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Recovery of Galactosyltransferase Activity from Sucrose Gradients During Isolation of Golgi Membranes

M. M. Mitranic, J. M. Sturgess, and M. A. Moscarello

Biochemistry Department and Pathology Department,
Research Institute, The Hospital for Sick Children, Toronto, Canada

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Summary. The purification of Golgi membranes has been characterized in membrane fractions isolated from rat liver by homogenization and by differential centrifugation on a discontinuous sucrose gradient. The purification of membranes, judged from the ratio of galactosyltransferase in the Golgi-rich fractions to that in the liver homogenate, correlated with the proportion of structurally intact isolated membranes. With increasing purification of membrane fractions, there was a corresponding increase in the total recovery of enzyme activity. It is postulated that this may reflect not only an increase in the purification of membranes but may involve the presence of specific galactosyltransferase inhibitors in intracellular membranes of the liver.

The biosynthesis of plasma glycoproteins requires the sequential addition of various monosaccharides to polypeptides after their release from the ribosome, as they pass through the membrane system of the cell. The enzyme responsible for the addition of galactose to the oligosaccharide chain, i.e. for the synthesis of galactosyl-(B,1-4)-N-acetylglucosamine linkage in plasma glycoproteins, is present in the Golgi apparatus of the liver cell. The evidence for this has been described in detail previously (Schachter, Jabbal, Hudgin, Pinteric, McGuire & Roseman, 1970).

In recent years, methods for the isolation of Golgi-rich fractions from rat liver have been reported (Fleischer & Fleischer, 1970; Leelavathi, Estes, Feingold & Lombardi, 1970; Morré, Hamilton, Mollenhauer, Mahley, Cunningham, Cheetham & Lequire, 1970; Ehrenreich, Bergeron, Siekevitz & Palade, 1973; Sturgess, Katona & Moscarello, 1973). In addition to morphological criteria of purity, assays for galactosyltransferase have been used as markers for Golgi membrane fractions. The purity of each preparation has been expressed in terms of a purification factor, representing the ratio of the specific activity of galactosyltransferase in the isolated Golgi

fraction to that in the liver homogenate. In addition to routine monitoring of each Golgi preparation with electron-microscopy, the percentage of total membranes which could be identified as Golgi membranes has been estimated using morphometric techniques.

In early studies, the purification factors were in the range between 20 and 40 whereas the percentage of Golgi membranes in these preparations varied from 60 to 70%. With more experience of the separation procedures, purification factors have increased steadily so that we have routinely obtained values of 60 to 100 and in some cases as high as 120. At the same time the recovery of galactosyltransferase activity has increased with increasing purification factor. This latter observation appears to be contrary to that usually observed when an enzyme is purified, i.e., the greater the purity, the less the amount of enzyme recovered. In this communication, we document these observations and suggest a possible explanation.

Materials and Methods

Male Wistar rats, 180 to 220 g were fasted for 15 hr and then anesthesized with ether and exsanguinated. The liver was resected quickly from each rat and after removing a slice of liver from the median lobe for electron-microscopy, the remainder was homogenized in 0.25 M sucrose in Tris buffer, pH 7.4, for the preparation of Golgi-rich fractions (Sturgess *et al.*, 1973). The liver homogenate was centrifuged at 10,000 rpm in the SW 25.1 rotor for 60 min. The supernatant resulting from this centrifugation was referred to as the first supernatant; the pellet as the first pellet. The membranes were removed from the top of the first pellet and this was used as the gradient charge. The charge was placed on a discontinuous sucrose gradient as described previously (Sturgess *et al.*, 1973).

Calculation of Purification Factor

Galactosyltransferase was assayed on the liver homogenate and Golgi fractions according to the method of Schachter *et al.* (1970). Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard. Enzyme activity was expressed as dpm transferred per 2-hr incubation at 37 °C per mg protein. Linearity of the assay was investigated separately in Golgi (Treloar, 1974) fractions and homogenates. The purification factor was calculated from the ratio of specific activity of galactosyltransferase in the fraction to the specific activity in the homogenate. The recovery of galactosyltransferase in the Golgi fraction or the homogenate was calculated as specific activity × total protein present in the fraction.

Quantitative Recovery of Galactosyltransferase Activity from the Sucrose Gradient

The appearance of the sucrose gradient showing separation of membrane bands is illustrated schematically in Fig. 1. From the gradient, three distinct membrane fractions

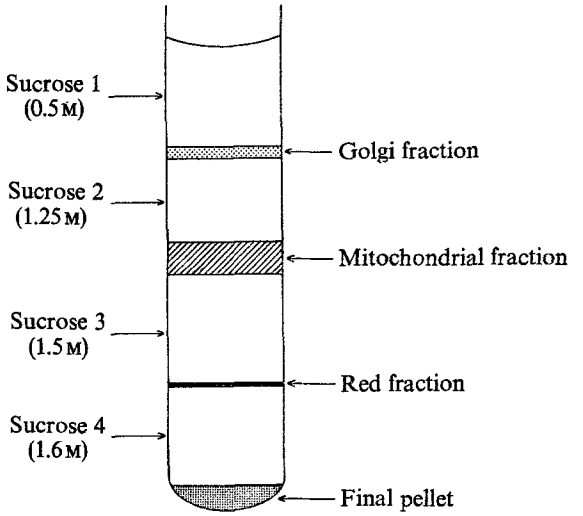


Fig. 1. Diagrammatic representation of the discontinuous sucrose gradient showing separation of membrane fractions

were separated, the Golgi-rich fraction at the interphase of 0.5 and 1.25 M sucrose, the mitochondrial fraction at the interphase of 1.25 and 1.5 M sucrose, and the red fraction at the interphase of 1.5 and 1.6 M sucrose. A pellet was obtained at the bottom of each tube. In addition to the membrane fractions isolated from the various interphases, the sucrose between the different interphases was also assayed. These included (1) 0.5 M sucrose, (2) 1.25 M sucrose, (3) 1.5 M sucrose, and (4) 1.6 M sucrose.

All fractions were assayed for galactosyltransferase activity to determine the recovery of this enzyme in various parts of the gradient. The high concentrations of sucrose from the gradient were found to interfere with the assay for the enzyme. Therefore, each fraction was diluted with distilled water and centrifuged at 30,000 rpm for 30 min in the SW 50.1 rotor. The pellet was suspended in distilled water and sonicated. Enzyme activity was determined on the sonicated pellet and on the supernatant from each centrifugation. Protein was measured using the ninhydrin method (Moore & Stein, 1954) after hydrolysis in 2 N NaOH for 2 hr at 95 °C. The method of Lowry *et al.* (1951) was not used on these fractions due to interference of sucrose.

Electron-Microscopy

Golgi-rich fractions were examined by negative staining and thin-sectioning techniques as described earlier (Sturgess *et al.*, 1973). Samples of sucrose solutions and membrane fractions from each gradient were diluted with 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and the membranes were pelleted by centrifugation into small flat-bottomed vials so that membranes formed a thin layer on the bottom of the tube. The samples were rinsed in 0.1 M sodium phosphate buffer, post-fixed in 1% osmium tetroxide in veronal acetate buffer, pH 7.4, and dehydrated in graded ethanol solutions. Samples of the membrane layer were embedded in Spurr low viscosity epoxy resin, oriented so that the section plane would pass perpendicularly through the membrane fraction.

Ultrathin sections were prepared on a Porter Blum MT-2 ultramicrotome using a diamond knife and contrast of the sections was enhanced with uranyl acetate and lead citrate. Samples were examined in a Philips EM 300 electron-microscope at 60 kV.

Morphometric Analysis of Golgi Membrane Fractions

Sections through the membrane pellet sampled a particulate layer representing the top to the bottom of the fraction. Electron-micrographs were recorded at random from each section at 14,000× magnification. For each fraction, sections were prepared from at least two samples of the particulate layer and 10 micrographs were recorded from each section. A point counting grid was overlaid on each micrograph and the membrane under each point was recorded. For quantitation purposes, the Golgi complex included all these smooth surface membranes arranged in stacks of parallel cisternae, the associated small vesicles and tubules, and the secretory vesicles (Sturgess & De la Iglesia, 1972). Smooth surface vesicles which were not closely associated with the cisternae were recorded as smooth endoplasmic reticulum and not as Golgi membranes. The number of points which included membranes characteristic of the Golgi complex were calculated and expressed as a percentage of the total membrane in the fraction.

Results

Relationship Between Percentage Golgi Membranes and Purification Factor

The relationship between the percentage of Golgi membranes in each fraction and the purification factor is shown in Fig. 2. A linear relationship was observed: when the percent Golgi membranes increased from 57 to 82% there was a corresponding increase in the purification factor from 20 to 100. The variation in purification factor reflected the relative yield of the Golgi membranes in the fractions.

Relationship Between Percent Recovery of Galactosyltransferase Activity and Purification Factor

The relationship between the recovery of galactosyltransferase in the Golgi fraction expressed as a percentage of activity in the homogenate and the purification factor is shown in Fig. 3. As the purification factor increased from 10 to 120 the percent recovery of galactosyltransferase increased from 5 to 80%. With the highest purification factors, 80% of the galactosyltransferase activity of the liver homogenate was recovered in the Golgi fraction.

Recovery of Galactosyltransferase from the Sucrose Gradient

The galactosyltransferase activity in the pelleted material and the supernatants from the sucrose gradients is summarized in Table 1. The total

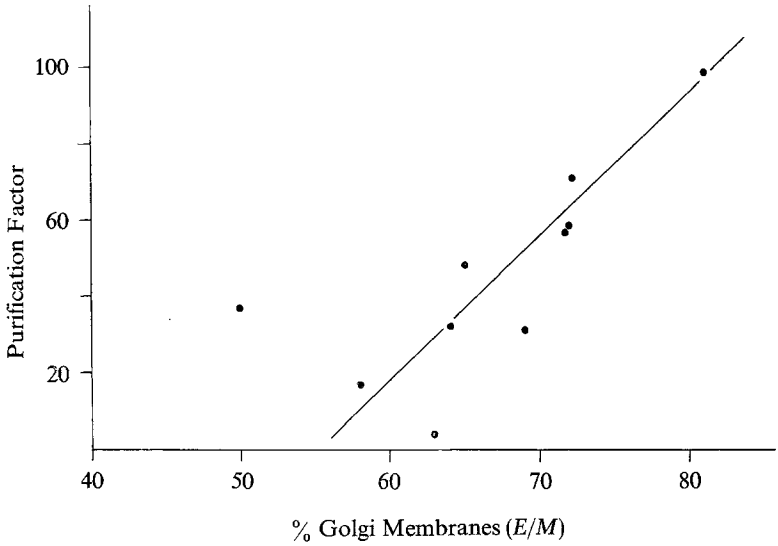


Fig. 2. Relationship between the purification factor (galactosyltransferase activity in the Golgi fraction/activity in the liver homogenate) and percentage of Golgi membranes in the isolated Golgi-rich fractions

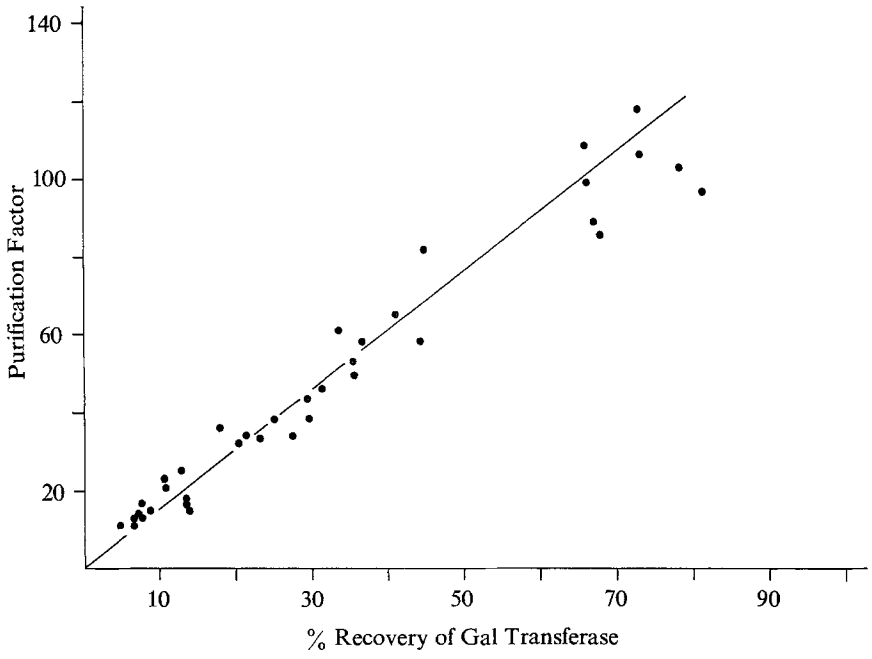


Fig. 3. Relationship between the purification factor (galactosyltransferase activity in the Golgi fraction/activity in the liver homogenate) and recovery of galactosyltransferase expressed as a percentage of activity in the homogenate

Table 1. Galactosyltransferase activity in various fractions from the sucrose gradient

Fraction	Specific activity of pellet ($\times 10^{-5}$)	Total activity in pellet [a] ($\times 10^{-5}$)	Total activity in supernatants [a] ($\times 10^{-5}$)	Sum of activity in supernatants + pellet ($\times 10^{-5}$)
Homogenate	0.035	39.90	0.24	40.14
1st Supernatant	0.006	2.95	0.05	3.00
1st Pellet	0.044	24.80	0.09	24.89
Gradient charge	0.248	16.20	0.03	16.23
Sucrose 1	0.047	0.19	0.25	0.44
Golgi fraction	3.825	16.45	0.27	16.72
Sucrose 2	0.24	0.23	—	0.23
Mitochondrial fraction	0.094	3.85	0.22	4.07
Sucrose 3	0.115	0.02	0.03	0.05
Red fraction	0.045	0.01	0.49	0.50
Sucrose 4	0.341	0.04	0.33	0.37
Final pellet	0.040	0.07	3.08	3.18

[a] Each fraction isolated from the gradient was centrifuged at 40,000 rpm for 30 min to remove the membrane fragments from sucrose. Galactosyltransferase was measured on each of the resulting pellets and supernatants.

activity recovered in the Golgi fraction from the gradient was 16.4×10^5 dpm which represented 100% of the gradient charge. However, other fractions, notably the mitochondrial fraction and the supernatant from the final pellet contained 4.1×10^5 and 3.2×10^5 dpm of total activity, respectively. The total recovery of galactosyltransferase in the Golgi and mitochondrial fractions and in the final pellet was 24×10^5 dpm which represented 150% of the gradient charge.

Electron-Microscopy of Gradient Fractions

The morphological characteristics of each fraction of the gradient were examined by electron-microscopy.

Sucrose 1. The characteristic membranes of the Sucrose 1 fraction are shown in Fig. 4. The sucrose at the top of the gradient contained mainly fine, granular material with small, smooth-surfaced vesicles 40 to 100 nm in diameter and some larger vesicles 300 to 500 nm in diameter. These vesicles included some secretory vesicles containing very low density lipoprotein (VLDL) type granules, and some vesicles devoid of contents and characteristics of autophagic vesicles.

Golgi Fraction. The characteristic membranes of the Golgi fraction are shown in Fig. 5. The Golgi fraction contained predominantly smooth-surfaced membranes, many arranged in stacks of disc-shaped cisternae associated with tubular and vesicular membranes characteristic of the Golgi complex. Other membranes included small vesicles of plasma membrane, smooth endoplasmic reticulum and some rough endoplasmic reticulum (Fig. 5).

Sucrose 2. The characteristic membranes in the sucrose layer between the Golgi and mitochondrial fraction are shown in Fig. 6. This fraction contained membrane vesicles with a gradation in membrane type from the top to the bottom of this sucrose layer. Towards the upper part, there were mainly smooth-surfaced membranes. Many of these appeared as flattened cisternae characteristic of the Golgi complex but the cisternae were devoid of osmiophilic material. Secretory vesicles were present also. Towards the lower part of the sucrose layer there were very small vesicles, mainly rough-surfaced, characteristic of rough endoplasmic reticulum.

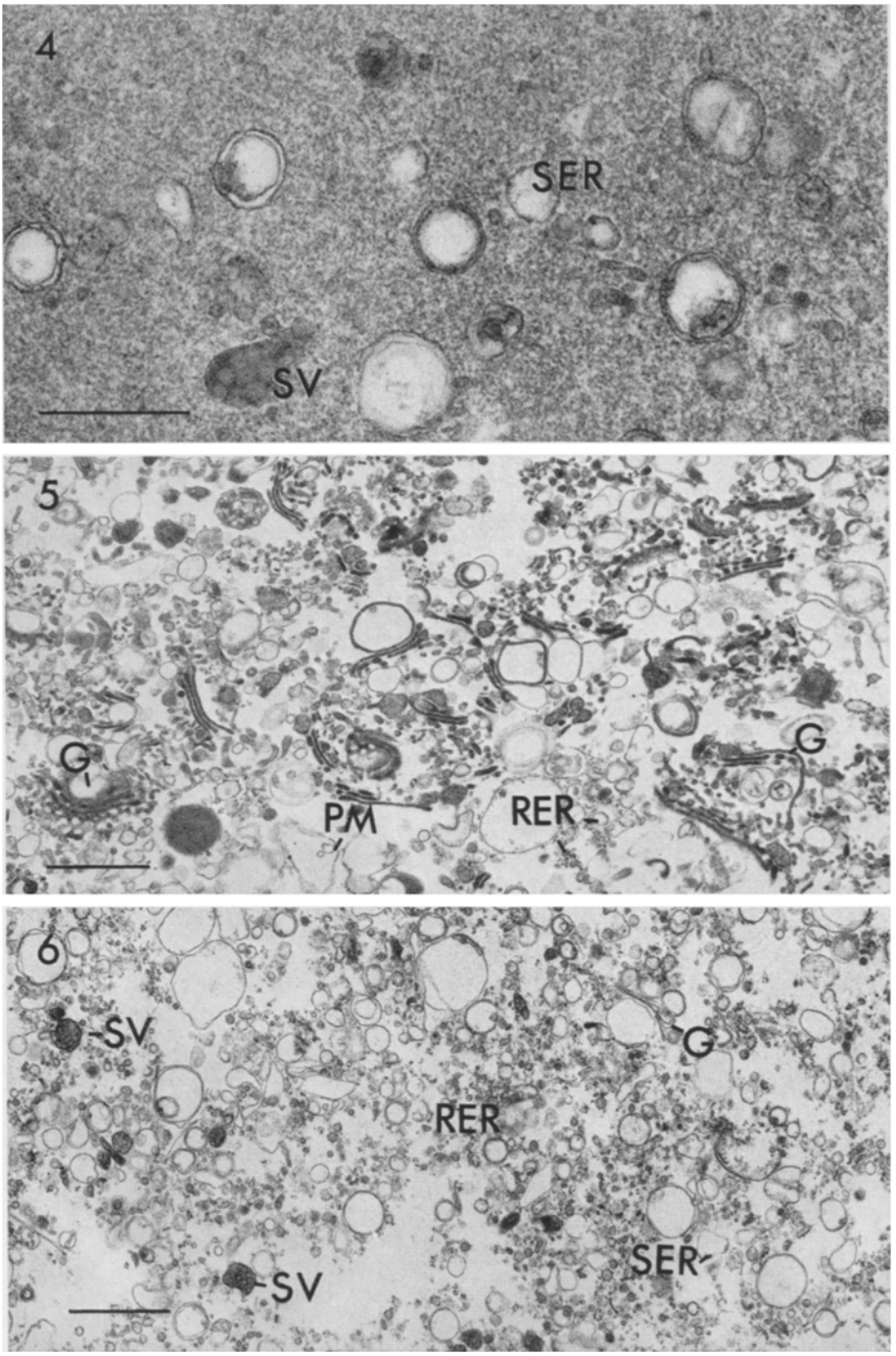
Mitochondrial Fraction. The characteristic membranes in the mitochondrial fraction are shown in Fig. 7. This fraction contained large amounts of membrane, predominantly composed of rough endoplasmic reticulum, and mitochondria.

Sucrose 3. The characteristic membranes of the Sucrose 3 fraction are shown in Fig. 8. The main components of this fraction were large mitochondria and aggregates of rough endoplasmic reticulum.

Red Fraction. The characteristic membranes of the red fraction are shown in Fig. 9. This fraction was diffuse and contained aggregates of intracellular membranes predominantly. The red color resulted from the presence of erythrocytes in the fraction.

Sucrose 4. The characteristic membranes in the Sucrose 4 fraction are shown in Fig. 10. This band was similar in composition to the red fraction and the final pellet. It contained nuclei, large aggregates of cytoplasmic membranes arranged in a similar form to that observed in the intact cell. Some erythrocytes and intact hepatocytes were present also.

Pellet. The characteristic material pelleted at the bottom of the sucrose gradient is shown in Fig. 11. The pellet contained intact cells, partially disrupted cells which showed large aggregates of cytoplasmic membranes which were intact and surrounding a central nucleus. Occasional free nuclei were present also.



Figs. 4—11. Electron-micrographs of the characteristic membranes which separate at the sucrose interphases and which are present in sucrose solutions of the gradient (*see* Fig. 1). *G*—Golgi membranes, *SV*—secretory vesicles, *RER*—rough endoplasmic reticulum, *SER*—smooth endoplasmic reticulum, *M*—Mitochondria, *PM*—plasma membrane, *CM*—cytoplasmic membranes, *N*—nucleus, *E*—erythrocyte. Line = 1 μm.

Discussion

A large number of galactosyltransferases are known to exist in various animal tissues but only one is reported from liver. Although these enzymes all catalyze the transfer of galactose from UDP-galactose to various acceptors (McGuire, Jourdian, Carlson & Roseman, 1965; Roseman, 1968; Schachter *et al.*, 1970), at least eight different enzymes have been reported (Schachter & Roden, 1973). The galactosyltransferase assayed in this study is responsible for the synthesis of the galactosyl-(B,1-4)-N-acetylglucosamine linkage.

Glycosyltransferases are localized mainly in the membranes of the Golgi apparatus but may be present in other intracellular membranes also. For example, glucosamine is added to the polypeptide chain in part at sites on the rough endoplasmic reticulum (Lawford & Schachter, 1966). Presumably a glycosyltransferase responsible for the synthesis of the asparagine-N-acetylglucosamine linkage must be present in this subcellular site.

Recent reports have indicated that galactosyltransferases may be present in plasma membranes of rat liver (Aronson, Tan & Peters, 1973). Although this observation needs more thorough documentation, it is possible that galactosyltransferase activity may be present in other intracellular membranes. The activity of these enzymes in sites other than the Golgi apparatus may be controlled by the presence of a specific galactosyltransferase inhibitor. An example of such a control mechanism occurs in the mammary gland. The A protein in mammary gland has galactosyltransferase activity transferring galactose to N-acetylglucosamine. Another mammary gland protein, α-lactalbumin has been shown to inhibit the galactosyltransferase activity of the A protein (Brew, Vanaman & Hill, 1968). At the same time transfer of galactose to glucose is stimulated, resulting in the synthesis of lactose. The synthesis of α-lactalbumin is induced in late pregnancy and occurs only in the mammary gland. Similar control mechanisms may operate in other tissues and different intracellular membranes may exhibit different ways of controlling glycosyltransferase activity.

In this study, a parallel increase is observed between the purification and yield of galactosyltransferase in Golgi membranes isolated from the liver. This is difficult to interpret on the basis of most enzyme purification data.

Fig. 4. Sucrose 1. × 21,000

Fig. 5. Golgi fraction. × 14,000

Fig. 6. Sucrose 2. × 14,000

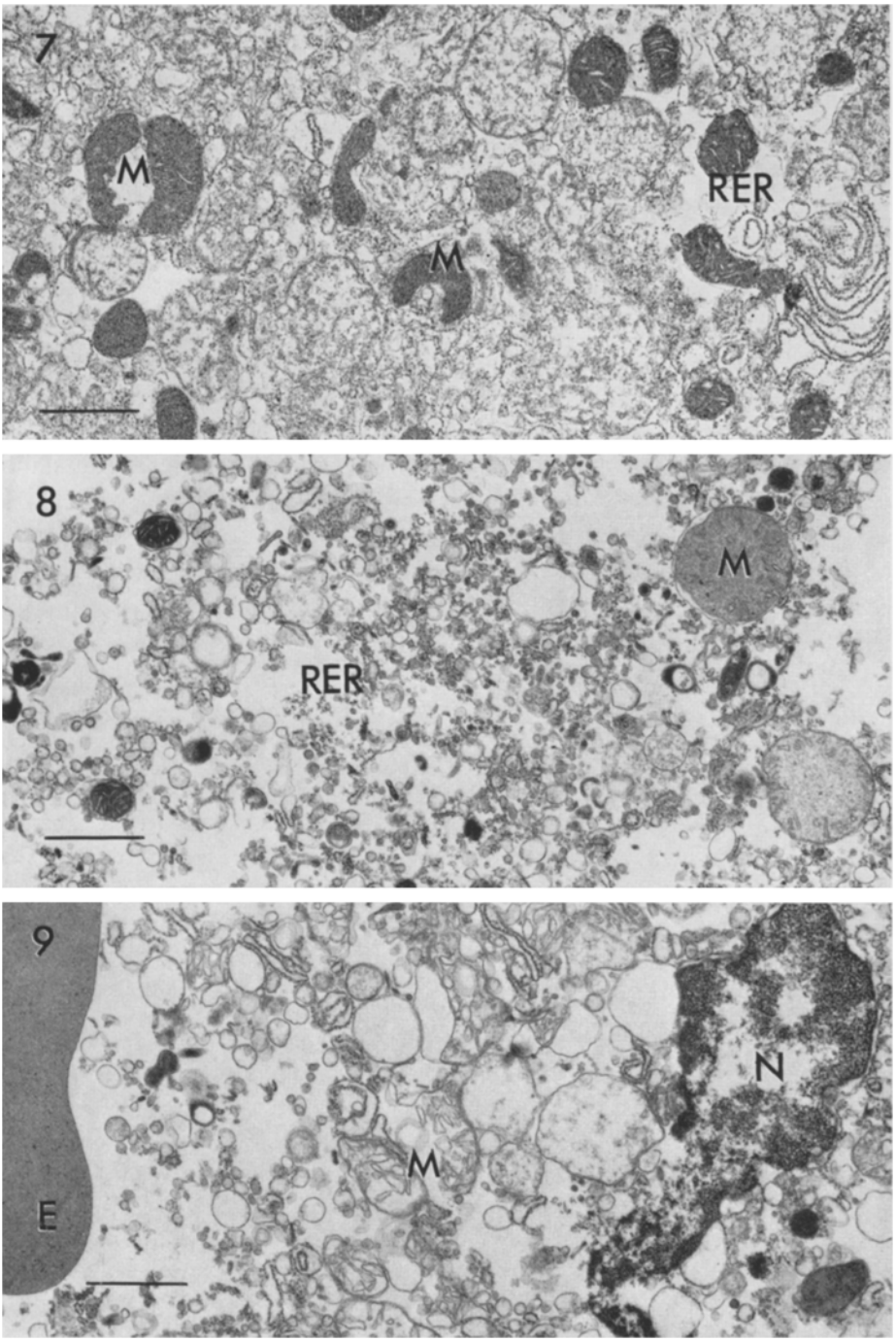


Fig. 7. Mitochondrial fraction. × 14,000
Fig. 8. Sucrose 3. × 14,000
Fig. 9. Red fraction. × 14,000

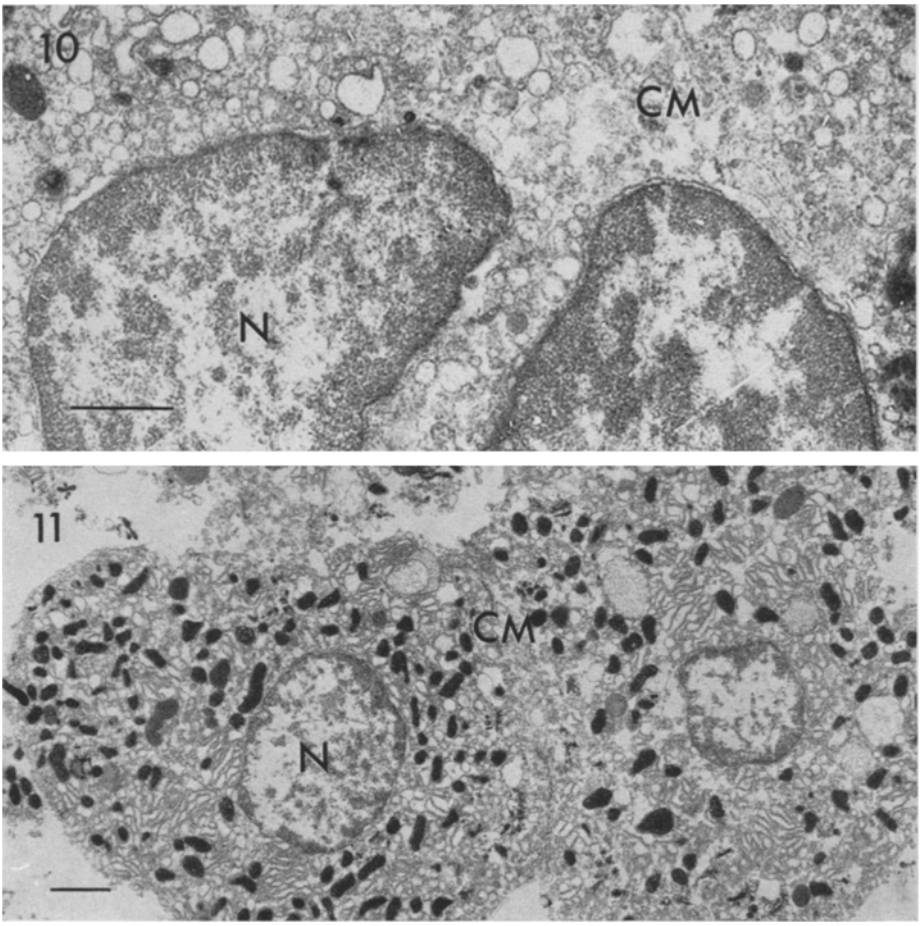


Fig. 10. Sucrose 4. × 14,000

Fig. 11. Pellet. × 8000

However, the increase in galactosyltransferase activity with increasing purification factor indicates the existence of an inhibitor which may be important in metabolic control. If we postulate that galactosyltransferase is not exclusive to the Golgi complex, although this is the major site of its action, then there must be a mechanism to inhibit the enzyme activity in the other membrane fractions and in serum. Galactosyltransferase activity has been found in various sera (Mookerjea, Michaels, Hudgin, Moscarello, Chow & Schachter, 1972), but the activity is much lower by about 100-fold compared to the Golgi. It may be that the enzyme which leaves the Golgi membranes is complexed with a specific inhibitor protein.

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