Mannitol and glucose movement across the Golgi membrane of lactating-rat mammary gland

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1. Purified Golgi-membrane vesicles of lactating-rat mammary gland were penetrated by glucose, 3-*O*-methylglucose, mannose, fructose, sorbitol and mannitol, but not by lactose or sucrose. 2. The kinetics of mannitol uptake and release were followed at 2–6°C with the aid of fine filters (0.45 µm pore size) to separate the vesicles from the medium. 3. Mannitol efflux exhibited apparent first-order kinetics with $k \approx 1 \text{ min}^{-1}$. Neither saturability, nor inhibition by excess sorbitol or glucose, could be observed. 4. Mannitol efflux at 18°C was about seven times faster than at 1°C, and rates at higher temperatures were too fast to be measured. The rate of glucose efflux at 2–6°C exceeded that of mannitol severalfold. 5. These findings imply a channel or carrier of definite, but limited, specificity straddling the Golgi membrane and able to supply glucose for lactose synthesis.

The conversion of glucose into lactose within the lumen of the Golgi apparatus in the lactating mammary gland necessitates its rapid movement through the Golgi membrane. A carrier for this purpose has been postulated as part of the uridine nucleotide cycle that supports lactose synthesis as a whole (Kuhn & White, 1977, 1980). The first direct study of this carrier employed a simple, particulate, preparation of lactating-rat mammary gland in which lactose-synthesizing vesicles, formed by the pinching-off of a region of Golgi dictyosome, could be loaded with [¹⁴C]lactose formed biosynthetically from glucose and UDP-[¹⁴C]galactose. Exposure of the preparation to iso-osmotic solutions of penetrating solutes resulted in the rupture of the vesicles by the water that accompanied the solute entry, with consequent release of [¹⁴C]lactose. From the extent of this release it could be shown that both D- and L-glucose, as well as a wide variety of uncharged polyols of similar size, readily penetrated the Golgi membrane. Nevertheless, positional isomers differed from one another in their rates of penetration, whereas disaccharides were essentially excluded (White *et al.*, 1980).

The above results suggested that if the transport could be slowed down by employing a low temperature, and using D-mannitol as a poor analogue of D-glucose, it might be possible to observe the direct influx and efflux of solute with purified vesicular preparations of Golgi membrane. The present paper reports the use of rapid filtration through cellulose nitrate filters to study the entry and

exit of mannitol and other polyols from such vesicles. A similar technique has been used to study Ca²⁺ uptake by Golgi membranes prepared in the same way (West, 1980).

Materials and methods

The source of animals and the preparation of Golgi-membrane vesicles have been described previously (Kuhn *et al.*, 1980). Vesicles were re-suspended in 0.25 M-lactose containing 50 mM-glycylglycine/KOH buffer, pH 7.0, to give a protein concentration of about 12 mg/ml as determined by the method of Peterson (1977). The vesicle preparation was used within 2 h.

Measurement of solute uptake and efflux

In a typical experiment, described here for 10 mM-mannitol, Golgi vesicles (100 µl) were mixed with sugar solution (20 µl) containing 0.25 M-lactose, 0.06 M-[¹⁴C]mannitol and 0.06 M-[³H]sucrose in a small tube. All operations were carried out in the cold room at 2–6°C. For measurements of mannitol uptake, duplicate samples (10 µl) were taken at various times after mixing; given that about 2 min was required to process each sample, several identical reaction mixtures were needed to build up a curve as in Fig. 2(a). Each sample was delivered into 1 ml of 0.25 M-lactose, pH 7.0, above a cellulose nitrate filter (2.5 cm diameter, 0.45 µm pore size)

resting on a sintered glass disc fitted to a Buchner funnel connected through a trap to a single stage Speedivac high-vacuum oil pump (Edwards High Vacuum, Crawley, Sussex, U.K.). Suction was immediately applied by opening a connecting tap and the filter was washed through with a further 5 ml of 0.25 M-lactose solution. Filtration typically took 10–15 s.

For efflux experiments a single reaction mixture was allowed to stand for 15–30 min to permit complete equilibration of the mannitol into the vesicles. Successive duplicate samples (10 μ l) were then taken for filtration as described above, except that suction was delayed for the period of efflux required, i.e. the efflux actually occurred in the 1 ml of lactose solution resting on the filter. For those experiments described in the legends to Figures as 'exchange efflux' the lactose solution additionally contained unlabelled sucrose and the unlabelled solute under study at the same concentrations as those to which the Golgi vesicles were exposed. This was essential to prevent osmotic lysis when high concentrations of transporting or competing solute were being used.

Filters were each dissolved in 10 ml of scintillation fluid (Kuhn & White, 1975) and the ^{14}C and ^3H isotopes were measured simultaneously at about 62 and 30% efficiency respectively in a suitably programmed Nuclear Chicago liquid-scintillation counter.

Calculation of specific solute retention

From the known specific radioactivities of the ^{14}C labelled solute and ^3H sucrose used, the amounts of each that were retained on the filter could be calculated. The excess of test solute over sucrose retained was taken as representing specific solute retention. All values shown are means of duplicate samples unless otherwise stated. The amount of protein retained on the filter was taken as being 88% of that applied. This factor was separately determined in six closely agreeing experiments in which known amounts of Golgi-membrane protein (100–200 μg) were applied to a filter and washed with 0.25 M-sucrose. The protein content of the filtrate was then determined by the method of Peterson (1977), in which sucrose gave a lower blank value than did lactose.

D-[^{14}C]Glucose, D-[1- ^{14}C]mannitol, D-[1- ^{14}C]mannose, D-[U- ^{14}C]fructose, D-[U- ^{14}C]sorbitol, [U- ^{14}C]sucrose, [D-glucose-1- ^{14}C]lactose, [6,6'-(n)- ^3H]sucrose and 3-O-methyl-D-[1- ^3H]glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled sugars were from BDH Chemicals, Poole, Dorset, U.K., or from Fisons Scientific Apparatus, Loughborough, Leics., U.K. Sartorius cellulose nitrate filters were supplied by V. A. Howe & Co., London, U.K.

Results

Experimental design

Two features of the technique described in the Materials and methods section were essential for the success of the transport studies given below. First, the use of fine filters facilitated the separation and washing of membrane vesicles within a time compatible with the rates of solute efflux. Secondly, the inclusion of [^3H]sucrose at a concentration equal to that of the test solute allowed correction for solute that had merely been adsorbed on to the vesicles or trapped within interstices between them. This correction usually amounted to about half of the retained solute and was often rather variable from one filtration to the next. However, such variability was satisfactorily coped with by the double-isotope design of the experiments. Very little solute was adsorbed by the filters themselves.

Penetration of Golgi vesicles by solutes

Table 1 shows the uptake and retention of various solutes incubated for 15 min with the Golgi vesicles. Glucose, 3-O-methylglucose, mannose, fructose, sorbitol and mannitol were all taken up, whereas lactose and (by definition) sucrose were excluded. Retention of glucose appeared to be rather less than that of mannitol and sorbitol, probably due to its greater loss during filtration as discussed below. A

Table 1. The retention of different solutes by Golgi-membrane vesicles

Vesicles were equilibrated with solute (10 mM) labelled with ^{14}C or, in the case of 3-O-methylglucose, with ^3H . Sucrose (10 mM) labelled with ^3H (or ^{14}C when 3-O-methylglucose was being used) was included as an extravascular marker. Protein retention was in the range 123–160 μg /filter. The results of three separate experiments are shown. In two of these, vesicles equilibrated with mannitol were additionally collected by filtration in lactose-free medium to cause osmotic rupture, or at room temperature (20°C). Further details are given in the Materials and methods section.

Expt. ...	Solute retention (nmol)		
	1	2	3
Lactose	0	0	0
Glucose	0.73	0.57	0.64
3-O-Methylglucose	0.73	0.73	0.67
Mannose	0.72	0.66	0.73
Fructose	0.79	0.71	0.73
Sorbitol	0.84	0.81	0.73
Mannitol	0.84	0.76	0.71
Mannitol (hypo-osmotic)	0.27	—	0.21
Mannitol (20°C)	0.38	—	0.45

similar explanation may account for the decreased mannitol retention at 20°C (see Fig. 5 below). Much less retention of mannitol was observed with vesicles that were osmotically ruptured by being washed with hypo-osmotic solution.

Proportionality of mannitol uptake to concentration of mannitol and vesicles

Fig. 1(a) shows that the amount of mannitol taken up and retained was linearly related to the concentration of Golgi vesicles. Fig. 1(b) shows linearity between mannitol uptake and its concentration in the medium over a 20-fold concentration range.

The results so far described, taken together with the sucrose correction that was routinely applied, are not consistent with mere adsorption of mannitol on to the membrane surfaces. Rather, they show that mannitol enters a membrane-bounded space that is not penetrated by sucrose.

Time course of mannitol uptake and release

Fig. 2(a) shows the time course of mannitol uptake and release by Golgi vesicles exposed to, or equilibrated with, 10 mM-mannitol at 2–6°C. The changes were very rapid despite the low temperature. Accurate timing of sampling was more easily achieved during efflux, so that this process was selected for the further studies described in the present paper. A semi-logarithmic replot of the efflux data (Fig. 2b) gave a straight line, consistent with first-order kinetics. An apparent first-order rate constant of $k = 1.16 \text{ min}^{-1}$ was derived from a plot of $\log[a/(a-x)]$ against time (where a = mannitol concentration measured at zero-time efflux and x = the amount of mannitol lost over time t). In the experiment shown in Fig. 2(a) the amount of mannitol observed at equilibrium of the vesicles with the 10 mM solution to which they were exposed

represented an intravesicular volume of $0.42 \mu\text{l}/\text{mg}$ of protein. In such experiments, however, the time of filtration (usually 10–15 s) was such that an appreciable efflux of solute must have been incurred. When the curve in Fig. 2(b) is extrapolated back to the 'true' zero time to allow for this, a 'true' intravesicular volume of $0.52 \mu\text{l}/\text{mg}$ of protein is obtained. Similar treatment of nine separate preparations in which the efflux of 1 or 10 mM-mannitol was measured gave a mean ($\pm$ S.E.M.) intravesicular volume of $0.84 \pm 0.07 \mu\text{l}/\text{mg}$ of protein.

Comparison of mannitol and glucose efflux

Fig. 3 shows a semi-logarithmic plot of glucose and mannitol efflux from a single preparation of Golgi vesicles. It should be noted that the efflux studied in this and in subsequent experiments was actually an exchange since the vesicles loaded with ^{14}C -labelled solute were diluted out into unlabelled solute of the same concentration. Efflux of glucose ($k = 2.85 \text{ min}^{-1}$) was about four times faster than that of mannitol ($k = 0.74 \text{ min}^{-1}$). The extrapolated curves intersect at about the time expected for the 'true' zero time described above, as would be expected for two substances effluxing from the same compartment. In a second such experiment the rate constants were 2.3 min^{-1} for glucose and 0.9 min^{-1} for mannitol.

Effect of concentration of mannitol on efflux rate

The rate of efflux of mannitol over a 100-fold range in its concentration was studied in five preparations, with the mean results shown in Fig. 4(a). The rate increased even up to 100 mM-mannitol,

Fig. 1. Proportionality of mannitol retention to (a) concentration of Golgi vesicles and (b) concentration of mannitol

Vesicles were equilibrated with mannitol for 15 min at (a) 1 mM-mannitol and the vesicular protein concentration shown, or (b) a vesicular protein concentration of approx. 10 mg/ml and the mannitol concentration shown.

Fig. 2. Time course of mannitol uptake and efflux (a) The retention of mannitol was measured either at various times after exposure of vesicles to 10 mM mannitol (O), or at various times after vesicles equilibrated with 10 mM-mannitol were diluted with mannitol-free solution (●). In both cases the filtration was carried out with 0.25 M-lactose. (b) Shows a semi-logarithmic plot of the efflux data in (a).

Fig. 3. *A comparison of glucose (O) and mannitol (●) efflux*

Golgi vesicles were equilibrated with 10 mM-glucose or 10 mM-mannitol. Exchange efflux was then measured after dilution of the vesicles into 0.25 M-lactose containing unlabelled glucose or mannitol, each at 10 mM.

Fig. 4. *Effect of mannitol concentration on rate of efflux*

Golgi vesicles were equilibrated with 1–100 mM-mannitol. Retained mannitol was then measured at 0 and 15 s after dilution of the vesicles into 0.25 M-lactose containing unlabelled 1–100 mM-mannitol, and the rate of exchange efflux was calculated from the difference between these two values. The means ($\pm$ S.E.M.) for five experiments are shown; two individual values were discarded as being implausibly high. (a) Rate of efflux at different concentrations of mannitol; (b) reciprocal plot of the data in (a).

with only a slight apparent tendency to fall off. An apparent K_m of 250 mM was derived from the reciprocal plot in Fig. 4(b).

Effects of competing solutes on mannitol efflux

Sorbitol and glucose were introduced at 100 mM concentration in an attempt to competitively inhibit the efflux of 1 mM-mannitol. Efflux was measured over 1 min at 2–6°C. The mean rate constants in five separate determinations were $0.82 \pm 0.10 \text{ min}^{-1}$ (no competitor), $0.81 \pm 0.10 \text{ min}^{-1}$ (with 100 mM-sorbitol) and $0.97 \pm 0.14 \text{ min}^{-1}$ (with 100 mM-glucose). Clearly there was no significant inhibition by these competitors, and the rate constants for efflux of 1 mM-mannitol compared closely with those given above for 10 mM-mannitol.

Effect of phloretin

In two separate experiments (results not shown) 1 mM-phloretin preincubated with Golgi vesicles for 15 min at 0–5°C inhibited subsequent efflux of mannitol by 28 and 65% respectively.

Effect of temperature on mannitol efflux

In a single experiment the efflux of 10 mM-

Fig. 5. *Effect of temperature on mannitol efflux* Vesicles equilibrated with 10 mM-mannitol were diluted into 0.25 M-lactose containing unlabelled 10 mM-mannitol at 1°C (●) or at 18°C (O), and mannitol retention was measured after various times of exchange efflux.

mannitol was measured at 1 and 18°C. The results shown as a semi-logarithmic plot in Fig. 5 yielded first-order rate constants of 0.67 and 4.91 min⁻¹ respectively. At higher temperatures the rate was too fast to measure by this technique.

Discussion

Validity of the method

The membrane preparations used here are such as we have previously described as being greatly enriched in galactosyltransferase (EC 2.4.1.22) and nucleoside diphosphatase (EC 3.6.1.6), and may therefore be considered to be of Golgi origin (Kuhn & White, 1977; Kuhn *et al.*, 1980). Nevertheless it cannot be taken for granted that the membrane vesicles that are here penetrated by mannitol and glucose are identical with those that synthesize lactose, since there is growing evidence for the heterogeneity of Golgi dictyosome membranes. It is therefore significant that the solute transport properties revealed by the present filtration method strongly resemble those shown by the osmotic lysis method, which, as previously pointed out (White *et al.*, 1980), monitors only lactose-synthesizing vesicles. Both techniques reveal a transport process that accepts monosaccharides and their similarly-sized, uncharged, derivatives, but not disaccharides. In both cases it has proved impossible to saturate the process with high concentrations of solute or to inhibit it with high concentrations of competing solute. In both cases the transport of D-glucose is clearly greater than that of D-mannitol. Finally, in both cases the transport is so rapid that satisfactory measurements have been realized only at low temperature. We therefore conclude that both techniques probably measure the same transport process in lactose-synthesizing Golgi vesicles.

The nature of the carrier

Our studies with the osmotic lysis technique (White *et al.*, 1980), and now by direct efflux, impute to this carrier properties that are distinctly different from those of better-studied monosaccharide carriers in cell outer membranes. Thus its lack of saturation and its resistance to competitive inhibition suggest a very weak binding of the solute being transported. Such properties, as well as the apparent first-order kinetics observed for net efflux of mannitol (Fig. 2a), could be ascribed to a simple pore were it not for the discrimination shown between glucose and mannitol or, indeed, between glucose and galactose, 2-deoxyglucose and 6-deoxyglucose, or 2-deoxygalactose and 6-deoxygalactose (White *et al.*, 1980). Therefore possible models include either a pore, or a

binding site, of dimensions that exclude disaccharides and of boundaries that are lined by hydrophilic groups able to form hydrogen bonds with a wide range of polyols. Only a protein could presumably account for the specificities described above. We consider the possibility that this is a primitive carrier not unlike that responsible for the passage of sugars across the lysosomal membrane (Docherty *et al.*, 1979). Whether the activity of this carrier in fact suffices to supply glucose for lactose synthesis can only be tested when it becomes possible to measure it at 37°C.

It is recognized that lactose synthetase is, through the special properties of its α -lactalbumin component, a recent evolutionary adaptation of a galactosyltransferase (EC 2.4.1.22) that is more widely employed in the synthesis of glycoprotein (Brew, 1979). It is therefore of interest that the monosaccharide transport system, investigated here in the context of lactose synthesis, might also have a role in glycoprotein synthesis. It appears that mannose and glucose residues, attached to protein in the rough endoplasmic reticulum, are subsequently partially hydrolysed before the attachment of other sugars by glycosyltransferases located in the Golgi apparatus (Kornfeld *et al.*, 1978; Tabas & Kornfeld, 1978). There is evidence that this hydrolysis may also take place in the Golgi apparatus (Tulsiani *et al.*, 1977), in which case a general sugar carrier might serve to return the free sugars to the cytosol.

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