Orientation and Role of Nucleosidediphosphatase and 5′-Nucleotidase in Golgi Vesicles from Rat Liver†

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ABSTRACT: The fate of UDP formed during the galactosylation of added *N*-acetylglucosamine in Golgi vesicles isolated from rat liver using D_2O–sucrose gradients has been determined. UDP-Gal labeled with [^{14}C]uracil was used, and the products of the reaction were separated and quantitated by using high-pressure liquid chromatography. [^{14}C]Uridine rather than [^{14}C]UDP or [^{14}C]UMP was found to accumulate, indicating the presence of both UDPase and UMPase activities in the Golgi. Golgi vesicles were shown to contain a nucleosidediphosphatase activity that is membrane bound. It appears to be located on the luminal face of the Golgi since it is activated 3–5-fold by detergents and 4-fold by treatment of the vesicles with Filipin. We have shown previously that Filipin disrupts the Golgi but does not solubilize membrane-bound enzymes. The nucleosidediphosphatase of the Golgi differs from that present in rough endoplasmic reticulum in its absolute requirement for Ca^{2+} for activity and in its substrate specificity that is higher for UDP than for IDP. Golgi vesicles also contain UMPase activity that is stimulated only 2-fold by detergents or Filipin. Concanavalin A inhibits this activity about 80% in both intact and detergent-treated vesicles. The Golgi UMPase is thus probably identical with 5′-nucleotidase. These results are consistent with histochemical evidence from other laboratories that indicate that 5′-nucleotidase is present on both sides of liver Golgi membranes. In the presence of concanavalin A and *N*-acetylglucosamine, intact Golgi vesicles were found to convert UDP-Gal to UMP. These findings indicate that UDP formed by galactosyltransferase in the lumen of the vesicles is rapidly converted to UMP by UDPase in the lumen but that UMP moves rapidly out of the lumen of the Golgi and is broken down to uridine by 5′-nucleotidase on the cytoplasmic side of the vesicles.

UDP-galactose:*N*-acetylglucosamine galactosyltransferase is one of the enzymes unique to the Golgi apparatus in a number of mammalian cells (Fleischer & Fleischer, 1970; Fleischer & Zambrano, 1974). The function of the enzyme is believed to be the modification of glycoproteins destined for secretion (Schachter et al., 1970; Schanbacher & Ebner, 1970). We have shown that in Golgi derived from either rat liver or kidney, the enzyme appears to be an intrinsic membrane protein (Fleischer & Smigel, 1978). As expected for an enzyme processing secretory proteins, the enzyme is mainly oriented toward the lumen of the Golgi apparatus (Kuhn & White, 1975; Fleischer, 1981a). This raises an interesting topological problem since UDP-Gal, the substrate for this enzyme (Morrison & Ebner, 1971), is probably synthesized only on the cytoplasmic side of the Golgi membrane (Coates et al., 1980). In addition, UDP-Gal is present at a lower concentration in the cytoplasm than is UDP-Glc (Murphy et al., 1973), a competitive inhibitor of galactosyltransferase (Morrison & Ebner, 1971). On the basis of inhibition studies of galactosyltransferase by UDP-Glc in intact vs. disrupted Golgi vesicles from the mammary gland, Kuhn and White have postulated that a specific transport of UDP-Gal must occur across the membrane (Kuhn & White, 1976).

Another important topological problem involved in the efficient functioning of the galactosylation system in the Golgi apparatus involves UDP, the product of the galactosyltransferase. Since UDP is highly inhibitory to this enzyme (Khatra et al., 1974), accumulation of UDP within the Golgi lumen would inhibit galactosylation of secreted products. However, high concentrations of UDP-Gal cause an apparent activation of galactosyltransferase in Golgi vesicles derived from rat mammary gland (Kuhn et al., 1980) rather than inhibition. In addition, Kuhn and White showed that UMP rather than UDP accumulates during lactose formation in this system due to the presence of UDPase activity in the Golgi (Kuhn & White, 1977).

In the present paper we have investigated that fate of UDP formed during galactosylation of added *N*-acetylglucosamine in the Golgi vesicles prepared from rat liver by using D_2O–sucrose gradients (Fleischer, 1981b). Such vesicles have undergone less osmotic shock than those prepared by using H_2O–sucrose gradients since shallower sucrose gradients are necessary for the isolation. The method also avoids osmotic shock in the last step of the preparation since Golgi fractions recovered from the gradients can be diluted for final recovery by centrifugation using H_2O–sucrose of similar osmolarity as that present in the fractions. The vesicles are more intact than those prepared by using H_2O–sucrose as measured by the increase of both galactosyl- and sialyltransferase activities after treatment with Triton X-100 (Fleischer, 1981b). Such vesicles appear to be ideal for studies aimed at determining the fate of the products formed by the glycosyltransferases in the lumen of liver Golgi vesicles and the orientation of the enzymes involved.

The results obtained in our studies indicate that UDP formed by the action of galactosyltransferase on the lumen face of the Golgi membranes is rapidly broken down to UMP by the nucleosidediphosphatase, also on the lumen side of the membrane, but that UMP moves out of the lumen of the Golgi and is hydrolyzed to uridine by 5′-nucleotidase, present on the cytoplasmic face of the Golgi membrane.

Experimental Procedures

Materials. Male Holtzman rats, 200–250 g, fed ad libitum were used. Golgi-rich fractions were prepared from the livers by using D_2O–sucrose step gradients as described previously (Fleischer, 1981b). Fractions 2 and 3 from the gradients were

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combined, diluted with an equal volume of 8.5% sucrose in H_2O, and centrifuged at 40000 rpm for 60 min in a 42.1 Spinco rotor. The pellet was resuspended with a Potter-Elvehjem homogenizer in 0.25 M sucrose that had been previously adjusted to pH 7.0 with KOH. Rough endoplasmic reticulum (RER) fractions were prepared by fractionation of a total microsomal pellet on a sucrose step gradient containing CsCl as described (Fleischer & Fleischer, 1969). All preparations and centrifugations were carried out at 6 °C. The sucrose used was obtained from EM Laboratories, Inc., and was a special grade designated for gradient centrifugation. D_2O (99.9%) was obtained from Bio-Rad. Crystalline bovine plasma albumin was obtained from Armour Pharmaceutical Co. Triton X-100 was obtained from Research Products International, Elkgrove Village, IL, and neutralized to pH 7.0 with NaOH before use. Lubrol WX and sodium dodecyl sulfate were obtained from Sigma. Concanavalin A was obtained from Pharmacia.

[2-^{14}C]UDP-Gal was prepared from [2-^{14}C]UTP (New England Nuclear) by using uridine diphosphoglucose pyrophosphohydrolase (Sigma) essentially as described by Kuhn & White (1977), except for the isolation of the product. For the isolation, the entire reaction mixture was streaked on Eastman cellulose paper (13255), air-dried, and developed for 6 h by using 95% ethanol–1 M NH_4OAc (7:3 by volume). The product was located by using UV light, and the band was cut out, placed into a tube, and extracted 3 times with 0.5 mL of distilled water at 37 °C for 5 min. The extracts were combined and lyophilized, and the product was redissolved in 0.5 mL of distilled water. The purity of the product was checked by using high-pressure liquid chromatography (Fleischer, 1981b). Greater than 98% of the radioactivity chromatographed as a single peak with a retention time corresponding to that of UDP-Gal. About 1.2% of the activity was present as UMP.

[2-^{14}C]UDP was prepared from [2-^{14}C]UTP by using Apyrase (Sigma). The reaction mixture contained, in order of addition, 1 μmol of sodium succinate, pH 6.5, 0.8 μmol of $CaCl_2$, 1.6 μmol of [2-^{14}C]UTP (6.3 mCi/mmol), and 20 μg of Apyrase in a final volume of 0.1 mL. The mixture was incubated for 3 h at 30 °C and then spotted on poly(ethylenimine)-impregnated cellulose plates, 20 × 20 cm (Polygram Cel 300 PEI plates, Brinkmann Instruments, Westbury, NY), that had been prewashed in 50% (v/v) methanol. The plates were developed in 0.75 M LiCl, and the spots corresponding to standard UMP and UDP were cut out and extracted with 2 mL of 1 M Tris-HCl,[1] pH 7.5, containing 70 mM $MgCl_2$ for 90 min at 37 °C with frequent shaking. The solids were removed by centrifugation at 6000 rpm for 15 min in a Beckman JA 20 rotor. The purity of the nucleotides was checked by using high-pressure liquid chromatography (Fleischer, 1981b). Greater than 91% of the UV-absorbing material was recovered in a single peak with the appropriate retention time. The only contaminant detectable in either preparation eluted with the solvent front. All other radioactive compounds used were obtained fron New England Nuclear.

Enzymatic Assays. UDP-Gal:*N*-acetylglucosamine galactosyltransferase was determined as described (Fleischer & Smigel, 1978). The preparations used in this study had galactosyltransferase activities of 985 ± 135 (n = 15) nmol h^{-1} (mg of protein)$^{-1}$ at 37 °C.

Assays for nucleosidediphosphatase activity contained, in order of addition, 20 μmol of imidazole hydrochloride, pH 7.4, 1 μmol of $CaCl_2$, 20–30 μg of protein, 40 μg of Lubrol WX, and 3.5 μmol of UDP in a total volume of 0.5 mL. After incubation for 10 min at 37 °C, the reaction was stopped by addition of 0.4 mL of cold 1.5 N perchloric acid. The precipitated protein was removed by centrifugation for 15 min in a clinical centrifuge and inorganic phosphate determined on an aliquot of the supernatant by a modification of the method of Fiske & SubbaRow (1925). In some experiments the amount and type of detergent used were varied as indicated in the individual experiments. When Triton X-100 or Lubrol WX was used in the assay mixture, 10 mg of sodium dodecyl sulfate was included in the colorimetric reaction.

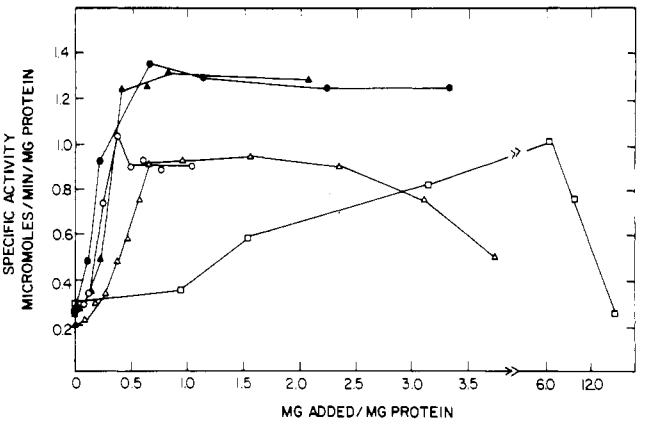

FIGURE 1: Effect of detergents and Filipin on UDPase activity of Golgi vesicles isolated from rat liver. The vesicles (20–30 μg of protein) were incubated with varying amounts of these agents for 15 min at 22 °C by using the standard assay conditions. UDP was then added and hydrolysis measured after 10 min at 37 °C as described under Experimental Procedures. (▲) Triton X-100; (●) Lubrol WX; (○) Filipin; (△) sodium dodecyl sulfate; (□) deoxycholate.

Assays for 5'-nucleotidase contained, in order of addition, 20 μmol of imidazole hydrochloride, pH 7.4, 20 μg of protein, 20 μg of Triton X-100, and 7 μmol of UMP in a final volume of 0.5 mL. After a 10-min incubation at 37 °C, the reaction was stopped by addition of 1 mL of cold 0.5 M HCl containing 3% (w/v) ascorbic acid and 0.5% (w/v) ammonium molybdate. Inorganic phosphorus was determined by the method of Ottolenghi (1975). In some experiments the amount and type of detergent used were varied as indicated in the individual experiments. When Triton X-100 was used in the assay, the reaction was stopped by adding sodium dodecyl sulfate to a final concentration of 0.5% (w/v) before adding the ascorbate–ammonium molybdate mixture.

Protein was determined by the method of Lowry et al. (1951) using crystalline bovine plasma albumin as a standard.

Results

Golgi vesicles derived from rat liver contain an active nucleosidediphosphatase that utilizes UDP as a substrate and is stimulated 3–5-fold by pretreatment of the vesicles with detergents (Figure 1). Highest activation was observed with nonionic detergents Triton X-100 and Lubrol WX at levels of 0.7–0.8 mg of detergent/mg of Golgi protein. Negatively charged detergents such as sodium dodecyl sulfate and deoxycholate also activated the preparation but to a lesser extent. At high concentrations, these detergents inactivated the enzyme. Filipin, a polyene antibiotic that disrupts Golgi vesicles by complexing with the cholesterol present in the membranes (Fleischer, 1981a), also activated the UDPase activity of Golgi vesicles. These results support the view that the enzyme is localized in the lumen of the Golgi vesicles.

Optimum activity for UDPase in the Golgi was obtained at pH 7.4 by using 0.1 M imidazole hydrochloride as the

[1] Abbreviations: RER, rough endoplasmic reticulum; Tris, tris(hydroxymethyl)aminomethane.

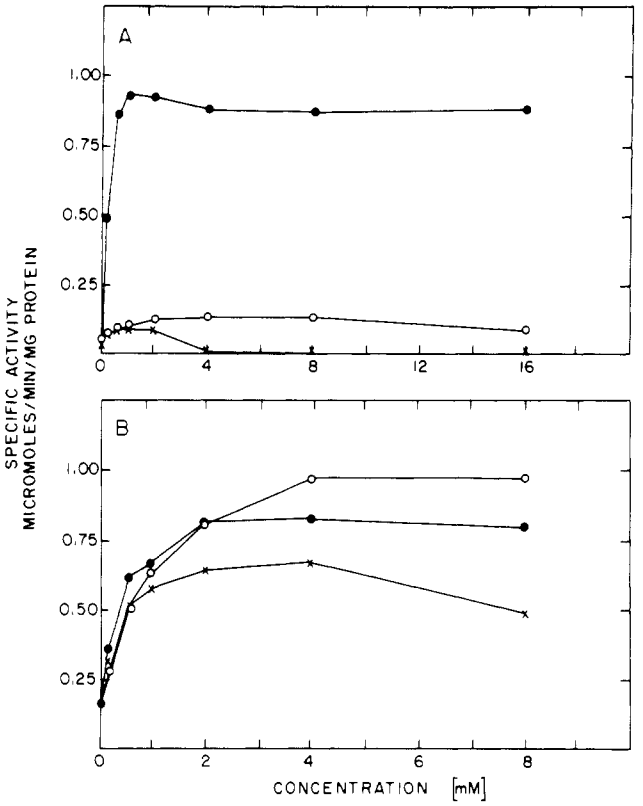

FIGURE 2: Effect of varying concentrations of divalent cations on the UDPase activity of Golgi vesicles (A) and rough endoplasmic reticulum (B) isolated from rat liver. Assays with either cell organelle were carried out as described under Experimental Procedures. In both cases the assay mixtures contained 20–30 μg of protein and 40 μg of Lubrol WX. (●) $CaCl_2$; (○) $MgCl_2$; (×) $MnCl_2$.

buffer. The curve was relatively flat between pH 6.5 and pH 7.5 but then decreased significantly above pH 8. The pH range 7.5–9.0 was studied by using 0.1 M Tris-HCl.

The UDPase activity of Golgi is specifically stimulated by Ca^{2+} but not by Mg^{2+} or Mn^{2+} (Figure 2A). Maximum activation is obtained at 1 mM Ca^{2+}. In contrast, UDPase activity present in the rough endoplasmic reticulum is stimulated by all these cations in the order $Mg^{2+} > Ca^{2+} > Mn^{2+}$ (Figure 2B). Maximum activation was found at 4 mM Mg^{2+}.

The activity in RER is stimulated maximally about 3-fold by Lubrol WX (2 mg/mg of protein) and about 2-fold by sodium dodecyl sulfate (4 mg/mg of protein). Higher concentrations of Lubrol WX were not inhibitory, whereas a 2.5-fold excess of sodium dodecyl sulfate inhibited the activity completely. Under similar assay conditions, that is, in the presence of Lubrol WX at pH 7.4 and 2 mM Ca^{2+} for Golgi and 4 mM Mg^{2+} for RER, both preparations showed simple Michaelis–Menton kinetics with increasing UDP concentrations. The apparent K_m for Golgi was 0.46 mM while that for RER was 1.6 mM. The possible effects of Lubrol WX on the apparent K_m for UDP of the two fractions was not investigated.

Comparison of the specificity of the nucleosidediphosphatase activities of the two preparations (Table I) illustrates further differences in the fractions. The activity in the Golgi fraction was highest with UDP as the substrate while the activity with IDP and GDP was somewhat lower. The activity of rough endoplasmic reticulum on the other hand was highest when IDP was used although UDP and GDP are also utilized fairly well. Pyrophosphate was not cleaved significantly by either preparation. To show that the enzyme was indeed a nucleosidediphosphatase, we incubated Golgi with [2-^{14}C]UDP at

Table I: Specificity of Phosphate Release by Golgi Compared to RER of Rat Liver[a]

substrate	sp act.[b] Golgi	RER
UDP	100	71 ± 6
IDP	91 ± 3	100
GDP	76 ± 14	88 ± 14
CDP	49 ± 4	6 ± 1
TPP[c]	13 ± 5	11 ± 4
UTP	13 ± 4	15 ± 8
ATP	12 ± 12	6 ± 6
CTP	10 ± 2	4 ± 0
CMP	8 ± 0	7 ± 7
GTP	4 ± 3	19 ± 6
UMP	3 ± 2	10 ± 4
PP_i[c]	3 ± 3	5 ± 1
ADP	2 ± 2	8 ± 4
UDP-Glc	0 ± 0	0 ± 0

[a] In all cases, 7 mM substrates were used. Other conditions of the assay were as described for the measurement of nucleosidediphosphatase activity in Golgi fractions given under Experimental Procedures. Values are average of results from two different enzyme preparations ± the standard deviation. [b] Expressed as percent of the highest specific activity observed. For Golgi the highest value was 1.2 ± 0.1 μmol of P_i released min^{-1} (mg of protein)$^{-1}$ at 37 °C while for RER the highest value was 1.2 ± 0.3 μmol min^{-1} (mg of protein)$^{-1}$. [c] TPP = thiamin pyrophosphate; PP_i = sodium pyrophosphate.

Table II: Effects of an Alkaline Salt Wash Followed by Extractions with Lubrol WX on the Solubility of UDPase Activity of Rat Liver Golgi Vesicles[a]

treatment	fraction	protein[b] (%)	UDPase sp act.[c]	UDPase total[d] (%)
none	Golgi	100	1.18	100
(A) $NaCl$-$NaHCO_3$ wash	pellet	60.2	1.54	78
	supernatant	30.9	0.00	0
(B) Lubrol WX (2 mg/mL) added to (A)	pellet	45.7	0.35	10.5
	supernatant	60.9	1.22	48.0

[a] In both treatments A and B, 1–2 mg of protein/mL was used. In step A the mixture was sedimented at 100000g_{max} for 60 min. In step B, the pellet from step A was resuspended in 0.25 M sucrose and treated with 2 mg/mL Lubrol WX. After 15 min at 0 °C, the mixture was sedimented for 1 h at 100000g_{max}. [b] Percentage of total protein (set at 100 for the original Golgi) present in the fraction. [c] Specific activity is expressed as micromoles of P_i released per minute per milligram of protein. [d] Percentage of total activity (set at 100 for the original Golgi) present in the fraction. All fractions were assayed in the presence of Lubrol WX as described under Experimental Procedures for Golgi.

37 °C for either 0 or 10 min using the standard assay conditions for UDPase. The reaction was stopped by addition of 0.25 mL of 50% trichloroacetic acid. Aliquots of the trichloroacetic acid soluble fractions were analyzed for their nucleotide content by using high-pressure liquid chromatography (Fleischer, 1981b). Two-milliliter fractions of the effluent were collected, and the distribution of radioactivity was determined. At zero time, 99% of the radioactivity was present in a peak with a retention time corresponding to that of UDP. After a 10-min incubation, the only product detectable migrated with a retention time corresponding to that of UMP.

For determination of whether the UDPase was membrane bound or free in the lumen of the Golgi vesicles, freshly prepared Golgi were first treated with hypotonic shock followed by a slightly alkaline salt wash to remove soluble Golgi contents or ionically bound proteins (Fleischer & Smigel, 1978;

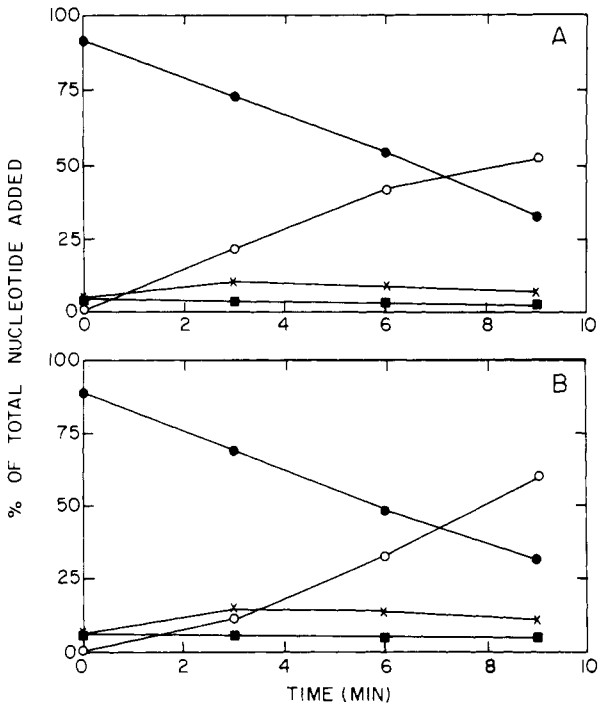

FIGURE 3: Further metabolism of UDP formed by galactosyltransferase of intact or solubilized rat liver Golgi vesicles. (A) Freshly prepared rat liver Golgi (900 μg of protein) was preincubated at 4 °C for 1 h in 1 mL of 0.08 M sucrose containing 55 μmol of sodium cacodylate, pH 6.5, 45 μmol of mercaptoethanol, 20 μmol of *N*-acetylglucosamine, 2 μmol of $MnCl_2$, and 1 μmol of $CaCl_2$. After the mixture was warmed to 37 °C, 140 nmol of [2-^{14}C]UDP-Gal, 0.82 mCi/mmol labeled in the uracil moiety, was added. The reaction was stopped at the indicated times by the addition of 1 mL of 50% (w/w) trichloroacetic acid, and the nucleotides were extracted and analyzed by using high-pressure liquid chromatography as described previously (Fleischer, 1981b). Two-milliliter samples were collected after chromatography, and their radioactivity was determined by adding 10 mL of scintillation cocktail to a 1-mL aliquot of each fraction and counting the mixture in a scintillation counter. (B) Freshly prepared rat liver Golgi (300 μg of protein) was incubated as in (A) except that 5 mg of Triton X-100 was also present and no preincubation at 0 °C was used. Less protein was used so that the same amount of breakdown of UDP-Gal would occur as in (A). The reaction was stopped and the products were extracted and analyzed as in (A). (●) UDP-Gal; (○) uridine; (×) UMP; (■) UDP.

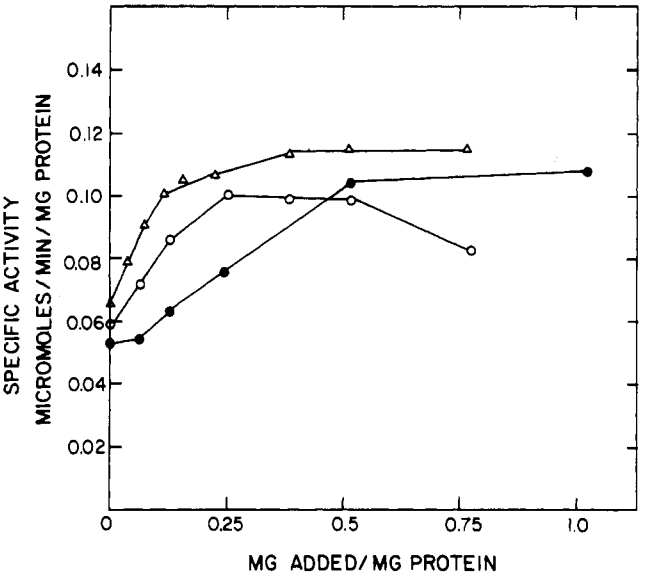

FIGURE 4: Effect of detergents and Filipin on UMPase activity of Golgi vesicles isolated from rat liver. The vesicles (20 μg of protein) were incubated with varying amounts of these agents for 15 min at 22 °C by using the standard assay conditions. UMP was then added and hydrolysis measured after 10 min at 37 °C as described under Experimental Procedures. (△) Sodium dodecyl sulfate; (○) Filipin; (●) Triton X-100.

Fleischer, 1974). After centrifugation to recover the membranes, the distribution of protein and total UDPase activity in the two fractions were determined (Table II). In all cases, Lubrol WX was present in the assays. The results show that UDPase activity remains bound to the membrane following the procedure although 31% of the protein of the preparation, mainly cisternal contents, is solubilized. Addition of the nonionic detergent Lubrol WX to the washed membranes results in the release of most of the nucleosidediphosphatase. About 14% of the activity remains membrane bound, even if the level of detergent used to solubilize the membrane is increased further. About 20% of the initial activity of the membranes was lost during each step in this procedure so that only about 60% of the original UDPase activity was recovered finally. The cause of the unstability of the enzyme during the lengthy treatments was not investigated.

The fate of UDP released during galactosylation of added *N*-acetylglucosamine was determined by using UDP-Gal radioactively labeled with ^{14}C in the uracil moiety. As shown in Figure 3, little or no UDP accumulated during the galactosylation reaction by either intact or detergent-solubilized Golgi vesicles. Surprisingly, only a low level of UMP accumulated during the reaction. The main product eluted with the solvent front in the high-pressure liquid chromatography of the fraction soluble in trichloroacetic acid. Thin-layer chromatography was used to further identify the major product formed during the incubation. After a 9-min incubation, the fraction soluble in cold 12.5% trichloroacetic acid was treated to remove the trichloroacetic acid as described previously (Fleischer, 1981b) and chromatographed on poly(ethylenimine)-impregnated cellulose plates prewashed in 50% (v/v) methanol. The plate was developed in 86% butanol in water (v/v). Sixty-three percent of the total counts applied migrated with the same mobility as uridine, 37% of the counts remained at the origin, and no counts were recovered at the area corresponding to uracil. A duplicate sample analyzed by high-pressure liquid chromatography yielded 64% of the total radioactivity applied migrating at the solvent front, 24.3% recovered as UDP-Gal, 5.8% recovered as UMP, and 2.2% recovered as UDP.

The rapid breakdown of added UDP-Gal all the way to uridine indicated the presence of UMPase activity in addition to UDPase in the Golgi preparation. Figure 4 summarizes the UMPase activity of the Golgi vesicles measured directly. In contrast to the UDPase, the UMPase activity of this fraction is activated only about 1.5–2-fold by addition of detergents or by Filipin. Histochemical localization of 5′-nucleotidase in isolated Golgi fractions (Farquhar et al., 1974; Little & Widnell, 1975) indicates the enzyme is present in the lumen of secretory vesicles and on the cytoplasmic surface of cisternal elements. Our results are compatible with such a distribution.

A characteristic of 5′-nucleotidase is its susceptibility to inhibition by concanavalin A (Riordan & Slavik, 1974; Little & Widnell, 1975). We therefore checked the effect of concanavalin A on the UMPase activity of the Golgi vesicles. Figure 5 shows about 80% of the total UMPase activity present in detergent-solubilized Golgi vesicles was inhibited by concanavalin A. The remaining 20% activity was not inhibited even at high levels of concanavalin A. In intact vesicles, about half of the total concanavalin A sensitive UMPase was inhibited. These results indicate that the UMPase we are measuring is due to 5′-nucleotidase and that, in this preparation

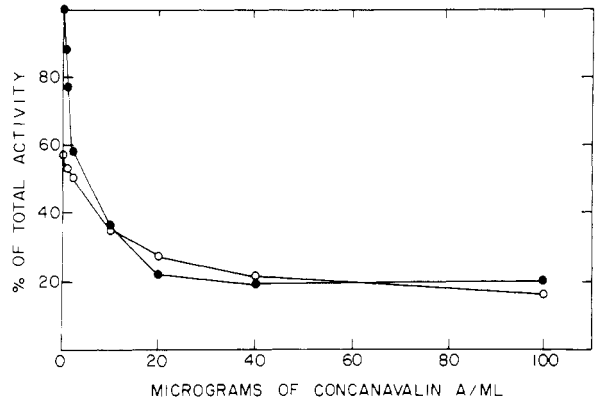

FIGURE 5: Effect of concanavalin A on UMPase activity of rat liver Golgi in the presence and absence of Triton X-100. The reaction mixture contained 20 μg of protein in a final volume of 0.5 mL, 40 mM imidazole hydrochloride, pH 7.4, 10 mM $CaCl_2$, 10 mM $MnCl_2$, and, when present, 20 μg of Triton X-100. After addition of concanavalin A, the mixture was preincubated for 45 min at 22 °C before addition of UMP and measurement of hydrolysis at 37 °C as described under Experimental Procedures. (●) Plus Triton X-100; (○) minus Triton X-100.

of Golgi vesicles, about half of the enzyme is oriented toward the lumen.

We next investigated whether concanavalin A had any effect on the formation of uridine during the breakdown of UDP-Gal by Golgi vesicles. The results are summarized in Figure 6. It can be seen that preincubation of intact Golgi vesicles with concanavalin A, which is accessible to and presumably could inhibit only the 5′-nucleotidase on the cytoplasmic side of the vesicles, resulted in the accumulation of UMP rather than uridine (Figure 6A). Similar results were obtained with Golgi solubilized with Triton X-100 (Figure 6B).

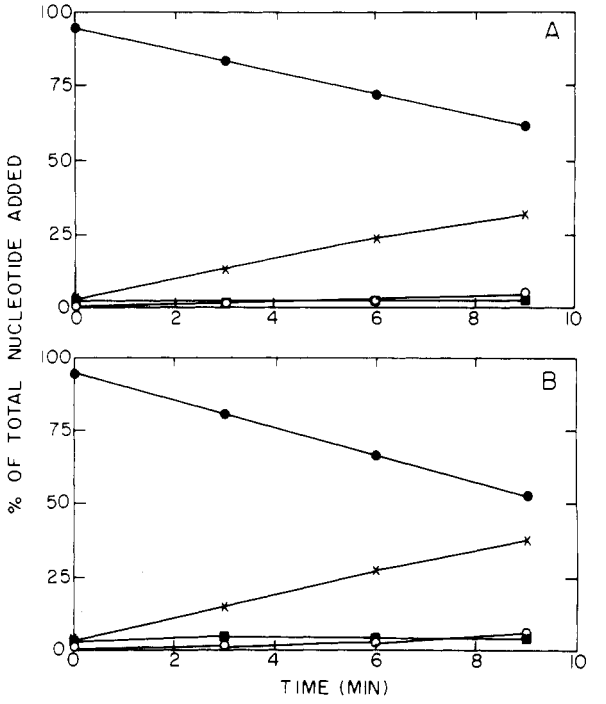

FIGURE 6: Effect of concanavalin A on metabolism of UDP formed by galactosyltransferase in intact or insolubilized rat liver Golgi vesicles. (A) Freshly prepared Golgi (900 μg of protein) was preincubated at 22 °C for 45 min in 1 mL of 0.14 M sucrose containing 900 μg of concanavalin A, 55 μmol of mercaptoethanol, 10 μmol of $MnCl_2$, 10 μmol of $CaCl_2$, and 20 μmol of *N*-acetylglucosamine. After the mixture was warmed to 37 °C, 140 nmol of [2-^{14}C]UDP-Gal, 0.82 mCi/mmol labeled in the uracil moiety, was added. The reaction was stopped at the indicated times with trichloroacetic acid and the nucleotides were extracted and analyzed as described in the legend to Figure 3. (B) Freshly prepared rat liver Golgi (300 μg of protein) was incubated as in (A) except that 300 μg of concanavalin A was used and 5 mg of Triton X-100 was also present. Less protein was used so that the same amount of breakdown of UDP-Gal would occur as in (A). The reaction was stopped and the products were analyzed as in (A). (●) UDP-Gal; (○) uridine; (×) UMP; (■) UDP.

Discussion

The data presented in this paper support the hypothesis first put forth by Kuhn & White (1977) that UDP formed by the galactosylation of secretory products by galactosyltransferase in the lumen of the Golgi apparatus is rapidly broken down by a nucleosidediphosphatase also present on the lumen side of the Golgi membrane. The presence of this pathway in Golgi vesicles from such diverse tissues as mammary gland and liver indicates it is a general mechanism of nucleotide metabolism in this organelle. The orientation of the nucleosidediphosphatase in our preparations of Golgi vesicles closely parallels that of galactosyltransferase and sialyltransferase, which we have shown to be oriented greater than 90% toward the lumen (Fleischer, 1981a). The nucleosidediphosphatase in intact Golgi vesicles from rat liver is stimulated 4–5-fold by nonionic detergents and about 3-fold by Filipin, a polyene antibiotic that disrupts the Golgi membrane but does not solubilize membrane-bound enzymes. This is consistent with our findings for galactosyltransferase and sialyltransferase in these vesicles. These activities are also stimulated about 5-fold by Triton X-100 when either *N*-acetylglucosamine or lactose is used as an acceptor (Fleischer, 1981a,b). The criterion of susceptibility to trypsin was not useful for determining the orientation of the nucleosidediphosphatase, since we found that the latter was not affected by treatment with trypsin regardless of the presence or absence of detergent.

Similar criteria have been used to localize nucleosidediphosphatase on the luminal face of the endoplasmic reticulum in rat liver (Kuriyama, 1972). It has been argued that the latency of the nucleosidediphosphatase (or inosine-5′-diphosphatase) of rat liver endoplasmic reticulum may be due to the presence of a noncompetitive inhibitor in the microsomal membrane (Little et al., 1976). However, no direct evidence for such an inhibitor has been presented.

A second important conclusion we can draw from our studies is that the nucleosidediphosphatase present in Golgi appears to be distinct from that present in endoplasmic reticulum in rat liver. It differs in its absolute requirement for Ca^{2+} and in its substrate specificity. The specificity with respect to Ca^{2+} may be unique to the liver Golgi enzyme. The UDPase activity of Golgi from rat mammary gland is activated equally well by Mn^{2+} and almost as well by Mg^{2+} (Kuhn & White, 1977). The purity of this preparation, however, has not been rigorously established, so that this difference may not be real. The rat liver Golgi enzyme may differ further from the microsomal enzyme in that we find its activity is not enhanced by ATP or by pyridoxal phosphate, which are allosteric effectors of the purified microsomal enzyme (Kawakita & Yamazaki, 1978, 1980). Both enzymes also cleave thiamin pyrophosphate although to a much lesser extent than UDP. The Golgi nucleosidediphosphatase is probably the enzyme responsible for the breakdown of thiamin pyrophosphate used extensively to reveal the Golgi apparatus histochemically in tissues such as rat liver (Goldfischer et al., 1964). Nucleoside diphosphates have also been used as substrates for the histochemical localization (Novikoff & Goldfischer, 1961). The fact that the endoplasmic reticulum is not stained by these procedures may be due to differences in the stability of the two enzymes

during the fixation procedure.

In contrast to the results of Kuhn and White using rat mammary gland Golgi (Kuhn & White, 1977), we find that uridine rather than UMP is the main product formed by rat liver Golgi during galactosylation of added substrate. This is probably due to the action of 5′-nucleotidase, which is present in liver but not in mammary gland Golgi. Addition of concanavalin A, an inhibitor of 5′-nucleotidase that does not penetrate the Golgi membrane (Little & Widnell, 1975), causes UMP to accumulate during galactosylation in our system. This occurs despite the presence of UMPase in the lumen of some of the vesicles, which has been demonstrated histochemically (Farquhar et al., 1974; Little & Widnell, 1975) and by our present results on the enhancement of UMPase in these vesicles by detergents or by Filipin. Although UMPase on the inside of the vesicles is not distributed homogeneously throughout the Golgi, it is present in secretory vesicles that also contain the highest concentration of galactosyltransferase activity (Bretz et al., 1980). Thus, a third important conclusion to be drawn from our data is that UMP must not accumulate within such vesicles to any extent but must move out rapidly into the medium where it is broken down to uridine by 5′-nucleotidase on the cytoplasmic side of the vesicles. A similar conclusion was drawn by Kuhn & White (1977) on the basis of the observation that externally added UMP was just as inhibitory to the galactosyltransferase reaction in intact mammary gland Golgi vesicles as in disrupted vesicles. The sidedness or intactness of the mammary gland vesicles was not clearly established, however, and alternative explanations of their kinetic results could be made.

Although UMP can leave the vesicles quickly enough during glycosylation to escape hydrolysis by the luminal 5′-nucleotidase, it apparently cannot enter the intact vesicles fast enough to be hydrolyzed by the luminal 5′-nucleotidase when assays are carried out in the absence of detergent. This apparent anomaly can be explained when the characteristics of UMP transport in Golgi vesicles are determined directly. Our preliminary results show that UMP is taken up by Golgi vesicles with an apparent K_m of 17 μM and a V_{max} of 2.1 nmol min^{-1} (mg of protein)$^{-1}$ (Brandan & Fleischer, 1981). This transport rate is of the same order of magnitude as the rate of UMP formation during the glycosylation reaction illustrated in Figure 6. The rate of UDP-Gal breakdown in this experiment is about 4 nmol min^{-1} (mg of protein)$^{-1}$. When UMPase activity is assayed by direct measurements, however, we are measuring rates of the order of 60–100 nmol min^{-1} (mg of protein)$^{-1}$. The transport of UMP into intact vesicles is too slow to mask the effect of the membrane on the overall hydrolysis rate in this case.

It is difficult to extrapolate the results found in the present in vitro studies to the situation in the cell. High concentrations of a permeable but unnatural substrate, *N*-acetylglucosamine, were used intentionally in our studies in order to see the effects of accumulation of products in the lumen of the vesicles. In the cell, galactosylation would probably proceed at a much lower rate in the Golgi lumen. The high concentration of UDPase in the lumen would still function to prevent accumulation of UDP, and the UMP formed would eventually move out of the lumen by an as yet undefined mechanism. Whether the UMP is then broken down to uridine by 5′-nucleotidase, which is present on the cytoplasmic face of not only the Golgi apparatus but also the large amount of endoplasmic reticulum in liver (Widnell, 1972), remains to be determined. In mammary gland, the cytoplasm contains an active UMP kinase that can rephosphorylate the UMP (Kuhn & White, 1977). In liver, the situation is more complex and requires further clarification.

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