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Methods

Communications

Isolation and Characterization of Proteolipids from Rat Liver (LSP) and Kidney (KSP)

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In this study, the biochemical characteristics of proteolipids of rat liver and kidney homogenates were compared. In all preparations, the proteo-lipids were eluted at the void volume of the Biogel A-15 m column after gel chromatography, and a molecular weight over 15×10^6 was estimated. Electron microscopy of the concentrated void volume fractions showed globular particles with diameters of 30–100 nm for liver (LSP) and 30–60 nm for kidney (KSP) preparations that might be formed from plasma membrane fragments. After ultracentrifugation in a CsCl gradient, rat LSP and KSP floated in a small density range from 1.16–1.17 g/ml. There was no significant difference between the relative percentages of phospholipids, triglycerides and cholesterol of both proteo-lipids. However, the relative mean amount of total protein in rat KSP (60.4% vs 50.4%) was significantly higher and the content of free fatty acids (4.3% vs 12.6%) significantly lower compared to rat LSP. SDS-PAGE revealed at least 12 protein subunits ranging from 15,000–130,000 in both preparations, but one protein of about M_r 49,000 might represent a liver specific component. The isolated proteo-lipids from rat liver and kidney homogenates showed similar biochemical characteristics as those from human sources, which could explain the known cross-reactivity of antibodies against these preparations.

The putative function of these proteo-lipids is not known, although there is some evidence from human studies that they carry receptor proteins.
Lipids 23, 410–414 (1988).

Cellular and humoral immune reactions against the liver-specific membrane lipoprotein complex (LSP) have been widely reported in patients with liver disease (1,2). LSP is a large, lipid-associated complex containing several antigens, including species-specific and species cross-reactive determinants, of which at least two seem to be liver specific, but their precise identity has not been established (3–5). Immunochemical and electron microscopical evidence suggests that LSP is derived from the liver cell plasma membrane (6–8). An important role of the plasma membrane is to transmit biochemical signals generated by ligand-receptor interaction at the cell surface across the lipid bilayer and into the cell (9). Recently, the hepatic asialo-glycoprotein receptor (hepatic lectin) has been detected as a minor component of liver-specific membrane lipoprotein (10). To our knowledge, this has been the first time that any component of LSP has been positively identified.

Further studies have shown that LSP is a far more complex particle than was first recognized. It has an apparent molecular weight (by gel filtration) of more than 20×10^6, contains phospholipids and triglycerides, and shows multiple protein subunits on SDS-polyacrylamide gel electrophoresis (4). Behrens and Paronetto (11) have demonstrated that kidney extract (KSP) handled in the same manner as liver extract has many similarities to LSP and that antibodies against LSP and KSP give the same immunofluorescence pattern in sections of liver, kidney and stomach. Furthermore, LSP and KSP cross-react in double gel diffusion.

These findings point against a complete organ specificity of LSP in humans in accordance with later studies by Riisom and Diederichsen (12). Hopf et al. tested LSP from different species and showed that rat LSP cross-reacted weakly with human LSP (6). However, Kakumu et al. demonstrated that antiserum to rat LSP, absorbed with normal human plasma, gave a single line on immunodiffusion against purified LSP from rat and human livers. Furthermore, antisera to rat and human LSP reacted almost equally with both rat and human hepatocytes, respectively (1). Whereas biochemical investigations have concentrated on human LSP and more recently KSP, there were to our knowledge no data on the biochemical characterization of these proteo-lipids in rat liver and kidney.

In view of the demonstrated shared antigenic properties, we performed an extensive characterization of these proteo-lipids in rats, which permits a comparative evaluation with the results of the previous studies in humans.

MATERIALS AND METHODS

Preparation of rat liver and kidney membrane lipoproteins. Rat livers and kidneys were obtained from male Sprague-Dawley rats weighing ca. 150 g. The preparation of the membrane lipoproteins was performed at +4 C according to Behrens and Paronetto (11) and McFarlane et al. (4). The liver and kidney tissue was cut into small pieces and washed for five hr in 0.25 M sucrose solution adjusted to pH 8.0 with 0.1 M NaOH, changing the solution every 20 min. For each of the 10 different preparations, 10 rat livers and 20 kidneys were pooled. After crushing the tissue pieces in 0.25 M sucrose solution (wt/vol, 1:1) with an Ultra-Turrax blender, homogenization in a potter homogenizer was performed. The homogenate was ultracentrifugated at 100,000 g for one hr using a Beckman 60 Ti-rotor. Two hundred IU/ml penicillin and 10 μg/ml gentamicin were added to the supernatant, and 4 ml were incubated at 20 C with 1.0 μCi of ethanolic [1,2-^{3}H]cholesterol (New England Nuclear, Boston, MA), which is incorporated in the protein lipid complexes and serves as an easily detectable, highly sensitive but not quantitative marker of the lipid moiety. Afterwards, gel chromatography was performed on a 95.0 × 3.0 cm Biogel A-15 m column with 0.1 M Tris HCl, pH 8.0, containing 0.2 M NaCl and 1 mM Na_2 EDTA as elution buffer.

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Abbreviations: KSP, kidney specific preparations; LSP, liver-specific preparations; PAG, polyacrimide gel; SDS-PAGE, sodium dodecyl sulfate polyacrimide gel electrophoresis.

Usually, 80 to 90 8.4 ml fractions were collected. The absorbance at 280 nm was recorded with a Beckman spectrophotometer. After addition of 5.0 ml Bray-scintillation solution to 0.1 ml aliquots of the fractions, the β-radiation emitted by [1,2-^{3}H]cholesterol was measured in a Betaszint BF 5000 counter. All fractions containing [1,2-^{3}H]cholesterol were pooled and concentrated to about 10% of the original volume using PM 10 membranes (Amicon, Witten, FRG). Further characterization was done by chemical assay, electron microscopy, density gradient ultracentrifugation and SDS-polyacrylamide gel electrophoresis.

Biochemical assays. Protein was measured by the method of Lowry after precipitation of the proteins with trichloracetic acid (13). Total cholesterol and triglyceride determinations were performed enzymatically with commercial test kits (Boehringer Mannheim, FRG) (14,15). The colorimetric NEFA C-test (Wako Chemicals, Osaka, Japan) was used for the quantification of unesterified fatty acids (16). The total phospholipids were determined in chloroform/methanol extracts (3:1, v/v) according to Fiske and Subbarow (17). Further fractionation of the phospholipids was achieved by thin layer chromatography on 20 × 20 cm glass plates coated with silica gel (Merck, Darmstadt, FRG).

Chloroform/methanol/water (65:35:8, v/v/v) was used as solvent system. The separated phospholipids were made visible with iodine vapor and stained individually. Cardiolipin, phosphatidylcholine, sphingomyelin, phosphatidylinositol and ethanolamine glycerophospholipids served as reference substances. Afterwards, a colorimetric phospholipid determination was performed. The accuracy was tested by Precilip E.L. standard sera (Boehringer, Mannheim, FRG).

Density gradient ultracentrifugation. One ml of the sample was added to 3.4 ml of CsCl-solution (density 1.25 g/ml). During 72 hr of ultracentrifugation at 4 C and 50,000 rpm in a Beckman SW-60 rotor, a concave gradient from 1.06 to 1.43 g/ml was established (18). Eleven 0.4 ml fractions were obtained by pipetting from the top. The densities were calculated by weighing 0.2 ml portions. The absorbance at 280 nm and the counts per min were

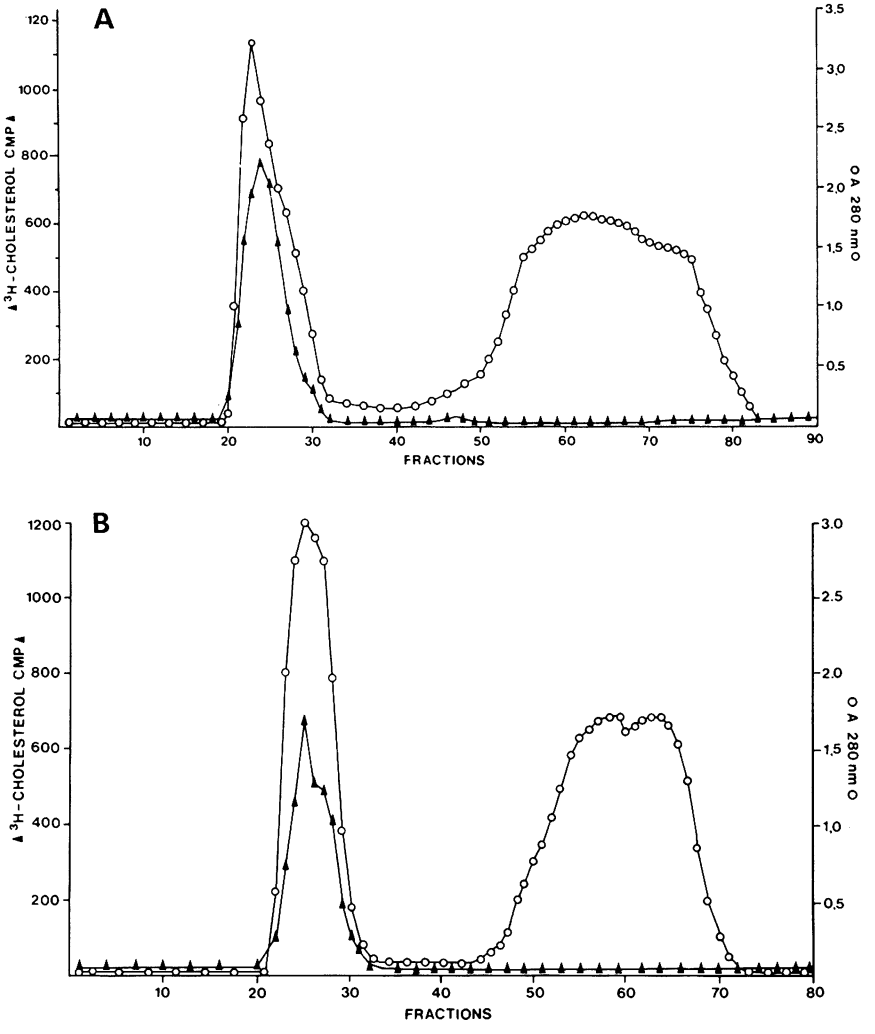

FIG. 1. Typical gel chromatographic elution profile of the concentrated 100,000 g supernatant of liver (A) and kidney homogenates (B) on Biogel A-15 m. Column size 95 × 3.0 cm. Points represent cpm emitted by [1,2-^{2}H]cholesterol (▲) and the absorbance at 280 nm (O) in each collected fraction. V_0, void volume.

measured in each fraction and compared with the position of the macroscopically visible turbid disk of ca. 1-mm height.

Electron microscopy. Electron micrographs were taken with a Siemens 101 electron microscope at 80 kV. The membrane lipoprotein preparations were negatively stained with 2% potassium phosphotungstate, pH 7.0 (19). A small drop of the suspension was placed on a 200 or 300 Formavar carbon-coated grid and examined after drying at room temperature.

SDS-Polyacrylamide gel electrophoresis. For SDS-PAGE, aliquots of the samples were diluted (1:4, v/v) with 37.5 mM Tris HCl buffer (pH 8.8) containing 1 g/dl SDS and 4% 2-mercaptoethanol. After incubation for three min at 95 C, the electrophoretic protein separation was performed in ultrathin (0.36 mm) SDS-polyacrylamide pore gradient gels (T = 4.0–22.5%) on PAG films. The protein subunits were stained with silver nitrate (20). The molecular weights were estimated by comparison with calibration standards (Pharmacia, Freiburg, FRG) according to the method of Weber and Osborne (21).

Statistical analysis. Using mean and standard deviation of the mean for further statistical analysis of our data, Student's t-test was applied.

RESULTS

The elution profile of the 100,000 g liver and kidney supernatants on a Biogel A-15 m column showed rising absorbance at 280 nm, coinciding with elevated counts/min in the first peak as well as high protein absorbance without evidence of radioactivity in the second peak (Fig. 1).

Liver (Fig. 1A, first peak) and kidney (Fig. 1B, first peak) proteo-lipids showed similar elution patterns. Both were eluted in the void volume of the Biogel A-15 m column, indicating a molecular weight of more than 15×10^6.

The determination of the void volume (v_0) had been performed with 10% Dextran 5000 (Serva, Heidelberg, FRG) composing macromolecules of up to 40×10^6.

The chemical analysis of the pooled and concentrated fractions revealed 50.4% protein, 23.2% phospholipids, 12.6% free fatty acids, 11.2% triglycerides and 5.6% total cholesterol (mean values) for liver and 60.4% protein, 20.5% phospholipids, 11.1% triglycerides, 4.7% total cholesterol and 4.3% free fatty acids (mean values) for kidney lipoproteins (Table 1).

The percentage of total protein in rat KSP was significantly ($p < 0.02$) higher and the percentage of free fatty acids significantly ($p < 0.001$) lower compared with the relative amounts in rat LSP. The composition of the phospholipid moiety fractionated by thin layer chromatography is illustrated in Table 2. There were no significant differences for cardiolipin, ethanolamine glycerophospolipids, phosphatidylcholine, phosphatidylinositol and sphingomyelin in both preparations.

An average density of 1.16 g/ml was measured for rat LSP and of 1.17 g/ml for rat KSP by CsCl-gradient ultracentrifugation (Fig. 2A and B). After negative staining, electron microscopy showed globular macromolecules with diameters of 30–100 nm for liver and of 30–60 nm for kidney preparations (Fig. 3A and B). Assuming that one spheric particle on the photograph corresponded to a single molecule, an average molecular weight of 100×10^6 and 50×10^6 was calculated for liver and kidney proteo-lipid complexes, respectively. The particles consisted of at least 12 protein subunits with a molecular weight ranging from 15,000 to 130,000, separated by SDS-PAGE (Fig. 4A and B).

The protein profiles on the gels following SDS-PAGE of rat LSP and rat KSP were similar but show one interesting difference. The protein at about M_r 49,000 appears to be more heavily stained on the liver sample compared with the kidney sample and also may be slightly smaller. The other differences in the electrophoretic profiles of both samples seemed to be caused by varying concentrations of corresponding subunits macroscopically visible by the slightly different intensity of the silver nitrate staining. These visual impressions have been confirmed by a quantitative densitometric scan of these gels.

DISCUSSION

The findings reported here indicate that LSP and KSP could be prepared by standard methods from rat liver and kidney. There were some variations in terms of lipid/protein ratio and the relative amount of the major lipids (phospholipids, free fatty acids, triglycerides, cholesterol) between the 10 different preparations of LSP and KSP. This might be caused by the isolation procedure that composed multiple steps as crushing of the tissue, homogenization, ultracentrifugation, gel chromatography, ultrafiltration and thin layer chromatography. In all preparations, rat LSP and rat KSP were eluted in a single peak at the void volume of the column after gel chromatography. From these results, a molecular weight of rat LSP and rat KSP over 15×10^6 is estimated. However, because of the hydrophobic nature, proteo-lipids may behave atypical on gel filtration, and aggregation of

TABLE 1

Relative Composition of Lipids and Protein in the Rat Liver and Kidney Membrane Lipoproteins of 10 Preparations (Mean ± SEM)

	Liver (%)	Kidney (%)	p-Value
Protein	50.4 ± 2.2	60.4 ± 3.2	<0.02
Phospholipids	23.2 ± 3.9	20.5 ± 2.6	n.s.
Free fatty acids	12.6 ± 1.1	4.3 ± 0.5	<0.001
Triglycerides	11.2 ± 1.8	11.1 ± 2.4	n.s.
Cholesterol	5.6 ± 0.5	4.7 ± 0.7	n.s.

TABLE 2

Relative Composition of Phospholipids in the Rat Liver and Kidney Membrane Lipoproteins of 10 Preparations (Mean ± SEM)

	Liver (%)	Kidney (%)	
Cardiolipin	5.4 ± 1.7	9.3 ± 3.0	n.s.
Ethanolamine glycerophospholipids	23.0 ± 7.6	24.7 ± 1.4	n.s.
Phosphatidylcholine	18.9 ± 7.4	30.8 ± 4.8	n.s.
Phosphatidylinositol	17.2 ± 8.3	19.1 ± 4.2	n.s.
Sphingomyelin	35.5 ± 12.1	15.6 ± 2.2	n.s.

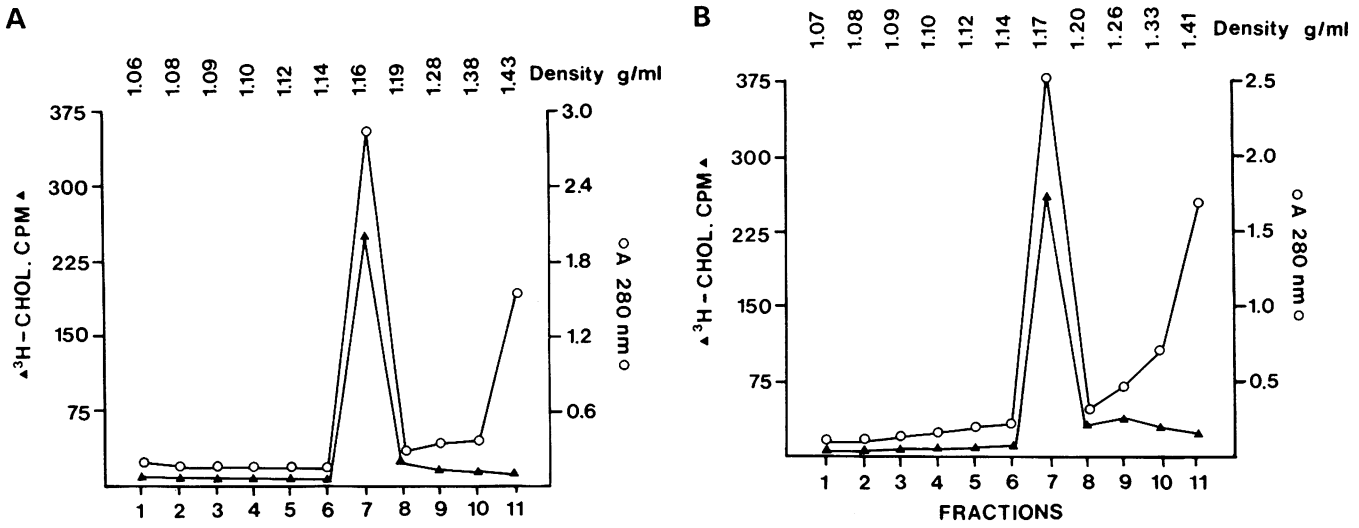

FIG. 2. Density determination of the isolated rat liver (A) and kidney membrane lipoproteins (B) by isopycnic ultracentrifugation in a CsCl gradient (50,000 rpm for 72 hr, 4 C). Points represent cpm emitted by [1,2-³H]cholesterol (▲) and the absorbance at 280 nm (○) recorded in each fraction pipetted from the top.

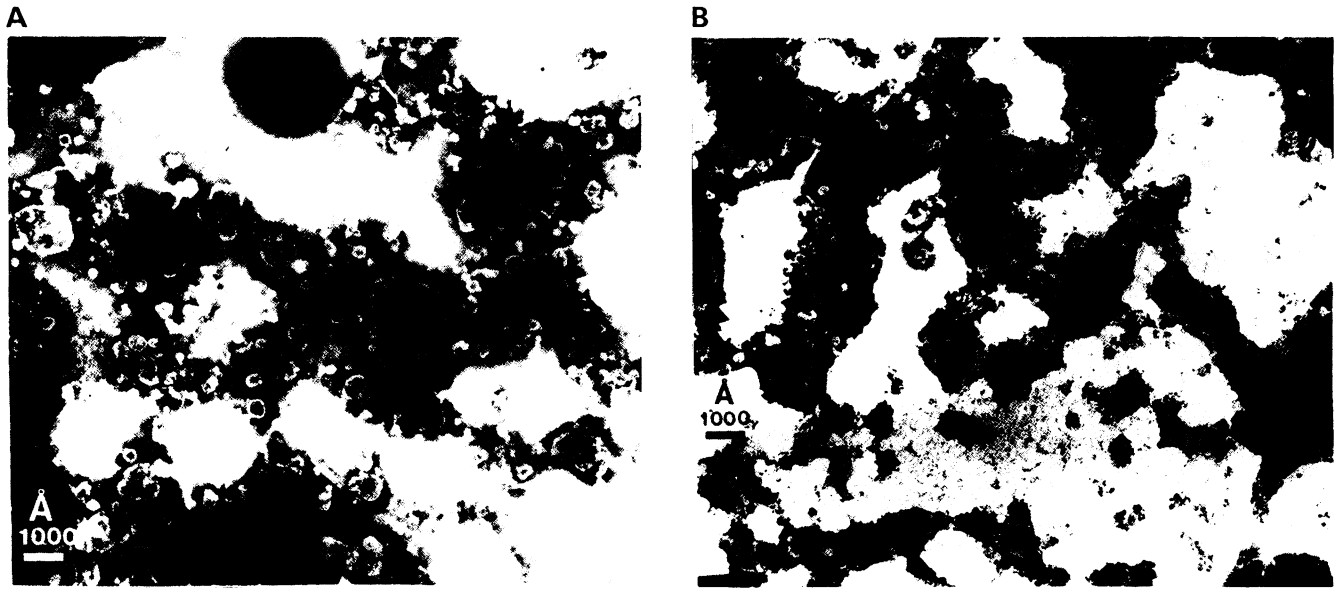

FIG. 3. Electron microscopy of the liver (A) and kidney (B) membrane lipoprotein preparation using a Siemens 101 electron microscope and a negative staining technique with 2% potassium phosphotungstate, pH 7.0 (×60,000).

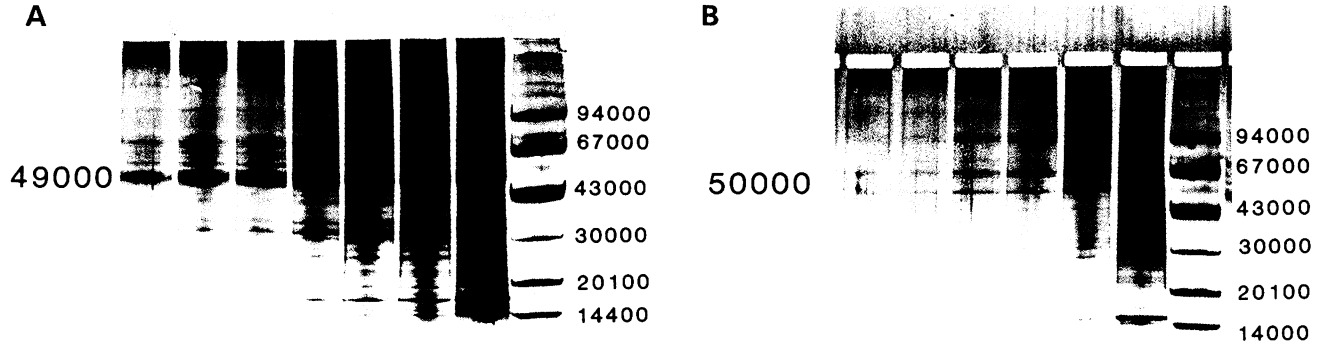

FIG. 4. SDS-PAGE of the liver (A) and kidney (B) membrane lipoprotein preparation on ultrathin (0.36 mm) pore gradient gels (T, 4.0-22.5%) in increasing concentrations. The standard calibration kit is shown in the right lane.

molecules to higher molecular weight complexes is likely. The electron microscopy of the concentrated void volume fractions showed globular particles with diameters of 30–100 nm for liver and 30–60 nm for kidney preparations. These particles could represent vesicles that might have been formed spontaneously from isolated plasma membrane fragments as has been suggested by others from electron microscopical studies on human LSP and KSP (22).

The density gradient ultracentrifugation revealed in all preparations a highly reproducible macroscopically visible turbid disk of ca. 1 mm height with a density between 1.16 g/ml and 1.17 g/ml. This reflects the rather constant protein/lipid ratio of all preparations.

The results of gel chromatography, electron microscopy and density gradient ultracentrifugation showed similar properties of rat LSP and rat KSP. However, further biochemical investigation demonstrated some significant differences between both preparations. The relative mean percentage of total protein in rat KSP (60.4 vs 50.4) was significantly higher and the percentage of free fatty acids (4.3 vs 12.6) significantly lower compared with the relative amounts in rat LSP. However, the free fatty acids might be formed as an artifact during the isolation procedure, and the difference between liver and kidney preparations could be due to different lipolytic activity in these samples. The SDS-PAGE revealed at least 12 protein subunits ranging from 15,000–130,000 in both preparations. One protein at about M_r 49,000 appeared to be more heavily stained on the liver sample compared with the kidney sample and also might be slightly smaller (49,000 vs 50,000). This protein could represent an organ-specific component.

Our results of the quantitative protein-lipid determination in rats were in accordance to the data in humans published by other authors. The average lipid protein ratio of 0.98 in the liver preparations fits in the range of 0.24–1.09 described by Lebwohl and Gerber (8). The mean protein content of 50.4% and the phospholipid moiety of 23.2% showed a similar ratio as reported by McFarlane et al. (4). The phospholipid distribution pattern on cardiolipin, ethanolamine glycerophospholipids, phosphatidylcholine, phosphatidylinositol, sphingomyelin as well as the mean relative amount of triglycerides (11.2%) corresponded to the results of human LSP preparations by Hütteroth and Meyer zum Büschenfelde (5).

Human KSP preparations showed similar gel chromatographic elution patterns and comparable densities compared with our data obtained in rat KSP (11). Furthermore, the lipid protein ratio and the lipid composition resembled those of membrane lipoproteins isolated from human urinary fluid (23). In view of the known cross-reactivity of anti-sera against human LSP and rat LSP, a comparison of the protein profiles of these preparations was of special interest. Studies from McFarlane et al. and Lebwohl and Gerber have indicated that human LSP may be comprised of up to 13 protein subunits of different molecular sizes (4,8). Behrens and Paronetto observed eight protein subunits with molecular weights of 40,000 to 96,000 in human LSP (11).

More recently, Riisom and Diederichsen separated in human LSP and KSP samples four major bands corresponding to molecular weights of 66,000, 60,000, 55,000 and 50,000, and a number of minor protein subunits with molecular weights between 350,000 and 29,000 (12). Our electrophoretic profiles of rat LSP and KSP resembled those of Riisom and Diederichsen very closely, and there was no evidence for a species-specific subunit. These strong similarities could explain the shared antigenic properties of human and rat LSP.

We conclude from our data that large molecular proteo-lipids can be highly reproducibly isolated from rat liver and rat kidney homogenates using standard procedures. Obviously, these proteo-lipids are easily soluble in the cytosol, but immunochemical and electromicroscopical studies of these proteo-lipids from other sources suggest that they constitute fragments of the plasma membrane. There were some differences between rat liver and rat kidney preparations in regard to the relative amounts of protein, free fatty acids and the electrophoretic profile. A 49,000 protein subunit in rat LSP might represent an organ-specific antigen. According to the similar biochemical properties, a close relationship between rat and human LSP and KSP must be assumed. The putative function of these proteo-lipids is not known, although there is some evidence from human studies that they carry receptor proteins for asialo-glycoproteins.

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