Glycosyl Transfer from Nucleotide Sugars to C₈₅- and C₅₅-Polyprenyl and Retinyl Phosphates by Microsomal Subfractions and Golgi Membranes of Rat Liver

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The capacity of isolated membrane fractions to catalyse transfer of sugars from sugar nucleotides to α -saturated and non-saturated forms of phosphorylated C₈₅ and C₅₅ polyprenols and retinyl phosphate was examined. The amount of endogenous lipid acceptor present for various sugars was also measured. It appears that the types and amounts of polyprenyl phosphates present in rough- and smooth-microsomal fractions and Golgi membranes are different and the individual polyprenyl phosphates exhibit specificity as sugar acceptors.

Two types of polyprenols have been identified as intermediates in the synthesis of certain types of glycoproteins; i.e. the dolichol and retinol types (Waechter & Lennarz, 1976; De Luca *et al.*, 1973). For dolichol it was demonstrated that dolichol phosphate monosaccharide may transfer its glycosyl unit directly to an oligosaccharide unit attached through a pyrophosphate linkage to another dolichol molecule. Consequently, at least four pathways for transferring saccharide moieties to protein may exist: transfer from the sugar nucleotide, from dolichol mono- or pyro-phosphate and, finally, from retinyl phosphate (De Luca, 1977).

There are two major groups of glycoproteins whose peptide and saccharide moieties are synthesized in the endoplasmic reticulum and Golgi apparatus: secretory proteins, which are subsequently secreted into the blood, and membrane glycoproteins, which are integral components of various cytoplasmic and plasma membranes. A large variety of sugar sequences are found in both of these types of glycoproteins and there are several possible biochemical mechanisms for the synthesis of these sequences. Some of the oligosaccharide chains may be completed in a sequential manner during the transport of the protein (Schachter *et al.*, 1970; Molnar, 1975), two or more transferases may mediate the transfer of specific saccharide residues to different protein acceptors (Wetmore *et al.*, 1974), and the biosynthesis of different types of glycoproteins may exhibit an asymmetric distribution in the transverse plane of the membrane (Nilsson *et al.*, 1976). It is also possible, however, that the specificity of sugar transfer is determined partially by the polyprenol utilized in the

reaction. Dolichols isolated from the liver are heterogeneous; the number of isoprene residues and the number and pattern of *cis*- and *trans*-double bonds in these compounds vary (Hemming, 1974). In the present paper we isolated rough- and smooth-microsomal fractions and Golgi membranes to examine the possibility that exogenous and endogenous lipid acceptors display specificity in accepting sugar residues from sugar nucleotides.

Materials and Methods

Rough- and smooth-microsomal fractions were prepared from starved adult male albino rats (Dallner, 1974). The smooth-microsomal fraction was floated in the same system that was used for the preparation of Golgi membranes to remove Golgi contaminants. The total Golgi fraction was prepared as described by Ehrenreich *et al.* (1973). All fractions were washed by centrifugation at 105 000g for 60 min in 0.15 M-Tris/HCl buffer, pH 8.0, containing 5 mM-MgCl₂, to remove adsorbed proteins. In experiments with dolichol phosphates the 400 μ l incubation mixture contained: 30 mM-Tris/HCl buffer, pH 7.8; 2.5 mM-EDTA; 10 mM-MnCl₂; ATP, 1.35 mM for the rough-microsomal fraction, 1.75 mM for the smooth-microsomal fraction, and 2.50 mM for Golgi membranes; GDP-[¹⁴C]mannose (sp. radioactivity 179 mCi/mmol), UDP-*N*-acetyl[¹⁴C]glucosamine (sp. radioactivity 300 mCi/mmol), UDP-[¹⁴C]glucose (sp. radioactivity 300 mCi/mmol), UDP-[¹⁴C]galactose (sp. radioactivity 321 mCi/mmol), or CMP-*N*-acetyl[¹⁴C]neuraminic acid (sp. radioactivity 260 mCi/mmol) (all from The Radiochemical Centre, Amer-

sham, Bucks., U.K.) corresponding to 100000 c.p.m. each; 0.4% Triton X-100; and 50 μ l of sample containing 1.5 mg (rough-microsomal fraction), 1.0 mg (smooth-microsomal fraction) or 0.6 mg (Golgi membranes) of protein. In experiments with isolated polyprenyl phosphates, 10 nmol of these compounds was added in 10 μ l of chloroform/methanol (2:1, v/v) to the incubation tube together with 2 μ l of 0.1 M-MnCl₂ and 5 μ l of 25 mM-EDTA, and this mixture was dried under vacuum. Incubation was carried out at 30°C for 15 min, followed by extraction with 3 $\times$ 3 ml of chloroform/methanol (2:1, v/v) at 40°C (lipid I fraction). The remaining fraction was extracted with 3 $\times$ 1 ml of chloroform/methanol/water (10:10:3, by vol.) (lipid II fraction). The protein pellet was washed with 1 ml of water and solubilized in 1 ml of 2% sodium dodecyl sulphate. The total radioactivity in the lipid extracts was routinely determined;

80–90% of the radioactivity was identified as dolichol monophosphate sugar and dolichol pyrophosphate oligosaccharide by t.l.c. (Oliver & Hemming, 1975). In experiments with retinyl phosphate the 200 μ l incubation mixture contained: 30 mM-Tris/HCl buffer, pH 7.8; 2.5 mM-EDTA; 10 mM-MnCl₂; 1.8 mM-ATP; 150 μ g of microsomal phospholipids (value obtained by multiplying the phosphorus content by 25) dissolved in 20 μ l of dimethyl sulphoxide with or without 20 μ g of retinyl phosphate; the nucleotide sugars corresponding to 200000 c.p.m. each; and 100 μ l of sample containing 3 mg (rough-microsomal fraction), 2 mg (smooth-microsomal fraction) or 1.2 mg (Golgi membranes) of protein. The incubation was carried out at 37°C for 15 min and terminated by the addition of 3 ml of chloroform/methanol (2:1, v/v). After centrifugation (2000g for 10 min) the total soluble phase was placed on a DEAE-cellulose

Table 1. *Incorporation of sugars into lipids and proteins in the presence of rough- and smooth-microsomal fractions in vitro*

The values in parentheses show the incorporation in c.p.m./mg of protein in the absence of added phosphorylated polyprenols. These values were taken as controls and the other values are expressed as the ratio between the value obtained in the presence of added polyprenyl phosphate and that of the control. The values obtained in the lipid II fractions in the case of UDP-Gal and CMP-AcNeu were very low and therefore only approximate values are given. Lipid I represents the chloroform/methanol (2:1, v/v) extract. Lipid II represents the radioactivity in the chloroform/methanol/water (10:10:3, by vol.) extract from the insoluble residue. The remaining radioactivity in the residue represents the incorporation into protein. C₈₅ and C₅₅ represent polyprenyl phosphates with the appropriate number of carbon atoms. α -diH-C₈₅ and α -diH-C₅₅ represent polyprenyl phosphates with saturated α -isoprene units.

		Stimulation of incorporation (fold increase over control shown in parentheses)					
Substrate	Addition	Rough-microsomal fraction			Smooth-microsomal fraction		
		Lipid I	Lipid II	Protein	Lipid I	Lipid II	Protein
GDP-Man	None	(4668)	(336)	(1617)	(3156)	(211)	(1310)
	C ₈₅	5.7	1.1	0.76	8.3	0.83	0.80
	α -diH-C ₈₅	11	1.3	0.48	21	1.0	0.62
	C ₅₅	3.1	0.98	0.85	8.9	0.97	0.79
	α -diH-C ₅₅	9.8	1.5	0.50	17	1.1	0.64
UDP-GlcNAc	None	(3648)	(67)	(466)	(2565)	(44)	(320)
	C ₈₅	1.2	0.92	0.96	1.2	1.3	0.82
	α -diH-C ₈₅	3.0	1.0	1.2	4.5	1.1	0.95
	C ₅₅	1.1	1.0	1.0	2.8	0.98	1.0
	α -diH-C ₅₅	2.7	1.1	1.2	4.7	1.3	1.1
UDP-Glc	None	(6097)	(555)	(971)	(6691)	(401)	(1126)
	C ₈₅	1.8	0.75	1.1	2.1	0.96	1.1
	α -diH-C ₈₅	2.2	0.72	1.3	2.9	0.85	0.95
	C ₅₅	1.7	0.86	1.0	1.8	0.92	1.0
	α -diH-C ₅₅	2.5	0.63	0.96	2.8	0.83	0.96
UDP-Gal	None	(272)	(~20)	(3101)	(463)	(~20)	(7516)
	C ₈₅	1.2	1	0.99	1.1	1	0.94
	α -diH-C ₈₅	0.98	1	1.0	1.2	1	0.99
	C ₅₅	1.0	1	1.1	1.1	1	1.0
	α -diH-C ₅₅	1.0	1	0.99	1.2	1	1.0
CMP-AcNeu	None	(56)	(~10)	(480)	(86)	(~10)	(1373)
	C ₈₅	1.2	1	1.0	1.0	1	1.1
	α -diH-C ₈₅	1.2	1	0.99	0.97	1	1.0
	C ₅₅	1.0	1	0.96	1.0	1	1.0
	α -diH-C ₅₅	1.3	1	1.0	1.0	1	1.1

column, chromatographed, and retinyl phosphate sugar was isolated by t.l.c. as described previously (Silverman-Jones *et al.*, 1976). All of these procedures were carried out in red light. Retinyl phosphate and the dolichol derivatives were prepared by published procedures (Frot-Coutaz & De Luca, 1976; Mańkowski *et al.*, 1976). Protein was determined by the biuret reaction (Gornall *et al.*, 1949). All experiments were repeated 4 to 7 times and the Tables give the median values obtained.

Results and Discussion

Transfer of mannose from GDP-mannose to added C₈₅ and C₅₅ dolichol phosphates is 10–20 times the rate of transfer to endogenous acceptors in rough- and smooth-microsomal fractions and Golgi membranes (Tables 1 and 2). This transfer rate is significantly less when the non-saturated prenol derivatives are added. Incorporation into the lipid II

fraction and protein is not stimulated and in some cases is inhibited. With UDP-*N*-acetylglucosamine as substrate, only the incorporation into the first lipid extract was increased (3–4-fold) by adding the two dolichol phosphates. Glucose incorporation into endogenous lipids is high for all three subcellular fractions, but stimulation by added polyprenyl phosphates is only 2-fold in the microsomal fractions and not detectable in Golgi membranes. Galactose and *N*-acetylneuraminic acid cannot be transferred to added polyprenyl phosphates in the presence of these subcellular membranes. All subcellular fractions were prepared with previously established procedures and the purity of these fractions has been discussed in detail in previous papers (Bergman & Dallner, 1976; DePierre & Dallner, 1976). The degree of cross-contamination was found to be low and the main contamination consisted of the presence of Golgi membranes in the smooth-microsomal fraction. For this reason, the smooth-microsomal fractions were floated on a discontinuous gradient that proved to be highly efficient for the removal of this contaminant.

In the present study the relation between various transferase activities is not the same as in several previously published studies. This could reflect differences in the preparation of subfractions and in the conditions used for the incubations *in vitro*. Specifically, the measures taken to remove (Tris-washing) and to inhibit (ATP) the antagonistic enzymes are important.

Microsomal subfractions and Golgi membranes isolated from rats not deficient in vitamin A stimulate the transfer of sugar into retinyl phosphate only from GDP-mannose (Table 3). This stimulation is greatest with the rough-microsomal fraction, intermediate with the smooth-microsomal fraction and least with Golgi membranes.

At present, no specific and sensitive method for determining the amounts of the different types of polyprenyl phosphates in isolated subfractions is

Table 2. Incorporation of sugars into lipids and proteins in the presence of Golgi membranes *in vitro*
Details were as in Table 1. The values obtained in the lipid I fraction in the case of CMP-AcNeu and in the lipid II fractions in the case of UDP-GlcNAc, UDP-Gal and CMP-AcNeu were very low and therefore only approximate values are given.

Substrate	Addition	Stimulation of incorporation (fold increase over control shown in parentheses)		
		Lipid I	Lipid II	Protein
GDP-Man	None	(209)	(82)	(591)
	C ₈₅	3.7	0.97	1.0
	α-diH-C ₈₅	13	1.1	1.1
	C ₅₅	3.4	0.96	0.97
	α-diH-C ₅₅	9.2	0.97	1.0
UDP-GlcNAc	None	(232)	(~15)	(965)
	C ₈₅	1.0	1	1.1
	α-diH-C ₈₅	3.1	1	1.2
	C ₅₅	0.97	1	1.0
	α-diH-C ₅₅	3.0	1	0.96
UDP-Glc	None	(600)	(222)	(1355)
	C ₈₅	1.0	1.0	1.3
	α-diH-C ₈₅	1.2	1.0	1.5
	C ₅₅	1.0	0.98	1.2
	α-diH-C ₅₅	0.90	0.90	1.4
UDP-Gal	None	(637)	(~15)	(6966)
	C ₈₅	1.0	1	1.0
	α-diH-C ₈₅	1.1	1	1.0
	C ₅₅	0.98	1	0.99
	α-diH-C ₅₅	0.99	1	0.97
CMP-AcNeu	None	(~15)	(~15)	(1798)
	C ₈₅	1	1	1.0
	α-diH-C ₈₅	1	1	1.1
	C ₅₅	1	1	1.1
	α-diH-C ₅₅	1	1	1.1

Table 3. Incorporation of sugars into retinyl phosphate in the presence of cytoplasmic membranes
Retinyl phosphate (RP) sugars were isolated as described in the Materials and Methods section.

Substrate	Sp. radioactivity (c.p.m./mg of protein)					
	Rough-microsomal fraction		Smooth-microsomal fraction		Golgi membranes	
	-RP	+RP	-RP	+RP	-RP	+RP
GDP-Man	74	1766	248	2710	106	584
UDP-GlcNAc	11	14	6	13	4	8
UDP-Glc	7	8	8	12	6	6
UDP-Gal	5	5	10	10	7	12
CMP-AcNeu	3	7	8	9	2	5

Fig. 1. Time course of mannose and *N*-acetylglucosamine transfer to endogenous lipid acceptors of the rough-microsomal fraction

The incubation mixture contained: 30mM-Tris/HCl buffer, pH7.8; 2.5mM-EDTA; 10mM-MnCl₂, 0.4% Triton X-100, 4nmol of GDP-mannose or UDP-*N*-acetylglucosamine and the amounts of ATP, radioactive substrates and protein that are given in the Materials and Methods section. After various times of incubation the radioactivity was determined in the chloroform/methanol (2:1, v/v) extract. Since the amount of radioactivity incorporated is proportional to the amount of sugars covalently bound to lipid intermediates, the absolute amount of sugar incorporated can be calculated. The radioactivity was associated with one single peak on t.l.c. corresponding to dolichol-sugar phosphate. Symbols: ●, mannose; ○, *N*-acetylglucosamine.

available, but their distribution among subcellular fractions is obviously broad (Dallner *et al.*, 1972). In an attempt to estimate the amount of endogenous lipid that functions as sugar acceptor and exhibits chromatographic properties similar to those of dolichol phosphate, an indirect procedure was used. Fig. 1 shows that in the presence of an excess of substrate the radioactivity in the lipid extract reached a maximum value after 5 min, i.e. saturation occurred. Presupposing a stoichiometric interaction between the appropriate dolichol phosphate and the sugar, the radioactivity incorporated after incubation for 15 min was taken as a measure of the functionally active dolichol phosphate present. The radioactive substance was also shown to behave chromatographically like dolichol phosphate. Table 4 shows the values obtained with the three subfractions and the three sugars. Each subfraction exhibits individual behaviour in accepting the various sugars, indicating that dolichol phosphates may be heterogeneously distributed.

The results in the present paper theoretically may be interpreted in several ways. The first is the possibility that a number of polyprenyl phosphates with acceptor specificity are participating in the glycosylation of proteins in the membrane of the endoplasmic reticulum and that the subcellular distribution of these polyprenols is heterogeneous. The

Table 4. Sugar transfer to endogenous lipid acceptors in isolated rough- and smooth-microsomal fractions and Golgi membranes

The incubation mixture is given in the legend of Fig. 1 and in the Materials and Methods section. The medium was supplemented with 4nmol of non-radioactive GDP-mannose, UDP-GlcNAc or UDP-Glc. Incubation was carried out at 30°C for 15 min, at which time all the available lipid intermediates were glycosylated. The values are calculated from the radioactivity present in the chloroform/methanol (2:1, v/v) extract as described in the legend of Fig. 1.

Fraction	Sugar transfer (pmol/mg of protein)		
	Man	GlcNAc	Glc
Rough-microsomal fraction	39	35	76
Smooth-microsomal fraction	19	15	47
Golgi membranes	11	5.1	25

second explanation could be that separate pools of polyprenyl phosphates exist for each type of transferase that vary in concentration in different membranes. Thus the results may be explained without assuming a difference in the structure of the lipid intermediates. Also one could claim that there are differences in the rate of entrance of the various exogenous polyprenyl phosphates into the membranes where they can react with the transferases. However, all the experiments described in this paper were performed by using a concentration of Triton X-100 that completely dissolves the membranes. Under these conditions no permeability barrier is present and the interaction of lipid intermediates with transferase enzymes is not limited by the presence of a membrane structure. C₈₅- and C₅₅-polyprenyl phosphates with saturated α -isoprene units, like retinyl phosphate, accept mannose effectively, but are much less effective acceptors for other sugars. The amount of endogenous acceptor for *N*-acetylglucosamine is, however, high in rough- and smooth-microsomal fractions, compared with the finding for Golgi membranes. The most effective lipid acceptor in the intracellular membranes examined in the present paper is that for glucose, but the polyprenyl phosphates added were less effective acceptors for this sugar than for mannose and glucosamine. It is possible that the amounts and types of polyprenyl phosphates present in cytoplasmic membranes vary in accordance with the type of biosynthetic pathway present.

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References

- Bergman, A. & Dallner, G. (1976) *Biochim. Biophys. Acta* **433**, 496–508
- Dallner, G. (1974) *Methods Enzymol.* **31A**, 191–201
- Dallner, G., Behrens, N. H., Parodi, A. J. & Leloir, L. F. (1972) *FEBS Lett.* **24**, 315–317
- De Luca, L. M. (1977) *Vitam. Horm. (N. Y.)*, **35**, 1–57
- De Luca, L. M., Maestri, N., Rosso, G. & Wolf, G. (1973) *J. Biol. Chem.* **248**, 641–648
- DePierre, J. W. & Dallner, G. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., ed.), pp. 79–131, Chapman and Hall, London
- Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. & Palade, G. E. (1973) *J. Cell Biol.* **59**, 45–72
- Frot-Coutaz, J. P. & De Luca, L. M. (1976) *Biochem. J.* **159**, 799–801
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Hemming, F. W. (1974) *MTP Int. Rev. Sci. Biochem. Ser. One* **4**, 39–97
- Mańkowski, T., Jankowski, W., Chojnacki, T. & Franke, P. (1976) *Biochemistry* **15**, 2125–2130
- Molnar, J. (1975) *Mol. Cell. Biochem.* **6**, 3–14
- Nilsson, O. S., Bergman, A. & Dallner, G. (1976) Abstr. *Int. Congr. Cell Biol. 1st, Boston*, p. 235
- Oliver, G. J. A. & Hemming, F. W. (1975) *Biochem. J.* **152**, 191–199
- Schachter, H., Jabbal, I., Hudgin, R. L., Pinteric, L., McGuire, E. J. & Roseman, S. (1970) *J. Biol. Chem.* **245**, 1090–1100
- Silverman-Jones, C. S., Frot-Coutaz, J. P. & De Luca, L. M. (1976) *Anal. Biochem.* **75**, 664–667
- Waechter, C. J. & Lennarz, W. J. (1976) *Annu. Rev. Biochem.* **45**, 95–112
- Wetmore, S., Mahley, R. W., Brown, W. V. & Schachter, H. (1974) *Can. J. Biochem.* **52**, 655–669