

Membrane Fusion and Glycosylation in the Rat Hepatic Golgi Apparatus

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ABSTRACT When purified Golgi fractions were incubated with UDP-[³H]galactose in the absence of Triton-X-100, radioactivity was incorporated into an endogenous lipid and several peptide acceptors.

Electron microscope analysis of Golgi fractions incubated in the endogenous galactosyl transferase assay medium revealed extensive fusion of Golgi saccules. Systematic removal of constituents in the galactosyl transferase assay medium showed enhanced (minus β -mercaptoethanol) or reduced (minus ATP, minus sodium cacodylate buffer or minus MnCl₂) fusion of Golgi membranes compared to the complete medium. Stereologic analysis revealed a correlation between membrane fusion and galactosyl transferase activity ($r = 0.99$, $P < 0.001$). Electron microscope radioautography was carried out after incubation of Golgi fractions with UDP-[³H]galactose. Silver grains were not observed over *trans* elements of Golgi but were revealed mainly over large fused saccules with the number of silver grains being proportionate to membrane fusion ($r = 0.92$, $P < 0.001$).

Bilayer destabilization at points of Golgi membrane fusion may act to translocate galactose across the Golgi membrane and thereby provide a fusion regulated substrate for terminal glycosylation.

Nucleotide sugar transfer to endogenous and exogenous acceptors by subcellular fractions *in vitro* is often studied using incubation medium containing detergents. Several studies using detergent solubilized membrane have been carried out on the incorporation of the core sugars *N*-acetylglucosamine and mannose (see reviews 19, 25 and 31 as well as references 4, 15, 22, 26, and 30) and the terminal sugars *N*-acetylglucosamine, galactose, and sialic acid (8, 29).

Recently, it was shown that the core sugars of oligosaccharide chains could be incorporated *in vitro* in endogenous acceptors of rough endoplasmic reticulum membranes under specific incubation conditions and in the absence of detergents (13, 14). As shown in the preceding paper (7) UDP-galactose (a distal sugar of oligosaccharide chains) can also be incorporated *in vitro* into endogenous acceptors of isolated Golgi apparatus incubated in the absence of detergent. Assuming that Golgi membranes are impermeable to exogenous nucleotide sugars, we are led to wonder just how these sugars are membrane translocated in the absence of detergents.

We report in this paper biochemical and stereological evidence revealing a correlation between glycosylation of Golgi

membranes and Golgi membrane fusion *in vitro*. Because of the high correlation observed between the two phenomena it is proposed that membrane fusion regulates the translocation of sugar across the Golgi membrane. Electron microscope radioautography of Golgi membranes incubated in the presence of UDP-[³H]galactose reveals concentrated sugar uptake only within fused membranes, consistent with the above proposal. Portions of this work have been presented in abstract form (28).

MATERIALS AND METHODS

Biochemical Procedures

The techniques for isolating Golgi fractions and other protocols such as SDS-PAGE¹ and fluorography were exactly as described in the companion paper (7). Endogenous glycosylation was also as described in reference 7; that is, without ovomucoid acceptor and at pH 7. For all experiments, Triton-X-100 was omitted

¹ Abbreviations are: ATP, adenosine triphosphate; Dol-P-gal, dolichyl galactosyl phosphate; PTA, phosphotungstic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein.

from the incubation mixture. Thus, the complete incubation medium for endogenous glycosylation consisted of 30 mM sodium cacodylate pH 7.0; 1.6 μ M UDP- 3 H]galactose (11.6 Ci/mmol); 30 mM $MnCl_2$; 2 mM ATP; 30 mM β -mercaptoethanol; Golgi fractions (10–100 μ g protein). The final volume of the incubation mixture was usually 0.1–0.5 ml. Endogenous glycosylation was high in the Golgi intermediate and intact Golgi fractions (7). Thus, the work described herein was restricted to these two fractions.

Organic extraction of incubations was carried out as described by Behrens et al. (4). Briefly, the incubation (0.5 ml) was stopped with 11.6 volumes of chloroform/methanol/4 mM $MgCl_2$ (3:2:0.8). This resulted in two phases. The lower phase was washed twice with 2.5 ml chloroform/methanol/4 mM $MgCl_2$ (1:16:16) and termed the monosaccharidal lipid fraction. The residual material at the interphase between the organic and aqueous phases was further extracted with three washes of 2.5 ml of chloroform/methanol/water (10:10:3). This combined extract was termed the oligosaccharidal lipid fraction. Final residual material (pellet) was termed the glycopeptide fraction.

Thin-layer chromatography was carried out on silica gel G thin-layer plastic chromatography plates (Eastman Kodak, Rochester, N. Y.) using as solvent (3) chloroform/methanol/water (60:25:4). Unsaturation was marked by staining with iodine and isoprenoids were revealed by anisaldehyde reagent (12) the latter resulting in a characteristic yellow-green colour. For experiments in which attempts were made to detect retinyl derivatives, thin-layer chromatograms were viewed after irradiation at wavelengths of 225 and 350 nm. In experiments involving analysis of retinyl compounds the entire isolation of Golgi fractions was carried out with fluorescent lights off and organic extractions were carried out under a yellow safety light. Radioactivity on thin-layer plates was assessed by automated scanning (Packard Radioscanner, Downers Grove, Ill.) as well as cutting silica gel plates into 1 cm segments and scintillation counting (Packard Spectrometer, model 3003).

Morphological Procedures

ELECTRON MICROSCOPY: Golgi fractions were incubated in the endogenous galactosyl transferase medium as described above then fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4. After fixation, Golgi fractions were recovered onto Millipore membranes (Millipore Corp., Bedford, Mass.) using the Baudhuin procedure (2) as modified by Wibo et al. (32) then postfixed in 1% OsO_4 and block stained in uranyl acetate. Membrane pellicles were dehydrated and embedded in Epon 812 following standard protocols. Thin sections (silver interference color) were prepared, grid-stained with uranyl acetate and lead citrate and examined in the electron microscope.

STEREOLOGICAL ANALYSIS OF GOLGI SUBFRACTIONS: Adjacent non-overlapping micrographs were taken at 10,000-times magnification covering the complete thickness of the membrane pellicles. The negatives were projected onto the translucent measuring tablet of a Zeiss MOP-3 digitizer (Carl Zeiss, Inc., Don Mills, Ontario) at a final magnification of 60,000 times. The contour of the membrane profiles was traced manually with the MOP-3 stylus directly yielding the profile perimeter lengths for the structures within the micrographs. The profile perimeter lengths were automatically stored and subdivided into classes. The sum of the profile lengths found within each class was expressed as a percent of the total length.

RADIOAUTOGRAPHY OF LABELED SUBCELLULAR FRACTIONS: For electron microscope radioautography subcellular fractions were incubated with UDP- 3 H]galactose for 30 min at either 0° or 37°C and fixed and embedded in Epon. Thin sections (silver interference color) were cut on an LKB-Huxley ultramicrotome and a monolayer of Ilford L-4 emulsion was applied (17). After exposure, the film was treated by gold intensification and subjected to "solution-physical" development in Agfa-Gevaert (Teterboro, N. J.) developer (18).

RESULTS

Biochemistry

The incorporation with UDP- 3 H]galactose onto endogenous acceptors of the Golgi intermediate or intact Golgi fraction revealed 75% of the incorporated radioactivity in glycopeptides with 5–10% in the oligosaccharidal lipid fraction and 16–20% in the monosaccharidal lipid fraction (Table I). SDS-PAGE (Fig. 1) documented a band of M_r 10,000 for the monosaccharidal lipid fraction. This band was absent from the glycopeptide fraction but was present in the parent Golgi fractions before lipid extraction.

Thin-layer chromatography of the radiolabeled monosaccharidal lipid fraction was carried out. The experiments were done on scaled-up incubation volumes to enable chemical as

TABLE I
Distribution of Radioactivity after Endogenous Incorporation with UDP- 3 H]galactose

Extraction	Golgi intermediate fraction	Intact Golgi fraction
	%	
Monosaccharidal lipid	16.0 \pm 1.2	20.2
Oligosaccharidal lipid	9.6 \pm 1.9	5.1
Glycopeptide	75.4 \pm 3.1	74.7

UDP- 3 H]galactose (2.5 μ Ci; s.a., 11.6 Ci/mmol) was incubated with Golgi intermediate and intact Golgi fractions (20–40 μ g protein) for 30 min at 37°C (100 μ l volume) without Triton X-100 as described in Materials and Methods. Chemical extraction of the incubation mixture was carried out as described by Behrens et al. (reference 4, see Material and Methods) and the results expressed as a percent of recovered radioactivity. Total recovered radioactivity was 90–114% of PTA pellets of the fractions.

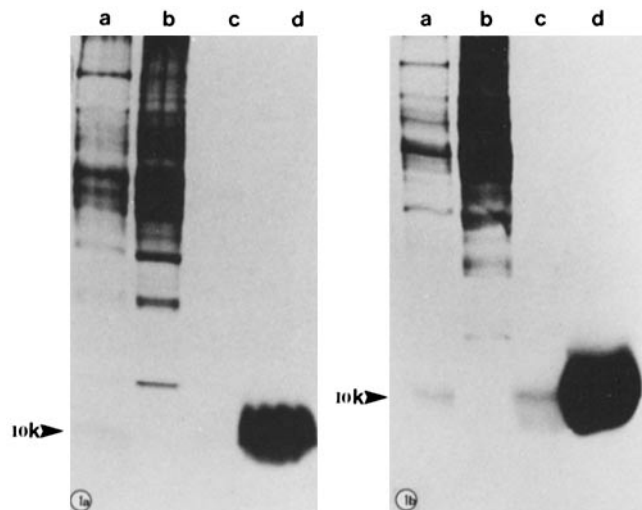


FIGURE 1 Fluorographic analysis of acceptors in intact Golgi fraction (1a) and Golgi intermediate fraction (1b) after endogenous glycosylation with UDP- 3 H]galactose as substrate. Incubations were carried out as described in the legend to Table I and lipid extraction following the procedure of Behrens et al. (4) as described in Materials and Methods. The following were electrophoresed; slot a, parent Golgi fraction (in complete medium); slot b, glycopeptide fraction after lipid extraction; slot c, oligosaccharidal lipid fraction; slot d, monosaccharidal lipid fraction. The arrow denotes the mobility of a band of M_r = 10,000 which is present in slots a, c, d but absent from slot b. Both slots b and d were overloaded to emphasize the extraction of the M_r 10,000 band. Exposure, 6 wk (Fig. 1 a) and 12 wk (Fig. 1 b).

well as radiological detection of lipids. The chromatograms revealed five unsaturated constituents (Table II). Radio-scanning (Fig. 2) revealed peaks only at the origin and in spot no. 4 which coincidentally stained yellow-green with anisaldehyde reagent (Table II). Separate analyses in which the chromatograms were sliced into 1-cm portions confirmed the exclusive location of radioactivity to the origin and spot no. 4 (Table II). Observation of thin-layer plates after exposure to wavelengths of 225 nm and 350 nm indicated no absorption at these wavelengths.

The systematic removal of each of the constituents of incubation medium for endogenous glycosylation was studied (Fig. 3). Maximal activity was observed when incubations were carried out in the absence of β -mercaptoethanol. Removal of either $MnCl_2$ or sodium cacodylate from the incubation mixture resulted in negligible incorporation of 3 H]galactose. Removal of ATP resulted in lower incorporation from maximal

TABLE II
Thin-layer Chromatographic Analysis of Monosaccharidal Lipid Fraction

Spot	R _f	Iodine	Anisaldehyde reagent	Radioactivity cpm
Origin	—	—	—	20,200
1	0.24	+	—	—
2	0.40	+	—	—
3	0.50	+	—	—
4	0.57	+	+	9,400
5	0.72	+	+	—

Golgi intermediate fraction (4 mg) was incubated for 30 min with 50 μ Ci of UDP-[³H]galactose (11.6 Ci/mmol) in the complete medium (3 ml) for endogenous glycosylation (described in Materials and Methods). After extraction of the monosaccharidal lipid fraction, an aliquot was concentrated and chromatographed on silica gel thin-layer chromatograms.

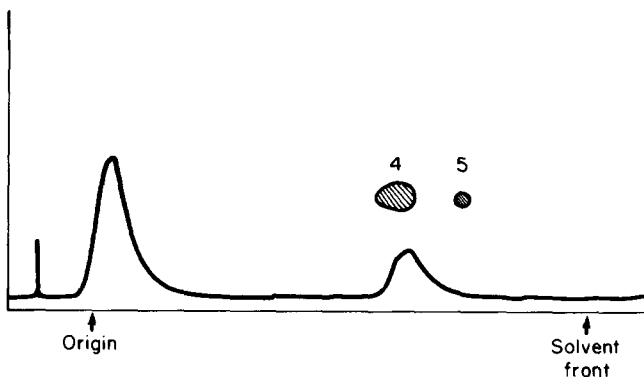


FIGURE 2 Radioscan of radioactivity distribution in monosaccharidal lipid fraction following thin-layer chromatography. The positions of the two anisaldehyde staining spots are indicated as 4 and 5 (see, as well, Table II). Spot 4 corresponds to the mobility of Dol-P-gal and spot 5 to dolichyl phosphate.

(i.e. minus β -mercaptoethanol) or control (complete medium). Similar results were obtained using the intact Golgi fraction (results not shown).

Morphology

Ultrastructural analysis of Golgi vesicles (Golgi intermediate fraction) under various incubation conditions were carried out (Fig. 4). Membrane fusion was observed for those incubations in which β -mercaptoethanol had been omitted (Fig. 4d). Golgi membrane fusion was also revealed when incubations were carried out in complete medium (Fig. 4b) or in the absence of ATP (Fig. 4f). In contrast, little qualitative difference in morphology was noted for Golgi vesicles in unincubated samples (Fig. 4a) or in samples where either $MnCl_2$ or sodium cacodylate had been removed from the medium (Fig. 4c and e). Similar findings were obtained with the intact Golgi fraction (not shown).

For each experimental condition, the amount of membrane fusion was assayed by measuring Golgi membrane lengths (Fig. 5). The mean perimeter of sectioned Golgi vesicles was compared to the degree of [³H]galactose incorporation that had been determined biochemically (Table III). From these observations, a regression line was calculated (Fig. 6). The coefficient of correlation of $r = 0.986$ was significant at $P < 0.001$ as evaluated by Student's t test.

Variable degrees of membrane damage were noted for the

various incubation conditions. Membranes with free ends were particularly evident when incubations were carried out in the absence of ATP (Table III). Membranes with free ends were also noticeable in preparations incubated in complete medium but not so evident in other preparations (Table III).

Radioautography

In an attempt to locate the sites of incorporation of [³H]-galactose Golgi fractions were processed for electron microscope radioautography after incubation in endogenous galactosyl transferase medium. Silver grains were observed not over mature VLDL-filled secretion vesicles but rather over large empty vesicles (Fig. 7a). Control experiments revealed few silver grains and the absence of large fused vesicles (Fig. 7b). Quantitative assessment of grain distribution along the membranes of the large vesicles (Table IV) indicated a high correlation between grain number ([³H]galactose incorporation) and membrane length. This correlation was calculated as significant ($P < 0.001$) based on a coefficient of correlation $r = 0.918$ (Fig. 8). Similar studies were carried out with the Golgi intermediate fraction and also revealed a significant correlation ($r = 0.92$, $P < 0.01$) between galactose incorporation and membrane fusion (not shown).

DISCUSSION

Paiement et al. (23) have documented a GTP-dependent fusion of rough microsomal (stripped) vesicles in vitro. They further discussed similarities between the requirements for membrane fusion and glycosylation occurring within the ER-derived membranes.

The present study reveals a quantitative relationship between

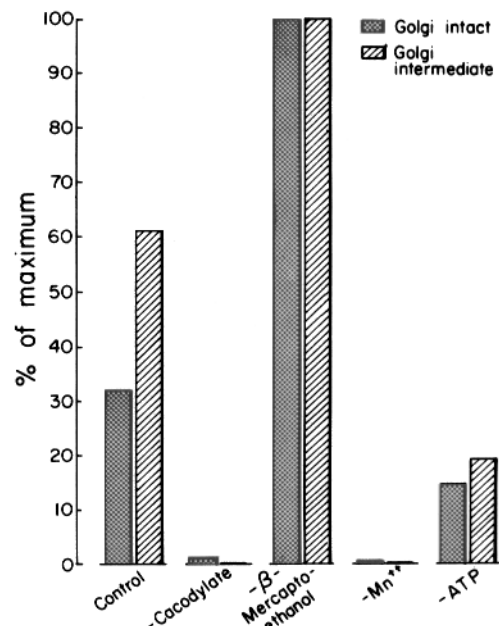
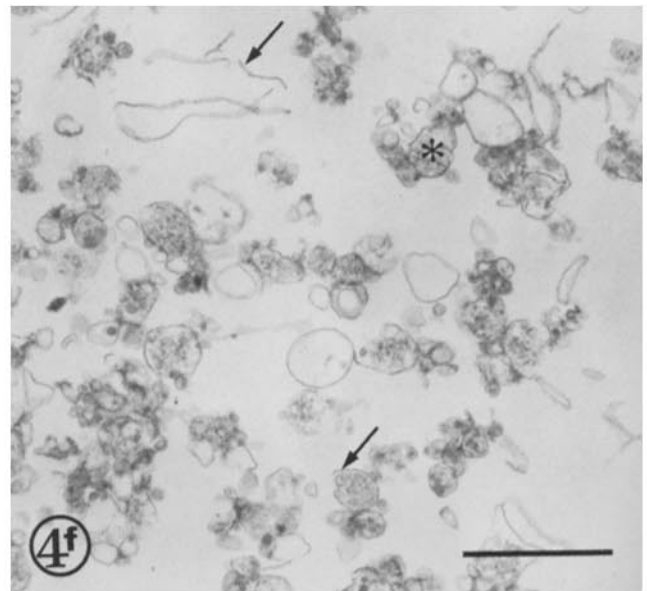
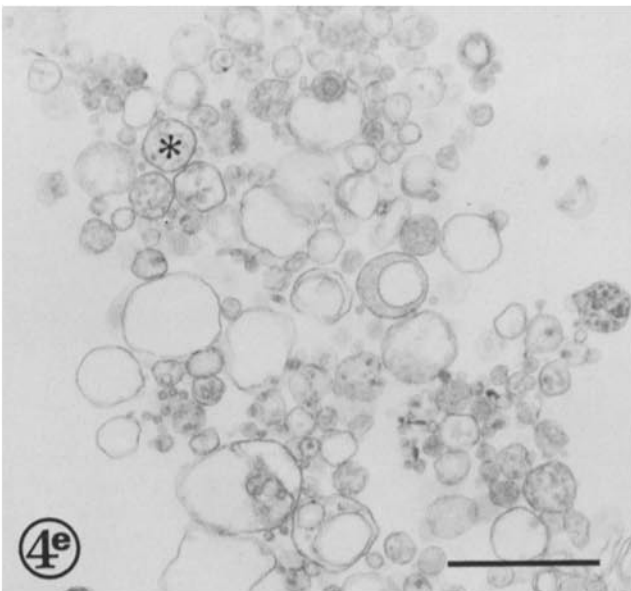
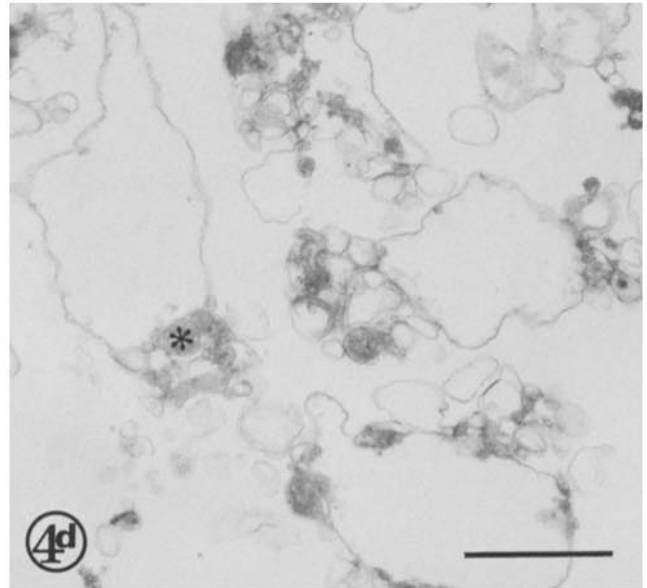
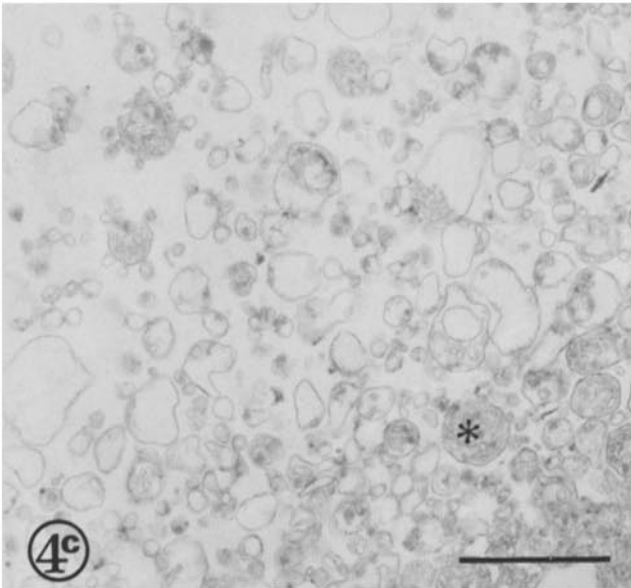
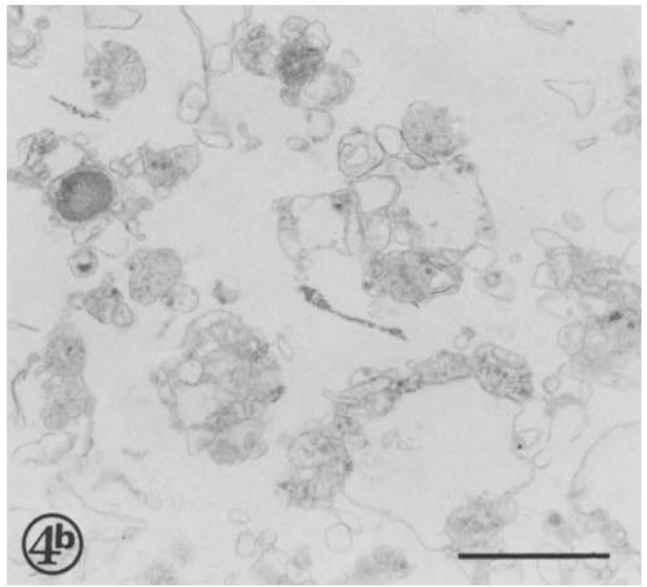
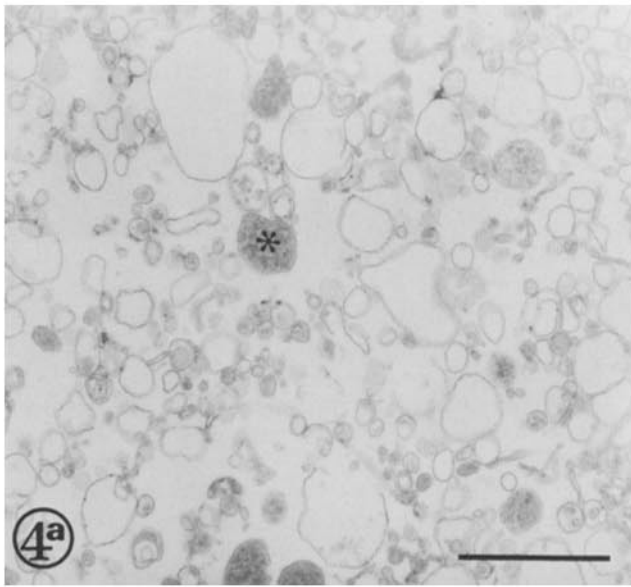


FIGURE 3 Effect of removing constituents of incubation medium on the incorporation with UDP-[³H]-galactose into Golgi fractions. Incubations (0.1 ml) were carried out on resuspended (13-45 μ g protein) Golgi intermediate or intact Golgi fraction for 60 min in the presence of 3.6 μ Ci of UDP-[³H]-galactose (11.6 Ci/mmol). Maximal incorporation was observed when the incubations were carried out in the absence of β -mercaptoethanol (intact Golgi fraction, 2.4 pmol galactose incorporated min^{-1} mg protein^{-1} , Golgi intermediate fraction 2.8 pmol min^{-1} mg protein^{-1}) and set at 100%.



requirements for endogenous glycosylation occurring with Golgi-derived membranes and membrane fusion occurring among the same membranes. Because of the correlation it is suggested that Golgi membrane fusion plays a role in glycosylation of endogenous Golgi proteins. These points are considered in further detail.

MORPHOMETRY OF Gi MEMBRANES

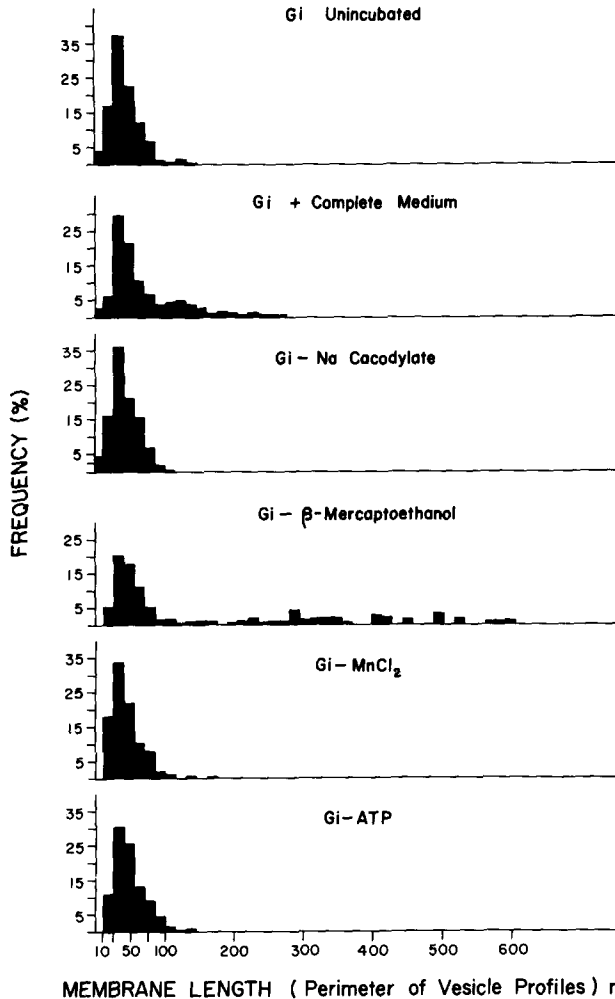


FIGURE 5 Distribution of Golgi membrane lengths (perimeters) for the conditions described in Fig. 4 as well as for freshly-prepared Golgi membranes, unincubated in galactosyl transferase medium (*Gi unincubated*). Stereological analysis was carried out as described in Materials and Methods on projected electron microscope negative images ($\times 60,000$) of randomly prepared pellicles of the Golgi intermediate fraction. The Golgi intermediate fraction represents a reasonably uniform population of vesicles thereby facilitating the quantitative analysis. Similar stereologic studies were not carried out on the intact Golgi fraction due to the complicated geometry of the starting preparations. Scale, 60 mm = 1 μ m.

Lipid Acceptor and Terminal Glycosylation

A glycolipid acceptor was observed after endogenous glycosylation of the Golgi fractions. The glycolipid was extracted

TABLE III
Effect of Galactosyl Transferase Constituents on Morphology and Biochemistry of Golgi Intermediate Fraction

Incubation condition	Galactose transfer $\mu\text{mol} \cdot \text{mg prot.}^{-1} \cdot 60 \text{ min}^{-1}$	Mean length* mm	Open membranes‡ %
None	Nil	34.5	3.5
Complete medium	102.40	46.8	19.3
Medium minus sodium cacodylate	0.19	34.5	1.3
Medium minus β -mercaptoethanol	167.90	58.4	6.1
Medium minus MnCl_2	0.72	30.3	4.0
Medium minus ATP	34.80	37.2	44.9

* Membrane lengths represent the circumference of the vesicle profiles as measured on micrographs at 60,000-times magnification. Greater than 50,000 mm of membrane were measured for each incubation condition and the number of vesicles ranged between 868 (minus β -mercaptoethanol) to 1,488 (minus MnCl_2).

‡ Greater than 1,000 membrane structures were examined in each case. The number of membranes with free ends was expressed as a percent of the total number of membranes examined.

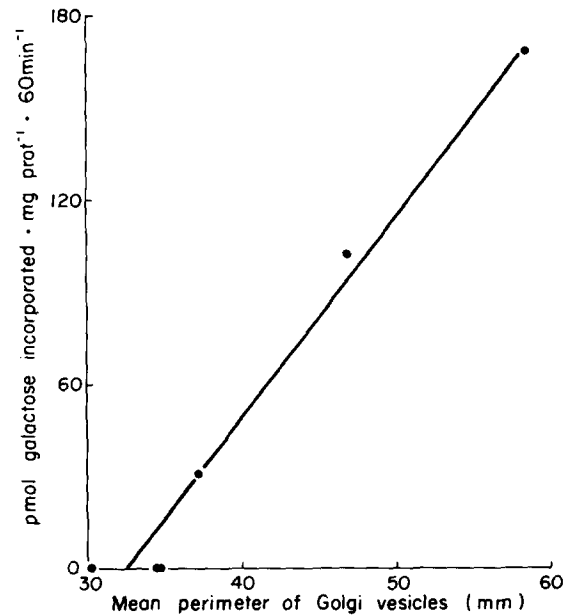


FIGURE 6 Relationship of average Golgi membrane length and endogenous galactosyl transferase activity of Golgi intermediate fraction. The data was obtained from Table III and a line calculated with a coefficient of correlation $r = 0.986$. Such a correlation was further calculated to be significant ($P < 0.001$) based on Student's double-tailed t test.

FIGURE 4 The appearance of Golgi intermediate fractions without (a) or with incubation (b-f) at 37°C for 60 min. Golgi vesicles are shown after incubation in the presence of all the constituents of the endogenous galactosyl transferase assay (b) and after incubation in medium lacking one of the following constituents: Sodium cacodylate (c), β -mercaptoethanol (d), MnCl_2 (e), ATP (f). Large vesicle profiles can be seen within the preparations incubated in complete medium (b) and in medium lacking β -mercaptoethanol (d). Smaller vesicles, some containing VLDL-like particles are evident at the periphery of the large vesicles. Golgi vesicles incubated in the absence of ATP (f) are also aggregated but contain vesicles that are smaller as well as numerous membranes with free ends (arrows). Golgi vesicles incubated in medium lacking Sodium cacodylate (c) or MnCl_2 (e) show very similar characteristics to vesicles that were not incubated (compare c and e with a). (*) VLDL-containing vesicles. Bar, 1 μ m. $\times 20,000$.

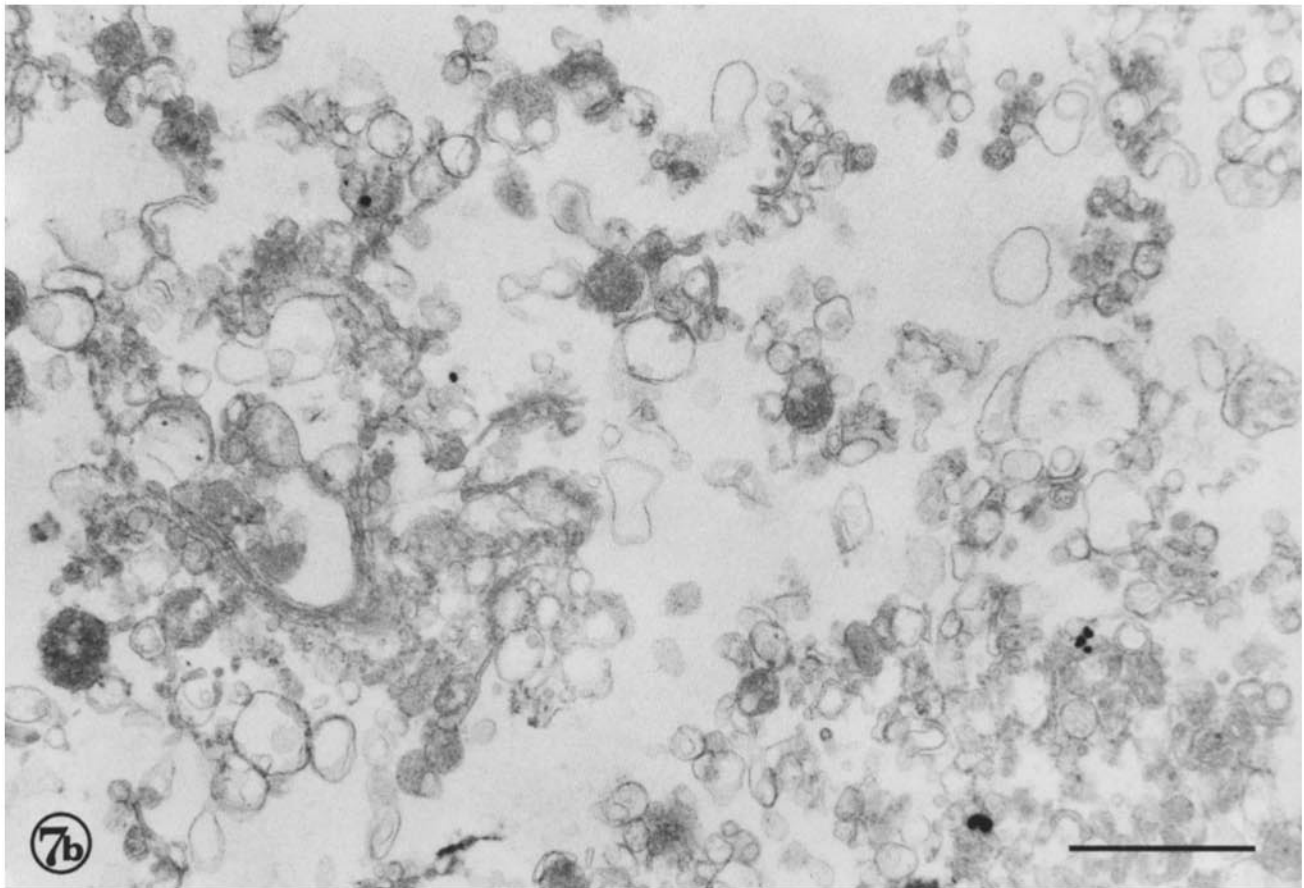
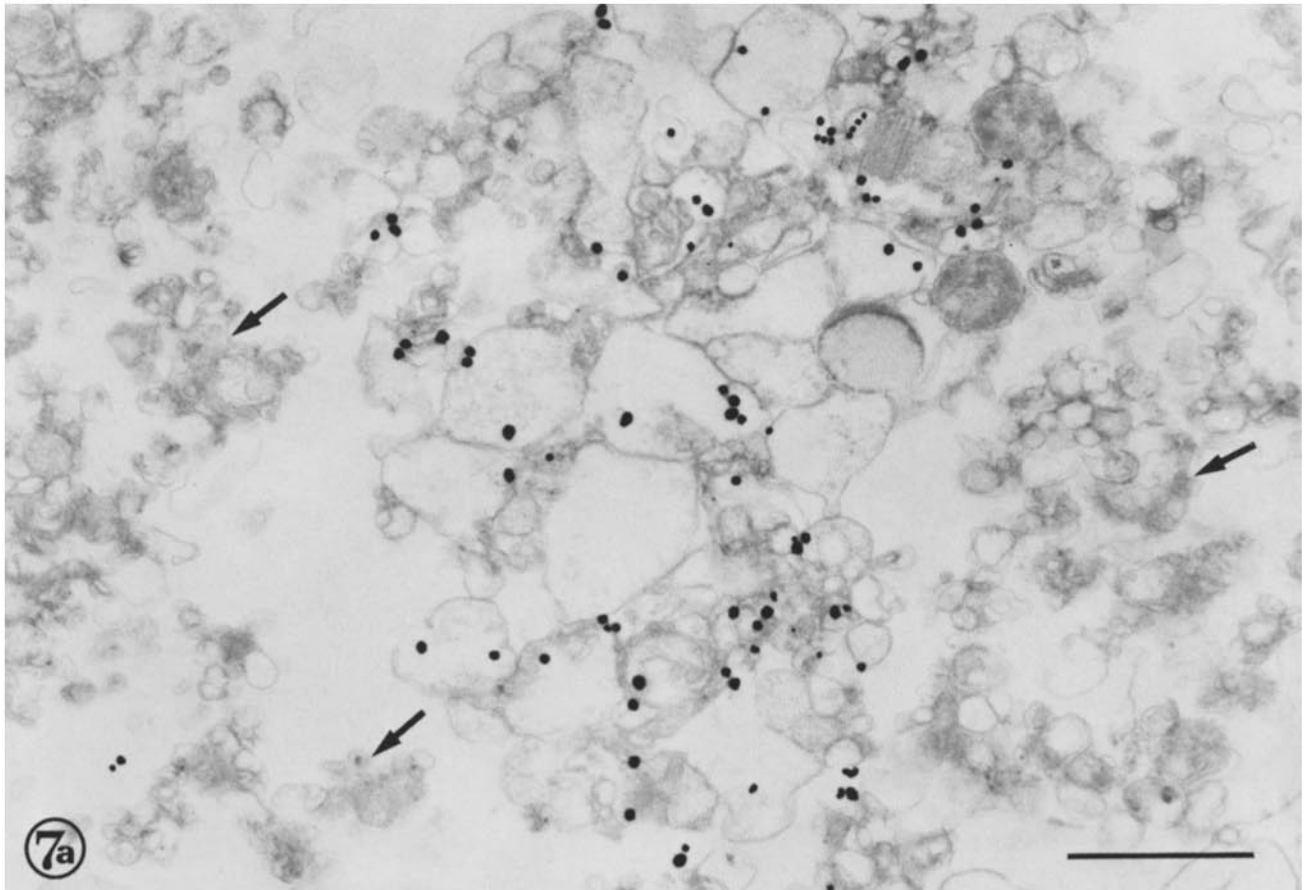


TABLE IV
Relationship of Silver Grains on Electron Microscope Radioautographs to Fused Golgi Membranes

Class of membrane lengths (mm)*	0-20	20-40	40-60	60-80	80-100	100-120	120-140	140-160	160-180
Grains within each class‡	7	130	154	113	30	53	11	21	10
Number of vesicles within each class	7	95	78	44	8	10	3	3	1
Mean number of grains per class	1.00	1.37	1.97	2.57	3.75	5.30	3.67	7.00	10.00

* Membrane lengths represent the circumference of the vesicle profiles, measured on micrographs magnified 30,000 times. Other experimental conditions were as described in the legend to Fig. 7a.

‡ Silver grains were counted as appearing over a membrane if any part of the grain touched the membrane or if it was included within the vesicular content (see references 6, 16, and 27). Extra-vesicular grains made up 13% of the grains associated with the total preparation and were excluded from the analysis.

using an organic solvent that isolates monosaccharidal lipids (24). It was chromatographed as a lipid that stains with anisaldehyde reagent and thus behaved like an isoprenoid (12). It further revealed a mobility ($R_f = 0.57$) equivalent to monosugar dolichyl phosphate standards (3). Based on these properties a tentative identification of dolichyl galactosyl phosphate has been made for the lipidic acceptor arising during endogenous glycosylation of Golgi fractions in vitro. Zatta et al. (33) have previously identified, on a tentative basis, a similar compound². However, this latter study was carried out on whole microsomes and our own study extends the finding to purified Golgi fractions.

Membrane Fusion

Turbidity of incubation media was observed during the biochemical studies involving endogenous glycosylation. This provided the impetus to document Golgi membrane structure during endogenous glycosylation. Structural alterations were obvious as indicated by the presence of large membrane fusion products. It was noteworthy that these large fusion products contained few or no lipoprotein particles. Indeed, Golgi vesicles densely packed with lipoprotein particles contributed very little to the fused structures. Rather, the fusing elements were either flattened saccular components of the intact Golgi fraction or disrupted saccular elements of the Golgi intermediate fraction. These saccular elements contained notably few lipoprotein particles (Figs. 4 and 7).

The Correlation of Membrane Fusion and Glycosylation

The removal of various constituents of the endogenous glycosylation medium was observed to modulate membrane fusion in direct proportion to galactose incorporation. This correlation was judged significant by statistical analysis.

The nature of the agents that promoted or inhibited Golgi membrane fusion was noteworthy. For example, the removal

² Bergeron et al. (5) have also provided evidence for the participation of a glycolipid acceptor in galactosyl transferase assays carried out with Golgi fractions isolated from liver microsomes. Appelkvist et al. (1) studying galactose transfer to endogenous acceptors of Golgi membranes were unable to detect glycolipid.

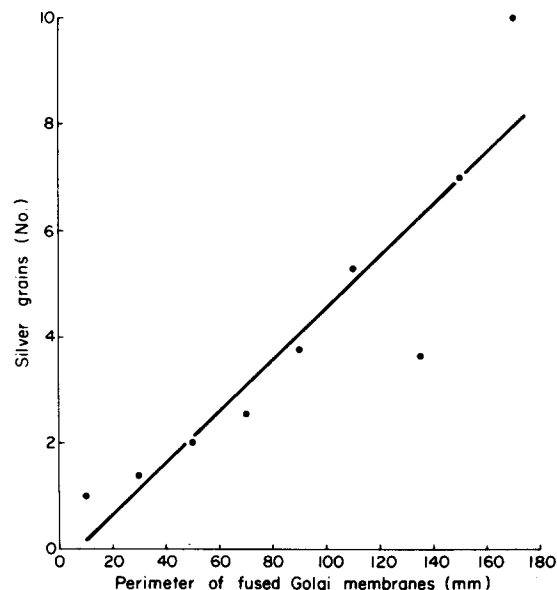


FIGURE 8 Relationship of Golgi membrane fusion and localization of silver grains by electron microscope radioautography. The data is derived from Table IV and calculated to a straight line with a coefficient of correlation $r = 0.918$, with a significance of $P < 0.001$ based on the Student's double-tailed t test.

of β -mercaptoethanol markedly promoted membrane fusion as well as galactose incorporation. Thus β -mercaptoethanol likely inhibits membrane fusion and glycosylation. This contrasts with ATP. Removal of this compound resulted in a marked increase in broken Golgi vesicles without stimulation (indeed an inhibition) of [³H]galactose incorporation. Broken vesicles were also observed after incubation in complete medium, yet galactose incorporation was less than maximal. These findings served to rule out simple permeability barrier breakdown as an explanation for increases in endogenous glycosylation. Indeed where large values of broken membranes were found ($-ATP$, see Table III) inhibition of galactose incorporation was noted. Thus ATP probably serves to stabilize fused membranes.

The near complete inhibition of fusion and galactose incorporation by removal of $MnCl_2$ or sodium cacodylate buffer may simply indicate the requirement of cations and pH for membrane fusion. Alternatively these agents may induce a

FIGURE 7 Radioautographs of intact Golgi fraction after incubation in galactosyl transferase medium. The fractions (180 μ g protein) were incubated 30 min in 0.5 ml of complete medium containing 17 μ Ci UDP-[³H]-galactose (11.6 Ci/mmol) and then fixed and processed for electron microscopic radioautography as outlined in the Materials and Methods. Radioautographs were exposed for 32 d. (a) Intact Golgi fraction incubated at 37°C for 30 min. Silver grains indicative of [³H]-galactose incorporation are only observed over fused VLDL-poor membranes. Other vesicle aggregates (arrows) are unmarked by silver grains. Bar, 1 μ m. \times 25,000. (b) Intact Golgi fraction incubated under identical conditions as in a except that the incubation was done at 0°C. Few silver grains lie over the membranes. Golgi stacks are still recognizable after the 30 min incubation. Bar, 1 μ m. \times 25,000.

conformational change in constituents of Golgi membranes which leads to fusion.

Galactose Incorporation Occurs within the Membranes of Large Fusion Products

Radioautography showed that sites of [³H]galactose incorporation were large membranes surrounding VLDL-poor vesicles that appeared only during incubation in endogenous galactosyl transferase medium at 37°C. The large membranes are a consequence of membrane fusion, thus galactose incorporation occurs within fused membranes. It appears therefore that glycosylation and fusion of Golgi membranes depend on the same factors and these phenomena occur within the same compartments. The fact that VLDL-rich *trans* Golgi elements did not incorporate [³H]galactose is consistent with the postulate of the first paper (7) that the site of galactose incorporation in vivo is in Golgi saccules.

Relation of Golgi Membrane Fusion to Endogenous Glycosylation

If membrane fusion plays a causal role in galactose transfer to endogenous acceptors of Golgi membranes one would predict that the sites of galactose incorporation are the fused membranes (large fusion products poor in VLDL content). This prediction was validated by the radioautographic studies. VLDL-rich *trans* Golgi elements did not incorporate [³H]galactose and only large empty fusion products did after incubation in endogenous galactosyl transferase medium at 37°C. Furthermore, if membrane fusion is causally linked to galactose transfer than one would also predict a correlation between membrane length (measure of membrane fusion) and number of silver grains (measure of galactose transfer). Indeed, a high correlation was observed between these two phenomena (Fig. 8).

We have concluded therefore that membrane fusion stimulates galactose transfer to endogenous acceptors of Golgi membranes. Lipid-micellar intermediates have been proposed to be formed at points of membrane fusion (9–11, 21). Inverted micelles, in particular, might enable translocation across the Golgi membrane of substrate for terminal galactosylation.

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