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INTERACTION OF LECTINS WITH PROTEINS OF THE ENDOPLASMIC RETICULUM AND GOLGI SYSTEM OF RAT LIVER

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SUMMARY

The interaction of glycoproteins of rough and smooth microsomal and Golgi membranes with Sepharose-bound lectins has been studied. One of these lectins was a crude preparation from wheat germ lipase which was found to bind primarily to *N*-acetyl neuraminic acid. Rough microsomes, smooth microsomes and Golgi membranes contain glycoproteins which bind to Concanavalin A (Con A specific for mannose residues) in decreasing amounts in the order indicated (rough, smooth and Golgi) and to wheat germ agglutinin (WGA, glucosamine-specific) and to the crude lipase preparation in increasing amounts in the order indicated. The small amount of binding of rough microsomes and Golgi membranes to *Crotalaria* (galactose-specific) increases substantially after neuraminidase treatment. Three submicrosomal particle preparations enriched either in AMPase or in NADH- or NADPH-oxidizing electron-transport enzymes contain glycoproteins which bind Con A and wheat germ agglutinin. The latter binding is sensitive to neuraminidase treatment. Two other submicrosomal particle preparations, both enriched in glucose-6-phosphatase activity, bind preferentially to WGA. This binding is, however, not sensitive to neuraminidase. Prolonged incubation with *Ervilia* lectin (mannose-specific) inhibits NADH-ferricyanide reductase activity, while the electron-transport chain involving cytochrome b_5 is also inhibited by *Crotalaria*, indicating that both the flavoprotein and the cytochrome b_5 are glycoproteins whose oligosaccharide chains have terminal mannose or galactose residues.

INTRODUCTION

The membranes of the endoplasmic reticulum contain a number of different enzymes for glycosylation of 2 types of proteins, secretory and membrane glycoproteins. With the exception of albumin all secretory proteins which are transported through the channel system of the endoplasmic reticulum through the Golgi apparatus to the blood contain covalently bound sugar residues. In contrast to the secretory proteins the nature and the function of membrane glycoproteins have not yet been established. There are indications that some microsomal enzymes and ribophorin are glycoproteins (Bischoff, Tran-thi & Decker, 1975; Evans & Gurd, 1973; Dean, 1974; Haugen & Coon, 1976; Grebenau, Sabatini & Kreibich, 1977).

The sugar content of endoplasmic reticulum membranes includes the neutral sugars mannose and galactose, the amino sugar glucosamine and the charged sialic acid (Miyajima, Tomikawa, Kawasaki & Yamashina, 1969; Bergman & Dallner, 1976). The individual glycoproteins have not yet been isolated and their oligosaccharide

sequences not yet characterized. However, it is possible to obtain information by using various lectins. The interaction of lectins with membranes has so far been studied mainly with plasma membranes (Cook & Stoddart, 1973). On the other hand, there are also studies which demonstrate that various intact intracellular membranes exhibit a relatively limited interaction with a few types of lectins (Nicolson, Lacourbière & Delmonte, 1972; Henning & Uhlenbruck, 1973; Monneron & Segretain, 1974; Keenan, Franke & Kartenbeck, 1974). If isolated intracellular membranes are solubilized, the ability of integral membrane glycoproteins to associate with lectins increases to a significant extent (Winqvist, Eriksson & Dallner, 1974).

In this paper we have investigated the interaction of a number of lectins with rough and smooth microsomal and Golgi membranes and also analysed lectin interaction with specific submicrosomal particles. It was found that some of the microsomal electron-transport enzymes are inhibited by lectins, which would suggest that they are glycoproteins.

MATERIALS AND METHODS

Fractionation

Male Sprague–Dawley rats weighing 180–200 g were starved for 20 h. Total microsomes and rough and smooth subfractions were prepared as described earlier (Dallner, 1974). For preparation of the Golgi fraction, rats were given 1·2 g 50% ethanol/100 g body weight by stomach tube 90 min before decapitation. That portion of the Golgi complex which has a density less than that of 0·86 M sucrose (Golgi I and II) was used in all experiments (Ehrenreich, Bergeron, Siekevitz & Palade, 1973). In order to remove adsorbed and luminal secretory proteins all fractions were subjected to the Tris-water-Tris washing procedure (Dallner, 1974). Submicrosomal particles were prepared by using washed total microsomes (Winqvist & Dallner, 1976). Fractionation was performed on a continuous gradient ranging between 0·29 M and 1·75 M sucrose and containing 0·19% deoxycholate. After isopycnic equilibration the 5 bands were collected. In *in vivo* experiments 300 μCi D-[1-^{3}H]glucosamine (2000 mCi/mmol, Radiochemical Centre, Amersham, England) were injected into the portal vein of rats under pentobarbital anaesthesia 60 min before decapitation. The specific activities of the washed fractions were: 19000 cpm/mg protein for rough membranes, 53000 cpm/mg protein for smooth membranes, and 12000 cpm/mg protein for Golgi membranes.

Incubation with lectins

The incubation mixture contained 0·3 ml of lectin-Sepharose suspended in 0·15 M Tris-Cl buffer, pH 7·8 (50% sedimented gel, vol/vol), 0·5 ml buffer or 0·5 ml 0·2 M lectin inhibitor in buffer, 0·05 ml 10% deoxycholate (excluded when intact membranes were studied) and 0·2 ml membrane fraction (about 2 mg protein). The amount of lectin used was in excess to the amount of lectin-binding components. The following lectin inhibitors were used: for Con A, α-methyl mannose; for WGA, *N*-acetyl glucosamine; for *Crotalaria* lactose and for crude lipase fraction, *N*-acetylneuraminic acid (NANA). The mixture was incubated for 30 min at 25 °C with gentle shaking. After incubation the lectin-Sepharose complex was washed 3 times by centrifugation using 5 ml Tris-Cl buffer containing 0·1% deoxycholate. The pellet was suspended in 0·2 ml water and transferred to a scintillation vial with 10 ml Bray's solution (Bray, 1960).

Galactose labelling

For labelling of terminal galactose of isolated and purified cytochrome b_5 (Ozols, 1974), 0·5 mg protein in 0·1 M phosphate buffer, pH 7·4 was incubated with 0·05 mg (6 units) galactose

oxidase (Worthington, Freehold, New Jersey) overnight at 37 °C. A small crystal containing about 2·5 mCi of sodium [^{3}H]borohydride (700 mCi/mmol, The Radiochemical Centre) was added to the mixture and the incubation continued for another 10 min at 37 °C. The suspension was applied to a Sephadex G-25 column equilibrated with water and the void volume was collected and freeze-dried. Part of the cytochrome b_5 was dissolved in 4 % sodium dodecyl sulphate sample buffer containing 5 % mercaptoethanol and used for polyacrylamide gel electrophoresis according to Weber & Osborn (1969). Another portion was dissolved in 0·1 M phosphate buffer, pH 7·4, and incubated with an excess of anti-cytochrome b_5 antiserum (kindly supplied by Dr Å. Elhammer) for 2 h at 25 °C and overnight at 4 °C. The mixture was then applied to a protein A-Sepharose column. After washing with 10 column volumes (no more radioactivity was eluted), the cytochrome b_5-antibody complex was eluted with 0·2 M citrate buffer, pH 3.

Chemical and enzymic analysis

Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. *N*-acetylneuraminic acid was liberated by hydrolysis in 0·05 M H_2SO_4, 60 min 80 °C, purified by ion exchange chromatography (Svennerholm, 1963) and the free sialic acid was measured with the Warren procedure (Warren, 1963). *N*-acetylneuraminic acid type VI from Sigma Co. (St Louis, Miss.) was used as a standard. The various enzyme activities were measured according to methods described earlier (Eriksson, 1973; Beaufay *et al.* 1974).

Lectins

Con A and WGA as well as Con A-Sepharose and WGA-Sepharose were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Lectins from Crotalaria, Vicia cracca, Vicia ervilia, Vicia sativa, Vicia villosa (mannose- or *N*-acetyl galactosamine-specific) were all kindly supplied by Dr B. Ersson, Uppsala University, Sweden.

For preparation of crude lipase lectin, 10 g wheat germ lipase (Type I, Sigma) were dissolved in 400 ml 0·05 M phosphate buffer, pH 7·4. After centrifugation for 30 min at 10000 **g** the supernatant was decanted and applied to an affinity column, where glucosamine had been coupled to CH-Sepharose (Pharmacia). After washing with 0·2 M NaCl the lectin was eluted with 0·15 M *N*-acetyl glucosamine in 0·05 M phosphate buffer, pH 7·4, dialysed against water and freeze-dried. Typically, 5 mg of protein were recovered. In sodium dodecyl sulphate-polyacrylamide electrophoresis, this preparation, designated as crude lipase lectin, gave 2 bands, a weak one at mol. wt 25000 and a strong one at mol. wt 38000.

The various lectins were coupled to CNBr-activated Sepharose according to the method of Cuatrecasas (Cuatrecasas, 1970).

RESULTS

Lectin binding to cytoplasmic membranes

Rough and smooth membranes labelled *in vivo* with glucosamine were incubated with Con A, WGA and *Crotalaria* lectins covalently bound to Sepharose. The intact membrane demonstrated a small amount of interaction, about half of which could be inhibited by the specific sugar inhibitors (Table 1). When, on the other hand, the membranes were solubilized with detergent prior to incubation, the percentages of the total radioactivity bound to Con A were 37, 28 and 20% in rough, smooth and Golgi membranes, respectively. Only an insignificant portion of this binding could not be specifically inhibited. Nor did Sepharose without lectin bind any significant amount of radioactivity (Winqvist *et al.* 1974). Binding to WGA was less than that to Con A and was lowest in rough, moderate in smooth, and highest in Golgi membranes. Binding to *Crotalaria* lectin was low in all 3 fractions.

Table 1. *Binding of membranes to lectin-Sepharose*

Fraction		Radioactivity bound, %		
		Con A	WGA	Crotalaria
Rough microsomes	Intact	6 ± 0·8	3 ± 0·2	1 ± 0·2
	Intact + inhibitor	2 ± 0·3	2 ± 0·2	1 ± 0·1
	Solubilized	37 ± 4·2	6 ± 0·9	3 ± 0·2
	Solubilized + inhibitor	3 ± 0·7	2 ± 0·3	2 ± 0·2
Smooth microsomes	Intact	3 ± 0·2	2 ± 0·3	2 ± 0·3
	Intact + inhibitor	2 ± 0·1	1 ± 0·2	1 ± 0·2
	Solubilized	28 ± 3·2	11 ± 1·0	3 ± 0·2
	Solubilized + inhibitor	4 ± 0·5	3 ± 0·4	1 ± 0·1
Golgi membranes	Intact	3 ± 0·2	2 ± 0·3	1 ± 0·1
	Intact + inhibitor	2 ± 0·2	2 ± 0·1	1 ± 0·2
	Solubilized	20 ± 1·8	14 ± 2·0	2 ± 0·2
	Solubilized + inhibitor	3 ± 0·4	2 ± 0·3	1 ± 0·1

300 μCi [^{3}H]glucosamine were injected into the portal vein of rats. The animals were decapitated 60 min later, and subfractions were prepared and subjected to the Tris-water-Tris washing procedure. The incubation was performed as described in Materials and methods. The values in the Table are the percentage of the total radioactivity that was bound to the lectin-Sepharose. Inhibitors used were: for Con A α-methyl mannose, for WGA *N*-acetylglucosamine and for *Crotalaria* lactose. The values are the means ± S.E.M. of 10 experiments.

In Table 2 are shown results using an additional lectin, i.e. crude lipase, which represents a partially purified preparation from commercial wheat germ lipase. Here substantial binding of all 3 fractions was observed. When the fractions were pretreated with neuraminidase, there were no changes in Con A-binding, whereas the increase in binding to *Crotalaria* indicates that the dominant sugar moiety next to terminal NANA in these membrane glycoproteins is galactose. Neuraminidase pretreatment decreased the WGA binding of all 3 fractions to some extent, while the binding of rough and Golgi membranes to crude lipase was greatly diminished. Pretreatment with low concentrations of deoxycholate, known to abolish the membrane permeability barrier (Kreibich, Debey & Sabatini, 1973), did not in itself alter the binding, but neuraminidase treatment in the presence of deoxycholate removed almost all the binding sites for crude lipase in smooth membranes.

The nature of the binding to crude lipase lectin

Affinity chromatography experiments were performed to investigate the binding to crude lipase lectin. Fetuin and asialofetuin (where the sialic acid had been removed by hydrolysis) were applied to a crude lipase-Sepharose column (Fig. 1.). When fetuin was used, almost none appeared in the eluate, but after application of NANA, all fetuin in the column was displaced and could be recovered in subsequent fractions. Asialofetuin, on the other hand, passed through the column and could be collected at the front. NANA could not displace any further amounts from the column. This indicates interaction of NANA with the crude lipase. To exclude the possibility that

Table 2. *Binding of glycoproteins in microsomes to lectin-Sepharose*

Treatment	Solubilized membrane fraction	Radioactivity bound, %			
		Con A	Crotalaria	WGA	Crude lipase
None	Rough	35 ± 3	1 ± 0·2	3 ± 0·4	11 ± 1·2
	Smooth	25 ± 2	1 ± 0·1	7 ± 0·6	13 ± 1·0
	Golgi	17 ± 2	1 ± 0·1	12 ± 1·3	16 ± 1·0
Neuraminidase	Rough	32 ± 2	5 ± 0·5	2 ± 0·1	1 ± 0·1
	Smooth	25 ± 2	3 ± 0·3	5 ± 0·4	10 ± 0·9
	Golgi	19 ± 2	8 ± 0·7	9 ± 0·7	4 ± 0·6
0·05 % Deoxycholate	Rough	25 ± 3	1 ± 0·2	2 ± 0·4	8 ± 0·7
	Smooth	25 ± 2	1 ± 0·1	6 ± 0·5	14 ± 1·6
	Golgi	15 ± 2	2 ± 0·2	10 ± 0·9	15 ± 1·2
0·05 % Deoxycholate + neuraminidase	Rough	20 ± 3	5 ± 0·8	2 ± 0·3	2 ± 0·3
	Smooth	25 ± 3	6 ± 0·7	4 ± 0·5	1 ± 0·1
	Golgi	14 ± 2	9 ± 0·7	9 ± 1·1	3 ± 0·3

In vivo labelling and preparation of membrane fractions was performed as described in Table 1. The fractions (4 mg protein/ml) were pretreated with either neuraminidase (*Cl. perfringens*, Sigma Co., 10 μg/mg protein), or 0·005 % deoxycholate-50 mM KCl, or both (Kreibich, Debey & Sabatini, 1973). After incubation at 37 °C for 10 min, the membranes were pelleted by centrifugation and resuspended in 0·15 M Tris-Cl, pH 8. Incubation with various Sepharose-bound lectins was then performed as in Materials and methods. Here all samples were solubilized by 0·5 % deoxycholate prior to lectin incubation. The values represent that part of the binding which can be inhibited by the specific inhibitors, i.e. 'Solubilized' minus 'Solubilized + inhibitor' values according to Table 1. The values are means ± S.E.M. (n = 7).

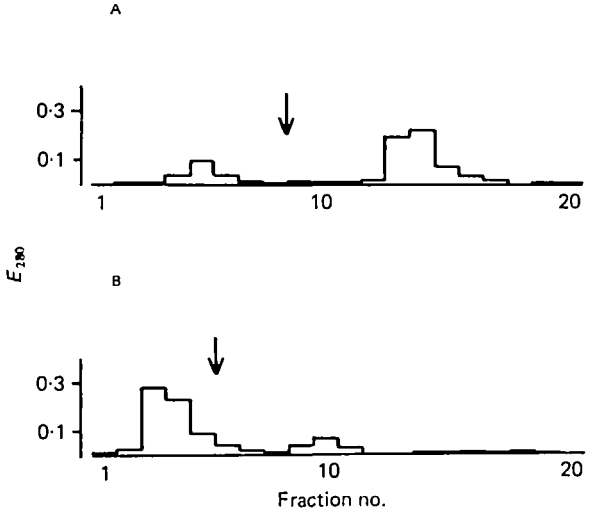

Fig. 1. Fetuin and asialofetuin adsorption to crude lipase-Sepharose column. (A) A small column (0·6 × 5 cm) was filled with Sepharose with covalently attached lectin prepared from wheat germ lipase (Sigma Co.) as described in Materials and methods. 1 mg of fetuin (type IV, Sigma Co.) dissolved in 0·5 ml Tris-Cl buffer (0·15 M, pH 7·8) was applied to the column, which was then eluted with the same buffer until absorption at 208 nm became negligible. 0·2 M NANA was used to displace the bound fetuin from the column (arrow in the figure). 0·5-ml fractions were collected and u.v. absorption at 280 nm was determined. (B) Details as in A but asialofetuin was substituted for fetuin. Asialofetuin was prepared by hydrolysis of fetuin in 0·05 M H_2SO_4 for 60 min at 80 °C. After neutralization and dialysis the asialofetuin was freeze-dried.

the neuraminidase employed is contaminated with glucosaminidase, thin-layer chromatography was run on the products of the hydrolysis (Fig. 2). The only sugar released by neuraminidase was NANA.

Chemical determination of NANA in rough and smooth microsomes after neuraminidase treatment also supports the view that crude lipase interacts with terminal NANA residues. This treatment removes a large part of the NANA from rough but not from smooth microsomes (Table 3). This Table also demonstrates that after pretreatment with protease, neuraminidase has access to additional NANA residues

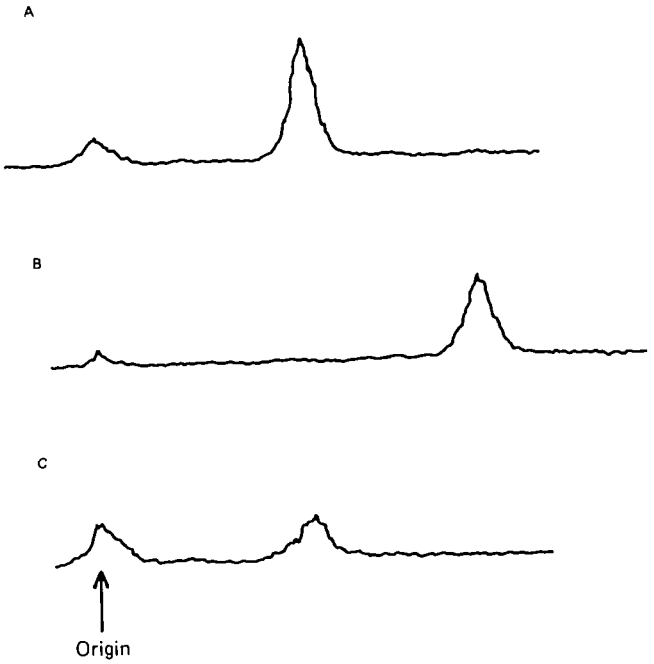

Fig. 2. Chromatography of material released by neuraminidase from microsomes. Microsomes were prepared from rats injected with [^{14}C]glucosamine and treated with 10 μg neuraminidase per mg protein. The microsomes were centrifuged for 4 h at 100000 g and the supernatant was freeze-dried and applied to a precoated cellulose plate (Merck, Darmstadt) (A) The thin layer chromatogram was developed by descending chromatography in butanol:propanol:ethanol:water:ammonia (20:40:20:20:1). As references [^{14}C]NANA (B) and [^{14}C]*N*-acetyl glucosamine (C) were used. The chromatogram was then scanned for radioactivity. The radioactivity spots were then scraped off and measured in scintillation vials. The sialic acid peak in A was found to contain 2600 cpm, the *N*-acetylglucosamine peak in B 1700 cpm, and the peak in C, corresponding to sialic acid, 800 cpm.

in rough microsomes and a large part of the NANA in smooth membranes can also be removed now. This combined treatment only affects the outer surface of the membrane, since permeability studies showed that the vesicles were still impermeable to large macromolecules such as Dextran 70000. Liver microsomes are known to retain their original inside-outside orientation even after various enzyme treatments (Nilsson *et al.* 1978).

Table 3. *NANA content of rough and smooth microsomal membranes*

Treatment	NANA (μg/mg protein) content in Rough microsomes	Smooth microsomes
None	6·0	13·8
Neuraminidase	2·9	12·8
Protease	4·6	12·7
Protease + neuraminidase	1·7	8·1

In the case of neuraminidase treatment microsomal subfractions were treated with 10 μg enzyme (2 units/mg enzyme protein) per mg microsomal protein.

When protease was used, non-specific protease from Streptomyces griseus (Sigma) was incubated with microsomes (20 μg enzyme protein/mg microsomal protein). In the case of combined treatment the fractions were first incubated with protease, then sedimented by centrifugation and the resuspended pellet was then incubated with neuraminidase. All enzyme treatments were carried out at 37 °C for 10 min. NANA was measured after acid hydrolysis with the Warren thiobarbiturate method (Svennerholm, 1963; Warren, 1963). The values represent the means of 4 experiments.

Effect of trypsin treatment on lectin binding

A sizeable fraction of the protein-bound neutral and amino sugar residues are contained in the trypsin-sensitive portion of intact cytoplasmic membranes (Bergman & Dallner, 1976). Therefore, trypsin-treated solubilized membranes were tested for lectin binding (Table 4). Trypsin treatment did not influence the amount of labelled glycoproteins interacting with Con A. On the other hand, proteolysis of both rough and smooth microsomes decrease their binding to WGA and *Crotalaria.* In fact, *Crotalaria* binding to smooth membranes from which NANA had been removed was almost abolished. The lack of effect of trypsin on binding to Con A does not necessarily exclude the presence of terminal mannose at the cytoplasmic surface of the membrane, since there may still be enough sugar left for binding.

In Table 5 the 3 types of membrane were solubilized and incubated with 5 different lectins all known to be inhibited by α-methyl mannose. Since both the amount and the pattern of binding vary with the lectin used, it would seem that the binding is influenced by several factors and not exclusively dependent on the presence of a specific sugar moiety (Allen, Neuburger & Sharon, 1973; Pereira, Kisailus, Gruezo & Kabat, 1978).

Submicrosomal particles

Submicrosomal particles may be prepared by isopycnic equilibration of microsomal membranes through a deoxycholate-containing continuous sucrose gradient (Winqvist & Dallner, 1976). Five separate fractions are recovered after centrifugation, with a phospholipid/protein ratio which decreases from 2·51 in the top fraction to 0·11 in the bottom one. The 5 fractions also exhibit specific patterns of enzyme activity (Table 6). AMPase and other microsomal hydrolases are mainly localized in fraction 1. The enzymes participating in NADH oxidation are enriched in fraction 2, and those

Table 4. *Binding of glycoproteins from trypsin-treated membranes to lectin-Sepharose*

Membrane fraction	Treatment	Radioactivity bound %		
		Con A	WGA	Crotalaria
Rough	None	34 ± 3	3 ± 0·2	5 ± 0·8
	Trypsin	37 ± 4	1 ± 0·1	3 ± 0·6
Smooth	None	25 ± 2	7 ± 1	6 ± 0·5
	Trypsin	23 ± 2	5 ± 0·7	1 ± 0·3
Golgi	None	17 ± 2	12 ± 0·8	18 ± 1
	Trypsin	17 ± 3	11 ± 0·9	17 ± 2

Membrane fractions were prepared from rats injected with [^{3}H]glucosamine and then treated with trypsin (50 μg/mg protein, 10 min, 37 °C). After centrifugation the pellets were suspended in Tris-Cl buffer (0·15 M, pH 7·8) dissolved in 0·5 % deoxycholate and incubated with lectin-Sepharose as described in Materials and methods. With *Crotalaria*, the membranes had first been treated with neuraminidase and thereafter with trypsin. The values are 'Solubilized' minus 'Solubilized + inhibitor' values as in Table 2. The data are given as mean values ± S.E.M. (n = 8).

Table 5. *Binding of glycoproteins to mannose-specific lectins*

Lectins	Radioactivity bound, %		
	Rough	Smooth	Golgi
Con A	35	25	17
Vicia crassa	23	19	7
V. ervilia	26	24	10
V. sativa	15	7	6
V. villosa	16	11	5

Dissolved membranes from rats injected with [^{3}H]glucosamine were incubated with mannose-specific lectin-Sepharose as described in Materials and methods. The Villosa lectin used here is mannose-specific. The figures represent 'Solubilized' minus 'Solubilized + inhibitor' as in Table 2. The values are means of 3 experiments.

involved in NADPH oxidation in fraction 3. Glucose-6-phosphatase activity is present in fractions 1 and 4, but the highest activity by far is in fraction 5.

After labelling *in vivo* all of these subfractions displayed protein-bound glucosamine label in decreasing amounts in the order 2, 1, 3, 4 and 5 (Table 7). Microsomes were also treated with neuraminidase in the presence of low concentrations of deoxycholate before preparing submicrosomal particles. The greatest losses of radioactivity were observed in fractions 1, 2 and 3.

Experiments with lectins show that the submicrosomal particles described above contain various types of glycoproteins (Table 8). Interaction with Con A occurs with the upper 3 fractions and is most pronounced in fraction 2. The picture with WGA is somewhat different. Here the interaction is more general, being highest in fractions 2, 3, and 4. Binding to *Crotalaria* and *Ervilia* lectins is low in the various fractions,

Table 6. *Distribution of some enzyme activities in submicrosomal particles (relative specific activity or amount)*

Fraction	AMPase	Cytochrome *c* reductase		Glucose-6-phospha-tase	Protein	Phospholipid/ protein ratio
		NADH-	NADPH-			
1	3·7	1·5	0·1	1·3	1·3	2·51
2	0·5	1·9	0·9	0·1	2·6	1·37
3	0·5	0·4	1·7	0·5	2·1	1·01
4	0·1	0·1	0·4	1·4	1·3	0·38
5	0·1	0·1	0·1	3·6	1·2	0·11

Fractions were collected after centrifugation in the deoxycholate-containing gradient and enzyme activities and protein content were determined (Eriksson, 1973; Beaufay *et al.* 1974). The relative specific activities were calculated, i.e. the specific activities from gradient fractions were divided by the activity in a reference tube where the original microsomal sample was homogeneously distributed. The values represent means (n = 8).

Table 7. [^3H]*glucosamine labelling of submicrosomal particles*

Microsomes	Fraction (cpm/mg protein)				
	1	2	3	4	5
Untreated	19700	24300	10200	6160	3740
Pretreated by neuraminidase in the presence of deoxycholate	16200	19000	7280	5360	3440

Rats were injected intraperitoneally with 150 μCi [^{3}H]glucosamine/100 g 1 h before decapitation. Total microsomes were prepared, treated with Tris-water-Tris (Materials and methods), and treated with neuraminidase (10 μg/mg protein, 10 min, 37 °C) in the presence of 0·05 % deoxycholate and 50 mM KCl with about 4 mg protein per ml (Kreibich *et al.* 1973). The treated microsomes and the non-treated control were fractionated on a deoxycholate-containing gradient (Winqvist & Dallner, 1976), and radiocativity was measured in the individual fractions. Each value is the mean of 3 experiments.

while binding to *N*-acetyl galactosamine-specific *Villosa* lectin is absent. Submicrosomal particles prepared from neuraminidase-treated microsomes exhibit a decreased WGA-binding by the upper 3 fractions, indicating that some of the interaction is due to NANA residues. The same 3 fractions display a highly significant (11- to 26-fold) increase in interaction with *Crotalaria* lectin after neuraminidase treatment. Thus, the submicrosomal fractions prepared show both specific enzyme patterns and characteristic lectin interactions.

Effects of lectins on microsomes

A number of experimental approaches are available which demonstrate that the interaction of lectins with the cell membrane has both structural and functional consequences. The behaviour of microsomal vesicles in the presence of various lectins was investigated (Table 9). In the presence of Con A, *Ervilia* and WGA all

Table 8. *Binding of glycoproteins from submicrosomal particles to various Sepharose-bound lectins*

	Fraction	Radioactivity bound (%)				
		Con A	WGA	Crotalaria	Ervilia	Villosa
Untreated	1	13 ± 1·0	13 ± 1·6	1 ± 0·1	2 ± 0·2	0 ± 0·1
	2	23 ± 1·8	24 ± 1·7	1 ± 0·2	3 ± 0·2	0 ± 0·1
	3	17 ± 2·1	19 ± 2·3	2 ± 0·2	4 ± 0·2	2 ± 0·3
	4	6 ± 0·8	21 ± 2·6	2 ± 0·1	1 ± 0·1	0 ± 0·2
	5	5 ± 0·6	11 ± 0·9	2 ± 0·2	3 ± 0·3	0 ± 0·1
Pretreated by neuraminidase in the presence of deoxycholate	1	14 ± 0·9	5 ± 0·4	11 ± 1·1	1 ± 0·2	0 ± 0·1
	2	34 ± 4·1	8 ± 1·1	26 ± 2·2	4 ± 0·5	0 ± 0·1
	3	26 ± 3·7	11 ± 0·9	24 ± 2·1	3 ± 0·3	1 ± 0·2
	4	16 ± 3·7	20 ± 1·8	10 ± 1·6	1 ± 0·2	1 ± 0·1
	5	1 ± 0·2	7 ± 0·6	6 ± 0·9	1 ± 0·1	0 ± 0·2

Total, Tris-water-Tris-washed microsomes were prepared from *in vivo* labelled rats and neuraminidase-treated as in Table 7 and submicrosomal particles were prepared on a deoxycholate-containing gradient (Winqvist & Dallner, 1976). The fractions were dissolved in 0·4% deoxycholate and incubated with Sepharose beads with covalently attached lectin (20 °C, 30 min). After incubation the beads were pelleted by centrifugation at 100 g for 5 min and the radioactivity in the pellet was measured. The Villosa lectin used here is *N*-acetyl galactosamine-specific. These figures represent 'solubilized' minus 'solubilized + inhibitor' as in Table 2. The values are the means + S.E.M. of 5 experiments.

Table 9. *Aggregation of microsomes by lectin*

Lectin	NADPH-cytochrome *c* reductase activity in filtrate, %
None	90
Con A, 1 mg/ml	87
Ervilia, 1 mg/ml	85
WGA, 1 mg/ml	86
Crotalaria, 0·25 mg/ml	81
Crotalaria, 0·5 mg/ml	67
Crotalaria, 1 mg/ml	44

Total microsomes (8 mg protein/ml) were incubated with various lectins at room temperature for 0·5 h. The incubation mixtures were then passed through a 0·65-μm Millipore filter (Millipore Filter Co., Bedford, Mass.) and the amount of NADPH-cytochrome *c* reductase recovered in the filtrate was determined. The means are given of 4 experiments.

microsomal vesicles existed as separate entities. On the other hand, with increasing concentrations of *Crotalaria* microsomes demonstrated a parallel increase in aggregation.

Several microsomal enzyme functions were investigated after incubation of intact vesicles with various lectins. Prolonged incubation with *Crotalaria* lectin does not interfere with NADH-ferricyanide reductase which, however, is almost completely inhibited in the presence of Ervilia lectin (Fig. 3). NADH-cytochrome *c* reductase activity, which involves the interaction of flavoprotein with cytochrome b_5, is inhibited

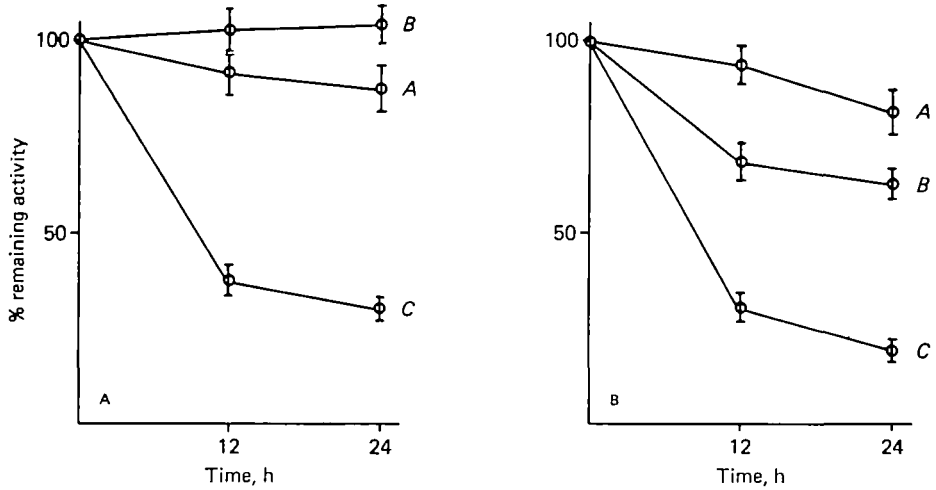

Fig. 3. Inhibition of microsomal NADH oxidation by lectins. Total microsomes (8 mg protein/ml in 0·25 M sucrose) were incubated with lectins (1 mg/ml) at 10 °C. After 12 and 24 h NADH-ferricyanide and -cytochrome *c* reductases were measured (Figs. A, B, respectively). The activities are expressed as percentage of the original. *A*, control; *B*, Crotalaria; *C*, Ervilia. The values represent 8 experiments, the vertical bars S.E.M.

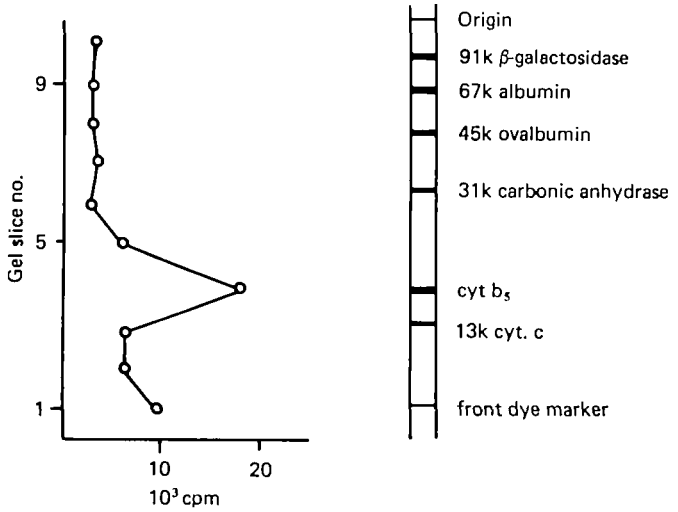

Fig. 4. Sodium dodecyl sulphate electrophoresis of isolated cytochrome b_5 treated with galactose oxidase and tritiated with borohydride. After the electrophoresis, the radioactivity in gel slices was determined after homogenization in Bray's solution. Reference proteins (including cytochrome b_5 itself) are shown on the right.

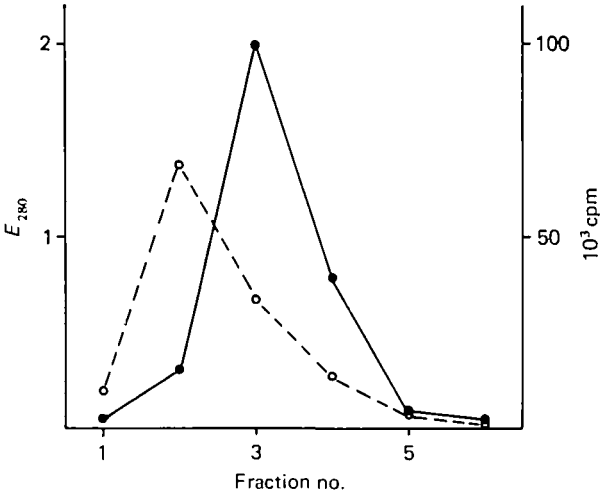

Fig. 5. Elution of galactose oxidase-treated cytochrome b_5 and anti-b_5 from protein A-Sepharose column. Isolated cytochrome b_5 was labelled with [^{3}H]borohydride after treatment with galactose oxidase and incubated with an excess of rabbit antibodies against b_5. The incubation mixture was applied to a protein A-Sepharose column, was then washed with 10 bed volumes of 0·1 M phosphate buffer, pH 7·5, and eluted with 0·2 M citrate buffer, pH 3. Extinction at 280 nm (—●—) and radioactivity (cpm) in the fractions (- - ○ - -) were due to excess of antibodies. The radioactive peak was identified as cytochrome b_5 by gel electrophoresis.

not only by *Ervilia* lectin but also by *Crotalaria*. One cannot expect a complete inhibition with *Crotalaria* lectin even if it interacts with cytochrome b_5, since the cytochrome is present in 10-fold excess over the reductase. The inhibitions of NADH-ferricyanide reductase with *Ervilia* lectin and of NADH-cytochrome *c* reductase with both *Ervilia* and *Crotalaria* lectins indicate that both the flavoprotein and the cytochrome are glycoproteins. Lectin inhibition, however, does not necessarily imply that the enzyme itself is a glycoprotein. The lectin could bind to an adjacent protein in the membrane and thus prevent the penetration of the substrate to the membrane enzyme, particularly in the case of large substrates such as the cytochromes. Further evidence for the glycoprotein nature of cytochrome b_5 is given by treatment with galactose oxidase followed by labelling with tritiated borohydride. Detergent-isolated cytochrome b_5 was labelled with the galactose oxidase procedure. Sodium dodecyl sulphate-gel electrophoresis gave a single labelled peak at the same position as the non-labelled cytochrome (Fig. 4).

The galactose oxidase-labelled cytochrome b_5 was also incubated with specific antibodies and the resulting complex was adsorbed onto a protein A-Sepharose column (Fig. 5). The cytochrome-antibody complex was eluted and the labelled protein peak was identified as cytochrome b_5 by gel electrophoresis.

DISCUSSION

In this study the interaction of glycoproteins of the endoplasmic reticulum with various types of lectins was investigated. Sepharose-bound lectins do not interact appreciably with intact microsomal or Golgi vesicles, probably for 2 reasons. First, the oligosaccharide residues of the membrane glycoproteins at the cytoplasmic surface appear to be short and thus may not reach the lectin-binding site. And secondly, interaction may be prevented because the microsomal vesicle is larger than the channels in the Sepharose bead and thus may not reach lectin molecules bound inside the bead.

Detergent-solubilized membranes contain glycoproteins which react with various types of lectins. Concanavalin A, *Crotalaria*, WGA and a crude fraction from wheat germ lipase were used. The specificity of Con A for terminal mannose is well established, both by X-ray analysis (Becker *et al.* 1975) and inhibition studies (Portez & Goldstein, 1970). *Crotalaria* lectin interacts mainly with galactose and lactose (Ersson, Aspberg & Porath, 1973), while WGA binds mainly *N*-acetyl glucosamine (Allen, Neuberger & Sharon, 1973). A number of factors influence the binding of glycoproteins to lectins (Lis & Sharon, 1973), e.g., the nature of the terminal sugar, the length and the composition of the oligosaccharide chain, the type of sugar interaction and the structure of the peptide. Still, the use of lectins is one of the most valuable methods for studying the nature of the terminal sugar residues of protein-bound oligosaccharide chains. Our experiments demonstrate that a crude preparation of wheat germ lipase contains a lectin interacting mainly with NANA. The preparation was not further purified and we cannot tell whether the binding is of the 'lock-and-key' type or involves a more unspecific electrostatic interaction. Nevertheless, the binding and inhibitor studies indicated that the preparation may be used as a lectin for probing terminal NANA residues. It should be added that pure WGA also interacts to some extent with terminal NANA, since neuraminidase treatment decreased binding of this lectin both to microsomes and submicrosomal particles. This is in agreement with previous studies (Allen *et al.* 1973; Greenaway & LeVine, 1973; Redwood & Polefka, 1976; Jordan, Basset & Redwood, 1977).

The dominating interaction in the case of membrane glycoproteins was that with Con A, indicating a large amount of terminal or 1→2-linked mannose (Goldstein, Reichert & Misaki, 1974), in decreasing amount in the order rough and smooth microsomes and Golgi membranes. The total amount of glycosamine and NANA residues increases in the order rough and smooth microsomes and Golgi membranes, as could be demonstrated with WGA and crude lipase. This type of sugar distribution is in agreement with the subcellular distribution of the glycosylation processes, since in contrast to GDP-mannosyl transferase both UDP-glycosaminyl and CMP-sialyl transferases are enriched in the Golgi system (Nilsson *et al.* 1978; Schachter *et al.* 1970). There is a small amount of binding to *Crotalaria* lectin with all 3 fractions, and this can be increased by pretreating the membranes with neuraminidase. Obviously, the sugar next to the terminal NANA in these membranes is galactose. Both chemical measurements and lectin interaction studies show that a large part of the

protein-bound oligosaccharide chains terminating with galactose-NANA are on the cytoplasmic side of rough and Golgi membranes, while in smooth microsomes about half of these chains are located on this side, but they appear to be buried by neighbouring protein molecules. Compartmentalization of the membrane glycoproteins was not studied in more detail, but is is clear from these treatments with various hydrolytic enzymes that some protein-bound sugar residues are located at the cytoplasmic side of the membrane.

High Con A-binding was observed with lipid- and AMPase-rich (fraction 1), NADH oxidase-rich (fraction 2) and NADPH oxidase-rich (fraction 3) submicrosomal particles. The same fractions also exhibited WGA binding which was mainly neuraminidase-sensitive. WGA-binding was also observed with the glucose-6-phosphatase-rich particles (fractions 4 and 5), but this binding was not influenced by neuraminidase treatment. Thus, glucosamine appears to be the dominating protein-bound sugar in these fractions. The high WGA-binding by submicrosomal particles and also the large amount of binding to *Crotalaria* lectin after neuraminidase treatment can be explained by the fact that the membranes were subjected to a long deoxycholate treatment (48 h) which should result in uncovering and unfolding of polypeptide chains.

In this study glycoproteins were labelled with glucosamine 1 h before decapitation. Consequently, we cannot expect labelling of all protein-bound oligosaccharide chains and furthermore, the label is not randomly distributed. Clearly, those glycoproteins which are newly synthesized contain all the label. In spite of the intensive movement of the glycoproteins, partly within the endoplasmic reticulum and partly between the membrane and cytoplasmic compartment (Omura & Kuriyama, 1971; Autuori, Svensson & Dallner, 1975), it is not possible to obtain a complete labelling or an even distribution of the label and, therefore, the approach employed here should be interpreted in qualitative terms.

The lectin-inhibition studies suggest that the NADH-oxidizing flavoprotein is a glycoprotein with a terminal mannose residue and that cytochrome b_5 also has an oligosaccharide chain with terminal galactose. Chromatographic and electrophoretic analyses of the galactose oxidase-labelled cytochrome b_5 also supported the idea that cytochrome b_5 has a covalently bound oligosaccharide chain. Other investigations have demonstrated that cytochrome b_5 reductase and cytochrome b_5 exhibit similar structures composed of a hydrophobic part in a lipid environment and a hydrophilic protruding into the water phase (Spatz & Strittmatter, 1971, 1973). It appears probable that the hydrophilic portion of these enzymes contains an oligosaccharide chain, which may represent some of the sugar residues of the outer surface of microsomal vesicles.

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