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Composition, stability and electrolyte permeability of Golgi membranes from lactating-rat mammary gland

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1. Golgi membrane vesicles, isolated from lactating-rat mammary gland and greatly enriched in galactosyltransferase (EC 2.4.1.22), contained over 40 separate bands of protein, including some periodic acid/Schiff-staining material and free thiol groups, when analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 2. The membrane lipids were enriched in phosphatidylcholine, phosphatidylethanolamine and unesterified cholesterol. 3. Membrane fluidity, as monitored by the fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene, increased linearly over 5–37°C. 4. The vesicle membranes were impermeable to lactose over a wide pH range, but admitted electrolytes of molecular weight below about 300. 5. These properties are discussed with respect to other cellular membranes and the secretion of milk products.

Galactosyltransferase (EC 2.4.1.22) synthesizes lactose in the presence of α-lactalbumin within dictyosomes of the Golgi apparatus of lactating mammary gland. Although detailed characteristics of the reaction have been elucidated with pure preparations of the enzyme from milk and colostrum, its operation in the framework of cell metabolism and structure has been studied with simple particulate preparations, exploiting the uniqueness of the lactose synthase reaction, or with purified membrane preparations greatly enriched in galactosyltransferase and nucleoside diphosphatase (EC 3.6.1.6) activities (Kuhn & White, 1975, 1977). These enzymes, which work in tandem, appear to reside on the luminal face of the dictyosome membrane. Therefore the properties of this membrane determine the accessibility of substrates and cofactors to the enzyme system, the removal of nucleotide products, and the retention of lactose for secretion (Kuhn *et al.*, 1980). Indirect evidence points to the specific transport of UDP-galactose and UMP (Kuhn & White, 1976), whereas studies with sugars and related uncharged polyols have suggested the presence of a pore, or carrier, that transmits small polyols of various structures but discriminates against disaccharides (White *et al.*, 1980, 1981).

Because the galactosyltransferase-enriched preparations referred to above contain vesicles arising by the pinching-off of dictyosome membranes rather than by the isolation of originally discrete organelles, electron microscopy is of limited value in assessing their purity. In order to characterize these preparations more fully we have therefore analysed their proteins by SDS/polyacrylamide-gel electrophoresis, their lipids by t.l.c., their lipid fluidity by fluorescence polarization and their thiol content by reaction with *N*-ethylmaleimide. Further studies on simple particulate preparations, centred around the retention of endogenously generated [^{14}C]lactose, have examined the stability towards pH and thiol reagents, and the penetration by charged solutes, of the membrane associated with lactose synthesis. A preliminary report of some of this work has appeared (White, 1981).

Materials and methods

Golgi membranes were purified from 13–18-day-lactating rats as previously described (Kuhn & White, 1977). They were used within 2h for experiments involving lactose retention, but were otherwise sometimes used after storage for a few days at −18°C. Particulate preparations were made as described by Kuhn & White (1975).

Abbreviations used: SDS, sodium dodecyl sulphate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid.

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Analysis of membranes by SDS/polyacrylamide-gel electrophoresis

The membranes (about 5mg of protein) were gently suspended in 0.5M-NaCl containing 1mM-EDTA, pH8.0 (7.5ml), with a syringe, and kept at 37°C for 30min. The membranes were then collected by centrifugation at 150000 $g_{av.}$ for 30min at 5°C and resuspended in 1mM-EDTA, pH8.0, at 37°C for a similar period, being again collected by centrifugation.

Samples were prepared for electrophoresis by heating at 100°C for 5min with 5% (w/v) SDS and 0.1% (w/v) mercaptoethanol. Samples (each 20–50μl, containing about 100μg of protein) were applied to wells at the top of a 5–15% (w/v) polyacrylamide linear gradient slab gel containing 0.1% (w/v) SDS. The gel was subjected to electrophoresis for about 8h at a constant current (10mA) with the discontinuous buffer system of Laemmli (1970) but without spacer gels. Proteins were stained with 0.1% (w/v) Coomassie Blue. Molecular weights of the polypeptide bands were estimated by comparison with human erythrocyte membrane proteins included in each slab gel.

Determination of thiol content

Golgi membranes (about 13μg of protein) were incubated in duplicate at 37°C with *N*-ethyl[2,3-^{14}C]maleimide (10mM, 2.1Ci/mol), Tes/NaOH buffer, pH7.5 (50mM) and with or without Triton X-100 (0.1%, w/v) in a final volume of 25μl. The reaction was stopped by the addition of 0.35M-cysteine (10μl), after which a sample (20μl) was spotted on to a numbered square (1.5cm^2) of Whatman no. 1 filter paper. This was immersed for 15min in a large volume of 10% (w/v) trichloroacetic acid, and then for 5min in each of two fresh such solutions. After being air-dried the paper was counted for radioactivity in the presence of 10ml of toluene-based scintillation fluid (Kuhn & White, 1977).

Lipid analysis

Membrane lipids extracted by the method of Folch *et al.* (1957) were chromatographed on thin layers of silica gel G in chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) to separate phospholipids (Skipski *et al.*, 1964), or in hexane/diethyl ether/acetic acid (80:20:1, by vol.) to separate neutral lipids (Wood *et al.*, 1964). Lipid spots were located with iodine vapour, and phospholipids were eluted from the gel twice with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.), once with methanol and once with methanol/acetic acid/water (94:1:5, by vol.). The pooled extracts were evaporated to dryness and assayed for total phosphorus (King, 1932). Recovery of applied lipid phosphorus averaged 96%. Authentic markers run alongside were used to identify each lipid, except for phosphatidylserine and phosphatidylinositol, which were assumed to run in the area preceding phosphatidylethanolamine. Cholesterol was determined directly on lipid extracts (Zlatkis *et al.*, 1953), visual examination of thin layers having revealed it to be present only in unesterified form.

Fluorescence polarization as a measure of membrane fluidity

The fluidity of the membrane lipids was monitored with the probe 1,6-diphenylhexa-1,3,5-triene essentially as described by Lowe & Coleman (1981). A freshly prepared solution of the probe (2μM) was incubated with an equal volume of Golgi membranes (0.2μmol of lipid phosphorus/ml) for 30min at 37°C. The fluorescence polarization was detected at 430nm with an excitation wavelength of 360nm, with the sample at temperatures from 5°C to 37°C.

Filtration of [^{14}C]lactose-containing vesicles

Samples of mammary particles (about 60μg of protein) suspended in 0.25osM- or 0.30osM-solute were diluted into 2ml of 0.25M- or 0.30M-lactose above a 25mm cellulose nitrate filter disc (Sartorius, 0.2μm pore size) on a simple Millipore filtration apparatus. The filter was sucked dry with a Speedivac high-vacuum oil pump (Edwards High Vacuum, Crawley, Sussex, U.K.) and washed with a further 5ml of 0.25M- or 0.30M-lactose at 5°C. Filtration took about 30s. The filter was then dissolved in 10ml of Triton/xylene scintillation fluid (Kuhn & White, 1975) and counted for radioactivity.

Results

Analysis of Golgi membrane proteins by SDS/polyacrylamide-gel electrophoresis

Golgi membranes, with specific galactosyltransferase activities of 200–600nmol/min per mg of protein (Kuhn & White, 1977), were analysed in polyacrylamide gels and showed more than 40 closely spaced bands ranging from 10000 to 200000 molecular weight (Fig. 1, track 1). No one band appeared to account for more than 5% of the total. Further enrichment of the transferase activity was achieved by sequential extraction of the membranes with buffers of high and low ionic strength designed to remove peripheral proteins and those trapped within vesicle lumina. The pattern of membrane proteins was not obviously altered by extraction with 0.5M-NaCl (track 2) followed by 1mM-EDTA (track 3), even though the somewhat different pattern of the eluted proteins obtained after concentration (tracks 4 and 5 respectively) indicated at least some selectivity of extraction. Analysis

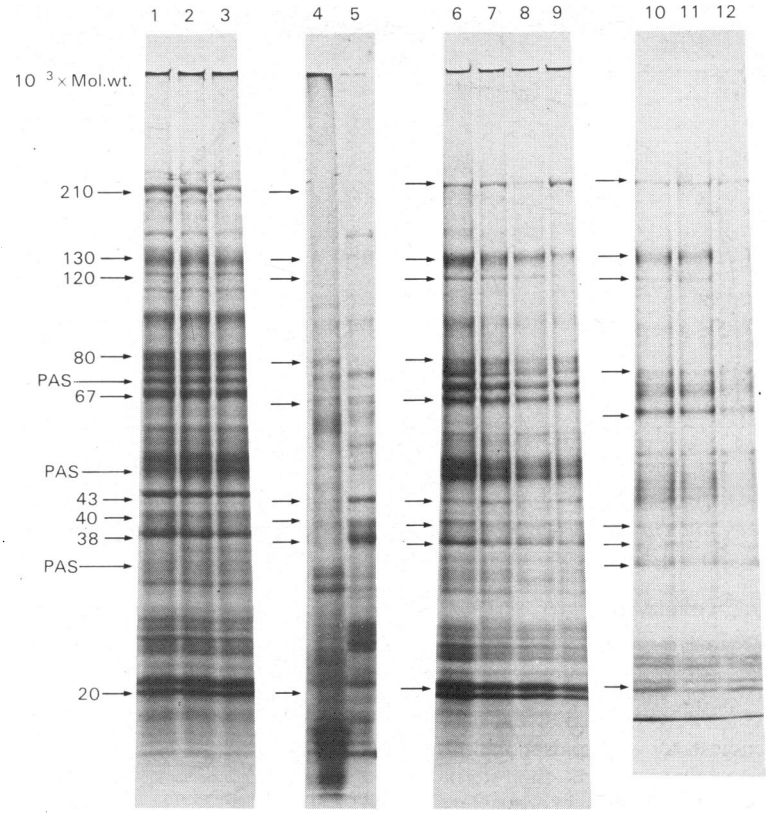

Fig. 1. *Separation of Golgi-membrane proteins by SDS/polyacrylamide-gel electrophoresis*
Proteins migrating from the cathode (top) to the anode (bottom) were stained with Coomassie Blue. Numbered tracks are described in the text, with arrows across the Figure indicating corresponding bands in different preparations. PAS indicates periodic acid/Schiff-staining regions.

of multiple preparations of extracted Golgi membranes revealed a reproducible pattern of bands (for examples, see tracks 6–9) showing, within the limitations of the technique, the reproducible nature of the membranes isolated from lactating mammary tissue by the procedure used. The main features of this pattern included two high-molecular-weight proteins of about 120000 and 210000, a diffuse band near 130000, and a group of four bands with approximate molecular weights in the range 67000–80000. Three proteins, of molecular weights about 38000, 40000 and 43000, were also predominant in most preparations. In the lower-molecular-weight range a pair of bands of molecular weight about 20000 were usually noted. Three periodic acid/Schiff-positive bands with apparent molecular weights about 30000, 45000 and 70000 were detected.

Up to 70% of Golgi membrane protein could be solubilized by 1% of either sodium cholate, sodium deoxycholate or Triton X-100 (tracks 10, 11 and 12), but only Triton gave evidence of any selective elution.

Thiol content

Binding of *N*-ethyl[^{14}C]maleimide presented to membrane preparations at different concentrations increased rapidly up to 2mM and more slowly up to 10mM (results not shown). Fig. 2(*a*) shows that binding was rapid over the first 10–20min at 37°C, thereafter proceeding more slowly up to at least 2h. Binding was greater at all times in the presence of Triton X-100. In three different preparations the amounts of *N*-ethyl[^{14}C]maleimide bound in 2 or 3h were 45, 55 and 37nmol/mg of protein respectively in the absence of Triton. This is equivalent to an average of 2–3 exposed thiol groups per protein molecule of molecular weight 50000, with about one further thiol group becoming exposed in the presence of Triton.

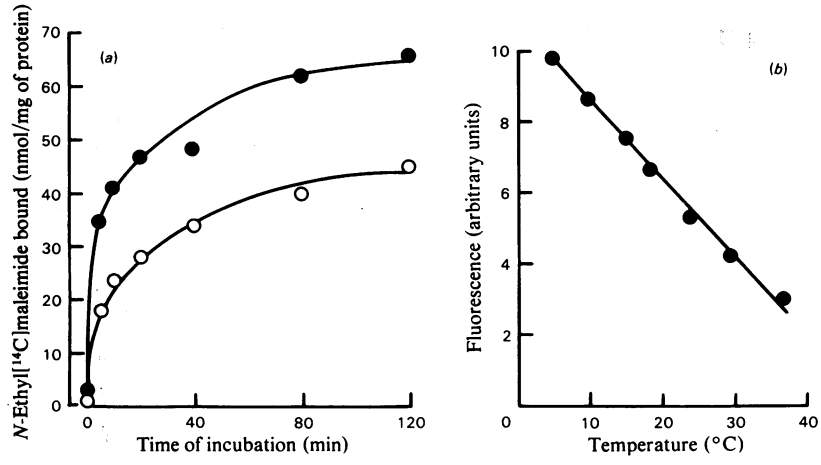

Fig. 2. (*a*) *Reactions of N-ethylmaleimide with Golgi membrane with* (●) *and without* (○) *Triton X-100, and* (*b*) *fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene in Golgi membrane at different temperatures*
Details are given in the Materials and methods section.

Lipid analysis

From the appearance of thin-layer chromatograms (not shown), chloroform/methanol extracts appeared to contain cholesterol and phospholipids, with only traces of triacylglycerols and cholesterol ester. The distribution of phospholipids, expressed as a percentage of total lipid phosphorus (±S.E.M.) in six preparations, was phosphatidylcholine (59.3 ± 1.5), phosphatidylethanolamine (26.5 ± 0.9), sphingomyelin (7.1 ± 0.7), phosphatidylserine plus phosphatidylinositol (4.4 ± 1.0), as well as unidentified material at the origin (3.5 ± 0.8) and solvent front (1.2 ± 0.4). The cholesterol content was 0.46 ± 0.03 mol/mol of phospholipid. From these data the calculated total lipid content of the membranes was 1.2 mg/mg of protein.

Membrane fluidity

In view of the high cholesterol content of the Golgi membrane, and because permeability studies have mainly had to be carried out at 0–5°C, it was decided to see whether the physical state of the membrane changed continuously or not with temperature. Fig. 2(*b*) shows that the fluorescence polarization of a membrane preparation exposed to 1,6-diphenylhexa-1,3,5-triene varied linearly over the temperature range 5–37°C. It was concluded that the fluidity of the membrane lipids probably also change continuously over this range.

[14C]Lactose retention as a measure of membrane integrity

Previous studies with both simple and purified mammary preparations have shown that the retention of newly synthesized [14C]lactose within vesicles collected by centrifugation can serve to monitor the intactness of the Golgi membrane (White *et al.*, 1980; Kuhn *et al.*, 1980). Fig. 3 now shows that

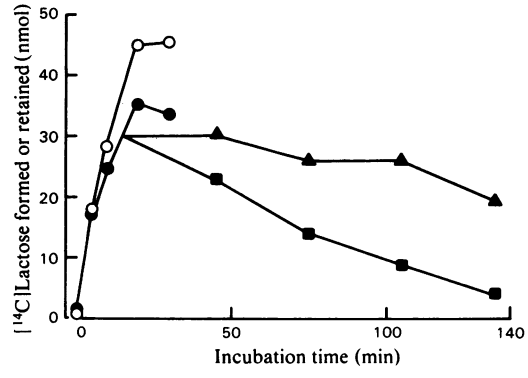

Fig. 3. *Formation of total* (○) *and filter-retained* (●) *[14C]lactose and the retention of lactose at 0°C* (▲) *and 37°C* (■)
Particulate preparation (about 3 mg of protein) was incubated with glycylglycine/KOH buffer (43 mM), $MnCl_2$ (5 mM), UDP-galactose (0.25 mM), [U-14C]-glucose (0.25 mM, 2 Ci/mol) and lactose (0.25 M) in a final volume of 0.4 ml at 37°C. Samples were taken at times up to 30 min for determination of total [14C]lactose (Kuhn & White, 1975) and of retained lactose. Further samples taken at 15 min were diluted with an equal volume of 0.25 M-lactose/10 mM-EDTA and kept at 0°C or 37°C for up to 135 min. Portions were withdrawn for filtration during this time. Results are expressed as nmol of [14C]lactose per original reaction mixture.

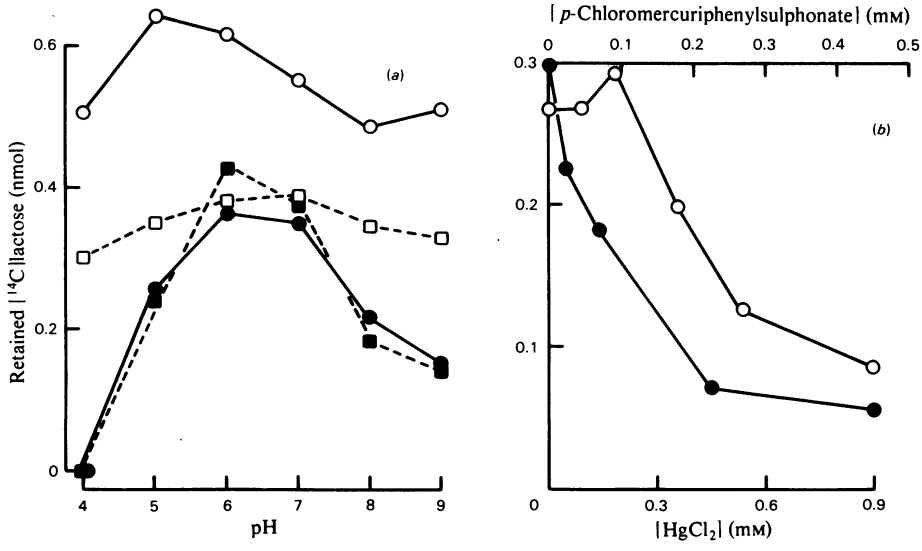

Fig. 4. *Effect of (a) pH and (b) mercurial reagents on the retention of [^{14}C]lactose by mammary particles*
Particulate preparations (0.4ml) were charged with [^{14}C]lactose as described in the legend to Fig. 3. After 20min they were chilled in ice and 100mM-EDTA/0.25M-lactose (0.04ml) was added. Portions (20μl) were diluted with 0.25M-lactose (180μl) containing 55mM-dimethylglutaric acid/NaOH buffer (pH4, 5, 6 or 7), 55mM-Tes/NaOH buffer (pH7 or 8), 55mM-Tris/HCl buffer (pH8 or 9), $HgCl_2$ (0.05–1.0mM) or *p*-chloromercuriphenylsulphonate (0.05–0.5mM), and maintained under the following conditions: (*a*) ○, □, 0°C for 30min; ●, ■, 37°C for 20min; (*b*) 0°C for 60min with $HgCl_2$ (●) or *p*-chloromercuriphenylsulphonate (○), before filtration of a suitable sample. Results are expressed as nmol of [^{14}C]lactose per 10μl of original reaction mixture. (*a*) shows the results of two separate experiments (circles and squares respectively). Buffers were adjusted to the desired pH values at the temperature at which they were to be used. The values obtained in (*a*) with dimethylglutarate and Tris buffers were adjusted by factors not exceeding 1.47, calculated by comparison with values from Tes buffer at overlapping pH values.

such lactose is also effectively retained by vesicles collected by rapid filtration, and that this technique is useful in following the leakage of lactose from vesicles kept at 0°C or 37°C over 2h.

Effect of pH and thiol reagents on Golgi-membrane stability

Fig. 4(*a*) shows the results of two experiments in which the retention of [^{14}C]lactose by filterable particles was measured after storage at different pH values. Lactose was effectively retained over the pH range 4–9 at 0°C, indicative of considerable membrane stability. At 37°C, however, the membrane appeared unstable beyond the range pH6–7, and lactose was completely lost at pH4. No experiments were carried out to test the possible contribution of endogenous enzymes to such instability.

Fig. 4(*b*) shows that at 0°C increasing concentrations of the thiol reagents *p*-chloromercuriphenylsulphonate and $HgCl_2$ caused increasing loss of the [^{14}C]lactose. By contrast, no loss occurred when particles were stored in the presence of *N*-ethylmaleimide (20mM) or 5,5′-dithiobis-(2-nitrobenzoic acid) (1mM) (results not shown).

Table 1. *Retention of [^{14}C]lactose by particles exposed to lactose, glucose or different salts*
The procedure followed that given in the legend to Fig. 5, with exposure to 0.30OSM solutions for 5min at 0°C in all cases. Retention of [^{14}C]lactose is expressed as a percentage of that in particles exposed to 0.3M-lactose. Values are means (±S.E.M.) of six separate determinations.

Lactose	100
Sodium metrizoate	97.1±3.6
Sodium lactobionate	90.3±3.1
Disodium AMP	76.9±4.0
D-Glucosamine hydrochloride	68.8±4.1
Potassium D-gluconate	67.3±3.0
Trisodium citrate	64.5±3.1
Choline chloride	63.2±5.2
Disodium L-malate	62.3±4.4
Disodium glycerol 3-phosphate	60.1±4.6
Disodium D-glucose 6-phosphate	58.4±4.0
KCl	51.0±2.0
NaCl	50.8±4.0
D-Glucose	25.7±3.0

Penetration of the Golgi membrane by salts

When vesicles loaded with [^{14}C]lactose are transferred to an iso-osmotic solution of a pene-

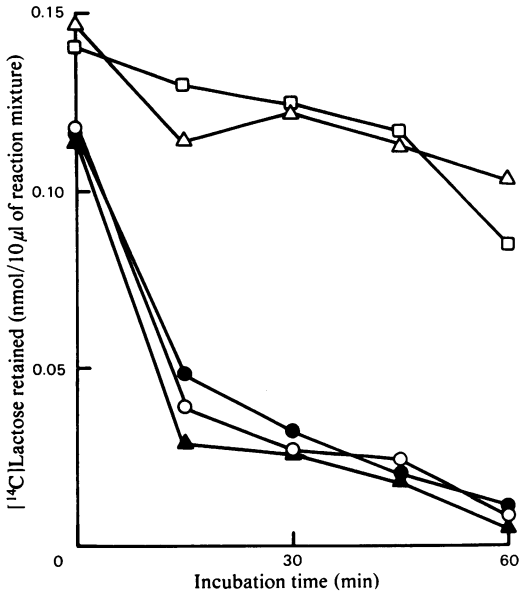

Fig. 5. *Osmotic lysis by LiCl, NaCl and KCl*
Retention of [^{14}C]lactose was measured in samples of particle preparation (20 μl) incubated for various times at 0°C, with 180 μl of 0.30sM solutions of lactose (△), sucrose (□), LiCl (▲), NaCl (●) or KCl (○). Results are expressed as nmol of [^{14}C]lactose retained per 10 μl of original reaction mixture.

trating non-electrolyte, they undergo osmotic lysis with consequent loss of [^{14}C]lactose (White *et al.*, 1980). Fig. 5 shows the time course of osmotic lysis by LiCl, NaCl and KCl compared with that by the relatively impermeant solutes lactose and sucrose. Table 1 records the retention of [^{14}C]lactose by vesicles exposed to a wide range of salts for 5 min at 0°C. Except for sodium metrizoate (anion molecular weight 627) and sodium lactobionate (anion molecular weight 355), all the salts caused substantial lysis, although none was as effective as glucose. In those cases tested (KCl, choline chloride, sodium citrate) the inclusion of 300mM-lactose gave complete osmotic protection, implying that the salts were not in themselves injurious to the membrane (results not shown).

Discussion

Analysis of the purified Golgi membranes prepared in this laboratory from lactating-rat mammary gland gives a picture of a cholesterol-rich, cardiolipin-poor, membrane that reproducibly contains at least 40 intrinsic polypeptides, many of which are likely to carry exposed thiol groups. This large number of polypeptides is not inconsistent with the increasing number of enzymic and transport functions connected with glycoprotein synthesis that are being ascribed to the Golgi apparatus. Whether the membranes are derived from the entire Golgi dictyosome or only from part of it cannot be said, although the same galactosyltransferase of HeLa cells has been localized to the distal cisterna (Roth & Berger, 1981). Keenan *et al.* (1972) have reported a lower cholesterol content and a much higher triacylglycerol content for rat mammary Golgi membranes, although their phospholipid patterns were similar. The greater cholesterol content of rat liver Golgi dictyosomes (Stremmel & Debruch, 1979) may owe something to the role of these dictyosomes in hepatic lipoprotein secretion. In any case the high cholesterol content of these membranes probably accounts for the smooth fluidity change with temperature, indicated by the smooth change in fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene.

Simple particulate preparations, in which the Golgi membrane-derived vesicles have been biosynthetically loaded with [^{14}C]lactose, form a handy but nevertheless specific and stringent means of studying the permeability properties of the membrane associated with lactose synthesis (White *et al.*, 1980, 1981). The usefulness of this method is enhanced by employing rapid filtration instead of centrifugation to collect the vesicles free from untrapped radioactivity. The impermeability of the Golgi membrane to lactose is believed to be an important facet of its role in the mammary gland, and presumably depends on its general structural integrity. This integrity appears to be maintained over a fairly wide range of pH, especially at 0°C, and is not impaired by reaction of free membrane thiol groups, except by mercurials.

With the technique of osmotic lysis it is now shown that the membrane is nevertheless permeable, not only to small non-electrolytes (White *et al.*, 1980), but also to small electrolytes. A wide range of electrolytes of molecular weight below about 300 readily penetrate the membrane, albeit not as rapidly as non-electrolytes of comparable molecular weight. This difference might reflect a larger hydration shell. The penetrability of AMP is therefore rather unexpected, since its molecular weight is similar to that of lactose.

It is not likely that many different electrolytes and non-electrolytes penetrate the Golgi membrane by different mechanisms, or even by a common mechanism that would involve specific binding to a carrier. Therefore we consider that a simple pore or channel, more improbably a mobile cavity, of appropriate dimensions may account for the admission of small solutes and the exclusion of disaccharides. This implies that the composition of the Golgi luminal fluid *in vivo* is similar to that of the

cytosol with respect to small solutes, and that no electrochemical or pH gradient exists across the membrane. Insofar as the aqueous phase of milk may arise from the contents of the Golgi lumen (Linzell & Peaker, 1971), this might account for the passage of KCl, NaCl, citrate and many other metabolites from the cytosol into the milk. Linzell *et al.* (1976) have reached this conclusion for the citrate of goat milk from studies of its rate of secretion. Thus the citrate/isocitrate ratio in goat milk, which exceeds the ratio in whole mammary tissue (Faulkner, 1980), might reflect its ratio in the cytosol. It is also possible, however, that the apical membrane of the secretory cell shows a similar permeability, as indeed it does to monosaccharides (Faulkner *et al.*, 1981).

In admitting the passage of both charged and uncharged molecules, the Golgi membrane is strikingly different from the hepatic lysosomal membrane, which also admits a wide range of non-electrolytes, but yet, consistent with its maintenance of a pH gradient, excludes ions even as small as lactate or glycerol phosphate (Lloyd, 1969; Applemans & de Duve, 1955). Rather it may resemble the outer membranes of mitochondria, and of bacteria, to which 'pore' proteins appear to lend the characteristic of a molecular sieve.

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