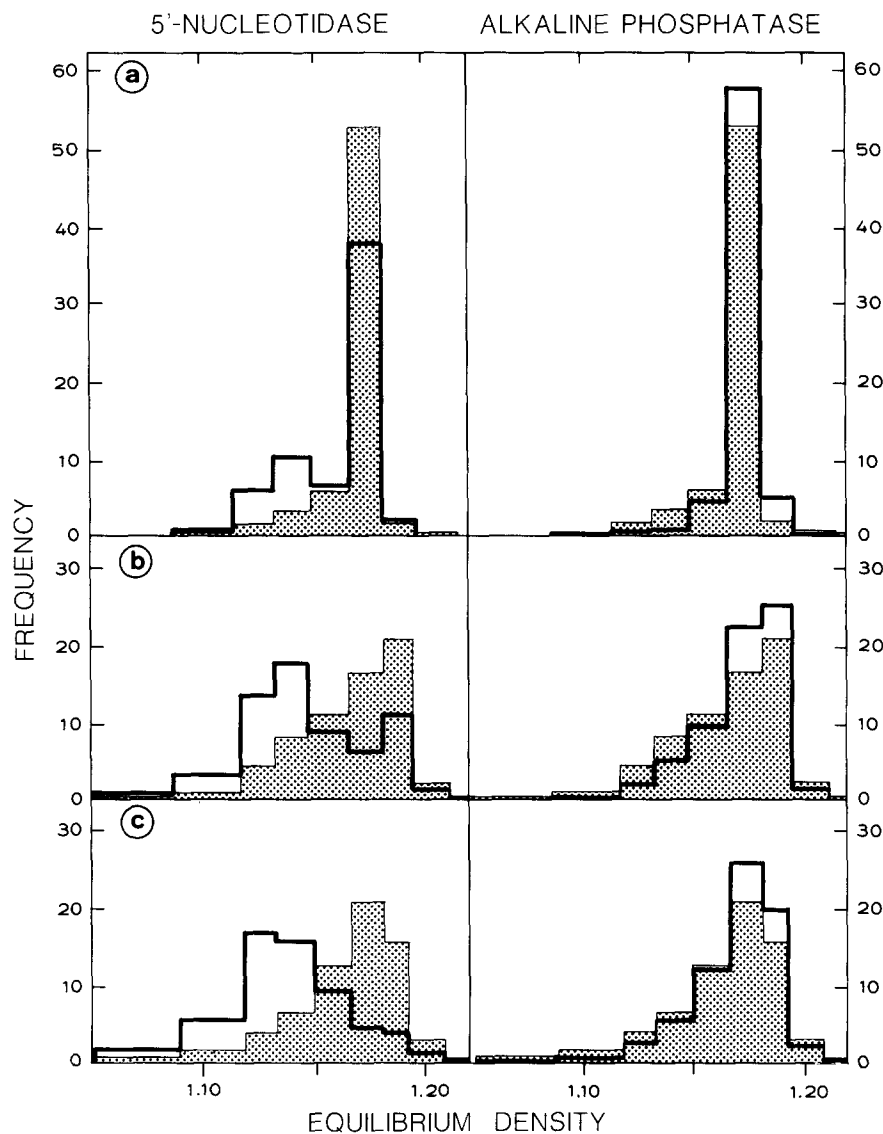

FIGURE 10   Effect of mechanical disruption on the density distributions of plasma membrane constituents. A first sample served as control (a). Two other samples (b and c) were treated in a 220-V-designed Ultra-Turrax, operated at 160 V for 10 s. Portion c was further treated three times at 180 V for 20 s. Isopycnic centrifugation was carried out in a SW65 rotor (65,000 rpm, 180 min). The shaded histograms are the distribution profiles of protein. Yields of 5'-nucleotidase, alkaline phosphatase, and protein were 25%, 15%, and 1.5% of the liver content, respectively.

of 1.16 after digitonin treatment. Moreover, the distribution profiles become narrower and overlap more markedly. Alkaline phosphodiesterase I, leucine-β-naphthylamidase, and the major part of the protein are also displaced to the same portion of the gradient (not shown).

GOLGI PREPARATIONS: The results of two isopycnic centrifugation experiments carried out on Golgi preparations are presented in Figs. 13 and 14. As described under Materials and Methods, the original Golgi preparation was first resuspended in 0.25 M sucrose–3 mM imidazole, pH 7.4. After addition of digitonin to a portion of the suspension, the samples were centrifuged and resuspended in digitonin-free 0.25 M sucrose. The sucrose-washed and digitonin-treated preparations layered on the gradients contained ~95% of the enzyme activities of the original Golgi preparation, together with, respectively, 85–90% and 75–80% of the protein. Presumably, owing to damage suffered in the course of this series of operations, Golgi elements released part of their secretory content.

In Fig. 13 (left-hand side) the distribution of galactosyltransferase (shaded) is compared to those of sialyltransferase and N-acetylglucosaminyltransferase. The three glycosyltransferases have nearly identical density distributions, in the control sample (a) as well as in the digitonin-treated sample (b). Digitonin treatment resulted in a shift of the three enzymes to higher densities. This shift (~0.02 density unit) is less than that reported in Table III for the enzymes characteristic of plasma membrane preparations. Its mechanism is still unknown. Both the formation of a complex with cholesterol (present either in the membrane or in the lumenal lipoprotein material) or a partial release of the lumenal content might result in an in-

creased density. Although the morphological alterations caused by digitonin are apparently restricted to some Golgi elements, the density distribution of the glycosyltransferases remains unimodal.

Because the yield of the three glycosyltransferases in Golgi preparations rarely exceeds 30% of the liver content, their density distributions have also been examined in microsomal fractions (Fig. 13, right-hand side). Consistently, 75–80% of these enzyme activities were recovered in such fractions. The density distributions obtained (c: control; d: digitonin) are essentially the same as those of the Golgi preparation, except that they are somewhat less dispersed around the peak densities. As in Golgi preparations, the distribution profiles of the three enzymes overlap almost perfectly, and digitonin induces a moderate increase in the equilibrium density.

Enzymes recovered with a low yield in Golgi preparations were also assayed in density gradient subfractions. The density distributions obtained from control and digitonin-treated preparations are compared in Fig. 14. In spite of the overlap (due to the isolation procedure which selects the material banding at a 1.07–1.14 density interface), the partial dissociation of these enzymes from galactosyltransferase contrasts with the much closer fit found within the glycosyltransferase group (Fig. 13). Most characteristically, the trace activities of 5'-nucleotidase and alkaline phosphodiesterase I show a distinctly greater shift (0.035 and 0.045 density unit, respectively) by digitonin than galactosyltransferase, whereas glucose-6-phosphatase and NADH cytochrome c reductase are practically unaffected by the digitonin treatment. Protein roughly follows galactosyltransferase. The excess over galactosyltransferase recovered in
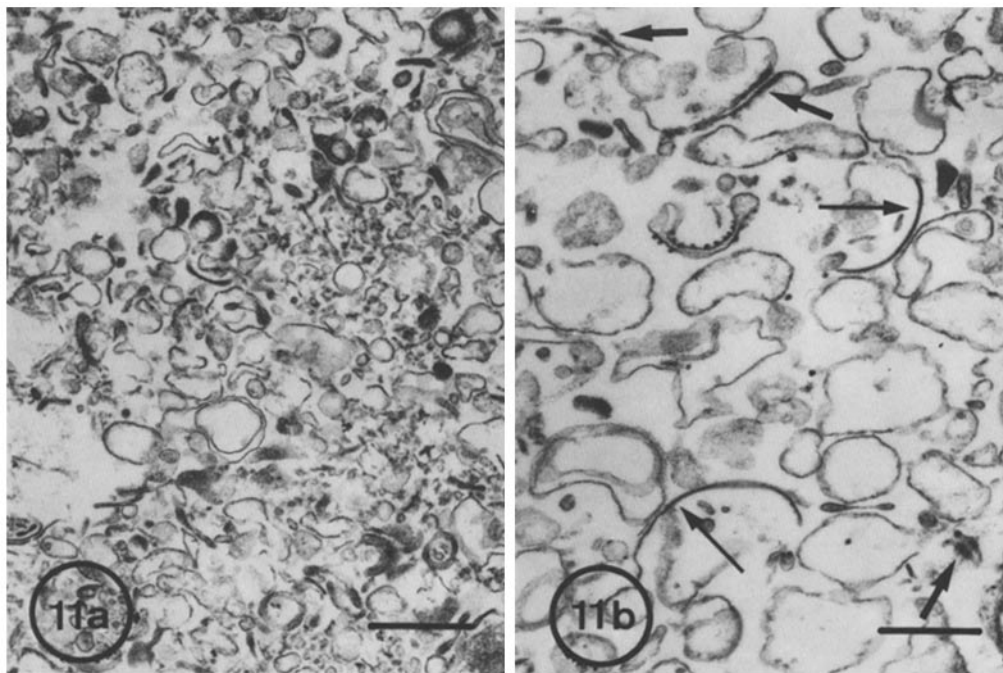

FIGURE 11 Morphological appearance of two subfractions from a disrupted plasma membrane preparation. The preparation was treated with an Ultra-Turrax operated at 220 V for 20 s, and then subfractionated by density equilibration. (a) Light subfraction (d = 1.11): it contains 0.07% of the protein in liver; the relative specific activities of 5'-nucleotidase and alkaline phosphatase are 39 and 3.2, respectively. This heterogeneous subfraction shows, in addition to medium-sized empty vesicles and amorphous material, numerous minute elements, either membrane fragments or tubules and vesicles, often containing electron-dense material. (b) Heavy subfraction (d = 1.17): it contains 0.15% of the protein in liver; the relative specific activities of 5'-nucleotidase and alkaline phosphatase are 5.3 and 11.3, respectively. Elements recovered in this subfraction are distinctly larger. Several desmosomes (short arrows) and gap junctions (long arrows) are recognizable. A rough cisterna is clearly visible. Bars, 0.5 μm. × 27,000.

the lightest subfractions might reflect additional release of protein from damaged vesicles. In other experiments (not shown), we found that cholesterol follows 5'-nucleotidase more closely than galactosyltransferase.

On electron microscope examination, the light subfractions are abundant in VLDL-rich vesicles (Fig. 15a), as expected. On the heavy side of the glycosyltransferase peak, the subfractions from the sucrose-washed preparations consist predominantly of profiles reminiscent of flat cisternae and tortuous interconnected tubules (Fig. 15b), whereas the subfractions from the digitonin-treated preparations characteristically contain many weakly contrasted tubular profiles (Fig. 16a). The densest subfractions (Figs. 15c and 16b) show a majority of non-Golgi elements, such as rough vesicles, dense bodies, peroxisomes, and peroxisomal cores. Some membranes with the discontinuous aspect seen in digitonin-treated plasma membranes (Fig. 2) are also recognizable in the heavy subfractions from digitonin-treated Golgi preparations (Fig. 16b).

OUTER MITOCHONDRIAL MEMBRANE PREPARATIONS: As in other membrane preparations, we found no evidence of a loss of enzyme activity after the treatment of outer mitochondrial membrane preparations with digitonin. After density equilibration (Fig. 17), monoamine oxidase and NADH cytochrome c reductase are clearly distinguished from the other enzymes. They are confined to the upper part of the gradient, at densities lower than 1.15, contrasting sharply with cytochrome c oxidase, which is recovered in the lower part of the gradient, and with esterase, which is spread over a wide density span between 1.10 and 1.25. The dissociation of monoamine oxidase and NADH cytochrome c reductase from 5'-nucleotidase, alkaline phosphodiesterase I, and cholesterol (not shown),

which all peak at 1.15–1.16 in the untreated preparation, is strongly strengthened after digitonin treatment. Indeed, these latter constituents are then shifted toward higher densities, by ~0.035 density unit, whereas the other enzymes retain their original equilibrium densities.

The specific activity of monoamine oxidase and NADH cytochrome c reductase is increased twofold in the upper part of the gradient. In these light subfractions from either untreated (not shown) or digitonin-treated (Fig. 18a) samples, the electron microscope shows essentially empty membranous bags of various shapes, 0.2–0.5 μm in size, some of which consist of 2 or 3 concentric membrane profiles. The main components of heavy subfractions are swollen mitochondrial ghosts stripped of their outer membrane and fragments of the rough endoplasmic reticulum. Besides, after digitonin treatment, broken-looking vesicles of the aspect described for plasma membranes are clearly recognizable (Fig. 18b). At the concentration used, digitonin has no detectable effect on the morphology of outer and inner mitochondrial membranes.

DISCUSSION

Behavior of Enzymes in Fractionation Experiments and Their Classification into Groups

The results reported above clearly show that, in general, each enzyme behaves in a similar fashion in every kind of membrane preparation analyzed, including microsome preparations. The differences noted (e.g., in the average densities) usually fall within the range expected for different, nonrandom samples of a polydisperse entity. As a consequence, the classification arrived at from our earlier studies on the microsome fraction (4, 10) is strengthened by these additional data.

GROUP a1: The distribution of monoamine oxidase in preparations of outer mitochondrial membranes centrifuged to equilibrium is almost identical to that of the microsomal activity reported earlier (4, 10). It is characterized by a low equilibrium density (~1.12), a sharp distribution profile, and a complete lack of sensitivity to digitonin (Fig. 17). In other membrane preparations, the level of monoamine oxidase is very low or similar to that of cytochrome c oxidase (Table I).

GROUP a2: In all the membrane preparations examined, 5'-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphatase have in common the distinguishing property of being shifted (0.03–0.04 density unit) to markedly higher equilibrium densities by digitonin. This property, originally observed in microsomes (4, 63), is demonstrated here for the plasma membrane (Figs. 8, 9, and 12), Golgi complex (Fig. 14), and outer mitochondrial membrane (Fig. 17) preparations. This group otherwise shows evidence of heterogeneity, especially in the plasma membrane–rich preparation subjected to shearing (Figs. 10 and 12), but also in the microsome fraction (10). On the average, the equilibrium density of group a2 enzymes is higher (~1.17; see Table III) in the plasma membrane preparations than in microsomes (~1.14 [10]). As suggested by Wisher and Evans (68), this higher density might result from the presence of protein-rich junctional complexes in plasma membranes prepared according to variations of Neville's method (50). This interpretation is consistent with the morphological data showing that junctional complexes are prominent in the heaviest subfractions from sheared plasma membranes (Fig. 11).

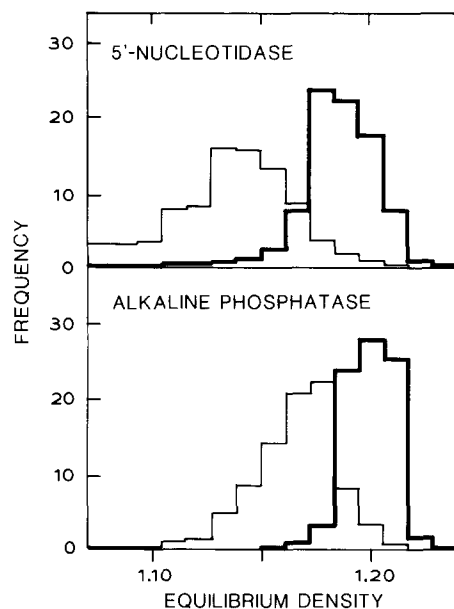GROUP a3: Group a3 includes three glycosyltransferase

FIGURE 12 Influence of digitonin on the density distributions of 5'-nucleotidase and alkaline phosphatase after mechanical disruption of membranes. The plasma membrane preparation was disrupted in an Ultra-Turrax (see Fig. 11) and a sample was treated with digitonin at a final concentration of 2.8 mg/ml (digitonin:cholesterol molar ratio of 1.1). Isopycnic centrifugation was carried out in a SW65 rotor (65,000 rpm, 180 min). Normalized histograms are given in thick line for the digitonin-treated preparation and in thin line for the control (no digitonin) sample. Yields of 5'-nucleotidase and alkaline phosphatase in the plasma membrane preparation were 23 and 11% of the liver content, respectively.

activities involved in the synthesis of the peripheral portion of the saccharide chain of complex glycoproteins. N-Acetylglucosaminyltransferase, galactosyltransferase, and sialyltransferase are recovered with high yield and increased specific activity in microsomes and in Golgi-rich fractions. In both membrane fractions, their equilibrium density in a sucrose gradient is low (1.12–1.13) and moderately sensitive to digitonin (Fig. 13). The digitonin shift (0.02–0.025) clearly differentiates the glycosyltransferases under study, from the a2 constituents on the one hand and from glucose-6-phosphatase and NADH cytochrome c reductase on the other (Fig. 14 and reference 4). As noted in another study (1), the digitonin shift experienced by galacto-

syltransferase in plasma membrane preparations is also less marked than that of 5'-nucleotidase and other group a2 enzymes. The fair enzymic homogeneity evidenced by the coincidence of the density profiles in Golgi preparations and in microsomes (Fig. 13) is in keeping with the data of Bretz et al. (17) on Golgi fractions isolated from ethanol-fed animals. It is an additional distinguishing feature of this group.

GROUPS b AND c: Taking into account the well-established presence of NADH cytochrome c reductase in the outer envelope of mitochondria (25, 60), it appears that glucose-6-phosphatase and other representatives of groups b and c are found only in trace amounts in the three membrane prepara-
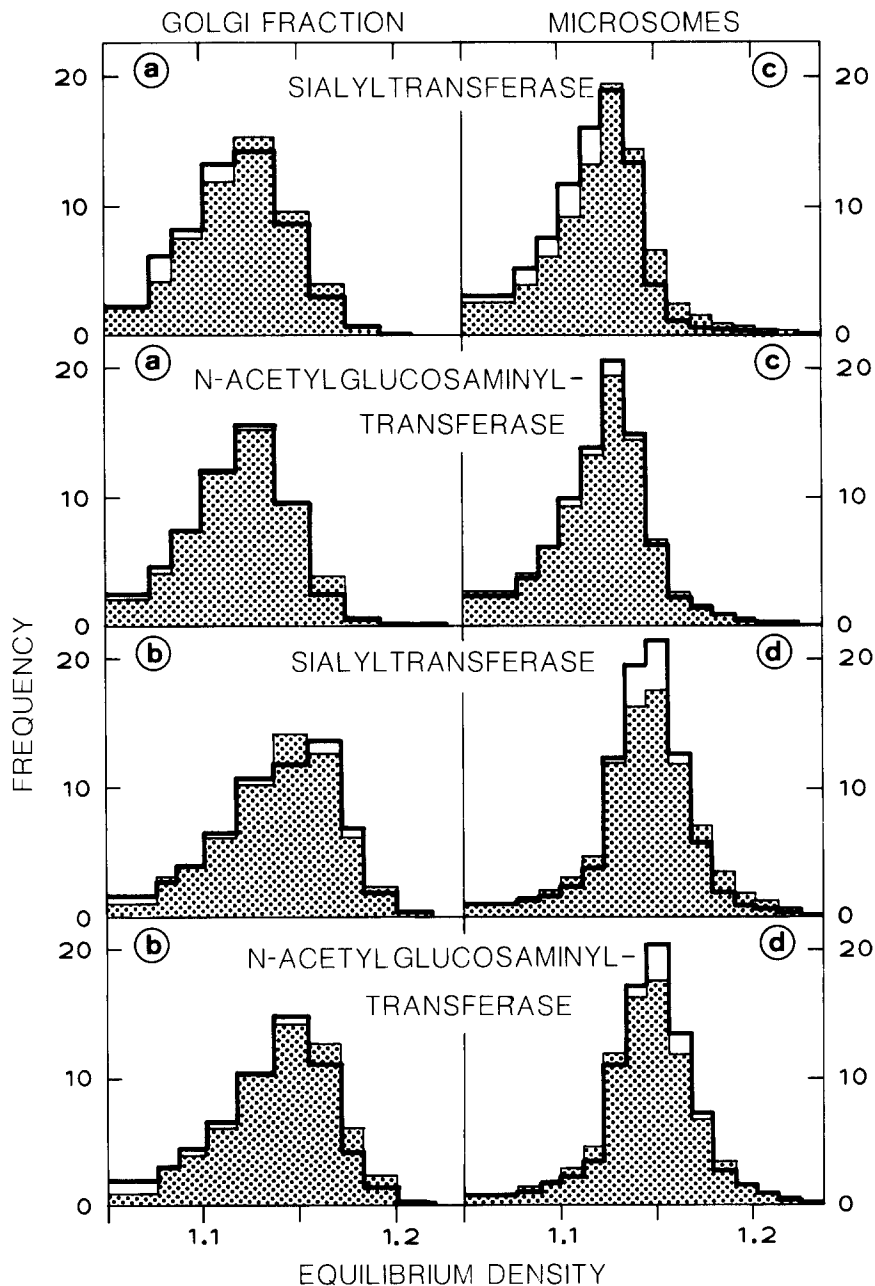

FIGURE 13 Density distributions of glycosyltransferases in Golgi complex preparations and microsomal fractions. The left-hand panels show the distribution profiles obtained with a Golgi preparation. The profiles given by the untreated and the digitonin-treated sample are indicated by a and b, respectively. The right-hand panels show normalized and averaged distribution patterns obtained from two to six untreated (c) or digitonin-treated (d) microsomal fractions. The distribution of galactosyltransferase is reproduced on each panel in shaded form. Isopycnic centrifugation was carried out in the E-40 rotor. The yields of glycosyltransferases were 15–21% of total activity in liver in the Golgi preparation, and 75–80% in the microsomes. The molar ratio digitonin: cholesterol was 0.9 and 1.4 for the Golgi preparation and the microsomal fractions, respectively.

tions (Table I). As a rule, their density distributions differ from those of the typical enzymes of these preparations, and they are not modified after digitonin treatment (Figs. 8, 14, and 17). The displacement of part of the glucose-6-phosphatase activity induced by digitonin in plasma membrane preparations apparently contradicts the usual behavior of this enzyme and would be compatible with 2-3% of the enzyme in liver being truly present in subcellular entities sensitive to digitonin. The question remains, however, whether the digitonin-sensitive activity corresponds to the authentic glucose-6-phosphatase or to another pH 5-labile phosphomonoesterase.

In contrast with our findings, Borgese and Meldolesi (16) have recently reported that part of the NADH cytochrome c reductase activity in their Golgi preparation, like galactosyltransferase, was shifted by digitonin. Moreover, NADH cytochrome c reductase attained a comparatively high specific activity in their preparation and seemed less stable than the homologous microsomal enzyme. This peculiar, digitonin-sensitive NADH cytochrome c reductase was not detected in this work, possibly due to differences in the experimental conditions, including animal strain, liver perfusion, and, as suggested by Borgese and Meldolesi (16), the initial method of selection of the material. Interestingly, NADPH cytochrome c reductase was not shifted by digitonin in the Golgi preparation analyzed by these authors (16).

The differences between microsomal groups b and c result from a heterogeneous distribution of the respective enzymes along the rough and smooth portions of endoplasmic reticulum
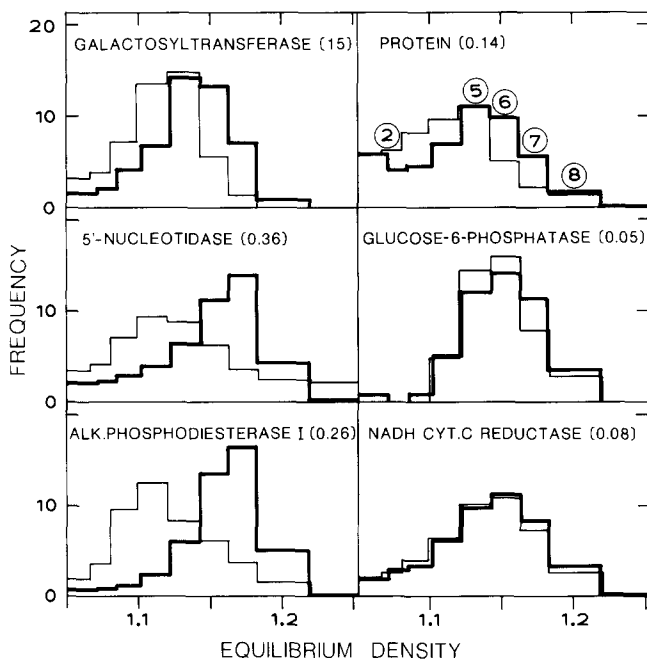

FIGURE 14 Density distributions of constituents in a Golgi complex preparation. Percent yields of constituents in the Golgi sample analyzed are indicated in parentheses. After isopycnic centrifugation in the E-40 rotor, nine subfractions were obtained from the untreated (thin line) and digitonin-treated (thick line) samples. The data for glucose-6-phosphatase correspond to the pH 5-labile activity (35% of the total activity in this Golgi preparation). In this experiment, 5'-nucleotidase was measured with a more sensitive assay. The usual incubation medium was supplemented with [³H]-AMP (0.2 Ci/mol), and the [³H]adenosine formed was determined according to Avruch and Wallach (5). The numbers in the protein panel refer to the subfractions illustrated in Figs. 15 and 16.

(55), which is perhaps related to their transmembrane topology (2). Otherwise the arrangement of microsomal enzymes into distinct groups has been interpreted as indicating the existence of a distinct membrane entity of the cell behind each group (4, 10). This interpretation is rendered more plausible by the finding that the various groups retain their identity in several different purified membrane preparations fairly well and further dissociate when these preparations are subjected to additional fractionation means. Moreover, noncentrifugal fractionation methods applied to Golgi preparations have yielded results that largely corroborate our own findings. Galactosyltransferase was markedly dissociated from NADH cytochrome c reductase and NADPH cytochrome c (P-450) reductase when Golgi preparations were reacted with anti-NADPH cytochrome c reductase-coated beads (41) or subfractionated by countercurrent distribution in an aqueous polymer two-phase system (35, 36).

Morphological Equivalents of the Biochemically Characterized Membrane Entities

Referring to the enzyme properties of purified membrane preparations reported by others, we have previously related (4, 10) the various entities of the microsome fraction with the outer mitochondrial membrane ($a1$), the plasma membrane ($a2$), the Golgi apparatus ($a3$), and the endoplasmic reticulum (b and c groups). These preliminary identifications are supported by our present biochemical and morphological data. Indeed, purified outer mitochondrial membranes are not affected by digitonin, in agreement with the behavior of the $a1$ microsomal component. Authentic plasma membrane elements are altered in their density and morphology in a manner nearly identical to that seen for the $a2$ entity in the microsome fraction. Likewise, as stressed above, the digitonin shift of characteristic Golgi elements is similar to that of the $a3$ microsomal entity. It is also significant that the extent of alteration in the morphology caused by digitonin is related to the density shift. Overtly apparent in plasma membranes (Fig. 2), morphological alteration is more subtle in Golgi components (Figs. 6 and 7) and undetectable in other membranes, including the outer and inner membranes of mitochondria (Fig. 18 a and b) and membranes derived from endoplasmic reticulum (4).

To decide whether the subcellular preparations are made up of sharply defined membrane entities, each of which coincides with one of the major membrane systems of the cell, we should be able to compare, on a quantitative basis, the composition of each preparation as it can be established from either its biochemical or its morphological characteristics. One difficulty with this comparison stems from the biochemical heterogeneity noted within the entities that we relate to the plasma membrane and endoplasmic reticulum. The major difficulty, however, lies in the identification of membranes in micrographs of subcellular fractions with respect to their origin in the cell (see reference 44). Although some contaminants, such as damaged mitochondria and rough microsomes, can be clearly recognized (Figs. 3, 4, 6, 11 b, 15 c, 16 b, and 18 b), many smooth membrane elements are barely identifiable in the absence of the topological features existing in the intact tissue. Vesicles that are altered by digitonin in the same way as the plasma membranes have been detected in Golgi- and outer mitochondrial membrane-rich preparations; after subfractionation in a sucrose gradient, these vesicles are particularly prominent in the subfractions endowed with a relatively high specific activity of 5'-nucleotidase and alkaline phosphodiesterase I (Figs. 16 b
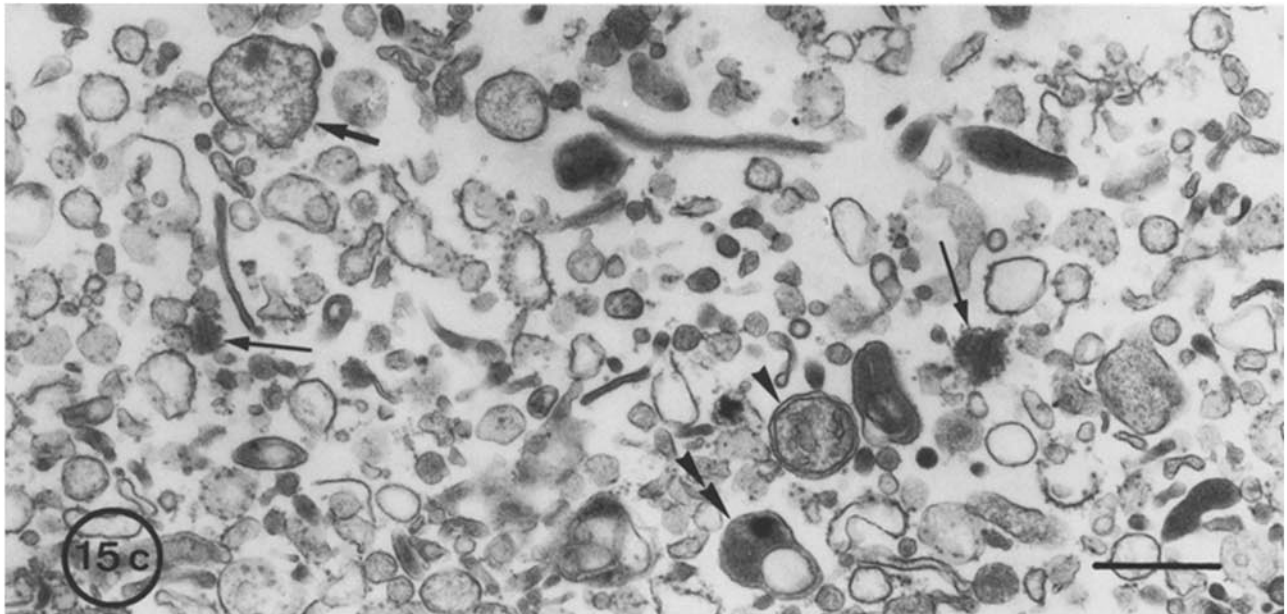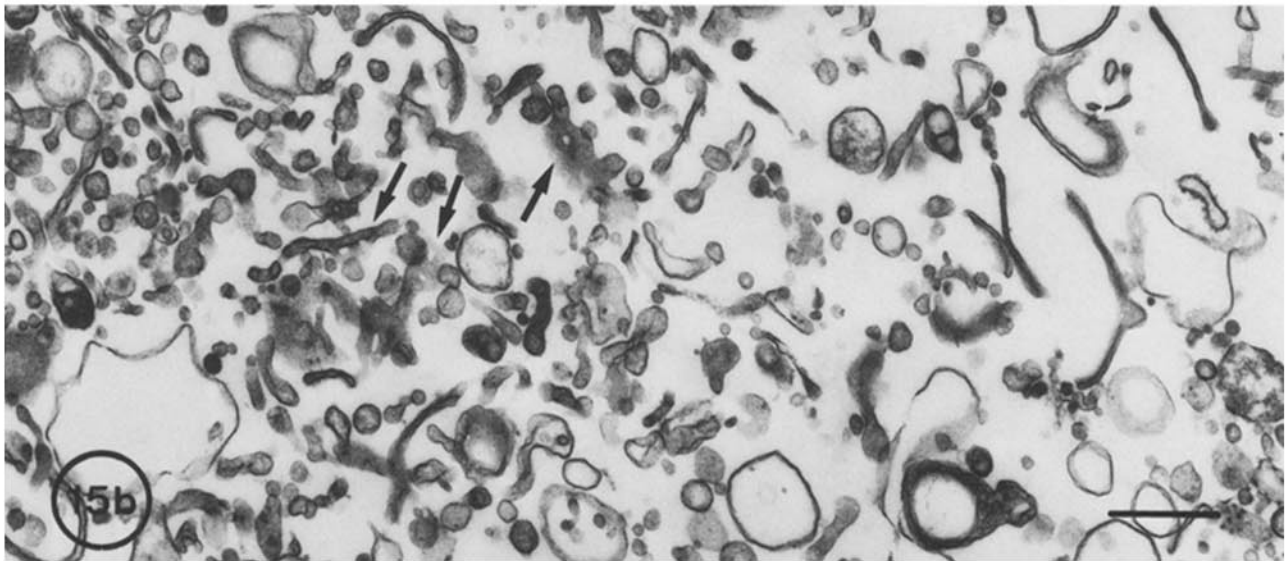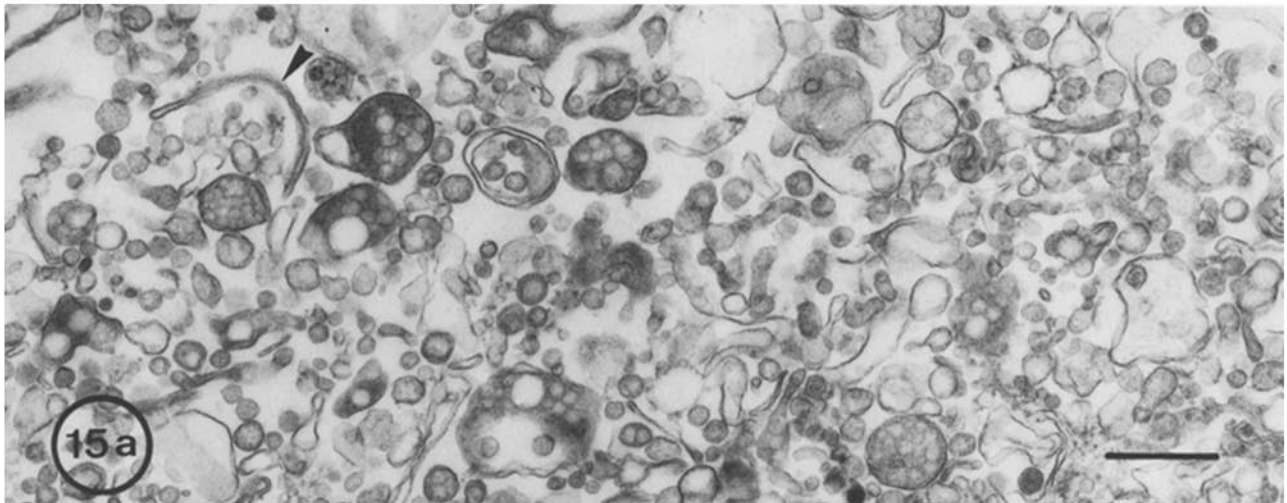
FIGURE 15   Micrographs of subfractions from a control Golgi complex preparation. See Fig. 14 for the density and the biochemical properties of these subfractions. (a) Subfraction 2: most vesicles and vacuoles contain VLDL particles. A few cisternal profiles (arrowhead) are present. × 30,500. (b) Subfraction 5: VLDL particles are nearly absent. Numerous profiles clearly originate from variously oriented sections through cisternal elements. Fenestrations are apparent in tangential sections (arrows). × 30,200. (c) Subfractions 7 and 8 (mixed): this highly heterogeneous fraction is abundant in rough vesicles. The micrograph also shows a peroxisome (short arrow), peroxisomal cores (long arrows), a mitochondrion (arrowhead), a lysosome (double arrowhead), and elongated cisternal profiles. × 33,000. Bars, 0.5 µm.
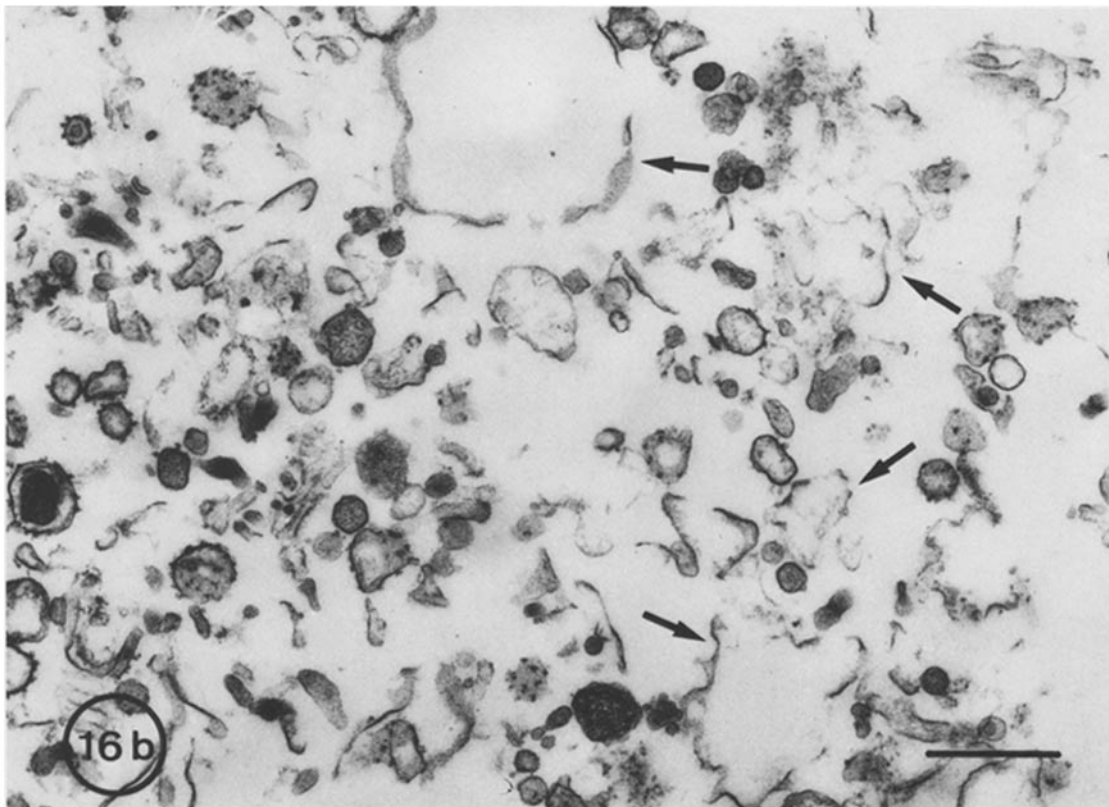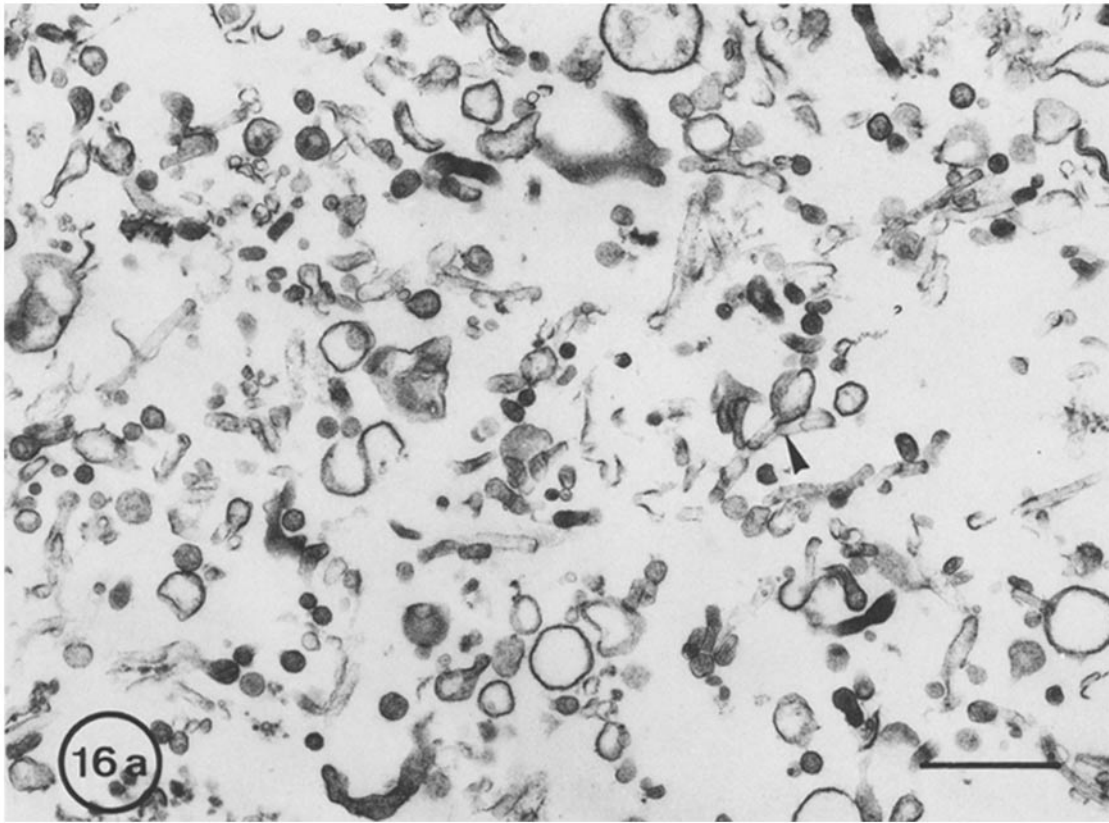
FIGURE 16 Micrographs of subfractions from a digitonin-treated Golgi preparation. See Fig. 14 for the density and the biochemical properties of these subfractions. (a) Subfraction 6: the small, elongated, poorly stained profiles described in Fig. 6 are a major component of the subfraction. Some aspects suggest a continuity between these elements and unmodified membranes (arrowhead). (b) Subfraction 8: in addition to numerous rough vesicles, this micrograph shows rather large elements limited by a discontinuous membrane (arrows). This aspect is characteristic of digitonin-treated plasma membranes (Fig. 2). Bars, 0.5 μm. × 36,000.
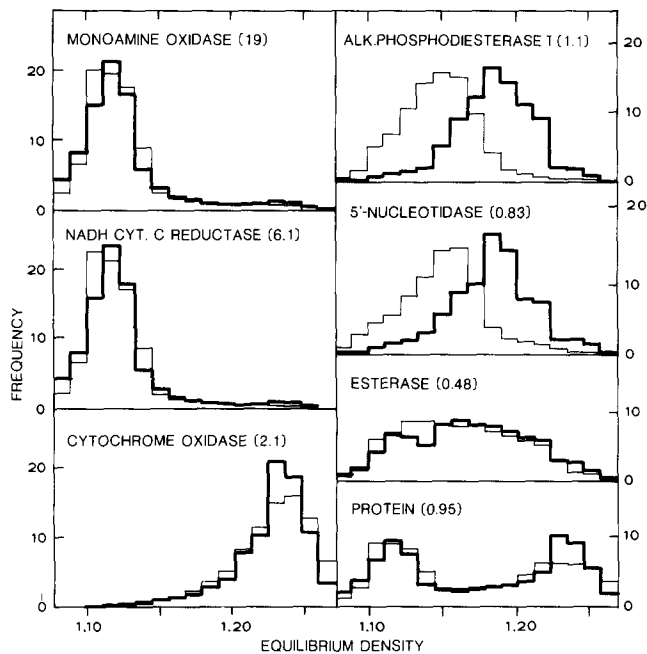
FIGURE 17 Density distributions of constituents in control and digitonin-treated samples from outer mitochondrial membrane preparations. Results from two experiments have been averaged. Yields of constituents are given in parentheses, in percent of the liver content. The distribution profiles obtained with the control (thin line) and the digitonin-treated (thick line) samples were superimposed after normalization.

and 18b; see also reference 4 for microsomes), demonstrating the occurrence of the a2 entity as a contaminant in the original membrane preparations. In the absence of digitonin treatment, this contamination would escape detection under the electron microscope. This fact illustrates the limitation of morphological methods in assessing the true degree of purification of subcellular membranes and casts doubts on the meaning of the morphometric data reported in some studies (42, 57).

The biochemical properties of Golgi-rich fractions indicate a significant degree of potential contamination (Table II). Because the relative specific activities of the enzymes of groups b and c are on the order of 1, the contribution of elements derived from the endoplasmic reticulum to the total protein may be about the same in these preparations as in the original homogenate. The maximal relative specific activity of glucose-6-phosphatase in microsome subfractions being ~5 (derived from references 3 and 10), a fair estimate is ~20%. Similar reasoning leads to the estimate that ~6% of the protein is contributed by the a2 entity. It is possibly less in view of the heterogeneity disclosed in this and in other works (28, 37, 64, 68) on plasma membranes. Although high, the value arrived at for contamination by the endoplasmic reticulum is not truly incompatible with morphology. Contrary to this interpretation, Hino et al. (35, 36) concluded that in their Golgi preparations endoplasmic reticulum and plasma membrane enzymes are not associated with contaminants, because in aqueous polymer two-phase systems they are distributed differently from their homologues in microsomal and plasma membrane-rich preparations. However, because they are recovered with low yield
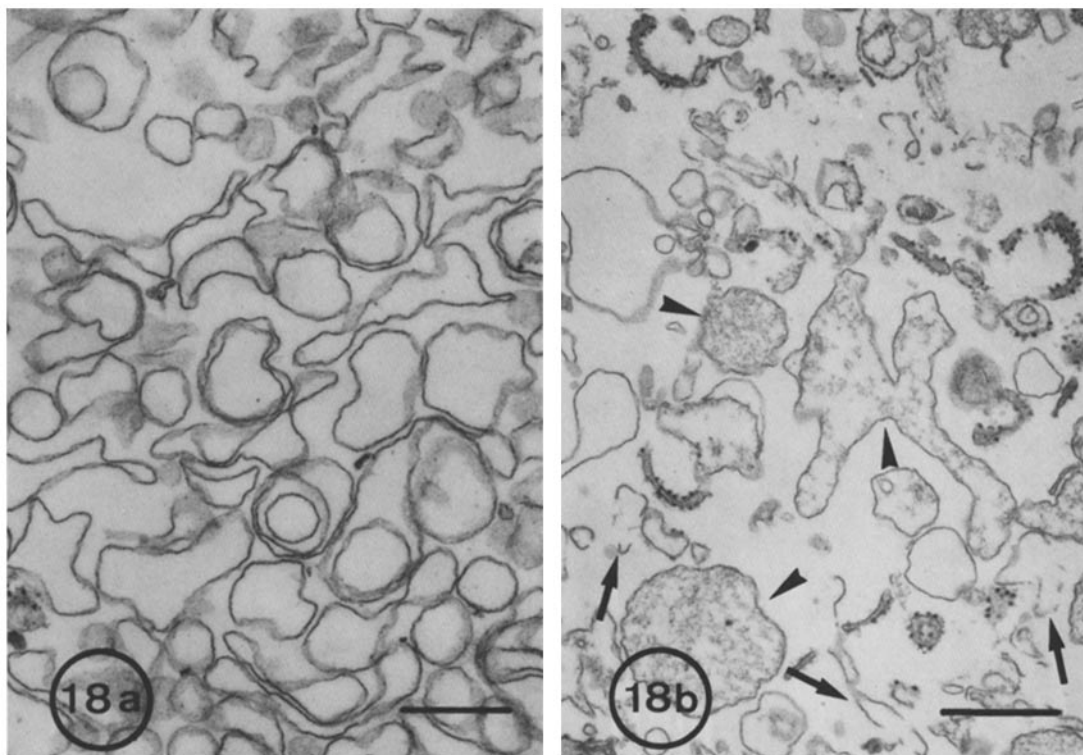

FIGURE 18 Micrographs of subfractions obtained after isopycnic centrifugation of digitonin-treated outer mitochondrial membrane preparations. (a) Light subfraction (d = 1.12): it is made up of empty membranous bags, some with internalized membranes. Identical pictures were obtained from control preparations. × 27,000. (b) Heavy subfraction (d = 1.19): this subfraction is clearly more heterogeneous. The large profiles containing a fuzzy material (arrowheads) probably correspond to swollen mitochondrial matrix compartments delimited by inner membranes (mitoplasts). Also noticeable are ribosome-coated elements and fenestrated profiles similar to those of digitonin-treated plasma membranes (arrows). × 31,500. Bars, 0.5 μm.

in Golgi-rich fractions, and because the putative contaminants are enzymically heterogeneous, the evidence is not conclusive. More impressive evidence in favor of the presence of endoplasmic reticulum and plasma membrane enzymes in bona fide Golgi elements are the following morphological arguments. (a) Cytochemical tests reveal 5'-nucleotidase (30) and adenylate cyclase (19) activity in VLDL-filled vacuoles (see also reference 43) and along the distended ends of cisternal profiles in Golgi preparations isolated from ethanol-treated rats. (b) Most of the elements specifically adsorbed on beads coated with anti-NADPH cytochrome c (P-450) reductase contain clustered VLDL particles (41), a typical feature of the Golgi complex. However, in intact cells, rows of VLDL particles have occasionally been detected in tubular profiles considered to be part of the smooth endoplasmic reticulum (20, 27, 52). Moreover, clustered VLDL particles have also been described in GERL, an organelle thought to be a specialized hydrolase-rich region of the endoplasmic reticulum (51). The use of lipoprotein particles as a hallmark of Golgi elements has been criticized by Bergeron (13), who found numerous such particles bound to the sinusoidal membrane. Upon homogenization, VLDL particles could remain entrapped within inside-out elements formed from this highly convoluted zone of the plasma membrane.

To reconcile these seemingly contradictory arguments, perhaps we must reconsider the view that each of the membrane entities evidenced by the centrifugal behavior of enzymes exactly corresponds to one of the structural entities defined on a purely morphological basis. Indeed, if Golgi preparations are taken to be completely pure, our results indicate that the Golgi apparatus consists of at least three biochemically distinct membrane domains: a specific domain, characterized by several glycosyltransferases apparently distributed in an identical manner along their host membranes, and two other domains that are biochemically related to the endoplasmic reticulum and to the plasma membrane, respectively. This kind of heterogeneity is compatible with the data from other laboratories (35, 36, 41, 45; see also reference 61) and with the Golgi apparatus acting as a functional link between the endoplasmic reticulum and the cell periphery in the secretory process (53). It challenges the concept of a gradual transition in the membranes from the biochemical characters of the endoplasmic reticulum to those of the cell periphery (48), or else implies that the transitional elements are only a tiny fraction of the cell membranes, which cannot presently be demonstrated by cell fractionation techniques. The semantic questions raised have been discussed elsewhere (8, 24).

The levels of some enzyme activities in plasma membrane preparations also suggest significant contamination by mitochondrial membranes and by endoplasmic reticulum elements, which, together, may account for ~40% of the protein. In agreement with this estimation, we found that after treatment with digitonin half of the protein dissociates from alkaline phosphatase (Fig. 8), which, as judged from its behavior in disaggregated membranes (Fig. 10), is the most satisfactory marker of the a2 entity in plasma membrane preparations. The proportion of nonfenestrated elements seen in typical preparations after digitonin treatment (Fig. 3) is also compatible with contamination by digitonin-insensitive membranes. Clearly, the bulk of NADH cytochrome c reductase is associated with such elements.

The exact delineation of the cell structures underlying entity a2 raises questions identical to those we discussed above. As previously envisaged (1, 4, 10), this entity may, in addition to the plasma membranes, include some particular endomembranes of the cell. The elements enriched with 5'-nucleotidase that are found in low-density subfractions after shearing of the plasma membrane preparations could originate from the bile canaliculi, as suggested by Wisher and Evans (68). It is unclear why alkaline phosphatase accompanied 5'-nucleotidase in the "zonal-light" subfraction described by these authors, whereas, in our experiments, it was recovered with the bulk of protein in heavy subfractions enriched with junctional complexes.

Thus, the information now available shows that enzymes are generally confined to sharply defined domains of the cell membranes. There are noticeable exceptions, in particular the association of NADH cytochrome c reductase and cytochrome b_5 with the endoplasmic reticulum and the outer mitochondrial membrane. The presence of this electron transport chain in other types of membranes is more controversial (16, 32, 39, 42, 46, and this work). Furthermore, even some established exceptions may turn out to be more apparent than real because, for instance, the microsomal cytochrome b_5 differs from the haemoprotein(s) located in the outer mitochondrial membrane (33, 40). The mechanisms by which this confinement of enzymes in specific membrane domains is established and preserved is still a challenging question (for a discussion, see reference 8).

REFERENCES

1. Amar-Costesec, A. 1981. Analytical study of microsomes and isolated subcellular membranes from rat liver. VII. The distribution of protein-bound sialic acid. *J. Cell Biol.* 89: 62–69.
2. Amar-Costesec, A., and H. Beaufay. 1981. A structural basis of enzymic heterogeneity within liver endoplasmic reticulum. *J. Theor. Biol.* 89:217–230.
3. Amar-Costesec, A., H. Beaufay, M. Wibo, D. Thinès-Sempoux, E. Feytmans, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* 61: 201–212.
4. Amar-Costesec, A., M. Wibo, D. Thinès-Sempoux, H. Beaufay, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. IV. Biochemical, physical, and morphological modifications of microsomal components induced by digitonin, EDTA, and pyrophosphate. *J. Cell Biol.* 62:717–745.
5. Avruch, J., and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim. Biophys. Acta.* 233:334–347.
6. Baudhuin, P., P. Evrard, and J. Berthet. 1967. Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. *J. Cell Biol.* 32:181–191.
7. Beaufay, H., and A. Amar-Costesec. 1976. Cell fractionation techniques. *In* Methods in Membrane Biology. E. D. Korn, editor. Plenum Press, New York-London. 6:1–100.
8. Beaufay, H., A. Amar-Costesec, and C. de Duve. 1981. The topology of enzymes in microsomes from liver. *In* Mitochondria and Microsomes. C. P. Lee, G. Schatz, and G. Dallner, editors. Addison-Wesley Publishing Company, Reading, Mass. In press.
9. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thinès-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* 61:188–200.
10. Beaufay, H., A. Amar-Costesec, D. Thinès-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. *J. Cell Biol.* 61:213–231.
11. Beaufay, H., H. G. Hers, J. Berthet, and C. de Duve. 1954. Le système hexose-phosphatasique. V. Influence de divers agents sur l'activité et la stabilité de la glucose-6-phosphatase. *Bull. Soc. Chim. Biol.* 36:1539–1550.
12. Beaufay, H., P. Jacques, P. Baudhuin, D. Z. Sellinger, J. Berthet, and C. de Duve. 1964. Tissue fractionation studies. 18. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. *Biochem. J.* 92:184–205.

13. Bergeron, J. J. M. 1979. Golgi fractions from livers of control and ethanol-intoxicated rats. Enzymic and morphologic properties following rapid isolation. *Biochim. Biophys. Acta.* 555:493–503.

14. Bergeron, J. J. M., J. H. Ehrenreich, P. Siekevitz, and G. E. Palade. 1973. Golgi fractions prepared from rat liver homogenates. II. Biochemical characterization. *J. Cell Biol.* 59:73–88.

15. Bezkorovainy, A., and R. J. Winzler. 1961. Isolation and characterization of an acid α_2-glycoprotein. *Biochim. Biophys. Acta.* 49:559–565.

16. Borgese, N., and J. Meldolesi. 1980. Localization and biosynthesis of NADH cytochrome b_5 reductase, an integral membrane protein, in rat liver cells. I. Distribution of the enzyme activity in microsomes, mitochondria, and Golgi complex. *J. Cell Biol.* 85:501–515.

17. Bretz, R., H. Bretz, and G. E. Palade. 1980. Distribution of terminal glycosyltransferases in hepatic Golgi fractions. *J. Cell Biol.* 84:87–101.

18. Cassidy, J. T., G. W. Jourdian, and S. Roseman. 1965. The sialic acids. VI. Purification and properties of sialidase from *Clostridium perfringens. J. Biol. Chem.* 240:3501–3506.

19. Cheng, H., and M. G. Farquhar. 1976. Presence of adenylate cyclase activity in Golgi and other fractions from rat liver. II. Cytochemical localization within Golgi and ER membranes. *J. Cell Biol.* 70:671–684.

20. Claude, A. 1970. Growth and differentiation of cytoplasmic membranes in the course of lipoprotein granule synthesis in the hepatic cell. I. Elaboration of elements of the Golgi complex. *J. Cell Biol.* 47:745–766.

21. Colbeau, A., J. Nachbaur, and P. M. Vignais. 1971. Enzymic characterization and lipid composition of rat liver subcellular membranes. *Biochim. Biophys. Acta.* 249:462–492.

22. de Duve, C. 1971. Tissue fractionation. Past and present. *J. Cell Biol.* 50:20D–55D.

23. de Duve, C. 1976. Enzymatic properties of rat-liver cytomembranes. *Pontif. Acad. Sci. Scr. Varia.* 40:47–64.

24. de Duve, C., and H. Beaufay. 1981. A short history of tissue fractionation. *J. Cell Biol.* In press.

25. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* 60:604–617.

26. DePierre, J. W., and M. L. Karnovsky. 1973. Plasma membranes of mammalian cells. A review of methods for their characterization and isolation. *J. Cell Biol.* 56:275–303.

27. Ehrenreich, J. H., J. J. M. Bergeron, P. Siekevitz, and G. E. Palade. 1973. Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. *J. Cell Biol.* 59:45–72.

28. Evans, W. H. 1970. Fractionation of liver plasma membranes prepared by zonal centrifugation. *Biochem. J.* 116:833–842.

29. Elias, P. M., J. Goerke, D. S. Friend, and B. E. Brown. 1978. Freeze-fracture identification of sterol-digitonin complexes in cell and liposome membranes. *J. Cell Biol.* 78:577–596.

30. Farquhar, M. G., J. J. M. Bergeron, and G. E. Palade. 1974. Cytochemistry of Golgi fractions prepared from rat liver. *J. Cell Biol.* 60:8–25.

31. Fleischer, B., S. Fleischer, and H. Ozawa. 1969. Isolation and characterization of Golgi membranes from bovine liver. *J. Cell Biol.* 43:59–79.

32. Fowler, S., J. Remacle, A. Trouet, H. Beaufay, J. Berthet, M. Wibo, and P. Hauser. 1976. Analytical study of microsomes and isolated subcellular membranes from rat liver. V. Immunological localization of cytochrome b_5 by electron microscopy: methodology and application to various subcellular fractions. *J. Cell Biol.* 71:535–550.

33. Fukushima, K., and R. Sato. 1973. Purification and characterization of cytochrome b_5-like hemoprotein associated with outer mitochondrial membrane of rat liver. *J. Biochem. (Tokyo).* 74:161–173.

34. Goldberg, J. A., and A. M. Rutenburg. 1958. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. *Cancer (Phila.).* 11:283–291.

35. Hino, Y., A. Asano, and R. Sato. 1978. Biochemical studies on rat liver Golgi apparatus. II. Further characterization of isolated Golgi fraction. *J. Biochem. (Tokyo).* 83:925–934.

36. Hino, Y., A. Asano, and R. Sato. 1978. Biochemical studies on rat liver Golgi apparatus. III. Subfractionation of fragmented Golgi apparatus by counter-current distribution. *J. Biochem. (Tokyo).* 83:935–942.

37. House, P. D. R., P. Poulis, and M. J. Weidemann. 1972. Isolation of a plasma-membrane subfraction from rat liver containing an insulin-sensitive cyclic-AMP phosphodiesterase. *Eur. J. Biochem.* 24:429–437.

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

39. Huang, C. M., H. Goldenberg, C. Frantz, D. J. Morré, T. W. Keenan, and F. L. Crane. 1979. Comparison of NADH-linked cytochrome c reductases of endoplasmic reticulum, Golgi apparatus and plasma membrane. *Int. J. Biochem.* 10:723–731.

40. Ito, A. 1980. Cytochrome b_5-like hemoprotein of outer mitochondrial membranes: OM cytochrome b. I. Purification of OM cytochrome b from rat liver mitochondria and comparison of its molecular properties with those of cytochrome b_5. *J. Biochem. (Tokyo).* 87:63–71.

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

42. Jarash, E.-D., J. Kartenbeck, G. Bruder, A. Fink, D. J. Morré, and W. W. Franke. 1979. B-type cytochromes in plasma membranes isolated from rat liver, in comparison with those of endomembranes. *J. Cell Biol.* 80:37–52.

43. Little, J. S., and C. C. Widnell. 1975. Evidence for the translocation of 5'-nucleotidase across hepatic membranes in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 72:4013–4017.

44. Losa, G., E. R. Weibel, and R. P. Bolender. 1978. Integrated stereological and biochemical studies on hepatocytic membranes. III. Relative surface of endoplasmic reticulum membranes in microsomal fractions estimated on freeze-fracture preparations. *J. Cell Biol.* 78:289–308.

45. Matsuura, S., and Y. Tashiro. 1979. Immunoelectron-microscopic studies of endoplasmic reticulum-Golgi relationships in the intracellular transport process of lipoprotein particles in rat hepatocytes. *J. Cell Sci.* 39:273–290.

46. Meldolesi, J., G. Corte, G. Pietrini, and N. Borgese. 1980. Localization and biosynthesis of NADH-cytochrome b_5 reductase, an integral membrane protein, in rat liver cells. II. Evidence that a single enzyme accounts for the activity in its various subcellular locations. *J. Cell Biol.* 85:516–526.

47. Morré, D. J., R. L. Hamilton, H. H. Mollenhauer, R. W. Mahley, W. P. Cunningham, R. D. Cheetham, and V. S. Lequire. 1970. Isolation of a Golgi apparatus-rich fraction from rat liver. I. Method and morphology. *J. Cell Biol.* 44:484–491.

48. Morré, D. J., J. Kartenbeck, and W. W. Franke. 1979. Membrane flow and interconversions among endomembranes. *Biochim. Biophys. Acta.* 559:71–152.

49. Morré, D. J., L. M. Merlin, and T. W. Keenan. 1969. Localization of glycosyltransferase activities in a Golgi apparatus-rich fraction isolated from rat liver. *Biochem. Biophys. Res. Commun.* 37:813–819.

50. Neville, D. M. 1960. The isolation of a cell membrane fraction from rat liver. *J. Biophys. Biochem. Cytol.* 8:413–422.

51. Novikoff, P. M., and A. Yam. 1978. Sites of lipoprotein particles in normal rat hepatocytes. *J. Cell Biol.* 76:1–11.

52. Ovtracht, L., D. J. Morré, R. D. Cheetham, and H. H. Mollenhauer. 1973. Subfractionation of Golgi apparatus from rat liver: method and morphology. *J. Microsc. (Paris).* 18:87–102.

53. Palade, G. 1975. Intracellular aspects of the process of protein synthesis. *Science (Wash. D. C.).* 189:347–358.

54. Parsons, D. F., G. R. Williams, and B. Chance. 1966. Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Ann. N. Y. Acad. Sci.* 137:643–666.

55. Remacle, J., S. Fowler, H. Beaufay, A. Amar-Costesec, and J. Berthet. 1976. Analytical study of microsomes and isolated subcellular membranes from rat liver. VI. Electron microscope examination of microsomes for cytochrome b_5 by means of a ferritin-labeled antibody. *J. Cell Biol.* 71:551–564.

56. Schachter, H., I. Jabbal, R. L. Hudgin, L. Pinteric, E. J. McGuire, and S. Roseman. 1970. Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. *J. Biol. Chem.* 245:1090–1100.

57. Schilling, E. E., H. Goldenberg, D. J. Morré, and F. L. Crane. 1979. Distribution of insulin receptors among mouse liver endomembranes. *Biochim. Biophys. Acta.* 555:504–511.

58. Schnaitman, C., V. G. Erwin, and J. W. Greenawalt. 1967. The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria. *J. Cell Biol.* 32:719–735.

59. Song, C. S., W. Rubin, A. B. Rifkind, and A. Kappas. 1969. Plasma membranes of the rat liver. Isolation and enzymatic characterization of a fraction rich in bile canaliculi. *J. Cell Biol.* 41:124–132.

60. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J. Cell Biol.* 32:415–438.

61. Tartakoff, A. M. 1980. The Golgi complex: crossroads for vesicular traffic. *Int. Rev. Exp. Pathol.* 22:227–251.

62. Thinès-Sempoux, D. 1972. A comparison between the lysosomal and the plasma membrane. *In* Lysosomes in Biology and Pathology. J. T. Dingle, editor. Elsevier North-Holland, Amsterdam, The Netherlands. 3:278–299.

63. Thinès-Sempoux, D., A. Amar-Costesec, H. Beaufay, and J. Berthet. 1969. The association of cholesterol, 5'-nucleotidase, and alkaline phosphodiesterase I with a distinct group of microsomal particles. *J. Cell Biol.* 43:189–192.

64. Toda, G., H. Oka, T. Oda, and Y. Ikeda. 1975. Subfractionation of rat liver plasma membrane. Uneven distribution of plasma membrane-bound enzymes on the liver cell surface. *Biochim. Biophys. Acta.* 413:52–64.

65. Weimer, H. E., J. W. Mehl, and R. J. Winzler. 1950. Studies on the mucoproteins of human plasma. V. Isolation and characterization of a homogeneous mucoprotein. *J. Biol. Chem.* 185:561–568.

66. Wibo, M., A. Amar-Costesec, J. Berthet, and H. Beaufay. 1971. Electron microscope examination of subcellular fractions. III. Quantitative analysis of the microsomal fraction isolated from rat liver. *J. Cell Biol.* 51:52–71.

67. Wibo, M., D. Godelaine, A. Amar-Costesec, and H. Beaufay. 1976. Subcellular location of glycosyltransferases in rat liver. *In* The Liver. Quantitative Aspects of Structure and Function. R. Preisig, J. Bircher, and G. Paumgartner, editors. Editio Cantor, Aulendorf, W. Germany. 70–83.

68. Wisher, M. H., and W. H. Evans. 1975. Functional polarity of the rat hepatocyte surface membrane. Isolation and characterization of plasma-membrane subfractions from the blood-sinusoidal, bile-canalicular and contiguous surfaces of the hepatocyte. *Biochem. J.* 146:375–388.