BIOGENESIS OF MICROSOMAL MEMBRANE GLYCOPROTEINS IN RAT LIVER

I. Presence of Glycoproteins in Microsomes and Cytosol

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ABSTRACT

The glycoproteins of microsomes and cytosol were studied. Various washing procedures did not release the proteins from the microsomes, and immunological tests demonstrated that the sialoproteins are not serum components. Low concentrations of deoxycholate and incubation in 0.25 M sucrose solution liberated a small amount of microsomal sialoprotein and this fraction exhibited a high degree of labeling of protein-bound *N*-acetylneuraminic acid. A part of the glycoprotein fraction could not be solubilized, even with a high concentration of the detergent. Thoroughly perfused rat liver contained sialoproteins in the particle-free supernate. The level of sialoprotein present could not be due to contamination with serum or broken organelles. The high in vivo incorporation of [^{3}H]glucosamine into protein-bound sialic acid of Golgi membranes and cytosol was paralleled by a delayed and lesser rate of incorporation into the rough and smooth microsomal membranes. This incorporation pattern suggests the possibility that the glycoproteins of cytosol and Golgi may later be incorporated into the membrane of the endoplasmic reticulum.

A large number of the proteins present in cells and tissues contain covalently bound carbohydrates. The glycoproteins of the extracellular space have been carefully investigated, since they are easily available and occur in large amounts (1). Studies performed in recent years have also demonstrated that a large number of intracellular structures in various animal tissues, such as the gastric mucosa (2), small intestine (3), brain (4), kidney (5), platelets (6), fibroblasts (7), many solid tumors, and ascites tumor cells (8, 9), contain glycoproteins. The nature of the association between the glycoproteins and these structures is not yet established; the main glycoprotein of the erythrocyte membrane appears to be an integral protein and bridges the membrane.

Many investigations have demonstrated that liver plasma membranes contain both glycoproteins and glycolipids (10, 11). The other membranes of the hepatocyte have been studied much less extensively, but it appears that outer and inner mitochondrial (12), lysosomal (13), and nuclear membranes (14) contain some glycoproteins.

With regard to the membranes of endoplasmic reticulum (ER)[1], several investigations have indi-

[1] *Abbreviations used in this paper:* DOC, deoxycholate; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; LDH, lactate dehydrogenase, NANA, *N*-acetylneuraminic acid; PLP, phospholipid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; and TWT, Tris-water-Tris.

cated that isolated microsomes and microsomal subfractions also contain glycoproteins that seem to be integral membrane proteins. The carbohydrate part of these glycoproteins consists of the neutral sugars mannose, galactose, and glucose, the amino sugars glucosamine and galactosamine, and sialic acid (15, 16).

The experimental difficulties in working with the glycoproteins of the ER include possible contamination of microsomes with other subcellular membranes or proteins of nonmembranous nature. The total microsomal fraction may be contaminated with both plasma and Golgi membranes, structures known to contain glycoproteins. In addition, 30–40% of the protein in the microsomal fraction is non-ER protein adsorbed to the surface of the microsomal membrane, mainly because of its high negative surface charge (17). Most of the blood proteins are synthesized in the liver ER, and after homogenization these proteins are enclosed in the lumen of the microsomal vesicles. Secretory proteins thus make up 10–15% of the total protein in the microsomal fraction.

In this paper, we present various types of experimental evidence that the glycoproteins of the microsomal fraction from rat liver are constitutive components of ER membranes. It is also shown that the particle-free supernate from rat liver contains glycoproteins which cannot be ascribed to remaining serum proteins or to components released from organelles during homogenization. A preliminary report of this work has appeared (18).

MATERIALS AND METHODS

Animals

Adult male albino rats weighing 160–180 g were used. All animals were starved for 20 h before sacrifice. Since blood serum contains large amounts of glycoprotein, the complete removal of blood from the liver is of basic importance in most experiments of the kind described here. Before perfusion, the animals were anesthetized by intraperitoneal injection of 0.1 ml (60 mg/ml) of Pentobarbital [5-Ethyl-5-(1-methylbutyl) barbituric acid]. For perfusion, a cannula was inserted into the portal vein and fixed with a ligature; both the superior and inferior vena cava were also ligated with a hemostat. The cannula was connected with plastic tubing to a 50-ml syringe filled with 0.25 M sucrose, and this solution was pumped into the blood vessels of the liver. A series of small cuts were made on the edges of all the lobes of the liver. It was necessary to perfuse the liver with 200–250 ml of cold sucrose under high pressure in order to obtain the light-brown color which is characteristic of this organ when it has been completely depleted of blood. The importance of perfusion is emphasized by the observations that after perfusion the protein content of the liver supernate decreases by 40% and the protein-associated *N*-acetylneuraminic acid (NANA) is only about 5 μg/g liver, which is 15% of the content obtained without perfusion.

For isolation of Golgi membranes, starved animals were given 1.2 g 50% ethanol per 100 g body weight by stomach tube 90 min before decapitation (19). In experiments where lysosomes were isolated, the rats were injected intraperitoneally with 1 ml Triton WR-1339 (oxyethylated *t*-actylphenol formaldehyde polymer, Rohm & Hass Co., Philadelphia, Pa.) (364 mg/ml) 4 days before sacrifice (20).

Fractionation

Total microsomes and microsomal subfractions were prepared as described previously (21). The microsomal pellet was suspended (microsomes from 2 g wet weight of liver per 10 ml) in 0.15 M Tris-HCl buffer, pH 8.0, and centrifuged at 105,000 *g* for 60 min in order to remove adsorbed proteins. The pellet and the centrifuge tube were rinsed three times with cold distilled water, and microsomes from 1 g liver (wet weight) were resuspended in 10 ml distilled water to give a protein concentration of 1.5 mg protein per ml. Microsomal subfractions and other subcellular particles were suspended to give the same final protein concentration. In some experiments, smooth microsomes were flotated as described below for the isolation of the Golgi fraction. These fractions were routinely incubated at 30°C for 15 min, cooled in an ice-water bath and subsequently centrifuged at 105,000 *g* for 60 min. This procedure removed the contents of the vesicles (17). For the final washing, the pellet was resuspended (20 mg protein/10 ml) in 0.15 M Tris-HCl buffer, pH 8.0, and centrifuged at 105,000 *g* for 60 min. The isolated pellets were finally resuspended either in 0.25 M sucrose or, in cases where sialic acid determination was to be carried out in distilled water, at a concentration of 5 mg protein per ml.

Golgi membranes were prepared according to the procedure of Ehrenreich et al. (19) using sucrose layers of 1.17 M, 1.15 M, and 0.25 M. The total Golgi fraction, floating on the top of the 1.15 M sucrose, was always used. Plasma membranes (22), mitochondria (23), and lysosomes (20) were isolated using procedures described earlier. The Golgi and plasma membrane fractions were also subjected to the Tris-water-Tris (TWT) washing procedure described above for microsomal particles.

For the preparation of particle-free supernate, the postmicrosomal supernate was centrifuged for an additional 4 h at 105,000 *g*. After the centrifugation, the top 0.5 ml of the tube contents was sucked off and discarded. The remaining supernate was removed with a Pasteur

pipette, leaving behind the last 0.5 ml at the bottom, which was also discarded.

Incorporation Experiments

D-[1-^{3}H]glucosamine (2,000 mCi/mmol) and/or DL-[1-^{14}C]leucine (200 mCi/mmol) from the Radiochemical Centre, Amersham, England were mixed with 9% NaCl solution to obtain a final concentration of 0.9% NaCl. Appropriate amounts of radioactivity in 0.2–0.5 ml volumes were injected into the portal vein under Pentobarbital anesthesia, and the abdomen was sutured shut until decapitation or perfusion. In time course experiments, nonlabeled glucosamine (20 mg in 0.2 ml of physiological solution) was also injected into the portal vein 10 min after pulse labeling. Protein-bound sialic acid was labeled with [^{3}H]glucosamine and not with labeled sialic acid, since sialic acid is taken up by hepatocytes in vivo only at a low rate (24).

For the analysis of total radioactivity in particle suspensions, aliquots were precipitated with 5% trichloroacetic acid (TCA); and after dissolving the protein in 1–2 ml 1% sodium dodecyl sulfate (SDS), 10–15 ml Bray solution (25) were added and the radioactivity determined by scintillation counting. In experiments where *N*-acetylneuraminic acid (NANA) was isolated before counting, aliquots of the sugar dissolved in water were mixed with 10 ml Bray solution supplemented with 0.1% bis-methylstyrylbenzene.

Antibody Treatment

Microsomal membranes (4 mg protein/ml) were treated with deoxycholate (DOC) (final concentration 0.5%) and centrifuged to remove insoluble material. In the case of supernate, no DOC was added. The dissolved membrane fraction or supernate was incubated with rabbit antiserum against rat serum protein; to control for nonspecific precipitation, rabbit antiserum against chicken serum protein was also used (26). The medium for the precipitation reaction contained 0.15 M Tris-HCl buffer, pH 7.5. The amount of antiserum required to obtain maximal precipitation was established by titration, and this amount of rabbit antiserum against rat serum was incubated with the sample for 60 min at 37°C. To achieve complete precipitation of all antigen-antibody complexes, purified sheep IgG against rabbit IgG was added in optimal amounts. The sheep antirabbit IgG was purified from serum from immunized animals by binding to rabbit antirat serum immunosorbent followed by dissociation with glycine buffer, pH 2.8, according to Avremas and Ternynck (27). The reaction mixture was then incubated for an additional 60 min at 37°C followed by storage at 4°C overnight. The precipitate was then separated by centrifugation. Radioactivity was measured both in the supernate and in the pellet, the latter after washing twice with 0.15 M KCl and dissolving in 1 ml 1% SDS.

Polyacrylamide SDS-gel Electrophoresis

SDS-gel electrophoresis was performed according to Weber and Osborn (28). 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS was used as the electrolyte buffer. The gels (10 cm × 0.5 cm) contained 10% acrylamide and the concentration of methylenebisacrylamide was 2.7% of that of acrylamide.

In preparing the sample membrane proteins were suspended at a concentration of 1 mg/ml in sodium phosphate buffer, 0.1 M, pH 7.0, 2% SDS, 10% β-mercaptoethanol, 0.05 M Na_2CO_3 and 10% sucrose solution. After addition of 50 μl 0.25% bromphenol blue (marker dye), the samples were incubated at 100°C for 2 min. Aliquots containing 50–200 μg protein were then layered on the gels. In the case of the supernatant proteins the SDS concentration was 0.4%. In other respects, the procedure was the same as for membrane proteins.

Electrophoresis was carried out at 5 mA/tube for 15 h. Under these conditions, the marker dye migrated to the lower end of the tube. The proteins were stained with Coomassie brilliant blue R 250 at a concentration of 0.4% in 50% methanol and 10% acetic acid for 20 min. The gels were destained in a solution of 8% acetic acid, 5% methanol overnight. Densitometric measurements were performed at 550 nm with a linear scanning attachment connected to Pye Unicam spectrophotometer (Pye Instruments, Cambridge, England), using an 8 mm × 10 cm quartz cuvette.

After the run, the gels were cut into 2 mm slices, dried, and transferred to a combustion cone together with cellulose powder and two drops of Combustaid (Packard Instrument Co., Inc.). The samples were then combusted in a Packard oxidizer (Packard Instrument Co., Inc., Downers Grove, Ill.). for 1.5–2.5 min; and vials containing either ^{3}H or ^{14}C were mixed with the scintillators from the oxidizer and measured in a Beckman LS-100 scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Counts were corrected for quenching, which was caused mainly by water in the tritium channel and by the Carbosorb (Packard Instrument Co., Inc.) used for elution of the carbon dioxide in the ^{14}C channel.

Since slices for the measurement of radioactivity originate from a gel duplicate of the one on which protein was stained and scanned, and since furthermore the slices were 2 mm thick and thus did not offer the same resolution as protein scanning, one cannot expect a complete overlap between the individual proteins and the corresponding peaks of radioactivity. No correction for this error has been made.

Chemical Determinations

Before determining sialic acid, the particle suspensions, supernate, and various chromatographic fractions

were precipitated with 6% TCA and extracted with 2 ml chloroform-ethanol, 1:1, per 0.5 g liver equivalent at 4°C overnight in order to remove lipids. We found this to be an important step, since lipids adversely affect the reliability of the Warren thiobarbituric acid assay for sialic acid (29). The Warren procedure results in high absorption not only at 549 nm but also at 532 nm if nonextracted microsomes are used, but if lipids were first removed with chloroform-ethanol, the absorption at 532 nm decreased to a great extent. The OD_{549}/OD_{532} ratio of samples is considerably in the latter case, corresponding to that obtained with pure neuraminic acid. Partition of the chloroform-ethanol extract was performed with cold 0.1 N HCl (5 ml HCl per 2 ml chloroform). The precipitated protein present at the interface was extracted once more in the same manner, except that the extraction time was decreased to 10 min. NANA was liberated by incubating the pellet 60 min at 80°C in 2.5 ml 0.05 M H_2SO_4. After centrifugation, the supernate was applied to a Dowex 2-X8 column (Dow Chemical Co., Midland, Mich.) and eluted with 8 ml 1 M sodium acetate buffer, pH 4.6, according to Svennerholm (30). The eluate was lyophilized, and the sample dissolved in water. Aliquots were assayed for sialic acid using the Warren procedure (29), and radioactivity was counted in other aliquots. It was necessary to modify the Warren test by doubling the sodium periodate-phosphoric acid concentration in order to obtain reproducible results.

Protein was determined routinely according to Lowry et al. (31) with bovine serum albumin as standard. In some cases protein measurements were made with the Biuret reaction (32). Phospholipids were analyzed as described previously (33).

RESULTS

Marker Enzymes

The rough microsomes prepared with a Cs^+-containing discontinuous sucrose gradient contain very limited amounts of membrane from other organelles, as demonstrated by the measurement of various marker enzymes (Table I). This fraction contains practically no CMP-sialic acid transferase activity, a Golgi marker, or cytochrome *c* oxidase present on the inner mitochondrial membrane. Some acid phosphatase (a marker for lysosomes) and AMPase activity are present; the latter may actually be localized to a certain extent on the ER itself, as well as on the plasma membrane. As expected, part of the Golgi fraction is recovered in the smooth microsomes; this contamination could, however, largely be removed by flotation.

Effect of Perfusion and Washing

Extensive perfusion of the liver was carried out, and the effectiveness of the perfusion was determined by measuring remaining radioactivity after injecting the rats with labeled serum proteins (Table II, exp 1). Only 0.5% of the total protein-bound label in the liver remained after perfusion of this organ. This finding indicates that a liver homogenate prepared after perfusion does not contain significant amounts of serum proteins.

One measure of the effectiveness of the TWT washing procedure consisted of adding [^{3}H]-leucine-labeled serum protein to the homogenate and subsequently isolating and washing the microsomal fraction (Table II, exp 2). Only about 0.3% of the added radioactivity was found associated with microsomes in the washed pellet, an amount which is far too low for contaminating serum proteins to interfere seriously with studies of microsomal glycoproteins.

TABLE I

Distribution of Marker Enzymes in Liver Subfractions

	G6Pase*	CMP-NANA‡ transferase	AMPase*	Cytochrome§ *c* oxidase	Acid phosphatase*
Rough microsomes	2.2	4.2	0.03	0.05	0.09
Smooth microsomes	2.4	40.8	0.06	0.04	0.18
Smooth microsomes (flotated)	2.5	13.1		0.04	0.16
Golgi fraction		862.0			
Mitochondria	0.5			5.01	
Lysosomes					1.12
Plasma membranes			0.73		

Smooth microsomes were flotated according to Ehrenreich et al. (19). For the measurement of CMP-NANA transferase desialidated fetuin was used as acceptor (35). The values are the means of 4–6 experiments.

* μmol P_i/min/mg protein.

‡ pmol NANA transferred/10 min/mg protein.

§ μmol O_2/min/mg protein.

TABLE II

Removal of Labeled Serum Protein by Perfusion and Removal of Adsorbed Serum Protein from Microsomes

Exp	Fraction	Total counts	
		cpm	%
1	Homogenate (no perfusion)	653,000	100
	Homogenate (perfusion)	3,300	0.5
	105,000 *g* supernate	450	0.07
2	Homogenate	936,000	100
	Microsomes	24,800	2.6
	TWT-washed microsomes	3,100	0.3

In exp 1 labeled total serum proteins were prepared by intraportal injection of 0.2 mCi [^{3}H]leucine followed by decapitation 60 min later. The serum was passed through a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and 0.5 ml aliquots from the protein peak were injected into the portal vein of rats. 10 min later the homogenate was prepared from one liver, and another was perfused. The perfused liver was homogenized, and supernate was prepared by centrifugation at 105,000 *g* for 2 h. In exp 2 labeled serum protein was prepared as in exp 1, as aliquot was mixed with liver homogenate and after preparation of microsomes TWT washing was performed (17). The values are the mean of three experiments.

Microsomal membranes are known to be highly charged, and this property causes surface adsorption of a number of cytoplasmic proteins during homogenization and centrifugation (34). In addition secretory proteins are found in the lumen of microsomal vesicles. It has been demonstrated previously that alkaline Tris buffer effectively removes adsorbed proteins, and incubation in distilled water at 30°C followed by cooling liberates nonmembranous secretory proteins (17). When isolated microsomes were subjected to the TWT washing procedure, more than 40% of microsomal protein, bound sialic acid, and total incorporated glucosamine, but none of the phospholipid (PLP), could be removed (Table III). We also subjected TWT microsomes to a number of additional treatments including repeated washing with alkaline or acid buffer, increasing ionic strength, low concentrations of DOC, sonication, and combinations of these treatments. The recovery of protein, PLP, and NANA varied, since membrane components were solubilized to a varying extent, but the ratio of NANA to PLP (microgram/milligram) was more or less unchanged by such treatments. This experiment demonstrates that after the TWT washing procedure it is not possible to remove more protein from the microsomes without releasing PLP. At the same time it can be concluded that the sialoprotein of the microsomes remaining after treatment with 0.2% DOC and sonication cannot be present in a free state in the microsomal lumen.

Treatment with Antibody

Secretory glycoproteins are not necessarily in a free form in the microsomal lumen (26), so another approach was also required. Washed and dissolved rough and smooth microsomal subfractions were treated with rabbit antiserum against rat serum protein (Table IV). When these microsomes were isolated from rats injected with [^{3}H]glucosamine 60 min before decapitation and then subjected to antibody treatment, about 20% of the label could be recovered in the precipitated pellet. This indicates that some glucosamine-containing serum proteins are still attached to washed microsomal membranes. On the other hand, if only the radioactivity in sialic acid moiety was counted, no label appeared in the precipitated pellet. These results are in agreement with the well documented fact that the sialic acid moiety can be attached to glycoproteins only within the Golgi complex (35). This experiment strongly indicates that the sialic acid-containing glycoproteins found in washed microsomes do not represent contamination by serum proteins.

Treatment with DOC and Incubation

In order to study the manner of association of sialoproteins with rough microsomal membranes, TWT microsomes were treated with increasing concentrations of DOC and then centrifuged. The decrease in PLP recovered in the pellet was paralleled by a decrease in protein-bound sialic acid (Fig. 1 A). It is remarkable that even at 0.4% DOC, when most of the membrane material was solubilized, part of the sialoprotein was still sedimentable. These experiments were performed with microsomes prepared from rats injected with [^{3}H]glucosamine, and, as is apparent from Fig. 1 A, the loosely bound proteins had a higher sialic acid-bound radioactivity than the proteins which were released by DOC concentrations of 0.2% or more.

TABLE III

Effect of Various Treatments on Microsomal Constituents

Exp	Treatment of total microsomal fraction	Protein	PLP	NANA	NANA, μg / PLP, mg	cpm in NANA / mg protein
		mg	*mg*	*μg*		
1	None	22.1	7.5	70.0	9.3	
2	TWT washing procedure	13.0	7.5	40.5	5.4	1,852
	+ Tris buffer (0.01 M Tris-HCl, pH 7.5)	12.2	7.5	37.7	5.0	1,905
	+ Tris buffer + 1.5 M KCl	12.1	7.5	36.8	4.9	1,930
	+ Tris buffer + 1.5 M KCl + 0.2% DOC	8.2	3.9	19.0	4.9	1,673
	+ Tris buffer + sonication	8.9	6.4	27.9	4.4	1,947
	+ Tris buffer + 1.5 M KCl + sonication	9.4	5.9	31.2	5.3	1,998
	+ Tris buffer + 1.5 M KCl + 0.2% DOC + sonication	8.2	3.7	19.4	5.2	1,703
	+ 0.5 M citrate-phosphate buffer, pH 5.0	12.0	5.8	28.9	5.1	1,214

Microsomes were prepared after injecting 0.2 mCi [^{3}H]glucosamine (10 Ci/mmol, Radiochemical Centre, Amersham, England) into the portal vein of 150-g rats 60 min before decapitation. Preparation of the total microsomal fraction and the TWT washing procedure are described in Materials and Methods. After washing the microsomes were subjected to different treatments and centrifuged (3 h at 105,000 *g*), the pellets were analyzed for protein, PLP, NANA and radioactivity in NANA as described in the Materials and Methods. In the case of DOC treatment, the concentration of protein was 5 mg/ml, and sonication was performed with a Branson sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at 3.5 A for 2 min.

TABLE IV

Treatment of Microsomes with Rabbit Antiserum to Rat Serum Protein

Fraction	Radioactivity in membranes: Total	Nonprecipitable with antiserum	Precipitable with antiserum	Radioactivity in NANA: Total	Non precipitable with antiserum	Precipitable with antiserum
	cpm/g liver	*cpm/g liver*	%	*cpm/g liver*	*cpm/g liver*	%
Total microsomes	232,000	161,000	44	9,322	10,241	0
Rough microsomes	108,000	73,000	22	2,747	2,890	0
Smooth microsomes	103,000	78,000	12	7,707	8,351	0

Rats were injected with [^{3}H]glucosamine as described in Table II, decapitated 60 min later, and the liver homogenates were subfractionated. TWT microsomes and microsomal subfractions were treated with antisera as described in Materials and Methods. Total, precipitable, and nonprecipitable radioactivity was determined. Also, NANA was extracted from these fractions for analysis of radioactivity. The values given represent the means of four experiments.

When the same DOC treatment was performed on rough microsomes previously incubated in 0.25 M sucrose for 60 min (Fig. 1 B), a somewhat higher detergent concentration was required to solubilize PLP and NANA, clearly because of the fact that loosely bound sialoproteins were already removed during the preincubation. In other respects the solubilization was very similar to that shown in Fig. 1 A.

To study the events occurring during the incubation of rough microsomes in 0.25 M sucrose, SDS-acrylamide gel electrophoresis was performed. In these experiments microsomes were isolated from rats injected directly into the portal vein with [^{3}H]glucosamine and [^{14}C]leucine. Analysis of TWT rough microsomes before incubation revealed most of the proteins to be present in the region of the gel where proteins of high molecular weight are found; the majority of the sugar and amino acid label could be recovered in this same region (Fig. 2 A). These findings are in agreement with those of Kreibich and Sabatini (36). Since our

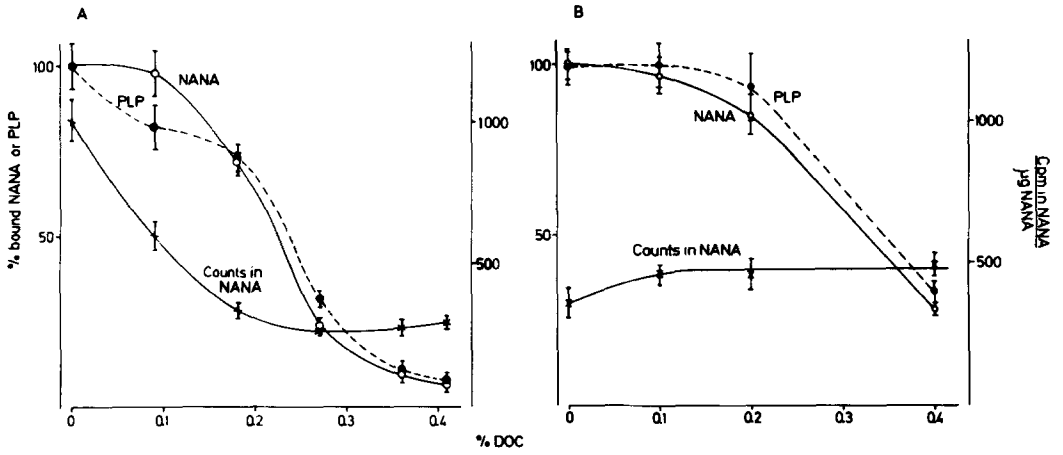

FIGURE 1 The effect of treating microsomes with DOC on membrane-bound NANA and PLP. Rough microsomes were prepared 30 min after injection of [^{3}H]glucosamine (0.5 mCi) into rats. Microsomes (1 mg protein/ml) without (A) or with preincubation (B) (37°C, 60 min, 0.25 M sucrose) were treated with increasing amounts of DOC. PLP, NANA, and NANA-associated radioactivity were determined in the pellet after centrifugation. The values are the results of five experiments; the vertical bars represent SEM.

washing treatment did not remove all of the bound ribosomes, a variable amount of nonlabeled protein always appeared in the lower molecular weight region of the gel. After incubation of rough microsomes in sucrose, the most significant change was a decrease in a protein highly labeled with [^{3}H]glucosamine in the 50,000 mol wt region (Fig. 2 B). As shown in Fig. 2 C, the gel pattern of the proteins released by incubation exhibited a number of protein peaks in the 50–70,000 mol wt region, with the highest sugar (^{3}H) and amino acid (^{14}C) label in the 50,000 dalton peak, corresponding to the peak in the microsomal pattern which is decreased by incubation.

In Vivo Labeling of Cytoplasmic Membranes

The incorporation rate of [^{3}H]glucosamine into protein-bound sialic acid of cytoplasmic membranes and supernate was followed after injection of the label into the portal vein. To obtain optimal and reliable results a pulse labeling was achieved by injecting a 10,000-fold excess of nonlabeled glucosamine 10 min after the injection of the radioactive substance. The liver was carefully perfused, and subcellular fractions were washed using the procedure described in Materials and Methods.

The labeling pattern of isolated fractions is shown in Fig. 3. The specific radioactivity (counts per minute in NANA/microgram NANA) of Golgi membrane sialoproteins rose to a maximum after no more than 30 min, followed by a rapid decay. The situation with the particle-free supernate was very similar: the specific radioactivity in protein-bound sialic acid reached an early maximum and decreased in a fashion parallel with that of the Golgi membranes. The time course of the appearance of label in rough and smooth I microsomes was very different from that of the Golgi membranes and supernate. The radioactivity in these fractions increased slowly and reached a maximum only after 3 h; but even at this time point the sp act was significantly below the maximum value obtained in Golgi membrane and supernate.

Presence of Sialoproteins in the Cytoplasm

A surprising finding in the above experiments was the presence of sialoproteins in the particle-free supernate. To further investigate this point, several control experiments were performed.

Table V describes the content of protein-bound sialic acid and the rate of incorporation of [^{3}H]glucosamine (injected into rats 30 min before sacrifice) in various subcellular fractions. The supernatant fraction contains only a small amount of protein-bound sialic acid, but its high specific radioactivity is approached only by Golgi mem-

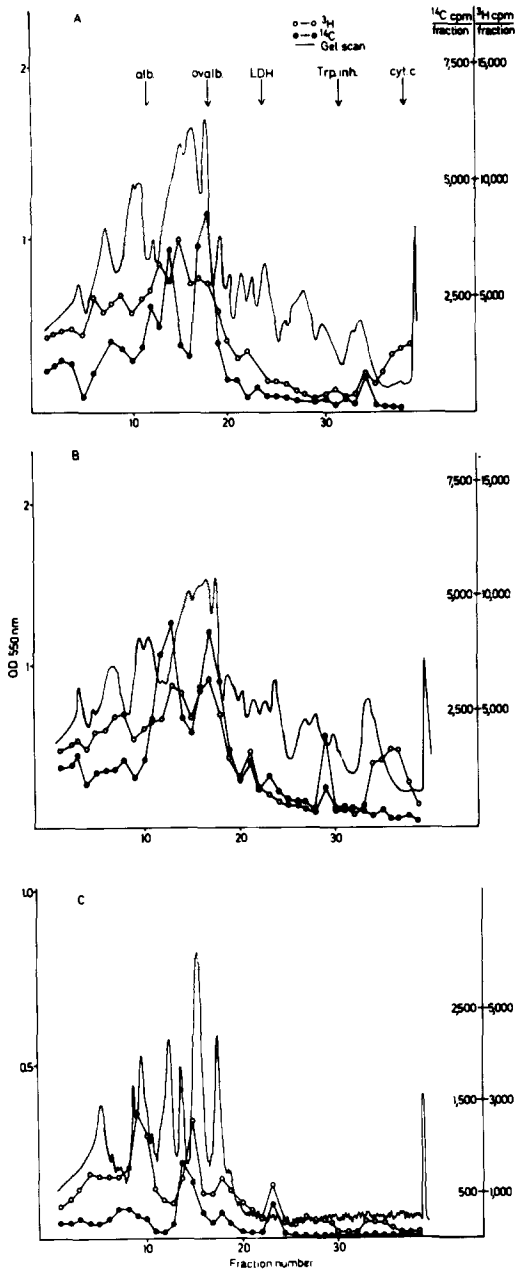

FIGURE 2 SDS-gel electrophoresis of rough microsomes labeled in vivo with [^{3}H]glucosamine and [^{14}C]leucine. Rats were injected intraportally with 1 mCi of [^{3}H]glucosamine and 250 μCi [^{14}C]leucine, and the liver was perfused 30 min later. Microsomes were isolated, suspended in 0.25 M sucrose (1 g/ml), and incubated at 37°C for 60 min. Aliquots of microsomes before (A) and after (B) incubation and the supernate (C) after incubation (~250 μg protein) were subjected to SDS-gel electrophoresis. The arrows in (A) give the position of the standards with known mol wt, i.e., albumin (67,000), ovalbumin (43,500), lactate dehydrogenase, LDH (36,000), trypsin inhibitor (21,000), and cytochrome *c* (13,400).

branes. The sialic acid content of smooth microsomes (flotated to remove Golgi contaminant) is significantly higher than that of their rough counter part, while in plasma membranes the relatively high NANA content is characterized by a lower radioactivity than in smooth microsomes. Since our smooth microsomal fraction as routinely prepared is seriously contaminated with Golgi vesicles, smooth microsomes were also prepared from ethanol-treated rats and subjected to flotation. This treatment removes 70% of the CMP-sialic acid transferase activity indicating effective removal of Golgi membranes (see Table I). As shown in Table V, the NANA content of smooth microsomes is practically the same after flotation as before this procedure, i.e., 5.7 μg/mg protein. Lysosomes are known to contain many glycoprotein enzymes (37), which may be released during homogenization into the supernate. The low incorporation rate of the lysosomes speaks against the possibility of serious interference in these experiments by contamination of the fractions with lysosomal glycoproteins. The situation is very similar with serum glycoproteins, where the low specific radioactivity of NANA speaks against serious interference from these glycoproteins.

The importance of careful perfusion when studying components of the supernate is apparent from Table VI. The protein content of the supernate decreases by 40% after perfusion; and, what is more important, protein-associated NANA after perfusion is only about 5 μg per g liver, which is 15% of the content without perfusion. The large majority of supernatant radioactivity can be removed by dialysis. When dialyzed supernate from perfused liver (removed from rats injected with [^{3}H]glucosamine) was treated with rabbit antiserum to rat serum proteins, precipitation did not exceed a few percent of the total protein-bound radioactivity. These immunoprecipitation analyses are in agreement with the above conclusions that after perfusion the remaining serum proteins represent very minor components of the total supernatant glycoproteins.

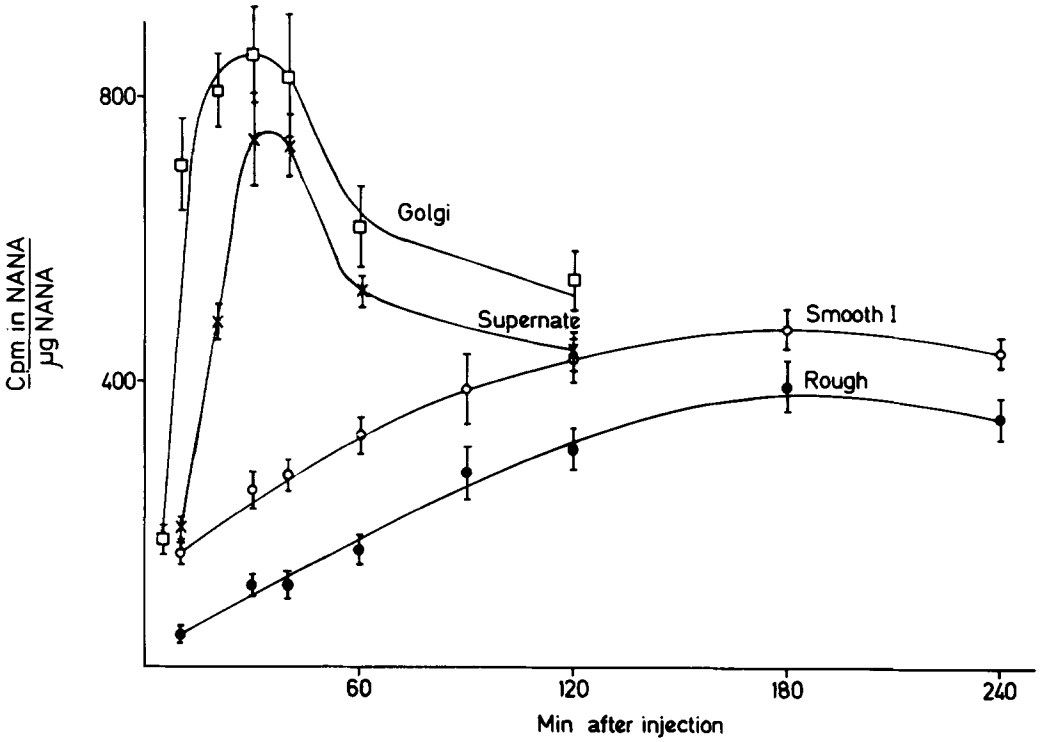

FIGURE 3 In vivo incorporation of[^{3}H]glucosamine into protein-bound NANA of different subcellular fractions. [^{3}H]glucosamine (0.1 mCi/rat) was injected intraportally. After 10 min, 20 mg of nonlabeled glucosamine was injected in the same manner. At appropriate time points, the livers were perfused, removed, homogenized, and subfractionated. NANA was isolated according to Svennerholm (30), and the specific radioactivity (cpm in NANA/µg NANA) was measured. The values are the results of six to nine experiments; the vertical bars represent SEM.

DISCUSSION

The results described here indicate the presence of glycoproteins in liver microsomal membranes and in the soluble cytoplasm. An important feature of the isolated microsomal vesicles is that many of their components are not related to the membrane itself but are adsorbed or luminal. Furthermore, fractionation by ultracentrifugation is based on physico-chemical properties such as size, density, permeability, and surface charge, with the result that no organelles or fragments of organelles can be isolated uncontaminated with other organelles. Obviously, all of these considerations become especially important when studying components present in low amounts, since in such cases even marginal contamination might compromise in the significance of the results.

One of the main sources of contamination in the microsomal fraction is that from serum protein. This can be of two types: serum proteins liberated from the blood vessels during homogenization and newly synthesized export proteins in the process of being transported through the ER lumen. Extensive perfusion under pressure removes all blood from the liver. The negative surface charge of microsomes, however, causes the adsorption of large amounts of soluble proteins, even in the absence of serum proteins. Since washing with alkaline Tris-buffer has previously been shown to be very effective in removing adsorbed proteins, this procedure was routinely applied in all of our studies. In the washing experiments (see Table III) Tris-washed microsomes were also treated with 0.2% DOC, which solubilized 40% of the PLP; in this case the presence of any remaining adsorbed protein is improbable. Even after DOC treatment the ratio of protein-bound NANA to PLP is unchanged, emphasizing again that glycoproteins in microsomal membranes do not represent an ar-

TABLE V

Subcellular Localization of Protein-Bound NANA and its Specific Radioactivity

Fractions	μg NANA / mg protein	cpm in NANA / μg NANA
Golgi membranes	1.80 ± 0.12	859 ± 80
Supernate	0.22 ± 0.02	757 ± 51
Smooth microsomes (flotated)	5.82 ± 0.35	250 ± 20
Smooth microsomes (ethanol treatment)	6.17 ± 0.71	260 ± 19
Smooth microsomes (ethanol treatment followed by flotation)	5.77 ± 0.70	247 ± 26
Rough microsomes	0.94 ± 0.01	113 ± 15
Plasma membranes	3.03 ± 0.20	116 ± 17
Lysosomes	8.55 ± 1.10	86 ± 15
Serum	13.60 ± 2.35	55 ± 12

Rats were injected in the portal vein with 0.1 mCi of [^{3}H]glucosamine. After 10 min, 20 mg of nonlabeled glucosamine were injected as chaser. 30 min later the livers were perfused. Subcellular fractions were prepared as described in Materials and Methods. Smooth microsomes, isolated both from nontreated and ethanol-treated rats were flotated in order to remove Golgi contamination as described earlier (19). All membrane fractions, with the exception of lysosomes were subjected to the TWT washing procedure, lysosomes were washed only with Tris buffer. Blood was taken from the venae cavae, and after clotting, serum was collected. After delipidation, NANA and counts per minute/microgram NANA were determined as earlier. The values are means ± SEM (n = 6).

tefact caused by adsorption. The control experiments involving intraportal injection of labeled serum proteins before perfusion and mixing of these labeled proteins with the homogenate before subfractionation and washing are also in agreement with this conclusion.

The large majority of blood glycoproteins are synthesized in the liver ER and transported through the lumen of the rough and smooth ER to the Golgi system. After homogenization these proteins are retained inside of the microsomal vesicles (35). In all our experiments the TWT washing procedure assured the effective removal of nonmembrane-bound luminal proteins. DOC also liberates the contents of microsomes (38), and TWT microsomes retained the major part of their sialoproteins even after detergent treatment. Tests using two-step antibody precipitation gave results which were in good agreement with the findings of Redman and Cherian (26) as well as with those of Kreibich and Sabatini (36). These tests gave a 10–20% precipitation of the total protein-bound [^{3}H]glucosamine. These precipitated proteins can be attributed to the presence of some bound serum proteins whose oligosaccharide chain is incomplete but which are still precipitable by antibiodies to rat serum proteins (39). On the other hand, the fact that no radioactive sialic acid was associated with the immunoprecipitate clearly demonstrated that the membranous sialoproteins represent constitutive components of the microsomal membrane. In addition, after the washing procedure we were unable to obtain any precipitation of dissolved Golgi membranes with rabbit antiserum against

TABLE VI

NANA and NANA-Associated Radioactivity in the Supernatant Fraction from Rat Liver

Liver	Supernatant fraction	Protein	Total radioactivity	Protein-bound NANA	Specific radioactivity
		mg/g liver	*cpm/mg protein*	*μg/g liver*	*(cpm in NANA)/(μg NANA)*
Not perfused	Not dialyzed	40.1	40,472	36.4	227
Perfused	Not dialyzed	24.7	36,321	5.5	971
Not perfused	Dialyzed	38.3	1,059	31.9	216
Perfused	Dialyzed	23.3	1,195	4.8	1,036
Perfused	Dialyzed + antibody-treated	22.2	1,114	4.5	987

Rats were injected in the portal vein with 125 μCi [^{3}H]glucosamine. After 30 min, some of the livers were perfused with cold 0.25 M sucrose. Particle-free supernates were prepared by centrifuging the microsomal supernate at 105,000 *g* for 4 h. The upper 0.5 ml at the top of the tube (neutral fat) and the 0.5 ml immediately above the pellet were discarded. In some cases the supernate was also dialyzed overnight against 5 mM Tris-HCl buffer, pH 7.5, 15 mM KCl with three changes of buffer. Each value represents the mean of three experiments.

rat serum proteins, a finding in agreement with those of Bizzi and Marsh (40).

The microsomal fraction isolated by differential centrifugation is contaminated by several other cytoplasmic organelles. This contamination is highly dependent on the type of homogenization employed. We employ mild and short homogenization, which results in a recovery of only 50–60% of the total hepatic ER, but at the same time gives much less contamination than most of the methods of preparation used in other laboratories (41). By measuring a number of marker enzymes in our rough microsomes (see Table I), only marginal activity of Golgi, mitochondrial, and lysosomal markers could be detected. The low AMPase activity is probably not a result of plasma membrane contamination. On the other hand, smooth microsomes contain Golgi membranes, which, however, can be removed by flotation; this procedure does not substantially decrease the amount of protein-bound sialic acid present in the smooth subfraction. In conclusion, the presence of sialoproteins in microsomal membranes cannot be explained by contamination with other organelles. This point is further strenghthened by the data on the distribution and specific labeling of NANA. The lower content of NANA relative to protein in Golgi and plasma membranes and the low incorporation rate of [^{3}H]glucosamine into lysosomal sialic acid indicate that microsomal glycoproteins do not originate from other contaminating organelles. In agreement with this idea is the time course of [^{3}H]glucosamine incorporation into sialic acid in vivo (Fig. 3).

The observation that the particle-free supernate from rat liver contains glycoproteins is surprising, since these proteins are generally regarded to be either membranous or present in and excreted through a channel system lacking direct connection with the cytoplasm. The nonsedimentability of these glycoproteins by high speed centrifugation overnight eliminated the possibility that they belong to membranes or membrane fragments with a low sedimentation velocity. On the other hand, several experimental findings indicate the absence of serum glycoproteins from the supernatant fraction, e.g., the inability to precipitate supernatant glycoproteins with rabbit antibodies to rat serum proteins and the observation that the specific radioactivity in protein-bound NANA in the supernate is more than 10 times that of the serum. In addition, experiments where labeled serum proteins were injected in vivo into rats demonstrated that less than 0.1% of label was recovered in the 105,000 *g* supernate after perfusion of the liver before homogenization. The low initial labeling of lysosomal sialoproteins speaks against the possibility that the lysosomal contents are released during homogenization.

These experiments strongly suggest that, using the definition of Singer and Nicolson (42), all the sialoproteins found in microsomes may be regarded as integral membrane proteins. By using a low amount of detergent it is possible to separate these proteins into two groups, one which is relatively loosely bound and one which is more tightly bound. Radioactive labeling and gel electrophoretic analyses yielded evidence that these two groups differ. The experiments reported here do not indicate whether the two groups contain different molecular species with well-defined functions, whether they are interrelated by gradual conversion, or whether they are located in different segments of the ER membrane system.

The finding that microsomes contain glycoproteins with terminal galactose and sialic acid without containing the glycosyl transferases required for attaching these moieties raises the question as to where these proteins are completed and how they are transported to their final localization. The experiments illustrated in Fig. 3 detected the presence of cytoplasmic glycoprotein which is rapidly labeled in contrast to the low initial label of rough and smooth microsomal glycoproteins. A reasonable working hypothesis can now be constructed for the biosynthesis of microsomal sialoproteins, including the key step of transferring a protein unit from the cytoplasm into the microsomal membranes. Experiments designed to demonstrate this process are described in the following paper (43).

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