UDP-GlcNAc Transport across the Golgi Membrane: Electroneutral Exchange for Dianionic UMP†

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ABSTRACT: We have examined the coupling and charge stoichiometry for UDP-GlcNAc transport into Golgi-enriched vesicles from rat liver. In the absence of added energy sources, these Golgi vesicles concentrate UDP-GlcNAc at least 20-fold, presumably by exchange with endogenous nucleotides. Under the conditions used, extravesicular degradation of UDP-GlcNAc has been eliminated, and less than 15% of the internalized radioactivity becomes associated with endogenous macromolecules. Of the remaining intravesicular label, 85% remains unmetabolized UDP-[^{3}H]GlcNAc, and approximately 15% is hydrolyzed to [^{3}H]GlcNAc-1-phosphate. Efflux of accumulated UDP-[^{3}H]GlcNAc is induced by addition of nonradioactive UDP-GlcNAc, UMP, UDP, or UDP-galactose to the external medium. Permeabilization of Golgi vesicles causes a rapid and nearly complete loss of internal UDP-[^{3}H]GlcNAc, indicating that the results reflect transport and not binding. Moreover, transport of UDP-[^{3}H]GlcNAc into these Golgi vesicles was stimulated up to 5-fold by mechanically preloading vesicles with either UDP-GlcNAc or UMP. The response of UMP/UMP exchange and UMP/UDP-GlcNAc exchange to alterations in intravesicular and extravesicular pH suggests that UDP-GlcNAc enters the Golgi apparatus in electroneutral exchange with the dianionic form of UMP.

Many glycosylation and sulfation reactions involved in the synthesis of all types of glycoconjugates occur in the Golgi apparatus lumen and require nucleotide sugars and 3′-phosphoadenosine-5′-phosphosulfate (PAPS)[1] as glycose and sulfate donors. These molecules are formed in the cytoplasm or nucleus (Kean, 1970; Coates et al., 1980) and therefore must cross the Golgi membrane to be utilized (Fleischer, 1983; Hirschberg & Snider, 1987). Several reports (Kuhn & White, 1976; Sommers & Hirschberg, 1982; Fleischer, 1983; Yusuf et al., 1983; Capasso & Hirschberg, 1984; Barthelson & Roth, 1985; Perez & Hirschberg, 1985) have suggested that nucleotide sugars and PAPS can be transported into vesicles derived from the Golgi apparatus. Association of nucleotide sugars or PAPS with membrane vesicles appears to be saturable, mediated by genetically distinct components (Deutscher et al., 1984; Deutscher & Hirschberg, 1986; Brandli et al., 1988), specific for the Golgi, and sensitive to proteolysis at the cytoplasmic face of the vesicle [see Hirschberg and Snider (1987) for a review]. However, these studies have not ruled out binding as a cause for the measured association nor have they revealed which driving forces are responsible.

A countertransport mechanism in which cytosolic nucleotide sugar exchanges for luminal nucleoside monophosphate has been proposed to explain entry of GDP-fucose into the Golgi apparatus and removal of the GMP product from the Golgi lumen (Capasso & Hirschberg, 1984). This conclusion was based on the observation that Golgi fractions which had accumulated [^{3}H]GDP-fucose released ^{3}H when incubated with an excess of GDP-[^{14}C]fucose. Since in these studies significant degradation of luminal GDP-fucose to GMP was observed, the assumption was made that the radioactivity leaving the vesicles was [^{3}H]GMP. With the use of similar experimental results and reasoning, this countertransport hypothesis was extended to include all the nucleotide transport systems. In all these studies, however, countertransport of nucleotide sugar for nucleoside monophosphate was not rigorously demonstrated. In fact, in preliminary studies, we found that the extent of degradation of intravesicular UDP-GlcNAc depends greatly upon the procedure employed to prepare the samples for analysis. Using procedures similar to those previously reported (Sommers & Hirschberg, 1982; Capasso & Hirschberg, 1984), we found that degradation of intravesicular UDP-GlcNAc was artificially elevated. If internal breakdown of GDP-fucose (and other nucleotide sugars) had been overestimated in previous studies, the results seen could easily be explained by self-exchange of internal [^{3}H]GDP-fucose for external GDP-[^{14}C]fucose.

Since the nucleotide sugar and PAPS substrates for glycosylation and sulfation reactions within the Golgi apparatus are ionized at physiological pH, their transport into the Golgi must involve charge compensation by counterion movement coupled either directly as part of the transporter's catalytic cycle or indirectly through another ion flux across the Golgi membrane. The nucleotide sugar transport systems may therefore be sensitive to perturbations of the ion gradients across the Golgi membrane. Indeed, ionophores such as monensin and nigericin drastically alter glycosylation and sulfation within the Golgi apparatus (Tartakoff, 1983).

In this paper, we investigate the possibility that a transmembrane pH difference or electrical potential may be involved in the regulation of the nucleotide sugar transport systems. We find that transport of UDP-GlcNAc^{2-} into Golgi vesicles occurs in exchange for luminal UMP^{2-}, indicating an

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[1] Abbreviations: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; AMP-PNP, 5′-adenylyl imidodiphosphate; SDS, sodium dodecyl sulfate; SD, standard deviation.

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electroneutral countertransport mechanism which may be regulated by the internal pH of the Golgi apparatus.

Materials and Methods

Chemicals and Radiochemicals. Yeast UDP-glucose pyrophosphorylase, yeast inorganic pyrophosphatase, acetic anhydride, EPPS, HEPES, MES, AMP-PNP, 2,3-dimercaptopropanol, filipin, ovalbumin (grade V), bovine serum albumin (fraction V), nucleotide sugars, and nucleoside mono- and diphosphates were from Sigma Chemical Co. (St. Louis, MO). Acridine orange was from Aldrich Chemical Co. (Milwaukee, WI). Yeast hexokinase and rabbit muscle phosphoglucomutase were from Boehringher Mannheim Biochemicals (Indianapolis, IN). Isethionic acid (potassium salt) was from Eastman Kodak Co. (Rochester, NY). [^{14}C]Sucrose (560 mCi/mmol) was obtained from ICN Radiochemicals (Irvine, CA). [5-^{3}H]UMP (24 Ci/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). D-[6-^{3}H]Glucosamine hydrochloride (27 Ci/mmol), uridine diphospho-D-[6-^{3}H]galactose (14.5 Ci/mmol), and $^{3}H_2O$ (5 mCi/mL) were from Amersham Corp. (Arlington Heights, IL).

Preparation of Golgi Vesicles. A fraction rich in Golgi apparatus membranes was isolated from rat liver according to the procedure of Leelavathi et al. (1970) with the following modifications. "Ultra-pure" sucrose (Schwarz/Mann Biotech, Cleveland, OH) was used for all the sucrose-containing buffers in this procedure. The minced liver was homogenized by using a Polytron PTA-20S generator (Brinkmann Instruments, Westbury, NY) at setting 4 for 45 s. All the centrifugation conditions and step gradients were equivalent to those described. After collection of the final Golgi fraction (the 0.5/1.1 M sucrose interface), it was gradually diluted 2-fold (over approximately 10 min) with the potassium phosphate buffer devoid of sucrose. The Golgi membranes were then pelleted by centrifugation in a type 75Ti rotor at 35 000 rpm for 1 h at 4 °C and gently resuspended in a minimal volume of 0.25 M sucrose containing 10 mM K^+-HEPES (final pH adjusted to 7.0). Samples (75 μL) were frozen in 1.5-mL microcentrifuge tubes in liquid nitrogen and stored at −80 °C. Direct comparison of the UDP-GlcNAc transport activity of fresh and previously frozen Golgi vesicles indicated that there was no significant difference in transport activity. Intravesicular volume (under conditions of the transport assays) was determined by using [^{14}C]sucrose and $^{3}H_2O$ as described (Rottenberg, 1979), except without extraction.

Analytical Procedures. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Golgi membrane enrichment of preparations was determined by the enrichment of galactosyltransferase activity (Brew et al., 1975) with the following modification. The final reaction mixture contained 7 mg/mL ovalbumin, 20 mM $MnCl_2$, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.1), 2 mM ATP, 0.1 mM UDP-[^{3}H]galactose, and the suitably diluted sample. Multiple samples (30 μL) were spotted onto Whatman 3MM paper sheets, dried, and precipitated with trichloroacetic acid (Corbin & Reimann, 1974). After being dried, the sheets were then submerged in 10% trichloroacetic acid/1% $NaPP_i$ to precipitate the protein, washed free of trichloroacetic acid by submersions (3 × 15 min) in 95% EtOH, and then dried. The areas containing the individual samples were cut apart, and the radioactivity associated with each piece was eluted in 0.5 mL of 1% SDS and detected by scintillation spectrometry. The integrity and topography were determined by the latency of galactosyltransferase activity. Golgi vesicle preparations were typically enriched 50–70-fold over the homogenate in galactosyltransferase activity, and were >95% sealed and right-side-out. Rough endoplasmic reticulum contamination was assessed by measurement of glucose-6-phosphatase activity (Aronson & Touster, 1974). Glucose-6-phosphatase activity was enriched only 2-fold over the homogenate, with <3% yield.

Synthesis of UDP-[^{3}H]-GlcNAc. Radioactive UDP-GlcNAc was synthesized from D-[6-^{3}H]glucosamine hydrochloride either as described by Owada and Neufeld (1982) with no modifications or as described by Lang and Kornfeld (1984) with the following modifications. The UDP-glucose pyrophosphorylase and inorganic pyrophosphatase were dialyzed (together) against the dialysis buffer described. Nonradioactive glucosamine was eliminated from the reaction. Aliquots of the UDP-glucose pyrophosphorylase/inorganic pyrophosphatase mixture were added to the reaction mixture at 2-h intervals until there was no further conversion to UDP-glucosamine, as judged by migration of radioactivity on either analytical thin-layer chromatography or descending paper chromatography. In order to maximize the yield, this reaction was sometimes incubated for several days before proceeding to the acetylation step. Preparative purification of UDP-[^{3}H]GlcNAc utilized only the two descending paper chromatography systems described (system II followed by system I).

Buffers. The following buffers were used for various procedures, as indicated. SHE = 0.4 m sucrose with 10 mM HEPES and 2 mM EDTA, adjusted to the indicated pH with KOH; STKM = 0.25 M sucrose with 10 mM Tris, 150 mM KCl, and 1 mM $MgCl_2$, adjusted to the indicated pH with HCl; STEK = 0.25 mM sucrose with 10 mM Tris, 2 mM EDTA, and 150 mM KCl, adjusted to the indicated pH with HCl; STE = 0.4 M sucrose with 10 mM Tris and 2 mM EDTA, adjusted to the indicated pH with HCl; SME = 0.4 M sucrose with 10 mM MES and 2 mM EDTA, adjusted to the indicated pH with KOH; STMEK = 0.1 M sucrose with 2 mM MES, 2 mM EDTA, and 150 mM potassium isethionate adjusted to the indicated pH with Tris base; STME = 0.4 M sucrose with 2 mM MES and 2 mM EDTA adjusted to the indicated pH with Tris base; stop buffer = reaction medium devoid of dimercaptopropanol and UDP-GlcNAc.

Filtration Transport Assay. Golgi vesicles (100 μg of protein) were incubated at 30 °C in a reaction mixture (200 μL) which routinely contained buffered sucrose and 0.5 mM dimercaptopropanol, 8 μM UDP-[^{3}H]GlcNAc (232 Ci/mol), and, in some experiments, other components (as indicated in the figure legends) for the times indicated. Reactions were initiated by the addition of membranes. To terminate the transport assay, the reaction mixture was diluted 10-fold with ice-cold stop buffer, and the entire reaction mixture was poured over a Gelman GN-6 filter (Gelman Sciences, Ann Arbor, MI) under vacuum. The reaction tube was rinsed with an additional 2-mL aliquot of ice-cold stop buffer which was also poured over the filter. The filter was then removed from the vacuum and dried, and the radioactivity trapped on the filter was determined by scintillation spectrometry using 5 mL of Betafluor (National Diagnostics, Manville, NJ) for each filter.

Centrifugation Transport Assay. The centrifugation transport assay was performed essentially as described (Perez & Hirschberg, 1985), except that the total radioactivity remaining in the washed pellet was determined and considered to be a measure of total transport activity.

Ion-Exchange Chromatography. The soluble radioactive species in either filtrates or filter extracts were separated by ion-exchange chromatography on 1-mL AG1-X8 columns (formate form) (Bio-Rad Laboratories, Richmond, CA). For

analysis of the radioactivity remaining in the extravesicular medium, the reaction was terminated by diluting the mixture only 2-fold with ice-cold stop buffer and filtered, and the filtrate was stored at −20 °C until chromatographic analysis. For analysis of internal soluble radioactivity, the reaction was terminated by filtration, and the filter was placed into a 20-mL scintillation vial containing 0.5 mL of 1% SDS, 0.1 M NaCl, 0.02% NaN_3, and nonradioactive *N*-acetylglucosamine (0.5 μmol), *N*-acetylglucosamine phosphate (0.1 μmol), and UDP-GlcNAc (0.5 μmol). The vial was immediately placed on a hot plate and allowed to boil for 1.5 min. The vial containing the filter extract was then stored at −20 °C until chromatographic analysis. After application of the samples (which always contained nonradioactive *N*-acetylglucosamine, *N*-acetylglucosamine phosphate, and UDP-GlcNAc), the columns were washed with distilled water to elute *N*-acetyl-[^{3}H]glucosamine, 3 N formic acid, or 0.1 M ammonium formate (pH 7) (as indicated in the figure) to elute *N*-acetyl[^{3}H]glucosamine phosphate, and finally 15 N formic acid or 0.2 M ammonium formate (pH 7) to elute UDP-[^{3}H]-GlcNAc.

Gel Filtration Chromatography. Transport reactions which were to be analyzed for transfer of radioactivity to macromolecular species were terminated as follows. The reaction was brought to 1% SDS and 0.1 M NaCl [with nonradioactive *N*-acetylglucosamine (0.5 μmol), *N*-acetylglucosamine phosphate (0.1 μmol), and UDP-GlcNAc (0.5 μmol) added] and was immediately boiled for 5 min. A sample (200 μL) of the reaction mixture was chromatographed either immediately or after storage at −20 °C on a column (0.7 cm × 18.8 cm) of Sephadex G-50 (fine) (Sigma Chemical Co.) equilibrated in 1% SDS, 0.1 M NaCl, and 0.02% NaN_3. Fractions (0.37 mL) were collected, and the amount of radioactivity eluting in each fraction was determined. The void volume (V_o) of the column was determined by using blue dextran (≥10 kDa). The internal volume (V_i) of the column was determined by using ^3H_2O. There was a 13-fraction separation between the V_o and the V_i.

Mechanical Loading Protocol. The mechanical loading protocol, which has been described in detail elsewhere (Waldman & Rudnick, 1989), is described briefly below. Throughout the procedure, the Golgi vesicles were kept at 0–4 °C. Golgi vesicles (approximately 1.7 mg of protein) were gently mixed with the desired intravesicular medium ("loading buffer"; as indicated in the figure legends) in a final volume of 220 μL in a 5 × 20 mm polyallomer centrifuge tube (Beckman Instruments, Palo Alto, CA) and centrifuged at 178000*g* for 5 min at 4 °C in the 30° rotor in a Beckman Airfuge. The supernatant was removed, and the pellet was quickly and rigorously resuspended with a 50-μL Hamilton syringe in a minimal volume of the corresponding loading buffer. Small samples (2 μL) of the mechanically loaded vesicles were routinely used in the filtration transport assay described above to minimize the addition of loading medium components to the reaction mixture. The protein concentration of the supernatant and pellet was determined for each individual resuspension; the pellet usually contained 76–91% of the recovered protein, and the recoveries were usually 75–92% of the total protein. The loading buffers always contained, in addition to the indicated buffer and other additions (such as UMP, UDP-GlcNAc, etc.), 0.5 mM dimercaptopropanol and AMP-PNP, unless otherwise indicated.

Acridine Orange Acidification Assay. Golgi vesicles (330 μg of protein) were incubated at room temperature in a cuvette containing 2 mL of 10 mM Tris–EPPS (pH 8.5), 0.4 M sucrose with 6 μM acridine orange, 25 mM KCl, and 5 mM ATP. The relative fluorescence of the mixture was measured by using an excitation wavelength of 490 nm and an emission wavelength of 526 nm on a Perkin-Elmer LS-5B luminescence spectrometer. After the base-line fluorescence stabilized, acidification was initiated by the addition of 6 mM $MgSO_4$, and the relative fluorescence was monitored. After there was no further detectable quenching, 10 mM NH_4Cl was added to neutralize the vesicle interior and reverse the quenching.

Results

To determine the charge stoichiometry of UDP-GlcNAc transport, it was important first to establish that the UDP-[^{3}H]GlcNAc that associated with Golgi vesicles was actually transported into the vesicle lumen. Our preliminary experiments revealed discrepancies with a previously published report (Perez & Hirschberg, 1985) of extensive metabolism of UDP-[^{3}H]GlcNAc associated with Golgi vesicles. We therefore sought conditions to minimize metabolism of internal and external UDP-[^{3}H]GlcNAc.

Evidence That UDP-GlcNAc Is Transported without Significant Metabolism, Transfer to Endogenous Acceptors, or Binding. To avoid the multiple time-consuming manipulations associated with the centrifugation assay (Perez & Hirschberg, 1987) previously used to measure nucleotide sugar transport into Golgi vesicles, we utilized a rapid filtration assay (see Materials and Methods for details). This assay is very rapid (typically requiring ≤30 s from the end of the incubation period to removing the washed filter from the vacuum apparatus) and gives the same results when compared directly with the centrifugation assay (data not shown). Thus, one potential problem with filtration, namely, that the vesicles might break on the filter and release their accumulated solutes, is not a significant factor. The association of radioactivity with the vesicles as measured by filtration was saturable with respect to both time and substrate concentration (Figure 1), with a K_m of 7.13 ± 1.62 μM and a V_{max} of 915 ± 87 pmol mg^{-1} min^{-1} for UDP-GlcNAc. This association was also temperature dependent, with transport being decreased at 0 °C (7.6% of that measured at 30 °C after a 10-min incubation).

(*A*) *Metabolism.* We first determined the extent of UDP-[^{3}H]GlcNAc metabolism in the external medium. Radioactivity in the external medium was analyzed by ion-exchange chromatography to separate the radioactive sugar, sugar–phosphate, and nucleotide sugar species, and the profiles are shown in Figure 2. Before incubation with membrane vesicles, greater than 99% of the radioactivity in the reaction mixture eluted with 15 N formic acid (Figure 2A, squares), as did the UDP-[^{3}H]GlcNAc standard on a parallel column. After a 10-min incubation at 30 °C, the external radioactivity in a reaction mixture containing Golgi vesicles eluted predominantly (approximately 80%) at the 3 N formic acid step (Figure 2A, circles), where the *N*-acetyl[^{3}H]glucosamine phosphate standard elutes from this column. The addition of either ATP (in the absence of Mg^{2+}) or AMP-PNP (a nonhydrolyzable analogue of ATP) resulted in essentially complete protection of the external UDP-[^{3}H]GlcNAc (Figure 2A, upright and inverted triangles, respectively), with no effect on the total amount of UDP-[^{3}H]GlcNAc transport (data not shown). Addition of 2 mM EDTA to the external medium also significantly protected (87%) the external UDP-[^{3}H]-GlcNAc from degradation (data not shown).

Figure 2B demonstrates that, under conditions (+AMP-PNP) which protected the medium UDP-[^{3}H]GlcNAc, Golgi vesicle associated UDP-[^{3}H]GlcNAc is not significantly metabolized. After a 20-min incubation at 30 °C, vesicles were

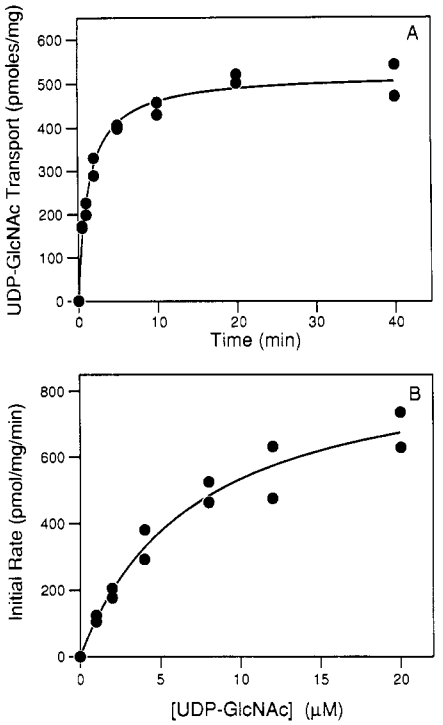

FIGURE 1: Time and substrate concentration dependence of UDP-[^{3}H]GlcNAc transport into Golgi vesicles. (A) Golgi vesicles were incubated with UDP-[^{3}H]GlcNAc in SHE buffer at pH 7.5 containing 0.1 μM AMP-PNP for the times indicated, and transport of UDP-[^{3}H]GlcNAc into the vesicles (picomoles per milligram of protein) was measured as described under Materials and Methods. Reactions were initiated by the addition of membranes and were terminated by filtration. The amount of radioactivity trapped on the filter at 0 min of incubation (background) was subtracted from each sample. (B) UDP-GlcNAc transport was assayed as described above, except that the concentrations of UDP-[^{3}H]GlcNAc were varied as indicated in the figure, and the incubations were for 15 s to determine the initial rate of transport. The amount of radioactivity trapped on the filter at 0 min of incubation for each concentration of UDP-GlcNAc was subtracted from the corresponding experimental values.

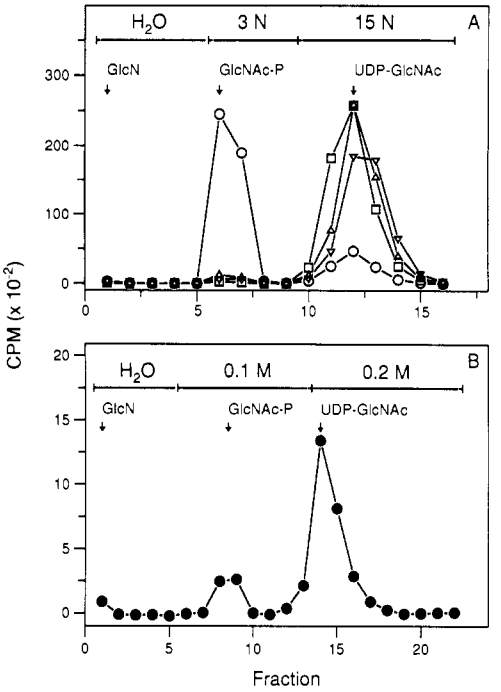

FIGURE 2: Analysis of external and internal radioactivity by ion-exchange chromatography. (A) Golgi vesicles were incubated in the UDP-[^{3}H]GlcNAc transport reaction mixture (STKM buffer at pH 7.5) as described in the legend to Figure 1 (panel A), except that the reaction mixture contained AMP-PNP only where indicated and was terminated and chromatographed as described under Materials and Methods. Nonradioactive *N*-acetylglucosamine, *N*-acetylglucosamine phosphate, and UDP-GlcNAc were added to the filtrates prior to chromatography. The elution steps are indicated in the figure. (Squares) Fresh unused reaction cocktail (containing UDP-[^{3}H]-GlcNAc); (circles) filtrate from a 10-min UDP-GlcNAc transport reaction; (triangles) filtrate from a 10-min UDP-GlcNAc transport reaction containing 1 mM ATP; (inverted triangles) filtrate from a 10-min UDP-[^{3}H]GlcNAc transport reaction containing 1 mM AMP-PNP. (B) Golgi vesicles were incubated for 20 min in the UDP-GlcNAc transport reaction mixture (SHE buffer at pH 7.5) as described in the legend to Figure 1 (panel A), except that the reaction mixture contained 1 mM AMP-PNP and the reaction was terminated and analyzed by ion-exchange chromatography as described under Materials and Methods. The elution steps are indicated in the figure. The elution positions of radioactive standards are indicated in the figure. GlcN, glucosamine; GlcNAc-P, *N*-acetylglucosamine phosphate; UDP-GlcNAc, uridine 5′-diphospho-*N*-acetylglucosamine.

collected by filtration, and the associated radioactive solutes were released from the filter by boiling in 1% SDS and analyzed by ion-exchange chromatography (Figure 2B). Approximately 85% of the soluble radioactivity associated with the vesicles was intact UDP-[^{3}H]GlcNAc, with some degradation to *N*-acetyl-[^{3}H]glucosamine phosphate and very little degradation to *N*-acetyl-[^{3}H]glucosamine.

(*B*) *Transfer to Endogenous Acceptors.* To evaluate the extent of *N*-acetyl-[^{3}H]glucosamine transfer from UDP-[^{3}H]GlcNAc to endogenous macromolecular acceptors, we incubated vesicles with UDP-[^{3}H]GlcNAc, terminated further *N*-acetylglucosaminyltransferase activity by boiling with SDS, and separated the radiolabeled components by gel filtration chromatography. This chromatographic procedure completely separated the macromolecular radioactive species (≥10 kDa) from the unused UDP-[^{3}H]GlcNAc and its degradation products. Figure 3 shows the time course of UDP-[^{3}H]-GlcNAc association with Golgi vesicles and the amount of [^{3}H]GlcNAc transfer to endogenous acceptors determined by gel filtration chromatography of parallel assays. Less than 15% of the vesicle-associated radioactivity eluted with the macromolecular species (presumably glycoproteins and glycolipids) even at the longest incubation period assayed.

(*C*) *Binding.* We next determined if the UDP-[^{3}H]GlcNAc associated with Golgi vesicles was free in the vesicle lumen. Vesicles were incubated in reaction medium containing different amounts of sucrose (to vary the osmolarity of the medium), and the accumulation of UDP-[^{3}H]GlcNAc was measured. Figure 4A shows the steady-state level of vesicle-associated UDP-[^{3}H]GlcNAc plotted against the reciprocal of the medium osmolarity. The linear relationship extrapolates to zero uptake at infinite osmolarity, as predicted for solute transport into the vesicle lumen (Kessler & Toggenburger, 1979). In further support of this conclusion, UDP-[^{3}H]-GlcNAc associated with Golgi vesicles was rapidly and almost completely released (Figure 4B) by filipin, a polyene antibiotic (Kinsky, 1970; De Kruijff et al., 1974) which has been previously shown to be an effective permeabilizing agent for Golgi apparatus membranes (Fleischer, 1983).

Considering the small amount of metabolism and transfer to endogenous acceptors, over 70% of the vesicle-associated UDP-[^{3}H]GlcNAc appears to be free in the vesicle interior. From the measured intravesicular volume (3 μL/mg of membrane protein), we calculate that UDP-[^{3}H]GlcNAc transport leads to at least a 20-fold concentration gradient. Since this is not a highly purified preparation of Golgi apparatus vesicles and all the vesicles may not contain the UDP-GlcNAc transporter, the actual concentration of UDP-GlcNAc within those vesicles capable of UDP-GlcNAc transport is probably higher than this estimate. Clearly, an

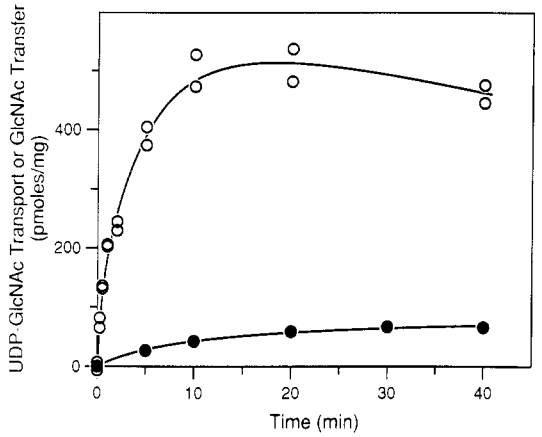

FIGURE 3: Accumulation of total and macromolecular radioactivity by Golgi vesicles. All transport reactions were incubated as described under Materials and Methods in SHE buffer at pH 7.5 containing 0.1 mM AMP-PNP for the times indicated. For each time point, triplicate reaction mixtures were incubated. Two of each were terminated by filtration to determine total transport at each time point. The other reaction was terminated and analyzed by gel filtration chromatography as described under Materials and Methods. The amount of radioactivity eluting with the void volume (fractions 8–14) for each sample was determined. (Open circles) Total radioactivity trapped on filter; (closed circles) macromolecular radioactivity.

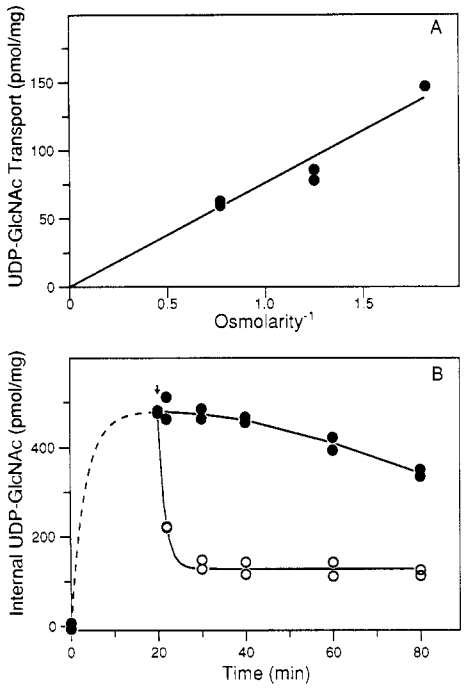

FIGURE 4: UDP-[^{3}H]GlcNAc is transported into the Golgi vesicle lumen and remains soluble. UDP-[^{3}H]GlcNAc transport into Golgi vesicles incubated in the indicated reaction mixtures was measured as described under Materials and Methods. (A) The sucrose concentration of the reaction mixture (STKM buffer at pH 7.5) was adjusted to vary the osmolarity. The steady-state level of UDP-[^{3}H]GlcNAc uptake was measured for several osmolarities and plotted against the reciprocal of the osmolarity of the reaction medium. (B) Golgi vesicles were incubated in STEK buffer at pH 7.5 (supplemented with 1 mM AMP-PNP) for 20 min prior to the addition of filipin (50 μg/assay in 5 μL of dimethylformamide) or dimethylformamide alone (5 μL). The reactions were terminated by filtration at the times indicated. The arrow indicates the time of addition of filipin in dimethylformamide (open circles) or dimethylformamide alone (closed circles). The dashed line represents typical update time course determined from previous experiments.

endogenous driving force is utilized to accumulate UDP-GlcNAc to these levels.

Mechanism of UDP-GlcNAc Transport into Golgi Vesicles.

Table I: Induced Efflux of Internal UDP-[^{3}H]GlcNAc[a]

countertransport "substrate"	UDP-[^{3}H]GlcNAc remaining in vesicles (pmol/mg)	efflux (%)
no addition	378.6	0
UDP-GlcNAc	85.4	77.4
UMP	117.5	69.0
UDP	153.4	59.5
UDP-Gal	187.4	50.5
AMP	349.2	7.8

[a] Golgi vesicles were incubated for 5 min (to steady state) in the UDP-[^{3}H]GlcNAc transport reaction mixture as described under Materials and Methods, in STEK buffer at pH 7.5 and supplemented with 1 mM AMP-PNP. Additions (to give 1 mM final external concentrations of each compound) were then made to the external medium, and the reactions were incubated for an additional 30 s before termination by filtration as described under Materials and Methods.

(*A*) *Countertransport for UMP.* Capasso and Hirschberg (1984) proposed obligatory exchange of lumenal GMP for cytoplasmic GDP-fucose to account for removal of the GMP product from the Golgi lumen as more glycose donor enters. This conclusion was based on the observation that Golgi fractions which had accumulated [^{3}H]GDP-fucose released ^{3}H when incubated with an excess of GDP-[^{14}C]fucose and the assumption that the radioactivity leaving the vesicles represented GMP formed by luminal metabolism. Our results (see Figure 2B) indicate that there is very little internal degradation of UDP-GlcNAc during the transport assay period. We found that other methods used to terminate the transport reaction and prepare the sample for analysis (methods which involve keeping the vesicles at 0–4 °C for 15–30 min) artifactually degrade internal UDP-[^{3}H]GlcNAc (data not shown). Our results suggest that the luminal degradation of GDP-fucose (and other nucleotide sugars) during the transport assay may have been overestimated in previous studies and therefore have led us to reexamine the nucleotide sugar/nucleoside monophosphate countertransport hypothesis in further detail.

To evaluate the possibility that internal UDP-GlcNAc (or its metabolites) acts as a driving force, we artificially imposed gradients of UDP-GlcNAc, UDP-Gal, ATP, AMP, UDP, UMP, uridine, or uracil and measured their ability to drive UDP-[^{3}H]GlcNAc efflux and influx. Table I shows the effect of externally added nucleotides on UDP-[^{3}H]GlcNAc efflux. Golgi vesicles which had accumulated UDP-[^{3}H]GlcNAc lost radioactivity when exposed to an inwardly directed gradient of some nucleoside derivatives (Table I). UDP-GlcNAc was most effective, as would be expected for self-exchange. UDP-Galactose, although less effective, also caused efflux. Of the nucleotides tested, UMP caused the most rapid efflux, UDP-induced efflux was somewhat slower, and AMP had almost no effect.

For influx measurements, Golgi vesicles that had been loaded by centrifugation and resuspension (see Materials and Methods for details) with the desired solutes were diluted into medium containing UDP-[^{3}H]GlcNAc. As shown in Figure 5 (panels A and B), both internal UDP-GlcNAc (Figure 5A) and internal UMP (Figure 5A,B) markedly stimulate UDP-[^{3}H]GlcNAc accumulation relative to vesicles loaded with only the assay buffer. Loading vesicles with ATP (Figure 5A) or with uridine or uracil (Figure 5B) did not stimulate UDP-[^{3}H]GlcNAc accumulation relative to buffer-loaded controls. In a separate experiment, we determined that the UMP loaded into these vesicles is not degraded under these conditions (data not shown). These results directly and conclusively demonstrate that UDP-GlcNAc exchanges for luminal UMP.

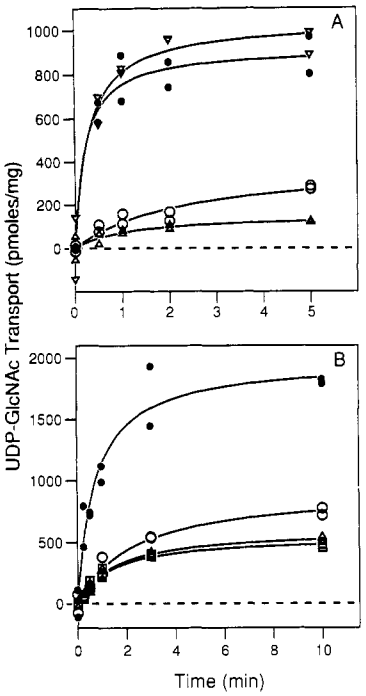

FIGURE 5: Mechanical loading of Golgi vesicles. Golgi vesicles were mechanically loaded (see Materials and Methods) with either STE buffer at pH 7.5 containing no AMP-PNP (panel A) or SHE buffer at pH 7.5 containing 6 μM AMP-PNP (panel B) and 1 mM of the desired potential countertransport substrates, as described under Materials and Methods. The vesicles used for the experiment shown in panel A were preincubated in STE buffer containing 10% glycerol and collected by centrifugation prior to the mechanical loading procedure. Samples (2 μL containing 40–50 μg of protein) were assayed for UDP-[^{3}H]GlcNAc transport activity as described in the legend to Figure 1, with 1 μM AMP-PNP in the reaction medium. (Panel A) (open circles) Buffer-loaded control; (closed circles) UMP; (inverted triangles) UDP-GlcNAc; (upright triangles) ATP. (Panel B) (open circles) Buffer-loaded control; (closed circles) UMP; (squares) uridine; (triangles) uracil.

Table II: Effect of Internal and External pH on UDP-GlcNAc Transport into UMP-Loaded Vesicles[a]

	initial rate of transport (pmol mg^{-1} min^{-1}) for external pH of	
internal pH	5.45	7.50
5.45	28.98 (±38.20)[b]	56.24 (±81.06)
7.50	516.08 (±7.64)	367.62 (±42.15)

[a] Standard deviation. [b] Golgi vesicles were mechanically loaded with either SME buffer at pH 5.45 or SHE buffer at pH 7.50, both containing 6 mM AMP-PNP (to prevent degradation of the UMP) and with or without 1 mM UMP, as described under Materials and Methods. Samples (2 μL containing 40–50 μg of protein) were assayed for 15 s to measure the initial rate of UDP-[^{3}H]GlcNAc transport as described under Materials and Methods. The external medium (200 μL) was similarly buffered at either pH 5.45 or pH 7.50, as indicated, and contained 1 μM AMP-PNP. The values represent the average of duplicates, after the subtraction of the corresponding buffer-loaded (without UMP) background values.

(B) Electroneutral Countertransport of UDP-GlcNAc^{2-} for UMP^{2-}. UDP-GlcNAc ($pK_a \sim 2$) could potentially exchange with either the monoanionic or the dianionic forms of UMP ($pK_a = 6.4$). We have used three experimental approaches to determine which ionic form of UMP is utilized.

First, we measured exchange of internal UMP with external UDP-[^{3}H]GlcNAc at high and low internal and external pH. As shown in Table II, vesicles loaded with UMP at pH 5.45 transported less UDP-[^{3}H]GlcNAc than those loaded with UMP at pH 7.50, consistant with UMP^{2-} being the ionic form involved in the countertransport reaction. The highest stimulation of UDP-[^{3}H]GlcNAc transport by internal UMP occurred when the internal pH was 7.50 and the external pH was 5.45. This transmembrane pH difference is expected to result in a greater driving force for UMP^{2-} efflux since the externalized UMP^{2-} (which could compete with UDP-GlcNAc for influx) will be protonated to the monoanionic form in the acidic external medium.

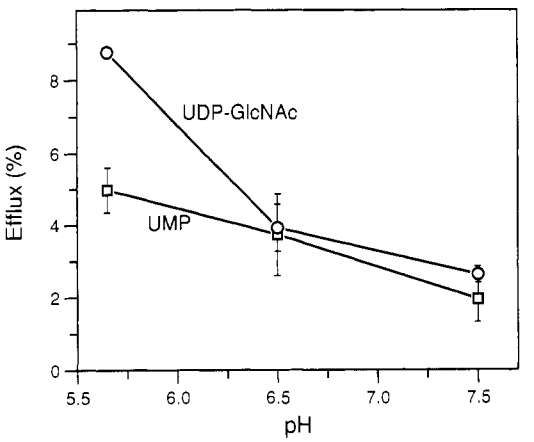

FIGURE 6: UDP-GlcNAc-induced and UMP-induced efflux of UDP-[^{3}H]GlcNAc from Golgi vesicles. Golgi vesicles were incubated in SHE buffer at pH 7.5 containing 1 mM AMP-PNP as described under Materials and Methods and allowed to take up UDP-[^{3}H]-GlcNAc to steady state (5 min). The reactions were then diluted 10-fold with buffer containing either 1 μM UDP-GlcNAc or 1 μM UMP at the pH values indicated. SHE buffer was used for the dilutions at pH 7.50, while SME buffer was used for the dilutions at pH 6.50 and 5.65. All dilution buffers contained 0.1 mM AMP-PNP and 0.5 μM dimercaptopropanol. The initial rates of efflux were measured at 5 s after dilution. Under these conditions, UDP-[^{3}H]-GlcNAc efflux was linear with external UDP-GlcNAc or UMP concentration and time. Each data point represents the average (±SD) of two separate experiments, each with four–five data points per condition. Error bars are included only where they exceed the symbol. The initial rate of efflux is the increase in radioactivity efflux relative to diluted controls (without external UMP or UDP-GlcNAc). (Circles) UDP-GlcNAc-induced UDP-[^{3}H]GlcNAc efflux; (squares) UMP-induced UDP-[^{3}H]GlcNAc efflux.

Using a second experimental approach, we determined the effect of external pH on the ability of external UMP to exchange with internal UDP-[^{3}H]GlcNAc. Golgi vesicles which had accumulated UDP-[^{3}H]GlcNAc to steady state were diluted with medium containing UMP at either pH 5.65, 6.50, or 7.50, and the initial rate of UDP-[^{3}H]GlcNAc efflux was measured. As a control for nonspecific effects of external pH on the UDP-GlcNAc transporter protein, external UDP-GlcNAc (which is not protonated throughout this pH range) was substituted for external UMP. Figure 6 shows that both UMP- and UDP-GlcNAc-induced UDP-[^{3}H]GlcNAc efflux increase as the external pH is decreased from 7.50 to 5.65. However, at pH 5.65, UMP-induced UDP-[^{3}H]GlcNAc efflux is diminished relative to the control (UDP-GlcNAc/UDP-GlcNAc exchange). Lowering the external pH alters the relative concentrations of UMP^{2-} and UMP$^-$ in the external medium, and only at the lowest external pH tested is UMP$^-$ the predominant form of UMP in the medium. These results, therefore, suggest that UMP$^-$ is a poor substrate for UDP-GlcNAc countertransport. It should be noted that in both this and the previous experiment (Table II), UMP/UDP-GlcNAc exchange was enhanced (relative to the corresponding controls) when UMP moved out of an alkaline compartment rather than an acidic compartment.

The third approach involved artificial generation of a transmembrane pH difference using nigericin. Golgi vesicles were loaded with UMP and K^+ ions at pH 5.26, where most

Table III: Effect of a Nigericin-Generated pH Gradient on UDP-GlcNAc/UMP and UMP/UMP Exchange[a]

reaction conditions	nigericin	UDP-GlcNAc transport (pmol/mg)	UMP transport (pmol/mg)
$[K^-]_{in} > [K^+]_{out}$	−	382.81 (±66.87)	531.41 (±55.91)
	+	191.91 (±19.16)	679.24 (±38.89)
$[K^+]_{in} = [K^+]_{out}$	−	495.21 (±84.87)	759.29 (±54.19)
	+	463.06 (±8.55)	817.65 (±15.92)

[a] Golgi vesicles were mechanically loaded with or without 1 mM UMP in STMEK buffer at pH 5.26 (containing 6 mM AMP-PNP and K^+) as described under Materials and Methods. Each pool of loaded vesicles was divided equally, and 0.8 μL of 4 mM nigericin (in 95% EtOH; final concentration of 40 μM nigericin and 0.95% EtOH) or 0.8 μL of 95% EtOH alone was added to one sample for each loading condition. Samples (2 μL containing approximately 50 μg of protein) were assayed for UDP-[^{3}H]GlcNAc transport or [^{3}H]UMP transport for 2 min as described under Materials and Methods, using reaction medium buffered either without K^+ (STME buffer) or with K^+ (STMEK buffer) at pH 5.26, as indicated, and containing 1 mM AMP-PNP. The data represent the average of triplicates (±SD), after subtraction of the corresponding buffer-loaded (without UMP) background values.

of the UMP is monoanionic, and then assayed at the same pH for UDP-[^{3}H]GlcNAc transport in either the presence or the absence of external K^+. In the absence of external K^+, nigericin converts the K^+ gradient into a transmembrane pH difference (inside acid) by H^+/K^+ exchange (Pressman, 1976). As shown in Table III, this nigericin-induced acidification decreases the ability of vesicles loaded with UMP to concentrate UDP-[^{3}H]GlcNAc, presumably by inhibiting the conversion of internal monoanionic UMP to the dianionic form. The effect of nigericin requires a K^+ gradient and thus is not observed in the presence of external K^+. As a control, we measured accumulation of [^{3}H]UMP under the same conditions. In this case, exchange will not lead to net H^+ appearance on the inside and disappearance from the medium as predicted for exchange of the dianionic forms of UMP and UDP-GlcNAc. The data in Table III show that the nigericin-induced transmembrane pH difference does not inhibit UMP/UMP exchange in either the presence or the absence of external K^+.

Discussion

The results in this paper demonstrate that rat liver Golgi vesicles transport UDP-GlcNAc without extensive degradation and that cytoplasmic UDP-GlcNAc probably exchanges for luminal dianionic UMP. Some of our results are contrary to those of Perez and Hirschberg (1985) where significant metabolism of UDP-GlcNAc both in the reaction medium and associated with the vesicles was reported. In the previous study (Perez & Hirschberg, 1985), approximately 30% of the external radioactive UDP-GlcNAc and approximately 75% of the internal soluble radioactive UDP-GlcNAc were degraded (mostly to UMP and GlcNAc-1-phosphate) after a 5-min incubation at 30 °C. Furthermore, approximately 25% of the vesicle-associated label was associated with acid-insoluble material (endogenous acceptors). In this paper, we demonstrate that degradation of external UDP-GlcNAc is blocked by addition of either ATP or AMP-PNP to the external medium and that luminal UDP-GlcNAc is degraded much less than previously reported.

Our results indicate that Golgi vesicles concentrate UDP-GlcNAc at least 20-fold in the absence of added energy sources. Since the preparation is likely to contain vesicles from non-Golgi sources also, the true concentration gradient is probably higher. The most likely driving force for this accumulation is a concentration gradient of endogenous nucleotides across the Golgi membrane. Rat liver Golgi vesicles have previously been found to contain significant amounts (0.1–6.3 nmol/mg of protein) of UMP, CMP, AMP, UDP, and perhaps UDP-Gal (Fleischer, 1981). On the basis of these estimated in vivo amounts and our intravesicular volume measurements on Golgi vesicles, this would at least be equivalent to 0.03–2.1 mM concentrations of these nucleoside derivatives. Except possibly for UDP-galactose (Brandan & Fleischer, 1981), all of the nucleotide sugar transport systems studied have apparent K_m values in the 1–10 μM range (4), suggesting that the luminal concentrations of nucleotide monophosphates in the Golgi apparatus should be sufficient to drive nucleotide sugar/nucleoside monophosphate countertransport systems at V_{max}. The glycosyltransferases which utilize the nucleotide sugars in the Golgi apparatus have apparent K_m values in the 0.1–1 mM range (Schachter et al., 1983) and in fact can be inhibited by the nucleoside di- and monophosphate products they generate (Khatra et al., 1974; Kuhn & White, 1977; Brandan & Fleischer, 1982). Countertransport of luminal monophosphates for cytoplasmic nucleotide sugars may also serve to minimize such inhibition in vivo.

The ability to artificially generate transmembrane gradients of nucleotides and nucleotide sugars under conditions minimizing substrate metabolism has allowed us to evaluate the coupling and charge stoichiometry of UDP-GlcNAc transport into the Golgi apparatus. The simplest mechanism consistent with our results is electroneutral exchange of cytoplasmic UDP-GlcNAc with dianionic UMP within the Golgi lumen. Perhaps the strongest prediction of this mechanism (verified by the data in Table III) is that a transmembrane pH difference (inside acid) should inhibit UDP-GlcNAc, but not UMP, influx at low pH. Under these conditions, exit of UMP^{2-} requires dissociation of an H^+ ion from internal UMP^- and should be followed by reprotonation of UMP^{2-} in the external medium. The resultant net entry of one H^+ ion is balanced by the exit of one H^+ ion when internal UMP exchanges with external [^{3}H]UMP. However, when UMP exit is coupled to entry of UDP-[^{3}H]GlcNAc (which is dianionic throughout the physiological pH range), net H^+ uptake should result. Luminal acidification should inhibit UMP/UDP-GlcNAc exchange by opposing this formal H^+ influx. Another prediction of this electroneutral mechanism is that UMP/UDP-GlcNAc exchange should be insensitive to the transmembrane electrical potential. As predicted, imposition of a K^+ diffusion potential using valinomycin at neutral pH had no effect on exchange (data not shown). Although UMP-UDP-GlcNac stoichiometry was not directly measured, electroneutral exchange suggests 1:1 stoichiometry.

Although a countertransport hypothesis predicts that UDP-GlcNAc and UMP should cause efflux by exchanging with internal UDP-[^{3}H]GlcNAc, it does not explain why UDP-galactose and UDP also caused significant efflux (see Table I). UDP-Galactose and UDP-GlcNAc are thought to be transported by separate systems (Deutscher et al., 1984; Deutscher & Hirschberg, 1986; Brandli et al., 1988). There are several possible explanations for the observed effects of UDP-galactose and UDP. First, the UDP-galactose-induced UDP-[^{3}H]GlcNAc efflux may be an indirect result of UDP-galactose transport into these vesicles. If UDP-[^{3}H]GlcNAc accumulation is maintained by an outwardly directed gradient of UMP, then UDP-galactose/UMP exchange could decrease the UMP gradient and decrease the driving force holding UDP-[^{3}H]GlcNAc inside. Alternatively, we cannot rule out

the possibility that there is a small amount of UDP or UDP-galactose hydrolysis to UMP under these conditions and that the effect is actually due to UMP. Although 8 mM UDP-GlcNAc is quite stable in the reaction medium under these conditions, hydrolysis of only about 0.1% of the 1 mM UDP or UDP-galactose could give rise to enough UMP to produce the results shown in Table I.

An H^+-pumping ATPase is found in the membrane of many intracellular organelles (Mellman et al., 1986; Rudnick, 1986). Little is known about the role of this enzyme in the Golgi apparatus. Sorting and processing steps are in some manner dependent on the action of the vacuolar H^+-ATPase, since Chinese hamster ovary cell mutants which are less able or unable to acidify endocytic vesicles demonstrate alterations in glycoprotein processing as well (similar to those seen in the parental cell line treated with monensin) (Robbins et al., 1984). The trans Golgi and the trans Golgi network have been shown to be somewhat acidic (Anderson & Pathak, 1985; Orci et al., 1985, 1986; Schwartz et al., 1985; Mellman et al., 1986), and the trans Golgi is thought to be the site at which monensin and chloroquine affect sorting processing and movement of proteins by neutralizing the cisternae of this organelle (Griffith et al., 1983; Moore et al., 1983; Quinn et al., 1983; Boss et al., 1984; Ledger & Tanner, 1984; Griffith & Simons, 1986; Wagner et al., 1986). Nonetheless, the specific role(s) of acidification in this organelle is (are) not clear.

From the results presented in this paper, it is unlikely that ATP-driven acidification is directly coupled to UDP-GlcNAc accumulation in the Golgi apparatus. At neutral pH, exchange of the dianionic forms of UMP and UDP-GlcNAc should be neither enhanced nor inhibited by the transmembrane electrical potential and pH difference which result from H^+ pumping. Only at acidic pH, where UMP exists predominantly as the monoanion, will acidification affect transport. Such inhibition was observed in Table III when the pH difference is imposed by using nigericin. This prediction is consistent also with the previously reported inability of ATP to affect nucleotide sugar transport at neutral pH (Capasso & Hirschberg, 1984), a phenomenon we have reproduced (data not shown). This lack of an ATP effect is not due to loss or denaturation of the H^+ pump during vesicle isolation since we observed ATP-driven acidification in this preparation using acridine orange fluorescence quenching (data not shown).

Because UMP has a pK_a of 6.4 (very near physiological pH), it may be that the various Golgi cisternae contain different concentrations of UMP^{2-} due to acidification by the H^+-pumping ATPase thought to be present in some Golgi regions (Glickman et al., 1983; Zhang & Schneider, 1983; Anderson & Pathak, 1985; Orci et al., 1985, 1986; Schwartz et al., 1985; Mellman et al., 1986). Most *N*-acetylglucosaminyltransferase activity is thought to take place in the cis and medial Golgi (Dunphy & Rothman, 1983, 1985), and our findings indicate that a neutral pH in these cisternae should promote UDP-GlcNAc/UMP countertransport provided the transporter is present in these cisternae. Although it is generally thought that the nucleotide sugar transporters, like many glycosyltransferases (Kornfeld & Kornfeld, 1985; Farquhar, 1986), are localized to specific cisternae of the Golgi apparatus, there is currently no evidence to support such localization. The UDP-GlcNAc transporter may in fact be present throughout the Golgi apparatus cisternae and may instead be regulated by the pH of the Golgi cisternae, being more active in the cis Golgi, where UMP^{2-} would be the predominant form of UMP present, and less active in the more acidic trans Golgi, where UMP^- would be the predominant form of UMP present. Although similar studies have not yet been performed with the other UDP-sugars, it is possible that other transporters, such as the UDP-galactose transporter, utilize UMP^- as their countertransport substrate and thus are more active in distal (and more acidic) cisternae of the Golgi apparatus. Nucleotide sugar transport (and thus glycosylation reactions which utilized the luminal nucleotide sugars) may be regulated by acidification of the Golgi apparatus due to the resident H^+-pumping ATPase activity.

Acknowledgments

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Registry No. UDP-GlcNAc, 528-04-1; UDP-[^{3}H]GlcNAc, 123857-49-8; 5′-UMP, 58-97-9; 5′-UDP, 58-98-0; 5′-AMP, 61-19-8; UDP-galactose, 2956-16-3; dianionic UMP, 5690-54-0.

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Melittin Binding to Mixed Phosphatidylglycerol/Phosphatidylcholine Membranes†

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ABSTRACT: The binding of bee venom melittin to negatively charged unilamellar vesicles and planar lipid bilayers composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) was studied with circular dichroism and deuterium NMR spectroscopy. The melittin binding isotherm was measured for small unilamellar vesicles containing 10 or 20 mol % POPG. Due to electrostatic attraction, binding of the positively charged melittin was much enhanced as compared to the binding to neutral lipid vesicles. However, after correction for electrostatic effects by means of the Gouy-Chapman theory, all melittin binding isotherms could be described by a partition equilibrium with the same surface partition constant of $K_P = (4.5 \pm 0.6) \times 10^4$ M^{-1}. It was estimated that about 50% of the total melittin surface was embedded in a hydrophobic environment. The melittin partition constant for small unilamellar vesicles was by a factor of 20 larger than that of planar bilayers and attests to the tighter lipid packing in the nonsonicated bilayers. Deuterium NMR studies were performed with coarse lipid dispersions. Binding of melittin to POPC/POPG (80/20 mol/mol) membranes caused systematic changes in the conformation of the phosphocholine and phosphoglycerol head groups which were ascribed to the influence of electrostatic charge on the choline dipole. While the negative charge of phosphatidylglycerol moved the N^+ end of the choline $^-P-N^+$ dipole toward the bilayer interior, the binding of melittin reversed this effect and rotated the N^+ end toward the aqueous phase. No specific melittin-POPG complexes could be detected. The phosphoglycerol head group was less affected by melittin binding than its choline counterpart.

Melittin, the main component of bee venom, is a cationic, amphiphilic peptide which binds to membranes and, at higher concentrations, disrupts the bilayer structure [cf. Batenburg et al. (1988), Altenbach and Hubbell (1988), and Dufourc et al. (1986a,b) and references cited therein]. Melittin carries a net charge of +5 to +6, and its binding to negatively charged membranes is distinctly enhanced compared to neutral membranes. This has been demonstrated experimentally in a series of binding studies on melittin-phosphatidylserine (Dufourcq & Faucon, 1977), melittin-cardiolipin (Batenburg et al., 1987a,b), melittin-phosphatidylglycerol (Batenburg et al., 1987c), and melittin-phosphatidylcholine/phosphatidylethanolamine membranes (Batenburg et al., 1988). The quantitative analysis of these binding isotherms was based on a chemical model with a defined number of lipids constituting a peptide "binding site". This type of approach corresponds to a Scatchard analysis and, though empirically useful, is inadequate to describe the binding of large ligands to a fluidlike

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