Coalescence of Microsomal Vesicles from Rat Liver: A Phenomenon Occurring in Parallel with Enhancement of the Glycosylation Activity during Incubation of Stripped Rough Microsomes with GTP

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ABSTRACT   Rough microsomes from rat liver have been subjected to various treatments and incubated afterwards with UDP-N-acetyl-[^{14}C]glucosamine and GDP-mannose in the presence of GTP (0.5 mM), or of other nucleotides. In agreement with earlier results from this laboratory, the preparations previously treated to strip off the ribosomes and incubated in the presence of GTP assembled dolichol-linked oligosaccharides and transferred these oligosaccharides to endogenous protein acceptors much more actively than untreated preparations, or stripped preparations incubated in the absence of GTP. Thin-section and freeze-fracture electron microscopy have revealed that pyrophosphate-treated preparations incubated with GTP are aggregated and contain numerous vesicles as large as 1–4 μm, or more. Such large vesicles were not present before incubation and thus were considered to have been formed through coalescence of regular-sized ones. Like glycosylation, the coalescence phenomenon depends upon the removal of ribosomes, because it occurred whether ribosomes had been stripped, at least partly, with pyrophosphate, KCl, or puromycin, but not when rough microsomes had been washed with 0.25 M sucrose or with KCl and MgCl$_2$. Like glycosylation, it also depends on the addition of GTP and was not induced by ATP, UTP, CTP, and nonhydrolysable analogues of GTP. Rough microsomes coalesced, however, when pyrophosphate-treated preparations were incubated with GTP in the absence of nucleotide sugars, or in the presence of tunicamycin, indicating that the coalescence phenomenon does not result from the glycosylation of some membrane constituents.

Glycosylation of secretory and other proteins is one of the key functions of the endoplasmic reticulum membranes. An oligosaccharide, whose inner portion will become the "core" portion of the saccharide chain in glycoproteins, is assembled on a lipid constituent of the membrane through a sequence of transfer reactions carried out by membrane-associated enzymes, and ultimately transferred to the acceptor protein (see reference 15 for a review). Investigations on the glycosylation of endogenous constituents of rough microsomes from rat liver have shown recently that, in vitro, this metabolic activity is markedly influenced by various factors in intact (as opposed to detergent-disrupted) membranes. Unless specific requirements are met, rough microsomes express only weakly their capacity of assembling dolichol-linked oligosaccharides from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and guanosine diphosphate mannose (GDP-Man) and of transferring such oligosaccharides to microsomal proteins. The main requirements are that ribosomes be partially removed by treatment with pyrophosphate (PP$_i$) (8–10), or with other reagents (19, 20), and that the microsomes be subsequently incubated with the nucleotide sugars in the presence of GTP (8–10). Rough microsomes, even when they are incubated with the nucleotide precursors in the presence of GTP, and stripped rough microsomes incubated with such precursors in the ab-

sence of GTP produce dolichyl phosphate mannose and, less efficiently, dolichyl diphosphate N-acetylglucosamine, but fail to form more complex glycoside derivatives of their endogenous dolichyl phosphate and also fail to glycosylate protein.

The hindrances which curb the glycosylation activity in intact rough microsomal membranes, and the mechanism by which these are overcome when stripped membranes are incubated in the presence of GTP are still unknown. In an effort to gain more insight into some of these questions we have examined the morphological characteristics of the microsomal membranes before and after incubation. In the electron microscope we observed that PP$_i$-treated preparations contain very large membrane-bounded vesicles after incubation with GTP. Such large vesicles are not present amongst unincubated preparations and arise during incubation by coalescence of regular-sized vesicles. This paper describes the characteristics of this morphological phenomenon, and reveals that it occurs under highly specific conditions, conditions that are identical to those which lead to efficient functioning of protein glycosylation through the dolichol pathway. Although both phenomena occur in parallel, the coalescence of microsomal membranes does not result as a consequence of the glycosylation of some membrane constituents. Part of these results have been reported in abstract form (14).

MATERIALS AND METHODS

Preparation and Treatment of Heavy Microsome Fractions

Microsomes from rat liver were prepared by differential centrifugation as described previously (2). Two subfractions, heavy and light microsomes, were separated from the unwashed microsomal pellets by density equilibration in a linear gradient of sucrose (8). Before incubation, heavy microsomes were diluted with 4 vol of ice-cold 0.25 M sucrose brought to pH 7.4 with 3 mM imidazole-HCl buffer, as such (a), or supplemented with 5 mM PP$_i$ (b), 0.75 M KCl (c), 0.75 M KCl and 5 mM MgCl$_2$ (d), or 0.75 M KCl, 5 mM MgCl$_2$, and 1 mM puromycin (e), and centrifuged for 30 min at 40,000 rpm in a No. 40 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) after standing at 0°C for 15 (a–d) or 30 min (e). The pellets were resuspended in the same medium, except those from treatment e which were brought in medium d. After centrifugation as described above, heavy microsomes were washed by resuspension in 0.25 M sucrose, centrifuged again, and finally resuspended in 0.25 M sucrose. For the sake of convenience, microsomes washed as in a–e were designated sucrose-washed, PP$_i$-treated, KCl-treated, KCl-MgCl$_2$-treated, and puromycin-treated, respectively. The puromycin treatment was similar but not identical to that outlined by Adelman et al. (1). In particular, the time and temperature conditions used in this study probably account for the lesser efficiency in releasing ribosomes (see Results). RNA associated with the microsomes after the various wash procedures was measured according to Fleck and Munro (7).

Incubation of Microsomes

Unless otherwise stated, washed heavy microsomes were incubated at 37°C, in a final volume of 0.5 ml, with 3.2 μM UDP-[^{14}C]GlcNAc, 5.6 μM GDP-Man, 40 mM Tris-HCl buffer at pH 7.4, 30 mM KCl, 7.5 mM MgCl$_2$, 2.5 mM MnCl$_2$, 2.5 mM dithiothreitol, 1 mM ATP, 0.5 mM GTP, 10 mM phosphoenolpyruvate, and 25 μg pyruvate kinase. The incorporation of labeled N-acetylglucosamine into various derivatives of the endogenous dolichyl monophosphate and into endogenous proteins was followed as described earlier (9).

Morphological Analysis

For thin-section electron microscopy, the reaction was stopped by mixing 50 μl of microsomes with 2.5 ml of ice-cold glutaraldehyde (1.5% in 0.05 M phosphate buffer, pH 7.4). After fixing overnight, the microsomes were recovered by filtration onto Millipore membranes (Millipore Corp., Bedford, Mass.) and subsequently postfixed with osmium tetroxide and processed as described previously (18).

For freeze-fracture electron microscopy, the reaction was first slowed on ice for 15 min and then stopped by spray-freezing (4). Spray-frozen droplets of the

microsomal suspension were embedded in butylbenzene at −88°C and mounted on supporting gold disks which were stored in liquid nitrogen until fracturing. The samples were fractured at −100°C and 10^{-6} torr in a Balzers freeze-etching apparatus (BA 360 M, Balzers AG, Balzers, Lichtenstein). Replicas were obtained by shadowing the fracture surface sequentially, first with platinum at a fixed angle of 45° followed with carbon at 90°, using electron gun evaporators and an oscillating quartz for monitoring thickness of the replica (12), 4 nm for platinum and 18 nm for carbon. Replicas were cleaned by floating on a bleach solution (1-3 h), washed with distilled water, and mounted on uncoated grids. The freeze-fracture electron micrographs were mounted with the shadow direction from bottom to top and the terminology used to identify the fracture faces and membrane surfaces was that suggested by Branton et al. (5).

Materials

UDP-[^{14}C]GlcNAc (300 Ci/mol) was purchased from the Radiochemical Centre (Amersham, England). GDP-Man, puromycin, and nucleotides were obtained from Sigma Chemical Co. (St. Louis, Mo.). Glutaraldehyde and osmium tetroxide came from E. Merck A.G. (Darmstadt, W. Germany). Guanylyl-imidodiphosphate (GppNp) and guanylyl-(β,γ-methylene)-diphosphonate (GppCp) were purchased from Boehringer Mannheim GmbH (Mannheim, W. Germany). Tunicamycin was a generous gift of Dr. R. L. Hamill (Eli Lilly and Co., Indianapolis, Ind.).

RESULTS

Incorporation of N-acetylglucosamine in Microsomal Acceptors

All microsomal preparations examined under the electron microscope were challenged for their capacity to synthesize lipid-linked oligosaccharides in vitro, and to transfer these oligosaccharides to protein. The results were in full agreement with the findings made previously in our laboratory (8, 9). In particular, rough microsomes treated with PP$_i$, with KCl, or with puromycin, and incubated afterwards with the nucleotide precursors in the presence of GTP showed high levels of N-acetylglucosamine incorporation into dolichyl diphoshate oligosaccharides and into protein (Table I). As shown for PP$_i$-treated heavy microsomes, this result is conditioned by the presence of GTP in the incubation medium. Much less label was also recovered in dolichol-linked oligosaccharides and in protein when sucrose-washed, or KCl-MgCl$_2$-treated preparations were incubated in the presence of GTP. As evidenced by

TABLE I

Effect of Various Treatments on the RNA Content and the Glycosylation Activity of Heavy Microsomes

Biochemical properties	Heavy microsomes treated with:*				
	Sucrose	PP$_i$	KCl	KCl-MgCl$_2$	Puromycin
RNA content, mg/g liver	2.63	0.90	0.39	1.95	0.85
Labeled products‡	nmol N-acetylglucosamine incorporated/g liver				
Dol-PP-GlcNAc	0.11	0.12 (0.12)§	0.19	0.23	0.17
Dol-PP-oligosaccharides‖	0.09	0.69 (0.05)	1.52	0.20	0.98
Protein	0.02	0.67 (0.02)	0.70	0.04	0.72

* Heavy microsome fractions have been washed with 0.25 M sucrose, or with other reagents as described in Materials and Methods.

‡ Incubation with the nucleotide precursors was carried out for 120 min at 37°C, in the presence of 0.5 mM GTP.

§ Values within brackets give the incorporation of label in the absence of GTP.

‖ Includes dolichyl diphosphate N,N'-diacetylchitobiose and the mannosylated derivatives of this glycolipid.
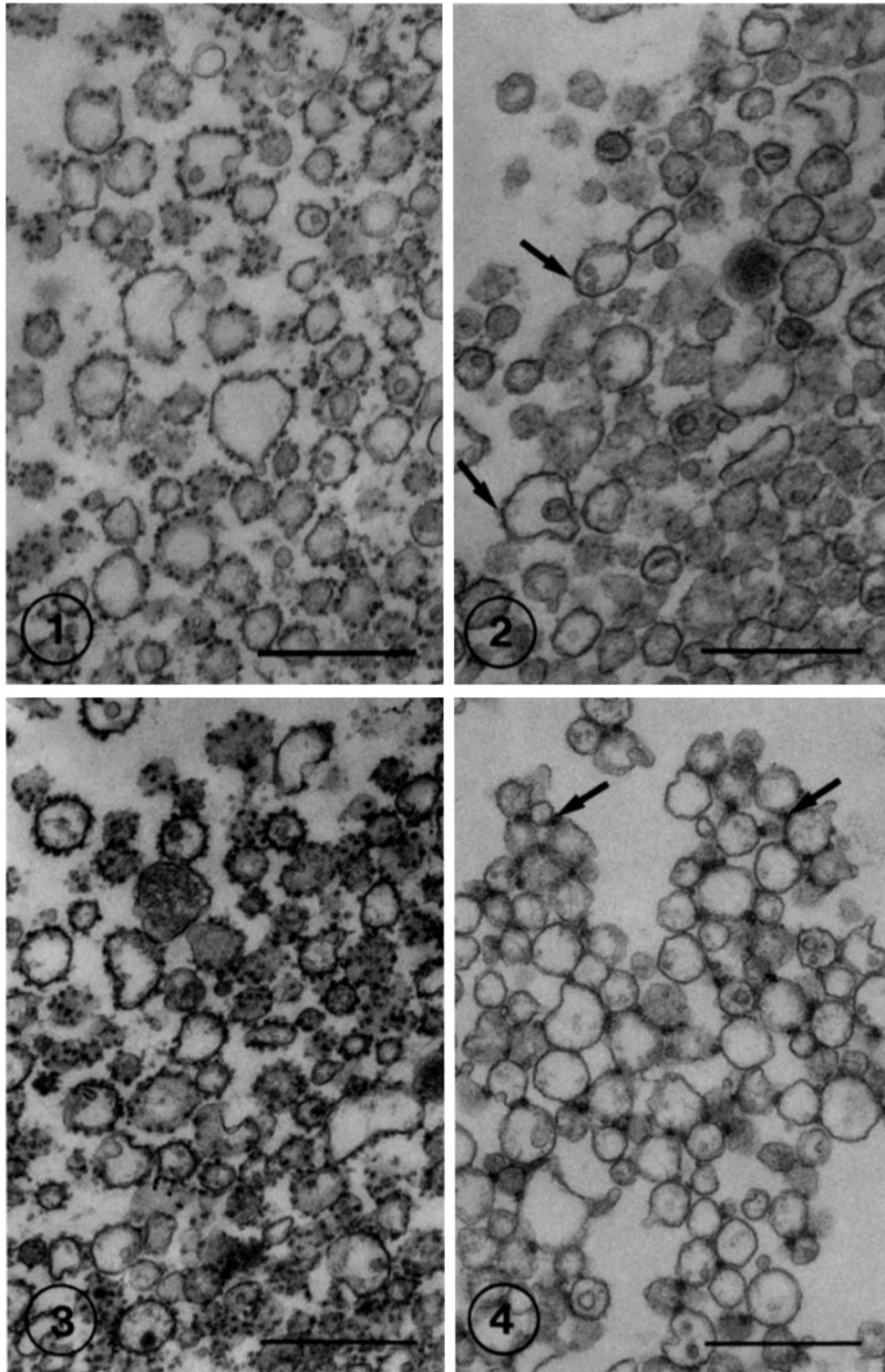
FIGURE 1 Electron micrograph showing heavy microsomes after washing with 0.25 M sucrose. The vesicle profiles are surrounded by numerous ribosomes and only rarely exceed 0.3 μm in size. Bar, 0.5 μm. × 45,000.

FIGURE 2 Electron micrograph showing heavy microsomes washed with 0.25 M sucrose containing 5 mM PP_i. The range of sizes of vesicle profiles is the same as in Fig. 1. Distinct ribosomal particles are no longer visible but remnants of ribosomal material can be seen normally distributed along the membranes (arrows). Bar, 0.5 μm. × 45,000.

FIGURE 3 Sucrose-washed heavy microsomes incubated at 37°C for 120 min with nucleotide sugars in the presence of GTP. The microsomes are like unincubated sucrose-washed microsomes with a normal complement of ribosomes. Similar images were obtained after incubation of sucrose-washed preparations in the absence of GTP. Bar, 0.5 μm. × 45,000.

FIGURE 4 PP_i-treated heavy microsomes incubated at 37°C for 120 min with nucleotide sugars in the absence of GTP. The membrane profiles are highly agglutinated and ribosomal remnants are no longer normally distributed along the membranes. Instead, electron-dense patches believed to be aggregates of ribosomal remnants are observed in regions of closely apposed membranes (arrows). Bar, 0.5 μm. × 45,000.

the release of RNA (Table I), the capacity of various preparations to incorporate N-acetylglucosamine into dolichol-linked oligosaccharides and into protein correlates with the removal of ribosomes. It has been found previously that the GTP-dependent glycosylation activity of rough microsomes requires the removal of 60% or more of the RNA (19, 20). The glycosylation capacity of PP_i-treated heavy microsomes was not revealed by ATP, CTP, UTP, and the nonhydrolyzable analogues GppCp and GppNp; however, ITP increased noticeably the amount of labeled dolichyl diphosphate N,N'-diacetylchitobiose formed (not shown).

Morphology of Sucrose-washed and PP_i-treated Heavy Microsomes and Effect of Incubation with GTP

Before incubation the sucrose-washed and the PP_i-treated heavy microsomes showed the morphological characteristics previously described (9). Sucrose-washed heavy microsomes (Fig. 1) consist of small membrane-bounded vesicles studded with numerous ribosomes and which only rarely exceed 0.3 μm in size. Mitochondria, peroxisomal cores, and small lysosomes were occasionally found amongst the rough vesicles. After treatment with PP_i (Fig. 2) the vesicles are similar in size to the sucrose-washed ones, but no longer have authentic ribosomes associated with them. Some residual electron-dense material is, however, noted on the membranes of PP_i-treated microsomes. The distribution of this material is similar to that of ribosomes in the sucrose-washed preparations.

Sucrose-washed microsomes incubated for 120 min at 37°C with nucleotide sugars and in the presence of GTP (Fig. 3)

exhibit the characteristics of unincubated preparations. They are similar in size and studded with numerous ribosomes which have apparently retained their original distribution. PP_i-treated heavy microsomes incubated as described above but in the absence of GTP are aggregated into clumps (Fig. 4). Small electron-dense patches are observed in regions of closely apposed vesicular membranes. These dense patches are thought to be aggregates of residual ribosomal material. The size of PP_i-treated vesicles is unaltered after incubation in the absence of GTP.

PP_i-treated heavy microsomes incubated in the presence of GTP are also aggregated into clumps (Fig. 5). However, the membrane-bounded vesicles are highly variable with respect to size, and vesicles with dimensions as large as 1-4 μm are then seen consistently amongst the aggregates of regular-sized vesicles. Small vesicles often completely or partially surround these large vesicular profiles. The membranes of the large vesicles are usually convex in regions associated with aggregates of small vesicles, and concave or deformed in regions located away from these aggregates. Electron-dense patches are evident within membrane invaginations of the large vesicles as well as along zones of close membrane contact between large and small vesicles. Some electron densities appear as discrete particles or stalks and separate apposing vesicle membranes by as much as 300 Å. They are believed to be aggregates of the ribosomal remnants still present on the vesicle membranes of PP_i-treated microsomes before incubation. Vesicle profiles observed within the lumen of the large vesicles often contain several small vesicles and thus probably represent transected invaginations of the large membranes (not shown). Contaminant organelles occasionally seen amongst the aggregates of
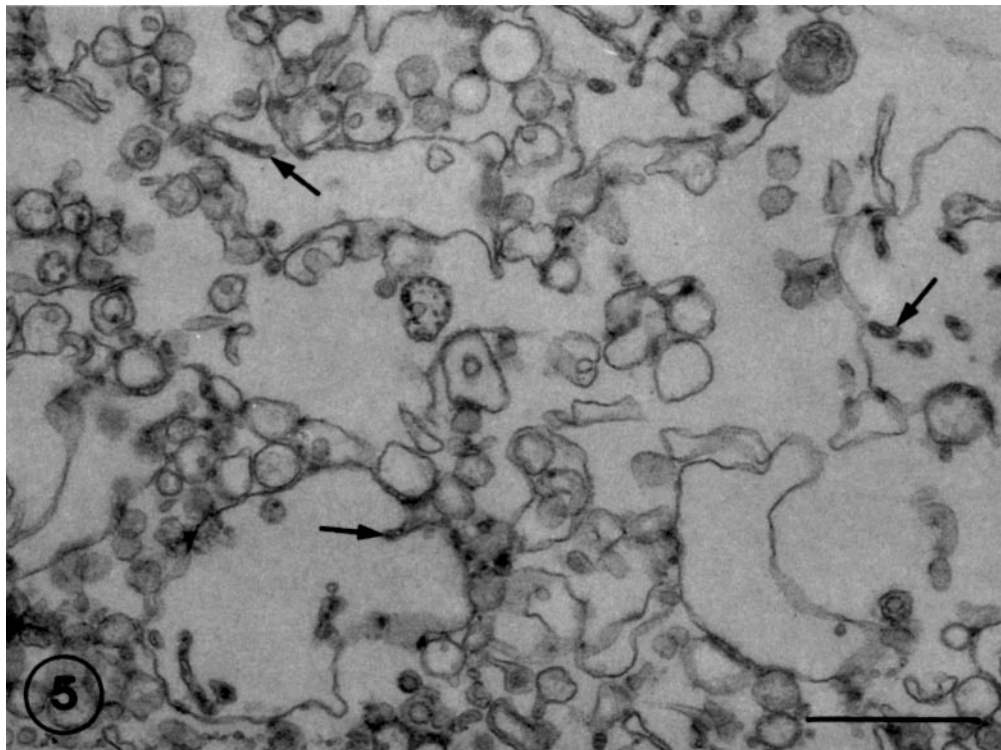

FIGURE 5 A survey electron micrograph showing PP_i-treated heavy microsomes incubated for 120 min at 37°C with nucleotide sugars in the presence of GTP. The vesicle profiles are aggregated, and amongst the aggregates can be seen profiles with dimensions as large as 1 μm. The membranes of the large vesicles closely apposed to those of the small vesicles are generally convex and show regions of invagination. Many of these invaginations contain electron densities believed to be aggregates of ribosomal remnants (arrows). Portions of large membranes away from vesicle aggregates are either concave or deformed in appearance. Bar, 0.5 μm. × 45,000.

microsomal vesicles were not located within invaginations of the large vesicles nor within their lumina.

Freeze-Fracture Electron Microscopy

Microsomes were also studied using replicas obtained from samples instantaneously frozen, unfixed, and still in the original incubation medium. PP_i-treated heavy microsomes incubated in the absence of GTP were clearly aggregated into clumps. Intramembrane particles were distributed in an apparently random manner along the concave cytoplasmic fracture faces (PF) as well as along the lumenal fracture faces (EF) and were more prominent on the PF of the vesicle membranes (results not shown), in agreement with other studies on rough microsomes from rat liver (11, 13).

PP_i-treated heavy microsomes incubated in the presence of GTP were also aggregated into clumps. The distinguishing feature of these aggregates is the presence of one or more, exceptionally large vesicles (Figs. 6 and 7). Normal-sized microsomal vesicles are often clumped to one side of the large vesicles. Irregularities, evident as small protuberances, were seen along the concave cytoplasmic fracture face of the large vesicles on the side facing the aggregates of smaller vesicles. Continuities between small vesicle membranes and those of large vesicles were occasionally recognized (for example, see Fig. 7). Such images of membrane continuity suggest incorporation of the small vesicle membranes into those of the large vesicles and support the idea that the large membrane-bounded structures formed during incubation in the presence of GTP do so as a result of fusion of the membranes of normal-sized vesicles. Because the freeze-fracture specimens were obtained from microsomes in an unfixed state, these results demonstrate that microsomes coalesce during incubation, not as a result of an artifact during fixation with glutaraldehyde. There was no overt evidence of a change in the distribution of intramembrane particles, but a clearcut conclusion cannot be drawn about this point and must await quantitative data.

Effect of Various Nucleotides on the Production of Large Vesicles by PP_i-treated Heavy Microsomes

In contrast to GTP, ATP does not cause the formation of large vesicles when PP_i-treated heavy microsomes are incubated with UDP-GlcNAc and GDP-Man. Indeed, stripped microsomes incubated in the absence of GTP but in the presence of 1 mM ATP retained their normal size (Fig. 4). The effect of other nucleotides and of nonhydrolyzable analogues of GTP has been investigated by substitution of CTP, UTP, GppCp, or GppNp for GTP. In no case did this lead to the formation of large vesicles, although large vesicles were formed in the presence of GTP, showing that the preparations were indeed reactive. After incubation in the presence of ITP, vesicles larger than the original ones but smaller than those formed in the presence of GTP were clearly apparent within the aggregates (not shown). Thus ITP is efficient, but distinctly less than GTP, in forming large vesicles among PP_i-treated heavy microsomes.

Effect of Various Pretreatments of Heavy Microsomes on the Production of Large Vesicles Induced by GTP

PP_i treatment of microsomes releases ribosomal material as well as a significant amount of nonribosomal protein (3, 9).

This prompted us to wonder whether it is the release of the ribosomes, or some other effect of PP_i on the microsomal membranes which causes them to undergo coalescence in the presence of GTP. We therefore examined the effects of other pretreatments of heavy microsomes on their property to form large vesicles.

Heavy microsomes treated with high salt (750 mM KCl) and then incubated in the presence of GTP, produce large vesicles which are characteristically associated with aggregates of smaller vesicles (Fig. 8). Ribosomal particles are not evident on the membranes of the vesicles, a fact consistent with the low RNA content assayed in this preparation (Table I). Heavy microsomes treated with high salt solution containing $MgCl_2$ (5 mM) lost only a small amount of RNA (Table I) and when incubated with GTP did not form large vesicles (Fig. 9). The vesicles appear normal in size, are not strongly agglutinated, and have numerous ribosomes. The distribution of ribosomes in this preparation is similar to that in unincubated sucrose-washed microsomes (compare Fig. 9 with Fig. 1).

Puromycin-treated heavy microsomes incubated in the presence of GTP produce large membrane-bounded structures (Fig. 10). The loss of ribosomal material is clearly seen, but it appears less than expected from the amount of RNA released (Table I). Ribosomes and ribosomelike particles are readily identifiable on both small and large vesicle membranes. Their distribution along the membranes of the large vesicles is often heterogeneous. They are concentrated in regions of membrane invagination and along areas of close apposition between the membranes of the large vesicles and those of small vesicles, leaving some parts of the membrane nearly ribosome free. Such images evoke the ribosome aggregation which occurs when rough microsomes are treated with ribonuclease and incubated afterwards above the thermotropic phase transition temperature of the microsomal phospholipids (13). Membrane-sidedness of the ribosomal material is conserved. Ribosome-like particles are always found on the extralumenal side of the membranes of both large and small vesicles.

Effect of Sugar Incorporation on the Formation of Large Vesicles by PP_i-treated Heavy Microsomes Incubated with GTP

The results reported above disclose a striking parallel between the morphological change and the glycosylation activity of heavy microsomes. Both are GTP-induced and, as far as we presently know, occur only when heavy microsomes have been stripped of ribosomes, at least partially. An explanation of the tight correlation between these two processes might be that the rearrangement of membranes into large vesicles results from the glycosylation of some constituents of the membrane. Consistent with this hypothesis was the observation that large vesicles appear and grow during incubation with nucleotide sugars and GTP at a rate (results not shown) resembling the kinetics of protein glycosylation previously reported (9, 10).

To check whether the glycosylation of membrane constituents is responsible for the morphological change, PP_i-treated heavy microsomes were incubated with GTP in the absence of nucleotide sugars, or in the presence of nucleotide sugars and tunicamycin, an antibiotic that inhibits protein glycosylation (16). The absence of exogenous nucleotide sugars in the incubation medium did not affect the formation of large vesicles in the presence of GTP (Fig. 11). Large vesicles were also formed when microsomes were incubated in the presence of nucleotide sugars and tunicamycin (not shown), despite the fact that N-
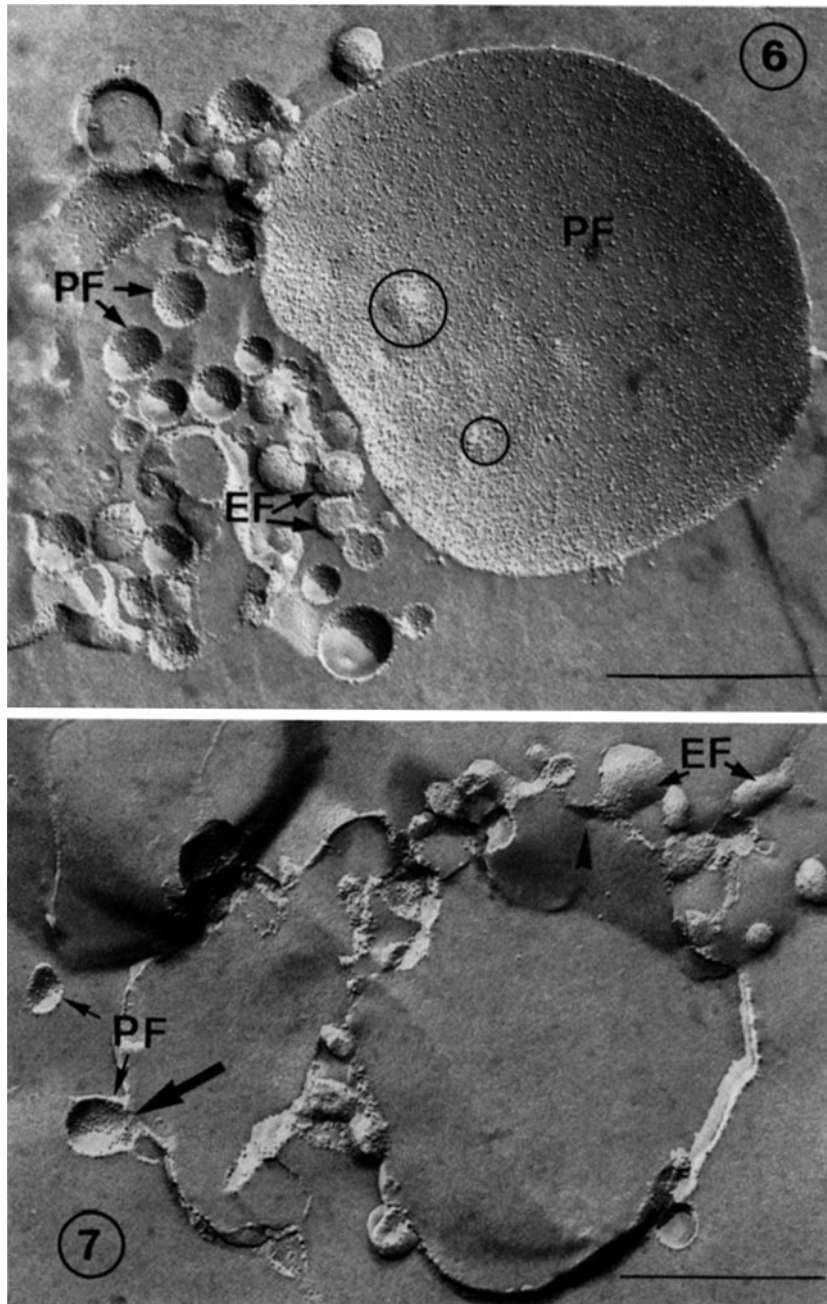
FIGURE 6 Freeze-fracture appearance of PPi-treated heavy microsomes incubated for 60 min at 37°C with nucleotide sugars in the presence of GTP. The concave cytoplasmic fracture face (PF) of a large vesicle membrane dominates the micrograph. This large membrane is closely associated with an aggregate of small vesicles. Intramembrane particles along the fracture face of the large vesicle membrane are numerous and appear homogeneous in distribution. The intramembrane particles observed along the PF and EF of the membranes of the small vesicles also appear homogeneously distributed. Small irregularities on the PF of the large vesicle membrane (encircled areas) are thought to be zones of contact between the large vesicle and regular-sized vesicles aggregated at its periphery. Bar, 0.5 μm. × 54,000.

FIGURE 7 Freeze-fracture appearance of PPi-treated microsomes incubated for 60 min at 37°C with nucleotide sugars in the presence of GTP. Two large vesicles are shown transected by the plane of fracture. The fracture faces (PF and EF) of membranes belonging to small vesicles aggregated at the periphery of the large ones are evident. Continuity of the content of a large vesicle can be followed where the cleavage plane deviates from the interior of the transected large vesicle to the cytoplasmic fracture face of a small vesicle (large arrow). This is interpreted as a fusion image where the membrane of a small vesicle is in the process of being incorporated into that of the large vesicle. Another small vesicle evident as a convex lumenal fracture face (arrowhead) is thought also to be in a similar stage of fusion. Bar, 0.5 μm. × 54,000.
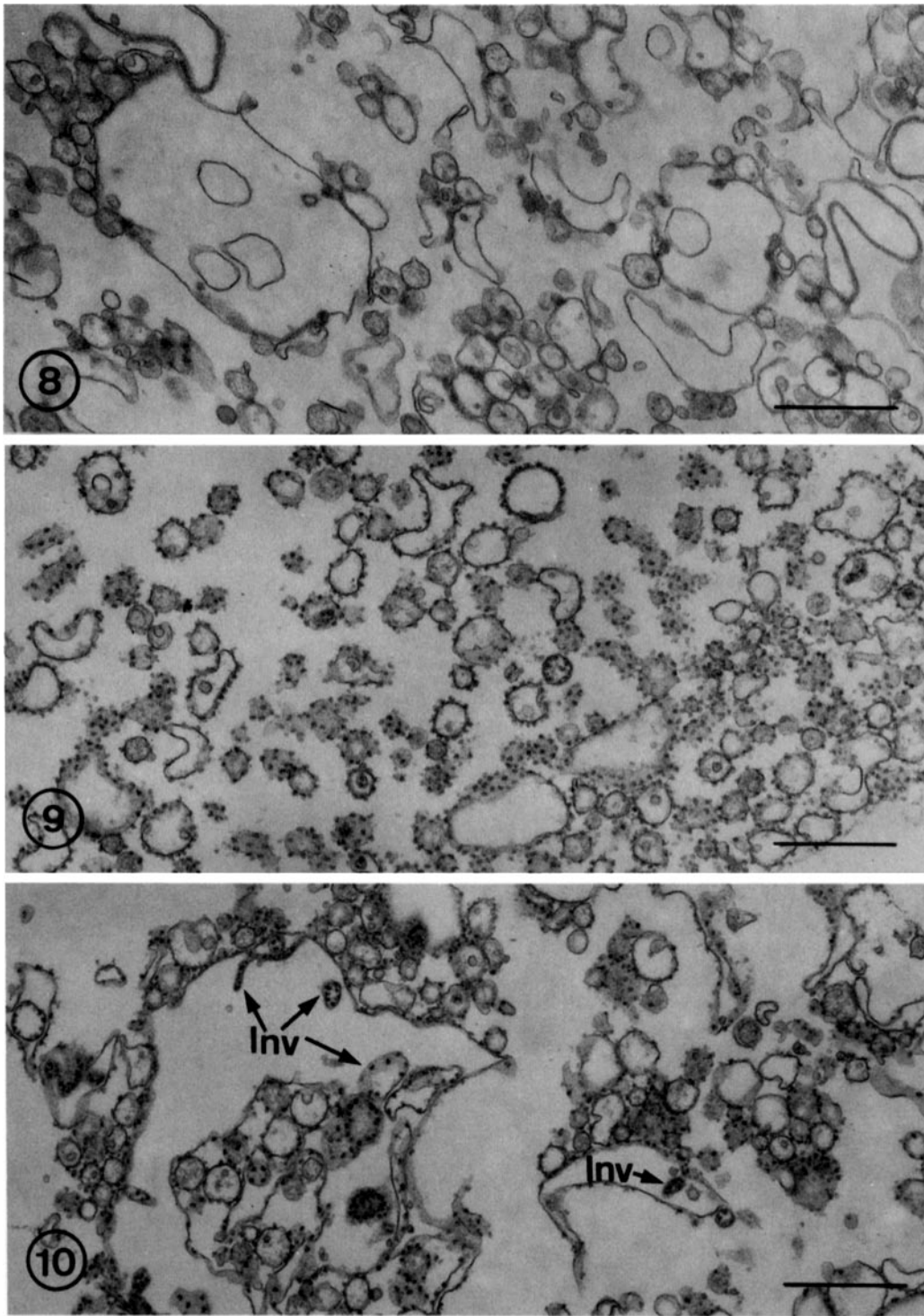
FIGURE 8 Appearance of KCl-treated heavy microsomes after incubation for 120 min at 37°C with nucleotide sugars in the presence of GTP. The vesicle profiles are devoid of distinct ribosomal particles, but show electron-dense patches in regions of closely apposed membranes. The vesicle profiles are aggregated and are highly variable in size. During incubation, large membrane-bounded vesicles were produced within aggregates of small vesicles. Bar, 0.5 μm. × 36,000.

FIGURE 9 KCl-MgCl$_2$-treated heavy microsomes are shown after incubation for 120 min at 37°C in the presence of nucleotide sugars and GTP. The vesicle profiles are not so variable in size as those observed in Fig. 8 and are surrounded by numerous ribosomes. Bar, 0.5 μm. × 36,000.

FIGURE 10 Puromycin-treated heavy microsomes are shown after incubation for 120 min at 37°C in the presence of nucleotide sugars and GTP. The vesicle profiles are aggregated. Amongst the aggregates can be seen very large membrane-bound structures and numerous small ones. In spite of the loss of 68% of the RNA, ribosome-like particles are still evident on the membranes of both large and small vesicles. They are particularly prominent in invaginations (Inv) of the large vesicle membranes. Bar, 0.5 μm. × 36,000.

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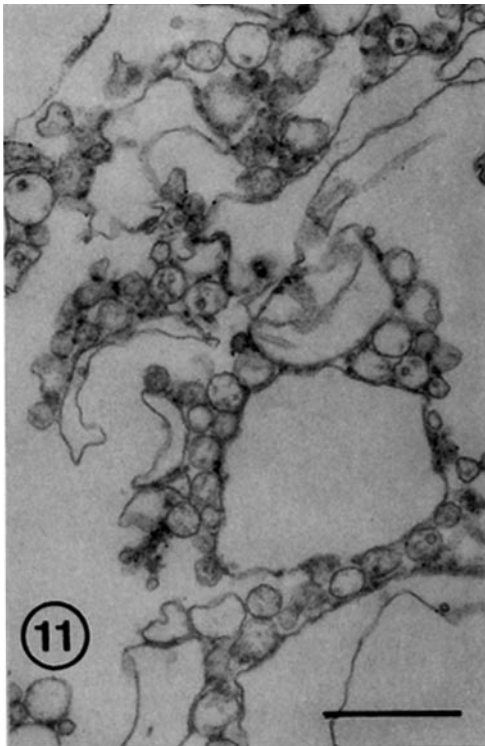
FIGURE 11 PP$_i$-treated heavy microsomes incubated for 120 min at 37°C, in the absence of nucleotide sugars. This omission had no effect on the GTP-induced formation of large vesicles. Large vesicle profiles are seen amongst aggregates of smaller vesicle profiles. Electron-dense material thought to represent ribosomal remnants appears selectively in zones of close membrane apposition. Bar, 0.5 μm. × 36,000.

acetylglucosamine incorporation into microsomal lipid and protein was nearly abolished (98% inhibition).

DISCUSSION

Thin-section and freeze-fracture electron microscopy of PP$_i$-treated heavy microsomes incubated with GTP have revealed the formation of aggregates which include numerous vesicles larger than 1 μm. These large vesicles arise during incubation by coalescence of smaller ones through a process of membrane fusion. Images showing continuity between membranes of small and large vesicles are consistent with this idea. The coalescence phenomenon depends on the removal of membrane-bound ribosomes, and on the presence of GTP in the incubation medium. It occurs whatever the means by which membranes are stripped and is induced to a much lesser extent by ITP than by GTP. Other nucleotides including nonhydrolyzable analogues of GTP are inactive. The effect of GTP is thus probably related to the phosphorylation potential of this nucleotide. The conditions that lead to coalescence of microsomes strikingly parallel the requirements for active protein glycosylation through the endogenous dolichol in rough microsomal membranes (8–10, 19, 20).

The meaning of the membrane fusion phenomenon has still to be established. The specificity of GTP and the fact that GTP acts at its normal concentration in hepatocytes, which is ~0.5 mM (6, 17), argue for a meaningful phenomenon demonstrating that some functions of the rough endoplasmic reticulum membranes depend upon this nucleotide. Nevertheless, it is uncertain whether the coalescence of vesicles per se has a functional equivalent in the intact cell. Because the rough endoplasmic reticulum consists of rather large cisternae, it is possible that some mechanism activated by GTP provides for its arrangement into a few, large structural entities, as opposed to numerous, small independent vesicles. Vesicular rough endoplasmic reticulum has been observed to fuse within intact cells to form large sheets (21), which is in accord with this suggestion.

Another riddle is the identity of the requirements for coalescence of microsomal vesicles and for efficient glycosylation of protein via endogenous dolichol intermediates. Membrane fusion is not conditioned by glycosylation of membrane constituents because microsomal vesicles coalesce in the absence of UDP-GlcNAc and GDP-Man, and in the presence of tunicamycin. Conversely, the enhancement of the glycosylation activity could be the result of the coalescence of microsomal vesicles, possibly through a change in permeability to activated sugars. This relationship is apparently not supported by the time-course of events, since evidence of fusion was not overtly apparent before 15 min, whereas the synthesis of dolichyl diphosphate N,N'-diacetylchitobiose is clearly enhanced earlier (9). Apparently, fusion and glycosylation both depend on an identical primary effect of GTP.

It is the removal of ribosomes, not another modification of the membrane by the agents used to remove the ribosomes, which acts in a cooperative manner with GTP. Most significantly, heavy microsomes treated with KCl, or with KCl, MgCl$_2$, and puromycin, have a reduced content in RNA and coalesce in the presence of GTP, whereas heavy microsomes treated with KCl and MgCl$_2$ retain a nearly normal content in RNA and fail to coalesce. It has been envisaged earlier that the removal of ribosomes unmasks the membrane sites where GTP acts (9). However, stripped membranes do not behave like the original rough membranes, even in the absence of GTP (Fig. 4). Microsomal vesicles do not aggregate and ribosomes retain their normal distribution along the membranes in sucrose-washed (Fig. 3) and in KCl-MgCl$_2$-treated (Fig. 9) preparations, whereas vesicles aggregate extensively and ribosomal remnants distinctly form clusters in stripped preparations. Thus, ribosomes or their residues, together with their binding sites in the membrane, display a greater lateral mobility in stripped membranes. Such may also be the case of other membrane constituents, since freeze-fracture studies have shown that the intramembrane particles aggregate at 24°C after complete removal of the ribosomes from the rough microsomes by treatment with puromycin in high salt medium (13). Conceivably, enough freedom of movement of membrane constituents may favor the fusion of vesicles and allow metabolic activities which involve several membrane-bound enzymes and substrates to proceed at a much faster rate. Aggregation of vesicles, which occurs in stripped preparations, might also facilitate initiation of membrane fusion, but is not relevant to glycosylation activity.

In addition to such factors, the structural and metabolic modifications observed tightly depend on GTP. The primary action of this nucleotide is unknown. Conceiving a single action for the coalescence of vesicles and for the enhancement of the glycosylation activity, one is led to speculate that GTP actively reorients, or translocates some constituent(s) of the membrane. Such an action might require enough freedom of movement within the membrane and, may be to this aim, the removal of ribosomes.

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