EFFECTS OF PHOSPHOTUNGSTATE NEGATIVE

STAINING ON THE MORPHOLOGY

OF THE ISOLATED GOLGI APPARATUS

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ABSTRACT

Isolated Golgi complexes can be recognized in phosphotungstate (PTA) negative
stain as stacks of membranous plates surrounded by a complex anastomosing
network of tubules and vesicles. The extent of this tubular network is, however,
much greater than can be observed in thin sections of whole cells. To determine
which of the steps leading to the final negatively stained image may produce the
observed changes, we have monitored each of the steps by other electron
microscope and biochemical methods. The first damage to the membranes seems
to occur during the initial isolation procedure as judged by the appearance of
smooth patches on the freeze-fractured membrane faces that are normally covered
with particles. Subsequent suspension of the Golgi fraction in water, to dilute the
sucrose for negative staining, leads to the disappearnce of the stacking, to some
tubulation and some vesiculation of the membranes as judged by thin section and
freeze-cleave microscopy. The latter technique also reveals an increase in
smooth-cleaving membrane faces. Application of the negative stain to the
water-washed Golgi fraction, finally, produces extensive tubular arrays and a
simultaneous decrease in the remaining large membranous vesicles. The freeze-
cleaved tubular membranes appear essentially smooth except for small patches of
aggregated particles. Parallel gel electrophoresis studies of the membranes and of
the water and negative stain wash extracts indicate that protein extraction is
involved in these morphological changes. PTA seems to be a particularly effective
solvent for certain membrane proteins that are not removed by the water wash.
These observations suggest that removal of membrane proteins alters structural
restraints on the membrane lipids so that they behave semiautonomously like
myelinics and form new artificial structures. This does not eliminate the possibility,
however, that some tubules also exist in the Golgi apparatus in vivo.

INTRODUCTION

The recognition of the characteristic appearance of isolated Golgi apparatus (GA) in negative stain (1) has greatly aided the development of isolation techniques for this interesting organelle (2–6). The tubular and fenestrate portions of Golgi apparatus cisternae, which are so evident in negative stain, have also been demonstrated in serial sections (7), by cytochemical staining (8), high-voltage electron micoscopy (9), and in freeze-etch replicas (10). These observations have led to theories about the function of a tubular network in interconnections between dictyosomes and as regions for specialized membrane transformation in secretion (11, 12).

The extent of the tubular network seen in negatively stained GA seems much greater, however, than can be accounted for by the tubules associated with the GA in sectioned tissue. Several previous reports have drawn attention to differences in the appearance of negatively stained membranes compared to thin-sectioned ones. The first study seriously to question the validity of the negatively stained image was that of Sjöstrand et al. (13), who observed an increase in the tubular membrane elements of mitochondria upon incubation in sucrose solutions after isolation from the cell. In a recent study by Terry (14), it has been shown that drying in the presence of silicotungstate causes the formation of highly specific tubular artifacts in membranes rich in unsaturated fatty acids.

In none of these studies, however, has each of the steps leading to the final negatively stained image been carefully monitored by other electron microscope and biochemical methods to determine which of the steps may produce the observed changes. To this end we have undertaken a comparative study of the electron microscope appearance and of the protein composition of GA membranes under a variety of conditions in order to determine to what extent fenestrae and tubules may arise as a result of the manipulation required for preparation of the electron microscope specimens.

GA membranes provide a useful model for studies of membrane structural transformations for two reasons. The first advantage of using the Golgi apparatus is the high degree of structural organization of the stacked cisternae, which makes identification of membranes possible under a variety of observational conditions. Second, the Golgi apparatus is a central element in the cytoplasmic membrane system and might be predisposed to undergo changes such as those observed in vivo between nuclear membrane, endoplasmic reticulum (ER), GA, secretory-vacuole, and plasma membrane.

MATERIALS AND METHODS

GA were isolated by a previously described technique (15). Briefly, this involved homogenization of excised rat testes in a solution containing 0.25 M sucrose, 50 mM Tris-maleate buffer, pH 6.5, 5 mM $MgCl_2$, and 1% Dextran (Sigma 200C, Sigma Chemical Co., St. Louis, Mo.). The lowest setting of a Polytron 10ST homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.) was used for 20 s. The homogenate was centrifuged at 2,000 g for 10 min, and the crude GA-mitochondrial fraction was resuspended in 1.1 M sucrose with buffer, Mg, and Dextran concentrations as above. This suspension was centrifuged in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 100,000 g for 90 min. The GA accumulated at the 1.1–1.25-M interface. They were collected, diluted to 0.25 M sucrose, and sedimented at 10,000 g for 10 min.

Negative staining was done by mixing a drop of a dilute suspension of GA (about 1 mg protein/ml) on a grid with 2% PTA, adjusted to pH 6.8 with KOH. The grid was blotted with filter paper and air dried. Samples to be thin sectioned were sedimented from buffered sucrose, or H_2O, or 1% PTA solution, depending on the experiment, and the pellet was cut into sectors before fixation in S-collidine-buffered 2.5% glutaraldehyde at 4°C for 1–2 h. Dehydration, embedding, and sectioning were by usual techniques.

Freeze-fracturing was performed according to the method of Moor and Mühlethaler (16) using a Balzers 360M apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). Replicas of samples of GA fractions subjected to the following treatments were examined: (a) suspended in either sucrose, H_2O, PTA, or 20% glycerol (vol/vol); (b) washed in H_2O, suspended in PTA; (c) fixed for 30 min in buffered 2.5% glutaraldehyde and slowly infiltrated with glycerol to 20%; (d) washed with either H_2O, PTA, or H_2O followed by PTA, and then fixed with buffered 2.5% glutaraladehyde and infiltrated with 20% glycerol.

The treated fractions were pelleted and frozen in liquid Freon 22 held at −150°C. Samples were fractured at −103°C, and replicas were made immediately without any etching. The replicas were cleaned on concentrated bleach and 40% CrO_3.

For acrylamide-gel electrophoresis, a combined GA fraction isolated from 24 rat testes was divided into unequal aliquots. One small aliquot was fixed immediately as an electron microscope control. Another was

dissolved in a sample buffer containing 5 mM Tris-HCl pH 8.8, 0.1% sodium dodecylsulfate, and 0.1% mercapto-ethanol before freezing. The remainder of the sample was resuspended in 5 ml of distilled, deionized H_2O and centrifuged at 50,000 g for 30 min. The supernate was saved, and the membrane residue was resuspended again in 5 ml of H_2O. The suspension was recentrifuged, and the supernate saved. The pH of the H_2O supernate after centrifugation was 7.0. The membrane residue from the second H_2O suspension was resuspended in 5 ml of 1% PTA freshly neutralized to pH 6.8 with KOH. The same centrifugation was repeated and the supernate (PTA wash) and membrane residue (final) were saved. The membrane residue was dissolved in the sample buffer described above.

The supernate were dialyzed separately against two changes of 1% SDS for 48 h at room temperature, concentrated to dryness against Dextran, and resuspended in 0.2 ml of sample buffer. Samples were then heated for 2 min in a boiling water bath, and equal volumes (20 μl) were placed on a 10% acrylamide slab gel, all according to the method of Laemmle (17). The gels were run at 15 mA for 5–6 h, stained with Coomassie Blue in 50% TCA, and destained in glacial acetic acid. p-Aminosalicylate (PAS)-glycoprotein analyses were done on cylindrical sodium dodecyl sulfate (SDS) gels, according to the procedures of Glossmann and Neville (18).

RESULTS

The size and complexity of the GA isolated from rat testes by the technique used in this study suggest that they are obtained from spermatocyte or early spermatid cells (5). The morphology of these GA in thin sections of whole cells has been recently described (19). Freeze-fracturing reveals a different view of cell components than does sectioning and avoids some of the artifacts incurred in fixation and embedding. A replica of a GA in a freeze-fractured spermatocyte cell is shown in Fig. 1. The stacks of cisternae are easily identified. Fenestrae or perforations (arrows) are seen at the margins of these cisternae, but any tubular network is very difficult to discern, whether or not the cells are fixed or only glycerinated before freezing. This failure may be partly due to the fact that the diameter of some of the tubules is only slightly larger than that of the irregularities seen in the surrounding cytoplasm. An even pattern of 60–80-Å granules can be seen on one of the membrane fracture faces.

Fig. 2 shows a thin section through a pellet of isolated spermatocyte GA. This pellet was spun down from the buffered sucrose homogenizing solution and was fixed in a buffered, isotonic glutaraldehyde solution. Portions of several Golgi bodies are present in this micrograph. The orientation of the cisternal stacks is preserved, along with the overall morphology of the organelles. There is a tendency for the GA to round up after isolation to form a sphere, as was previously described by Morré and Mollenhauer (20). Note the spiny-coated vesicles (indicated by the arrow in Fig. 2) that are characteristic of the GA observed in thin sections. The major contaminant of these fractions consists of large cytoplasmic droplets or residual bodies which are shed during spermatogenesis. To a lesser extent, the fraction also contains mitochondria, ER, and a large number of unidentifiable membranous vesicles in and around the isolated GA. There is also a very extensive tubular network associated with one GA. The extent of the tubular plexus appears to be inversely proportional to the number of cisternae in the stacks and to vary from preparation to preparation. As will be discussd later, we interpret this extensive tubular network to be indicative of damage to the organelle.

Fig. 3 shows an example of the appearance of isolated rat spermatocyte GA when negatively stained with neutral PTA. Several stacked cisternae may be identified in this complex. The reticulate periphery of the cisternae traps stain and is seen through the solid membrane portion so that it is difficult to tell which is on top and which is on the bottom. Stereomicrographs fail to increase the three-dimensional resolution. Apparently the membranes are strongly flattened during drying.

The cisternae of negatively stained specimens (Fig. 3) are usually fenestrated along thin edges, and the fenestrated portions are usually connected to the extensive, anastomosing tubular network previously described (1). Occasionally the fenestrae are so numerous and compact that the membranes become lacy or weblike. This particular GA is less tubate than most, so that more of the central platelike region and the fenestrate edges are visible. In general, the number of fenestrated cisternae of a GA is inversely proportional to the number of tubules present.

A number of small, blebbed vesicles are also present around the GA, as shown in Fig. 3. Morphologically they resemble the ER vesicles previously described (21), but the similarity between ER and GA in this procedure makes their positive identification difficult in the absence of the total morphology of GA described above. They
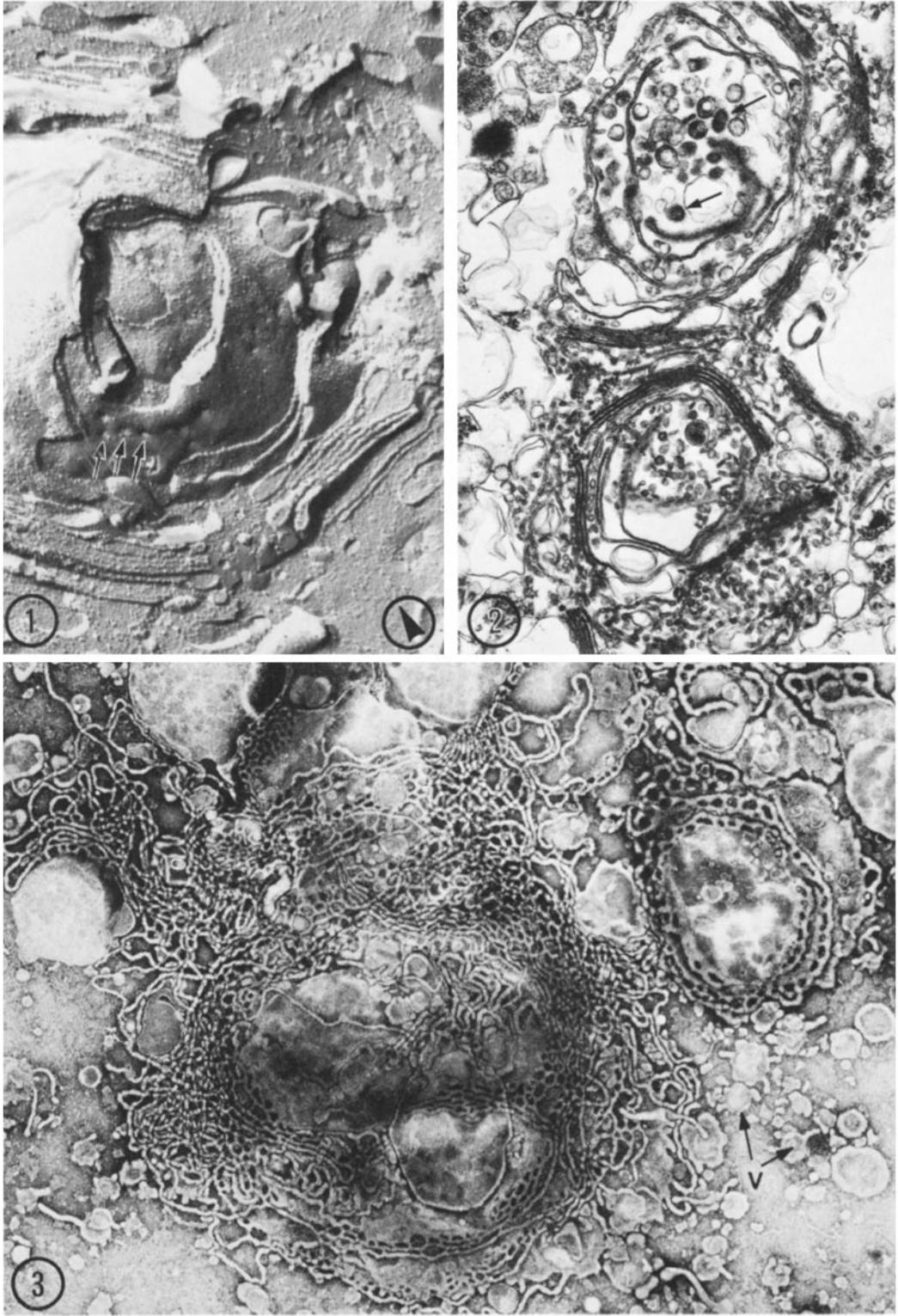
could be ER, but they could also be small frag-
ments of GA. As has been previously discussed
(15), the presence of tubules alone in negative stain
is also not sufficient evidence to identify GA.

The extent of the central plate-like portion of
cisternae seen in negative stain does not seem
nearly as large as the cisternal profiles seen in thin
sections. This impression is borne out in cases
where one observes individual cisternae which have
slid out of the cisternal stack. Fig. 4 shows such
cisternae. An important clue to the origin of the
tubules can be seen in the pattern of size and
distribution of the fenestrae. There is an expanding
mesh of perforations. The tubular interstices of
this web are lumpy or irregular, especially around
the larger, outer holes. There is a very strong
suggestion that the lumps (see double arrows)
represent connections in the network which have
broken, giving rise to longer tubes. Thin places in
the tubular skein (single arrows) suggest incipient
breaks.

Fig. 5 shows a high-magnification view of the
margin of two Golgi cisternae dried quickly in
PTA. We interpret the regular appearance of the
meshwork and the relatively small size of the
fenestrae as indications of a minimally damaged
specimen. Because of this good preservation of the
gross membrane morphology, it is of interest to
note the presence of small myelin figures in and
around the fenestrae. These myelin figures are not
often seen, and when they do appear it is nearly
always in conjunction with "well-preserved" cister-
nae. We therefore are inclined towards interpreting
these small myelin figures as transient structures
related to the first steps in the loosening and
breaking down of the structure of Golgi mem-
branes in PTA. The fact that these myelin figures
have not been reported in previous studies of

negatively stained Golgi membranes suggests that
they may occur only briefly or are washed off when
the grids are blotted.

In order to test the hypothesis that the tubular
reticulum of isolated GA may be increased by the
negative staining procedure, we have prepared
isolated organelles as if for negative staining, but
then we have observed them by other techniques.
Fig. 6 shows a fraction which was diluted with
distilled water to reduce the sucrose concentration
for negative staining, then sedimented, fixed, em-
bedded, and sectioned. It can be clearly seen that
the cisternae have mostly been replaced by vesicles
and tubules. The proportion of intact sheets,
tubules, and vesicles depends on the vigor with
which the membranes are dispersed and the length
of time in water. Very brief or gentle dispersal
leads to less change in the membranes than shown
here because many large clumps of organelles
remain in such a suspension, where their mem-
branes are in all likelihood protected from osmotic
stress. Very vigorous dispersal or long exposure to
water results in a population composed entirely of
vesicles. The GA which are dispersed enough to be
seen in negative stain are probably exposed to
stresses at least as severe as that shown in Fig. 6.

Figs. 7–11 show freeze-fracture replicas of iso-
lated GA before and after osmotic lysis and PTA
extraction. Fig. 7 shows an isolated fraction which
was fixed in isotonic, buffered glutaraldehyde, then
glycerinated and frozen. The overall morphology
of the organelles is very similar to that shown in
Figs. 1 and 2. At higher magnifications face views
of the membranes of the Golgi cisternae exhibit
two noticeable features. The first is illustrated in
Fig. 8 and pertains to the distribution of particles
on the fracture faces. The GA in well-preserved
tissues exhibits more or less randomly distributed

FIGURE 1   Freeze-fracture image of GA in a rat testis cell. The outer cisternae of the GA have been cross
fractured, while those in the center appear in face views. The arrows point to small fenestrae. Note the
absence of an extensive tubular system. × 40,000.

FIGURE 2   Section through a pellet of isolated rat spermatocyte GA. These organelles were fixed in the
presence of the buffered sucrose isolation medium but some damage has occurred, perhaps during isolation,
and the tubular network is very extensive. Note the spiny-coated vesicles (arrow) in the center of the upper
organelle. × 23,000.

FIGURE 3   PTA negatively stained isolated GA fraction. Note platelike central portions of cisternae,
fenestrated edges, and tubular network. This GA is relatively well preserved as judged by the relatively large
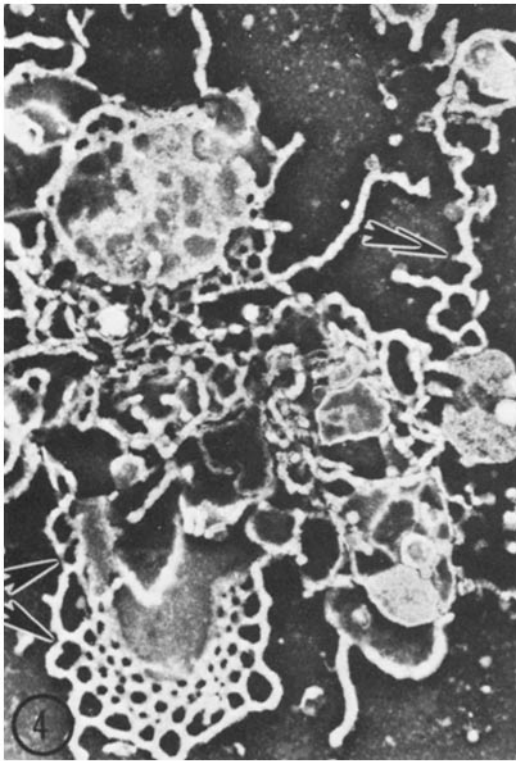cisternae and sparse tubules. (V) = membrane vesicles possibly derived from ER. × 24,000.

FIGURE 4 PTA negative stain of cisternae from unstacked GA. Note expanding meshwork of fenestrae from the center to the edges of the cisternae. The bumpiness of the tubules (double arrow) suggests broken interconnections in the web. Thin places in the web appear to be incipient breaks (single arrows). × 40,000.

FIGURE 5 High magnification of margin of cisternae of negatively stained GA. Note small, myelin-like figures in fenestrae (arrows) and apparent breaks in tubular mesh. × 125,000.
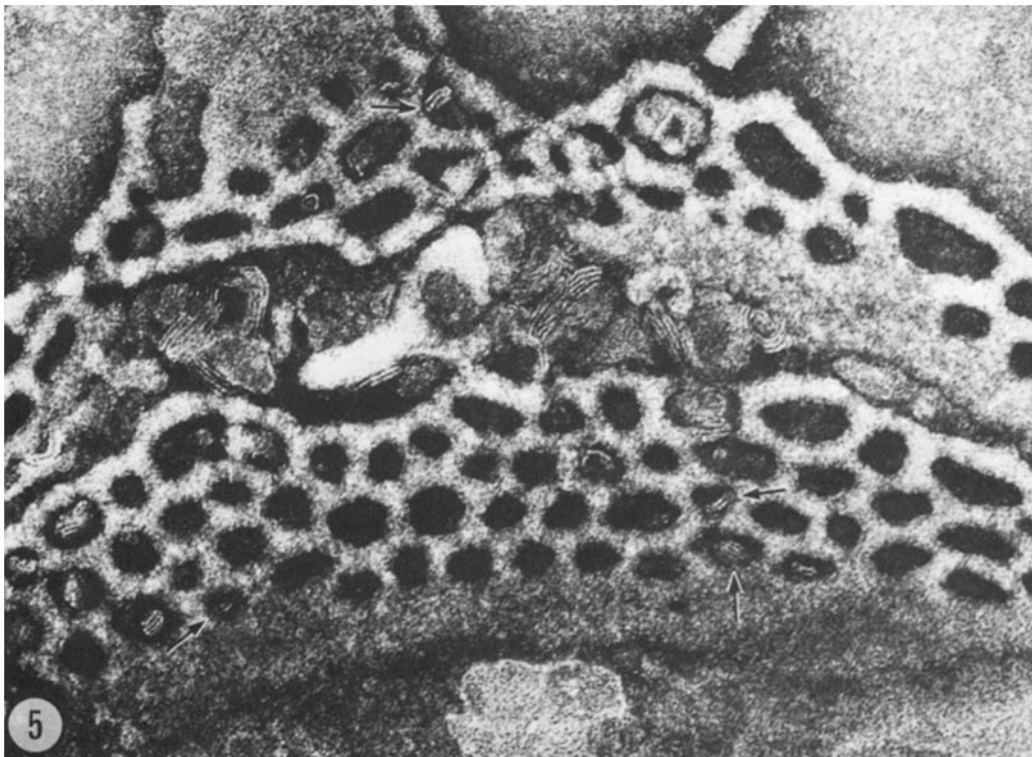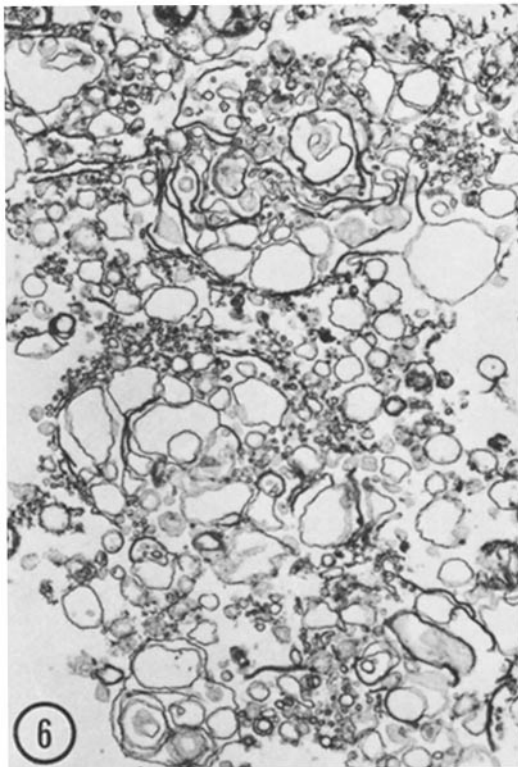
FIGURE 6 Section through a pellet of isolated GA which were diluted with distilled water to reduce the sucrose concentration to a level suitable for negative staining, then sedimented, fixed, embedded, and sectioned. Most of the cisterna membranes in these two organelles have been converted into tubules and vesicles. × 25,000.

FIGURE 7 Low-power micrograph of a freeze-fracture replica of an isolated GA fraction after glutaraldehyde fixation (in the buffered sucrose isolation medium) and glycerol infiltration before freezing. The overall morphology of the Golgi membranes is well preserved. However, upon close examination some membrane faces appear smooth and devoid of particles, while on other faces the particles appear in patches (compare with Figs. 1 and 8). A mitochondrial contaminant is seen in the center of the micrograph. × 29,000.
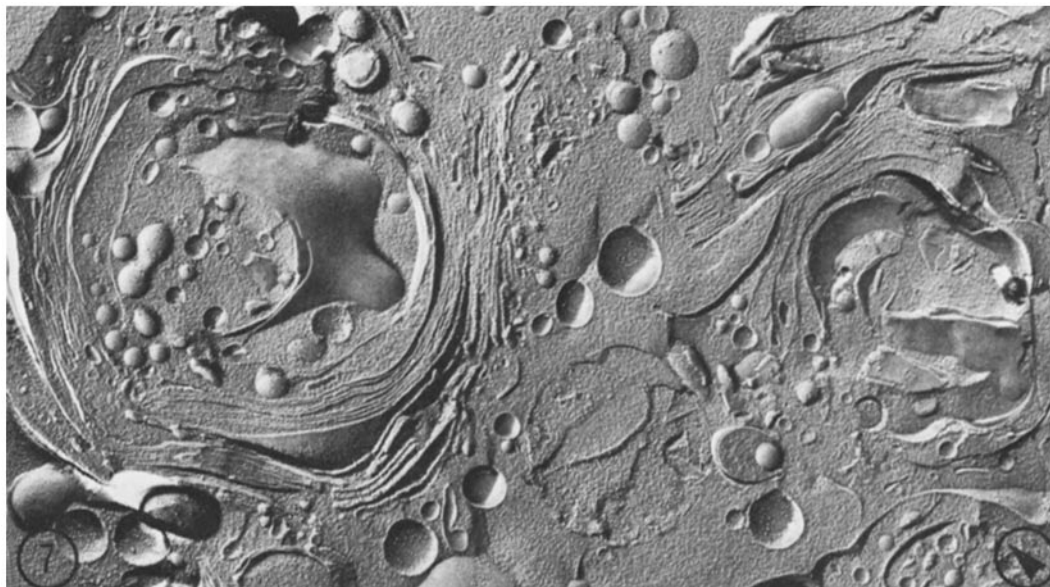
particles on both its A and B fracture faces (10).[1] In preparations of isolated GA, on the other hand, the particle-covered faces are invariably inter-

rupted by patches of material with very smooth surfaces that resemble pure lipid bilayers in their cleaving behavior. Notice also in Fig. 8 the relatively small fenestrae in the margin of one of the cisternae on the left side of the micrograph. A

[1] L. A. Stachelin. Unpublished observations.

larger field of fenestrae is demonstrated in Fig. 9. Although some of the fenestrae possess internal diameters of between 600 and 800 Å, the majority appear much smaller and have diameters of less than 300 Å.

When the sucrose medium is diluted out and the GA are suspended and then frozen in distilled water, the morphology of the organelles is seen in freeze-fracture to be greatly changed (Fig. 10). Some of the changes can be attributed to the compression of the remaining cisternae by the relatively large ice crystals. In addition, however, it is seen that while some membranes still retain a nearly normal fracture-face morphology, many others appear to be completely devoid of particles or to exhibit small patches of closely spaced, aggregated particles. Furthermore, many tubular membrane elements begin to appear. After resuspension in PTA (Fig. 11), the GA are completely transformed into a system of tubules and interconnected blebs, the fracture faces of which contain few individual particles or particulate aggregates. The junctions between the blebs are composed of flattened surfaces similar to the contact faces of soap bubbles. The blebs are much wider than the tubular network seen in negative stain. Perhaps freezing causes swelling, or alternatively, drying in phosphotungstate causes shrinkage. Fixation and glycerination of the membranes after water or PTA washing did not help to preserve the typical GA structure; on the contrary, these treatments resulted mostly in a further degradation of the membranes into microsomal-type vesicles.

The extraction of the membranes with PTA shown in Fig. 11 is not exactly comparable with the exposure to PTA in negative staining. In this case the membranes were resuspended in a large volume of cold, dilute PTA for several minutes and then centrifuged for 30 min before freezing. Ordinarily, membranes are exposed to a small volume of PTA, the concentration of which increases rapidly until the structures are embedded in a PTA glass after a few seconds. When isolated GA are exposed to dilute (1%) PTA as in Fig. 11, but then negatively stained by the normal procedure after centrifugation, very few tubules are observed and the preparation is seen to be composed mainly of a mixed population of vesicles (Fig. 12).

In order to test whether there may be alterations in the membrane composition which accompany or institute the morphological alterations described, we examined the protein content of the membranes and supernate washes using SDS-acrylamide gel electrophoresis. Fig. 13 shows an example of a gel on which four samples were run. The first column represents the total protein from the GA reaction after SDS solubilization. Approximately 30 bands could be identified on the original. Since the starting material contained other organelles, we cannot be certain which of these are GA proteins. The second column shows proteins released by water washing. These represent a subset of proteins, mostly of a higher molecular weight, which are probably secretory, matrix, or loosely bound membrane proteins.

When the membrane residue from water washing is reextracted with dilute PTA (conditions identical to those in Figs. 11 and 12) and the PTA extract is run on the gel, a pattern like that in column no. 3 is observed. This fraction is a subset of proteins distinctly different from those released by water. At least 8–10 bands in each of fractions nos. 2 and 3 are nonhomologous. Finally, if the membrane residue after PTA extraction is dissolved in SDS and run on the gels, still another subset of large proteins is seen (column no. 4).

When the Golgi fraction was resuspended in 0.25 M sucrose and then spun down at 100,000 g for 1 h, numerous peptides (not shown) remained in the supernate. Although most of these peptides coelectrophoresed with those from the water wash, some were of different molecular weight. If cylindrical SDS gels were run with any of these fractions (after extraction by the various procedures outlined) and stained for glycoproteins, three major glycoproteins of 66,000, 79,000, and 87,000 mol wt could be identified. These peptides were not found in the water, sucrose, or PTA supernates and therefore remained attached to the membranes during these treatments.

DISCUSSION

The results of this study show that the structural organization of GA membranes in flattened cisternal stacks, as seen in thin sections in electron microscopy, is subject to very drastic modification after organelles are extracted from the cell. Our interpretation is that a progressive transformation occurs beginning at the periphery of the cisternae and moving toward the center of the stack. First, enlarged fenestrae are seen, these grow further into a tubular meshwork, the meshwork breaks down to form long tubes, and finally, the tubes break into short segments to form vesicles. This transition occurs spontaneously after isolation, even in a buffered sucrose solution at low temperature. It is
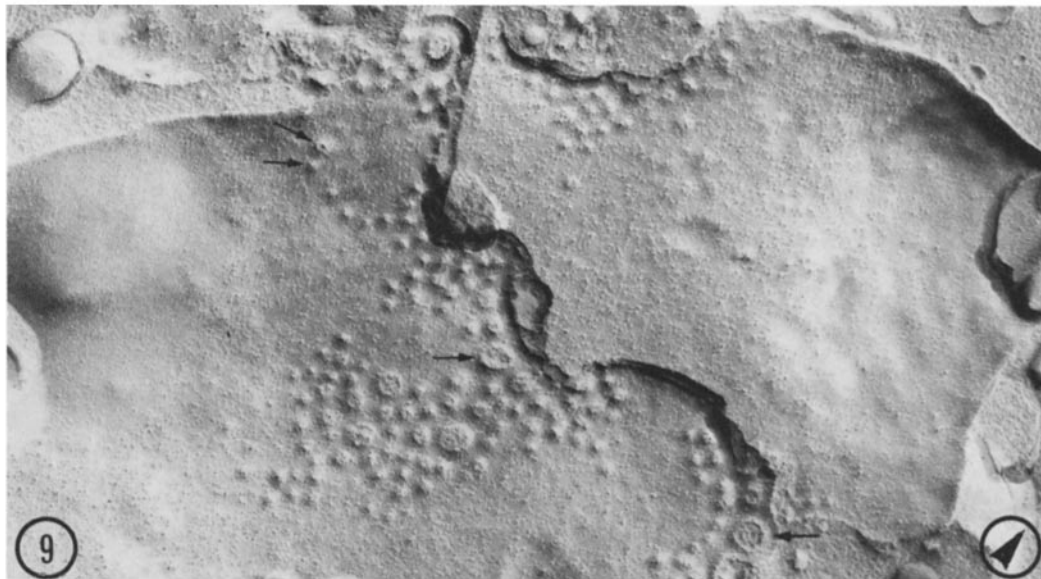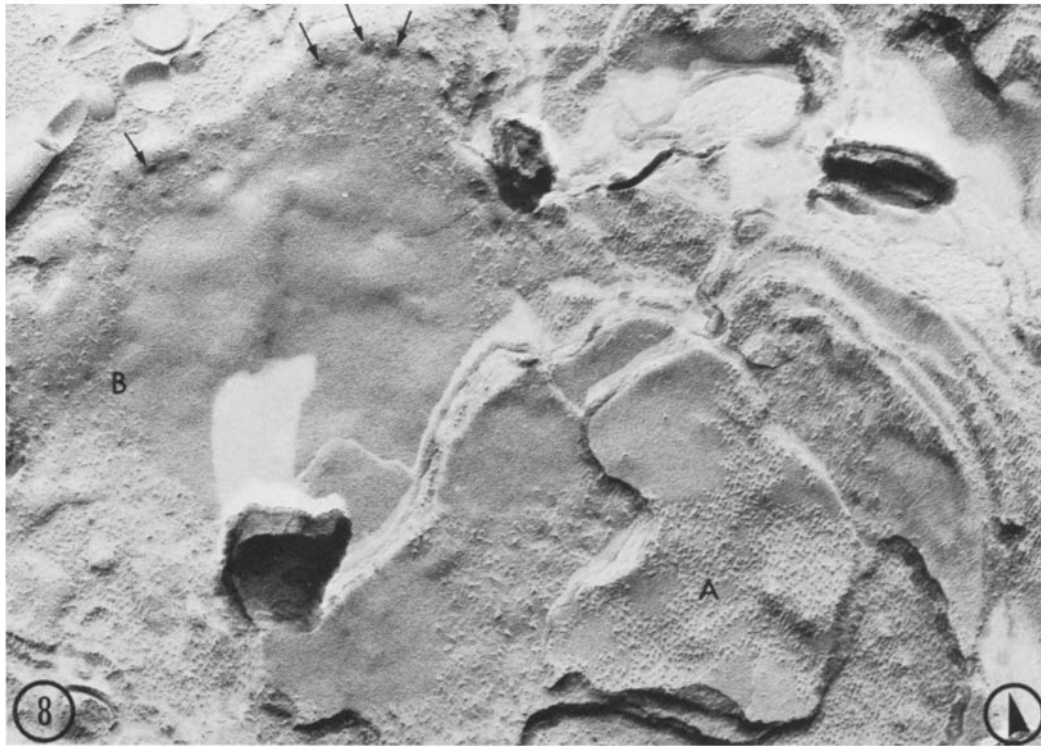
FIGURE 8 Higher magnification of an isolated GA from the same fraction as shown in Fig. 7. In contrast to the Golgi cisternae of well-preserved tissues, which exhibit more or less randomly distributed particles on both A and B fracture faces (Fig. 1), the particle-covered fracture faces of isolated GA are interrupted by patches of material with smooth surfaces and a resemblance in their cleaving behavior to pure lipid bilayers. This alteration in the distribution of membrane particles is the most sensitive indicator of damage we have found in isolated Golgi bodies. The small fenestrae (arrows) in the margin of one of the cisternae appear unaltered. × 72,000.

FIGURE 9 Two cisternal faces of an isolated GA processed as indicated in Fig. 7 and revealing extensive fields of fenestrae (arrows). While some of the fenestrae possess internal diameters of between 600 and 800 Å the majority appear much smaller and have diameters of less than 300 Å. Compare these fenestrae with those shown in Fig. 5. × 58,000.
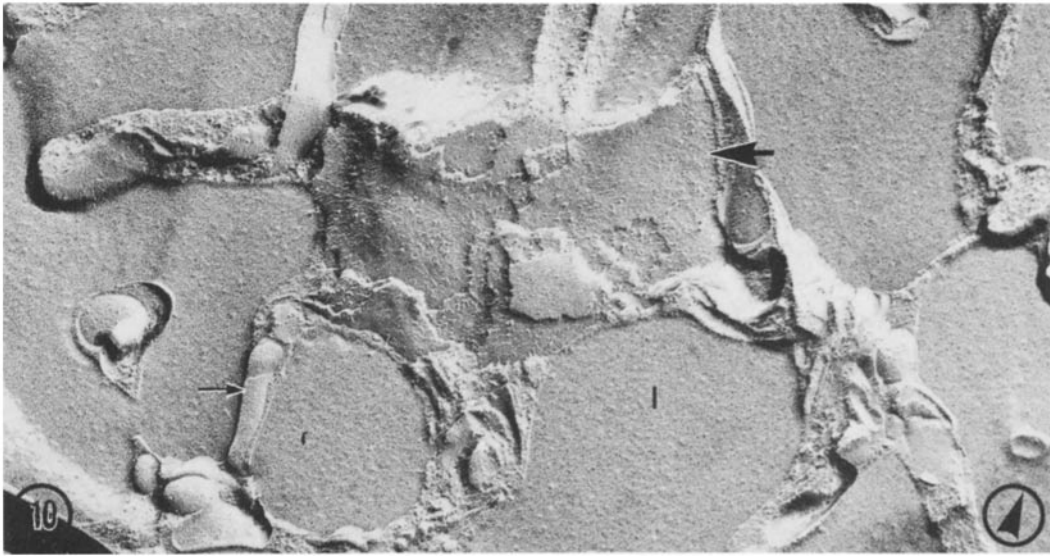
FIGURE 10   Freeze-cleaved membranes of isolated GA suspended and frozen in distilled water. Although the membranes have been compressed by the large ice crystals (1), it can be seen that while many of the larger membrane elements (large arrow) retain a nearly normal fracture morphology, the smaller and more tubular ones (small arrow) appear nearly devoid of particles. × 54,000.

FIGURE 11   Isolated Golgi membranes washed in water and resuspended and frozen in PTA. Only tubules and rows of interconnected blebs may be recognized, the fracture faces of which appear smooth except for a few individual patches of aggregated particles (arrows). The junctions between the blebs are composed of flattened surfaces (arrowheads) similar to the contact faces of soap bubbles. × 38,000.
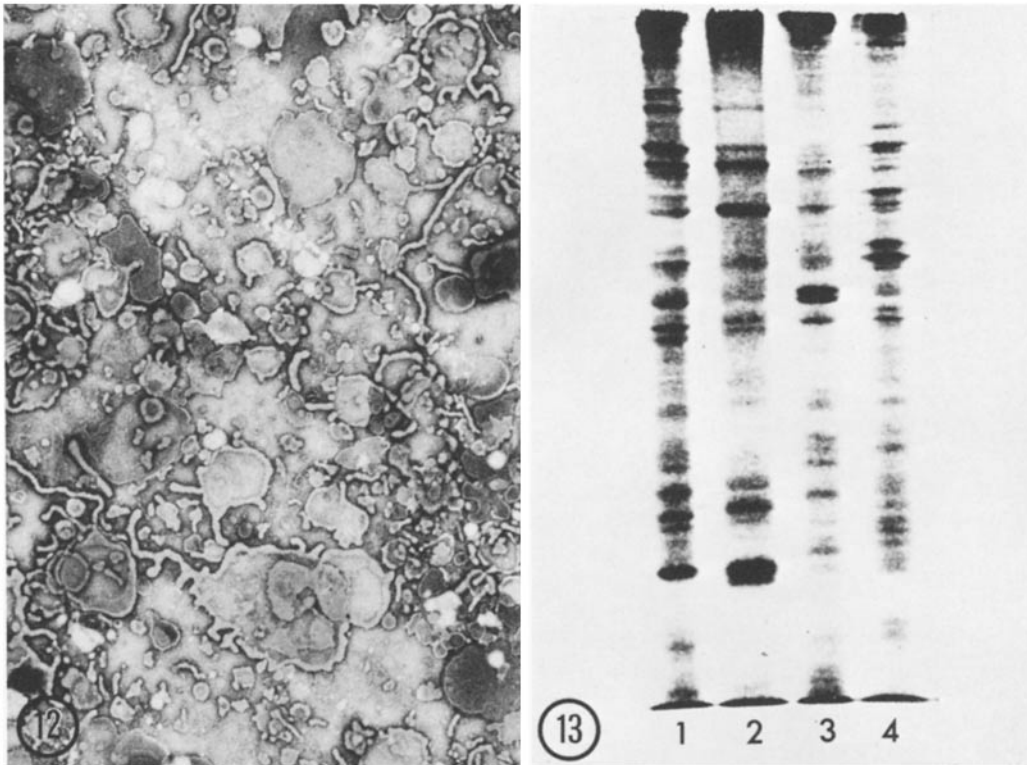
FIGURE 12   Negative stain image of Golgi membranes which were exposed for 30 min to a large volume of 1% neutral PTA in solution and then dried on a grid. Note that all semblance of normal Golgi morphology has been destroyed and only vesicles remain. × 25,000.

FIGURE 13   Photograph of SDS acrylamide slab-gel. Column no. 1 is total protein from a GA fraction extractable in 1% SDS. Column no. 2 is protein extracted by distilled water from a Golgi fraction. This presumably represents matrix or loosely bound proteins. Column no. 3 is proteins extracted by 1% neutral PTA from GA membranes *after* the water extraction in column no. 2. Column no. 4 is the final membrane protein residue after both water and PTA washing. Note the different populations of proteins extracted by water and PTA. Origin at top of picture. Approximately 0.9 original size.

greatly enhanced, however, by the manipulations required for PTA negative staining, namely, dilution of sucrose and buffer to low osmolarities, exposure to PTA, and drying.

Tubules are observed in isolated GA even when glutaraldehyde is added to the homogenizing medium and membrane proteins are fixed very quickly after the cells are broken (1). There are several possible explanations for this observation: (a) the tubules are a real feature of GA structure; (b) the sheet-to-tubule transition occurs very rapidly after cells are damaged, and glutaraldehyde fixation traps it in progress; or (c) glutaraldehyde fixation immobilizes proteins but leaves membrane lipids free to undergo rearrangement. Our studies

suggest that the extent of the tubular network associated with isolated GA is an indication of the amount of stress and damage to which the organelles have been subjected. It is difficult to extrapolate backwards from these observations to infer the true structure of the GA in vivo. There are many observations of fenestrae and tubular networks associated with GA in thin sections (1, 4, 7–9, 11, 12, 22–26), and in freeze-etch replicas (10, 27). Does the presence of these structures indicate fixation or freezing artifacts? Or are these normal features of the GA produced by natural mechanisms, the action of which is speeded up when the cells are disrupted? We would tend at this point to lean towards the latter explanation but with some

reservations. One would normally expect to find some fenestrae and tubules associated with the GA, and only an excessive number represent damage.

The earliest indication of damage which we have been able to identify in isolated GA is the rearrangement of membrane-intercalated granules seen in freeze fracture. Even those GA which show after isolation large stacked cisternae with small fenestrae and few tubules exhibit membrane fracture faces with large smooth areas (Fig. 8). These granule-free patches look very much like artificial lipid membrane layers after freeze-fracture. We interpret them as an indication of alteration in the molecular organization of the membranes, due to aggregation and probably extraction of proteins.

That protein extraction is involved in these morphological changes is indicated by our gel electrophoresis studies. Since these fractions are not pure GA, we cannot identify with certainty which of the bands observed come from the GA. We would like to be able to identify the proteins released by osmotic lysis in water as secretory products, but that is impossible at this point. We can, however, say that since the GA is the major component of this fraction and since the proteins released by water represent a substantial proportion of the total fraction, at least some of these proteins must have been in the lumen of the GA cisternae or loosely bound to the GA membranes.

In the same way, we cannot be sure which of the proteins extracted by PTA are GA proteins, but we can say that PTA does extract proteins from membranes that are not released by water, and that in the case of the GA, this extraction is concomitant with structural modifications. Other membranes may undergo similar alterations, but in the present study we did not attempt to follow their fate.

It seems surprising that PTA is so effective as a protein solvent in the concentrations used here. A 1% solution of PTA is only about 2 mM. Similar extractive properties for PTA have been reported, however, for proteins of ciliary microtubules and basal bodies from *Tetrahymena* (28), rat liver mitochondria (29), and bacteriophage (30). This solvent property may help explain why neutral PTA produces such even spreading of organelles and stain, since the dissolved proteins would lower the surface tension of the solution and promote spreading. PTA is not nearly so efficacious a stain if the membranes have been fixed in glutaraldehyde before staining. The high surface tension

after fixation can be overcome by adding albumin or other soluble protein to the stain (1).

Protein alteration may also be involved in the spontaneous tubulation of isolated GA when they are allowed to sit in buffered sucrose solutions for long time periods. Many of the secretory products of the GA are lysosomal in nature, and there is undoubtedly a high protease activity in cell-free GA suspensions. If unfixed GA fractions are allowed to stand overnight at 4°C, the transformations described here occur, and eventually membrane structure disappears completely.

Similar transitions in membrane structure from continuous sheets to tubules to vesicles, under the influence either of PTA or simply of incubation at low temperatures, have been previously reported for red blood cells (31) and for mitochondria (13). Intricate, myelin-like structures have been observed in negatively stained preparations of lipids (14, 32). Mollenhauer[2] has observed similar myelin-like forms in isolated GA and interprets them as possibly arising from membrane lipids. Our interpretation of this phenomenon is that extraction, aggregation, or hydrolysis of membrane proteins alters structural restraints on the membrane lipids so that they behave semiautonomously like meylinics and form new, artificial structures. In the process of seeking the lowest free-energy states, the membrane lipids go through several unstable intermediates which we recognize as fenestrated sheets, tubular webs, and tubes. The most stable form, which has the highest surface-to-volume ratio, is that of small spherical vesicles. Why webs form, structures which would appear to be less stable than either sheets or vesicles, is not clear. As might be expected, this reaction is strongly temperature dependent.

The effects of drying membranes in the presence of high concentrations of PTA is not as disruptive as one might expect. The reaction does not necessarily proceed to the vesicle state as it does in solution. It may be that drying occurs so quickly that the membranes are frozen in a PTA glass before the transitions are completed. A protective effect of this sort was originally proposed by Brenner and Horne (33) and supporting evidence for this theory has been reported by Van Brugen et al. for hemocyanin (34).

The electrophoresis studies described here also point out some interesting features of the GA. It is apparent that no matter how the GA is treated

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[2] H. H. Mollenhauer. Personal communication.

(sucrose, water, PTA, etc), a continuous extraction of peptides occurs. Since this is true even after lysis in water, it *appears* that many of these peptides are not simply soluble components of the Golgi cisternae. It would therefore appear that at least partial solubilization is an unavoidable aspect of the Golgi fraction isolation procedure. Finally, it is also evident that the major glycoproteins of the GA remain in the particulate fraction even after extensive extraction with water, sucrose, or PTA. It is interesting to speculate that the major glycoproteins of the GA constitute tightly bound membrane components which function as an integral part of the structural membrane. Hence these glycoproteins may be regarded as integral membrane proteins according to the classification of Singer and Nicolson (35).

CONCLUSION

In conclusion, we believe that while negative staining with PTA provides an extremely useful tool for the identification of isolated organelle membranes in biochemical preparations, the structures observed in such preparations must be interpreted with caution. The artifacts produced by negative staining, particularly those in isolated GA, may be of some help in revealing the relationship between membrane composition and structure. Any electron microscope study which seeks to determine the three-dimensional structure of organelle membranes in vitro should depend most on thin sections and freeze-fracturing.

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