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INTRAMEMBRANOUS ARRANGEMENT OF THE GLYCOSYLATING SYSTEMS IN ROUGH AND SMOOTH MICROSOMES FROM RAT LIVER

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The distribution of mannosyl-, glucosaminyl- and glucosyltransferases in rough and smooth microsomes isolated from rat liver homogenate has been investigated. Amphomycin and tunicamycin were used as inhibitors of dolichol-mediated glycosylation, and diazobenzene sulfonate and proteolytic enzymes were used as nonpenetrating surface probes. Under in vitro conditions only 20–30% of the proteins glycosylated are of the secretory type. Nonpenetrating surface probes, which interact with components on the outer surface of rough microsomal vesicles, decrease glycosylation of both secretory and membrane proteins to a great extent. Inhibitor sensitive glycosylation is present in both the outer and inner compartments of the microsomal membranes. In contrast, the surface probes and the inhibitors of dolichol-mediated glycosylation do not significantly affect protein glycosylation in smooth microsomes. When dolichol phosphate sugars were used as substrates, instead of nucleotide sugars, the probes used inhibited protein glycosylation in both subfractions. Glycosylation of externally added Lipidex-bound dolichol monophosphate and of ovalbumin were in agreement with the above results. It appears that both rough and smooth microsomes may possess several types of glycosylating pathways. The most prominent of these in rough microsomes under the conditions used is the dolichol mono- and pyrophosphate-mediated glycosylation of endogenous proteins, where the enzymes involved in the inital steps are distributed at the outer surfaces of the microsomal vesicles. The dominating pathway in smooth microsomes appears to function in completion of the oligosaccharide chain of the protein and this process does not involve lipid intermediates and cannot be influenced by nonpenetrating surface probes.

Introduction

The processes of core oligosaccharide assembly and of oligosaccharide transfer to protein, in which the *N*-glycosidic linkage is established between glucosamine and asparagine have been intensively studied in recent years [1–5]. One molecule of dolichol pyrophosphate accumulates two GlcNAc residues from UDP-GlcNAc, followed by transfer of nine mannose residues, five from GDPmannose and four from dolichol phosphate mannose. Finally, three glucose residues are added from dolichol phosphate glucose. The oligosaccharide chain bound in the protein is processed through a number of characterized steps and terminal sugars are added in the completion process [6].

The substrates in the glycosylating reactions are nucleotide-activated sugars, which are synthesized in the cytoplasm and as charged substances, cannot penetrate the membrane of the endoplasmic reticulum [7]. This fact may necessitate that the

Abbreviation: GlcNAc, *N*-acetylglucosamine.

first transferases of the glycosylating complex are in a position which allows them to interact with the substrate at the cytoplasmic surface. In addition, some of the enzymes at the outer surface of microsomes may have short covalently-bound oligosaccharide chains [8–11].

For these reasons one would expect that a part of the glycosyltransferase systems is associated with that compartment of endoplasmic membranes which faces the cytoplasmic environment. Previous studies have demonstrated that mannosyl-, glucosyl- and glucosaminyltransferase reactions are sensitive to protease treatment, and consequently, that at least a part of these transferase systems is associated with the outer surface [12–16]. A similar conclusion was arrived at by studying the effect of detergent on microsomal transferase reactions [17]. Concerning the position of the diacetylchitobiose and the localization of the enzyme mediating transfer of the lipid-linked oligosaccharide to the protein, it was concluded that, at least in the oviduct, these components are distributed at the inner, luminal face of the endoplasmic reticulum membranes [18,19].

In this paper the intramembranous distribution of glucosyl transfer reactions was studied in intact rough and smooth microsomes. For this purpose in vitro studies were conducted using proteases, inhibitors, and nonpermeable surface probes. The results suggest that glycosylation may proceed along several pathways, which are different in rough and smooth microsomes. Preliminary reports of this work have appeared elsewhere [20,21].

Materials and Methods

Animals and fractionation

Adult male albino rats weighing 160–180 g were used. All animals were starved for 20 h before killing.

Rough and smooth microsomes were prepared as described previously [22]. The fractions were suspended (microsomes from 1 g wet weight liver in 5 ml) in 0.15 M Tris-HCl, pH 8.0, and centrifuged at $105000 \times g$ for 60 min in order to remove adsorbed, cytoplasmic proteins. This washing step is necessary to remove the majority of pyrophosphatases splitting the nucleotide sugar substrates and also to eliminate cytoplasmic enzymes leading to epimerization of sugars. On the other hand, the latter type of reaction is known to be absent when microsomes are incubated in vitro with nucleotide sugars [23,24].

p-Diazobenzene sulfonate and proteolytic treatment

Treatment with proteases was performed in a medium containing 50 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.45 mg trypsin (Boehringer-Mannheim), 0.45 mg unspecific protease (type VII from *Bacillus amyloliquefaciens*, Sigma, St. Louis) and 18 mg microsomal protein in a final volume of 3 ml. The incubation was carried out for 10 min at 37°C and was terminated by the addition of 5 ml cold 0.25 M sucrose and centrifugation for 60 min at $105000 \times g$. Higher concentrations of proteases or longer incubation time did not increase the effects on glycosyltransferases.

p-Diazobenzene sulfonate was prepared as described previously [25]. The incubation mixture contained 50 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.75 mM *p*-diazobenzene sulfonate and 18 mg microsomal protein in a final volume of 3 ml. The incubation time was 2 min in an ice-water bath and the process was terminated by the addition of 5 ml cold 0.25 M sucrose containing 13 mM $CaCl_2$ and 8 mM $MgCl_2$ and centrifugation at $40000 \times g$ for 15 min.

Incubations

To follow glycosylation with GDP[^{14}C]mannose the incubation mixture contained 30 mM Tris-HCl, pH 7.8, 1 mM $MnCl_2$, 12.5 mM mercaptoethanol, 2 mM AMP, 0.4 μCi GDP[^{14}C]mannose (80 mCi/mmol, Radiochemical Centre, Amersham) and 2.5 mg microsomal protein in a total volume of 1 ml. The incubation mixture for UDP[^{14}C]GlcNAc as substrate contained 30 mM Tris-HCl, pH 7.8, 2.5 mM EDTA, 12.5 mM mercaptoethanol, 10 mM $MnCl_2$, 1.5 mM ATP, 0.8 μCi UDP[^{14}C]GlcNAc (300 mCi/mmol, Radiochemical Centre, Amersham) and 2.5 mg microsomal protein in a total volume of 1 ml. In the case of UDP[^{14}C]glucose as substrate the incubation mixture contained 30 mM Tris-HCl, pH 7.8, 12.5 mM mercaptoethanol, 10 mM $MnCl_2$, 1.5 mM ATP, 0.8 μCi UDP[^{14}C]glucose (200 mCi/mmol) and 2.5 mg microsomal protein in a total volume of 1 ml. When indicated,

incubation was performed in the presence of amphomycin (400 μg/ml) or tunicamycin (10 μg/ml). Microsomes were preincubated with these inhibitors for 5 min and the reaction was started by the addition of radioactive substrate. At the end of all experimental series incubations were performed using a range of substrate and varying protein concentrations. This was done to ensure that the results obtained are not caused by artifacts such as rate limiting substrate amounts during incubation. Under all these conditions the experimental results obtained gave similar patterns.

When dolichol phosphate sugar was used as substrate, the incubation mixture was the same as in the case of the corresponding sugar nucleotide. However, the radioactive substrate (40000 cpm) in chloroform/methanol (2 : 1, v/v) was supplemented with 4 μl of 0.1 M $MnCl_2$ and 10 μl of 25 mM EDTA and evaporated under vacuum. After addition of 80 μl 5% Triton X-100, the tube was vortexed for 1 min and supplemented with the rest of the incubation mixture.

The data given in tables and figures are the results obtained after 30 min incubation at 30°C. However, time curves done routinely demonstrated that shorter incubations gave principally the same results. The chloroform-methanol and the chloroform-methanol-water extracts were analyzed by chromatographic and thin-layer procedures [26] and were found in all cases to contain predominantly dolichol phosphate sugar and dolichol pyrophosphate oligosaccharide, respectively. Experiments were also routinely performed where the radioactivity was not measured directly in the extracts, but in dolichol monophosphate sugar and dolichol pyrophosphate oligosaccharide, which were isolated by chromatography. The results were similar in both procedures. All values given in the tables and figures are the means of 5–9 experiments.

Antibody precipitation

When nucleotide sugars were used as substrates, the mixtures were cooled at the end of incubation. 200 μl 25 mM EDTA was added and the mixture was centrifuged without dilution at 105000 × *g* for 45 min; this procedure leaves the non-protein bound oligosaccharides in the supernatant. In those experiments in which secretory and membrane proteins were separated the pellet was resuspended in 2.8 ml 0.25 M sucrose containing 2 mM EDTA and 0.5% Triton X-100. This mixture was centrifuged at 6000 × *g* for 15 min and the supernatant was supplemented with an excess of goat antiserum IgG fraction against total rat serum proteins (United States Biochemical Corp., Cleveland). Incubation was performed in rotating system, first 1 h at room temperature and subsequently at +4°C over night. The mixture was then supplemented with 100 mg protein A-Sepharose (Pharmacia, Uppsala), dissolved in 1 ml 0.2 M Tris-HCl buffer, pH 7.8, and the rotation was continued at room temperature for 30 min. The mixture was centrifuged thereafter at 600 × *g* for 20 min to separate the supernatant (membrane proteins) from the pellet (secretory proteins). The pellet was washed twice with distilled water by recentrifugation.

Lipid extraction

Extraction of dolichol phosphate derivatives was performed by chloroform/methanol [26]. The pellet was suspended in 6 ml chloroform/methanol (2:1, v/v), 0.5 ml 0.1 M $MgCl_2$ was added and incubation in a water-bath at 40°C for 20 min was performed with continuous mixing. After centrifugation the upper water-methanol phase was removed and the surface of the lower phase rinsed with 'upper phase'. The chloroform phase was then decanted, mixed with 1.5 ml upper phase, centrifuged, and the surface again rinsed with upper phase. This washing procedure was repeated twice. The remaining protein was washed two times with 1.5 ml upper phase and extracted once more with 6 ml chloroform/methanol (2 : 1, v/v) at 40°C for 20 min. The two chloroform-methanol extracts were pooled (dolichol phosphate sugar). The remaining fraction was extracted two times with 3 ml chloroform/methanol/H_2O (1 : 1 : 0.3, v/v) (dolichol pyrophosphate oligosaccharide). When the protein did not sediment completely during centrifugation, additional methanol was added before centrifugation. The protein pellet was washed with 1 ml H_2O and solubilized with 2.5 ml 2% sodium dodecyl sulfate. The two lipid extracts were evaporated and radioactivity determined after the addition of 10 ml Aqualuma Plus (Lumac, Basel). For extraction of the supernatant 6 ml chloroform/methanol (2:1, v/v) was added fol-

lowed by 0.5 ml 0.1 M $MgCl_2$ and the same extraction procedure was performed as in the case of the pellet.

Dolichol sugars

Dolichol pyrophosphate GlcNAc, dolichol phosphate mannose and dolichol phosphate glucose were prepared enzymatically and the two latter compounds were also obtained commercially (New England Nuclear). The enzymatically glycosylated dolichol phosphate was extracted, isolated with DEAE-cellulose chromatography, and identified on thin-layer chromatography as described earlier [26]. In order to present dolichol phosphate or dolichol phosphate sugars to rough microsomes without using detergents, these compounds were attached to Lipidex-1000 (Packard) which is an alkoxy group derivative of Sephadex LH-20 and highly hydrophobic. Dolichol(55), dolichol(55) phosphate or the appropriate dolichol phosphate sugar derivative in chloroform-methanol were evaporated and supplemented with 3 ml 95% ethanol and 2 ml Lipidex-1000 and mixed with vortex. Subsequently, 3.4 ml of H_2O was added dropwise. The mixture was centrifuged at $600 \times g$ for 10 min and the pellet was washed with water by centrifugation. The dolichol derivatives of the Lipidex were suspended in 5 ml H_2O and aliquots of this suspension used in incubations.

In those experiments where glycosylation of Lipidex-bound dolichol phosphate was studied, the incubation mixture was diluted with 5 ml cold H_2O and centrifuged at $600 \times g$ for 10 min. The supernatant was discarded and the pellet washed twice with 0.15 M Tris-HCl, pH 8.0, and finally, with water. Both electron microscopic and enzymic control were performed to ensure that the pellet was free from membranous components. Extraction of dolichol phosphate from Lipidex was carried out with chloroform/methanol (2:1, v/v). In these experiments the microsomal vesicles were also separated at the end of the incubation and found to possess preserved impermeability.

Ovalbumin as acceptor

Native ovalbumin or ovalbumin treated with β-glucosaminidase was used as acceptor protein in some experiments. At the end of the incubation the ovalbumin was isolated either by immunoprecipitation or by thin-layer gel filtration.

In the case of immunoprecipitation at the end of the incubation the mixture containing 0.2 mg ovalbumin was centrifuged at $105000 \times g$ for 3 h and the supernatant was supplemented with excess of rabbit antiserum IgG against chicken egg (United States Biochemical Corp.) and incubated at room temperature during 1 h and after in a cold room overnight under continuous rotation. The incubated mixture then was centrifuged at $105000 \times g$ for 1 h and the pellet was successively washed with water, 12%, 5%, 5% trichloroacetic acid and finally with water. The pellet was solubilized in 2% sodium dodecyl sulfate and radioactivity was measured after the addition of Aqualuma Plus scintillator.

Thin-layer gel filtration

In the case of thin-layer gel filtration the incubation mixture was centrifuged at $105000 \times g$ for 1 h, the supernatant freeze-dried and after dissolving 200 μg was applied on the plate. The plates were coated with Sephadex G-200 Superfine (Pharmacia, Uppsala) and were equilibrated with 50 mM Tris-HCl (pH 8.0) +50 mM NaCl. The samples were run for 6 h at 15° angle at room temperature. The protein spots were transferred to a paper replica (Whatman 3 MM) and developed in Coomassie Brilliant Blue R 250 solution according to Radola [27].

Chemical and other measurements

Protein determination was performed with the Biuret reaction [28] and phospholipids were analyzed as described earlier [29]. The various enzyme activities or amounts were measured as previously [30,31]. The intramicrosomal water was estimated by a centrifugation procedure [7].

Results

Effect of surface treatment on the microsomes

One approach to study the transverse topology of intact vesicles is by applying enzymic or chemical reagents which can interact with surface components but cannot cross the membrane. The outer surface of microsomal vesicles can be digested with suitable proteolytic enzymes which affect exclusively the proteins on this side of the membrane

[32–34]. Previous data have demonstrated that diazobenzene sulfonate may be used as an effective probe for surface proteins [25,35]. This probe has a negative charge on its sulfonate group, which strongly limits its permeability through membranes. The azo moiety of this substance reacts with a number of groups in proteins, including sulfhydryl, amino and hydroxyl groups [36,37]. One advantage of the diazobenzene sulfonate is its low molecular weight (186), so from the steric point of view it reacts easily with functional groups on proteins. On the other hand, the attack of proteases on the peptide bonds may be limited by steric hindrance. Earlier experiments demonstrated that diazobenzene sulfonate is a valuable tool to probe protein topography in microsomal membranes, but one has to carefully maintain well-defined conditions during the incubation [25,38]. The amount of protein present, the time of incubation and the temperature are important factors within narrow limits. If this probe is applied and the membrane barrier is not destroyed, the results concerning protein distribution agree well with those obtained by other procedures.

The validity and the effectiveness of the methods used here is demonstrated in Table I. Proteolysis removes about 35% of the microsomal protein and neither this nor *p*-diazobenzene sulfonate treatment liberate any of the phospholipid components. It is obvious that all the enzymes studied here are distributed in a completely asymmetric manner. The three electron transport enzymes are found on the cytoplasmic surface, while glucose-6-phosphatase, β-glucuronidase and *p*-nitrophenyl-propionate esterase are at the luminal surface. This enzyme distribution in microsomes is supported by different types of experimental evidence [12].

The basic condition for this approach is that the treatment employed does not change membrane permeability. Both *p*-diazobenzene sulfonate and proteolytic treatment are sufficiently mild not to affect microsomal permeability. The hydrolytic site of mannose-6-phosphatase is at the luminal surface and the glucose-6-phosphatase carrier does not transport substrate of this enzyme [39]. Consequently, in the absence of detergent, this enzyme is almost totally latent (Table II). *p*-Diazobenzene sulfonate and proteolytic treatment do not in-

TABLE I

EFFECTS OF *p*-DIAZOBENZENE SULFONATE AND PROTEOLYTIC TREATMENT ON MICROSOMAL ENZYMES

Microsomes were incubated in the presence of trypsin and unspecific protease (25 μg of each per mg protein) for 10 min at 37°C or in the presence of *p*-diazobenzene sulfonate (0.75 mM) for 2 min at 0°C. After pelleting and washing by recentrifugation enzyme activities or amounts were measured as described in Materials and Methods.

	Activity or amount	% remaining activity or amount after treatment with	
		p-diazobenzene sulfonate	proteases
Protein [a]	18.3	98	63
Phospholipid [a]	5.9	100	98
NADH-cytochrome *c* reductase [b]	1.82	7	10
NADPH-cytochrome *c* reductase [b]	0.12	8	8
Cytochrome *P*-450 [c]	1.60	16	19
Glucose-6-phosphatase [d]	1.26	85	82
β-Glucuronidase [e]	0.18	94	83
p-Nitrophenylpropionate esterase [e]	24.1	94	96

[a] mg/g liver.
[b] μmol NADH or NADPH oxidized per min per mg phospholipid.
[c] nmol/mg phospholipid.
[d] μmol P_i/min per mg phospholipid.
[e] μmol *p*-nitrophenyl liberated per min per mg phospholipid.

TABLE II

EFFECTS OF PROTEOLYTIC AND *p*-DIAZOBENZENE SULFONATE TREATMENT ON MICROSOMAL MEMBRANE INTEGRITY

Treatment	Mannose-6-phosphatase			Nucleoside diphosphatase (μmol P_i/min per mg phospholipid)	Intra-microsomal water (μl/mg dry weight)
	Activity(μmol P_i/min per mg phospholipid)		% latency		
	+Triton	−Triton			
None	0.87	0.04	95	1.57	1.29
p-Diazobenzene sulfonate	0.71	0.09	89	1.48	1.23
Proteolysis	0.82	0.07	91	1.48	1.20

crease microsomal permeability to mannose 6-phosphate, which has a molecular weight of 275. Nucleotide diphosphatase is loosely attached to the inner surface of the microsomal vesicle and easily released from the vesicle upon damage [40]. Again, this enzyme is not released by the treatments used. Another reliable approach for determining the condition of the microsomal vesicle is to determine the intramicrosomal water space [7]. This space was not affected by the treatment employed, which again shows that the vesicles remained intact.

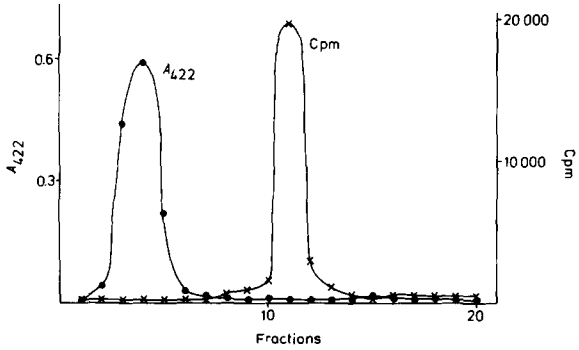

Fig. 1. Chromatographic separation of *p*-diazobenzene sulfonate-treated dolichol(55) [^{32}P]phosphate. Dolichol(55) [^{32}P]phosphate was micellized by sonication in the presence of egg phosphatidylcholine and incubated 2 min at 4°C with 1 mM *p*-diazobenzene sulfonate. The reaction was stopped by the addition of cold water and supplemented with Lipidex-1000. After equilibration of the column with 50% aceton-water the Lipidex-1000 associated sample was placed on the Lipidex column and eluated with 50% aceton-water. Fractions were collected and the distribution of radioactivity and the 422 nm absorbing material were determined.

In this paper *p*-diazobenzene sulfonate was used to probe proteins and to get information about their topology in the membrane. On the other hand, *p*-diazobenzene sulfonate reacts with some of the phospholipids and the possibility arose that it may react with dolichol phosphate in the membrane. Such an interaction could prevent dolichol phosphate from accepting sugar and render the procedure unsuitable for our purposes. In order to investigate this question dolichol(55) [^{32}P]phosphate was incubated with *p*-diazobenzene sulfonate and the mixture subjected to chromatography on Lipidex-1000 (Fig. 1). A complete separation of *p*-diazobenzene sulfonate and dolichol phosphate was obtained, indicating lack of interaction of the lipid with the probe.

Mannosyltransferases

Endogenous dolichol phosphate of rough microsomes in the absence of Triton or other detergents is a good acceptor of mannose from GDP-mannose (Table III). Under the conditions employed about 5–10% of the radioactive mannose is recovered in the dolichol pyrophosphate oligosaccharide fraction. Under in vitro conditions in rough microsomes from rat liver the protein glycosylated is mostly membrane protein and only about 15% are secretory proteins. Tunicamycin, a well established inhibitor of glucosamine transfer to dolichol phosphate [41–43] does not interfere with mannosylation, whereas amphomycin [44,45] inhibits mannosyl transfer reaction to dolichol phosphate completely and to dolichol pyrophosphate oligosaccharide to a large extent. Mannosylation of both secretory and membrane proteins is also in-

TABLE III

MANNOSYLATION OF ENDOGENOUS LIPIDS, SECRETORY AND MEMBRANE PROTEINS OF ROUGH MICROSOMES WITH GDP-MANNOSE AS SUBSTRATE

The values in parenthesis show the incorporation in cpm/mg protein and are taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of the control ×100.

Treatment	Inhibitor	% of control			
		Dol-*P*-Man [a]	Dol-*PP*-O [b]	Secretory protein	Membrane protein
None	–	100 (2216)	100 (166)	100 (68)	100 (461)
	Tunicamycin [c]	98	96	97	94
	Amphomycin [d]	2	21	56	52
p-Diazobenzene sulfonate	–	61	43	60	58
	Amphomycin [d]	2	12	40	41
Proteolysis	–	65	52	57	52

[a] Dol-*P*-Man, dolichol phosphate mannose.
[b] Dol-*PP*-O, dolichol pyrophosphate oligosaccharide.
[c] 10 μg per ml incubation medium.
[d] 400 μg per ml incubation medium.

hibited 50%. When the outer surface of rough microsomes is treated with *p*-diazobenzene sulfonate or proteolytic enzymes, the mannosylation of both lipids and proteins decrease about 40%. The incorporation into proteins remaining after *p*-diazobenzene sulfonate treatment was decreased another 20% by amphomycin, indicating that dolichol phosphate-mediated mannosylation is present both in the *p*-diazobenzene sulfonate-sensitive and insensitive membrane compartments.

In the case of smooth microsomes the situation is very different (Table IV). Smooth microsomes also exhibit an active incorporation of mannose into the dolichol phosphate fraction and again the majority of proteins mannosylated are membrane proteins. However, amphomycin, which inhibits

TABLE IV

MANNOSYLATION OF ENDOGENOUS LIPIDS, SECRETORY AND MEMBRANE PROTEINS OF SMOOTH MICROSOMES WITH GDP-MANNOSE AS SUBSTRATE

The values in parenthesis show the incorporation in cpm/mg protein and are taken as 100%. The other values are expressed as the ratio between the values obtained in the individual experiment and that of control ×100.

Treatment	Inhibitor	% of control			
		Dol-*P*-Man [a]	Dol-*PP*-O [b]	Secretory protein	Membrane protein
None	–	100 (928)	100 (85)	100 (76)	100 (408)
	Tunicamycin [c]	95	99	92	89
	Amphomycin [d]	5	30	96	98
p-Diazobenzene sulfonate	–	77	84	91	94
Proteolysis	–	76	89	89	87

[a] Dol-*P*-Man, dolichol phosphate mannose.
[b] Dol-*PP*-O, dolichol pyrophosphate oligosaccharide.
[c] 10 μg per ml incubation medium.
[d] 400 μg per ml incubation medium.

TABLE V

GLYCOSYLATION OF ENDOGENOUS LIPIDS AND PROTEINS OF ROUGH (RM) AND SMOOTH (SM) MICROSOMES WITH DOLICHOL PHOSPHATE MANNOSE AS SUBSTRATE

Rough and smooth microsomes were treated with *p*-diazobenzene sulfonate or proteolytic enzymes and recentrifuged before incubation as described in the Materials and Methods. Triton was included in the incubation mixture. The values in parenthesis show the incorporation in cpm/mg protein and are taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of the control ×100.

Expt.	Micro-somes	Treatment	Substrate	Triton (%)	% of control	
					Dol-*PP*-O [a]	Protein
1	RM	None	Dol-*P*-Man [b]	0.4	100 (114)	100 (457)
		p-Diazobenzene sulfonate		0.4	60	64
		Proteolysis		0.4	52	66
2	RM	None	Lipidex-Dol-*P*-Man	0	100 (78)	100 (296)
		Proteolysis		0	45	57
3	SM	None	Dol-*P*-Man	0.4	100 (165)	100 (390)
		p-Diazobenzene sulfonate		0.4	30	44
		Proteolysis		0.4	36	34

[a] Dol-*PP*-O, dolichol pyrophosphate oligosaccharide.
[b] Dol-*P*-Man, dolichol phosphate mannose.

mannosylation of the first lipid intermediate completely and of the second lipid intermediate partially, has no effect on mannosyl transfer to protein. *p*-Diazobenzene sulfonate and proteolytic treatment influence to some extent mannose transfer to lipid intermediates, but they interfere little, if at all, with mannosylation of endogenous protein.

In order to study the specificity of mannose transfer from dolichol phosphate to the bound oligosaccharide, rough and smooth microsomes were pretreated with *p*-diazobenzene sulfonate or proteolytic enzymes and incubation of rough microsomes with dolichol phosphate mannose was performed in the presence of Triton (Table V). The treatment with *p*-diazobenzene sulfonate or proteolytic enzymes preceeding incubation resulted in a decrease in protein glycosylation of about 40%. In order to avoid detergent, which destroys membrane structures, this substrate was also presented in Lipidex-bound form, which makes it 'water soluble'. Even in this case mannosylation was partially inhibited by proteolysis.

Mannosylated dolichol was also incubated with smooth microsomes and the result observed was unexpected. The substrate was effective in protein glycosylation and attack on surface proteins resulted in an inhibition of the mannosyl transfer reactions.

Glucosaminyltransferases

Incubation of rough microsomes with UDP-GlcNAc in the absence of Triton gives high incorporation into lipid intermediates; 95% of the radioactivity incorporated is associated with the first lipid intermediate and 5% with the second carrier (Table VI). As with mannose, only about 20% of the glucosamine is incorporated into secretory proteins; the rest is in membrane proteins. Tunicamycin inhibits not only incorporation into lipids, but to some extent into proteins as well. Interference with surface protein components eliminates about half of the incorporation into proteins. Tunicamycin causes some further decrease of sugar transfer into proteins of *p*-diazobenzene sulfonate -treated microsomes, indicating the presence of a dolichol-mediated system in the *p*-diazobenzene sulfonate-insensitive membrane compartment.

Glucosamine incorporation into smooth microsomal proteins differs from that of the rough counterpart, since about 40% of the radioactivity incorporated is found in secretory proteins (Table

TABLE VI

TRANSFER OF GlcNAc FROM UDP-GlcNAc TO ENDOGENOUS LIPIDS, SECRETORY AND MEMBRANE PROTEINS OF ROUGH (RM) AND SMOOTH (SM) MICROSOMES

The values in parenthesis show the incorporation in cpm/mg protein and are taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of control $\times 100$.

Expt.	Microsomes	Treatment	Inhibitor	% of control			
				Dol-*PP*-GlcNAc [a]	Dol-*PP*-O [b]	Secretory protein	Membrane protein
1	RM	None	–	100 (1181)	100 (68)	100 (134)	100 (631)
			Tunicamycin [c]	5	17	65	51
		p-Diazobenzene sulfonate	–	35	40	48	46
			Tunicamycin [c]	7	20	38	35
		Proteolysis	–	36	39	50	45
2	SM	None	–	100 (970)	100 (59)	100 (298)	100 (492)
			Tunicamycin [c]	16	26	89	87
		p-Diazobenzene sulfonate	–	50	47	91	94
		Proteolysis	–	46	51	91	89

[a] Dol-*PP*-GlcNAc, dolichol pyrophosphate *N*-acetylglucosamine.
[b] Dol-*PP*-O, dolichol pyrophosphate oligosaccharide.
[c] 10 μg per ml incubation medium.

VI). Tunicamycin does not seem to inhibit glucosaminyl transfer to proteins, suggesting the lack of involvement of dolichol phosphate in this glycosylation reaction. *p*-Diazobenzene sulfonate and proteolytic treatment eliminate about half of the glucosamine transfer to lipid intermediates, but have no substantial effect on protein glycosylation.

Transfer in both rough and smooth microsomes

TABLE VII

GLYCOSYLATION OF ENDOGENOUS LIPIDS AND PROTEINS OF ROUGH (RM) AND SMOOTH (SM) MICROSOMES WITH DOLICHOL PYROPHOSPHATE GlcNAc AS SUBSTRATE

The values in parenthesis show the incorporation in cpm/mg protein and are taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of control $\times 100$. All tubes contained 0.4% Triton X-100.

Expt.	Microsomes	Treatment	Substrate	Inhibitor	% of control	
					Dol-*PP*-O [a]	Total protein
1	RM	None	Dol-*PP*-GlcNAc [b]	–	100 (39)	100 (231)
				Tunicamycin [c]	92	90
		p-Diazobenzene sulfonate		–	48	39
		Proteolysis		–	50	41
2	SM	None	Dol-*PP*-GlcNAc [b]	–	100 (27)	100 (86)
		Proteolysis		–	44	44

[a] Dol-*PP*-O, dolichol pyrophosphate oligosaccharide.
[b] Dol-*PP*-GlcNAc, dolichol pyrophosphate *N*-acetylglucosamine.
[c] 10 μg per ml incubation medium.

TABLE VIII

TRANSFER OF GLUCOSE TO ENDOGENOUS LIPIDS, SECRETORY AND MEMBRANE PROTEINS OF ROUGH (RM) AND SMOOTH (SM) MICROSOMES

The values in parenthesis show the incorporation in cpm/mg protein and are taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of control $\times 100$.

Expt.	Microsomes	Treatment	Substrate	Triton (%)	Dol-*P*-Glc [a]	% of control			
						Dol-*PP*-O [b]	Secretory protein	Membrane protein	Total protein
1	RM	None	UDP-Glc	0	100 (516)	100 (53)	100 (488)	100 (534)	
		p-Diazobenzene sulfonate		0	34	54	53	45	
2	RM	None	Dol-*P*-Glc	0.4		100(146)			100 (852)
		p-Diazobenzene sulfonate		0.4	36				37
3	SM	None	UDP-Glc	0	100 (374)	100 (44)	100 (90)	100 (189)	
		p-Diazobenzene sulfonate		0	47	51	91	89	
4	SM	None	Dol-*P*-Glc	0.4		100 (46)			100 (232)
		p-Diazobenzene sulfonate		0.4		41			42

[a] Dol-*P*-Glc, dolichol phosphate glucose.

[b] Dol-*PP*-O, dolichol pyrophosphate oligosaccharide.

was tested with dolichol pyrophosphate GlcNAc, which proved to be good substrate (Table VII). Treatment of vesicles with *p*-diazobenzene sulfonate or proteolytic enzymes reduced glucosamine incorporation to a substantial extent. Tunicamycin has no effect on this sequence of reactions, which agrees with the known mechanism of inhibition by this compound.

Glucosyltransferases

Incubation of rough and smooth microsomes with UDPglucose results in incorporation of the sugar into both lipid intermediates (Table VIII). Transfer into proteins follows a different pattern in comparison with that of mannose and glucosamine, since as much as 50% and 30% of the sugar incorporated was found to be associated with secretory proteins of rough and smooth microsomes, respectively. *p*-Diazobenzene sulfonate treatment gave a sizeable decrease of incorporation into lipids of both microsomal subfractions, while the incorporation into proteins of smooth microsomes was not effected by this treatment. As in the case of the other sugars, dolichol monophosphate-bound glucose could also be used in the glycosylation of proteins, but in this case *p*-diazobenzene sulfonate treatment decreased the transfer by 50% or more.

Glycosylation of exogenous ovalbumin

The experiments described above indicated that some or a part of the glycosylating system in rough microsomes is present at the cytoplasmic surface. To obtain further information about the localization of the glycosylating system without perturbing the membranes, we used two approaches not requiring any treatment of the microsomes.

Ovalbumin which has been partially deglycosylated is a suitable sugar acceptor in microsomal systems [30]. If rough microsomes are incubated in the presence of sugar nucleotides and ovalbumin, glycosyltransferases on the outer surface of the vesicles may mediate sugar transfer to the exogenous protein. The microsomes can be removed after incubation by centrifugation and ovalbumin may be separated by chromatography or immunoprecipitation.

The pattern obtained by thin-layer gel chromatography of the supernatant after incubation on Sephadex G-200 is shown in Fig 2. This procedure made possible an effective and rapid separation of the acceptor from the incubation medium without contamination by other proteins. Using UDP-GlcNAc as substrate, immunoprecipitated ovalbumin was glycosylated by intact rough microsomes (Table IX). If the microsomes used were pretreated by *p*-diazobenzene sulfonate or proteo-

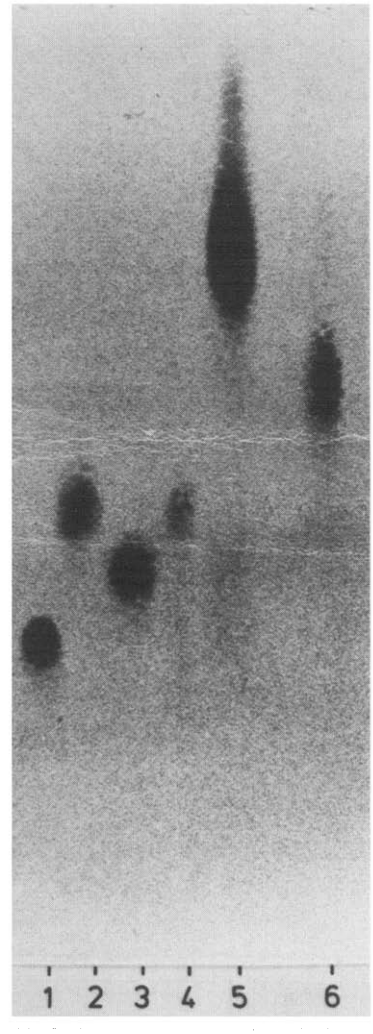

Fig. 2. Thin-layer gel filtration of ovalbumin. Ovalbumin was incubated as described and subsequently separated by thin-layer gel filtration on Sephadex G-200 Superfine. 1, cytochrome *c* (mol. wt. 12400); 2, ovalbumin (reference, mol. wt. 43000); 3, trypsin inhibitor (mol. wt. 21000); 4, the sample (ovalbumin); 5, human gammaglobulin (mol. wt. 157000); 6, bovine serum albumin (mol. wt. 67000).

TABLE IX

GLYCOSYLATION OF OVALBUMIN BY INTACT ROUGH MICROSOMES

At the end of the incubation ovalbumin was isolated by immunoprecipitation or by thin-layer gel filtration (TLG).

Expt.	Treatment	Substrate	Triton (%)	Ovalbumin (cpm/mg protein)	
				Antibody precipitate	Spot in TLG
1	None	UDP-GlcNAc	0	202	
	p-Diazobenzene sulfonate		0	15	
	Proteolysis		0	12	
2	None	UDP-GlcNAc + GDP-Man + UDP-Glc	0	436	410
			0.07	670	662
	Proteolysis		0	73	66
			0.07	493	508

lytic enzymes, the glucosamine transfer was inhibited completely. As expected an increased degree of glycosylation was obtained when the three labeled nucleotide-activated sugars of the core structure were used as substrate. A 50% increase in sugar transfer could be obtained by elimination of the permeability barrier with Triton. Proteolytic treatment of the microsomes decreases the glycosylation reaction to a great extent, but the original incorporation into ovalbumin could be largely re-established by addition of 0.4% Triton.

The results were similar using either immunoprecipitation or thin-layer gel chromatography of the particle-free supernatant. This experiment demonstrates that glycosyltransferases on the cytoplasmic surface of the microsomes are able to mediate sugar transfer to exogenous proteins. If these enzymes are inactivated by proteolysis, the glycosylating system on the luminal side can be used for the same purpose if the membrane permeability is eliminated by detergent.

TABLE X

GLYCOSYLATION OF LIPIDEX-BOUND DOLICHOL(55) PHOSPHATE BY ROUGH MICROSOMES (RM)

The values in parenthesis show the incorporation in cpm/mg protein and are taken as 100%. The other values are expressed as the ratio between the value obtained in the individual experiment and that of control × 100.

Expt.	Microsomes	Treatment	Substrate	Inhibitor	% of control, sugar incorporation into Lipidex-bound dolichol-phosphate
1	RM	None	UDP-GlcNAc	–	100 (910)
				Tunicamycin [a]	23
		p-Diazobenzene sulfonate		–	29
		Proteolysis		–	17
2	RM	None	GDP-Man	–	100 (412)
				Amphomycin [b]	10
		Proteolysis		–	11
3	RM	None	UDP-Glc	–	100 (1230)
		p-Diazobenzene sulfonate		–	11

[a] 10 μg per ml incubation medium.
[b] 400 μg per ml incubation medium.

Glycosylation of Lipidex-bound dolichol phosphate

Glycosylation of exogenous dolichol phosphate requires its solubilization by detergent, which also disrupts membrane structure. By using a hydrophobic matrix, such as Lipidex-1000, dolichol phosphate (in our case chemically phosphorylated dolichol(55)) can be presented to the microsomes in the water phase without the presence of detergent. Dolichol(55) phosphate accepts GlcNAc, mannose and glucose from appropriate nucleotides mediated by the glycosyltransferases of intact microsomes (Table X). Tunicamycin and amphomycin exert the same inhibitory effect as in the system involving endogenous dolichol phosphate and intact microsomes, and *p*-diazobenzene sulfonate or proteolytic treatment prevent the transfer of all three sugars to the exogenous dolichol phosphate. These experiments demonstrate that the glycosylating system on the surface of the intact microsomes can transfer activated sugars to exogenously added lipid intermediates.

The use of this Lipidex-technique to study glycosylation of exogenous dolichol phosphate is very effective and useful, but it requires that the hydrophobic interaction between the Lipidex-matrix and the lipid carrier is stable throughout the incubation period. Theoretically, it is possible that the endogenous and exogenous polyprenols in this system are exchangeable, which would make the approach unsuitable for our purposes. Chloroform-methanol removes dolichol phosphate quantitatively from Lipidex and this was the procedure used here to determine radioactivity at the end of the incubation (Table XI). Lipidex-dolichol phosphate does not react with GDPmannose in the absence of enzyme and Lipidex does not bind microsomal mannosylated polyprenols. Lipidex-bound dolichol phosphate is glycosylated with mannose and glucosamine in the presence of rough microsomes and the endogenous carrier is simultaneously glycosylated. On the other hand, when the endogenous dolichol phosphate of microsomes prelabeled with glucosamine is incubated with Lipidex or Lipidex-dolichol phosphate, no labeled lipid could be found on the Lipidex particles. Thus, these experiments demonstrate that Lipidex-bound dolichol phosphate does not exchange with its microsomal counterpart and that its glycosyla-

TABLE XI

INTERACTION OF DOLICHOL(55) WITH LIPIDEX-1000

In Expt. 1 Dolichol [^{32}P]phosphate-Lipidex-1000 was centrifuged at 500×*g* for 10 min and the pellet was washed with distilled water by recentrifugation. Dolichol [^{32}P]phosphate was released with chloroform/methanol (2:1, v/v). In Expt. 2 Lipidex-1000, rough microsomes (RM) and GDP[^{14}C]mannose were incubated together and the radioactivity in the Lipidex pellet and in the microsomal extract was measured. Lipidex-Dol(55)-*P* and GDP[^{14}C]mannose were also incubated in the absence or presence of rough microsomes. In the latter case radioactivity was measured both in the Lipidex pellet and in the microsomes. In Expt. 3 Lipidex-Dol-*P* was incubated with rough microsomes and UDP[^{14}C]GlcNAc and radioactivity was measured both in the Lipidex pellet and in the microsomal extract. Rough microsomes were also prelabeled by in vitro incubation with UDP[^{14}C]GlcNAc and then incubated with Lipidex or Lipidex-Dol-*P* [a].

Expt.	cpm	
	Lipidex-1000	Dol-*P*-sugar in microsomes
1 Dol-[^{32}P]*P*-Lipidex, pellet after washing	2850	
Supernatant after chloroform/methanol (2:1, v/v) treatment of pellet	2726	
2 Lipidex-Dol-*P* incubated with GDP[^{14}C]mannose	92	
Lipidex incubated with RM and GDP[^{14}C]mannose	102	2230
Lipidex-Dol-*P* incubated with RM and GDP[^{14}C]mannose	1405	2285
3 Lipidex-Dol-*P* incubated with RM and UDP[^{14}C]GlcNAc	1032	1815
Lipidex incubated with [^{14}C]GlcNAc prelabeled RM	52	
Lipidex-Dol-*P* incubated with [^{14}C]GlcNAc prelabeled RM	46	

[a] Dol-*P*, dolichol phosphate.

tion reflects an interaction with the glycosyltransferases on the outer surface of rough microsomes.

Discussion

In this paper the intramembranous distribution of microsomal glycosyltransferases was studied in vitro using inhibitors and impermeable protein probes. The glycosylating systems in liver microsomes are characterized by multiplicity and broad distribution, both in the lateral and in the transverse plane of the membrane, and the topographical localization is clearly one of the factors in regulation of glycoprotein synthesis.

Rough and smooth microsomes prepared from rat liver homogenate under appropriate conditions contain vesicles all of which possess identical outside-inside orientations [12]. The presence of bound ribosomes only on the outer surface, the complete asymmetry of some of the microsomal enzymes, and the quantitative retainment of secretory proteins in the vesicle lumen after homogenization are some of the observations supporting the idea that the vesicle population is homogenous in this respect. Both before and after treatment with surface probes the vesicles exhibit complete impermeability both to small substrates and to proteins, as demonstrated by the measurement of certain enzyme activities and of the intramicrosomal water space. Consequently, the results obtained in this paper would seem not to be influenced by artifacts such as inside-outside inversion of some vesicles and damage to the permeability barrier in others.

A summary of the glycosylating reactions which appear to take place in rough and smooth microsomes is given in Table XII. This schematic representation disregards the possibility that the various sugars may follow somewhat different pathways. Transfer of sugars from the outer water surface to dolichol in rough microsomes is mediated to large extent by transferases located at the outer surface. This was demonstrated by the fact that tunicamycin- and amphomycin-sensitive reactions were also sensitive to *p*-diazobenzene sulfonate and proteolytic treatment and also by the experiments with ovalbumin and Lipidex. A certain amount of dolichol glycosylation also occurs in the *p*-diazobenzene sulfonate-insensitive membrane compartment as well, i.e. inside the vesicles, as shown by the use of the inhibitors. These experiments suggest that to some extent direct glycosylation of proteins at the luminal surface may take place and the proteins glycosylated in this manner are localized at the inner surface of the endoplasmic membranes. However, these minor pathways proposed here must be demonstrated in the future by analysis of glycosylation of specific secretory and membrane proteins.

In contrast to rough microsomes, one pathway dominates in smooth microsomes, i.e., glycosylation of proteins at the inner compartment without involvement of lipid intermediates. This raises the question how the nucleotide sugar reaches the

TABLE XII

GLYCOSYLATING PATHWAYS IN ROUGH AND SMOOTH MICROSOMES

	Cytoplasm	*p*-Diazobenzene sulfonate-sensitive compartment	*p*-Diazobenzene sulfonate-insensitive compartment	Lumen
Rough microsomes				
Major pathway	Sugar →	Dolichol →	Dolichol →	Protein
Minor pathways	Sugar →	Dolichol-Protein		
	Sugar →	Protein		
	Sugar →		Dolichol →	Protein
Smooth microsomes				
Major pathway	Sugar →			Protein
Minor pathways	Sugar →	Dolichol →	Dolichol →	Protein
	Sugar →	Protein		

transferase at the luminal side, since microsomal membranes are impermeable to charged substances of any size [7]. It is possible that protein carriers, like those in Golgi membranes [46–48], exist in smooth microsomes. Such a carrier may be difficult to test in an experimental system, since its function could be the transfer of sugar to the location of the enzyme in the membrane and not transfer of the sugar in the free luminal compartment. Experiments with dolichol phosphate sugars as substrates indicate that under certain circumstances lipid carrier-mediated glycosylation may function. However, we cannot rule out the possibility that a part of these latter reactions are present on contaminating membranes. Surface probing agents decrease the amount of glycosylated proteins in smooth microsomes by only 10%, but this may reflect direct glycosylation of a few proteins at this location.

The fact that the initial part of the glycosylating system, that is the sugar nucleotide-dolichol monophosphate transferase, is associated with the cytoplasmic surface of the microsomal vesicles is not surprising. The nucleotide-activated sugars used in this investigation are synthesized in the cytoplasm and in the absence of membrane carriers they would be expected to interact with the cytoplasmic surface of the endoplasmic reticulum, since microsomal membranes are completely impermeable to charged substances. Surface probes also significantly decrease transfer of sugar from dolichol phosphate to dolichol pyrophosphate oligosaccharide, indicating that these dolichol phosphate sugar transferases are also associated with the outer surface. On the other hand, the transfer of the oligosaccharide core to protein occurs, at least to a large extent, at the inner, luminal surface [19]. Obviously, some protein glycosylation also occurs at the cytoplasmic surface. The main task of the glycosylating system in rough microsomes appears to be synthesis and transfer of the core to the protein acceptor. In contrast, the lack of involvement of dolichol phosphate in sugar transfer in smooth microsomes, together with the relative insensitivity to non-permeable surface probing agents suggest the glycosyltransferases at this location mainly mediate completion of the core oligosaccharide on the protein. The limited influence of surface probes on dolichol phosphate sugar synthesis in smooth microsomes, however, may be a reflection of core glycosylation of a few proteins at this location. Previous data indicate that liver smooth microsomes [49], in contrast to oviduct smooth microsomes [50], contain proteins which are acceptors of core oligosaccharides which indicates that under certain conditions the dolichol pathway may be involved in initial glycosylation reactions. The pathways which are designated as minor in Table XII may be necessary for glycosylation of certain membrane proteins, as separate from the pathway designed for secretory proteins. Microsomal cytochrome *P*-450, which has been highly purified in both Coon's and Sato's laboratories, contains one glucosamine and two mannose residues per mol protein [9,11]. The enzyme is localized on the cytoplasmic surface of the microsomes [25], where the hydrophilic sugar residue is also expected to be situated. The absence of a diacetylchitobiose unit suggests that the glycosylation process does not follow the pathway of core completion and processing and may occur without or with involvement of dolichol phosphate. In this example sugar transfer should occur by transferases exclusively distributed in the outer compartment of the membrane, since the newly synthesized enzyme is inserted directly and remains at this location [51,52].

It is difficult to judge the importance of the different glycosylation pathways in microsomes at present. The reactions studied in this investigation have only been measured qualitatively; the limitation of endogenous acceptors, among other things, does not allow quantitation in such a system. The obvious difference between nucleotide sugars and dolichol-bound sugars as substrates may result from the fact that Triton was absent in the former but present in the latter incubation medium. Some protein glycosylation occurs even in *p*-diazobenzene sulfonate-treated microsomes in the presence of inhibitors. However, our experiments could not exclude that this glycosylation occurred via pre-existing dolichol pyrophosphate oligosaccharide.

Immunoprecipitation was used to separate secretory and membrane proteins and under our conditions both types of proteins are glycosylated with the involvement of the dolichol system. This observation is in agreement with that previously

reported for the hen oviduct system [5]. What is surprising and obviously different from events in vivo is that the large majority of the proteins mannosylated and glucosaminidated in vitro are membrane proteins. One explanation for this finding is that under in vitro conditions continuous protein synthesis is absent and the available protein acceptors are mainly of the membrane type. This explanation, however, is hypothetical at present, since we do not know the number and nature of the microsomal proteins which are glycosylated. It is also very probable that the number and the length of the oligosaccharide chains which are present on different microsomal proteins are very different, which could cause problems in quantitation. Some of the secretory proteins such as fibrinogen or prothrombin are sugar-rich [53], while some of the membrane proteins, such as cytochrome *P*-450 [9], contain only a few sugars per mol of protein.

It may well happen that the glycosylating reactions in the various membrane systems are characterized by a diversity and multiplicity rather than by unifying and common principles. A reaction pattern established for a certain type of membrane in a tissue may not be valid for the same membrane in another tissue. Several of the active glycosylation reactions present in the smooth endoplasmic reticulum in rat liver are absent in the smooth microsomal fraction of the oviduct, where Golgi and plasma membranes are the dominating components. Those proteins which are not transported but remain in the endoplasmic reticulum as membrane components are present in small amounts and their glycosylation cannot be investigated in such a relatively simple way as those of many secretory and viral proteins. The small amount of these proteins, their slow turnover and limited distribution may be the reason why a number of glycosylating patterns are not apparent or can be questioned. Refined analytical methods and new approaches will be necessary in the future for the analysis of the multiple glycosylating reactions in various membranes.

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