Biochimica et Biophysica Acta, 455 (1976) 383–398

BBA 77519

BIOGENESIS OF MICROSOMAL MEMBRANE GLYCOPROTEINS IN RAT LIVER

IV. CHARACTERISTICS OF A CYTOPLASMIC LIPOPROTEIN HAVING PROPERTIES OF A MEMBRANE PRECURSOR

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(Received February 5th, 1976)
(Revised manuscript received June 30th, 1976)

SUMMARY

The supernatant fraction from a rat liver homogenate contains a lipoprotein complex which can be incorporated into microsomal membranes in vitro. The lipoprotein can be purified by gel filtration and flotation in a NaBr solution. The isolated lipoprotein has an equilibrium density in the range of 1.07–1.14 g/ml, a sedimentation coefficient of 4.9 S and a calculated molecular weight of 210 000. The isolated complex contains 44 % lipid by weight, half of which is phospholipid. The protein also has covalently bound sugar moieties, namely mannose, galactose, glucosamine, and sialic acid. The complex is unstable and may dissociate during the isolation procedure. The complex and its fragments contain two peptides with molecular weights of 11–13000 and 67–69000. These two components have practically identical amino acid compositions.

INTRODUCTION

Biosynthesis of the membrane of the endoplasmic reticulum has been investigated to only a very limited extent. Bound ribosomes of the rough membranes are involved in the synthesis of a large amount of secretory proteins and probably also some membrane proteins [1]. The concept that free ribosomes play the major role in synthesis of the cell's own proteins has been proposed previously [2]. Available reports indicate that in hepatocytes and other cells proteins synthesized on free ribosomes are later transferred to the endoplasmic reticulum and to other cellular membranes [3–5].

The membrane glycoproteins of the endoplasmic reticulum represent a special biosynthetic problem since the terminal sugars of the protein-bound oligosaccharide are added in the Golgi system [6]. Consequently, either the complete glycoprotein molecule or the oligosaccharide chain has to be transported from the Golgi complex

back to the endoplasmic reticulum membrane. Experimental findings seem to indicate that several microsomal enzymes or at least their apoprotein parts are transferred to the membrane from cytoplasmic pools [4, 5, 7]. Therefore, in previous investigations a similar pathway for the transfer of soluble glycoproteins into microsomes was studied in vitro [8–10]. It could be shown that cytoplasmic sialoproteins labeled in vivo with [^{3}H]glucosamine and [^{14}C]leucine can be incorporated into the microsomal membrane upon incubation with rough microsomes and that they can not subsequently be removed by various washing procedures and detergent treatments. The sodium dodecyl sulfate gel electrophoretic pattern of the protein incorporated in vitro was very similar to that found after in vivo labeling. Upon incubation of Golgi membranes in 0.25 M sucrose, sialoproteins were released which were partly similar to the protein previously found in the cytoplasm and some of these sialoproteins exhibited pronounced specificity in incorporation into microsomes in vitro.

The properties of the supernatant sialoproteins that can be incorporated into microsomes have been studied here. A lipoprotein complex exhibiting great instability and high degree of incorporation was isolated and some of its properties were determined.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats weighing 160–180 g were used. All animals were starved for 20 h before sacrifice. The livers were perfused carefully and extensively as described earlier [8]. For labeling of supernatant glycoproteins, [^{3}H]-glucosamine (2 Ci/mmol) from the Radiochemical Centre, Amersham, U.K., was injected, 0.5 mCi/rat, into the portal vein 30 min before decapitation. In the case of [^{14}C]leucine (200 μCi/mol, from the Radiochemical Centre) 50 μCi/rat was injected into the portal vein.

Fractionations. Rough microsomes were prepared as described previously [8]. The microsomes were washed with 0.15 M Tris · HCl buffer, pH 8.0, and the pellets after centrifugation were suspended in 0.25 M sucrose to a concentration of 10 mg protein per ml.

For preparation of particle-free supernatant the perfused rat livers were homogenized in a glass-Teflon homogenizer with four strokes at 440 rev./min. The large particles were removed by centrifugation at $10000 \times g$ for 20 min, and the resulting supernatant was again centrifuged at $105000 \times g$ for 60 min. The upper 3 ml was removed with a Pasteur pipette and discarded. The rest of the supernatant was then sucked off leaving behind the last 3 ml, which was discarded. After repeating the centrifugation at $105000 \times g$ for 4 h the supernatant was again collected in a similar manner. The top layer contains mainly floating cytoplasmic neutral lipids and the bottom layer may contain small cytoplasmic particles.

The particle-free supernatant was subjected to gel filtration on Sephadex G-25 in order to remove nonprotein components and the proteins appearing in the void volume were used for further analysis.

In order to purify lipoproteins that could be incorporated into microsomes, flotation in NaBr solution was performed. About 90 ml of the protein fraction from Sephadex G-25 gel filtration containing about 2 mg protein/ml in 10 mM Tris · HCl buffer, pH 8.0 was supplemented with 8.55 g solid sucrose and 24 g NaBr and the

volume was adjusted with water to 100 ml at 4 °C. Thus, the final concentration of sucrose was 0.25 M and of NaBr 2.33 M, resulting in a density of 1.21 g/ml in the solution. After centrifugation at 124000 × *g* for 48 h in a 42.1 rotor (Spinco Beckman L2-65B centrifuge) the top 4 ml of the solution was removed with a syringe connected to a bent needle. This fraction was designated "lipoprotein" (LP) fraction. The NaBr was routinely removed from this fraction by dialysis against 10 mM Tris · HCl buffer, pH 8.0 overnight (4 changes).

Sephadex G-25 chromatography. In order to remove small labeled components (glucosamine and CMP-AcNeu), 150 ml of liver supernatant prepared from injected rats was pumped onto a Sephadex G-25 column (5.0 × 100 cm). The sample was eluted with 10 mM Tris · HCl, pH 8.0 and with a pumping speed of 1.2 ml/min.

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). Elution of the sample was performed with 50 mM Tris · HCl, pH 8.0 + 50 mM NaCl at a pumping speed of 0.4 ml/min.

To separate the subunits of the lipoprotein complex, a freeze-dried preparation (2 mg protein) was dissolved in 1 ml 40 mM Tris · sulfate, pH 8.0 + 0.2 % sodium dodecyl sulfate and pumped onto a column of Sephadex G-100 (1.0 × 95 cm). Elution of the sample was carried out with the sodium dodecyl sulfate-containing buffer using a pumping speed of 0.1 ml/min.

Sephadex G-200 chromatography. Sephadex G-200 was used for estimation of molecular weight. The size of the column was 1.0 × 90 cm, the elution buffer 50 mM Tris · HCl, pH 8.0, and the pumping speed was 0.05 ml/min. The enzyme activity of reference proteins was measured as described earlier [11–13].

Thin-layer gel filtration. To obtain a reliable value for the molecular weight of the smaller peptide, thin layer-gel filtration with Sephadex G-75 was performed. 20 × 40 cm glass plates were coated with a 0.6 mm thick layer of a Sephadex G-75 superfine slurry in 50 mM Tris · HCl, pH 8.0 + 50 ml NaCl. After equilibration overnight the samples (approx. 50 μg protein in a volume of 10 μl) were applied to the gel. The samples were run for 6 h at a 15° angle at room temperature. The protein spots were transferred to a paper replica (Whatman 3MM) and developed in Coomassie Brilliant Blue R 250 solution according to Radola [14].

Polyacrylamide sodium dodecyl sulfate gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed according to Weber and Osborn [15]. The gels (10 × 0.5 cm) contained 10 % acrylamide and the concentration of methylenebisacrylamide was 2.7 % of that of the total amount of acrylamide. The gels were loaded with approx. 100 μg protein. In all other aspects electrophoresis, staining of gels and measurements of radioactivity in gels were performed as described previously [9].

Determination of density and sedimentation coefficient. For determination of the equilibrium density of the lipoprotein complex an aliquot of the top layer after NaBr centrifugation, containing about 600 μg protein in a total volume of 3 ml, was placed in the bottom of an SW 40 centrifuge tube. If necessary the density of the sample was corrected to 1.2 g/ml with solid NaBr. On top of this layer a linear gradient was formed ranging between $\rho = 1.00$ g/ml and $\rho = 1.20$ g/ml, using NaBr in 10 mM Tris · HCl buffer, pH 8.0. The gradient volume was 8 ml. After centrifugation in an SW 40 rotor at 198000 × *g* for 40 h the content of the tube was fractionated by

puncturing the bottom of the tube and collecting 0.25-ml fractions. The absorbance, radioactivity and refractive index of the fractions were measured.

The sedimentation coefficient was determined by centrifugation on a sucrose gradient according to McEwen [16]. A linear gradient ranging from 0.145 to 0.464 M sucrose was used for the analyses. The lipoprotein fraction obtained by NaBr centrifugation was dialyzed against 10 mM Tris · HCl buffer, pH 8.0, for 90 min. This dialysis decreased the salt concentration sufficiently to allow layering on the sucrose gradient. 0.5 ml of the lipoprotein fraction containing 120 μg protein was placed on top of the gradient in a tube of the Spinco SW 40 rotor. After centrifugation at 36000 for 41 h the gradient was fractionated by collecting 0.4 ml fractions through the bottom of the tube. Radioactivity and protein were measured in these fractions. Calculation of $s_{20,w}$ was performed according to McEwen [16]. Molecular weight was determined according to Cox and Tanford [17].

Chemical determination. Protein was determined according to Lowry et al. with bovine serum albumin as standard [18]. Phospholipids, cholesterol and triglycerides were measured as described earlier [19]. The individual phospholipids were separated by silica gel H thin-layer chromatography according to Parker and Peterson [20] and the amount of phosphate was measured after extraction from the gel [19].

Neutral sugars were analyzed by gas-liquid chromatography of the alditol acetate derivatives [21]. Amino sugars were determined by amino acid analyzer [22]. The sialic acid content was estimated by the Warren procedure after Dowex 2-X8 ion exchange chromatography according to Svennerholm [23, 24].

RESULTS

Incorporation of lipoprotein

Previously it was demonstrated that glycoproteins in the supernatant and in certain fractions purified from the supernatant are readily incorporated into rough microsomes under in vitro conditions [9]. Table I shows three fractions isolated from the liver supernatant of rats injected with [^{3}H]glucosamine and [^{14}C]leucine. All of the soluble proteins, separated from small molecules, were obtained by gel filtration on a Sephadex G-25 column and are designated as the "G-25 pool". This fraction was further purified on a Sephadex G-100 column (for further details, see Fig. 3). Peak 1 is designated as the "G-100 pool". The supernatant lipoprotein, LP fraction, was isolated by separation procedure based on density (see Materials and Methods). The increasing purification of the sialolipoprotein in question is demonstrated by the dramatic increase of the specific amount of protein-bound *N*-acetylneuraminic acid from the G-25 pool to the G-100 pool and finally to the LP fraction. Similarly, the specific activity of the glucosamine label also increased; this, however, was not the case with the leucine label. Upon in vitro incubation of rough microsomes with all the supernatant fractions both labels were incorporated. The amount of protein-bound glucosamine incorporated was 23 % for the G-25 pool, 28 % for the G-100 pool and 41 % for the LP fraction. The per cent incorporation of the leucine label followed a similar pattern but was about half that of the glucosamine label. Radioactivity in the microsomes was determined after the Tris-water-Tris-washing procedure to avoid interference from adsorption of non-membranous proteins. Also,

TABLE I

INCORPORATION OF GLYCOPROTEINS FROM DIFFERENT FRACTIONS INTO ROUGH MICROSOMES

The incubation medium contained washed rough microsomes (5 mg protein), 20 mM Tris · HCl buffer, pH 7.5, 65 mM KCl, 0.4 mM CMP, 10 mM EDTA, 0.25 M sucrose and an aliquot of the G-25 pool, the G-100 pool or the lipoprotein fraction (2 mg, 1 mg, or 0.4 mg, respectively). After incubation at 37 °C for 60 min the suspension was cooled, centrifuged at 105 000 × *g* for 60 min and submitted to the Tris-water-Tris washing procedure [8]. Aliquots of the incubation mixture or microsomal suspension were supplemented with 2 % sodium dodecyl sulfate and radioactivity was measured after addition of Bray's solution [25].

	G-25 pool	G-100 pool	LP fraction
Amount of NANA			
(μg NANA/mg protein)	0.30	0.96	38.1
Total radioactivity in the incubation mixture			
[^{3}H]Gln, cpm	3610	7790	11 425
[^{14}C]Leucine, cpm	4410		4 277
Transferred radioactivity to microsomes			
[^{3}H]Gln, cpm	843	2160	4 650
[^{3}H]Gln, %	23.3	27.8	40.7
[^{14}C]Leucine, cpm	554		840
[^{14}C]Leucine, %	12.5		19.6

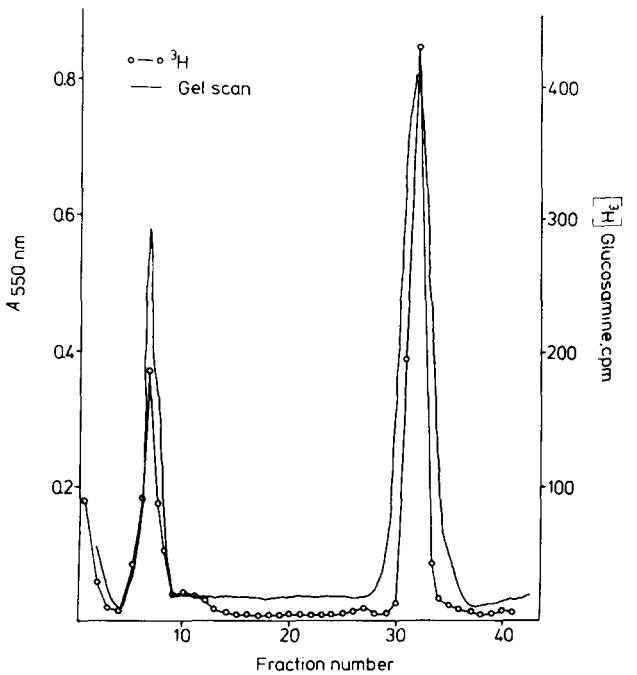

Fig. 1. Sodium dodecyl sulfate gel electrophoresis of the supernatant lipoprotein (LP). The labeled lipoprotein fraction was prepared by the NaBr-flotation procedure.

evidence has previously been presented that the incorporation does not represent an adsorption phenomenon, but that the sialolipoprotein incorporated is associated with the microsomal membrane as an integral protein [9].

Gel electrophoresis of LP fraction

The isolated lipoprotein fraction removed from the top part of the tube after NaBr centrifugation was subjected to sodium dodecyl sulfate-acrylamide gel electrophoresis (Fig. 1). Only two protein peaks could be detected in the gel, one with a molecular weight around 70000, and one around 10000*. When the LP fraction was isolated from rats injected with [^{3}H]glucosamine, two peaks of radioactivity appeared on the gel, both coincident with the protein peaks. Obviously, the lipoprotein complex isolated by the flotation procedure contains two peptides, both of which contain sugar moieties.

Properties of LP fraction

The livers used for the preparation were carefully perfused, thus making it improbable that contaminating serum lipoproteins would be present in the LP fraction. Previous model experiments, as well as gel electrophoretic characterization of various serum lipoproteins, excluded the possibility that the LP fraction which is incorporated into microsomes originates from the serum [8, 9]. To substantiate this point further, a two-step immunoprecipitation procedure was applied. Both the G-25 pool and the isolated LP fraction were incubated with a titrated amount of rabbit antiserum against rat serum proteins. In order to obtain complete precipitation of the antigen-antibody complex purified sheep IgG against rabbit IgG was added. As is demonstrated in Table II with lipoproteins labeled in vivo, only 0.6 % of the protein-bound label in the G-25 pool and 4.8 % from the LP fraction are precipitated by the antiserum. Thus, a significant contamination of our LP fraction with serum proteins seems to be excluded.

Chemical analysis of the LP fraction demonstrates that it contains 56 % protein and 44 % lipid by weight (Table III). Approximately half of the lipids are neutral and they can be divided into two equal components: esterified and non-

TABLE II

ANTIBODY PRECIPITATION OF G-25 POOL AND SUPERNATANT LIPOPROTEIN WITH RABBIT ANTISERUM AGAINST RAT SERUM PROTEINS

The labeled Sephadex G-25 pool and lipoprotein fraction were subjected to a two-step antibody precipitation using rabbit antiserum against rat serum proteins and sheep IGg against rabbit IGg [8]. To measure the unspecific precipitation antiserum against chick serum proteins was used.

	[^{3}H]Glucosamine total (cpm)	[^{3}H]Glucosamine precipitated (cpm)	[^{3}H]Glucosamine unspecifically precipitated (cpm)	[^{3}H]Glucosamine specifically precipitated (%)
G-25 pool	5090	163	31	0.6
LP fraction	913	125	85	4.8

* The relative amounts of the two peptides present in various preparations varied greatly.

TABLE III

COMPOSITION OF LP FRACTION

Component	Per cent of weight	Per cent of total
Total protein	56	
Total lipid	44	
Cholesterol and cholesterol esters	10	
Triglycerides	13	
Phospholipids	22	100
Phosphatidylcholine		72.4
Lysophosphatidylcholine		5.2
Sphingomyelin		9.7
Phosphatidylethanolamine		8.9
Phosphatidylinositol		3.9

esterified cholesterol on the one hand and triglycerides on the other. One fourth of the weight of the fraction is made up by phospholipids and Table III also shows the amount of the individual phospholipids present. Interestingly, approx. 90 % of the phospholipids are choline containing: 72 % is phosphatidylcholine, while two minor components are lysophosphatidylcholine and sphingomyelin. Phosphatidylethanolamine and phosphatidylinositol make up not more than 13 % of the total phospholipid content. The lipid pattern identified in the isolated fraction is dissimilar to that found in high density lipoproteins of the serum and the phospholipid composition resembles that of the isolated microsomal fraction [19, 26].

The LP fraction contains mannose and galactose as neutral sugars, glucosamine as amino sugar and also a sizeable amount of sialic acid (Table IV). Thus, qualitatively this isolated protein has a sugar composition similar to that of the microsomal membrane [27, 28]. The exact amount of sugars, particularly those of the mannose and galactose, is uncertain. The liver supernatant contains various glycosidases, either as an inherent enzyme [29] or as a result of lysosomal rupture, therefore some hydrolysis of the terminal sugars may occur during the long isolation procedure.

TABLE IV

CARBOHYDRATE CONTENT OF LP FRACTION

Determination of various sugars is given in Materials and Methods. The values are the means ±S.E. ($n = 7$).

Component	μg/mg protein
Mannose	23.5±4.2
Galactose	19.6±3.1
Glucosamine	36.5±5.1
Galactosamine	trace amount
N-Acetylneuraminic acid	38.1±3.9

Physical characteristics of LP fraction

The density of the isolated LP complex was determined by centrifugation on a salt gradient (ρ 1.0–1.2 g/ml) for 40 h (this time was necessary to attain equilibrium) (Fig. 2). The complex displayed a relatively broad distribution in the density range of 1.07–1.14 g/ml*. When all the fractions in this density range were collected and analyzed on sodium dodecyl sulfate gel the picture was identical with that of the complex shown in Fig. 1. Some protein was also present in the bottom fractions, probably because of aggregation of desintegrated complex and the presence of other contaminating proteins.

Isolation of the supernatant lipoprotein complex in a pure form requires a number of centrifugations and chromatographic steps. These procedures may affect complex lipoproteins. In order to determine the minimal size of the lipoprotein complex present in the G-25 pool, this pool was passed through a Sephadex G-100 column. Using a medium containing Tris and NaCl for elution, a protein peak exhibiting a high level of [^{3}H]glucosamine label was found in the void volume; while the larger part of the protein, some of which contained very little label was eluted in a stepwise manner (Fig. 3). When the fractions were incubated with microsomes, protein-bound sialic acid could be incorporated only by using the peak I fractions, namely fractions 45–55. This gel filtration experiment demonstrates that the lipoprotein complexes that can be incorporated into rough microsomes are localized exclusively in the void volume. Consequently, their molecular weight can not be lower than 150000.

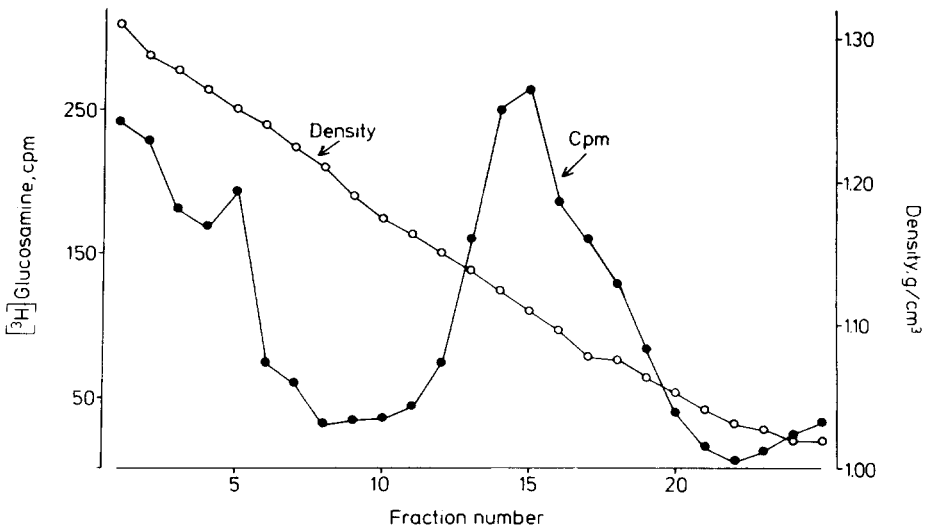

Fig. 2. Equilibrium density centrifugation of supernatant lipoprotein. Aliquots of the lipoprotein complex from the NaBr-flotation procedure were centrifuged to equilibrium in a NaBr gradient as described in Materials and Methods. The density in the individual fractions after centrifugation was measured with a refractometer and the proteins were localized by measuring radioactivity in the individual fractions.

* Similar density values could also be obtained after 75 h centrifugation on a sucrose gradient.

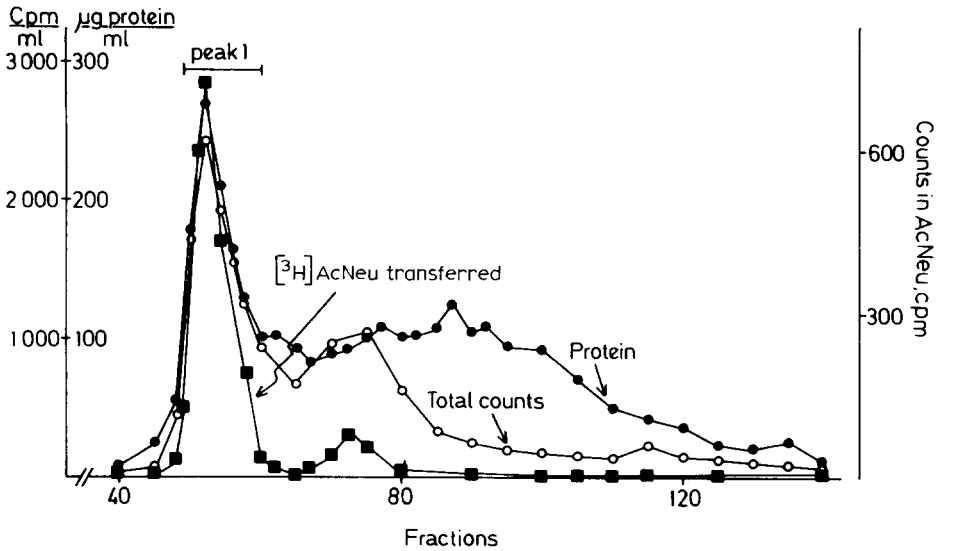

Fig. 3. Sephadex G-100 chromatography of the Sephadex G-25 pool. The distribution of radioactivity ([^{3}H]glucosamine), the protein amount and incorporation of [^{3}H]AcNeu into microsomes were measured for the different fractions. For the latter measurement 0.5 mg protein from each fraction was incubated with 5 mg microsomal protein as described in Materials and Methods. After incubation the microsomal AcNeu was isolated and radioactivity was determined. Peak I consists of the pooled fractions 45–55.

The sedimentation coefficient of the LP fraction was determined according to McEwen [16]. The isolated LP fraction was layered directly on a linear gradient in a preparative rotor. Calculation of the sedimentation coefficient for the LP fraction according to McEwen [16] gave a value of 4.9 S*.

A minimum value for the molecular weight of the lipoprotein was calculated from its $s_{20,w}$ using the equation

$$M = 12.72\,\pi\, N[s\eta(\bar{v}+\delta\bar{v}^0)^{\frac{1}{3}}/(1-\bar{v}\rho)]^{\frac{3}{2}}$$

[17], where M is the molecular weight for a spherical particle, η is the viscosity of the solvent, δ is the weight fraction of solvent bound to particle, $\bar{v}^0$ is the specific volume of the solvent, ρ is the density of the solvent, $\bar{v}$ is the partial specific volume of the lipoprotein and N is Avogadro's number. The partial specific volume could be estimated from the density obtained by gradient centrifugation in NaBr solution. The value, however, should be regarded as approximative since the density distribution of the LP fraction is relatively broad. This latter phenomenon may be explained by a partial redistribution of lipids among individual particles. The distribution pattern necessitated the use of the median density of 1.11. Since low protein concentration was used in the determination of density, the partial specific volume is approximately equal to the inverse of the density, i.e., 0.901. The hydration of the

* Determination of the sedimentation coefficient was also performed by the isokinetic gradient of Noll [30]. This procedure gave a value of 5.3S.

particle is not known, and therefore, the molecular weight for the unhydrated sphere was calculated [31], assuming $\delta = 0$. The values for η and ρ were taken from the literature [32]. With this formula a minimum molecular weight of 210000 could be calculated.

Additional experiments involving gel filtration on agarose and acrylamide gradient gel electrophoresis (not shown in figure) suggested a molecular weight of about 210000.

Properties of LP_1 and LP_2 fractions

The sodium dodecyl sulfate gel electrophoretic pattern of the isolated LP fraction illustrated in Fig. 1 demonstrated the presence of two peptides in the molecular regions of 70000 and 10000. In order to separate these two peptides the flotated LP fraction was freeze-dried and chromatographed on Sephadex G-100 in the presence of sodium dodecyl sulfate (Fig. 4). This resulted in two separated protein peaks, one in the high and one in the lower molecular weight region. Both these protein peaks were associated with [^{3}H]glucosamine, indicating the glycoprotein nature of the two peptides. Sodium dodecyl sulfate gel electrophoretic analysis of these peaks demonstrated the presence of relatively homogenous fractions consisting of only one main band (Fig. 5). The smaller molecular weight component (LP_1) displayed a molecular weight of 13100 and the larger one (LP_2) of 69000.

Gel chromatography of the freeze-dried preparation was also performed in the absence of sodium dodecyl sulfate (not shown in Fig. 5). The result was very similar to that described in Fig. 4. Obviously, the gel filtration procedure in the absence

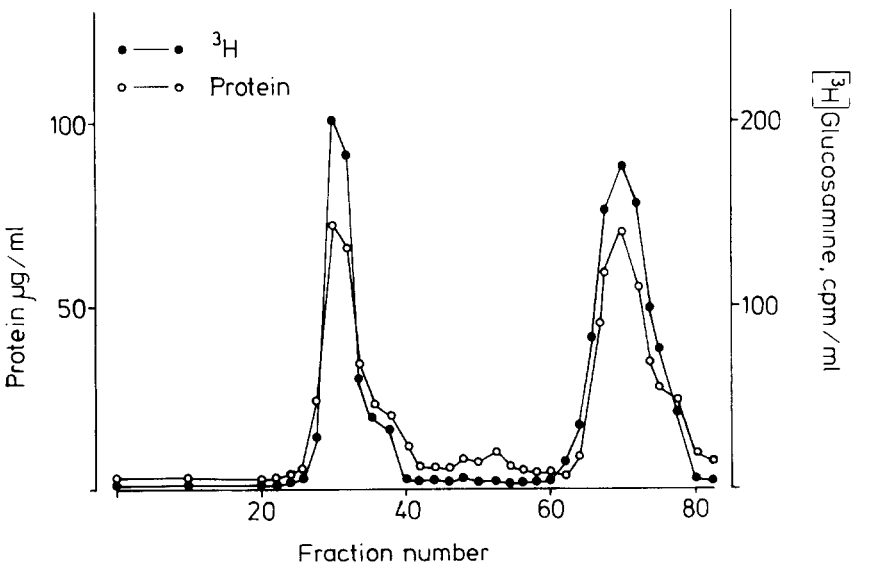

Fig. 4. Sephadex G-100 chromatography of supernatant lipoprotein. The lipoprotein isolated by the NaBr-flotation procedure was freeze-dried. The protein (1 mg) was dissolved in 0.2 % sodium dodecyl sulfate/40 mM Tris-sulfate, pH 8.0 and applied to the column. The sample was eluted with the same buffer. The individual fractions obtained after chromatography were analyzed for protein and radioactivity.

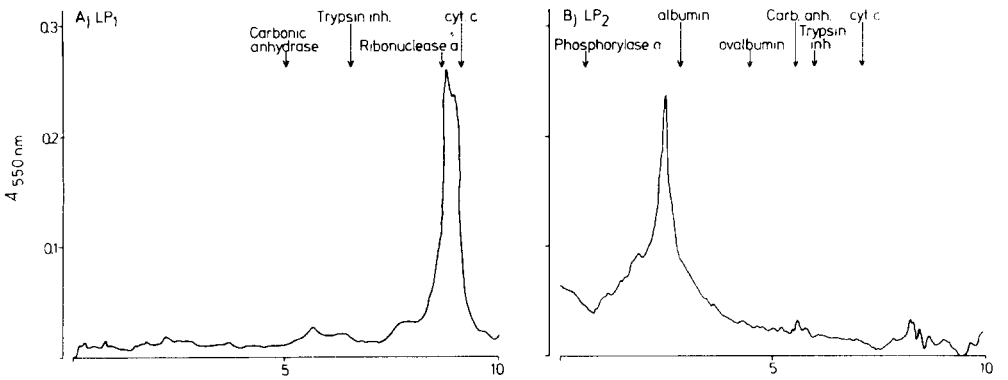

Fig. 5. Sodium dodecyl sulfate gel electrophoresis of the separated peptides of the lipoprotein. The two protein pools from Sephadex G-100 chromatography were concentrated by freeze-drying and 100 μg protein was applied to the gel. (A) The low molecular weight fraction (LP_1); (B) the high molecular weight fraction (LP_2) from Sephadex G-100 chromatography. The references used were: carbonic anhydrase (mol. wt. 29 000), trypsin inhibitor (mol. wt. 21 000), ribonuclease a (mol. wt. 13 700), cytochrome *c* (mol. wt. 12 400), phosphorylase *a* (mol. wt. 94 000), bovine serum albumin (mol. wt. 67 000), ovalbumin (mol. wt. 43 000). The gel system used in these experiments contained 15 % acrylamide (A) and 10 % acrylamide (B), respectively.

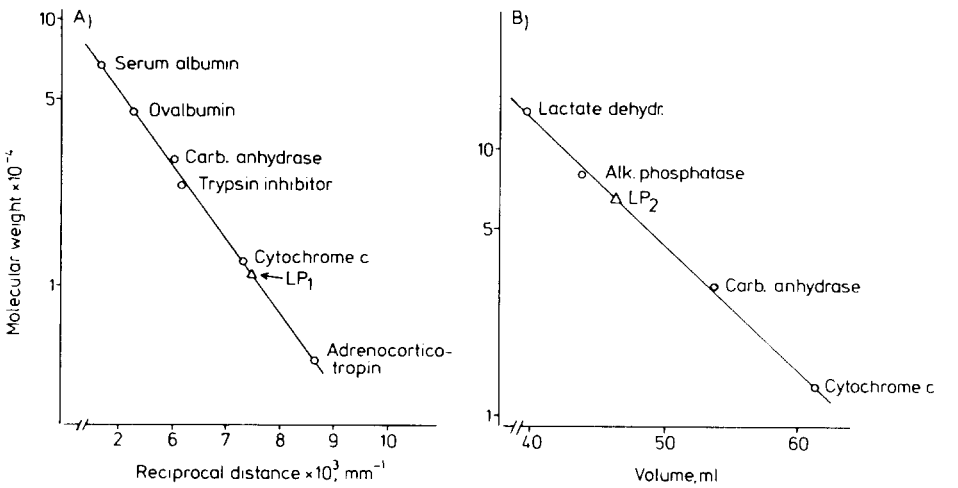

Fig. 6. Molecular weight estimation of the two peptides of the lipoprotein complex by gel filtration. (A) The smaller peptide (LP_1) from the Sephadex G-100 chromatography was subjected to thin layer gel filtration using Sephadex G-75 superfine. The references used were bovine serum albumin (mol. wt. 67 000), ovalbumin (mol. wt. 43 000), carbonic anhydrase (mol. wt. 29 000), trypsin inhibitor (mol. wt. 21 000), cytochrome *c* (mol. wt. 12 400) and adrenocorticotropin (mol. wt. 5260). The position of LP_1 is marked with a triangle. (B) The large molecular weight peptide (LP_2) from the Sephadex G-100 chromatography was rechromatographed on a Sephadex G-200 column. The references were lactate dehydrogenase (mol. wt. 138 000), alkaline phosphatase (mol. wt. 80 000), carbonic anhydrase (mol. wt. 29 000) and cyrochrome *c* (mol. wt. 12 400). The triangle marks the position of LP_2.

of detergent is sufficient to remove enough lipids to dissociate the complex into individual peptides. For this reason, in the following chromatographic analyses the detergent was omitted. Thus, this procedure gives two separated peptides similar to those two peptides revealed by sodium dodecyl sulfate gel electrophoresis of the intact complex.

Also, Sephadex thin-layer chromatography was used for determining the molecular weight of LP_1 fraction [14]. When the LP_1 fraction was run on a Sephadex G-75 layer, the LP protein spot corresponded to a molecular weight of 11000 (Fig. 6a). Thus, this method gave a somewhat lower value than sodium dodecyl sulfate gel electrophoresis (Fig. 5a). The LP_2 component was run on a Sephadex G-200 column (Fig. 6b). The column technique was used for this protein because it is easily detected on the basis of its high specific labeling. The single protein peak displayed a molecular weight of 67000. Again, this figure is somewhat lower than that obtained from the sodium dodecyl sulfate gel. The difference in the molecular weight values obtained from these two different methods may be due to the fact that the carbohydrate chain is known to interfere with the chromatographic and electrophoretic properties of the proteins [33]. The small differences found here indicate that the carbohydrate chains are relatively short.

Amino acid composition of LP_1 and LP_2.

Sodium dodecyl sulfate gel electrophoresis of microsomes incubated in vitro with labeled supernatant lipoprotein gave radioactive peaks in six regions, correspond-

TABLE V

AMINO ACID COMPOSITION OF LP_1 AND LP_2 FRACTIONS

The total composition of the pure peptides was obtained using a Beckman model 120-B amino acid analyzer after hydrolysis at 110 °C with 6 M HCl containing 1 % phenol for 24 h [22].

Amino acid	LP_1		LP_2	
	Residues per 100 residues	Nearest integer	Residues per 100 residues	Nearest integer
Aspartic acid	10.4	5.2	9.8	6.5
Threonine	5.4	2.7	6.0	4.0
Serine	6.8	3.4	7.5	5.0
Glutamic acid	15.7	7.8	13.1	8.7
Proline	4.7	2.3	4.0	2.7
Glycine	9.5	4.7	9.3	6.2
Alanine	8.3	4.1	9.4	6.1
Valine	5.5	2.7	6.4	4.3
Methionine	1.8	0.9	1.8	1.2
Isoleucine	4.3	2.1	4.7	3.1
Leucine	8.3	4.1	9.4	7.8
Tyrosine	2.7	1.3	2.5	1.7
Phenylalanine	3.6	1.8	3.9	2.6
Tryptophan	—	—	—	—
Lysine	6.8	3.4	6.4	4.3
Histidine	2.0	1.0	1.5	1.0
Arginine	4.5	2.3	4.9	3.7

ing to molecular weights of approximately 13, 23, 35, 46, 59 and 69000 [9]. A similar observation was made with the purified supernatant lipoprotein if it was mixed with sodium dodecyl sulfate at room temperature and was subjected directly to gel electrophoresis. In addition the quantitative relationship between LP_1 and LP_2 varied to a considerable degree for different treatments of the same sample and for different samples. These results suggest that within one complex only one protein is present and that the two proteins obtained both by gel filtration and sodium dodecyl sulfate gel electrophoresis originate from the same peptide but the larger represents a polymer of the smaller one. This point was further investigated by determining the amino acid composition of LP_1 and LP_2 fractions (Table V). The amino acids of the high and low molecular weight proteins were recovered in about the same amounts, and it appears that the two peptides have both qualitatively and quantitatively similar amino acid compositions. The similarity of the isolated proteins was tested by using the deviation function (D) defined by Harris et al. [34]: $D = [\Sigma(x_{1,i}-x_{2,i})^2]^{\frac{1}{2}}$, where $x_{1,i}$ represents the mole fraction of one amino acid in protein 1 and $x_{2,i}$ represents the mole fraction of the same acid in protein 2. A deviation number for LP_1 and LP_2 of 0.036 indicate the identity of the peptides, since a number lower than 0.1 characterizes proteins of the same origin.

DISCUSSION

The results of this study indicate that rat liver cytoplasm contains at least one sialolipoprotein which appears to be an intermediate in biosynthesis of the membrane of the endoplasmic reticulum. The purified form of this protein is incorporated into isolated microsomal membranes in vitro and this process may offer a reasonable explanation as to how proteins with completed oligosaccharide chains are incorporated into the endoplasmic reticulum membrane in vivo.

A study of the mechanism of membrane biosynthesis requires separation of the individual precursor components in intact form and in amounts sufficient for various physical and chemical characterizations. Clearly, the cytoplasmic lipoprotein complexes studied here are both unstable and present only in small amounts. The procedures used for isolation (i.e., salt gradients and gel filtrations), concentration of the material (i.e., lyophilization and diaflo filtration), and storage between isolation and characterization proved to be deleterious for the complex, leading to dissociation. The low stability of the complex may be attributed to the low net negative surface charge of the intact complex, the cause of which will be discussed below; but this property may on the other hand be essential for interaction of the complex with the microsomal membrane.

The intact lipoprotein complex has a molecular weight of approx. 210000. This appears to be the principal unit active in incorporation in vivo. The fact that the endoplasmic reticulum has all the enzymes necessary for synthesizing lipids [35] makes it improbable that this incorporation serves for the renewal of microsomal phospholipids. Instead, the function of the lipid is probably two-fold: it holds the complex together and it creates a hydrophobic environment for the hydrophobic part of the protein to exist in*. Since only about 50 % of the complex is lipid, it may

* Upon shaking in a $CHCl_3$/methanol/water system 67 % of the protein-bound radioactivity proved to be soluble in the organic phase. Also, after complete removal of lipids from the lipoprotein complex the apoprotein was insoluble in water.

be regarded as relatively lipid poor. Probably the amount of lipid present is limited to that amount necessary to build a stable micelle. The phospholipid composition, which is not very different from that of the microsomal membrane [36], is dominated by choline-containing lipids which make up about 90 % of the total amount. The amphoteric nature of these lipids, together with the high amount of neutral lipids present, is effective in decreasing the surface charge and should facilitate the incorporation of the complex into the microsomal membrane. The basic peptide in the complex has a molecular weight of 11000, which implies that every peptide is associated with about 6–7 phospholipid molecules. This is a number comparable to that calculated for many biological membranes.

Membrane glycoproteins are known to contain relatively low amounts of sugar, 1–2 % by weight, and the four major sugars are mannose, galactose, glucosamine and sialic acid [27, 28]. The complex studied here has a sugar composition which is similar to that of isolated membrane glycoproteins. The presence of sugar offers a relatively simple marker for following the fate of the protein in question. We have, however, at present no data indicating that the specificity of the incorporation depends on the composition and length of the oligosaccharide chain. Future investigation would be facilitated if the function of microsomal membrane glycoproteins could be elucidated, since at present we have practically no information about the role of the carbohydrate moiety of glycoproteins in the endoplasmic reticulum. It has been proposed [37] that the carbohydrate chain may increase the polarity of the protein facilitating an interaction with the hydrophilic environment of the membrane. This in turn contributes to the stability of the membrane structure by anchoring the protein in its right position and prevents its transverse movement.

Estimation of the molecular weight of the lipoprotein complex by centrifugation, agarose gel filtration and Sephadex gel filtration of the G-25 pool demonstrated that it is not below 200000. When sodium dodecyl sulfate gel electrophoresis and gel filtration were applied after flotation, or several consecutive steps were employed for the isolation of the complex, two peptides with molecular weights of 13000 and 69000 were found. These two components were not only variable in their relative amounts and interconvertible with each other in the various experiments, but also exhibited a very similar amino acid composition. Consequently, it is very probable that only one peptide is present in the complex studied here and the larger component with a molecular weight of 69000 represents an aggregation product of the smaller peptide. Such an aggregation may reflect the natural state of the peptide in the membrane, a situation similar to that described for detergent-isolated cytochrome b_5 [38]. Alternatively, the polymerization may represent an artefact caused by the presence of sodium dodecyl sulfate or by delipidation during the treatment of the sample before electrophoresis. A similar situation, that is, creation of a stable aggregate from a monomer unit, was observed by Shannon and Hill [39] during their studies of a cytoplasmic protein from *Neurospora crassa*. This protein also exhibited properties very similar to those of proteins isolated from cellular membranes.

Taking into consideration the results discussed above, it is plausible to suppose that the basic unit in the isolated lipoprotein complex is the peptide with 11000 molecular weight and that 10–12 peptides are arranged into a large complex by being embedded in lipid micelles. These peptides may be distributed as individual

"islands" in the surrounding lipids [40], a structure proposed by the fluid mosaic model of the membrane [41].

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Hans Jörnvall for amino acid analyses and Dr. Torvard Laurent and Dr. Håkan Pertoft for valuable discussions. This work has been supported by grants from the Swedish Medical Research Council and the Magnus Bergvall Foundation. Dr. F. Autuori was on leave of absence from the Institute of Histology and Embryology, Faculty of Science, University of Rome, Italy.

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