BIOGENESIS OF MICROSOMAL MEMBRANE GLYCOPROTEINS
IN RAT LIVER

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

FRANCESCO AUTUORI, HANS SVENSSON, and GUSTAV DALLNER

From the Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, and the
Department of Pathology at Sabbatsberg Hospital, Karolinska Institutet, S-104 05 Stockholm, Sweden

ABSTRACT

Sialoproteins isolated from the soluble fraction of rat liver could be incorporated
into microsomal membranes. This incorporation was dependent on protein con-
centration, time, and temperature. Sodium dodecyl sulfate gel electrophoresis
of membrane proteins after in vitro incorporation showed four major sugar-con-
taining 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 cyto-
solic glycoproteins that could be incorporated into microsomes. During pro-
longed centrifugation in a KBr solution with a density of 1.21 a highly labeled
([3H]glucosamine) protein (mol wt ~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 micro-
somal 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). Micro-
somal 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 re-
newal and insertion of new microsomal membrane
glycoproteins. Chemically, microsomal protein-
bound oligosaccharides consist of mannose, galac-
tose, 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 bio-
synthetic 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 al-
bumin, serum glycoproteins, various types of lipo-
proteins, and cholesterol, all of which are synthe-

1 Abbreviations used in this paper: DOC, deoxycholate;
EDTA, ethylenediamine tetraacetate; ER, endoplasmic
reticulum; GIN, glucosamine; HDL, high density lipo-
protein; 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 is suggested by the demonstration of a structure similar to that of the serum (12) and by recent evidence that serum HDL charged from the Golgi system and form a cytosolic pool for subsequent incorporation into ER membranes (7).

4. 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.

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>
<thead>
<tr>
<th>Table I</th>
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<tbody>
<tr>
<td><strong>Incubation of Microsomes with the Liver Supernatant Fraction from Rats Injected with [3H]Glucosamine</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Additions</th>
<th>cpm in NANA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernate</td>
<td>Total</td>
<td>None</td>
</tr>
<tr>
<td>“”</td>
<td>“”</td>
<td>CMP, 0.4 mM + EDTA, 10 mM</td>
</tr>
<tr>
<td>Rough</td>
<td>None</td>
<td>250 ± 19</td>
</tr>
<tr>
<td>Sephadex G-25 pool</td>
<td>Total</td>
<td>None</td>
</tr>
<tr>
<td>“”</td>
<td>CMP, 0.4 mM + EDTA, 10 mM</td>
<td>234 ± 19</td>
</tr>
</tbody>
</table>

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 microosomal supernatant 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>
<thead>
<tr>
<th>Table II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>The Effect of Incubation with Rough Microsomes on the Composition and Radioactivity of the Supernate</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>NANA</th>
<th>mg</th>
<th>µg NANA</th>
<th>µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation</td>
<td>20.0 ± 1.6</td>
<td>6.8 ± 0.43</td>
<td>2,145 ± 389</td>
<td></td>
</tr>
<tr>
<td>After incubation</td>
<td>21.1 ± 1.1</td>
<td>6.9 ± 0.68</td>
<td>1,170 ± 205</td>
<td></td>
</tr>
</tbody>
</table>

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).
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

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.
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.\(^6\)

\(^6\) 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.

**FIGURE 2** SDS-gel electrophoresis of double-labeled supernate. Rats were injected intraportally with 1 mCi [\(\text{H}\)]glucosamine and 250 \(\mu\)Ci [\(\text{C}\)]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 (∼100 \(\mu\)g protein/gel).
**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 \[^{3}H\]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 \[^{3}H\]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 \[^{3}H\]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 \[^{3}H\]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-
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-
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 \[3H\]glucosamine, while \[14C\]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 microsomes, 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
prolonged centrifugation (48 h). About 2% of the total protein was present in layer I, which contained 6% of the total incorporable \([^1\text{H}]\text{glucosamine}\) (Table IV) and displayed a specific activity of incorporation exceeding that of the other fractions three- to fivefold. The large majority of the protein sedimented to the lower part of the tube. As regards the incorporation of \([^{14}\text{C}]\text{leucine}\), there were only small variations among the various fractions.

The gel electrophoretic pattern of the total protein suspension before centrifugation in KBr solution has its main peak of radioactivity associated with fraction 9 (Fig. 2). The gel electrophoretic pattern of layer I 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**

<table>
<thead>
<tr>
<th>Pool</th>
<th>Total radioactivity in the incubation mixture</th>
<th>Total radioactivity in microsomes</th>
<th>Radioactivity transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cpm</td>
<td>[(^3)H]GIN</td>
<td>[(^{14})C]Leu</td>
</tr>
<tr>
<td>Pool I</td>
<td>8,590</td>
<td>5,036</td>
<td>793</td>
</tr>
<tr>
<td>&quot; II</td>
<td>14,650</td>
<td>5,421</td>
<td>426</td>
</tr>
<tr>
<td>&quot; III</td>
<td>6,850</td>
<td>797</td>
<td>484</td>
</tr>
<tr>
<td>&quot; IV</td>
<td>1,645</td>
<td>171</td>
<td>209</td>
</tr>
<tr>
<td>&quot; V</td>
<td>7,843</td>
<td>2,542</td>
<td>166</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>Layer</th>
<th>Protein mg</th>
<th>[(^3)H]GIN</th>
<th>[(^{14})C]Leu</th>
<th>[(^3)H]GIN</th>
<th>[(^{14})C]Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>cpm</td>
<td>cpm</td>
<td>%</td>
<td>cpm</td>
</tr>
<tr>
<td>Total</td>
<td>33.0</td>
<td>211,134</td>
<td>254,199</td>
<td>6,398</td>
<td>7,703</td>
</tr>
<tr>
<td>Layer 1</td>
<td>0.55</td>
<td>12,567</td>
<td>4,705</td>
<td>22,850</td>
<td>8,554</td>
</tr>
<tr>
<td>&quot; 2</td>
<td>0.72</td>
<td>5,056</td>
<td>3,025</td>
<td>7,023</td>
<td>4,202</td>
</tr>
<tr>
<td>&quot; 3</td>
<td>1.07</td>
<td>4,542</td>
<td>5,724</td>
<td>4,245</td>
<td>5,350</td>
</tr>
<tr>
<td>&quot; 4</td>
<td>1.84</td>
<td>7,509</td>
<td>11,183</td>
<td>4,081</td>
<td>6,078</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>4.03</td>
<td>17,897</td>
<td>33,686</td>
<td>4,441</td>
<td>8,359</td>
</tr>
<tr>
<td>&quot; 6</td>
<td>20.84</td>
<td>127,332</td>
<td>170,033</td>
<td>6,110</td>
<td>8,159</td>
</tr>
<tr>
<td>Pellet</td>
<td>0.95</td>
<td>16,193</td>
<td>8,717</td>
<td>17,048</td>
<td>9,176</td>
</tr>
</tbody>
</table>

Recovery

<table>
<thead>
<tr>
<th>Layer</th>
<th>Protein mg</th>
<th>[(^3)H]GIN</th>
<th>[(^{14})C]Leu</th>
</tr>
</thead>
</table>
|       | total | cpm | cpm |%
| Total | 30.0 | 191,096 | 237,073 | (91%) (90.5%) (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 [\(^3\)H]glucosamine and [\(^{14}\)C]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. Interestingly, the percentage of the protein a Layer I 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 supernatant 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 [H]glucosamine and [14C]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 [14C]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

<table>
<thead>
<tr>
<th></th>
<th>Total radioactivity in the incubation mixture</th>
<th>Total radioactivity in microsomes</th>
<th>Radioactivity transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]GIN  cpm</td>
<td>[14C]Leu  cpm</td>
<td>[3H]GIN  cpm</td>
</tr>
<tr>
<td>Total</td>
<td>4,478</td>
<td>5,392</td>
<td>764</td>
</tr>
<tr>
<td>Layer 1</td>
<td>11,425</td>
<td>4,277</td>
<td>4,650</td>
</tr>
<tr>
<td>&quot; 2</td>
<td>3,511</td>
<td>2,101</td>
<td>446</td>
</tr>
<tr>
<td>&quot; 3</td>
<td>2,122</td>
<td>2,675</td>
<td>282</td>
</tr>
<tr>
<td>&quot; 4</td>
<td>2,040</td>
<td>3,039</td>
<td>270</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>2,205</td>
<td>4,179</td>
<td>208</td>
</tr>
<tr>
<td>&quot; 6</td>
<td>3,055</td>
<td>4,079</td>
<td>268</td>
</tr>
</tbody>
</table>

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 supernatate. 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 supernatate 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 supernatate.

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 μg protein was applied to the gel.

<table>
<thead>
<tr>
<th>Exp</th>
<th>Lipoprotein</th>
<th>Radioactivity in LP [^3H]GIN (cpm/mg protein)</th>
<th>Radioactivity transferred to microsomes [[^14C]Leu (cpm/mg protein)]</th>
<th>%</th>
<th>Radioactivity transferred to microsomes [[^3H]GIN (cpm/mg protein)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LP from supernatant fraction</td>
<td>29,850</td>
<td>7,820</td>
<td>26.2 ± 3.1</td>
<td>809</td>
</tr>
<tr>
<td>2</td>
<td>HDL from serum</td>
<td>18,820</td>
<td>9,600</td>
<td>2.2 ± 2.9</td>
<td>163</td>
</tr>
<tr>
<td>3</td>
<td>LDL from serum</td>
<td>102,444</td>
<td>14,440</td>
<td>0.9 ± 0.11</td>
<td>158</td>
</tr>
</tbody>
</table>

Rats were injected with[^3H]glucosamine (125 μCi/rat) and with[^14C]leucine (80 μCi/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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