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Topology of UDP-galactose cleavage in relation to *N*-acetyl-lactosamine formation in Golgi vesicles

Translocation of activated galactose

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UDP-galactose appears to be produced on one side of a membrane barrier, opposite the galactosyltransferases that use it as a sugar donor. The translocation of activated galactose across membranes was studied in rat submaxillary-gland microsomal vesicles and in rat liver Golgi vesicles. When these intact vesicles containing the acceptor, *N*-acetylglucosamine, were incubated in the presence of UDP-galactose and two inhibitors of galactosyltransferase activity, the product, *N*-acetyl-lactosamine, formed within the vesicles. Thus at least the galactose moiety of UDP-galactose crossed the membranes. When intact Golgi vesicles were incubated with UDP-galactose labelled in both the uridine and the galactose moieties, labelled *N*-acetyl-lactosamine was again produced in the vesicles, but less than stoichiometric amounts of the uridine label was found there. Calculation of internal and external concentrations of UMP, a major product released from the cleaved uridine moiety, showed that the vesicles were actually enriched in UMP. When free UMP was incubated with the vesicles, this enrichment did not occur. This result was direct evidence for facilitated transport of UDP-galactose into the Golgi for use by galactosyltransferase.

Sugar nucleotides are used routinely to assay glycosyltransferases. In many cells, most glycosyltransferase activity exists within the lumens of the Golgi apparatus or endoplasmic reticulum (Fleischer, 1981), or outside the plasma membrane (Pierce *et al.*, 1980), whereas the sugar nucleotides are produced on the other side of a membrane barrier, in the cytoplasm (Schacter & Roseman, 1980; Snider *et al.*, 1980; Carey *et al.*, 1980; Hanover & Lennarz, 1982).

For the activated sugars to cross membranes, the simplest model requires that the sugar nucleotides be transported directly across the Golgi, endoplasmic-reticulum or plasma membranes to be used by the glycosyltransferases. Kuhn & White (1976, 1977) studied lactose synthesis in intact mammary-gland microsomal fractions and used UDP-galactose as a sugar donor. They concluded that: (*a*) UDP-galactose enters microsomal fractions directly and is cleaved to UDP by a galactosyltransferase, (*b*) UDP is in turn converted into UMP, and (*c*) UMP is rapidly transported out of the vesicles. Corroborative evidence for this model comes from the work of Roth & Berger (1982), Brandan & Fleischer (1980, 1981, 1982) and Hirschberg and co-workers (Carey & Hirschberg, 1980; Sommers & Hirschberg, 1982). The actual transport of UDP-galactose, however, has not been previously demonstrated.

A more complex model could involve the formation of a lipid–sugar intermediate that traverses the membranes (DeLuca, 1977; DeLuca *et al.*, 1979; Hubbard & Ivatt, 1981; Haselbeck & Tanner, 1982; Snider & Rogers, 1984). Galactosylated lipids with chromatographic properties of polyisoprene compounds have been isolated (Zatta *et al.*, 1975; Peterson *et al.*, 1976; DeLuca *et al.*, 1977; Adamo *et al.*, 1979).

The present paper reports an assay *in vitro* for the transmembrane movement of activated galactose, resulting in the synthesis of *N*-acetyl-lactosamine. The assay is performed by first loading microsomal vesicles with *N*-acetylglucosamine, the acceptor for a microsomal galactosyltransferase. Second, UDP-galactose, β-galactosidase and α-lactalbumin are added to the mixture for the final incubation. The β-galactosidase and the α-lactalbumin prevent the accumulation of *N*-acetyl-lactosamine owing to galactosyltransferases in ruptured or on everted vesicles. After incubation, *N*-acetyl-lactosamine is

assayed specifically. The formation of this product necessitates the translocation of some form of activated galactose, for example nucleotide–sugar or lipid–sugar, across microsomal membranes.

The assay has been also conducted with (1) [^{14}C]UDP-galactose instead of UDP-[^{3}H]-galactose alone and (2) Golgi-enriched vesicles instead of microsomal vesicles. Labelling the uridine moiety permits the identification of its point of cleavage from the galactose moiety. [^{14}C]UMP accumulates in the vesicles during these incubations, whereas such accumulation does not occur when the vesicles are incubated directly with labelled UMP. This result strongly supports the simple, direct, transport of UDP-galactose, and therefore the Kuhn & White (1977) model presented above.

Experimental

Materials

All non-radioactive reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. UDP-D-[6-^{3}H]galactose, [4-^{14}C]UTP and [5-^{3}H]-UDP were obtained from Amersham, Arlington Heights, IL, U.S.A. [^{3}H]Poly(ethylene glycol) (4000) and D-[^{14}C]glucose were products of New England Nuclear, Boston, MA, U.S.A. [^{14}C]UMP was obtained from Research Products International Corp., Mount Prospect, IL, U.S.A. Silicone oil was received as a sample from General Electric Co., Silicone Products Division, Camden, NJ, U.S.A.

Microsomal preparation

Submaxillary glands were removed from adult male Lewis rats after they were killed by CO_2 inhalation. The glands were minced with scissors and homogenized in a tissue grinder with a motor-driven serrated Teflon pestle. The homogenization buffer (approx. 10–15ml/rat) contained 0.01M-Tris/HCl, pH7.0, 0.25M-sucrose, 1mM-EDTA and 1mM-2-mercaptoethanol (Blobel & Dobberstein, 1975). The glands were further homogenized in a Dounce homogenizer. Glands and homogenates were kept at 4°C. To remove cell debris and nuclei, the homogenate was then centrifuged for 15min at $3000g_{av.}$. The resulting supernatant was removed and centrifuged at $120000g_{av.}$ for 35min. The pellet was resuspended in homogenization buffer at a ratio of approx. 300μl per gland and immediately frozen in batches.

Preparation of Golgi vesicles from rat liver

Golgi vesicles were prepared by the procedure of Bergeron (1979) and Bergeron *et al.* (1982), with some minor modifications. Male and female Lewis rats were killed by CO_2 inhalation and their livers perfused with homogenization buffer. The homogenization buffer consisted of 0.25M-sucrose adjusted to pH7.0 with 0.01M-Tris/HCl buffer and was added to the liver to give a final concentration of 20% (w/v) liver. The livers were homogenized in a Potter–Elvehjem homogenizer with a modified clearance of 0.66mm (0.026in) (Fleischer, 1974) for three strokes at 500rev./min and then with a stock pestle for five strokes. The homogenate was centrifuged at $10000g_{av.}$ for 10min. The pellet was washed and the supernatants were combined for pelleting at $90000g_{av.}$ for 70min. The pellet was resuspended in 7.67ml of 0.25M-sucrose, homogenized in a Dounce homogenizer (with the A pestle) and 18.88ml of 2.0M-sucrose was added. The volume was adjusted to 34.5ml and the refractive index was checked. Water or 2.0M-sucrose was added to give a final concentration of sucrose of 1.15M. Approx. 8.6ml of this was put at the bottom of each of four tubes of a Beckman SW 27 rotor: 1.0M-, 0.86M- and 0.25M-sucrose (10.8, 9.5 and 9.7ml respectively) were layered over this, and the gradient was centrifuged for 3h at 26000 rev./min ($90000g_{av.}$). The resulting interface fractions were removed and pelleted ($90000g$ for 70min). The pellets were resuspended and frozen at −20°C. The pellet under the gradient was also resuspended and kept for analysis. When Golgi vesicles were prepared for experimental use, the first two interface fractions were taken, pelleted together, resuspended in a small volume of 0.25M-sucrose and stored frozen in batches in liquid N_2.

Assay of gradient fractions for marker enzymes

5′-Nucleotidase. A 10μl sample was incubated in a 100μl mixture containing 5.0mM-5′-AMP, 5mM-$MgSO_4$, 0.1M-KCl, 0.05M-Tris/HCl, pH7.5, and 1mg of bovine serum albumin/ml. For the control incubations 5.0mM-2′-AMP replaced 5′-AMP. The experimental and control tubes were incubated for 15min at 37°C; then 200μl of cold 15% (w/v) trichloroacetic acid was added. The samples were centrifuged for 10min at $1000g$, and 200μl samples of the supernatants were assayed for P_i by the procedure of Chen *et al.* (1956). The difference between the values of the experimental and control incubations represented the specific 5′-nucleotidase activity.

Glucose-6-phosphatase. A 10μl sample was incubated in a 100μl mixture containing 0.05M-Mes (4-morpholine-ethanesulphonic acid) buffer, pH6.5, 5mM-glucose 6-phosphate, 1mM-$MgSO_4$, 0.2% Nonidet P-40 and 1mg of bovine serum albumin/ml. After incubation for 15min at 37°C, 200μl, of 15% trichloroacetic acid was added, the mixture was centrifuged as above, and a 200μl sample was for analysis for P_i.

Galactosyltransferase. This was assayed in the absence of vesicles in 50μl samples containing 10mM-$MnCl_2$, 0.02M-Mes buffer, pH6.5, 3mM-5′-AMP, 0.5% Nonidet P-40, 200μM-UDP-[^{3}H]galactose (23.8Ci/mol), 10mM-*N*-acetylglucosamine and approx. 10μg of microsomal protein. Samples were incubated at 37°C for 12min and terminated with 7μl of 0.025M-EDTA. The 50μl samples were spotted on 45.6cm × 56cm (18in × 22in) sheets of Whatman 3MM paper and electrophoresed towards the anode in 1% sodium borate buffer (pH9) as described previously (Roth *et al.*, 1971). Material remaining 0.64cm (0.25in) below the origin and 3.18cm (1.25in) above it was counted for radioactivity in toluene-based fluor. All counting was done in a Searle mark III scintillation counter for 2min. Control assays lacking *N*-acetylglucosamine were run simultaneously, and the resulting endogenous acceptor activity was subtracted from the values for the experimental incubations.

Protein was assayed by the Lowry method at 560nm, with bovine serum albumin as the standard.

N-Acetyl-lactosamine synthesis with intact microsomal vesicles

Transferase assays with intact microsomal vesicles were designed so that *N*-acetyl-lactosamine synthesis had to occur within intact vesicles. The assays were performed by combining 36μl of freshly thawed microsomal vesicles (submaxillary gland; 10mg of protein/ml) with 0.075M-Mes buffer, pH6.5, 2.25mM-$MnCl_2$, 1.5mM-$MgSO_4$, 4.5mM-5′-AMP, 0.1M-KCl and 15mM-*N*-acetylglucosamine in a final volume of 70μl. This mixture was incubated for 20min at 37°C and then added to 35μl of a solution containing 570 lactose units (μmol of lactose hydrolysed in 30min at 37°C) of β-galactosidase/ml, 21mg of α-lactalbumin/ml, 120μM-UDP-[^{3}H]galactose (95Ci/mol) and 0.1M-KCl. Immediately, 55μl was removed and added to 1.4μl of 20% Nonidet P-40. Both samples were incubated for 5min at 37°C, and the reaction was terminated by the addition of 7μl of 0.025M-EDTA and by immediately heating to 95°C for 3min. Samples were then assayed for *N*-acetyl-[^{3}H]lactosamine by the method described below.

Two-step chromotography (assay for N-acetyl-lactosamine)

To separate *N*-acetyl-lactosamine from high-M_r acceptors in galactosyltransferase assays, an extra step was added before sodium borate electrophoresis. On Whatman 3MM paper, the samples were first subjected to descending paper chromatography in butan-1-ol/pyridine/water (6:4:3, by vol.) for 5–6h [the front moves 20–23cm (8–9in) from the origin]. After drying the paper, the sodium borate electrophoresis step was carried out as described above. After being dried, the paper was cut into 1.9cm ($\frac{3}{4}$in) strips for scintillation counting. The *N*-acetyl-lactosamine peak migrated 5.7–11.4cm (2.25–4.5in) from the origin, whereas the high-M_r material stayed near the origin, and both galactose and UDP-galactose were found much further from the origin [approx. 20.3cm (8in)].

Synthesis of [^{14}C]UDP-galactose

[^{14}C]UDP-galactose was synthesized enzymically from [^{14}C]UTP as described by Anderson *et al.* (1959). The final product was purified by separation by electrophoresis in ammonium formate (0.15M, pH3.6) (Neufeld *et al.*, 1957). The region that co-migrated with authentic UDP-galactose (detected under u.v. light) on the dried paper chromatogram was removed and UDP-galactose was eluted with a descending siphoning wash of water. The eluate was freeze-dried and tested for contamination with [^{14}C]UDP-glucose by treatment with UDP-glucose dehydrogenase, followed by electrophoresis in ammonium formate. A UDP-[^{14}C]glucose standard treated identically shifted totally its migration to that of UDP-glucuronic acid. The eluate was also examined for contamination with other uridine-containing compounds by electrophoresis in ammonium formate. No UDP-glucose or uridine was detected (< 0.2%), although small amounts of UMP (1.6%) and UDP (1%) were found.

Use of [^{14}C]UDP-galactose and UDP-[^{3}H]galactose in a galactosyltransferase assay

[^{14}C]UDP-galactose was tested in combination with UDP-[^{3}H]galactose in a detergent-solubilized microsomal galactosyltransferase assay. Incubation mixtures were as follows: 85μg of submaxillary-gland microsomal protein/ml, 10mM-$MnCl_2$, 0.04M-Mes buffer, pH6.5, 3mM-5′-AMP, 10mM-*N*-acetylglucosamine, 0.5% Nonidet P-40 and 38.3mM-UDP-galactose (15 c.p.m. of UDP-[^{3}H]galactose/pmol, 18c.p.m. of [^{14}C]UDP-galactose/pmol). A parallel incubation mixture was run in the absence of *N*-acetylglucosamine. Mixtures were incubated at 37°C and two 30μl samples were taken at 0, 5 and 10min. One sample from each time was subjected to ammonium formate electrophoresis (Neufeld *et al.*, 1957) to determine UDP, UMP, uridine and UDP-galactose contents. The second sample was subjected to the two-step chromatography described above to determine *N*-acetyl-lactosamine content.

Incubation of Golgi vesicles with [^{14}C]UDP-galactose and UDP-[^{3}H]galactose

Golgi vesicles were separated from the supernatant of reaction mixtures by centrifuging them at low speed through a layer of silicone oil. This technique was used previously by Ballas & Arion (1977) and Hanover & Lennarz (1982) with Ca^{2+}- or Mg^{2+}-aggregated microsomal fractions. In this case the Golgi vesicles in 0.25M-sucrose were pre-incubated in 333.3μl of 0.02M-Mes/8mM-$MgCl_2$/2mM-$MnCl_2$/10mM-*N*-acetylglucosamine/0.08M-KCl (final concn. 0.06M-sucrose) for 20min at 37°C before adding a mixture (166.7μl) that had the same concentrations of buffer, $MnCl_2$ and $MgCl_2$, but which also contained α-lactalbumin, 3mM-adenosine 5'-[β,γ-imido]triphosphate, 3mM-2,3-dimercaptopropanol, 0.67μCi of [^{14}C]UDP-galactose, 3.0μCi of UDP-[^{3}H]galactose, unlabelled UDP-galactose (final concn. 57.3μM) and KCl to the same osmolarity as the first mixture (300mosM). After incubation for 3min at 37°C, two 200μl samples were layered in a 400μl polypropylene tube above a layer of 75μl of silicone oil (F-50) resting on a 50μl layer of termination medium (chilled to 0°C). Two different termination media were used: (A) 2% $HClO_4$ in 17% (w/v) sucrose and (B) 0.1M-Tris/HCl (pH7.4)/20mM-EDTA/20mM-UDP/20mM-UMP mixed 1:1 (v/v) with ethylene glycol. The samples were centrifuged in a Beckman Microfuge at 140V for 1–1.5min at room temperature. At 10s after starting the centrifuge, more samples were taken of the remaining reaction mixtures and added to $HClO_4$ or to a sample of termination medium B, concentrated to give the same final concentration. These second samples serve for measuring the concentration of solute in the supernatant. The centrifuge tubes were frozen and cut at the silicone-oil layer. The tubes containing medium B were frozen in liquid N_2, the others in a freezer (−20°C). The samples in $HClO_4$ were neutralized by adding 7μl of 3M-K_2CO_3/0.5M-triethanolamine to 100μl of incubation mixture. These samples were subjected to sodium borate electrophoresis to determine the total amount of [^{3}H]galactose transferred. The samples in ethylene glycol were subjected to ammonium formate electrophoresis to determine the amounts of labelled UDP, UMP, uridine and UDP-galactose present. For control experiments, the following labelled compounds were incubated under the same conditions used for the double-labelling experiment, except that the final volume was 313μl: (1) 0.25μCi of [^{3}H]poly(ethylene glycol) 4000, 0.11μCi of D-[^{14}C]glucose; (2) 0.68μCi of [^{3}H]uridine (10.9Ci/mmol) (+3μM unlabelled uridine); (3) 0.35μCi of UMP (450Ci/mol) (2.5μM final concn.). After incubation, 130μl samples were centrifuged as above. Samples (10μl) were taken of each incubation mixture 10s after starting the centrifuge. All reactions except that with UMP were terminated with 2% $HClO_4$. The neutralized samples were counted directly in Triton-containing toluene-based fluor. The UMP incubation was terminated in ethylene glycol and electrophoresed in ammonium formate to separate UMP and uridine.

Incubation of Golgi vesicles with [^{14}C]UDP-galactose and [^{3}H]UMP

Intact Golgi vesicles were incubated with [^{14}C]UDP-galactose and [^{3}H]UMP by following the extract protocol given above for incubation with [^{14}C]UDP-galactose and UDP-[^{3}H]galactose, with the following changes.

(1) UDP-galactose in the incubations consisted of 0.34 (Expt. 1) or 0.68 (Expts. 2–5) μCi of [^{14}C]UDP-galactose adjusted to a final concentration of 57.3μM with unlabelled UDP-galactose.

(2) [^{3}H]UMP (0.6μCi) was added to the incubation and adjusted to a final concentration of 8 (Expts. 2–5) or 12 (Expt. 1) μM with unlabelled UMP.

(3) The incubation time for Expt. 4 was 5min.

(4) In Expt. 5, *N*-acetylglucosamine was replaced by the same concentration of sucrose.

The [^{3}H]UMP in these experiments was prepared by heating [^{3}H]UDP (10.9Ci/mmol) in 0.1M-formic acid in a boiling-water bath for 12min. The formic acid was removed by freeze-drying.

Calculations (see Scheme 1)

All terms used were defined and calculated as given by Ballas & Arion (1977). The term V represents the solute-accessible space, or the theoretical volume of solution that should be associated with the pellet to give it the number of counts observed. V is calculated for glucose and poly(ethylene glycol) 4000, which are used as permeant and non-permeant markers respectively.

Results

Galactosyltransferase assays using intact microsomal vesicles (see Table 1) demonstrate that *N*-acetyl-lactosamine synthesis within intact vesicles can be assayed by using inhibitors to minimize net synthesis outside the vesicles. Without the two functional inhibitors (α-lactalbumin and β-galactosidase), activity is higher and is stimulated by detergent. In the presence of the inhibitors, detergent decreases *N*-acetyl-lactosamine formation by a factor of 10. These data indicate that α-lactalbumin and β-galactosidase can limit most of the galactosyltransferase activity in these incuba-

$$V = \frac{\dfrac{\text{(c.p.m. in pellet sample)}}{\text{(c.p.m. in reaction mixture sample)}} \times (\mu\text{l in reaction-mixture sample})}{\text{mg of protein in pellet sample}}$$

V_t = total solute-accessible space = intra-+extra-vesicular spaces ≡ glucose-accessible space
V_0 = extravesicular space ≡ poly(ethylene glycol) 4000-accessible space
V_i = intravesicular space = $V_t - V_0$
$[S]_m$ = [solute in reaction mixture]
S_t = total solute associated with the pellet = $S_0 + S_i + S_a$
$S_i + S_a = S_t - S_0$
$S_0 = V_0 \times [S]_m$

$$[S]_i + [S]_a = \text{concn. of solute inside vesicles (+adsorbed solute)} = \frac{S_i + S_a}{V_i}$$

$$\frac{[S]_i + [S]}{[S]_m} = \text{ratio of vesicle-associated concentration to bulk concentration}$$

Scheme 1.

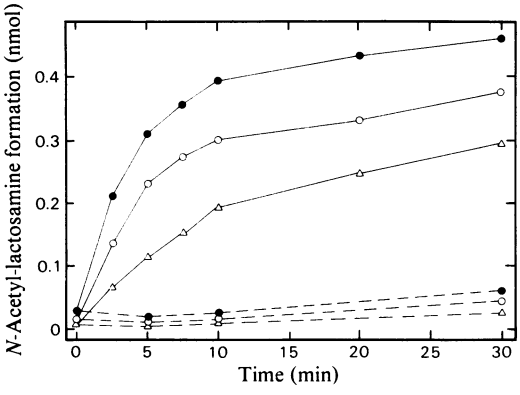

Fig. 1. *N-Acetyl-lactosamine synthesis in intact microsomal vesicles*

Assays were performed at three different concentrations of microsomal vesicles with the same protocol given in the Experimental section except that sample volume was 35 μl and UDP-galactose concentration was 40 μm. The final microsomal protein concentrations were: ●——●, 3.9 mg/ml; ○——○, 2.6 mg/ml; △——△, 1.3 mg/ml. Detergent-containing control samples were taken every other time an experimental sample was taken: ●--●, 3.9 mg of protein/ml; ○--○, 2.6 mg/ml; △--△, 1.3 mg/ml.

Table 1. *N-Acetyl-lactosamine synthesis with and without β-galactosidase and α-lactalbumin*

Results are expressed as c.p.m. of [^{3}H]*N*-acetyl-lactosamine synthesized. Assay conditions are given in the Experimental section.

	α-Lactalbumin and β-galactosidase	
Conditions	Absent	Present
Intact microsomal vesicles	6200	3270
Lysed vesicles	21600	296

tions, but cannot enter the intact microsomal vesicles.

Fig. 1 shows *N*-acetyl-lactosamine synthesis in intact microsomal vesicles as a function of microsomal protein concentration. The initial rates are proportional to the protein concentration.

When both [^{14}C]UDP-galactose and UDP-[^{3}H]-galactose are used in a galactosyltransferase assay with detergent (Fig. 2), UDP is released concomitant with transfer of galactose to *N*-acetyl-glucosamine. In incubations without *N*-acetyl-glucosamine, UDP and UMP concentrations are constant and are one-tenth of those formed in 10 min with acceptor present. No labelled uridine is found.

Transport of activated galactose can also be assayed when Golgi vesicles are incubated with UDP-galactose. The vesicles are prepared by a procedure developed by Bergeron (1979) and Bergeron *et al.* (1982) that uses sucrose-density-gradient centrifugation. Activities of some typical enzyme markers (see Table 2) are comparable with those obtained by Bergeron *et al.* (1982). The microsomal marker glucose-6-phosphatase and the plasma-membrane marker 5′-nucleotidase are present in the Golgi fractions at suitably low activities. Galactosyltransferase, a typical Golgi

marker, is enriched about 60-fold compared with the pellet, which sould contain the rough microsomal fraction, and over 65-fold compared with the homogenate.

When intact Golgi-enriched vesicles containing *N*-acetylglucosamine are incubated with [^{14}C]-UDP-galactose and UDP-[^{3}H]galactose, product is formed, as determined by sodium borate electrophoresis, but practically no UDP can be found in the incubation mixture. UMP appears both inside and outside the vesicles, but, in comparison with *N*-acetyl-lactosamine, less than stoichiometric amounts of UMP are associated with the vesicles, as shown in Table 3. Nevertheless, when the internal and external concentrations of UMP released from UDP-galactose are calculated, UMP appears to be enriched in the vesicles. Internal and external concentrations of UMP and uridine are determined as described in the Experimental section. The internal volume and the extravesicular volume of the pellets are determined by using D-glucose and poly(ethylene glycol) 4000 respectively as permeant and non-permeant markers. Table 3 shows that, after the vesicles are incubated with UDP-galactose, the ratio of internal to external concentrations of UMP, $([UMP]_i+[UMP]_a)/[UMP]_m$, is greater than unity. More important, this enrichment is not seen when UMP, instead of UDP-galactose, is incubated directly with the vesicles. The apparent internal concentration of UMP becomes less than zero, meaning that the amount of UMP associated with the vesicles is less than what would be predicted to be in the extravesicular space.

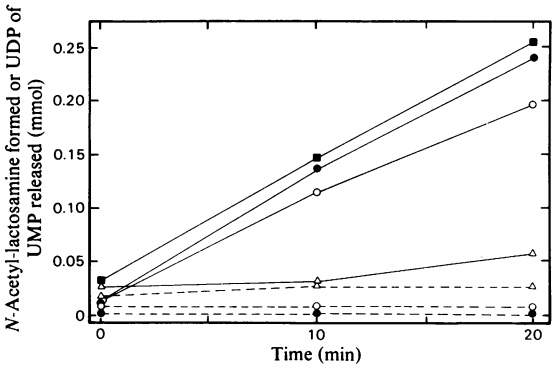

Fig. 2. *Use of [^{14}C]UDP-galactose and UDP-[^{3}H]galactose in a galactosyltransferase assay with detergent*
The synthesis of *N*-acetyl-lactosamine (●——●) was compared with the release of UDP (○——○) and UMP (△——△). The numbers for UDP and UMP were combined for further comparison (■——■). Incubation conditions are given in the Experimental section. A parallel set of samples was taken from an incubation mixture without *N*-acetylglucosamine (broken lines).

Table 2. *Marker enzyme activities in sucrose-gradient fractions*
For further details see the text. Results are means ± S.D. for three different experiments.

Fraction (interface)	Protein (%)	5′-Nucleotidase (nmol/min per μg)	Glucose-6-phosphatase (nmol/min per μg)	Galactosyltransferase (nmol/min per mg)
1 Golgi	0.55 ± 0.07	0.24 ± 0.09	0.05 ± 0.02	4.7 ± 1.6
2	1.06 ± 0.16	0.53 ± 0.24	0.11 ± 0.06	1.8 ± 0.9
3	3.2 ± 0.18	0.59 ± 0.34	0.13 ± 0.09	2.0 ± 0.7
4	31 ± 15	0.32 ± 0.15	0.19 ± 0.10	0.45 ± 0.04
5 (pellet)	65 ± 15	0.08 ± 0.06	0.24 ± 0.07	0.08 ± 0.03

Table 3. *Incubation of intact Golgi vesicles with [^{14}C]UDP-galactose and UDP-[^{3}H]galactose*
The concentrations of the labelled solutes inside and outside the vesicles were calculated as given in the Experimental section. Each value represents the mean ± S.D. for *n* experiments. Each experiment was performed at separate times with a different batch of Golgi vesicles. Negative quantities result when S_o is greater than S_i; the negative sign is carried through the rest of the calculations.

Labelled solute added	Labelled solute assayed	$[S]_m$ (μM)	$[S]_i+[S]_a$ (μM)	$\frac{[S]_i+[S]_a}{[S]_m}$
[^{14}C]UDP-galactose + UDP-[^{3}H]galactose ($n=3$)	[^{14}C]UMP	2.6 ± 0.5	17.0 ± 11	6.5
	[^{14}C]Uridine	10.7 ± 0.8	75.8 ± 31	7.1
	[^{14}C]UDP-galactose	40.7 ± 3.5	−68[c] ± 11	−1.7
	[^{3}H]Galactose transferred	3.0 ± 0.9	130 ± 26	44
[^{14}C]UMP ($n=4$)	[^{14}C]UMP	0.679 ± 0.11	−0.80 ± 0.97	−1.2
	[^{14}C]Uridine	1.78 ± 0.11	8.1 ± 1.3	4.6
[^{3}H]Uridine ($n=2$)	[^{3}H]Uridine	3.2 ± 0	13.8 ± 2.3	4.3

Table 4. *Incubation of intact Golgi vesicles with [^{14}C]UDP-galactose and [^{3}H]UMP*
Pellet values are corrected for the contamination of the pellet with supernatant by using the same method of calculation as given for Table 3. Further details are given in the Experimental section.

Expt. no.	Solute assayed	$^{14}C/^{3}H$ in supernatant	$^{14}C/^{3}H$ in pellet	$^{14}C/^{3}H$ in pellet / $^{14}C/^{3}H$ in supernatant
1	UMP	0.527	0.680	1.3
	Uridine	0.627	1.33	2.1
	UMP+uridine combined	0.562	1.11	2.0
2	UMP	0.634	2.08	3.3
	Uridine	1.19	1.79	1.5
	UMP+uridine combined	0.935	1.84	2.0
3	UMP	1.07	1.15	1.1
	Uridine	1.43	3.12	2.2
	UMP+uridine combined	1.21	2.37	2.0
4	UMP	1.46	2.99	2.1
	Uridine	2.01	3.61	1.8
	UMP+uridine combined	1.76	3.46	2.0
5 Minus *N*-acetyl-glucosamine	UMP	0.395	0.692	1.8
	Uridine	0.520	0.681	1.3
	UMP+uridine combined	0.449	0.684	1.5

Table 5. *Pellet/supernatant $^{14}C/^{3}H$ ratios when Golgi vesicles of different volumes are incubated with [^{3}H]UMP*

Pellet / supernatant	Vesicle volume (μl/mg of protein in incubation)
−0.87	0.66
−2.21	1.17
−2.49	1.17
0.12	1.17
0.30	1.44
−1.29	1.58
0.49	1.67
0.65	1.84
0.12	2.41

Uridine also is enriched in the pellet such that the internal concentration appears greater than that in the surrounding supernatant. This is true whether UDP-galactose, UMP or uridine is the source of the uridine that is assayed after the incubation. The enrichment is greatest, however, when UDP-galactose is the source. Table 3 also shows that there is no enrichment of UDP-galactose itself in the vesicles after the incubations.

Table 4 shows how the experimental and control incubations in Table 3 are combined to be performed simultaneously in a new set of experiments. The Golgi vesicles are incubated with [^{14}C]UDP-galactose and [^{3}H]UMP in order to compare the direct uptake of UMP and uridine from the supernatant with their uptake by way of UDP-galactose. The greater uptake of UMP and uridine from UDP-galactose can be seen from the $^{14}C/^{3}H$ ratios of the pellet and the supernatant respectively. In other words, the $^{14}C/^{3}H$ ratios for both UMP and uridine in the pellet are consistently greater than the same ratios for the supernatant. The ratio of the $^{14}C/^{3}H$ ratios is always greater than unity. When UMP and uridine concentrations are summed, in order to calculate the ratio for all uridine derivatives present, the ratio of the ratios is consistently 2.0. The exception occurs when *N*-acetylglucosamine, the exogenous acceptor, is omitted. In this case, the total concentration of UMP and uridine is lower and the ratio of the $^{14}C/^{3}H$ ratios for UMP and uridine combined falls from 2.0 to 1.5.

The increase in intravesicular UMP and uridine when the starting compound is UDP-galactose occurs over vesicle concentrations ranging from less than 1% to almost 10% of the incubation volumes. In fact, all of the ratios vary independently of fairly wide intravesicular concentrations. Table 5 shows the pellet/supernatant ratios obtained for seven different Golgi vesicle preparations when [^{3}H]UMP is the starting compound as well as the compound assayed after incubation.

Discussion

The experiments presented above demonstrate directly that UDP-galactose permeates Golgi membranes for use by a galactosyltransferase. Therefore the data validate the model proposed by Kuhn & White (1975, 1976, 1977). To demonstrate this transport of UDP-galactose, a new assay was developed that can be used to measure uptake of activated galactose, in whatever form, by membrane vesicles.

The transport of activated galactose can be assayed by measuring the synthesis of *N*-acetyl-

lactosamine inside intact vesicles. When intact microsomal vesicles are incubated without α-lactalbumin, which decreases the enzyme's affinity for *N*-acetylglucosamine, and β-galactosidase, which hydrolyses any *N*-acetyl-lactosamine that may still be produced, the large difference between *N*-acetyl-lactosamine synthesis with and without detergent implies that, without detergent, the vesicles are largely intact. The percentage of broken or everted vesicles can be calculated by subtracting the galactosyltransferase activity insensitive to the inhibitors α-lactalbumin and β-galactosidase from the activity without inhibitors (Table 1, 6200 − 3270), and dividing the difference by the total activity (21600), which yields 14%, a typical value for the microsomal vesicles.

With detergent, product synthesis occurs more than 3 times more rapidly, suggesting that the vesicular membranes present a rate-limiting barrier to activated galactose. With the inhibitors present, *N*-acetyl-lactosamine synthesis occurs only within intact vesicles, and the rate of synthesis represents the movement of activated galactose across the membranes. Because *N*-acetyl-lactosamine synthesis is observed under these conditions, activated galactose must be crossing the membranes, but the form the activated galactose takes during passage is not revealed by these experiments.

When intact Golgi-enriched vesicles containing *N*-acetylglucosamine are incubated with [^{14}C]-UDP-galactose and UDP-[^{3}H]galactose, product, pimarily *N*-acetyl-lactosamine, is formed and concentrated inside the vesicles. UMP appears both inside and outside the vesicles, but is concentrated inside the vesicles. Control incubations starting with UMP give very different results, with less UMP associated with the vesicles than the minimum expected to be carried in the extravesicular volume. This condition may result from 5′-nucleotidase activity associated with the vesicles. The low uptake of UMP from free UMP shows tha UDP-galactose, and not UMP in the surrounding medium, is the source of the vesicular UMP. This result suggests that UDP-galactose is transplanted directly across Golgi membranes.

Because the data are less variable when UMP and uridine are considered together, it is likely that UMP is hydrolysed while the vesicles are being centrifuged. Ballas & Arion (1977) suggest similarly that hydrolysis of glucose 6-phosphate occurs in their experiments, because the vesicle protein is concentrated into a few microlitres during transit through the silicone oil. Such hydrolysis could convert the UMP associated with the vesicles into uridine to different extents, and thereby change the appropriate ^{14}C/^{3}H ratios in a variable fashion. Combining the data for UMP and uridine simply focuses on all uridine products released from UDP-galactose.

When *N*-acetylglucosamine is omitted from the incubation mixture, the ^{14}C/^{3}H ratio falls from 2.0 to 1.5. This decrease shows that UDP-galactose must enter the vesicles to be cleaved by galactosyltransferase. A ratio of unity would not be expected, because of endogenous acceptor amounts in these vesicles. Without *N*-acetylglucosamine, galactosyltransferase activity is decreased to one-sixth the activity with *N*-acetylglucosamine (results not shown). The change in the ratio when exogenous acceptor is omitted suggests that the increased ratio in the pellet is dependent on *N*-acetyl-lactosamine formation. Therefore it appears that specifically this enzyme, UDP-galactose: *N*-acetylglucosamine 4-galactosyltransferase (EC 2.4.1.22), is receiving activated galactose in the form of UDP-galactose.

Overall, the data support the model proposed by Kuhn & White (1975, 1976, 1977). Their model includes: (1) the transport of UDP-galactose into the Golgi; (2) the hydrolysis of UDP by a nucleoside diphosphatase; and (3) the transport of UMP out of the Golgi. The model explains the accumulation of UMP outside, rather than inside, the Golgi vesicles, when they are incubated with UDP-galactose (and a galactosyltransferase reaction is occurring inside). This effect has been observed by Kuhn & White (1977) in rat mammary-gland Golgi preparations, and by Brandan & Fleischer (1982) in rat liver Golgi vesicles.

Even the rapid technique used in these current experiments, which sediments the vesicles in as little time as 10s (Ballas & Arion, 1977), shows relatively small amounts of UMP and uridine accumulating in the vesicles in comparison with *N*-acetyl-lactosamine (Table 3). It appears that the outward movement of UMP is almost co-ordinate with the transferase reaction.

The synthesis, itself, of *N*-acetyl-lactosamine within the vesicles indicates that the galactose moiety of UDP-galactose penetrates the Golgi membranes. Passage of the activated galactose is probably rate-limiting *in vitro*. Clearly the cell could control the activities of its different transferases by regulating the availability of their substrates. Although the galactosyltransferase studied here transfers galactose to *N*-acetylglucosamine, there are other galactosyltransferases that form different linkages, and that may use different sugar donors. For some transferases, lipid–galactose intermediates might function in an alternate pathway for transport of activated galactose. For UDP-galactose: *N*-acetylglucosamine 4-galactosyltransferase, the simplest and most probable explanation is the proposed direct transport of UDP-galactose.

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