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

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ABSTRACT

The subcellular distribution of NADH-cytochrome b_5 reductase in rat liver cells was reinvestigated. In fresh heavy and light Golgi fractions (GF_3 and GF_{1+2}) and in mitochondria, the specific activity of rotenone-insensitive NADH-cytochrome *c* reductase was ~100, 60, and 30%, respectively, of the value found in microsomes. However, the Golgi enzyme was unstable inasmuch as pelleting and resuspending the fresh fractions resulted in a considerable inactivation (40–60%), which was further increased with subsequent storage at 4°C. A similar inactivation was observed using cytochrome b_5 but not ferricyanide as electron acceptor. The inactivation of Golgi NADH-cytochrome *c* reductase activity was independent of the protein concentration of the fractions during storage, was unaffected by the addition of the antioxidant butylated hydroxytoluene, but was partly prevented by buffering the fractions at neutral pH and by storage at −20°C. A total Golgi fraction was analyzed by density equilibration on continuous sucrose gradients after exposure to digitonin. As expected, the distribution of both protein and galactosyl transferase were shifted to higher densities by this treatment. However, not all galactosyl transferase-bearing elements were shifted to the same extent by exposure to the detergent, suggesting a biochemical heterogeneity of the Golgi complex. In contrast to their behavior in microsomes, the distribution of NADH-cytochrome *c* reductase and cytochrome b_5 of Golgi fractions was shifted by digitonin, although to a lesser extent than that of galactosyl transferase. These results indicate that NADH-cytochrome b_5 reductase is an authentic component of Golgi membranes, as well as of microsomes and of mitochondria. The conflicting results reported in the past on the Golgi localization of the enzyme could be due, on the one hand, to the differential lability of the activity in its various subcellular locations and, on the other, to the heterogeneity of the Golgi complex in terms of both cholesterol and enzyme distribution.

Over the past years, differing views on membrane turnover (biogenesis and degradation of membrane components) and, in particular, on the biogenetic relations among various cell membranes have been presented. For instance, both free (8) and membrane-bound (48) polyribosomes have been proposed as the exclusive sites of synthesis of integral membrane proteins. Moreover, one point of view holds that all membranes derive from the rough-surfaced endoplasmic reticulum (ER)[1] by a combination of the processes of gradual transformation (differentiation) and flow (40), whereas, according to another opinion (36, 37), a greater degree of independent biogenesis might exist among the various membrane types.

Of course, such differing views on membrane biogenesis coexist because of the relative sparsity of reliable experimental data in this field. Technical problems in the study of the biosynthesis of membrane proteins are enormous because of the complexity of membrane protein composition and because of the relatively low turnover of most membrane proteins (see reference 55 for a recent review). On the one hand, purification of most membrane proteins is difficult because of their hydrophobic nature and their low concentration in the cell fractions used; on the other hand, contamination by even minute traces of rapidly synthesized proteins, such as secretory proteins, is likely to mask completely the behavior of the membrane protein being studied (10, 35).

Recently, important progress in the field has come from studies on the biosynthesis of viral membrane proteins and from the development of efficient cell-free translation systems (3, 25, 28, 29, 31, 54). The results of these studies are consistent with the idea (6, 32) that proteins restricted to the cytoplasmic face of membranes are made by free polyribosomes and inserted into the bilayer from the cytoplasm, whereas proteins spanning the membrane or exposed at the lumenal or external surfaces (which must, therefore, transfer a hydrophylic portion across the hydrophobic bilayer to reach their final destination) are made by bound polyribosomes and use the same mechanism as secretory proteins for their cotranslational transmembrane transfer (6, 7). These two classes of proteins have been designated endo- and ectoproteins, respectively (46). Clearly, both ecto- and endoproteins can be either integral or peripheral membrane proteins.

These considerations emphasize the need to study the biosynthesis of individual membrane proteins and to characterize them in terms of their localization with respect to the membrane bilayer. We have chosen to study the biosynthesis of a rat liver membrane protein, the flavoprotein NADH-cytochrome b_5 reductase, which is part of an electron transport chain involved in important processes such as fatty acid desaturation (50) and hydroxylation reactions (19). This protein is an integral membrane protein probably restricted to the cytoplasmic half of the bilayer (13). Moreover, it may have a multicompartment subcellular distribution inasmuch as the corresponding enzyme activity has been detected in several isolated cell fractions: microsomes, outer mitochondrial membranes, and Golgi fractions (5, 16, 20, 22, 24, 41, 45, 49). Because of its multicompartment distribution, NADH-cytochrome b_5 reductase appeared to be an ideal candidate for the study of biogenetic relations among membranes; on the other hand, as an endoprotein, it was suited to test the hypothesis that these proteins are synthesized by free polyribosomes and directly inserted at their final membrane location.

Because the Golgi location of NADH-cytochrome b_5 reductase has been questioned (1, 57), it appeared necessary to reinvestigate the subcellular distribution of the enzyme. The results of this part of the work are reported in this paper. In the following paper (38), we present evidence supporting the idea that the enzyme activity found in the various cell fractions is not due to isoenzymes but is attributable to a single enzyme protein. In the final paper[2], we report the results of the studies on the biosynthesis of the enzyme obtained with pulse-labeling and turnover experiments carried out in vivo.

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[1] *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; ER, endoplasmic reticulum; GF_{1+2} and GF_3, Golgi light and heavy fractions, respectively; MR, microsomes; PAGE, polyacrylamide gel electrophoresis; PCA, perchloric acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TH, total homogenate; VLDL, very low density lipoprotein.

[2] Borgese, N., G. Pietrini, and J. Meldolesi. 1980. Localization and biosynthesis of NADH-cytochrome b_5 reductase, an integral membrane protein, in rat liver cells. III. Evidence for the independent insertion and turnover of the enzyme in various subcellular compartments. *J. Cell Biol.* In press.

MATERIALS AND METHODS

General

Unless otherwise stated, sucrose solutions used were made up in double distilled water without added ions. "Buffered sucrose" refers to sucrose solutions containing 3 mM imidazole-HCl, pH 7.4. For spectrophotometric determinations, a Guilford type 2400 (Guilford Instrument Lab., Oberlin, Ohio) or a double beam Unicam SP 1800 (Pye Unicam Ltd., Cambridge, England) spectrophotometer was used. Ultracentrifugations were carried out in Spinco-Beckman ultracentrifuges (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Radioactivity counting was performed in an Intertechnique SL-30 (Intertechnique, Plaisir, France) liquid scintillation spectrometer.

Cell Fractionation

Male Sprague-Dawley rats, weighing 150–250 g and starved overnight, were sacrificed by decapitation. The livers were removed and immersed in ice-cold 0.25 M sucrose. All subsequent operations were carried out at 4°C.

After retrograde perfusion with cold 0.25 M sucrose, the livers were minced, passed through a tissue press, and homogenized in 0.25 M sucrose (4 vol) in a Potter-Elvehjem homogenizer with 10 strokes of a motor-driven (1,500 rpm) teflon pestle. Nuclei and cell debris were removed by low-speed centrifugation (10 min at 500 g_{av}), and the pellet was washed once by resuspension-sedimentation with a small volume of 0.25 M sucrose (1 mg/g equivalent of liver). The resulting postnuclear supernate was centrifuged for 10 min at 12,000 rpm in the 50 Ti rotor to obtain a crude mitochondrial pellet, which was washed once with 0.25 M sucrose (1 ml/g equivalent of liver; 12,000 rpm, 10 min, in the 50 Ti rotor) and three times with large volumes of 0.4 M sucrose (6 ml/g equivalent of liver; 8,500 rpm, 10 min, in the 30 rotor) to yield the final mitochondrial fraction.

The postmitochondrial supernate, obtained by pooling the two supernatant fractions of the 12,000 rpm spins, was centrifuged at 42,000 rpm for 1 h in the 60 Ti rotor to obtain the total microsomal (MR) fraction, which was used to prepare a light (GF_{1+2}) and heavy (GF_3) Golgi fraction, essentially as described by Ehrenreich et al. (14), with the difference that the rats had not been pretreated with ethanol, and with the following modifications in the discontinuous sucrose gradient. The MR pellet obtained from one liver was resuspended by hand in a Potter-Elvehjem homogenizer with 0.25 M sucrose to a volume of 4.5 ml. This suspension was brought to a concentration of 1.15 M sucrose by the addition of 2.0 M sucrose and layered over a 2.0-ml cushion of 2.0 M sucrose in a cellulose nitrate tube of the SW 27 rotor. A discontinuous sucrose gradient (8.0 ml of 1.10 M, 2 ml of 1.07 M, 8 ml of 0.86 M sucrose) was constructed over the microsomal suspension. The tube was filled with 0.25 M sucrose, and the gradients were centrifuged for 4 h at 25,000 rpm. The material banding at the 0.86–0.25 M sucrose interface was taken as the GF_{1+2} fraction, and the material in the 1.07 M sucrose layer and banding at the 1.07–0.86 M sucrose interface as the GF_3 fraction. The 1.10 M sucrose layer was discarded, and the material remaining in the load zone was kept as residual MR. These fractions, collected by U-shaped siliconized Pasteur pipettes, were diluted with two volumes of distilled water and sedimented into pellets (40,000 rpm, 1 h, in the 60 Ti rotor). Pelleted fractions were resuspended in small volumes of 0.25 M sucrose with a Dounce homogenizer and used immediately for enzyme assays. Unless otherwise specified, the MR fraction used in the experiments described in this and the other papers in this series (reference 38 and footnote 2) corresponds to the residual MR fraction collected from the discontinuous sucrose gradient.

Treatment of Cell Fractions with Digitonin

Microsomes and Golgi fractions were treated with digitonin essentially as described by Amar-Costesec et al. (1) and then analyzed on continuous sucrose gradients.

MICROSOMES: A total microsomal fraction prepared in buffered sucrose solutions by differential centrifugation from 5 g of liver was resuspended to a total volume of 2.6 ml with 0.25 M buffered sucrose and divided into two equal parts. 3.24 ml of 0.235% digitonin made up in 0.25 M buffered sucrose were added dropwise under continuous stirring to one part, and the other part (untreated) was similarly mixed with 0.25 M buffered sucrose not containing digitonin. Both suspensions were then incubated for 15 min in an ice bath. After centrifugation for 1 h at 40,000 rpm in the 50 Ti rotor, the digitonin-treated and untreated microsomal pellets were resuspended to a volume of 2.6 ml with 0.25 M buffered sucrose, and 1.0-ml aliquots were analyzed on continuous sucrose gradients (see below).

GOLGI: A total Golgi fraction was used. It was prepared essentially as described above in *Cell Fractionation*, but the gradient layered over the microsomal suspension consisted of 15 ml of 1.10 M sucrose and 15 ml of 0.4 M sucrose. The material banding between these two layers was taken as the total Golgi fraction. All sucrose solutions were buffered.

The material collected from the gradients was divided into two parts: one was supplemented with digitonin (65 μg/mg of protein, corresponding to a digitonin:cholesterol ratio of ~0.8) as described above, and the other part was left untreated. After the incubation, the suspensions were diluted with two volumes of 3 mM imidazole-HCl, pH 7.4, and the membranes were sedimented into a pellet (45,000 rpm, 1 h, in the 60 Ti rotor). Pellets were resuspended in a small volume of 0.25 M buffered sucrose and analyzed on continuous sucrose gradients.

CONTINUOUS SUCROSE GRADIENT CENTRIFUGATION: 1-ml aliquots were analyzed on 11-ml linear 0.77–1.5 M sucrose gradients made up in SW 41 cellulose nitrate tubes with buffered sucrose solutions and layered over an 0.5-ml cushion of 2.5 M buffered sucrose. Gradients were centrifuged at 25,000 rpm at 3°C for 14 h and stopped without braking. Fifteen fractions were collected with an Auto Densiflow probe (Buchler Instruments Inc., Fort Lee, N. J.) connected to a peristaltic pump. Enzymes were assayed on each fraction. Recoveries with respect to material loaded onto the gradients ranged between 60 and 100%.

In all experiments, a gradient not containing membranes was run in parallel. The densities indicated in Fig. 4 were obtained from the refractive indices of the "empty" gradient fractions.

Analytical Methods

The following enzyme activities were assayed by the indicated published procedures: rotenone-insensitive NADH-cytochrome *c* reductase (EC 1.6.2.2) and NADPH-cytochrome *c* reductase (EC 1.6.2.4.) as described by Sottocasa et al. (49); NADH-ferricyanide reductase and NADH-cytochrome b_5 reductase (EC 1.6.2.2) according to Takesue and Omura (52); cytochrome oxidase (EC 1.9.3.1) by the method of Cooperstein and Lazarow (11) except that samples were cleared with 0.2% Lubrol PX 5 min before the assay; glucose-6-phosphatase (EC 3.1.3.9) and 5′-nucleotidase (EC 3.1.3.5) were assayed as described by Swanson (51) and Widnell and Unkeless (58), respectively. The reactions

were stopped by TCA precipitation, and the inorganic phosphate released was determined by the method of Ames (2). For 5′-nucleotidase, a blank containing enzyme and 2′-AMP was used to correct for nonspecific phosphatase activity. Cytochrome b_5 was determined by the difference spectrum between the oxidized and the reduced form as described by Ernster et al. (15) with a mM^{-1} extinction coefficient of 163/Δ OD 424–409 (18). Galactosyl transferase (EC 2.4.1.38) was assayed as described by Beaufay et al. (4), but reaction mixtures of 40 μl contained 65 mM 2-*N*-morpholino ethane sulfonic acid buffer, pH 6.0, 1.35 mM β-mercaptoethanol, 0.65% Triton X-100, 6.5 mM ATP, 13 mM $MnCl_2$, 5 mM UDP-[^{14}C]galactose (specific activity 76 μCi/mmol), 12.5 mg/ml of protein acceptor, and the enzyme. In most experiments the acceptor used was sialidase–β-galactosidase-treated α_1 acid glycoprotein (47), but in the digitonin experiments ovalbumin was used as the acceptor (4). The reaction was allowed to proceed for 1 h at 37°C and was stopped by the addition of cold 10% TCA–0.5% perchloric acid (PCA). Blanks were incubated in parallel, but the protein acceptor was added after the TCA-PCA to correct for nonspecific precipitation of radioactivity. After centrifugation, the pellets were washed twice with 5% TCA–0.5% PCA, solubilized with Protosol, and counted in toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene.

Monoamine oxidase (EC 1.4.3.4.) was assayed as described by Yasonobu and Smith (59), modified as follows: incubation mixtures of 0.530 ml contained 75 mM phosphate buffer, pH 7.2, 3.8 mM benzylamine, and enzyme. Incubation was allowed to proceed for 2 h at 30°C. Ultraviolet light absorption of the samples was read at 250 nm against blanks to which 3.8 mM benzylamine has been added just before reading.

Protein and cholesterol were measured by the methods of Lowry et al. (34) and Watson (56), respectively.

Electron Microscopy

Aliquots of GF_3 and GF_{1+2} collected from the discontinuous sucrose gradient (see *Cell Fractionation* above) were fixed directly in suspension by the addition of an equal volume of 4% OsO_4 in cellulose nitrate tubes of the SW 50.1 rotor. The tubes were filled with water and centrifuged for 20 min at 30,000 rpm. Samples of the digitonin experiments were pelleted and fixed *in situ* with 1% OsO_4 made up in 0.1 M cacodylate buffer, pH 7.2. Blocks were stained with 0.5% magnesium uranyl acetate and embedded in Epon 812. Thin sections were cut with Reichert (C. Reichert AG, Vienna, Austria) and LKB (LKB-Produkter AB, Bromma, Sweden) ultramicrotomes, stained with uranyl acetate and lead citrate, and examined with a Philips 200 or 400 electron microscope.

Materials

The following reagents were purchased from the indicated sources: Lubrol PX, yeast 5′ and 2′ AMP, equine muscle ATP, fraction V bovine serum albumin, grade III ovalbumin, rotenone and UDP-galactose, Sigma Chemical Co., St. Louis, Mo.; Triton X-100, β-mercaptoethanol and benzylamine, B. D. H. Poole, Dorset, England; NADH, NADPH, horse heart cytochrome *c* (grade I) and glucose-6-phosphate, Boehringer Mannheim, Mannheim, W. Germany; uniformly labeled UDP-[^{14}C]galactose (200 mCi/mmol) and Protosol, New England Nuclear, Langen, W. Germany; 25% purified glutaraldehyde for electron microscopy, Fluka AG, Buchs, Switzerland; sucrose (special enzyme grade): Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. All other reagents were analytical grade and were purchased from Merck AG, Darmstadt, W. Germany.

Sialidase–β-galactosidase-treated α_1 acid glycoprotein was the kind gift of Dr. H. Schachter, University of Toronto. Trypsin-solubilized cytochrome b_5 was purified from rat liver microsomes according to Omura et al. (44).

Male Sprague-Dawley rats were a gift of Selvi Drug Corp., Milan, Italy.

RESULTS

Characterization of Cell Fractions

The results of enzyme assays carried out on our cell fractions are shown in Table I. In agreement with previous results (5), we found that Golgi fractions are approximately 45-fold enriched in galactosyl transferase activity. Recoveries of the enzyme in GF_3 and GF_{1+2} were, respectively, 20 and 6% of the activity in the total homogenate (not shown). The NADPH-cytochrome *c* reductase activity in GF_3 and GF_{1+2} was approximately 40 and 25%, respectively, of that observed in microsomes, and glucose-6-phosphatase activity was very low. NADH-cytochrome *c* reductase and cytochrome b_5 appeared unequally distributed in the two Golgi fractions: their specific activity was high in GF_3 and distinctly lower in GF_{1+2} (~78 and 25%, respectively, of the microsomal values).

The last column of Table I shows that, on the basis of glucose-6-phosphatase and NADPH-cytochrome *c* reductase activities, which are considered to be microsomal markers, the mitochondrial fraction was contamined ~5% by ER elements. The fraction was approximately fivefold enriched in cytochrome *c* oxidase over the homogenate, and its rotenone-insensitive NADH-cytochrome *c* reductase specific activity was approximately one-third of that found in microsomes, which is in good agreement with other workers' findings (49).

Because the NADH-dependent reductase-cytochrome b_5 system is located on the outer mitochondrial membrane (45, 49), we checked the possibility that the high activity found in Golgi fractions was due to contaminating mitochondrial fragments. GF_{1+2} and GF_3 contained only about 1% of the internal mitochondrial membrane marker, cytochrome *c* oxidase, as compared with the mitochondrial fraction. However, the outer membrane marker enzyme, monoamine oxidase, showed quite a different behavior: it was enriched only 3.2-fold over the homogenate in the mitochondrial fraction, suggesting the loss of outer membranes from this fraction, and was found in considerable amounts in GF_3 and GF_{1+2} (~20 and 5%, respectively, of the mitochondrial specific activity). However, when the specific activities of rotenone-insensitive NADH-cytochrome *c* reduc-

TABLE I

Enzyme Activities of Rat Liver Cell Fractions Prepared in Unbuffered Sucrose Solutions

	Total homogenate	Microsomes	GF_3	GF_{1+2}	Mitochondria
Glucose-6-phosphatase*	74 ± 9 (10)	260 ± 33 (10)	72 ± 11 (9)	11 ± 2 (10)	19 ± 4 (5)
NADH-cytochrome *c* reductase*	254 ± 28 (12)	831 ± 101 (12)	638 ± 96 (12)	220 ± 26 (12)	255 ± 42 (7)
Cytochrome b_5‡		0.51 ± 0.03 (4)	0.48 ± 0.07 (4)	0.13 ± 0.01 (4)	
NADPH-cytochrome *c* reductase*	17.7 ± 2.4 (8)	82.3 ± 10.8 (9)	36.2 ± 6.5 (9)	23.4 ± 8.6 (3)	4.4 ± 1.0 (5)
Galactosyltransferase§	43 ± 3 (3)	66 ± 20 (4)	1916 ± 212 (4)	1953 ± 106 (4)	
5′-Nucleotidase*	44 ± 12 (3)	66 ± 17 (3)	249 ± 95 (3)	131 ± 75 (3)	
Cytochrome-oxidase*	421 (1)	5 (1)	29 (1)	12 (1)	2,136 (1)
Monoamine oxidase*	8.7 (1)	1.4 (1)	6.1 (1)	1.3 (1)	29 (1)

Results shown are averages ± SE. The number of experiments is shown in parentheses.

* Nanomoles of product formed per minute per milligram of protein.

‡ Nanomoles of cytochrome b_5 per milligram of protein.

§ Nanomoles of galactose transferred per hour per milligram of protein.

tase and monoamine oxidase in mitochondrial and Golgi fractions are compared, it can be seen that only a small percentage of the reductase activity found in the latter can be accounted for by outer mitochondrial membranes (~5%) (Table I).

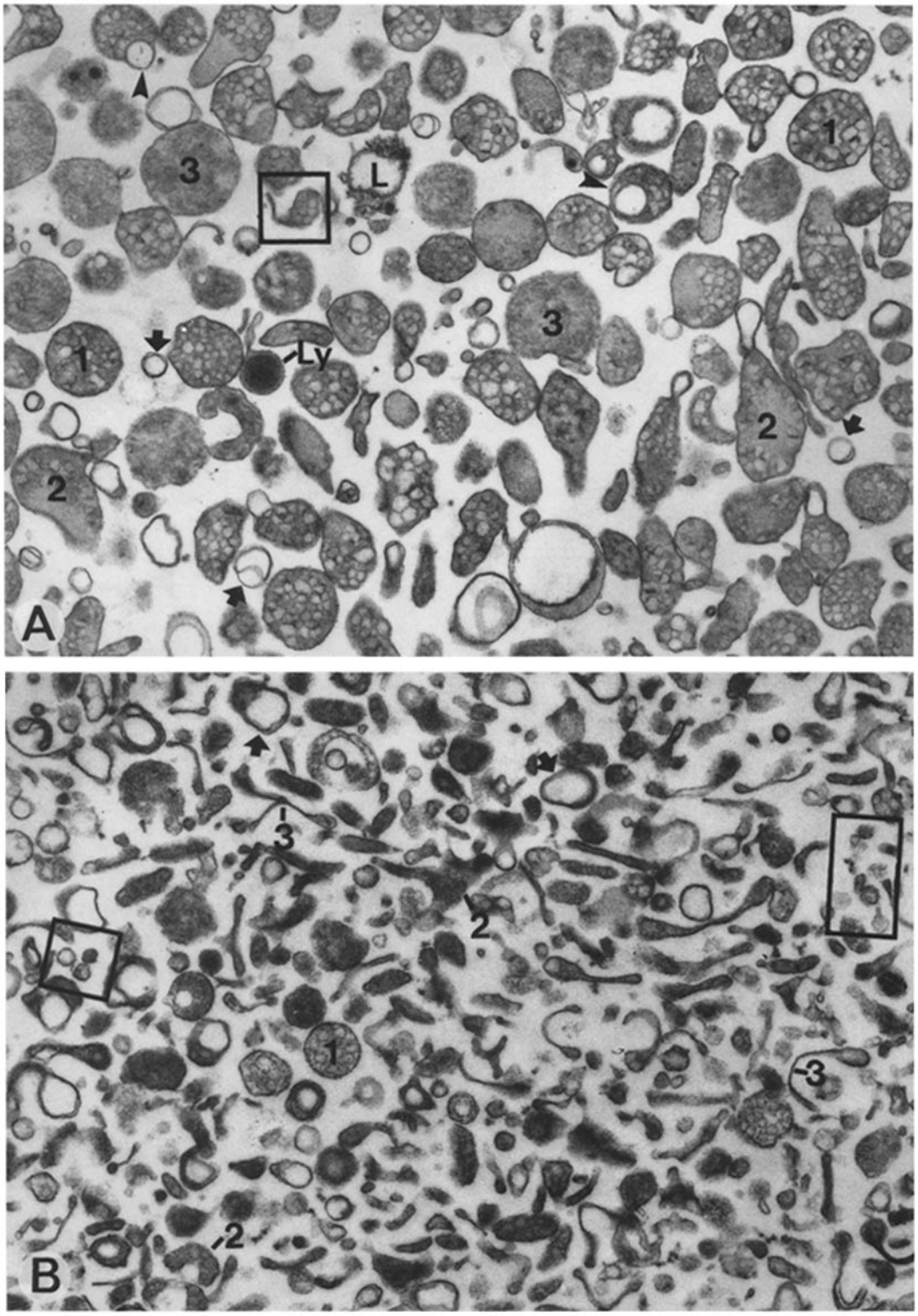
GF_3 and GF_{1+2} were also examined by electron microscopy (Fig. 1). Both fractions showed the characteristic morphology originally described by Ehrenreich et al. (14), with GF_3 rich in cisternal elements, and GF_{1+2} containing mostly VLDL-loaded vacuoles.

Aging of Enzyme Activities in Golgi Fractions

Widely divergent values for the specific activity of the NADH-cytochrome b_5 reductase in rat liver Golgi fractions have been reported in the literature, ranging from 15% (5, 41) to 80% (24) of the values of microsomal fractions. Such variability might indicate that artifacts are involved. In fact, it is known that enzyme activities can be variously affected during storage of the cell fractions (39). Inasmuch as it has recently been reported that some activities in Golgi fractions are particularly labile (23), we thought it possible that the divergent values for NADH-cytochrome *c* reductase are the result of its more rapid aging in Golgi fractions than in the other fractions. If this were the case, differences in the length of time the Golgi fractions were stored before the enzyme assays were carried out might account for the divergent values found.

The results shown in Fig. 2, *left*, show that the activity of rotenone-insensitive NADH-cytochrome *c* reductase indeed does decay with time in our Golgi fractions. When the enzyme was assayed immediately on the material collected from the discontinuous gradient, without pelleting of the fractions (−2.5-h time point), it was as high in GF_3 as in MR and ~60% of this value in GF_{1+2}. During the following 2.5 h (the time necessary for dilution, recentrifugation, and resuspension of the cell fractions), the activity in Golgi fractions decayed rapidly (in the experiments illustrated in Fig. 2 by ~50%), whereas it remained constant in the microsomes. This time point (0 time point) corresponds roughly to the time at which enzymes were routinely assayed in the experiments described in *Characterization of Cell Fractions*. Thereafter, the activity continued to decay slowly, more markedly in GF_{1+2} than in GF_3, whereas it remained approximately constant in the MR fraction. The behavior of NADPH-cytochrome *c* reductase was quite different (Fig. 2, *right*) inasmuch as no appreciable decay could be detected in any of the cell fractions investigated.

Because the reduction of added cytochrome *c* in the NADH-cytochrome *c* reductase assay involves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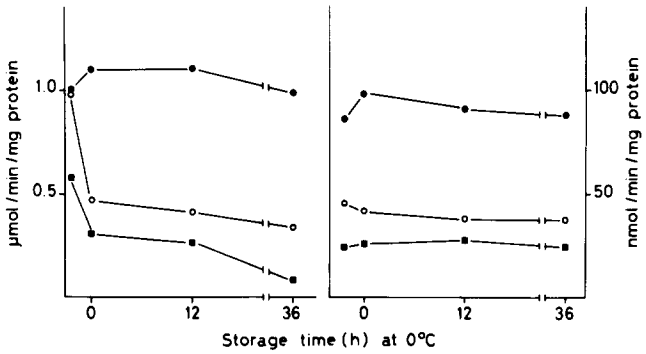


FIGURE 2 Aging of enzymes in MR and Golgi fractions. Cell fractions were prepared as described in Materials and Methods. After separation of the Golgi fractions by discontinuous sucrose gradient centrifugation, the bands were collected and an aliquot of each fraction was used for enzyme assay and protein determination (−2.5-h time point). Approximate protein concentrations in these samples were: MR, 10 mg/ml; GF_3, 0.5 mg/ml; GF_{1+2}, 0.3 mg/ml. The remaining material was diluted, pelleted and resuspended (see *Cell Fractionation* under Materials and Methods) in 0.25 M sucrose to an approximate protein concentration of: MR, 20 mg/ml; GF_3, 6 mg/ml; GF_{1+2}, 3 mg/ml. The data presented are averages of three experiments. (*Left*) Rotenone-insensitive NADH-cytochrome *c* reductase. (*Right*) NADPH-cytochrome *c* reductase. (●) MR. (○) GF_3. (■) GF_{1+2}.

the transfer of electrons through cytochrome b_5 to the final acceptor, it is conceivable that the decay of this enzyme activity might be due, rather than to an inactivation of the reductase itself, to a loss in the ability of Golgi-associated cytochrome b_5 to accept electrons from the reductase or to an alteration in membrane structure such that the two enzymes could no longer interact. That this is not the case can be seen from Table II, which shows that the same aging of enzyme activity was observed when trypsin-solubilized cytochrome b_5 was used as the final acceptor. In contrast, the ability of the reductase to transfer electrons to the low molecular weight acceptor ferricyanide did not decrease with time (column 3 of Table II).

In an attempt to elucidate the mechanism of the inactivation, we searched for conditions that could protect the reductase (Table III). Because Golgi fractions were routinely stored as more dilute suspensions than were MR fractions, we tested whether dilution might favor inactivation. Exp. II of Table III shows that dilution had no effect on the aging process. The anti-oxidant butylated hydroxytoluene (BHT) was also without effect (Exp. III). In contrast, storing the fractions at −20°C instead of at 0°C (Exp. I) and buffering all solutions at pH 7.4 throughout the fractionation procedure and during storage (Exp. IV) were both effective in protecting the enzyme activity. The effect of buffering at pH 7.4 is better seen in Fig.

FIGURE 1 (*A*) GF_{1+2} fraction. This field, which is representative of the lower half of the pellet, is occupied primarily by large Golgi vacuoles. In some of these vacuoles the content is entirely accounted for by tightly packed, recognizable VLDL particles (*1*); in others, VLDL particles or particle clusters are copacked with an amorphous material of similar electron density (*2*). In yet another group (*3*), the entire content appears amorphous. The shape of the vacuoles varies from rounded to highly irregular. The ring-shaped images (arrowheads) result from cutting, in part, through the VLDL-filled rims and, in part, through the empty bottoms of Golgi cisternae (box). The field also includes vesicles, many of which are double walled (arrows). The vesicles might derive from collapsed Golgi cisternae. *L* is probably a lipid droplet, and *Ly*, a lysosome. (*B*) GF_3 fraction. This field is from the middle of the pellet. VLDL-filled vacuoles (*1*) are much smaller than in GF_{1+2} and relatively sparse. The predominant components are cisternal elements (*2*) of various sizes and configurations, some of which appear partially collapsed (*3*). Annular profiles (arrows) are sections through buckled cisternae. Thin tubules and small vesicular images (probably originating from multiple sections of twisted tubules) are also visible (boxes). (*A* and *B*) × 32,500.

TABLE II

Effect of Storage at 0°C on the Ability of NADH-Cytochrome b_5 Reductase to Transfer Electrons to Different Acceptors

	Enzyme assayed					
	NADH-cytochrome *c* reductase*		NADH-cytochrome b_5 reductase*		NADH-FeCN reductase*	
	fresh‡	36 h	fresh‡	36 h	fresh‡	36 h
Microsomes	936	1,015	64.8	67.0	3,462	3,600
GF_3	1,051	358	82.9	35.9	3,200	2,896
GF_{1+2}	623	175	55.5	30.5	2,649	2,956

* Nanomoles of acceptor reduced per minute per milligram of protein.

‡ The term "fresh" refers to material collected directly from the discontinuous sucrose gradient (−2.5 h time point of Fig. 3).

3. At all time points, the Golgi fractions prepared with buffered solutions showed a much higher NADH-cytochrome *c* reductase activity than their counterparts prepared in unbuffered solutions (Fig. 3*b*). However, a decrease in the enzyme activity was observed after 36 h of storage, even in the presence of buffer, especially in GF_{1+2}. Buffering the sucrose solutions also partially protected glucose-6-phosphatase activity in GF_3 (Fig. 3*a*). As expected, the buffer had little or no effect on enzyme activities that are not labile under our experimental conditions, i.e., on microsomal enzyme activities and on Golgi NADPH-cytochrome *c* reductase and NADH-ferricyanide reductase (Fig. 3*c* and *d*).

The fact that the labile Golgi enzymes are protected by keeping the pH at 7.4 suggests that lysosomal enzymes might be involved in the inactivation process. If this is so, however, the lysosomal action does not involve the proteolytic cleavage of the reductase resulting in its detachment from membranes (52). In fact, ultracentrifugation of Golgi fractions after 36 h of storage in an unbuffered solution resulted in the recovery of all the NADH-ferricyanide reductase activity in the membrane pellet (not shown).

Effect of Digitonin on the Buoyancy of the Constituents of MR and Golgi Fractions

Taken together, the results presented in the preceding two sections of Results suggest that NADH-cytochrome b_5 reductase is an authentic component of Golgi membranes. However, the alternative possibility should also be considered, i.e., that the high specific activity found in fresh Golgi fractions was due to contaminants highly enriched in the enzyme. To check this possibility, we applied the digitonin technique (1) to a total Golgi fraction (GF_{1+2+3}). Digitonin has been shown to bind to membranes carrying Golgi and plasma membrane marker enzymes and, thus, to shift their buoyant densities to higher values. The effect of digitonin on these membranes is related to their cholesterol content and/or to the detergent properties of digitonin (1, 57). By comparing the distribution of the individual components of digitonin-treated and untreated cell fractions on equilibrium sucrose density gradients, it is thus possible to obtain information on their subcellular localization.

When the total MR fraction was analyzed (not shown), the data obtained were in good agreement with those of Amar-Costesec et al. (1) because the distribution of NADH- and NADPH-cytochrome *c* reductases were not altered by digitonin treatment (median buoyant densities,[3] 1.152 and 1.161, respectively), whereas the buoyancy of 5′-nucleotidase-bearing elements was increased (approximate median density shifted from 1.140 to 1.175). The median density of galactosyl transferase-bearing elements also increased, although to a lesser extent (from 1.132 to 1.160). However, the behavior of NADH-cytochrome *c* reductase in a total MR fraction cannot be expected to give any information concerning its Golgi location because Golgi elements constitute a small minority of the total MR fraction and, therefore, should be ex-

[3] The buoyant density values given must be considered as approximations because they were derived from the refractive indices of fractions of "empty" sucrose gradients run in parallel with those containing the cell fractions.

pected to contribute only a small part of the total NADH-cytochrome *c* reductase activity.

Quite different results were obtained when a total Golgi fraction (GF_{1+2+3}) was subjected to a similar analysis (Fig. 4). As expected, the distribution of galactosyl transferase was shifted by the digitonin treatment (from a median density of 1.121 to 1.137; Fig. 4*a*). The lower median density, compared with that of the same enzyme in a total MR fraction, is not surprising because the cell fractionation procedure we used for Golgi preparation would be expected to result in the enrichment of light elements. The data in Fig. 4 also indicate that not all the galactosyl transferase was affected by the digitonin treatment inasmuch as some enzyme activity remained even in the very light fractions of the gradient. This behavior, which was also observed by Wibo et al. (57), suggests heterogeneity among Golgi elements with regard to digitonin binding. Fig. 4*b*, *d*, and *f* shows the behavior of the components of the NADH-cytochrome b_5 reductase system, i.e., cytochrome b_5 (Fig. 4*f*) and NADH-cytochrome b_5 reductase, assayed with cytochrome *c* (Fig. 4*b*) or FeCN (Fig. 4*f*) as the electron acceptor. The results indicate that: (*a*) The distributions of the two enzyme activities and of cytochrome b_5, with and without digitonin treatment, are very similar to each other and differ from the distribution of galactosyl transferase, as well as from that of NADPH-cytochrome *c* reductase (Fig. 4*e*). The enzymes of the NADH-dependent system in the untreated sample were enriched in the very light gradient fractions (median buoyant density 1.116). (*b*) The distribution of these enzymes was affected by the digitonin treatment (the median buoyant density shifted to 1.126), although to a lesser extent than that of galactosyl transferase. Because, however, a considerable proportion of the enzyme contained in the upper fractions of the gradient was not shifted, bimodal distributions were obtained in digitonin-treated samples. The behavior of NADPH-cytochrome *c* reductase is shown in Fig. 4*e*. Its median buoyant density (1.120) was higher than that of the NADH-dependent reductase and that of cytochrome b_5, and the buoyancy of elements carrying this enzyme was little affected by digitonin. The distribution of protein (Fig. 4*c*) closely resembled that of galactosyl transferase, suggesting that galactosyl transferase activity is distributed fairly uniformly among all elements of the Golgi fraction.

The results shown in Fig. 4 cannot be explained by a preferential extraction of certain enzymes from the lighter components of the Golgi fraction. Although the analyses of the treated or untreated fractions showed that digitonin did affect a mild extraction of enzymes as well as of protein (from 10 to 25%, not shown), there was no correlation between the extent of the extraction and the effect on the buoyancy of the constituents.

Fig. 5 shows the morphology of Golgi elements treated (*B* and *D*) or not treated (*A* and *C*) with digitonin and separated on a continuous sucrose gradient, as were the elements of Fig. 4. A light fraction (fraction 3, Fig. 5*A*) showed an abundance in VLDL-filled granules and empty granules, whereas a heavier fraction (fraction 7, Fig. 5*C*) was enriched in cisternae, rows of small vesicles (probably twisted tubules), and double-

TABLE III

Effect of Various Treatments on Aging of NADH-Cytochrome c Reductase

Experiment	Fraction	Treatment	% activity after 36 h*
I	GF_{1+2}	None	37.8
	GF_{1+2}	Freezing‡	92.8
II	Microsomes (21 mg protein/ml)	None	97.4
	Microsomes (4.2 mg protein/ml)	Dilution§	115.0
	GF_3 (6.9 mg protein/ml)	None	61.7
	GF_3 (1.4 mg protein/ml)	Dilution§	65.9
III	GF_3	None	43.0
	GF_3	BHT‖	40.4
	GF_{1+2}	None	31.2
	GF_{1+2}	BHT‖	33.8
IV	GF_3	None	50.0
	GF_3	Buffer¶	72.4
	GF_{1+2}	None	12.1
	GF_{1+2}	Buffer¶	55.6

* Percent activity compared to activity measured immediately in pelleted and resuspended cell fractions (0 time point of Fig. 3).

‡ An aliquot of the resuspended fractions was frozen at −20°C and stored at that temperature.

§ An aliquot of the resuspended fractions was diluted to the indicated concentration and stored at that concentration at 0°C.

‖ All solutions for tissue homogenization, cell fractionation, and fraction storage contained BHT at 1 mg/liter.

¶ All solutions for tissue homogenization, cell fractionation, and fraction storage contained 3 mM imidazole-HCl, pH 7.4.

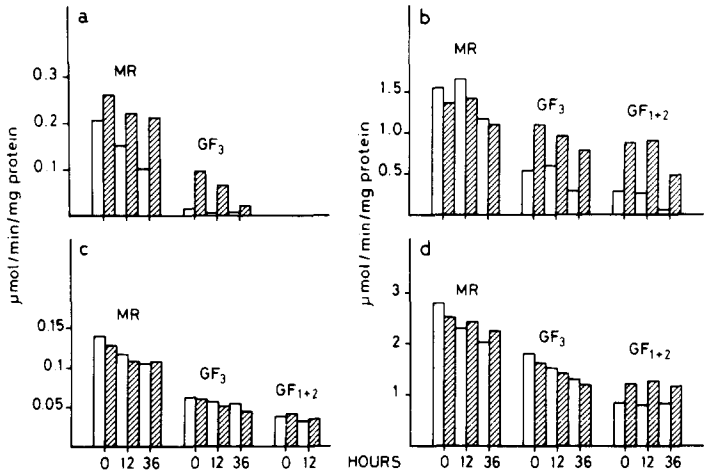


FIGURE 3 Effect of buffer on aging of enzymes in MR and Golgi fractions. Preparation of MR and Golgi fractions was carried out as described in Materials and Methods with either sucrose solutions buffered at pH 7.4 with 3 mM imidazole-HCl throughout the cell fractionation procedure (hatched bars) or unbuffered sucrose solutions (open bars). Protein concentrations were: MR, 21 mg/ml; GF_3, 6 mg/ml; GF_{1+2}, 1.1 mg/ml. (*a*) Glucose-6-phosphatase; (*b*) Rotenone-insensitive NADH-cytochrome *c* reductase; (*c*) NADPH-cytochrome *c* reductase; (*d*) NADH-FeCN reductase.

walled annular profiles. Digitonin treatment (Fig. 5 *B* and *D*) caused a partial extraction of content, which was confirmed by SDS-PAGE (not shown), and which appears to be analogous to the extraction that occurs in microsomes with low concentrations of other detergents (30). A second effect of digitonin was the production of numerous elements with a "broken" appearance, which is a known effect of digitonin binding (1). However, other recognizable Golgi elements failed to exhibit the typical "interruptions" (see *insets* in Fig. 5 *D*). If the broken appearance is the result of the formation of nonosmiophilic aggregates of digitonin-cholesterol complexes, as has been suggested (1), our results with Golgi fractions suggest that cholesterol is not distributed uniformly among Golgi elements, with some elements having a lower content than others. Such an unequal distribution would be one reason why not all the galactosyl transferase activity is shifted by digitonin to a higher buoyant density.

DISCUSSION

In this study, we have reinvestigated the subcellular distribution of rotenone-insensitive NADH-cytochrome *c* reductase activity. The results confirm the microsomal and mitochondrial localization of rotenone-insensitive NADH-cytochrome *c* reductase and indicate that this enzyme is endogenous also to Golgi elements because: (*a*) in fresh Golgi fractions collected from a discontinuous sucrose gradient or in pelleted and resuspended material prepared in the presence of 3 mM imidazole-HCl, pH 7.4, the specific activity of the enzyme was as high, or nearly as high, as in the MR fraction and could not be accounted for by contaminating outer mitochondrial membranes; and (*b*) in contrast to the result obtained with MR, the distribution of the reductase- and cytochrome b_5-bearing elements was shifted to a higher buoyant density when a total Golgi fraction was treated with digitonin.

In addition to the localizations reported here, both NADH-cytochrome b_5 reductase and cytochrome b_5 have been reported to be present in liver plasma membranes (27, 33, 43). However, the plasmalemma NADH-dependent reductase activity might be due to an enzyme different from the microsomal one because it has been reported that the acceptor specificities are not the same (12). On the other hand, because the plasma membrane activity is low, it has been attributed to microsomal contamination (53).

Our results on aging of enzymatic activities in Golgi fractions partially disagree with those recently reported by Howell et al. (23). In agreement with these authors, we observed aging of glucose-6-phosphatase in Golgi fractions. However, the

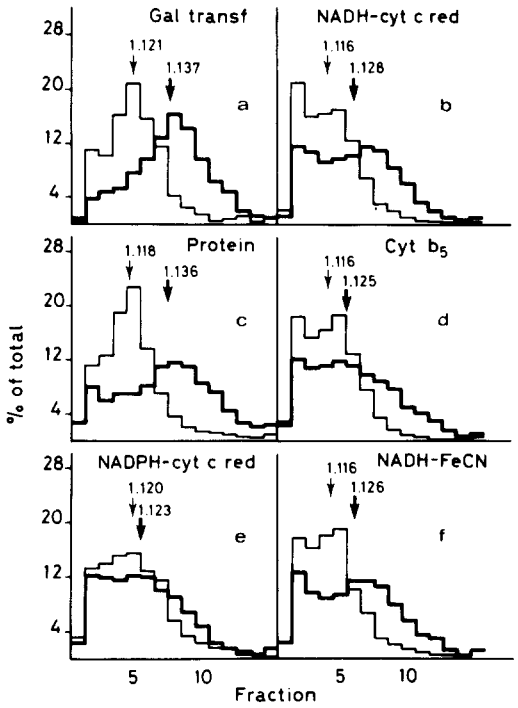
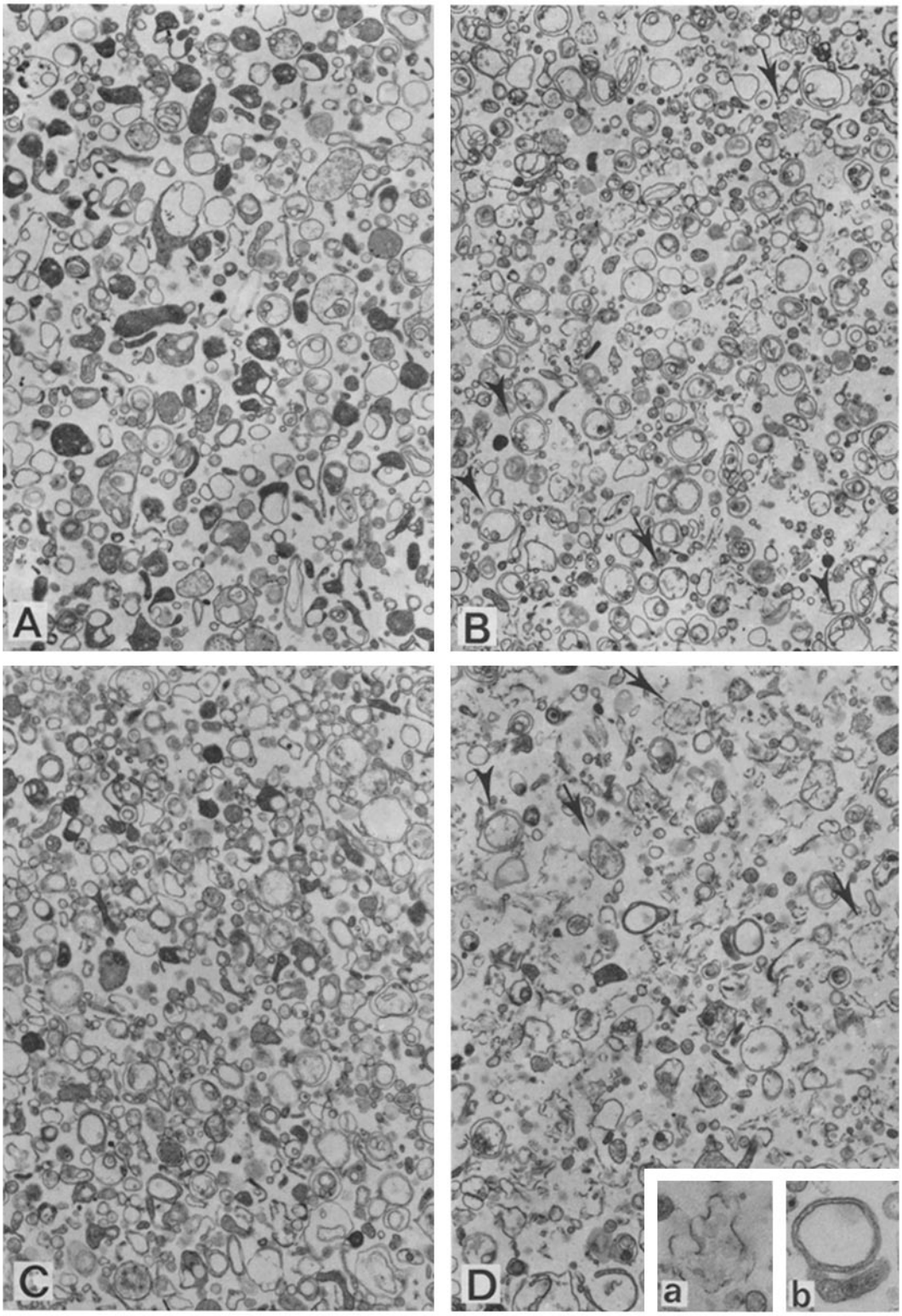


FIGURE 4 Effect of digitonin on the distribution of constituents of a total Golgi fraction on sucrose density gradients. A total Golgi fraction was prepared, treated with digitonin or left untreated, and analyzed by sucrose density gradient centrifugation, as described in Materials and Methods (*Treatment of Cell Fractions with Digitonin*). Results are presented as percent enzyme activity of the total recovered in each fraction on the gradient. Light lines, no digitonin treatment; heavy lines, samples treated with digitonin. Light and heavy arrows indicate the median buoyant densities of untreated and treated samples, respectively. (*a*) Galactosyl transferase. (*b*) Rotenone-insensitive NADH-cytochrome *c* reductase. (*c*) Protein. (*d*) Cytochrome b_5. (*e*) NADPH-cytochrome *c* reductase. (*f*) NADH-FeCN reductase. Data shown represent averages of values from two experiments, except for cytochrome b_5 (data from the second experiment only). In experiment I, the treated and untreated samples were isolated from 45 g of liver, and each sample was analyzed on a single gradient. In experiment II, the samples were isolated from 110 g of liver, and each was run on two separate gradients; corresponding fractions of these two parallel gradients were combined before the biochemical determinations were carried out. Amounts recovered on the gradients with and without digitonin, respectively, were: NADH-cytochrome *c* reductase (micromoles of cytochrome *c* reduced per minute): exp. I, 3.7 and 2.97; exp. II, 20.2 and 16.4; NADH-FeCN reductase (micromoles of FeCN reduced per minute): exp. I, 13.25 and 10.3; exp. II, 66.7 and 55.5; NADPH-cytochrome *c* reductase (micromoles of cytochrome *c* reduced per minute): exp. I, 0.16 and 0.147; exp. II, 0.798 and 0.763; galactosyl transferase (nanomoles of galactose transferred to ovalbumin per hour): exp. I, 355 and 376.5; exp. II, 1207 and 1244; protein (milligrams): exp. I, 6.17 and 4.96; exp. II, 20.4 and 16; cytochrome b_5 (nanomoles): exp. II, 8.5 and 6.3.

behavior of the NADH- and NADPH-cytochrome *c* reductases in our fractions was the opposite of what they observed. They found that NADH-cytochrome *c* reductase activity was stable at 0°C for as long as 24 h, whereas the NADPH-dependent activity decreased to very low levels during the same length of time. In addition to the fact that the Golgi fractions of Howell et al. (23) were obtained from ethanol-intoxicated rats, small differences in the way in which cell fractions were handled and stored may account for the discrepancies. Our Golgi fractions were stored at concentrations at least fourfold higher than theirs, and inactivation of NADPH-cytochrome *c* reductase appears to proceed at a higher rate in dilute samples (23). Moreover, NADH-cytochrome *c* reductase is inactivated the most when fractions are diluted, pelleted, and resuspended (this paper), steps that are not included in the fractionation procedure of Howell et al. (23). In any case, the mechanisms of aging of the two reductases are probably different because the inactivation of the NADPH-dependent enzyme may have involved lipid peroxidation, as suggested by the protection by catalase, EDTA, and antioxidants (23), whereas the NADH-dependent enzyme was protected by buffering all sucrose solutions at pH 7.4, suggesting the participation of lysosomes in the inactivation. Stabilization of sugar transferases by keeping solutions at pH 7 has also been recently reported (9).

The analysis of a Golgi fraction, treated or not treated with digitonin, by equilibration on continuous sucrose density gradients, in addition to providing evidence that the NADH-cytochrome b_5 reductase system is endogenous to Golgi elements, also yielded information on the distribution of this system within the Golgi fraction. We found that reductase-cytochrome b_5-bearing elements equilibrated at lower densities than those carrying galactosyl transferase, and that they were less affected by the digitonin treatment. A partial explanation for this behavior is that a portion of the electron transport enzymes in our Golgi fraction was contributed by contaminants whose buoyancy is unaffected by digitonin. These light elements,


A
B
C
D
a
b

even though accounting for a relatively small fraction (5–10%) of the Golgi activity, could be the cause of the bimodal distribution appearing after digitonin treatment. Possible contaminants with these characteristics are outer mitochondrial membranes and light microsomes. On the basis of our enzyme data (Table I), the contribution of outer mitochondrial membranes to NADH-cytochrome *c* reductase activity in Golgi fractions should be around 5%. Because of the lack of markers whose localization has been proved to be restricted to ER membranes, it is impossible to evaluate the contribution of microsomal contaminants. If NADPH-cytochrome *c* reductase were accepted as a bona fide microsomal marker enzyme, one should conclude that at least 50% of the NADH-dependent activity found in fresh Golgi fractions is endogenous to Golgi elements. However, the percent is most probably considerably higher because some NADPH-cytochrome *c* reductase activity is also endogenous to Golgi membranes (23, 26), and because the good correlation we observed between protein and galactosyl transferase distribution on continuous sucrose gradients in Golgi fractions, treated or untreated with digitonin, speaks for the purity of our fractions. An alternative or, at least, an additional explanation, which we favor, for the behavior of the NADH-cytochrome b_5 reductase system in our digitonin experiments is that the localization of these enzymes in the Golgi complex is restricted to a minority of elements characterized by the relative sparsity of cholesterol in their limiting membrane. The morphological analysis of digitonin-treated fractions, as well as the effect of digitonin on the buoyancy of galactosyl transferase-bearing elements, does indeed suggest that cholesterol is distributed nonuniformly among Golgi elements.

The restriction of the NADH-dependent electron transport system to only a fraction of Golgi elements may be the cause of the discrepancy between our results and those reported by Wibo et al. (57), who found that digitonin treatment did not alter the buoyant density of NADH-cytochrome *c* reductase-bearing elements of a rat liver Golgi fraction. However, their fraction, prepared by a procedure quite different from ours, is enriched in rapidly sedimenting empiled cisternae (42), which presumably are poor in reductase, as suggested also by the immunoelectronmicroscopy studies of Fowler et al. (17). Therefore, the NADH-cytochrome *c* reductase activity found by Wibo et al. (57) in their Golgi fraction may mainly have been contributed by contaminant MR.

Our results can be added to the already large body of evidence in favor of the biochemical heterogeneity of the Golgi complex (see reference 37 for a review). A heterogeneous intra-Golgi distribution of both NADH- and NADPH-cytochrome *c* reductases has recently also been reported by Hino et al. (20, 22) and by Ito and Palade (26) on the basis of results obtained with countercurrent distribution and immunoadsorption experiments, respectively. The latter authors separated a population of Golgi elements containing ~60% of the reductases and only ~20% of the galactosyl transferase present in the fraction. It should be mentioned that, according to these studies, the two reductases are distributed in parallel, whereas we found that the two enzymes could be differentiated on the basis of their digitonin-induced buoyancy shift. How much of the NADPH-

FIGURE 5 Electron microscopy of Golgi subfractions treated or not treated with digitonin. This figure illustrates the morphology of Golgi subfractions, with and without digitonin, separated by equilibration on continuous sucrose gradients (see Fig. 4). (*A* and *B*) Material equilibrated at ρ = 1.092-1.114. (*C* and *D*) Material equilibrated at ρ = 1.135-1.140. (*A* and *C*) Material not treated with digitonin. (*B* and *D*) Material treated with digitonin. The subfraction shown in *A* is similar to GF_{1+2} as illustrated in Fig. 1*A*. However, the density of many VLDL-filled vacuoles is considerably lower, suggesting a partial release of the segregated content. The heavier subfraction (*C*) is rich in cisternae, vesicles, and vacuoles, and often contains material of moderate density. In the samples treated with digitonin, illustrated in *B* and *D*, the dense vacuoles are drastically diminished and are replaced by elements containing fewer, smaller, less dense and ill-defined particles, probably originating by the partial solubilization and extraction of VLDL (arrows). Swollen vacuoles are numerous and many are double walled, encircling one or more discrete vesicles (arrowheads). Some elements of the digitonin-treated samples, especially those illustrated in *D*, show fenestrated membranes (*inset a*). These are often seen bounding vacuoles containing the disrupted VLDL particles. However, in other recognizable Golgi elements, the membranes appear continuous (*inset b*). *Insets a* and *b* are enlargements from *D*. (*A–C*) × 13,000. (*D*) × 14,500. (*insets*) × 37,500.

dependent reductase activity is attributable to microsomal contamination remains to be established.

From a consideration of this discussion, it may be concluded that the quite divergent values of enzyme specific activities reported for Golgi fractions in the literature may primarily be a consequence of the lability of many Golgi enzymes on the one hand, and, on the other hand, of incomplete recovery of Golgi elements deriving from an extremely heterogeneous organelle. Thus, the various fractions isolated by the available techniques appear not to be representative of the whole complex but are primarily enriched either in stacked cisternae (42) or in smaller vesicles, tubules, and VLDL-filled vacuoles, as found in our study. In conclusion, NADH-cytochrome b_5 reductase appears to be localized in three different organelles of rat liver cells. It remained to be established whether the activity in the various compartments was due to the same enzyme molecule. This question is dealt with in the next article in this series (38).

The skillful technical assistance of Mrs. G. Pietrini and the photographic assistance of Mr. P. Tinelli and Mr. F. Crippa are gratefully acknowledged. Special thanks to Dr. A. Catapano for carrying out the cholesterol assays.

Received for publication 22 May 1979, and in revised form 8 January 1980.

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