

BIOGENESIS OF MICROSOMAL MEMBRANE GLYCOPROTEINS IN RAT LIVER

II. Purification of Soluble Glycoproteins and their Incorporation into Microsomal Membranes

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ABSTRACT

Sialoproteins isolated from the soluble fraction of rat liver could be incorporated into microsomal membranes. This incorporation was dependent on protein concentration, time, and temperature. Sodium dodecyl sulfate gel electrophoresis of membrane proteins after *in vitro* incorporation showed four major sugar-containing peaks and was similar to that found after *in vivo* labeling. Most of the incorporated protein was tightly bound to the microsomal membrane. Gel filtration and ion-exchange chromatography revealed the presence of several cytosolic glycoproteins that could be incorporated into microsomes. During prolonged centrifugation in a KBr solution with a density of 1.21 a highly labeled ($[^3\text{H}]$ glucosamine) protein (mol wt $\sim 70,000$) that was actively incorporated into microsomes could be recovered in the upper region of the tube. These results demonstrate that several cytoplasmic glycoproteins of rat liver are transferred into microsomal membranes and that one of these is a lipoprotein.

The protein and lipid components of liver microsomal membranes turn over rapidly (1). The half-life of the lipid components ranges from 20 to 50 h, while that of the proteins, with a few exceptions, ranges from 40 to 120 h (2). Microsomal glycoproteins also display a relatively rapid turnover; their half-life is around 60 h (3). This high turnover rate indicates the presence of an effective and continuous mechanism for the renewal and insertion of new microsomal membrane glycoproteins. Chemically, microsomal protein-bound oligosaccharides consist of mannose, galactose, glucosamine, galactosamine, and sialic acid; of these sugars, galactose and sialic acid must be added to the growing oligosaccharide chain in the

Golgi complex (4). This arrangement of the biosynthetic system requires transfer of completed membrane glycoproteins from the Golgi back to the ER.¹ Such transfer contrasts with the well-established unidirectional transport of serum albumin, serum glycoproteins, various types of lipoproteins, and cholesterol, all of which are synthe-

¹ *Abbreviations used in this paper:* DOC, deoxycholate; EDTA, ethylenediamine tetraacetate; ER, endoplasmic reticulum; GlN, glucosamine; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; Leu, leucine; LP, lipoprotein; NANA, *N*-acetylneuraminic acid; PLP, phospholipid; and TWT, Tris-water-Tris.

sized in the ER and pass through the Golgi system on their way to the blood (5, 6).

Previous experiments indicated that newly synthesized membrane components might be discharged from the Golgi system and form a cytoplasmic pool for subsequent incorporation into ER membranes (7). In fact, this mode of synthesis of membrane glycoproteins was proposed by Bosmann et al. (8) during their extensive experimentation with HeLa cell membranes. Recently, Kim and Perdomo (9) also arrived at similar conclusions by studying glycosyl transferases of the subcellular membranes of rat small intestine and also by following the turnover rate of glycoproteins. In agreement with the above conclusions are those of Molnar and Sy (10) as well as those of Hallinan et al. (11).

Testing the hypothesis that supernatant glycoproteins are incorporated into the ER requires experiments with specific glycoproteins which have been purified to a reasonable degree. It can be expected that an integral protein has surface areas which can participate in hydrophobic interactions and that these areas are surrounded by lipids even when the protein is not present in a membrane. A protein with this kind of hydrophobic surface properties is difficult to purify since it easily aggregates, may associate with other proteins with appropriate physical characteristics, and also interacts with column material during chromatography. The possibility that membrane precursors exist in lipoprotein form is suggested by the structure of the high density lipoproteins (HDL) of serum (12) and by recent evidence that serum HDL demonstrates a structure similar to that of the main protein in the erythrocyte membrane (13). Lipoproteins can preferentially be isolated by flotation in salt solution, as is well illustrated in schemes used to purify lipoproteins.

In this paper, we describe the conditions for incorporation of cytoplasmic glycoproteins into microsomal membranes and at the same time present evidence for the integral nature of these proteins. Glycoproteins were also isolated by various chromatographic procedures and also by ultracentrifugation in a salt solution. The partially purified components were characterized. Preliminary reports of this work have appeared earlier (14, 15).

MATERIALS AND METHODS

The treatment of animals, fractionations, incorporation experiments, polyacrylamide gel electrophoresis, and

chemical determinations were as described in the preceding paper (7). The amount of [³H]glucosamine injected into the portal vein to prepare labeled supernate for incubation experiments was 0.5 mCi if not otherwise stated.

Incubation

The standard incubation medium contained Tris-HCl buffer-washed microsomes, 20 mM Tris-HCl buffer, pH 8.0, 65 mM KCl, 0.4 mM CMP, 10 mM ethylenediamine tetraacetate (EDTA), 0.25 M sucrose, and supernatant protein, in a final volume of 11.5 ml. The amount of microsomal and supernatant protein is given in individual experiments.

After incubation at 37°C for 60 min, the suspension was cooled in an ice-water bath, and the microsomes were separated by centrifugation (105,000 g, 60 min) and submitted to the Tris-water-Tris (TWT) washing procedure described previously (16).

Sephadex G-25 Chromatography

In order to remove small labeled components (glucosamine and CMP-NANA [*N*-acetylneuraminic acid]), 25 ml of liver supernate prepared from injected rats was pumped into a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) (3.2 × 70 cm). The Sephadex G-25 was swollen in 10 mM Tris-HCl, pH 8.0, and for elution the same buffer was used. The effluent was passed through a Uvicord (LKB-Beckman Instruments AB, Stockholm), and the absorption at 254 nm was recorded. The pumping speed was 0.5 ml/min, and 7-ml fractions were collected. The protein peak appearing at the void volume was pooled.

Sephadex G-100 Chromatography

20 ml of the pooled fractions from Sephadex G-25 chromatography was pumped onto a column of Sephadex G-100 (3.2 × 90 cm), pre-swollen in 50 mM Tris-HCl, pH 8.0 + 50 mM NaCl. Elution of the sample was performed with the same buffer at a pumping speed of 0.4 ml/min. The effluent was passed through a Uvicord, and absorption at 254 nm was measured. Fractions of 6 ml were collected and analyzed. The protein in the void volume, which contained 30% of the total protein put on the column, was used in further purification experiments.

DEAE-Sephadex Chromatography

The protein peak in the void volume from Sephadex G-100 chromatography was pumped onto a DEAE-Sephadex A-25 column (1 × 15 cm) at a pumping speed of 0.2 ml/min. The ion exchange medium was pre-swollen in 50 mM Tris-HCl, pH 8.0 + 50 mM NaCl, and the same tris buffer was also used to wash through nonbound protein and radioactivity. The bound protein was eluted

with a linear salt gradient ranging from 50 mM to 500 mM NaCl in 50 mM Tris-HCl, pH 8.0, over a period of 24 h. The remaining protein was washed out with 0.8 M NaCl in the same buffer. The pumping speed was 0.1 ml/min, and 2.4-ml fractions were collected.

Centrifugation in KBr

In order to separate the lipoproteins of the supernate, the Sephadex G-25 pool (about 10 mg protein) in 10 mM Tris-HCl, pH 8.0, was supplemented with solid sucrose and solid KBr to give a final concentration of 0.25 M sucrose and 2.18 M KBr and a final volume of 12 ml. The density of this mixture was 1.21. After centrifugation at 152,000 g for 48 h in a 50 Ti rotor on the Spinco-Beckman L2-65B centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), fractions of 2 ml each were removed with a syringe connected to a bent needle, starting from the top. There was always a small pellet, which was resuspended in 10 mM tris buffer, pH 8.0. To remove KBr, each fraction was dialyzed against the same tris buffer.

For isolation of serum, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL, the KBr centrifugation procedure of Lo and Marsh was followed (17).

RESULTS

Incubation of Microsomes with Supernate

Experiments with supernate and microsomes were designed to study the possible incorporation of cytoplasmic glycoproteins into microsomal membranes. Rats were injected with [³H]glucosamine, and the supernatant fraction was prepared from perfused liver. CMP-NANA was present in the supernate from perfused liver; and when total microsomes were incubated with this supernate, protein-bound NANA appeared (Table I). Our total microsomal preparation exhibited CMP-NANA transferase activity, indicating the pres-

TABLE I
Incubation of Microsomes with the Liver Supernatant Fraction from Rats Injected with [³H]Glucosamine

	Microsomes	Additions	cpm in NANA
			20 mg microsomal protein
Supernate	Total	None	413 ± 46
"	"	CMP, 0.4 mM + EDTA, 10 mM	234 ± 20
"	Rough	None	250 ± 19
"	"	CMP, 0.4 mM + EDTA, 10 mM	236 ± 15
Sephadex G-25 pool	Total	None	247 ± 28
" "	Total	CMP, 0.4 mM + EDTA, 10 mM	234 ± 19

Rats were injected with [³H]glucosamine (125 μCi/rat) through the portal vein. After 30 min, 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 on the top (neutral fat) and the last 0.5 ml above the pellet were discarded. The preparation of the Sephadex G-25 pool is described in Materials and Methods. Microsomes (20 mg protein) were incubated with supernate or Sephadex G-25 pool (15 mg protein). The values are the means ± SEM (n = 6).

TABLE II
The Effect of Incubation with Rough Microsomes on the Composition and Radioactivity of the Supernate

	Protein	NANA	cpm in NANA
			μg NANA
	mg	μg	
Before incubation	20.0 ± 1.6	6.8 ± 0.43	2,145 ± 389
After incubation	21.1 ± 1.1	6.9 ± 0.68	1,170 ± 205

Experimental conditions as in Table I. The supernates (prepared by Sephadex G-25 chromatography) were analyzed before and after incubation with nonlabeled microsomes. The values shown are means ± SEM (n = 7).

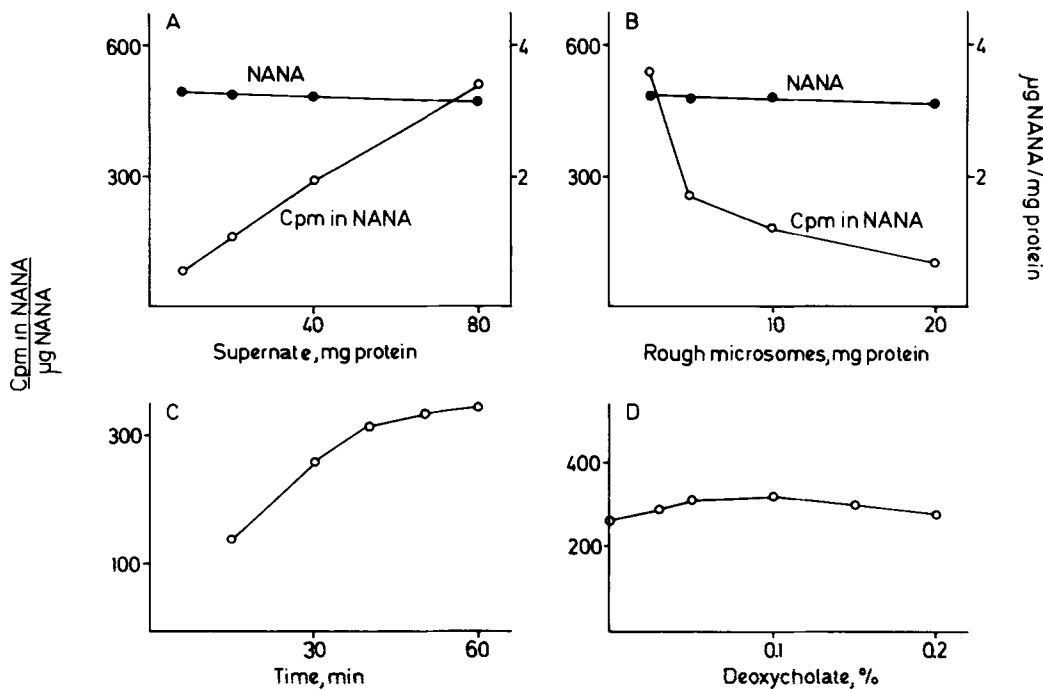


FIGURE 1 Parameters for the incorporation of supernatant glycoproteins into rough microsomes. (A) Fixed amount of rough microsomal protein (5 mg) incubated with increasing amounts of supernatant protein (incubation time 60 min); (B) fixed amount of supernatant protein (40 mg) incubated with increasing amounts of rough microsomes (incubation time 60 min); (C) time course; and (D) effect of DOC. In (A) when the maximum amount of supernate (80 mg) was used 52% of the total protein bound NANA-radioactivity was incorporated into microsomes. In (C and D) 5-mg microsomal protein was incubated with 40 mg supernatant protein. Supernate was subjected to Sephadex G-25 chromatography before use.

ence of Golgi membranes (4). For this reason, the incubation was repeated and CMP + EDTA were added to the incubation medium in a concentration known to inhibit the transferase activity (18). The radioactivity appearing in microsomes was now reduced by 50%. When rough microsomes were incubated with labeled supernate in the absence or presence of CMP and EDTA, the same specific activity of protein-bound NANA was found as in the case when total microsomes were incubated in the presence of transferase inhibitors. Thus, as expected because of the absence of transferase activity, rough microsomal incorporation was not affected by inhibitors. In all the following experiments CMP-NANA was removed completely by Sephadex G-25 chromatography of the supernatant fraction from perfused liver. As shown in Table I the incorporation of radioactivity from the supernate into total microsomes after Sephadex G-25 chromatography was no longer inhibited by CMP and EDTA. Consequently, the results

could not be explained as a simple transfer of sugar, but are interpreted as incorporation of a complete glycoprotein molecule from the supernate into microsomes.

The composition of the supernate before and after incubation with rough microsomes was determined. As shown in Table II, the amount of protein and protein-bound NANA in the supernate was unchanged after incubation, in spite of the fact that protein-bound radioactive NANA disappeared from the supernate and was incorporated into microsomal membranes.²

² In an attempt to modify the soluble protein and the microsomal membrane before incorporation, both supernate and microsomes were treated with trypsin at 4°C overnight. When this hydrolyzed supernate was incubated with nontreated microsomes, sialoprotein incorporation decreased more than 40%. On the other hand, trypsin treatment of the microsomes alone did not influence the incorporation rate. Clearly, it is necessary to have an intact glycoprotein for efficient incorporation.

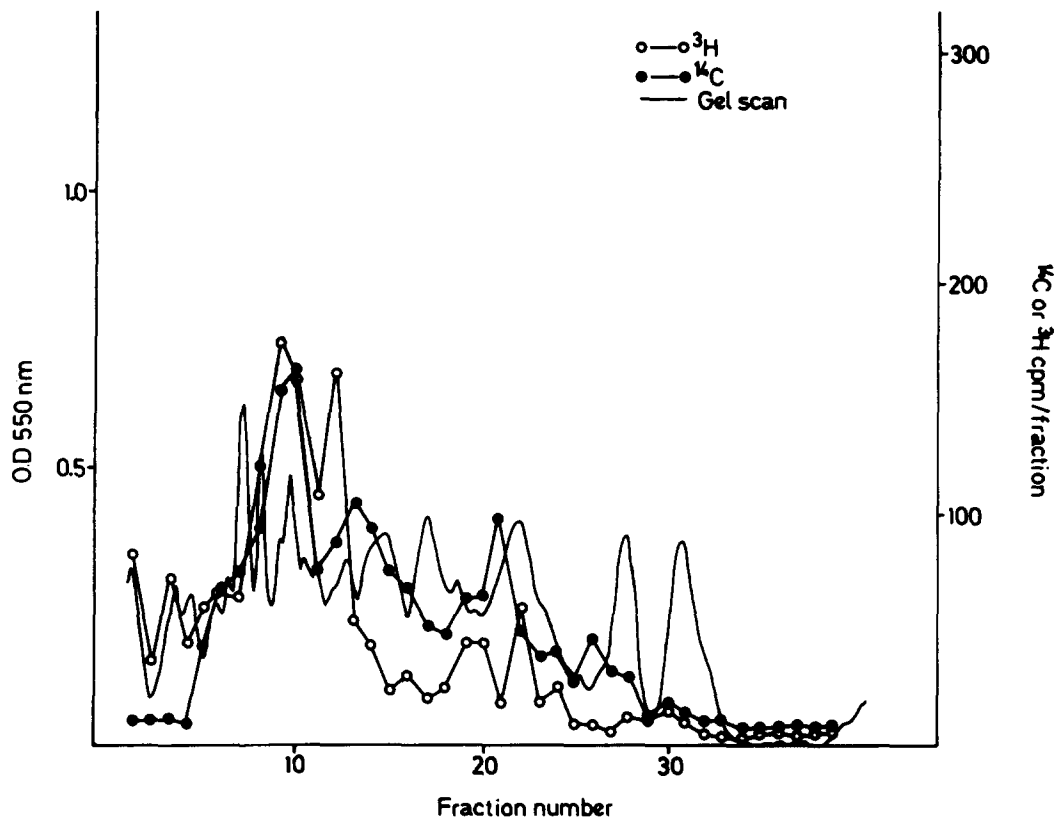


FIGURE 2 SDS-gel electrophoresis of double-labeled supernate. Rats were injected intraperitoneally with 1 mCi [^3H]glucosamine and 250 μCi [^{14}C]leucine, and the liver was perfused 30 min later. The particle-free supernate after centrifugation at 105,000 g for 4 h was subjected to SDS-gel electrophoresis ($\sim 100 \mu\text{g}$ protein/gel).

Some of the parameters for the incorporation of supernatant glycoproteins into rough microsomes were investigated. Fig. 1 shows the content and radioactivity of NANA in the washed microsomal pellet after incubation. The amount of incorporation of protein-bound NANA was dependent on the amount of supernatant protein present during incubation over a wide range (Fig. 1 A). The amount of NANA in the microsomal pellet was not detectably changed regardless of the extent of incorporation. If increasing amounts of microsomes were incubated with a fixed amount of supernatant protein, the incorporation on a protein basis exhibited an appropriate decrease (Fig. 1 B). The incorporation was time-dependent, at least in its early phase (Fig. 1 C), and it was uninfluenced by the presence of detergents such as DOC up to a final concentration of 0.2% (Fig. 1 D).

In order to achieve optimal incorporation, the temperature during incubation was maintained at

37°C in all experiments. The transfer reaction was highly dependent on temperature, decreasing as the temperature was lowered. All incubations were performed in the absence of divalent cations and in the presence of EDTA and CMP.

Besides washing according to Materials and Methods, a number of other washing procedures were tested after completion of incubation. These included washing with high concentrations of monovalent cations (1 M NaCl), which, however, did not change the results.³

³ Both the labeled supernate and microsomes after incubation with labeled supernate were extracted with chloroform-methanol (2:1) and with chloroform-methanol-water (1:1:0.3) in order to remove quantitatively both types of dolichol derivatives participating in glycoprotein synthesis (19). These extractions did not remove any of the radioactivity of the supernate or microsomes, showing that the sugar part of the supernatant sialoproteins is not transferred alone.

Gel Electrophoresis

In order to obtain information about the proteins incorporated into microsomes under *in vitro* conditions, the SDS-gel electrophoretic pattern of both the supernate before and of the microsomal pellet after incubation were studied. Supernate was prepared by Sephadex G-25 chromatography from rats injected with [^3H]glucosamine and [^{14}C]leucine (Fig. 2). A large variety of protein species in the mol wt range of 20,000 and 100,000 were seen. A number of proteins contained [^3H]glucosamine and [^{14}C]leucine, the highest amount of both labels was in fraction 9, corresponding to the 70,000 mol wt region.

After incubation of the double-labeled supernate with rough microsomes for 60 min, a number of labeled proteins and glycoproteins appeared to be incorporated (Fig. 3). There were four major bands with high [^3H]glucosamine activity, namely those in fractions 5, 9, 12, and 24; all four corresponded

to bands present in the supernate before incubation. Furthermore, there was a transfer of a few proteins containing low ratios of [^3H]glucosamine to [^{14}C]leucine from the supernate to the microsomes, but the nature of these peptides was not investigated further.

DOC Treatment

Deoxycholate (DOC) was used previously in the study of the interaction between glycoproteins and the microsomal membrane after *in vivo* labeling (7); similar investigations were now performed after *in vitro* labeling of microsomes. Increasing concentrations of DOC liberated sialoproteins from rough microsomes in a gradual manner; as with microsomes from rats injected with radioactive precursor, even the highest DOC concentration (0.4%) did not completely solubilize the incorporated sialoprotein (Fig. 4). This finding constitutes evidence that the glycoprotein incorpo-

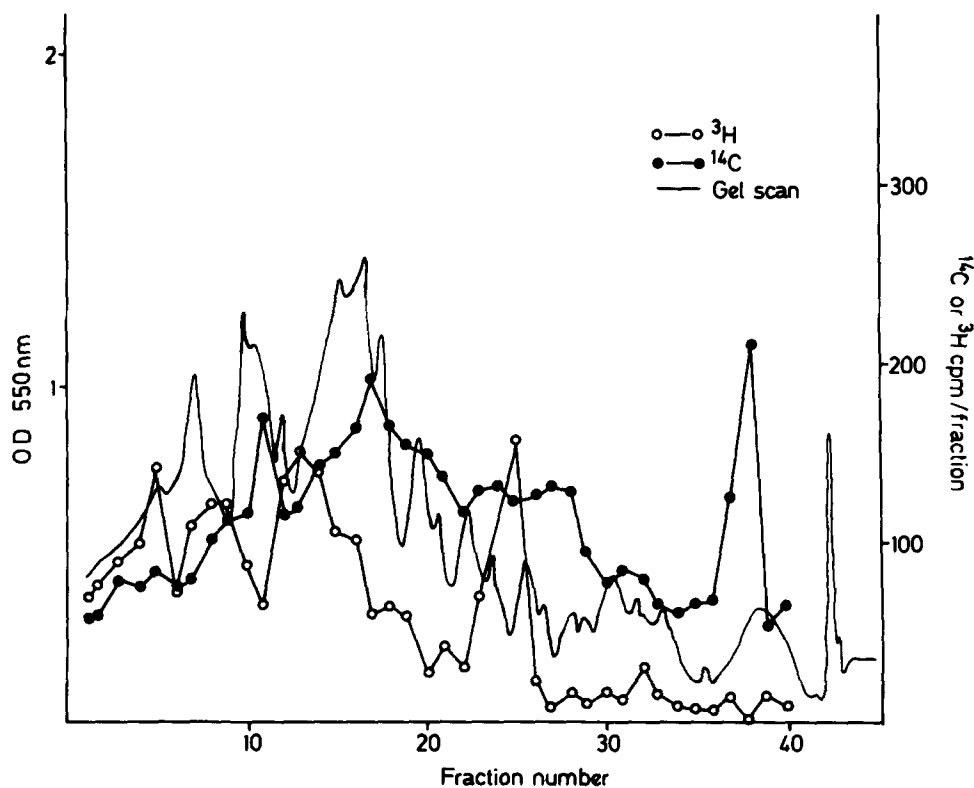


FIGURE 3 SDS-gel electrophoresis of rough microsomes after incubation with double-labeled supernate. Double-labeled supernate was prepared as described in the legend to Fig. 2 and incubated with rough microsomes (80 mg supernate and 10 mg microsomal protein). After incubation, microsomes were washed and subjected to SDS-gel electrophoresis ($\sim 250 \mu\text{g}$ protein/gel).

rated *in vitro* is an integral membrane constituent.

The protein pattern of *in vitro* labeled rough microsomal membranes after treatment with 0.3% DOC was analyzed by performing SDS-gel electrophoresis both on the pellet and on the soluble fraction (Fig. 5 A and B). A concentration of DOC that removed about 50% of the microsomal phospholipid (PLP) did not release several glycoproteins, particularly in the high mol wt region (between about 50,000–80,000 daltons). The soluble fraction demonstrated three peaks, with the major peak in fraction 15. This fraction was highly labeled with both [³H]glucosamine and [¹⁴C]leucine. For comparison, rough microsomes isolated after *in vivo* labeling were also treated with the same DOC concentration (0.3%); both the soluble and insoluble fractions were examined by SDS-gel electrophoresis (Fig. 5 C and D). Again, the glycoproteins which were not solubilized were concentrated in the high mol wt region. The gel pattern of the solubilized proteins demonstrated several distinct peaks, the major one again appearing in fraction 15. This peak not only contained a relatively large amount of protein, but also relatively large amounts of both labels.

Purification of the Supernatant Glycoproteins

The experiments described above demonstrate that sialoproteins can be incorporated into microsomal membranes *in vitro* and are also present in microsomes after *in vivo* labeling. These findings could be further substantiated by isolating a specific glycoprotein component from the cytoplasm and incorporating it into microsomes.

The supernate obtained after *in vivo* labeling was chromatographed on a Sephadex G-25 column, and the proteins in the void volume were chromatographed on a Sephadex G-100 column. When the fractions from Sephadex G-100 chromatography were analyzed, only proteins in the void volume could be incorporated into microsomes. The protein in the void volume was chromatographed on a DEAE-Sephadex column using an NaCl gradient. Radioactivity peaks were present around fraction 20 and two further peaks were found in the eluate at high NaCl concentration (Fig. 6). The peak of radioactivity around fraction 20 was not coincident with the main protein peak.

The combined Sephadex G-100 and DEAE-

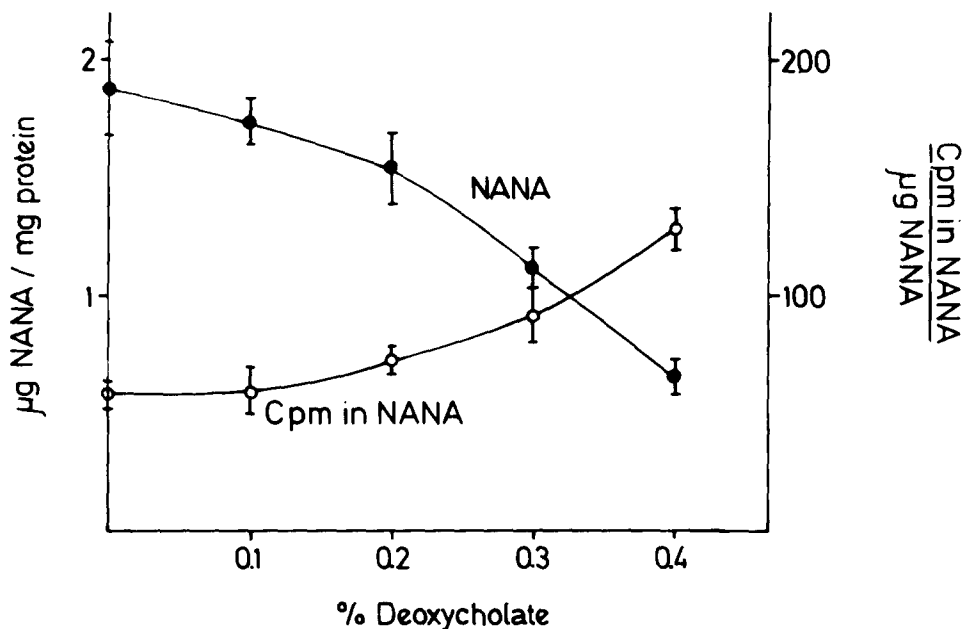


FIGURE 4 Effect of DOC on the release of sialoproteins from rough microsomes incubated *in vitro* with labeled supernate. 45 mg protein of supernate (from rats injected with 0.5 mCi [³H]glucosamine 30 min before decapitation) and 10 mg microsomal protein were incubated under the standard conditions. After incubation, the microsomes (1 mg protein/ml) were washed and treated with increasing amounts of DOC. After centrifugation, the pellet was analyzed for NANA and counts in NANA.

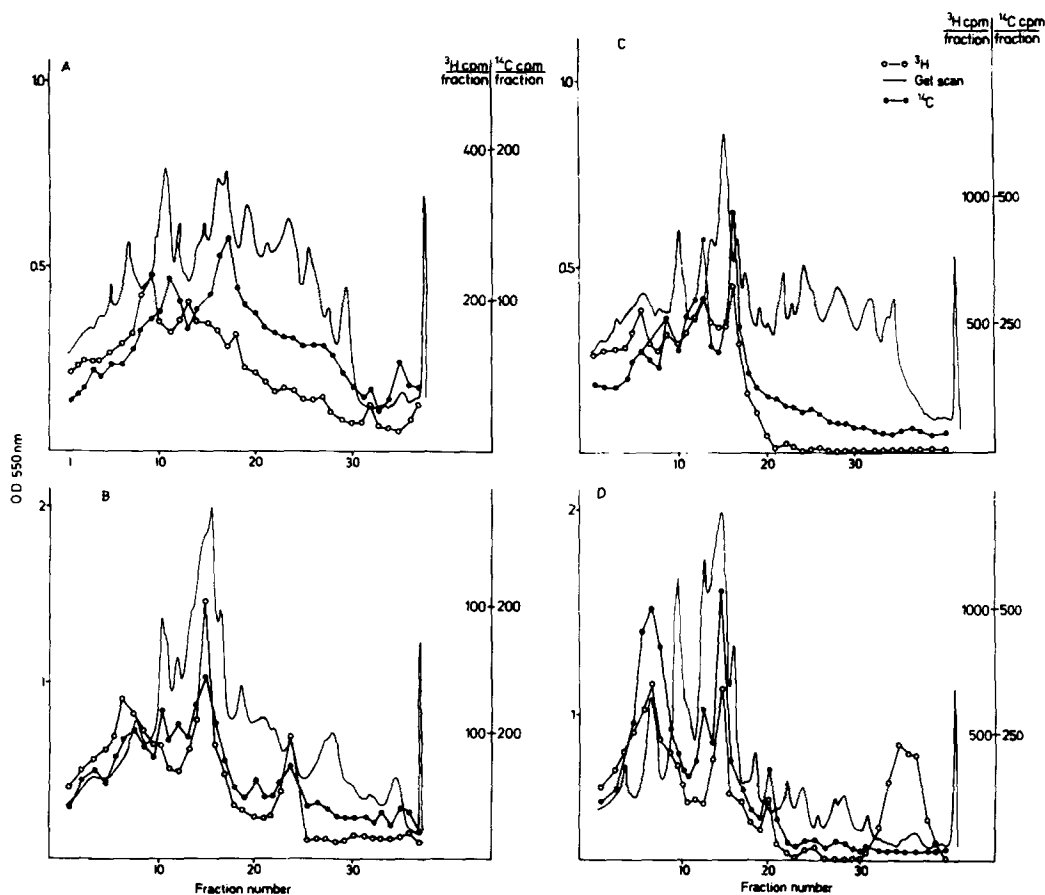


FIGURE 5 SDS-gel electrophoresis of double-labeled rough microsomes after treatment with 0.3% DOC (A and B) Double-labeled supernate was prepared as described in Materials and Methods and used for incubation with rough microsomes (45 mg supernate and 10 mg microsomal protein). After incubation (37°C, 60 min), the microsomes (1 mg protein/ml) were washed and treated with 0.3% DOC. 200 μ g protein was used in the SDS-gel electrophoretic system. A = pellet, B = supernate of the DOC-treated microsomes. In (C and D) rats were injected with 0.2 mCi [3 H]glucosamine and 80 μ Ci [14 C]leucine in the portal vein, and livers were perfused 30 min later. The rough microsomes (1 mg protein/ml) were treated as in (A and B). C = pellet, D = supernate of the DOC-treated microsomes.

Sephadex procedure was repeated with double-labeled supernate and the preparation obtained was incubated with microsomes. The heterogeneous composition of the pooled fractions is apparent from the analysis of the incorporation data (Table III). The total radioactivity incorporated varied between 2,000 and 15,000 counts in the case of [3 H]glucosamine, while [14 C]leucine exhibited a much more even incorporation. Pool I displayed a unique feature: more than 50% of both labels were transferred to microsomes upon incubation for 60 min. The other pools showed significantly less and unequal incorporation of the two labels into mi-

croosomes, thereby indicating the presence of several protein and glycoprotein components that were not incorporated.

Centrifugation in KBr Solution

The possibility that the specific glycoproteins being incorporated are associated with lipids was tested by flotation of the Sephadex G-25 pool in KBr solution. The system which proved to be most efficient for our purpose was the same as that generally used for the isolation of high density serum lipoproteins, i.e., suspension of the protein in KBr at a density of 1.21 and flotation by

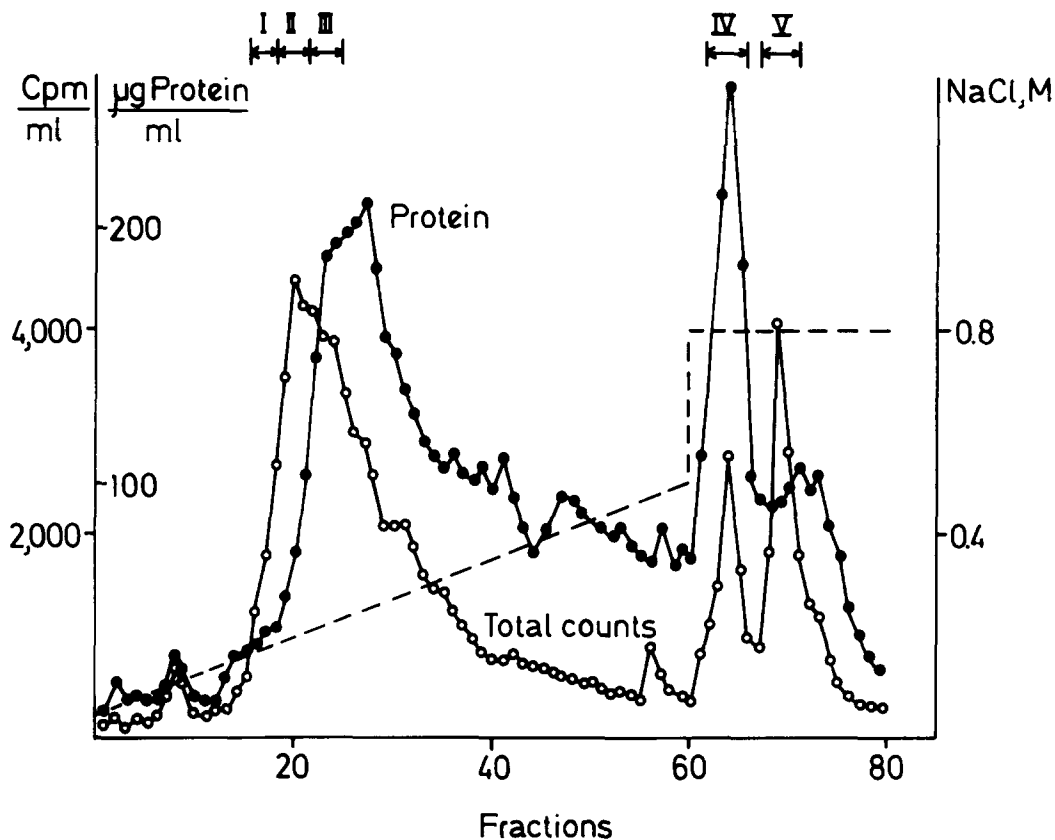


FIGURE 6 DEAE-Sephadex chromatography of proteins in the void volume after chromatography on Sephadex G-100. For preparation of supernate 200 μ Ci glucosamine was injected into the portal vein, the liver was perfused 30 min later, and the supernate was prepared. The supernate was first subjected to Sephadex G-25 chromatography followed by chromatography of the protein pool on Sephadex G-100 as described in Materials and Methods. The proteins in the void volume (fractions 45-55) were fractionated on a DEAE-Sephadex column. The following fractions were pooled: 16-18 (I); 19-21 (II); 22-24 (III); 61-65 (IV); and 67-71 (V).

prolonged centrifugation (48 h). About 2% of the total protein was present in layer 1, which contained 6% of the total incorporable [3 H]glucosamine (Table IV) and displayed a specific activity of incorporation exceeding that of the other fractions three- to fivefold.⁴ The large majority of the protein sedimented to the lower part of the tube. As regards the incorporation of [14 C]leucine, there were only small variations among the various fractions.

The gel electrophoretic pattern of the total protein suspension before centrifugation in KBr

⁴ The chemical composition of layer 1 was also studied. It contained both neutral and amino sugars as well as sialic acid and PLP, but the individual phosphatides were not isolated.

solution has its main peak of radioactivity associated with fraction 9 (Fig. 2). The gel electrophoretic pattern of layer 1 after KBr centrifugation showed two protein peaks, one which corresponded to fraction 9 and another near the end of the gel (Fig. 7 A). It is possible that the high mol wt peak of layer 1 is identical with the major labeled peak of the Sephadex G-25 pool (see Fig. 2), which also corresponded to fraction 9. The other layers had a more complex composition.

Since the supernatant glycolipoproteins exhibited flotation properties similar to those of the serum lipoproteins, KBr flotations were performed with serum. In this way direct comparison could be carried out in order to exclude the presence of any serum lipoproteins in the top layer after KBr flotation of the supernate. In agreement with

TABLE III
Incubation of the Double-Labeled Fractions from DEAE-Sephadex Chromatography with Total Microsomes

	Total radioactivity in the incubation mixture		Total radioactivity in microsomes		Radioactivity transfer	
	[³ H]GIN*	[¹⁴ C]Leu	[³ H]GIN	[¹⁴ C]Leu	[³ H]GIN	[¹⁴ C]Leu
	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	%	%
Pool I	8,590	1,480	5,036	793	59 ± 7.3	54 ± 6.5
“ II	14,650	2,520	5,421	426	37 ± 3.7	17 ± 1.8
“ III	6,850	2,850	797	484	12 ± 1.6	17 ± 1.4
“ IV	1,645	1,032	171	209	10 ± 0.82	20 ± 3.0
“ V	7,843	762	2,542	166	33 ± 2.5	22 ± 2.9

The following fractions were pooled from the DEAE-Sephadex chromatography of the protein in the Sephadex G-100 void volume (Fig. 6): Fractions 16-18 (I); 19-21 (II); 22-24 (III); 61-65 (IV); 67-71 (V). The incubation mixture is described in Materials and Methods. The microsomal and supernatant proteins were 6 mg and 0.3 mg, respectively, per 11.5 ml. Each value gives the mean of four experiments. The maximal deviation from the mean value of the percent transfer is given.

* GIN represents glucosamine.

TABLE IV
Distribution of Protein and Radioactivity after Centrifugation of Supernate in KBr Solution

	Protein	[³ H]GIN	[¹⁴ C]Leu	[³ H]GIN	[¹⁴ C]Leu
	<i>mg/total</i>	<i>cpm/total</i>	<i>cpm/total</i>	<i>cpm/mg protein</i>	<i>cpm/mg protein</i>
Total	33.0	211,134	254,199	6,398	7,703
Layer 1	0.55	12,567	4,705	22,850	8,554
“ 2	0.72	5,056	3,025	7,023	4,202
“ 3	1.07	4,542	5,724	4,245	5,350
“ 4	1.84	7,509	11,183	4,081	6,078
“ 5	4.03	17,897	33,686	4,441	8,359
“ 6	20.84	127,332	170,033	6,110	8,159
Pellet	0.95	16,193	8,717	17,046	9,176
Recovery	30.0 (91%)	191,096 (90.5%)	237,073 (93.3%)	—	—

The Sephadex G-25 pool was adjusted to 0.25 M sucrose and 2.18 M KBr and 12-ml tubes in the 50 Ti rotor were centrifuged at 152,000 *g* for 48 h. 2-ml layers were collected by suction, each pellet was suspended in 2 ml water and the corresponding fractions from different tubes were mixed. The samples were dialyzed overnight against Tris-HCl, 10 mM, pH 8.0 + 50 mM KCl. Each value represents the mean of six experiments.

previous data, serum VLDL, LDL and HDL displayed several components upon SDS-gel electrophoresis (20). It is evident that the SDS-gel electrophoresis pattern of the supernatant lipoprotein was very different from those of serum VLDL, LDL and HDL (Fig. 7).

An aliquot corresponding to 0.5 mg double-labeled protein from each layer after KBr fractionation was incubated with rough microsomes (2 mg protein), and the amounts of protein-bound [³H]glucosamine and [¹⁴C]leucine transferred to microsomes were determined (Table V). Highest

incorporation was achieved with layer 1, where the percent transfer of incorporated glucosamine was three to four times higher than that of the other layers.⁵ Interestingly, the percentage of the protein

⁵ Layer 1 was also incubated with isolated liver mitochondria. Under the same conditions the transfer of protein-bound glucosamine radioactivity to mitochondria was much less, i.e., only 40% that with liver microsomes. The specific transfer to the mitochondria was even less when the microsomal contamination in our mitochondrial fraction (about 7%) is taken into consideration.

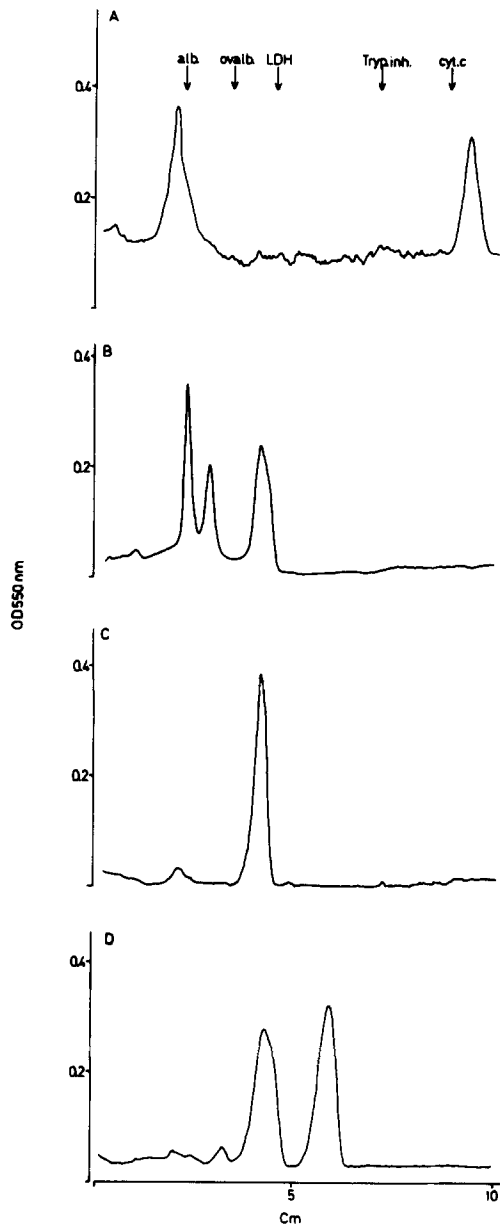


FIGURE 7 SDS-gel electrophoresis of supernate and serum lipoproteins. (A) Supernatant lipoprotein; (B) very low density (VLDL); (C) low density (LDL); and (D) high density lipoproteins (HDL) of the serum. All four lipoproteins were prepared by flotation on KBr solutions, as described in Materials and Methods. The arrows in Fig. 7A 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).

label incorporated was only half that of the sugar label.

The gel electrophoretic picture of rough microsomes after incubation with layer 1 from KBr flotation is of special interest. Fraction 9 contained not only the highest protein peak, but also the protein peak associated with the highest incorporation of [^3H]glucosamine and [^{14}C]leucine, suggesting that the whole glycoprotein unit is incorporated (Fig. 8). Additional minor peaks containing both labels were present in the lower molecular weight regions of the gel; and at the lower end of the gel (in fraction 35), sugar labeling without protein labeling could be seen. This labeling was probably in a glycolipid.

The gel electrophoretic pattern in Fig. 7 demonstrates that VLDL, LDL, and HDL have electrophoretic mobilities different than that of the supernatant lipoprotein (LP) and, consequently, that they are not the same proteins. As an additional control, double-labeled serum lipoproteins (HDL and LDL) and supernatant LP were prepared by gel filtration and centrifugation in the KBr systems, as described in Materials and Methods. In agreement with previous experiments, 26% and 15% of the total [^3H]glucosamine and [^{14}C]leucine, respectively, of the top layer of the floated supernate were incorporated (Table VI). The incorporation obtained with high and low density serum lipoproteins was almost insignificant, in spite of the high specific labeling of both protein and sugar components in these proteins.

DISCUSSION

The experiments described here indicate that certain cytoplasmic glycoproteins are incorporated into microsomal membranes upon *in vitro* incubation, thus suggesting a possible mechanism of renewal of at least some of the membrane proteins.

The hypothesis that cytoplasmic glycoproteins are incorporated into microsomal membranes seem to be a reasonable one, since the synthesis of these macromolecules is carried out by several different intracellular structures. The first step is the synthesis of the polypeptide on bound ribosomes, which is followed by the gradual completion of the oligosaccharide chain during transport from rough to smooth ER and to the Golgi system. It is obvious that much evidence is required to demonstrate that the *in vitro* incorporation of cytoplasmic proteins into microsomes is not due to

TABLE V
Incorporation of Glycoproteins in the Layers Obtained by KBr Flotation into Rough Microsomes

	Total radioactivity in the incubation mixture		Total radioactivity in microsomes		Radioactivity transfer	
	[³ H]GIN	[¹⁴ C]Leu	[³ H]GIN	[¹⁴ C]Leu	[³ H]GIN	[¹⁴ C]Leu
	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	%	%
Total	4,478	5,392	764	616	17.1 ± 1.8	11.4 ± 0.9
Layer 1	11,425	4,277	4,650	840	40.7 ± 5.2	19.6 ± 1.6
“ 2	3,511	2,101	446	86	12.7 ± 2.3	4.1 ± 0.71
“ 3	2,122	2,675	282	168	13.2 ± 1.1	6.3 ± 0.65
“ 4	2,040	3,039	270	284	13.2 ± 1.5	9.3 ± 1.12
“ 5	2,205	4,179	208	424	9.4 ± 1.5	10.1 ± 1.6
“ 6	3,055	4,079	268	456	8.8 ± 1.7	11.2 ± 1.3

The Sephadex G-25 pool was processed as described in the legend to Table IV. The 5-ml incubation mixture contained 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.4 mM CMP, 150 mM KCl, 2 mg microsomal protein and 0.5 mg protein for layers 1-6 or 0.7 mg protein for Total. After incubation at 37°C for 60 min, the mixtures were centrifuged at 105,000 *g* for 60 min, and the pellets were washed with Tris-HCl buffer, 0.15 M, pH 8.0, by centrifugation. Aliquots of the layers before and after incubation with the microsomes were used for determination of radioactivity. Each value represents the means of six experiments. The maximal deviation from the mean value of the percent transfer is given.

some unspecific phenomenon such as adsorption. A variety of experimental data argue against this possibility. The microsomal pellets after incubation were carefully washed in our experiments using a procedure known to remove adsorbed proteins (16). When an additional wash with a medium of high ionic strength was used, the incorporated protein still could not be removed. Incorporation was dependent on time, concentration, and temperature, characteristics of an active process. Similar to the findings from *in vivo* studies, protein-bound radioactivity incorporated *in vitro* could be only partially removed using a low concentration of DOC. Also, the electrophoretic pattern of the microsomal membranes after *in vitro* incorporation followed by treatment with 0.3% DOC was similar to that of membranes labeled *in vivo* and treated with the same detergent concentration.

Since several proteins of the cytoplasm are incorporated, it is important to purify them in order to demonstrate the specificity of the reaction. Two of the isolation procedures employed were successful, DEAE-Sephadex chromatography of the protein peak in the void volume after chromatography on Sephadex G-100 and centrifugation in KBr solutions. The presence of serum lipoproteins both in the lumen of the ER and in

serum raised the possibility that in spite of the effective perfusion a small amount of serum lipoprotein still might be present in the supernate. A comparison of the supernatant LP with those of the serum by gel electrophoresis argued against this possibility. The gel patterns of all the serum lipoproteins (VLDL, LDL, HDL) were very characteristic and very unlike that of supernatant LP. Our experiments in this respect are in complete agreement with the data in the literature (20). Incorporation experiments gave further evidence for this difference, since serum lipoproteins were incorporated very inefficiently. Immunological tests with antibodies against total rat serum protein revealed little or no contamination of supernate with serum proteins (7). However, the two-step antibody precipitation procedure does not provide absolute evidence for the complete absence of all serum lipoproteins in the supernate.

The specificity of the incorporation is further demonstrated by the results of the double-labeling experiments, which showed simultaneous incorporation of both protein and sugar moieties of the lipoprotein. Equal effectiveness with rough microsomes or in the presence of inhibitors of CMP-sialic acid transferase excluded the possibility that the incorporation studied involved only the terminal sugar moiety.

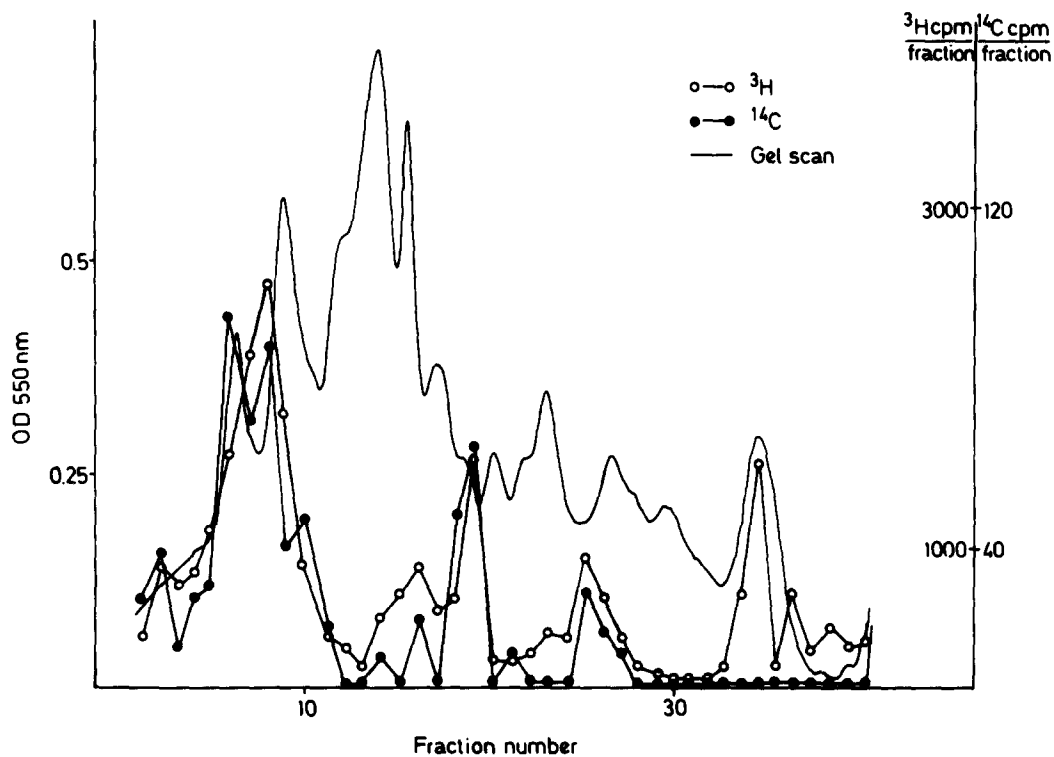


FIGURE 8 Pattern obtained by SDS-gel electrophoresis of rough microsomes after *in vitro* incubation with double-labeled layer from KBr flotation. Double-labeled supernate was prepared as described in Fig. 5, and after centrifugation in KBr solution the top layer was used for incubation with rough microsomes as in Table V. 250 μ g protein was applied to the gel.

TABLE VI
Incorporation of Various Lipoproteins into Rough Microsomes

Exp	Lipoprotein	Radioactivity in LP		Radioactivity transferred to microsomes			
		[³ H]GIN	[¹⁴ C]Leu	[³ H]GIN	%		[¹⁴ C]Leu
		<i>cpm/mg protein</i>		<i>cpm/mg protein</i>			<i>cpm/mg protein</i>
1	LP from supernatant fraction	29,850	5,220	7,820	26.2 ± 3.1		809
2	HDL from serum	18,820	9,600	414	2.2 ± 2.9		163
3	LDL from serum	102,444	14,440	921	0.9 ± 0.11		158

Rats were injected with [³H]glucosamine (125 μ Ci/rat) and with [¹⁴C]leucine (80 μ Ci/rat). In the case of supernatant LP the liver was perfused 30 min later and in the case of serum lipoproteins the rats were decapitated 90 min later. The incubation mixture contained 1 mg of lipoprotein + 1 mg microsomal protein in 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 0.15 M KCl, 10 mM EDTA, and 0.4 mM CMP. Incubation was carried out at 37°C for 60 min. After incubation, the fractions were washed with 0.15 M Tris-HCl, pH 8.0, and the pellets were dissolved in 0.3 ml of 2% sodium dodecyl sulfate and burned in a Packard oxidizer (Packard Instrument Co., Inc., Downers Grove, Ill.) before counting. Each value gives the mean of five experiments. The maximal deviation from the mean value of the percent transfer is given.

An important feature of the process studied here is that it is probably an exchange type of reaction. Analyses of the supernate and the microsomes before and after incubation did not show any change in the amount of protein and NANA of these fractions even when a large part of the radioactivity in NANA was incorporated. This finding fits well with the concept of a highly dynamic membrane whose components are being continuously renewed (21). It is well established that the phospholipids of intracellular membranes are also renewed by an exchange type of reaction without change in the qualitative or quantitative lipid composition of the membrane (22). In this case, the exchange is mediated by protein carriers.

An interesting aspect of the incorporation process studied here became apparent upon centrifugation in KBr solutions. The most active glycoprotein unit was found in the top layer, indicating the presence of protein-associated lipids, an indication supported by subsequent analysis. Phospholipids in micellar form may assemble several protein molecules into one complex.

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