Orientation of Glycoprotein Galactosyltransferase and Sialyltransferase Enzymes in Vesicles Derived from Rat Liver Golgi Apparatus

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ABSTRACT UDP-galactose : *N*-acetylglucosamine galactosyltransferase (GT) and CMP-sialic : desialylated transferrin sialyltransferase (ST) activities of rat liver Golgi apparatus are membrane-bound enzymes that can be released by treatment with Triton X-100. When protein substrates are used to assay these enzymes in freshly prepared Golgi vesicles, both activities are enhanced about eightfold by the addition of Triton X-100. When small molecular weight substrates are used, however, both activities are only enhanced about twofold by the addition of detergent. The enzymes remain inaccessible to large protein substrates even after freezing and storage of the Golgi preparation for 2 mo in liquid nitrogen. Accessibility to small molecular weight substrates increases significantly after such storage. GT and ST activities in Golgi vesicles are not destroyed by treatment with trypsin, but are destroyed by this treatment if the vesicles are first disrupted with Triton X-100. Treatment of Golgi vesicles with low levels of filipin, a polyene antibiotic known to complex with cholesterol in biological membranes, also results in enhanced trypsin susceptibility of both glycosyltransferases. Maximum destruction of the glycosyltransferase activities by trypsin is obtained at filipin to total cholesterol weight ratios of ~1.6 or molar ratios of ~1. This level of filipin does not solubilize the enzymes but causes both puckering of Golgi membranes visible by electron microscopy and disruption of the Golgi vesicles as measured by release of serum albumin. When isolated Golgi apparatus is fixed with glutaraldehyde to maintain the three-dimensional orientation of cisternae and secretory vesicles, and then treated with filipin, cisternal membranes on both cis and trans faces of the apparatus as well as secretory granule membranes appear to be affected about equally. These results indicate that liver Golgi vesicles as isolated are largely oriented with GT and ST on the luminal side of the membranes, which corresponds to the cisternal compartment of the Golgi apparatus in the hepatocyte. Cholesterol is an integral part of the membranes of the Golgi apparatus and its distribution throughout the apparatus is similar to that of both transferases.

The Golgi apparatus is the main locus in liver cells for glycosyltransferases that sequentially add the penultimate galactose and terminal sialic acid moieties to secreted glycoproteins (17–19, 35, 40, 41). In Golgi apparatus from rat liver, both galactosyltransferase (GT) and sialyltransferase (ST) are relatively nonspecific with respect to acceptor molecules. GT will transfer galactose from uridine diphosphogalactose (UDP-Gal) to either a small molecular weight acceptor, such as *N*-acetylglucosamine, to form *N*-acetyllactosamine or analogously to a free *N*-acetylglucosamine group on a glycoprotein such as ovalbumin (42). ST of rat liver Golgi apparatus also can utilize lactose or *N*-acetyllactosamine as an acceptor of sialic acid from cytidine monophosphosialic acid (CMP-NAN[*N*-acetylneuraminic acid]) to form the corresponding trisaccharide or to protein substrate such as desialylated serum transferrin containing the appropriate terminal *N*-acetyllactosamine group. More than one enzyme may be involved, because both the 2→6 and 2→3 isomers of sialyllactose are formed (37).

Both GT and ST of rat liver Golgi are membrane-bound enzymes that can be solubilized by use of Triton X-100. When treated in this manner, GT forms a complex with Triton X-100 consisting of 52% by weight detergent (20). Such high deter-

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gent-binding capability is one criterion of an intrinsic membrane protein (22, 44).

The main biological role of these glycosyltransferases appears to be the modification of secreted glycoproteins (17, 40). Thus the orientation of these enzymes in the Golgi apparatus is most probably towards the lumen. Some evidence for such an orientation has been obtained. In vesicles derived from Golgi apparatus of rat mammary gland, Kuhn and White (28) showed that newly synthesized lactose, a product of the GT-lactalbumin complex (8), was largely formed within the vesicles. Using Golgi apparatus isolated from rat liver, Hino et al. (23) showed that galactosyltransferase activity was enhanced 26-fold by treatment with Triton X-100 when ovalbumin was used as substrate.

Golgi apparatus isolated by our procedure, though largely intact when fixed directly from the sucrose step gradient, is vesiculated after dilution with distilled water and subsequently recovered by centrifugation and resuspended by gentle homogenization (16). In the present studies we have attempted to ascertain the orientation of GT and ST activities in these vesicles. We have three lines of evidence that support the hypothesis that both transferases are mainly oriented towards the luminal side of these vesicles. These are: (*a*) the effects of Triton X-100 on the transferase activities of the vesicles; (*b*) the effect of trypsin on the transferase activity of the vesicles; and (*c*) the effect of the polyene antibiotic filipin on the trypsin susceptibility of the transferases in the vesicles.

MATERIALS AND METHODS

Male Holtzmann rats, weighing 200–250 g and fed ad libitum, were used. Golgi apparatus–rich fractions were prepared from livers as described previously (16). They were used either freshly prepared or after quick-freezing and storage in liquid nitrogen. All radioactive compounds were purchased from New England Nuclear, Boston, Mass. UDP-Gal uniformly labeled with ^{14}C in galactose (~0.3 Ci/mmol) was diluted with carrier (obtained from Calbiochem-Behring Corp., American Hoschst Corp., San Diego, Calif.) to ~0.3 mCi/mmol. CMP-[^{14}C]-NAN (sialic -4 [^{14}C]; ~1 mCi/mmol) was used without addition of carrier. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co., Phoenix, Ariz., and crystalline ovalbumin (grade VI) human transferrin and soybean trypsin inhibitor, type 1-S, from Sigma Chemical Co., St. Louis, Mo. Transferrin was desialylated before use as described (20). Trypsin (grade B, essentially free of chymotrypsin) was obtained from Calbiochem-Behring Corp. It was dissolved (1 mg/ml) in 1 mM $CaCl_2$, 10 mM KCl, and the pH was adjusted to 3.0 with HCl. After storage for 3 h at room temperature to inactivate any residual chymotrypsin, it was stored at 4°C overnight before use. Filipin was the kind gift of Dr. Joseph Grady, The Upjohn Co., Kalamazoo, Mich. Just before use it was dissolved in dimethylformamide at 10 mg/ml. Triton X-100 was obtained from Research Products International Corp., Elk Grove Village, Ill., and was neutralized to pH 7 with KOH before use.

UDP-Gal : *N*-acetylglucosamine galactosyltransferase activity (GT) was determined as described previously (20). When ovalbumin was used as acceptor, 4 mg of ovalbumin, made up as 20% wt/vol in 0.1 M sodium cacodylate, pH 6.5, was added instead of *N*-acetylglucosamine. After incubation at 37°C for 1 h, the reaction (final volume, 95 μl) was stopped by the addition of 20 μl of cold 0.3 M EDTA, pH 7.5, and the samples were placed on ice. Aliquots of the reaction mix were spotted on Whatman No. 3 filter paper disks and dried, and the protein-bound radioactivity was determined as described by Mans and Novelli (32).

CMP-NAN : glycoprotein sialyltransferase was assayed by our modification (20) of the method of Schachter et al. (41). When lactose was used as acceptor, the method of Paulson et al. (37) was modified as follows. The assay mixture contained, in order of addition, 5 μmol of sodium phosphate, pH 6.8, 10 μg of protein, 300 μg of Triton X-100, 10 μmol of lactose, and 25 μmol of CMP-[^{14}C]-NAN, in a total volume of 50 μl. After incubation at 37°C for 1 h, 0.02 ml of cold 0.3 M EDTA was added and the samples were placed on ice. [^{14}C]Sialyllactose was separated from the reaction mix as described by Paulson et al. (37) and collected directly into scintillation vials containing 10 ml of scintillation fluid (Aqueous Counting Scintillant; Amersham/Searle Corp., Arlington Heights, Ill.).

To measure trypsin susceptibility, we suspended Golgi vesicles in 20 mM Tris-HCl, pH 8.1, at a protein concentration of 1–2 mg/ml. The suspension was allowed to come to room temperature (23°C) in the presence or absence of 1.2 mg/ml Triton X-100. Trypsin, prepared as described above, was then added to a final concentration of 8–10 μg/ml, and incubation continued at room temperature. At given times, aliquots were removed from the mix, added to 0.1 vol of soybean trypsin inhibitor, 5 mg/ml, and the aliquots were kept on ice until assayed for both galactosyl and sialyltransferase activities. All assays were carried out in the presence of added Triton X-100.

In some experiments the Golgi vesicles were pretreated with filipin before measurement of the trypsin susceptibility of the glycosyltransferases. Filipin, freshly dissolved in dimethylformamide, was added to the Golgi vesicles in amounts indicated in the individual experiments. In all cases the amount of dimethylformamide added was kept 5% by volume. Controls were treated with dimethylformamide alone. The mixture was incubated at 37°C for 15 min to allow complex formation between filipin and cholesterol (13). It was then cooled to 23°C before treatment with trypsin as described above.

The ability of filipin to release serum albumin from the lumen of the Golgi vesicles was quantitated using rocket immunoelectrophoresis (34, 46). The gels consisted of 1.2% agarose in 38 mM Tris-glycine, pH 8.6, containing 2 mg/ml Triton X-100, and 85 μg/ml rabbit anti-rat serum albumin antibody (lot no. 12029; Cappel Laboratories Inc., Cochranville, Pa.). After treatment with filipin as described above, the Golgi vesicles were sedimented by centrifugation at 80,000 *g* for 30 min. The supernates were removed and the pellets resuspended in 0.25 M sucrose. Supernates and pellets were treated with Triton X-100 at a final concentration of 0.6%, and 1–10 μg of protein were added to each well. Electrophoresis was carried out at a constant 100 V for 3.5 h. The gels were incubated at room temperature for 2 d in a humid atmosphere. Excess unreacted antibody was removed by washing with 0.9% NaCl followed by distilled water. The gels were stained with Coomassie Brilliant Blue, and the area under each peak was determined. Albumin concentration was calculated by comparison to a standard curve obtained by treating known concentrations of rat serum albumin in the same manner.

Electron microscopy of the Golgi vesicles before and after treatment with filipin was carried out after fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, followed by fixation in 1% OsO_4, dehydration, and sectioning as described previously (16). Negative staining with phosphotungstic acid has been described (19).

In the final experiment, we attempted to localize the filipin-cholesterol complex in the intact Golgi apparatus. To retain its structure, we fixed the preparation of Golgi apparatus as it was recovered from a D_2O-sucrose step gradient, before dilution with 0.25 M sucrose in H_2O.[1] This was done by mixing the Golgi apparatus in suspension (2 mg of protein) with 0.1 vol of 25% glutaraldehyde in 0.67 M cacodylate buffer, pH 7.4, and letting the mixture stand overnight at 4°C. The fixed sample was then diluted with 1 vol of 0.25 M sucrose in H_2O and centrifuged at 10,000 rpm for 10 min in a Spinco Ti 50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellet was washed once by gentle suspension in 0.25 M sucrose, pH 7.4, using a stirring rod, and recentrifuged as before. The pellet was finally suspended as before in 0.25 M sucrose, pH 7.4, and divided into two equal portions. To one portion was added 7 μl of dimethylformamide (control), whereas 7 μl of dimethylformamide containing 0.21 mg of filipin was added to the other. Both samples were incubated for 5 min at 37°C. The Golgi apparatus was recovered by centrifugation for 10,000 rpm for 10 min as before and supernates were discarded. The pellets were postfixed in 1% OsO_4, dehydrated, and sectioned as described previously (19).

Protein was determined by the procedure of Lowry et al. (31) and phosphorus by a modification of the method of Chen et al. (9).

RESULTS

Table I illustrates the fact that the glycosyltransferases in freshly prepared rat liver Golgi vesicles are largely inaccessible to protein substrates such as ovalbumin and desialylated transferrin. Specific activities of these enzymes can be enhanced about eightfold by the addition of Triton X-100. Smaller molecular weight substrates, such as *N*-acetylglucosamine or lactose, are more accessible to the enzymes in vesicles. Activity is stimulated only about twofold by the addition of detergent. Quick-freezing followed by short-term (24 h) storage in liquid nitrogen does not change these properties significantly. Long-term storage in liquid nitrogen causes an increase in accessibility of the enzymes to the small molecular weight substrates while the large molecular weight substrates are still largely

[1] B. Fleischer. The nucleotide content of rat liver Golgi vesicles. Manuscript in preparation.

inaccessible to the enzymes unless detergent is added.

Further evidence for the luminal localization of these glycosyltransferases was obtained by studying the action of trypsin on Golgi vesicles. A typical experiment is illustrated in Fig. 1. Fig. 1 *A* shows that 10 μg/ml trypsin at room temperature has little effect on galactosyltransferase activity of freshly prepared Golgi vesicles. Addition of Triton X-100, however, causes complete inactivation of this enzyme by trypsin after a 20-min incubation. In the absence of added *N*-acetylglucosamine, rat liver Golgi apparatus preparations catalyze the hydrolysis of UDP-Gal to give free galactose (17). The activity is not appreciably enhanced by the addition of Triton X-100, indicating that the enzyme in intact vesicles is more accessible to added UDP-Gal than is GT. Treatment with trypsin had no effect on this activity regardless of the addition of detergent, making it impossible to draw any conclusion as to its accessibility to the protease.

Fig. 1 *B* shows the effect of the trypsin treatment on sialyltransferase activity of the same Golgi vesicles. This enzyme is also resistant to trypsin unless detergent is added. It is somewhat less readily destroyed by trypsin than is galactosyltransferase. Under the same conditions of treatment, it takes 40 min to inactivate the sialyltransferase completely, whereas the galactosyltransferase is inactivated in 20 min. The apparent slight increase in sialyltransferase in the vesicles incubated with trypsin in the absence of Triton X-100 seen in this experiment is not typical (see, for example, Fig. 2 *B*). In five additional experiments, in which different Golgi preparations were used, sialyltransferase of the Golgi apparatus decreased 9.6 ± 4.0% after 40 min with this level of trypsin. Galactosyltransferase in these experiments decreased 10.2 ± 6.8%.

Because we have shown previously that Triton X-100 releases galactosyltransferase and sialyltransferase activity from the membrane, the increased trypsin susceptibility produced by

TABLE I

Effect of Triton X-100 on Accessibility of Substrates to Glycosyltransferases of Golgi Vesicles

	Specific Activity								
	Fresh			Stored in liquid nitrogen (24 h)			Stored in liquid nitrogen (2 mo)		
Triton X-100	−	+	+/−	−	+	+/−	−	+	+/−
Galactosyltransferase									
GlcNAc	320	531	1.7	322	595	1.8	510	609	1.2
Ovalbumin	21	163	7.9	29	174	6.0	29	143	4.9
Sialyltransferase									
Lactose	56	107	1.9	ND	ND	ND	80	97	1.2
Transferrin	56	439	7.8	ND	ND	ND	61	414	6.9

Specific activity is expressed as nmoles transferred per hour per milligram of protein at 37°C. Assays were carried out in the presence (+) or absence (−) of 7 mg/ml Triton X-100 in the assay medium. +/− is the ratio of specific activity in the presence and absence of Triton X-100. *ND*, not determined.

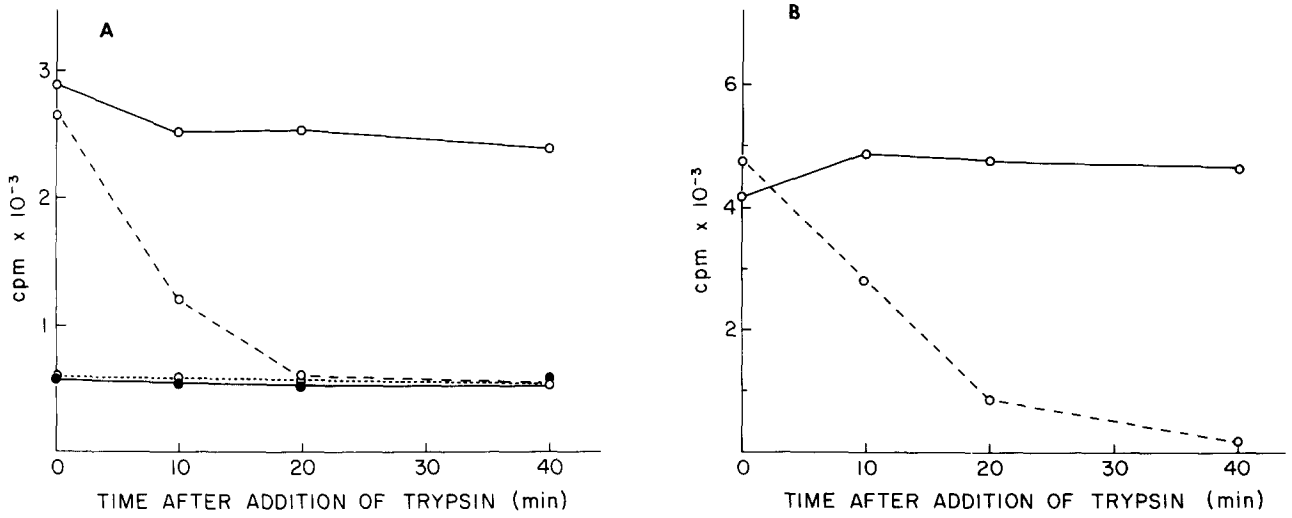

FIGURE 1 Golgi vesicles (0.5 ml, 0.77 mg of protein) were treated with 5 μg of trypsin at 23°C in the presence and absence of 0.6 mg of Triton X-100. At given times after addition of trypsin, 0.09-ml samples were removed, added to 0.01 ml solution of soybean trypsin inhibitor, 5 mg/ml water and assayed for glycosyltransferase activities. (*A*) Effect of trypsin on galactosyltransferase activity of rat liver Golgi vesicles. Galactosyltransferase activity of Golgi vesicles with GlcNAc as substrate after treatment with trypsin in the presence (O- - -O) and absence (O——O) of Triton X-100. Hydrolysis of UDP-Gal was measured in the absence of acceptor (−GlcNAc) after treatment with trypsin in the presence (O···O) and absence (●——●) of Triton X-100. All assays carried out in the presence of 7 mg/ml Triton X-100. (*B*) Effect of trypsin on sialyltransferase activity of rat liver Golgi vesicles. Sialyltransferase activity of Golgi vesicles with desialylated transferrin as substrate after treatment with trypsin in the presence (O- - -O) and absence (O——O) of Triton X-100. Assays contain 7 mg/ml Triton X-100.

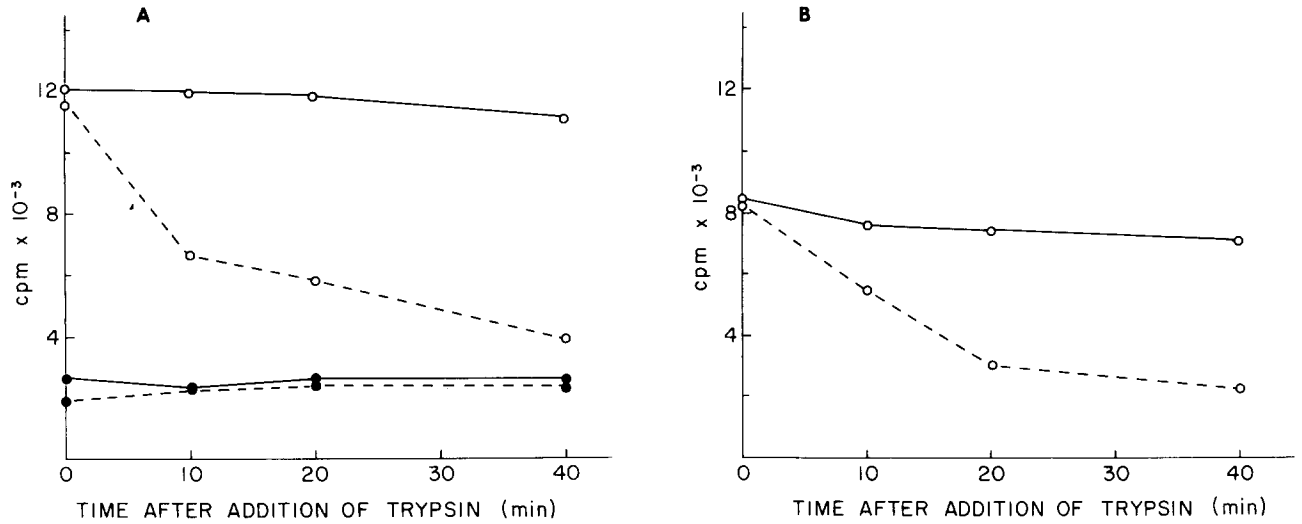

FIGURE 2 Golgi vesicles (0.95 mg of protein in 0.5 ml of 0.25 M sucrose) were incubated in the presence and absence of filipin (100 μg) for 15 min at 37°C. Both samples were then treated with 5 μg of trypsin at 23°C. At given times after addition of trypsin, 0.09 ml was removed, added to 0.01 ml solution of soybean trypsin inhibitor, 5 mg/ml water and assayed for glycosyltransferase activities. (*A*) Effect of filipin on trypsin susceptibility of galactosyltransferase activities of Golgi vesicles. Galactosyltransferase activity of Golgi vesicles pretreated with (O- - -O) and without (O——O) filipin. Assayed with GlcNAc as substrate in the presence of 7 mg/ml Triton X-100. Hydrolysis of UDP-Gal was measured in the absence of acceptor (−GlcNAc) after treatment with trypsin in the presence (●- - -●) and absence (●——●) of filipin. (*B*) Effect of filipin on trypsin susceptibility of sialyltransferase activity of Golgi vesicles. Sialyltransferase activity of Golgi vesicles pretreated with (O- - -O) and without (O——O) filipin. Assayed with desialylated transferrin as substrate in the presence of 7 mg/ml Triton X-100.

this treatment, though suggestive, was not a compelling argument for the luminal localization of these enzymes. We looked therefore for an agent that would have the specific effect of disrupting the Golgi membrane enough to make it permeable to various probes without solubilizing the proteins of the membrane.

We have shown that Golgi membranes, in contrast to other cytoplasmic membranes from liver, contain significant levels of cholesterol (49). Filipin, a polyene antibiotic, has been shown to complex with cholesterol present in membranes and cause lysis of erythrocyte membranes and increased permeability of phospholipid vesicles containing cholesterol (27, 36). We therefore investigated the effect of filipin treatment on the trypsin susceptibility of the glycosyltransferases in Golgi vesicles.

Golgi vesicles were treated with 0.1 mg of filipin/mg of protein at 37°C for 15 min. They were then treated with trypsin as before and assayed for activity in the presence of Triton X-100. Fig. 2*A* shows that filipin treatment causes galactosyltransferase activity of the vesicles to become trypsin sensitive. As with detergent treatment, no effect of trypsin was seen on the hydrolysis of UDP-galactose in vesicles pretreated with filipin. Fig. 2*B* shows that sialyltransferase activity also becomes trypsin sensitive after pretreatment of Golgi vesicles with filipin.

Using trypsin susceptibility as a gauge of filipin interaction with the membranes, it is possible to titrate the amount of filipin necessary for maximum disruption of the Golgi vesicles. Fig. 3 shows the results of such a titration. In this experiment, trypsin treatment was carried out for only 10 min so that not all activity would be destroyed even under optimal conditions. It can be seen that maximum destruction of the glycosyltransferases is obtained at ~1.6 mg of filipin added/mg of total cholesterol present in the Golgi preparations, or a molar ratio of filipin to cholesterol of ~1.

This level of filipin does not cause a significant release of

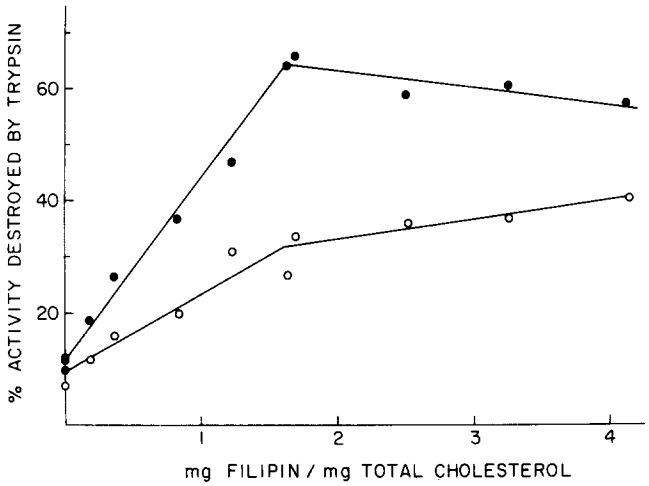

FIGURE 3 Effect of filipin on trypsin susceptibility of glycosyltransferase activities of Golgi vesicles. Golgi vesicles were treated as described in Fig. 2 with various ratios of filipin to protein. Each sample was then incubated in the presence of 5 μg of trypsin for 10 min at 23°C. At 0 time and at 10 min, 0.09-ml samples were added to 0.01 ml solution of soybean trypsin inhibitor, 5 mg/ml water. The samples were then assayed for galactosyltransferase (●) and sialyltransferase (O) activities as in Fig. 2. The percent activity destroyed by trypsin was calculated using the 0-time trypsin treatments as controls.

glycosyltransferases from the Golgi membrane but does cause significant release of Golgi contents as measured by the release of rat serum albumin (Table II). Addition of 0.11 mg of filipin/mg of protein, which corresponds to a ratio of 1.6 mg of filipin/mg of total cholesterol, the optimum level of filipin for trypsin sensitivity of the glycosyltransferases, leaves 93–96% of the enzymes membrane-bound and causes release of ~63% of the total albumin contained originally in the Golgi cisternae.

The effect of filipin on the Golgi vesicles can be visualized

TABLE II

Effect of Filipin on the Release of Glycosyltransferases from Golgi Membranes

Filipin added*	GT activity‡			ST activity‡			Albumin§	
	Pellet	Supernate	Recovery	Pellet	Supernate	Recovery	Pellet	Supernate
mg/mg protein								
0	98.4	1.6	100	97.3	2.7	100	82.9	17.1
0.11	95.9	4.1	112	93.2	6.8	117	37.5	62.5
0.21	92.6	7.4	108	88.9	11.1	104	26.3	73.7

* Golgi vesicles (0.95 mg of protein in 0.5 ml) were treated with various amounts of filipin dissolved in 20 μl of dimethylformamide for 15 min at 37°C, followed by centrifugation at 145,000 g_{max} for 60 min at 4°C to sediment the membranes. The membranes were resuspended in 0.25 M sucrose. Golgi vesicles contain 0.07 mg of cholesterol/mg of protein (49).

‡ Activity expressed as percent of total activity recovered in pellets plus supernates. GT activity was measured with GlcNAc, and ST activity was measured with disialylated transferrin as acceptor in the presence of Triton X-100. Recovery is expressed as: ([activity recovered in pellets plus supernates]/[activity originally added to the tube]) × 100.

§ In a separate series of experiments, the amount of rat serum albumin remaining in the Golgi vesicles after treatment with filipin and centrifugation was determined by use of rocket immunoelectrophoresis (see Materials and Methods).

by electron microscopy. Fig. 4*A* and *B* shows fixed and embedded sections of the Golgi vesicles preparations used in these studies. They are largely empty vesicles heterogeneous in size. Some filled vesicles derived from Golgi secretory vesicles can be seen and some membrane fragments and broken vesicles are also present.

Fig. 5*A* and *B* shows a similar view of filipin-treated vesicles. The membranes are still largely vesicular although smaller in size than the untreated preparation. The most striking effect of filipin is that the membranes appear to be puckered. This is emphasized in Fig. 5*C*, which shows either the surface of a puckered vesicle or a multilayered vesicle. Modification of the membrane can also be seen by negative staining. Fig. 6*A* shows the normal appearance of Golgi vesicles by negative staining with phosphotungstic acid. Fig. 6*B* shows the filipin-treated membranes after similar negative staining. Pore-like structures ~250 Å in diameter can be seen randomly dispersed in the membranes. Such structures have been described previously in lecithin vesicles containing cholesterol after treatment with filipin and are believed to be linear arrays of filipin-cholesterol aggregates rather than pores in the membrane (11). In our view also, the appearance of puckers in treated membranes seen in the embedded and sectioned samples is more consistent with local distortions of the membrane structure brought about by filipin-cholesterol clusters rather than pore structures through the membrane.

Isolated Golgi apparatus, when fixed with glutaraldehyde before dilution to recover them from sucrose gradient solutions, retain their three-dimensional structure (Fig. 7*A*). When such a fixed sample is treated with filipin, membrane alterations characteristic of filipin-cholesterol interactions can still be seen (Fig. 7*B*). Puckered membranes are found both on cis and trans cisternae as well as on secretory vesicles of the complex. In a qualitative way, at least, filipin interacts with all the membranes, which suggests that cholesterol is present throughout the membrane of the Golgi stack as well as in secretory vesicle membranes.

The puckering seen in the membranes of filipin-treated Golgi preparations is not uniformly distributed. The level of filipin used in these experiments was selected as the minimum necessary to allow access of trypsin into all the vesicles containing glycosyltransferases under the conditions of concentration, time, and temperature arbitrarily selected. Higher levels of filipin cause more extensive puckering and fragmentation (data not shown). Thus, the heterogeneity seen at this level of filipin is attributable mainly to the low level of filipin we have used. Some heterogeneity is also attributable to the fact that ~10–15% of the vesicles present are derived from endoplasmic reticulum and contain very low amounts of cholesterol (49).

DISCUSSION

One function of the Golgi apparatus in liver is the terminal glycosylation of proteins destined for secretion into the blood. There is general agreement that such proteins are synthesized on the membrane-bound ribosomes of rough endoplasmic reticulum (4). During translation, the proteins pass through the membrane where glycosylation begins with the addition of core sugars. This involves a dolichol phosphate oligosaccharide as intermediate (for a review of this pathway, see reference 45). The newly formed glycoproteins are then transferred to the Golgi apparatus, probably to the cis side (2). During the progress of the glycoproteins through the Golgi apparatus towards eventual secretion, glycosylation is completed by galactosyltransferase and sialyltransferases using nucleotide sugars as donors (17–19, 35, 40, 41). Both glycosyltransferases are membrane bound (20) and occur throughout the Golgi apparatus, although apparently in higher concentrations in trans elements than in cis elements (7, 33).

The Golgi apparatus can be separated from other organelles of liver in largely intact form by zonal centrifugation in a sucrose step gradient. Upon dilution, centrifugation, and rehomogenization, however, it becomes vesiculated (16). Although vesiculated, this preparation retains a significant proportion of secretory contents including albumin (38), proalbumin (14), and, when prepared from newborn rats, α-fetoprotein (1). About 30% of the total protein is attributable to soluble proteins of the lumen (16). This fraction represents ~25% of the total Golgi apparatus present in the liver homogenate. The results presented in this paper show that in this preparation both galactosyl and sialyltransferase are largely inaccessible to large molecular weight substrates or to inactivation by trypsin unless the vesicles are disrupted either with a nonspecific detergent such as Triton X-100 or with a more selective agent such as filipin. Thus, these vesicles are largely oriented with both galactosyltransferase and sialyltransferase facing the lumen of the vesicles, the orientation expected for enzymes that process secreted proteins. Even in freshly prepared vesicles, ~10% of the enzymes are accessible to substrates of large molecular weight and ~10% of both glycosyltransferases are sensitive to trypsin. It is very likely that this represents damaged or "open" vesicles, because some open-ended membranes are

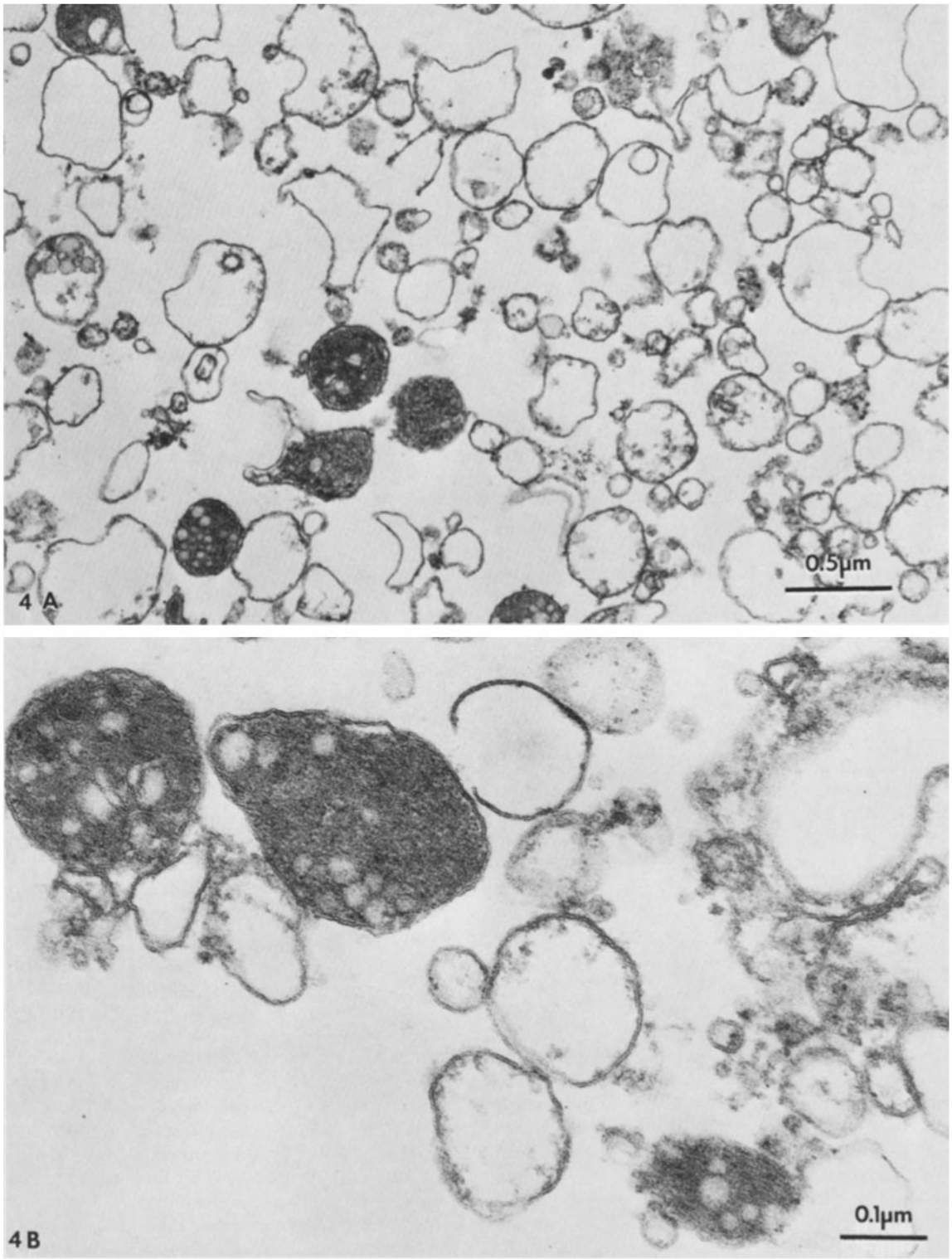

FIGURE 4 Electron micrographs of sections of fixed and embedded samples of normal rat liver Golgi vesicles isolated after osmotic shock. (*A*) × 35,000; (*B*) × 125,000.

seen by electron microscopy of fixed, embedded, and sectioned samples of these preparations, and serum albumin can be detected in supernates from these fractions. It should be possible to improve the isolation of Golgi apparatus to reduce the proportion of damaged vesicles. Such a preparation would be useful for the study of the transport properties of Golgi membranes. We are currently investigating the use of D_2O-sucrose gradients for this purpose.

It can be argued that filipin increases the trypsin sensitivity of the glycosyltransferases by changing the membrane structure so that these enzymes, which are really on the cytoplasmic side but somehow cryptic, are now accessible to substrate and trypsin. However, we have found that filipin activates nucleoside diphosphatase activity of isolated Golgi vesicles about fourfold without causing the enzyme to become sensitive to trypsin (6). This enzyme has been shown histochemically to be

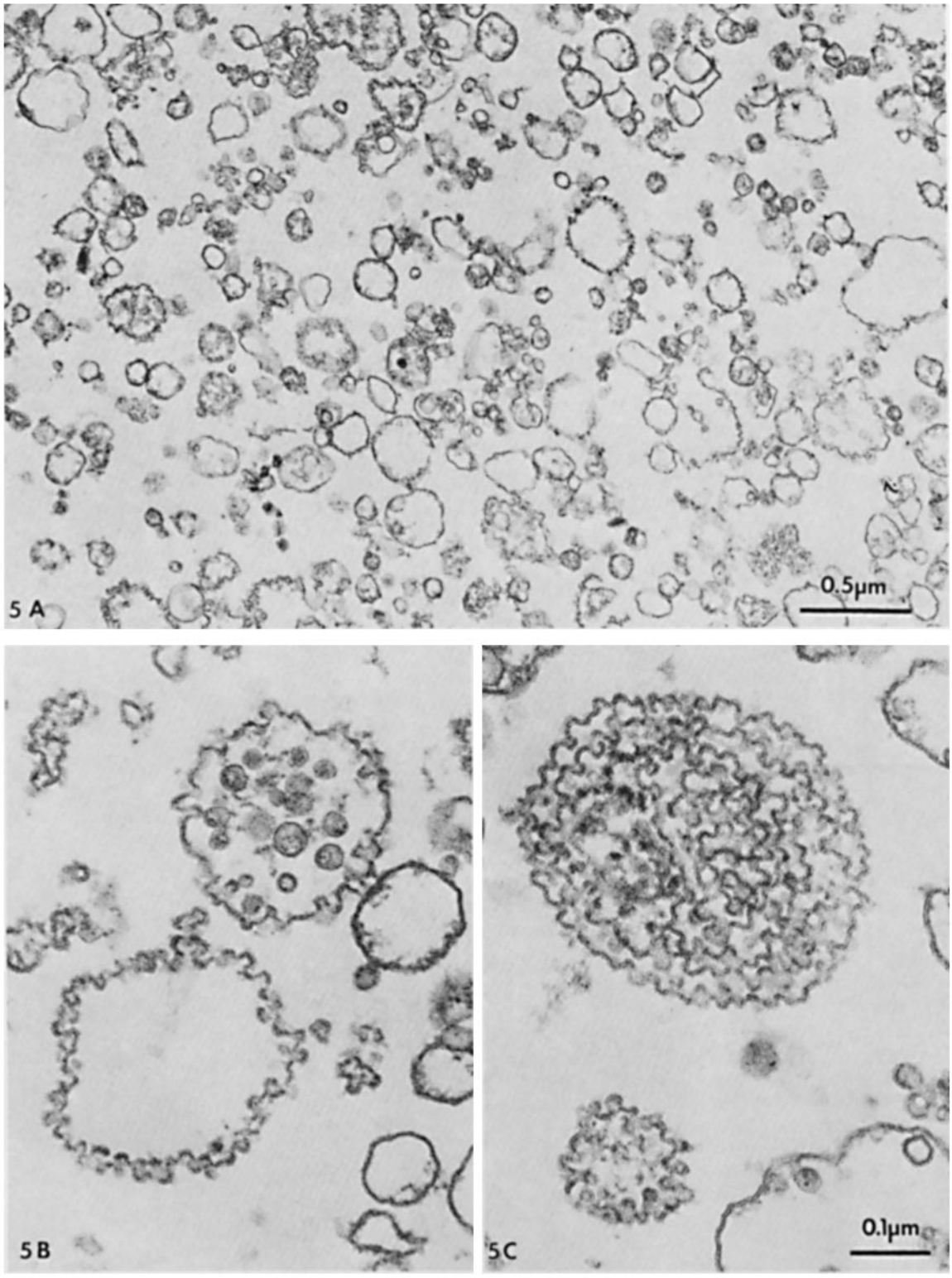

FIGURE 5 Electron micrographs of sections of fixed and embedded samples of rat liver Golgi vesicles treated with 0.11 mg of filipin/mg of protein. (*A*) × 35,000; (*B* and *C*) × 125,000.

localized on the lumen side of the Golgi membrane (21).

The demonstration that the polyene antibiotic filipin can be used to disrupt Golgi membranes confirms our previous finding that cholesterol is present in Golgi membranes rather than only in Golgi secretory products (49). Filipin is a useful tool for disrupting the membrane without solubilizing membrane-bound enzymes and makes it possible to study the orientation of various components in Golgi membranes. Filipin will complex with cholesterol in fixed membranes as well as unfixed materials, and, because the filipin-cholesterol complexes can be visualized by a number of electron microscopy techniques, this is a useful method for localizing cholesterol in membrane structures (39). We have used this approach to show that cholesterol is present throughout the Golgi apparatus mem-

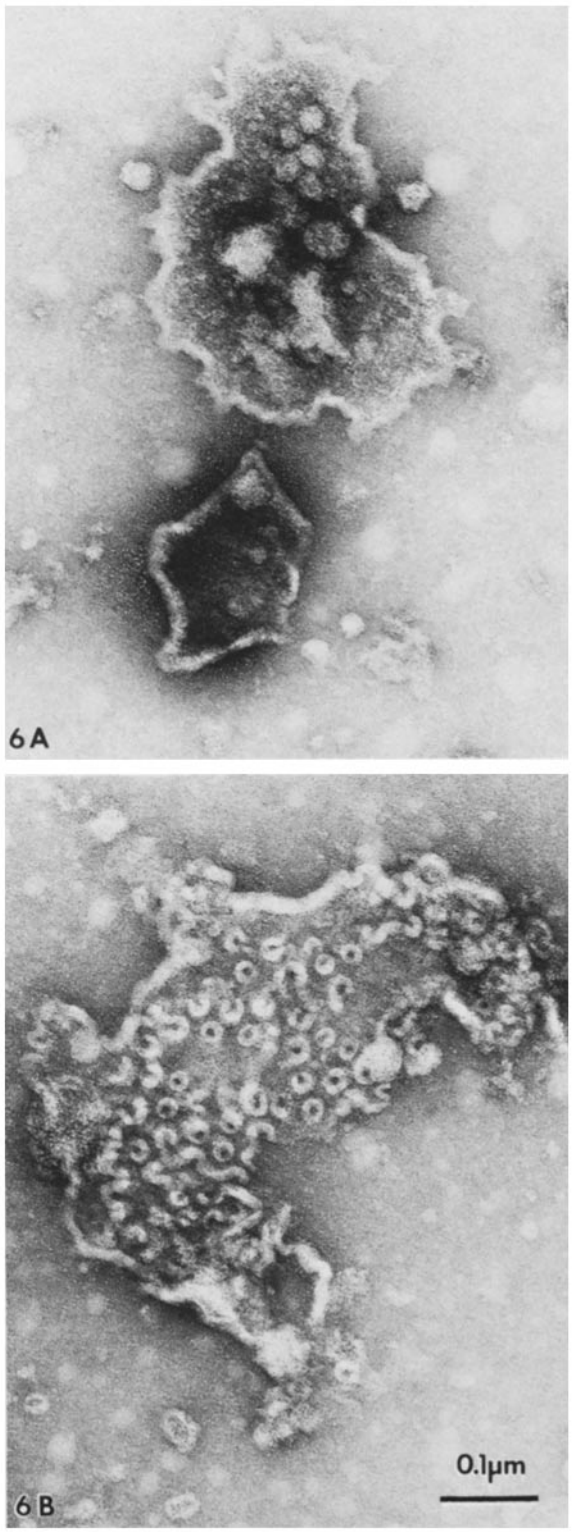

FIGURE 6 Electron micrographs of Golgi vesicles after negative staining with 2% phosphotungstic acid. (*A*) Normal vesicles. (*B*) Vesicles after treatment with 0.11 mg of filipin/mg of protein. × 125,000.

brane, in both cis and trans cisternae, and in secretory vesicle membranes as well.

Maximum disruption of the Golgi vesicles, as measured by the susceptibility of both galactosyltransferases and sialyltransferases to trypsin, is achieved at filipin to total cholesterol molar ratios of ~1. This is in agreement with the stoichiometry of the filipin-cholesterol complex observed in solution by fluorescence spectroscopy by Schroeder et al. (43) as well as by differential scanning calorimetry on liposomes containing cholesterol as determined by Van Deenen's laboratory (36). This may be fortuitous because, in solution, filipin does not appear to complex with cholesterol esters (43). Golgi vesicles contain 30% of their total cholesterol in the form of cholesterol esters (26, 49) so that the molar ratio of filipin to free cholesterol that produces maximum disruption is 1.5. Similar ratios have been reported to cause both maximum K^+ efflux and release of large cytoplasmic proteins from *Acholeplasma laidlawii* cells (12). In this respect filipin differs from other polyene antibiotics such as amphotericin B in its effects. The latter is believed to create aqueous pores of a specific size in cell membranes containing cholesterol (12). Using stopped-flow rapid kinetic measurements of filipin binding to intact and unsealed erythrocyte ghosts, Blau and Bittman (3) have concluded that cholesterol is distributed about equally on the outside and inside of the membrane, presumably in equal amounts in the two halves of the lipid bilayer.

Information is slowly accumulating regarding the arrangement of some enzymes present in preparations of Golgi apparatus–rich fractions from rat livers. Using antibodies specific for electron-transfer components of endoplasmic reticulum (ER) membranes, Borgese et al. found cytochrome b_5 and NADH–cytochrome b_5 reductase activities of Golgi vesicles isolated from ethanol-treated rats to be inhibited, indicating localization on the cytoplasmic side of the vesicles (5). At present there is considerable uncertainty as to whether these components are actually present in Golgi membranes or whether they result from contamination of the preparations with ER (24). It has been shown in some vesiculated preparations (25) that most of the ER-type activities are on a distinct subpopulation of vesicles in the fraction, compared with the galactosyltransferase activity.

There is general agreement that both Golgi apparatus and ER contain some 5′-nucleotidase activity, an enzyme activity commonly used as a "marker" enzyme for liver plasma membranes. In ER, the enzyme is localized exclusively on the cytoplasmic side of the membrane (47). In Golgi apparatus, it appears to be on the cytoplasmic side in the dilated cisternal rims but on the lumen side in the secretory vesicles (15, 30). Adenylate cyclase, also widely used as a marker for plasma membrane, has also been demonstrated histochemically on the cytoplasmic side of both Golgi membranes and smooth ER (10).

Thiamine pyrophosphatase, or nucleoside diphosphatase (48), activity is present on the luminal side of both Golgi apparatus and ER in liver (21). Kuhn and White (29) have proposed that, in Golgi apparatus, the enzyme is probably involved in the breakdown of UDP formed in the lumen by the action of galactosyltransferase and *N*-acetylglucosamine transferase on their respective nucleotide sugars.

Our studies provide strong evidence that isolated rat liver Golgi vesicles are mainly oriented with galactosyltransferase and sialyltransferase activities on the luminal face of the membranes, which corresponds to the cisternal side of the Golgi apparatus in the hepatocyte. Furthermore, they show that cholesterol is an integral part of the membranes of the Golgi apparatus and that its distribution throughout the Golgi apparatus is similar to that of both transferases. Cholesterol is probably present throughout the Golgi stack as well as in secretory vesicle membranes.

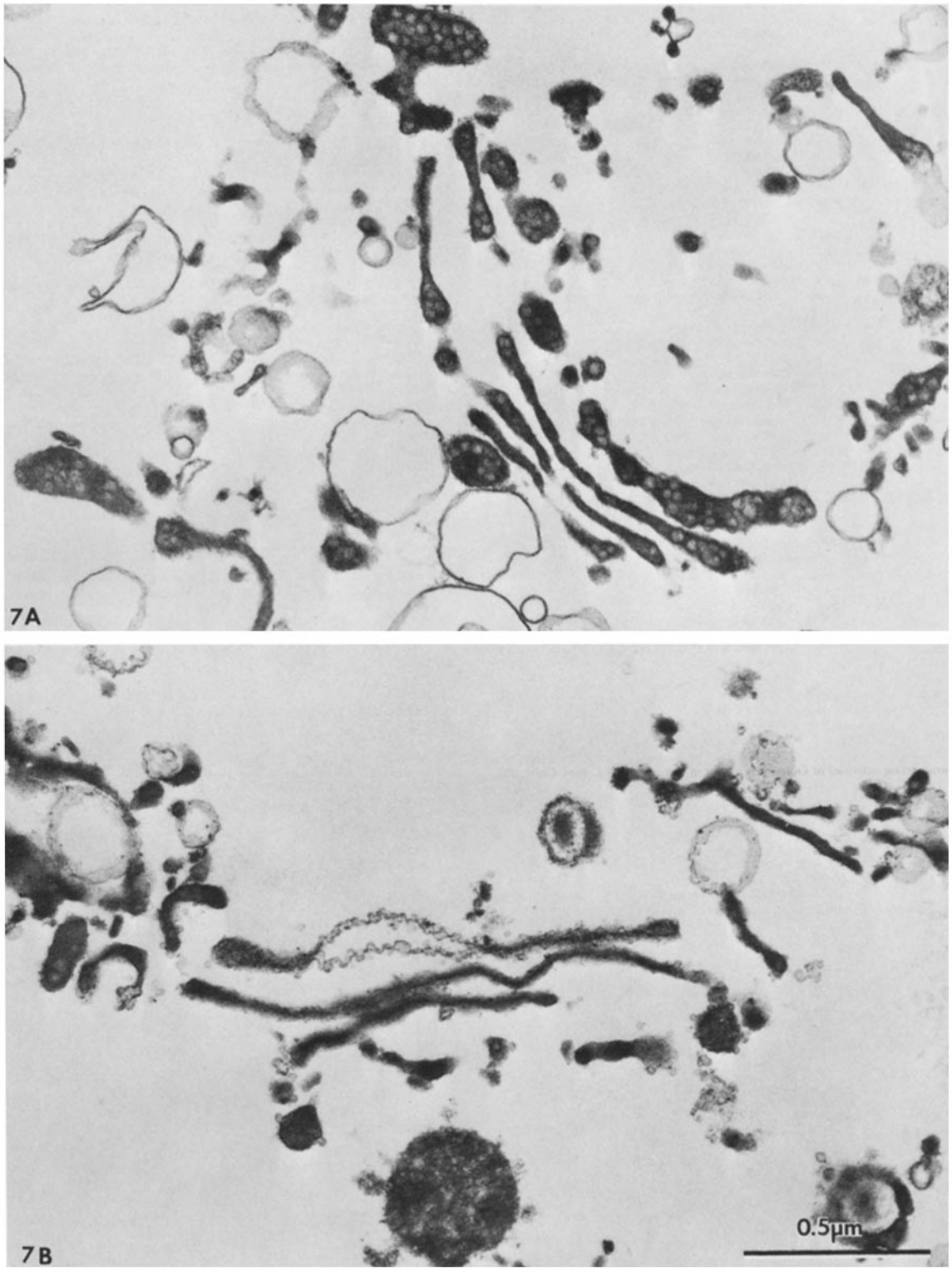

FIGURE 7 Electron micrographs of Golgi apparatus fixed with 2.5% glutaraldehyde before treatment with filipin. (*A*) Control treated with dimethylformamide after fixation. (*B*) Sample treated with 0.2 mg of filipin in dimethylformamide/mg of protein after fixation. × 60,000.

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