Transport of Sugar Nucleotides into Rat Liver Golgi

A NEW GOLGI MARKER ACTIVITY

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Following incubation of sealed, "right side out," rat liver Golgi-derived vesicles with a mixture of [14C]UDP-fucose and GDP-\[^{3}H\]fucose, the difference in the \(^{3}H\) to \(^{14}C\) ratio between the incubation medium and the lumen of the vesicles was less than 11%, suggesting that the sugar nucleotide was transported intact into the vesicles. Transport of GDP-fucose was temperature-dependent, saturable, with an apparent \(K_m\) of 7.5 \(\mu\)M and a \(V_{max}\) of 14 pmol/mg of protein/10 min and inhibited by substrate analogues. Pretreatment of intact Golgi vesicles with pronase inhibited transport by 80% under conditions in which sialyltransferases (a lumenal marker) were not inhibited. This result is consistent with a sugar nucleotide carrier protein, portions of which face the cytoplasmic side of Golgi vesicles.

Previous studies from this laboratory had shown that CMP-N-acetylneuraminic acid (CMP-NeuAc) could penetrate mouse liver microsomes in a manner suggesting carrier-mediated transport (Carey, D. J., Sommers, L. W., and Hirschberg, C. B. (1980) Cell 19, 597-605). Subsequent to transport, a portion of the N-acetylneuraminic acid moiety became covalently linked to proteins facing the lumen of Golgi vesicles (Carey, D. J., and Hirschberg, C. B. (1981) J. Biol. Chem. 256, 989-993).

Upon subfractionation of rat liver into vesicles derived from the smooth and rough endoplasmic reticulum and Golgi, both the highest CMP-NeuAc specific transport activity and total transport activity were localized in the Golgi. Any transport activity in the other vesicles could be accounted for by contamination with Golgi, as activity and total transport activity were localized in Golgi, both the highest CMP-NeuAc specific transport activity and glycoprotein acceptors which face the Golgi lumen. Previous studies in vitro from this laboratory (11) have demonstrated that mouse liver microsomes can transfer CMP-NeuAc from the incubation medium into a lumenal compartment in a manner suggesting carrier-mediated transport. The purposes of this study were to refine the location of the CMP-NeuAc transport process from a general microsomal location to a specific organelle and to obtain evidence for similar transport of other sugar nucleotides.

MATERIALS AND METHODS

Radioactive Sugars and Nucleotides

The following radioactive sugars and sugar nucleotides were used:

- CMP-[\(^{14}C\)]NeuAc, New England Nuclear (1.6 mCi/mmol); CMP-[\(^{3}H\)]NeuAc, Amersham (9.0 mCi/mmol); GDP-[\(^{3}H\)]NeuAc (19 Ci/mmol) prepared as previously described (11); GDP-[\(^{14}C\)]fucose, Amersham (17.5 Ci/mmol); GDP-[\(^{3}H\)]fucose, New England Nuclear (192 mCi/mmol); [\(^{3}H\)]fucose, New England Nuclear (13.2 Ci/mmol); [\(^{3}H\)]GMP, Amersham (52 Ci/mmol).

Synthesis and Purification of [\(^{3}H\)]GDP-fucose and [\(^{14}C\)]Fucose 1-Phosphate

GDP-fucose labeled in the guanosine moiety was prepared by enzymatic synthesis according to a procedure kindly obtained from Drs. J. A. Munro and H. Schachter (University of Toronto). The reaction mixture contained the following in a final volume of 7.5 ml: fucose 1-phosphate, prepared as described below (5 \(\mu\)mol); Tris-HCl, pH 8.0 (250 \(\mu\)mol); KF (62.5 \(\mu\)mol); MgCl\(_2\) (31.25 \(\mu\)mol); [\(^{3}H\)]GTP (7.5 \(\mu\)mol, 1875 \(\mu\)Ci); and GDP-fucose pyrophosphorylase, prepared as described below (4 ml). Following incubation for 4 h at 37 \(^\circ\)C, the reaction was stopped by addition of 5 volumes of 90% ethanol. The mixture was centrifuged at 23,000 \(\times\) \(g\) and the supernatant solution was evaporated under reduced pressure. The residue was dissolved in water (100 ml), GDP-[\(^{14}C\)]fucose (0.7 \(\mu\)Ci) added as marker, and the mixture purified on a Dowex 1-X2 chloride column (2.5 \(\times\) 30 cm) equilibrated with 5 \(\mu\)M Tris-HCl, pH 7.4. The column was eluted with a linear gradient of 1 liter of 5 \(\mu\)M Tris-HCl, pH 7.4, and 1 liter of buffer containing 1.2 M KCl. The peak containing GDP-fucose was collected.

The plasma membrane of mammalian cells is highly enriched in glycoproteins which are oriented asymetrically in the membrane, their oligosaccharide moieties on the cytoplasmic side (1-3). This topographic orientation is established at the time of glycosylation of the nascent polypeptide chains (4-7) and is maintained during subsequent glycosylation in the Golgi (6-9). It has been shown that if the permeability barrier of the Golgi membrane is disrupted, the glycosyltransferases catalyzing the transfer of sialic acid and galactose to proteins and lipids are inactivated by proteases; this suggests that the active sites of these glycosyltransferases are facing the lumen of the Golgi (8-10). Studies in vitro have shown that CMP-NeuAc, a substrate for sialyltransferases, is present in the lumen of mouse liver microsome mixtures of vesicles derived from the smooth and rough endoplasmic reticulum and Golgi (11). Since CMP-NeuAc and other sugar nucleotides are not synthesized in the Golgi lumen (21), the question arises of how such substrates become available to glycosyltransferases and glycoprotein acceptors which face the Golgi lumen. Previous studies in vitro from this laboratory (11) have demonstrated that mouse liver microsomes can transfer CMP-NeuAc from the incubation medium into a lumenal compartment in a manner suggesting carrier-mediated transport. The purposes of this study were to refine the location of the CMP-NeuAc transport process from a general microsomal location to a specific organelle and to obtain evidence for similar transport of other sugar nucleotides.
pool, evaporated, and desalted on a Sephadex G-10 column (1.5 × 45 cm), equilibrated in 50 mM NH₄Cl, followed by preparative ascending chromatography on Whatman No. 3MM paper using ethanol/ammonium acetate (1 M, pH 7.5) (3:2) as solvent. One symmetrical peak was obtained which co-migrated with standard GDP-[¹⁴C]fucose by Carey and Hirschberg (12) using a modification of the subcellular fractionation procedure originally described by Fleischer and Kervina (13). Smooth endoplasmic reticulum-derived vesicles were enriched 3.5-fold over homogenate in glucose-6-phosphate activity (6% yield of total homogenate activity), 1.2-fold in sialyltransferase activity (2% yield), and 2.7-fold in 5' nucleotide activity (5% yield). of these preparations were very similar to those published by Leelavathi (13) and Kervina (13) and Leelavathi (14) (not shown).

Isolation and Topography of Vesicles Derived from Rat Liver Smooth and Rough Endoplasmic Reticulum and Golgi

The above three fractions were obtained as previously described by Carey and Hirschberg (12) using a modification of the subcellular fractionation procedure originally described by Fleischer and Kervina (13). Smooth endoplasmic reticulum-derived vesicles were enriched 3.5-fold over homogenate in glucose-6-phosphate activity (6% yield of total homogenate activity), 1.2-fold in sialyltransferase activity (2% yield), and 2.7-fold in 5' nucleotide activity (5% yield). Rough endoplasmic reticulum-derived vesicles were enriched 3.4-fold over homogenate in glucose-6-phosphate activity (12% yield of total homogenate activity), 0.35-fold in sialyltransferase activity (1% yield), and 1.3-fold in 5' nucleotide activity (5% yield). Intensity of smooth and rough endoplasmic reticulum-derived vesicles, as determined by latency of mannose-6-phosphate (17), was found to be 92% and 86%, respectively.

Golgi-derived vesicles were enriched 1.4-fold in glucose-6-phosphate activity (0.5% yield), 2.3-fold over homogenate in sialyltransferase activity (14% yield of total homogenate activity), and 5.4-fold in 5' nucleotide activity (2% yield). Vesicle integrity as determined by latency toward neuraminidase treatment of vesicles prelabeled with CMP-[¹⁴C]NeuAc (17) was found to be 98% for all the vesicles isolated according to the procedure of Leelavathi et al. (14) were enriched 35-fold in sialyltransferase activity (20% yield of total homogenate activity) and 2.5-fold in NADH-cytochrome c reductase activity (0.5% yield of total homogenate activity). Vesicle integrity was 95%. Electron micrographs of these preparations were very similar to those published by Fleischer and Kervina (13) and Leelavathi et al. (14) (not shown).

**Assays for Marker Enzymes and Protein**

Activities of 5' nucleotide and glucose-6-phosphatase (10), NADH cytochrome c reductase (16), sialyltransferase (8), topography of Golgi vesicles (8), and topography of endoplasmic reticulum-derived vesicles (17) were assayed by published procedures without modification. Protein was determined according to Peterson (18).

**Isolation and Topography of Vesicles Derived from Rat Liver Smooth and Rough Endoplasmic Reticulum and Golgi**
Sugar Nucleotide Transport into Golgi

Phosphatidic moieties contain diphosphate linkages. It was, therefore, of interest to determine whether a similar transport system existed for these sugar diphosphate nucleotides. Studies in vitro and in vivo have shown that fucose is transferred to glycoproteins in the Golgi (19, 20). This transfer is thought to occur in the lumen. GDP-fucose, which is synthesized in the cytoplasm (21), could be expected to be transported into the Golgi lumen via a carrier protein system similar to that suggested for CMP-NeuAc. The following experiments were designed to substantiate this hypothesis. A fraction highly enriched in Golgi-derived vesicles, as determined by enrichment in marker enzymes (see "Materials and Methods") was used for subsequent studies. At least 90% of these vesicles were sealed and of the same membrane topography as in vivo. This was determined by measuring latency of neuraminidase catalyzed removal of labeled sialic acid from Golgi vesicles prelabeled with radioactive CMP-NeuAc (11; see "Materials and Methods").

Incubation of GDP-[14C]fucose with Golgi vesicles resulted in the accumulation of radioactive solutes within such vesicles (Fig. 1). The total amount of solutes inside the vesicles, the penetration index $S_v$, was determined by subtracting the total solutes outside vesicles in the pellet, $S_o$, from the total solutes (inside and outside) of the vesicle pellet, $S_v$. Detailed calculations are described under "Materials and Methods." This accumulation was linear with time and protein (see below) and was saturable with an apparent $K_m$ of 7.5 mM (based on the initial concentration of GDP-fucose in the incubation medium) and a $V_{max}$ of 14 pmol/10 min/mg of protein. Transport at 0 °C was less than 10% that at 23 °C (not shown; see Table V).

Chemical analyses of the radioactive solutes in the incubation medium and Golgi-vesicle pellet, following a 10-min incubation, showed that both compartments contained GDP-[14C]fucose, [14C]fucose, and [14C]fucose 1-phosphate, although each component was present in strikingly different ratios. The major radioactive species in the reaction medium was fucose 1-phosphate (72% of total); however, it was only 15% of the soluble radioactive species in the Golgi pellet. The major soluble radioactive species of the Golgi pellet was free fucose (80% of total); however, it was only 3% of the radioactive species in the reaction medium. GDP-fucose was 25% of the radioactivity in the reaction medium and 5% of the Golgi pellet.

For each of the above solutes of the Golgi pellet, we determined their penetration index, $S_v$, and their concentration within Golgi vesicles and the reaction medium (see "Calculations" under "Materials and Methods"). As shown in Table I, Experiment 1, only fucose was accumulated to a significant extent within Golgi vesicles (over 100-fold). Radioactive fucose 1-phosphate and GDP-fucose in the Golgi pellet could be accounted for as solutes trapped between vesicles. The accumulation of fucose within Golgi vesicles could have been the result of transport of GDP-fucose from the medium and subsequent breakdown within vesicles; however, it could also have arisen from transport of free fucose or fucose 1-phosphate, following exogenous breakdown of GDP-fucose. To determine to what extent this latter event was contributing to the accumulation of free fucose within vesicles, Golgi preparations were incubated with fucose or fucose 1-phosphate at concentrations comparable to those found in the incubation medium at the beginning and end of the reaction described in Table I, Experiment 1. Table I, Experiment 2, shows that no large increase in concentration of radioactive solutes occurred within Golgi vesicles, compared to the incubation medium, regardless of the initial concentrations of fucose and fucose 1-phosphate in the medium. This strongly suggests that the accumulation of fucose within Golgi vesicles observed in Experiment 1 was probably the result of the transport of GDP-fucose and not that of free fucose or fucose 1-phosphate.

Direct evidence that GDP-fucose was being transported by Golgi vesicles was obtained from incubations with a mixture of [H]GDP-fucose and GDP-[14C]fucose. Following a 10-min incubation, the $H$ to $^{14}$C ratio in the incubation medium was 4.8 while that in the Golgi pellet was 5.4 (Table II, Experiment 1). Analyses of the soluble radioactive species in the medium showed these to be GDP-[14C]fucose, [14C]fucose 1-phosphate, [14C]fucose, [H]GMP, [H]guanosine, and [H]GDP-fucose (Table III). The principal soluble radioactive species within and between the Golgi vesicles of the pellet were [14C]fucose and [H]GMP. It was determined from calculations of the penetration indices of each radioactive solute within Golgi
vesicles that only [14C]fucose and [3H]GMP occurred in higher concentration within the Golgi vesicles than in the incubation medium (Table III). Their enrichment in concentration within the Golgi vesicles relative to the medium were not equal because their concentrations in the medium following a 10-min incubation are different, and a significant portion of the fucose has become covalently bound to Golgi proteins (see below).

To determine whether the free fucose and GMP within the Golgi were due to the transport of free fucose or fucose 1-phosphate (Table I), strongly suggest that GDP-fucose is transported intact by Golgi vesicles, even though the intact sugar nucleotide could not be detected within such vesicles. Although the possibility of cleavage of the sugar nucleotide on the cytoplasmic surface of the Golgi vesicles followed by a simultaneous translocation of GMP and fucose (perhaps as an energy-rich intermediate) across the Golgi membrane cannot be ruled out, such translocations would have to be occurring at similar rates in order to maintain a 4H to 4C ratio in the medium within 11% of that of the Golgi vesicles (Table II, Experiment 1). This appears to be unlikely.

In addition to the soluble fucose derived from GDP-fucose within the lumen of the vesicles, a portion of fucose has been transferred covalently to glycoproteins. In a representative experiment, 59% of the radioactivity in the pellet (derived from GDP-[14C]fucose) was perchloric acid-insoluble following a 10-min incubation at 23 °C. Evidence suggesting a lumenal orientation of this macromolecular-bound radioactivity was obtained by the failure of pronase to solubilize the radioactivity unless the permeability barrier of the membrane has been disrupted with detergents (8, not shown). Mammalian and bacterial fucosidases (kindly obtained from Dr. D. Aminoff, University of Michigan) were inactive in solubilizing macromolecular-bound fucose.

On the assumption that a cytoplasmic-facing carrier protein is involved in GDP-fucose transport, intact Golgi vesicles were pretreated with pronase and sugar nucleotide transport measured. Under conditions in which transport was inhibited by more than 79%, the activity of sialyltransferase (presumably a luminal protein marker (8-10)) was not decreased (Table IV). Higher concentrations of pronase inactivated both activities (not shown). While this experiment suggests that at least a portion of the putative carrier protein faces the cytoplasm, it does not rule out the possibility that both activities being assayed have the same membrane topography but different susceptibilities to proteases. Transport of GDP-fucose was inhibited by GMP (Table VII).

### Table II

| Incubation mixture | Reaction medium before incubation | Reaction medium after incubation | Golgi pellet | Concentration of radioactive solutes in medium (mean ± S.D.) |
|--------------------|----------------------------------|---------------------------------|--------------|---------------------------------------------------------------|
| [3H]GDP-fucose + [3H]fucose (10 µM) | 9 ± 0 | 4 ± 2 | 1 ± 2 |
| [14C]GMP | 47 ± 3 | 11 ± 0 | 0 |
| GDP-[14C]fucose | 9 ± 1 | 0 | 0 |
| GDP-[14C]fucose | 4 ± 1 | 83 ± 9 | 133 ± 29 |
| GDP-[14C]fucose | 87 ± 2 | 7 ± 2 | 0 |

A calculated (as described under “Materials and Methods”) from the retention indices and accessible volumes of each solute. Values shown are the means of three separate determinations. 
Solutes within and between vesicles.

### Table III

| Transport of a mixture of [14C]fucose and GDP-[14C]fucose into Golgi vesicles: concentration of radioactive solutes within vesicles following a 10-min incubation |
|----------------------------------|---------------------------------|-------------------------------|
| Radioactive solutes following 10-min incubation. Per cent of total of each radioactive solute, mean ± S.D. | Concentration of radioactive solutes within vesicles in medium. |
| [3H]GDP-fucose | 9 ± 0 | 4 ± 2 | 1 ± 2 |
| [14C]fucose | 47 ± 3 | 11 ± 0 | 0 |
| GDP-[14C]fucose | 9 ± 1 | 0 | 0 |
| GDP-[14C]fucose | 4 ± 1 | 83 ± 9 | 133 ± 29 |
| GDP-[14C]fucose | 87 ± 2 | 7 ± 2 | 0 |

Values shown are the means of three separate determinations.

### Table IV

| Inhibition of sugar nucleotide transport following pretreatment of Golgi vesicles with pronase |
|----------------------------------|---------------------------------|-------------------------------|
| Sugar nucleotide | Concentration in the medium | Activity | Sialyltransferase | % of Control |
|-------------------|-------------------------------|-----------|-----------------|-------------|
| CMP-NeuAc | 1.3 | 96 | 106 |
| GDP-fucose | 0.6 | 21 | 106 |

**Note:** Values shown are the averages of two separate determinations.
Transport of CMP-N-Acetylneuraminic Acid into Golgi Vesicles—Previous studies from this laboratory showed that CMP-NeuAc could penetrate liver microsomes in vitro (11). These microsomes contained a mixture of vesicles derived from the smooth and rough endoplasmic reticulum and Golgi. It was, therefore, important to determine whether transport of CMP-NeuAc was occurring in one or more types of the above mentioned vesicles. Fractions highly enriched in vesicles from the above organelles were obtained (see "Materials and Methods"). At least 90% of each of the three types of vesicles used in subsequent transport studies were sealed and of the same membrane topography as in vivo. This was determined by measuring latency of mannose-6-phosphatase for smooth and rough endoplasmic reticulum-derived vesicles and latency of neuraminidase catalyzed removal of labeled sialic acid from Golgi vesicles prelabeled with radioactive CMP-NeuAc (see "Materials and Methods").

The above three types of vesicles were then incubated separately with CMP-NeuAc for 10 min at 0 and 23 °C and reisolated. As shown in Table V, columns 2 and 3, the penetration indices at 23 and 0 °C were approximately 90 times higher for Golgi than for SER-derived vesicles at the same temperature. The penetration index of SER vesicles was in turn four times higher than that of the RER-derived vesicles at 23 °C. From the penetration index and the volume within vesicles, the concentrations of solutes within vesicles were calculated. As shown in Table V, columns 4 and 5, only Golgi-derived vesicles were able to concentrate solutes from the incubation medium, and this occurred to a much larger extent (over 90%) at 23 than at 0 °C. The specific transport activity has, therefore, been defined as the difference between penetration indices at 23 and 0 °C (Table V, column 6). Golgi vesicles were enriched approximately 25-fold in this activity over SER-derived vesicles; RER-derived vesicles were inactive. The total transport activity/organelle without correction for contamination with other organelles was then calculated (Table V, column 7). Eighty-seven percent of the uncorrected CMP-NeuAc transport activity of the total homogenate was in the Golgi and 13% in the combined SER- and RER-derived fraction. The question of whether the transport activity in these latter fractions could be accounted for by contamination with Golgi was then addressed. Based on the units of sialyltransferase activity (a Golgi marker) contaminating the isolated SER- and RER-derived fractions, these fractions would be expected to contain 4 pmol/10 min of CMP-NeuAc transport activity. This is within experimental error of the 3.4 pmol/10 min of CMP-NeuAc transport activity which was actually measured in these fractions (Table V, column 7). These results strongly suggest that all the CMP-NeuAc transport activity is in the Golgi-derived vesicles and that the activity detected in SER- and RER-derived vesicles can be fully accounted for by Golgi contamination of these organelles (Table V, column 8).

A comparison of the specific and total activities of sialyltransferases and CMP-NeuAc transport among the three types of vesicles is shown in Table VI. The relative enrichment of Golgi vesicles in both activities was very similar. As these values were obtained independently from one another, these results strongly suggest that, for this particular subcellular fractionation procedure, both activities were copurifying in

### Table V

| Vesicles | Protein/fraction (μg) | Penetration index, S, | Concentration solutes within vesicles/concentration solutes in incubation medium | Specific transport activity (S, (23 °C) - S, (0 °C)) | Total transport activity (uncorrected for contamination) (corrected) | Total transport activity (corrected) (8) |
|----------|----------------------|-----------------------|--------------------------------------------------------------------------------|-----------------------------------------------|-------------------------------------------------|---------------------------------------------|
|          |                      |                       | 23 °C (2) | 0 °C (3) | 23 °C (4) | 0 °C (5) | pmol/mg/10 min | pmol/mg/10 min | pmol/mg/10 min | pmol/mg/10 min |
| RER      | 103                  | 0.0031b              | 0.0047f  | 0.488    | 0.329    | 0        | 0.124         | 0.043         | 22.4          | 22.4          |
| SER      | 49.8                 | 0.01299              | 0.00056  | 1.70      | 0.004   | 0.0124   | 0.289         | 0.100         | 13.2          | 13.2          |
| Golgi    | 10.6                 | 0.319d              | 0.030d   | 75.1      | 3.22    | 0.289    | 22.4          | 10.0          | 86.8         | 86.8         |
| Golgi (Ref. 14) |                  |                      |           |          |          |          |               |               |              |

* Numbers in parentheses are column numbers.
* Means of three separate determinations.
* Values obtained by multiplying the specific transport activity of each fraction by the mg of protein recovered of each (column 1) and dividing by the yield of the corresponding marker enzyme activity recovered for each fraction (18.1% of total homogenate glucose-6-phosphatase activity for SER- and RER-derived vesicles and 13.6% of total homogenerate sialyltransferase activity for Golgi).
* Means of two separate determinations.

### Table VI

Relative activities of sialyltransferase and CMP-NeuAc transport in rat liver vesicles derived from the smooth and rough endoplasmic reticulum and Golgi

| Vesicles | Sialyltransferase | CMP-NeuAc transport | Total activity | Relative % |
|----------|-------------------|---------------------|---------------|------------|
|          | Specific activity | Relative specific activity | Total activity | Relative % |
|          | pmol/mg/min       |                      | pmol/mg/min    |            |
| RER      | 14.8              | 0.000               | 15            | 0          |
| SER      | 49.4              | 0.031               | 0.124         | 0.043      |
| Golgi    | 1,590             | 1.000               | 0.289         | 1.000      | 86.8      |
similar yield. Golgi vesicles obtained by the procedure of Leelavathi et al. (14) were also highly active in CMP-NeuAc transport, as shown in Table V, column 4.

Characterization of CMP-NeuAc Transport into Golgi Vesicles—The above results showed that transport of CMP-NeuAc was occurring solely into Golgi-derived vesicles and not into vesicles derived from the smooth or rough endoplasmic reticulum. Transport of CMP-NeuAc into Golgi at 0°C was less than 10% of that measured at 23°C (Table V); therefore, transport in subsequent experiments was measured only at this latter temperature since the correction for 0°C was very small. Whether the penetration index at 0°C represents true transport, adsorption, or a combination of both events is not clear. Transport of CMP-NeuAc into Golgi vesicles was linear with time for at least 10 min and was linear with protein concentrations between 0.4 and 4.0 mg/ml (not shown).

Transport of CMP-NeuAc into Golgi was saturable with increasing concentrations of CMP-NeuAc in the incubation medium (not shown); the apparent \( K_m \) was 2.4 \( \mu \)M (based on the initial concentration of CMP-NeuAc in the incubation medium) and the \( V_{max} \) was 150 pmol/10 min/mg of protein.

As with GDP-fucose, the transport of CMP-NeuAc was inhibited (60%) by pronase pretreatment of the Golgi vesicles while sialyltransferase activity was not affected (Table IV) thus suggesting the involvement of a cytoplasmic-facing carrier protein in CMP-NeuAc transport across the Golgi membrane. Transport of CMP-NeuAc was inhibited by substrate analogues such as 5'-CMP (Table VII, Experiment 1).

**DISCUSSION**

The results of this paper demonstrate that Golgi membranes can transport CMP-NeuAc and GDP-fucose from the incubation medium into their lumen. As the vesicles used in this study are of the same topographical orientation as in vivo (6) and the above sugar nucleotides are not synthesized in the Golgi (21), the in vitro transport system described here is postulated to be the same system that translocates the sugar nucleotides in vivo.

The transport of CMP-NeuAc into a mixture of vesicles derived from the RER, SER, and Golgi has been previously described (11); in these studies the transport of CMP-NeuAc from the medium into RER and SER vesicles followed by the fusion of these endoplasmic reticulum-derived vesicles with existing Golgi elements could not be ruled out. The results shown in Tables V and VI strongly suggest that the transport activity for CMP-NeuAc is contained only in Golgi vesicles; therefore, the measurements of such transport activity can be used as a marker for Golgi vesicles. It had also been previously shown, using CMP-NeuAc labeled in both the sugar and nucleotide moieties, that the plasma membrane was impermeable to this sugar nucleotide (34).

It must be emphasized, however, that for the above Golgi transport measurements to be valid, one must first prove that the vesicle population being assayed is sealed and “right side out”; i.e. it must have the same topography as in vivo. Studies in this laboratory (8) as well as those of Howell et al. (23) have previously shown that freezing of such vesicles prior to transport assays can lead to vesicle breakage unless precautions, e.g. bovine serum albumin (10 mg/ml) in the buffer, are taken.

The Golgi specificity and the observations that transport is temperature-dependent, saturable, and inhibited by substrate analogues and proteases (under conditions which presumably only cleave cytoplasmic proteins in the Golgi vesicles (8)) all strongly suggest that the transport has a protein component, e.g. sugar nucleotide carrier protein(\(s\)).

The apparent \( K_m \) (2.4 \( \mu \)M) and \( V_{max} \) (150 pmol/10 min/mg of protein) obtained for CMP-NeuAc transport into Golgi vesicles are in good agreement with those values previously obtained using liver microsomes, a mixture of vesicles derived from the endoplasmic reticulum and Golgi (11); in these vesicles the apparent \( K_m \) for CMP-NeuAc transport was 0.6 \( \mu \)M and the \( V_{max} \) was 12 pmol/10 min/mg of protein. Since approximately 10% of this microsomal protein was due to Golgi, one would expect a \( V_{max} \) for a Golgi-enriched fraction to be in the range of 120 pmol/10 min/mg of protein. This value is in close agreement with the 150 pmol/10 min/mg of protein found here.

It has been previously shown in this laboratory, using the sugar nucleotide labeled in both the sugar and nucleotide moiety, that CMP-NeuAc was transported intact into microsomes (11). This fact, in light of the current findings that only Golgi vesicles within microsomes are active in transport, strongly suggests that CMP-NeuAc is transported as a unit into Golgi vesicles.

Previous studies with Golgi have shown that a portion of the neuraminic acid (derived from CMP-NeuAc) was transferred to glycoproteins and that the covalently linked N-acetylneuraminic acid was oriented toward the lumen of such vesicles (8). In a representative experiment with Golgi vesicles, after a 10-min incubation at 23°C, 83% of the radioactivity in the pellet was covalently bound, the remainder being soluble. Previous studies also suggested that the active sites of sialyltransferases were oriented toward the lumen of Golgi vesicles (8–10).

In the present studies with Golgi, as well as in previous ones with microsomes, it was found that a portion of CMP-NeuAc (up to 40%) had broken down in the reaction medium to CMP and N-acetylneuraminic acid (11). In both microsomes and Golgi vesicles the soluble species derived from CMP-NeuAc within vesicles were CMP-NeuAc, cytidine, and NeuAc (11). These latter two compounds most likely arise from a combination of 1) transfer of the sugar moiety from the sugar nucleotide to appropriate glycoprotein and glycolipid acceptors, 2) transfer of the sugar to water (hydrolysis), and 3) a lumenal phosphatase activity. The Golgi membrane is impermeable to both N-acetylneuraminic acid and cytidine (11).

It has also been shown in this study that GDP-fucose enters Golgi vesicles via a transport mechanism which is temperature-dependent, saturable, and inhibited by substrate ana-
logs and protease pretreatment as described above. The studies with GDP-fucose labeled in both the sugar and nucleotide moieties also strongly suggest that the sugar nucleotide is transported intact into the Golgi lumen in a manner analogous to CMP-NeuAc. Following transport, a portion of the sugar moiety is transferred to glycoproteins while the remainder of the sugar nucleotide is hydrolyzed to GMP and free fucose. It is not known at this time whether this latter reaction is catalyzed by a pyrophosphatase and phosphatase or by a fucosyltransferase acting as a hydrolase in the absence of appropriate acceptors. As shown in Table II, the enrichment of GMP and fucose within the Golgi lumen over that in the incubation medium cannot be accounted for by transport of GMP, fucose, or fucose 1-phosphate.

Concentrations of GDP-fucose up to 250 μM did not inhibit CMP-NeuAc transport (at 2 μM), suggesting that the two sugar nucleotides are not being transported by the same carrier protein. In addition, these two carrier proteins must have recognition entities facing the cytoplasmic side of the Golgi membrane; prior to this, only proteins with recognition sites facing the Golgi lumen (glycosyltransferases (9-10) and thiamine and nucleotide diphosphatase (24-26)) had been described.

In contrast to CMP-NeuAc, significant transport of GDP-fucose into microsomes containing a mixture of vesicles derived from the RER, SER, and Golgi was not detected (data not shown), probably because the Vₘₐₓ for GDP-fucose transport into Golgi is approximately 10-fold less than that of CMP-NeuAc.

Kuhn and White (27, 28) and Kuhn et al. (29) have previously presented indirect evidence for transport of UDP-galactose by rat mammary gland Golgi membranes. These investigators, as well as Brandan and Fleischer (30), have also shown transport of 5’-UMP by Golgi vesicles.

The above studies should encourage further investigation into the localization of other sugar nucleotide transport activities in functional regions of the Golgi. Although highly speculative, the carrier proteins for UDP-galactose and UDP-GlcNAc could logically be envisioned in Golgi regions closer to the forming face (cis), while the carrier proteins for the terminal sugars GDP-fucose and CMP-NeuAc could be closer to the mature face (trans). Bretz et al. (31) have found 3-fold differences in specific activities of glycosyltransferases in different Golgi vesicles whose isolation was based on differences in density; however, total activity measurements of the glycosyltransferases showed no differences within the different vesicle populations.

Another interesting question raised by these studies is whether the transport into endoplasmic reticulum-derived vesicles is required for UDP-glucose, GDP-mannose, and UDP-GlcNAc, sugars which are added via dolichol to asparagine-linked nascent polypeptide chains. Hanover and Lennarz (32) have presented evidence showing that in rough microsomes chitobiosyl-PP-dolichol is localized toward the lumen. One possible explanation for such a topographic arrangement would be that RER membranes transport UDP-GlcNAc into their lumen. It is not clear at this time whether GDP-mannose and UDP-glucose must also enter the lumen of the RER prior to sugar transfer to dolichol oligosaccharides. Some of the enzymes involved in such transfers can be inactivated with proteases without apparent disruption of the permeability barrier of the vesicles (33), suggesting that their active sites may be facing the cytoplasm. In such cases, no intralumenal transport for the above sugar nucleotides would be necessary. Clarification of this problem as well as further characterization of the above described transport activities are needed.

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