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STUDIES ON PLASMA MEMBRANES FROM LIVER CELLS SEPARATION AND CHARACTERIZATION OF LIPOPROTEIN SUBUNITS OF THE ISOLATED PLASMA MEMBRANES

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SUMMARY

1. High-density lipoproteins were separated from plasma membranes solubilized by brief sonication.

2. The three more soluble lipoproteins have increasingly higher hydrated densities along with decreasingly lower lipid–protein ratios and flotation properties (s_f) in the analytical ultracentrifuge.

3. A less soluble lipoprotein, the pellicle, floats out mainly at solution density 1.17 g/ml. It appears as quasicrystalline needles which have a lamellar appearance under phase contrast microscopy.

4. The greatest specific activities for the two principal membrane-localized enzymes, 5′-nucleotidase (EC 3.1.3.5) and (Na^+–K^+)-ATPase (EC 3.6.1.3) are associated with the soluble lipoprotein which has the lowest density, *e.g.* $d < 1.125$ g/ml. Different and decreasing amounts of activity are associated with the other soluble lipoproteins as their densities increase. The pellicle, also, has significant specific activity.

5. The three soluble lipoproteins are present in liver plasma membranes from normal young rats in the following proportional quantities: lipoproteins (A) with $d < 1.125$, 25–30 %; (B) with $d < 1.17$, 55–60 %; and (C) with $d < 1.21$ g/ml, 10–15 %. These amounts occur with regularity and are reproducible from experiment to experiment when the rats are young. In older (at least 1 year) and somewhat heavier rats, the majority of the total lipoprotein content is shifted to lipoprotein A.

6. The reproducibility of the quantities as well as the association of the activities for the two enzymes, suggest that the plasma membranes from young rat liver may be composed of units or subunits which are present in a pattern in the structural organization and that they have different biochemical properties.

INTRODUCTION

The DANIELLI AND DAVSON[1] and ROBERTSON[2] models of membranes, representing the bimolecular leaflet and unit structure, were postulated on the basis of

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permeability studies and microscopic observations. These may not conform with some data which have been presented recently by LUCY[3] and WOLMAN[4]. An example might be the impermeability of bimolecular leaflets to aqueous-soluble substances, water and ions, in disagreement with processes which undoubtedly do occur in biological membranes. Permeability through these structures was presumed to occur through pores incorporated at intervals.

FERNÁNDEZ-MORÁN[5] proposed, on the basis of his observations on the structure of the inner membrane of the mitochondrion, that membranes may be composed of globular subunits. This concept has been enlarged by GREEN AND PERDUE[6] who suggested that mitochondrial membranes were composed of repeating "lipoprotein" units arranged side by side. LUCY[3] has proposed globular micelles which have inherent pores and thus are more permeable.

Recently there has been an attempt to integrate these possible structures into one which would represent both the bimolecular leaflet and the globular micelle (WOLMAN[4]). As LUCY[3] states... "it may be unwise to assume that any one system... can be regarded as a universal model except in quite general terms... It may perhaps be suggested that some membranes contain globular micelles of lipid and separate globular molecules of protein in the plane of the lipid micelles, while other membranes are composed of globular lipoprotein units and yet others contain all three species".

In our preliminary communication[7], we first demonstrated that it was possible to isolate soluble lipoproteins from plasma membranes of rat liver (membranes that were not treated with detergents), but essentially none of their physicochemical characteristics were presented. MORGAN AND HANAHAN[8] also had shown that it was possible to isolate lipoprotein macromolecules from membranes of the erythrocyte.

This paper presents many of the characteristics of the lipoprotein macromolecules which can be separated from plasma membranes. A study of the lipoproteins of membranes is a new approach to the elucidation of the plasma membrane structural organization. Along with electron microscopic observations and results from other physical measurements, the lipoprotein studies will advance our knowledge from speculation to reality of the structure of membranes on a molecular level.

METHODS

Plasma membranes of the parenchymal cells were isolated from livers of rats. Male Sprague–Dawley rats aged 8–10 weeks (weight 250–350 g) were decapitated, exsanguinated and the liver weighed into 5-g portions that were then homogenized in 25 ml 1 mM $NaHCO_3$; 20 g (4 homogenizations) were pooled, stirred for 2 min in 500 ml 1 mM $NaHCO_3$, filtered successively through 2 meshes (90 and 120) of cheese cloth and centrifuged as described by NEVILLE[9]. Two changes were made: (1) homogenization of the liver was reduced to 10 strokes in a loosely fitting Potter–Elvehjem Teflon–glass homogenizer, essentially as described by EMMELOT AND BOS[10]; and (2) no further homogenization was done. Instead, the pellets from each washing were re-suspended gently but completely with a Pasteur pipette. The washing volumes (of 1 mM $NaHCO_3$) were successively decreased from 45 to 39 ml and the membranes were re-suspended in 10 ml for the final washing. Occasionally, and especially if livers from old rats were utilized, it was desirable to wash twice with 39 ml. It was essential to remove only the upper tan "fluffy" layer, avoiding the lower dark and viscous

layer, before re-suspending in the final 10 ml 1 mM $NaHCO_3$. The 3.1 ml of the "membranes" removed from the top of the final pellet, were mixed with 5.8 ml sucrose of density 1.34 g/ml and discontinuous sucrose gradients formed as described by EMMELOT *et al.*[11] and BARCLAY *et al.*[7] except that now an increased volume (1.3 ml) of the 1.18 g/ml sucrose was used. After centrifugation for 75 min at 30000 rev./min in the Sw-39 rotor (Beckman-Spinco) the membranes, usually a well-packed layer, could be removed easily. When necessary, these were stored in sucrose at 4°.

When enzyme assays were to be made to monitor for contamination and to estimate yield of membranes, the membranes were washed free of sucrose as soon as they were obtained. Three washings with 1 mM $NaHCO_3$, each followed by centrifugation at 40000 rev./min for 15 min in the Beckman–Spinco No. 40 rotor, were sufficient for most experiments. Membranes were suspended in Tris–HCl, pH 7.6, when the activities of (Na^+–K^+)-ATPase[12] (EC 3.6.1.3) and 5'-nucleotidase[13] (EC 3.1.3.5) were to be measured.

The following procedures for enzyme assays were used: a sample of whole liver homogenate was tested immediately for cytochrome *c* oxidase[14] (EC 1.9.3.1), glucose-6-phosphatase[15] (EC 3.1.3.9), ATPase and 5'-nucleotidase. As soon as the membranes were isolated and washed essentially free of sucrose, they were tested for activities of cytochrome *c* oxidase, malate dehydrogenase[16,17] (EC 1.1.1.3.7) and monoamine oxidase[18,19] (EC 1.4.3.4). Activities for the more stable enzymes, 5'-nucleotidase, glucose-6-phosphatase and ATPase, could be measured the following day. The mitochondrial layer which was collected from the interphase between density solutions 1.18 and 1.22 g/ml was occasionally monitored for the activities of cytochrome *c* oxidase, monoamine oxidase, and malate dehydrogenase.

In addition to enzyme assays, the membranes were observed routinely under the phase contrast microscope, and occasionally pellets were prepared for examination with the electron microscope.

The diphenylamine test[20] was used to determine the presence or absence of DNA, and the orcinol test[21] was used for RNA on 1-ml samples containing 3–5 mg membrane protein. These tests were performed immediately after washing the membranes in order to minimize nucleic acid enzyme effects.

After at least 10–14 preparations had been isolated (they were stored at 4° in the sucrose of the gradient), the membranes were washed with 1 mM $NaHCO_3$, pH 7.5. They were then suspended in phosphate buffer, pH 7.4, *I* 0.05, and treated with ultrasonic irradiation in 3-ml samples. An M.S.E. ultrasonic disintegrator, 60 W, 20 kcycles/sec was used. The probe was chilled in an ethanol–ice bath at —10° and the final temperature of the sonicated solution did not exceed 10°. The probe and samples were chilled at intervals when long sonication times were tried. Different time intervals (1.5, 3.0, 6.0 and 9.0 min) were used to study effects of ultrasonic irradiation on the solubilization of membrane suspensions, and 10 or 90 sec exposure upon the lipoprotein fractions isolated from the membranes as described in Fig. 1.

In addition to sonication in the phosphate buffer, other solvents were tried: 0.25 M sucrose, distilled water, the phosphate buffer prepared in ^2H_2O and 0.15 M NaCl–phosphate buffer, pH 7.4. When enzyme assays were planned, the membranes were suspended in the buffers appropriate for the particular enzymes to be studied, and then sonicated. Membranes solubilized by sonication were examined in the analytical ultracentrifuge (for either sedimenting or floating components) in the phos-

phate buffer and also after raising the solution density with KBr. Two different salts, solid KBr and NaCl, were used in some experiments to prepare the density gradients for separation of the different lipoproteins. Crystalline sucrose was also used. Ficoll solutions proved too viscous at higher concentrations to obtain accurate measurements of solution densities with a micropycnometer at 25°.

Separation of lipoprotein subunits. Fig. 1 illustrates the details of the separation of the lipoprotein subunits. Preparative separations were performed for 19.5 h at 15° (except lipoprotein C which took 21 h) in the 50 rotor at 50000 rev./min (g_{av} 150925) in the Beckman–Spinco Model L preparative ultracentrifuge. These conditions provided sufficient force to concentrate the lipoproteins into the top 1 or 2 ml. The densities selected finally, after testing many, separated the lipoprotein components sufficiently well that, for example, the third ml down the rotor tube would not contain any of the lipoprotein.

Usually a pool of 10–14 or more membrane preparations yielded sufficient amounts of the different lipoproteins to produce adequate and reproducible boundaries with schlieren optics in a double sector cell with the analytical ultracentrifuge at a usual speed of 31410 rev./min. Comparable salt reference solutions were carried along and identical volumes were removed to provide accurate base lines for calculations. These served also as solvent references in the determinations of viscosities, refractive indices, and hydrated densities of the lipoproteins (see below, *Other physical and chemical characteristics*).

The final infranatant solution (d>1.21 g/ml, Fig. 1, Step III) contained soluble protein*, the sedimentation coefficient and electrophoretic mobility of which were determined.

Lipid to protein ratios in lipoprotein subunits. Lipoproteins from several preparations were studied after dialysis against distilled water. Each floating pellicle, if present (see Fig. 1, Step II), was removed entirely from all samples to be tested and was studied independently. Total protein was determined with the procedure of LOWRY *et al.*[22] on a portion of the sample. The known remainder was extracted with chloroform–methanol as described previously for total lipid of whole membranes[23]. The lipid extracts were transferred to weighed tubes, evaporated and weighed to constant weight.

Other physical and chemical characteristics. Sephadex G-100, G-200 and Bio-gel 300 columns were used to separate and characterize approximate molecular weights of solubilized lipoproteins. All Sephadexes were used in a column (0.9 cm × 15 cm) with collection of 1-ml samples. Total protein was determined on the total sample applied to the column and on each fraction. A larger column, 1.5 cm × 60 cm, was also utilized. Moving boundary electrophoresis in phosphate–NaCl buffer, pH 7.5, I 0.2, at 1° in the Perkin–Elmer instrument 238 was employed to study some of the membrane proteins and/or lipoproteins.

Viscosities were measured at 25° in an Ostwald capillary tube viscometer and refractive index increments in the Abbé refractometer at the same temperature.

The lipoproteins of each class as defined by density were studied in the analytical

* Chemical analyses showed that all isolated components contained both protein and lipid. The ratios varied greatly, from 0.5 to 3. Components which sedimented in a solution density as high as 1.21 g/ml are usually referred to herein as proteins or "soluble proteins" and those which floated, as lipoproteins. However, all are lipid–protein complexes.

ultracentrifuge at two or three different densities, and a plot of nS_t *versus* density was prepared to estimate hydrated densities.

Specific refractive increments of lipoproteins A, B and C were determined from the refractive index increments according to LINDGREN *et al.*[24] so that concentrations of the different lipoproteins could be calculated.

When isolated lipoproteins were re-centrifuged, this was done in the Spinco 50 rotor at 50000 rev./min for 19.5 h using adaptors with tubes accommodating smaller volumes, usually 2–4 ml.

Effects of sonication and re-sonication upon the different lipoproteins were studied with ultracentrifuge analyses. Activities of 5'-nucleotidase and ATPase were measured before and after sonication for different time periods. The specific activities of these enzymes were measured in the different lipoproteins and proteins after dialysis against Tris–HCl buffer, pH 7.5, to remove the KBr.

Effects of freezing. Four membrane preparations were suspended in phosphate buffer and divided into two parts of equal protein concentrations and densities. One was frozen at —20° for three days, then allowed to thaw at 20°. This was repeated. Both samples were then sonicated, adjusted to the same solution density, 1.21 g/ml, and studied in the analytical ultracentrifuge to test the effects of freezing upon membrane lipoproteins.

Amino acid analyses of solubilized lipoproteins. Amino acid analyses were performed on lipoproteins separated at densities 1.125, 1.17 and 1.21 g/ml, on the pellicle lipoprotein which separates out principally at density 1.17 g/ml, and on the remaining soluble protein obtained during these separations (consult Fig. 1). Before the lipoproteins and proteins were studied for their amino acid content, they were dialyzed and de-lipidized according to the procedure of GRANDA AND SCANU[25].

The amino acids were determined as described by SALSER* AND BALIS[26] on acid-hydrolyzed samples run through the Beckman–Spinco Amino Acid Analyzer. The hydrolysis proceeded for 24 h at 110° under reduced pressure with 6 M HCl, which was removed subsequently by lyophilization. The residue was dissolved in citrate-diluting buffer, pH 2 (Beckman *Procedures Manual*). The amino acid analyzer had been modified for greater sensitivity and increased resolution. Duplicate analyses were carried out.

RESULTS

Isolation, as described above, of plasma membranes from rats 8–10 weeks of age and weighing 250–350 g yielded at least 1 mg membranes from 1 g fresh liver. If younger, more immature, rats (less than 8 weeks of age and weighing about 150 g) were used, the yield of membranes was diminished. From somewhat older, greater than 35 weeks, heavier rats, one obtained quantities of membranes comparable to younger animals, but the lipoprotein levels were greatly changed as discussed below.

Essential elimination of homogenization reduced fragmentation of the membranes and very few mitochondria were evident when preparations were monitored by phase or electron microscopy. The addition of a greater volume of the sucrose, density 1.18 g/ml, and the subsequently wider area between the membrane and mito-

* The authors are grateful to Dr. Josephine Salser for performing the amino acid analyses.

chondrial layers was a simple device to reduce the presence of mitochondria within the membrane layer and avoid the risk of contamination during removal from the gradient. In addition, the values for cytochrome *c* oxidase activity in membranes were routinely low, averaging 0.01 ΔA unit/mg protein per min compared with 3.1 ΔA units in liver homogenate and 45 units in isolated mitochondria. There probably was a minimum of mitochondrial fragmentation as suggested by the low values for the enzyme localized in the inner mitochondrial membrane (0.4 ΔA unit of malate dehydrogenase in plasma membranes compared with 5.9 units in mitochondria) and the enzyme localized in the outer mitochondrial membrane (0.003 ΔA unit of monoamine oxidase in plasma membranes compared with 0.25 ΔA unit in mitochondria).

The two enzymes which serve as principal markers for plasma membranes from liver parenchymal cells[27] had sufficiently greater specific activities in the isolated membranes than in the liver homogenate to show accumulation of membranes. After elimination of homogenization during the washing steps, the values for 5'-nucleotidase were routinely higher (ranging from 1.99 to 2.10 μmoles P/mg protein per min in 6 experiments), than they had been previously (1.34–1.50 μmoles in 5 experiments). The values in whole liver homogenate ranged from 9 to 17.0 μmoles.

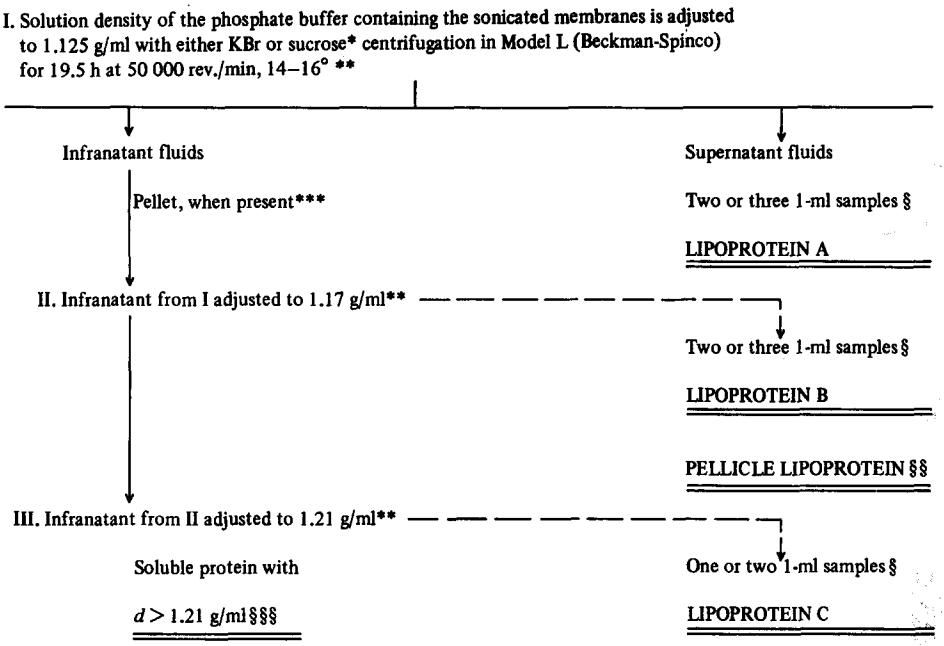
The values for ATPase were not increased by changing the technique; they ranged from 50 to 62 (mean 58) μmoles P/mg protein per h in membranes from young rats with a mean of 4.6 moles in whole liver homogenate.

Seven different membrane preparations, over a period of 1 year, were tested for the presence of nucleic acids. Neither DNA nor RNA were detected by the diphenylamine or orcinol reactions in any preparation even when 5 mg membrane protein was tested, although the orcinol method readily detected 25 μg RNA in a standard.

When whole membrane preparations, washed and suspended in phosphate buffer, pH 7.4, or Tris–HCl buffer, pH 7.5, were sonicated from 1.5 to 3 min at 10° or less, the cloudy suspension became a clear opalescent solution. It then resembled the appearance of the soluble lipoproteins in serum. The density of the solution after sonication varied with the number of preparations used. After adjustment to the density 1.063 g/ml, which should have permitted the separation of the usual soluble low-density lipoproteins, the solution was centrifuged for 19.5 h at 50000 rev./min. The top 1 ml contained no floating lipoproteins when examined in the analytical ultracentrifuge. None was observed in two successive 1-ml samples removed from the tops of the tubes containing 6 ml total solution. Thus it appeared that there were no classical low-density lipoproteins of the type separated from serum. On the other hand, higher densities and ultracentrifugation revealed the presence of high-density lipoproteins.

Fig. 1 shows the separation procedure for these high-density lipoproteins, *e.g.* those with densities >1.063 g/ml, using the standard classification for lipoproteins in blood plasma. Fig. 2 illustrates some of the evidence that lipoprotein complexes can be isolated from membranes by this procedure. The membrane components which had lower lipid to protein ratios and behaved as proteins under the experimental conditions used are shown also, *e.g.* Figs. 2-II and 2-VI.

Fig. 2-I shows lipoprotein A floating in a density slightly less than 1.125 g/ml. It had an $s_{f\,(1.125)} = 11.35$ under these conditions. This represented 25–30% of the total A + B + C (see Fig. 1) exclusive of other components in a sonicated solution



* Either sucrose or KBr in phosphate buffer at pH 7.4 can be used, but sucrose gives distorted schlieren boundaries with lipoproteins in the analytical ultracentrifuge.

** Runs in the preparative ultracentrifuge were done under these conditions, except for lipoprotein C which was separated after 21 h.

*** Re-suspended in phosphate buffer, pH 7.4, re-sonicated and studied. See text.

§ Individual samples were analyzed in the Model E Ultracentrifuge for the presence or absence of components, usually in the double sector cell at 31 410 rev./min.

§§ Pellicle collected, re-suspended in phosphate buffer, pH 7.4, re-sonicated and studied. See text.

§§§ Although this material has a lipid to protein ratio 0.50, it behaves as a protein under the separation density conditions used.

Fig. 1. Scheme for the density gradient separation of lipoproteins and proteins from plasma membranes solubilized by 3-min ultrasonic vibration.

containing sufficient membranes to produce a reliable boundary for component C.

Fig. 2-II shows the protein or "lipoprotein" which was in the insoluble pellet after separation, Step I on Fig. 1. This material was solubilized only by sonication. When the density was increased to 1.125 g/ml, this now solubilized protein sedimented.

Fig. 2-III illustrates the floating lipoprotein B with $d<1.17$ g/ml obtained after Step II on Fig. 1. It had an s_f (1.17) = 9.0 and, of the soluble lipoproteins, was present in the largest quantity (exclusive of the pellicle lipoprotein), approx. 55–60 % of the total lipoproteins A, B, and C. The boundary formed was sharper than those of either lipoproteins A or C.

Whereas lipoprotein B (Fig. 2-III) was in solution after flotation in d = 1.17 g/ml, the lipoprotein pellicle floated as a membranous layer on the surface. This was quantitatively removed. When suspended in phosphate buffer and sonicated for 10 sec the pellicle became soluble. Upon examination in the analytical ultracentrifuge, the large floating boundary seen in Fig. 2-IV was seen. There was usually a substantial

amount of this lipoprotein and it had a rapid flotation rate, $s_{f\,(1.17)} = 40.2$ when centrifuged at $d = 1.17$ g/ml. Since the specific refractive increment was not determined, the exact amounts obtained were not calculated, but qualitatively there was obviously an amount equal to or greater than that of lipoprotein B.

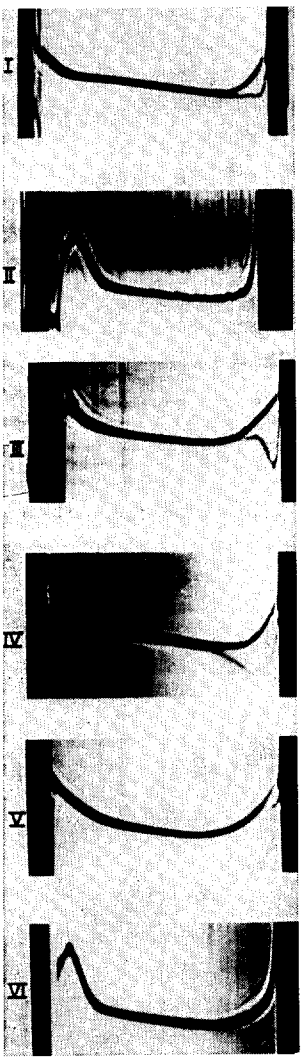


Fig. 2. Analytical ultracentrifuge pictures of lipoproteins and proteins isolated according to the scheme in Fig. 1. The boundaries in I, III, IV and V are moving from right to left; those in II and VI from left to right. (I) Lipoprotein A. Soluble lipoprotein floating in 1.125 g/ml KBr, 4 min after reaching 31410 rev./min. (II) Protein insoluble in KBr. Resuspended in phosphate buffer, sonicated and density increased to 1.125 g/ml with KBr, 48 min after reaching 31410 rev./min. (III) Lipoprotein B. Soluble lipoprotein floating in 1.170 g/ml KBr, 18 min after reaching 31410 rev./min. (IV) Pellicle. Insoluble lipoprotein floating in 1.170 g/ml suspended in phosphate buffer, pH 7.4, sonicated and density increased to 1.170 g/ml with KBr, 4 min after reaching 31140 rev./min. (V) Lipoprotein C. Soluble lipoprotein floating in 1.210 g/ml KBr, 20 min after reaching 31410 rev./min. (VI) Residual soluble protein. Dialyzed against phosphate buffer to remove KBr. Protein sedimenting in phosphate buffer at 16 min after reaching 59780 rev./min.

Fig. 2-V shows the proportionately smaller boundary obtained with soluble lipoprotein C. It moved (floated) more slowly, even at the high solution density of 1.21 g/ml, than either lipoproteins A or B at their respective densities. The quantity, 10–15 %, was smaller than that of A or B, and at least 14 membrane preparations were necessary to obtain a sufficient quantity to see boundaries with schlieren optics.

The proportional amounts (B>A>C) for lipoproteins A, B and C were obtained regularly when rats weighing 250 to 350 g were used. However, when the rats were large (550–675 g), and at least 1 year old, there was a marked alteration in the quantitative distribution of these lipoproteins. The average of five typical experiments with older, heavier rats showed 91.7 % for lipoprotein A; 8.3 % for lipoprotein B and no lipoprotein C.

After the above soluble lipoproteins and insoluble protein were removed, the protein or "lipoprotein" shown in Fig. 2-VI remained in the infranatant solution. After dialysis against phosphate buffer, pH 7.4, to remove the KBr, and analytical ultracentrifugation, it appeared as a slowly moving component with an $s = 2.67$ in phosphate buffer.

In several typical experiments in which fractions were separated (Fig. 1), there was good recovery of total protein, about 89 %, considering the amount of necessary manipulation.

The lipid to protein ratios for lipoproteins A, B and C were 2.17, 1.38 and 1.02, respectively. These were consistent with the flotation properties of the lipoproteins (see Table II).

Lipoprotein A had a higher specific activity for 5'-nucleotidase than either lipoproteins B, C or the pellicle. Similar results were obtained for the activity of ATPase (Table I). In whole membranes, the activity of this enzyme was decreased after sonication, for example, from a value 51 μmoles P per mg protein without sonication, to 7.2 μmoles after 9 min sonication. Therefore, all values in the lipoprotein subunits were undoubtedly lower than in the native, unsonicated lipoproteins. Sonication, even for 9 min, did not decrease the specific activity of the 5'-nucleotidase.

In Table II are shown other properties of the lipoproteins. The hydrated densities increased as the s_f properties decreased. The concentrations for lipoproteins A, B and C reflected the proportional quantities routinely obtained from analyses with the ultracentrifuge and also from the proportional amounts of total protein in the fractions isolated according to Fig. 1. For example, average total protein values for

TABLE I

ENZYME ACTIVITIES OF RAT LIVER PLASMA MEMBRANE LIPOPROTEINS

Whole membranes or lipoprotein subunits	*5'-Nucleotidase (μmoles P/mg protein per min)*	*(Na^+–K^+)-ATPase (μmoles/mg protein per h)*
Whole membranes	2.0	51.1
Whole membranes, sonicated 3 min	2.0	43.0
Lipoprotein A	3.8	33.7
Lipoprotein B	1.7	15.9
Lipoprotein C	0.8	7.7
Pellicle lipoprotein	1.6	6.2

lipoproteins B, A and C were 9.54, 4.32 and 2.35 mg of a total of 76.8 mg in this 14-membrane experiment.

An intriguing component was the so-called pellicle lipoprotein which floated as a film when the density was increased to $\geqslant$ 1.17 g/ml. The pellicle had a quasi-crystalline structure. It separated into long needles when the film was disturbed. Fig. 3 is a phase contrast microscopic picture taken at a magnification of 80 ×. It was necessary to view and photograph these structures without a cover glass because of their fragility. A lamellar structure was suggested from the appearance at the edges

TABLE II

SOME PHYSICOCHEMICAL PROPERTIES OF LIPOPROTEINS FROM MEMBRANES

Component	*Hydrated density (g/ml)*	*Specific refractive increment (Δn/g per 100 ml)*	*Lipoprotein* concentration (mg/100 ml)*	$-s_f$** × 10^{-13}	s** × 10^{-13}
Lipoprotein A	1.074–1.076	0.00072	119.3	9.5–14.0	
Lipoprotein B	1.120–1.128	0.00052	244.6	7.0– 9.0	
Lipoprotein C	1.171–1.179	0.00122	54.7	3.0– 4.8	
Insoluble pellet					2.66
Pellicle lipoprotein			39.0–42.0		
Soluble protein					2.67

* For these experiments, 40 preparations gave 100 ml sonicate, equivalent to about 800 mg membranes.

** Svedberg unit of sedimentation (or flotation) coefficient.

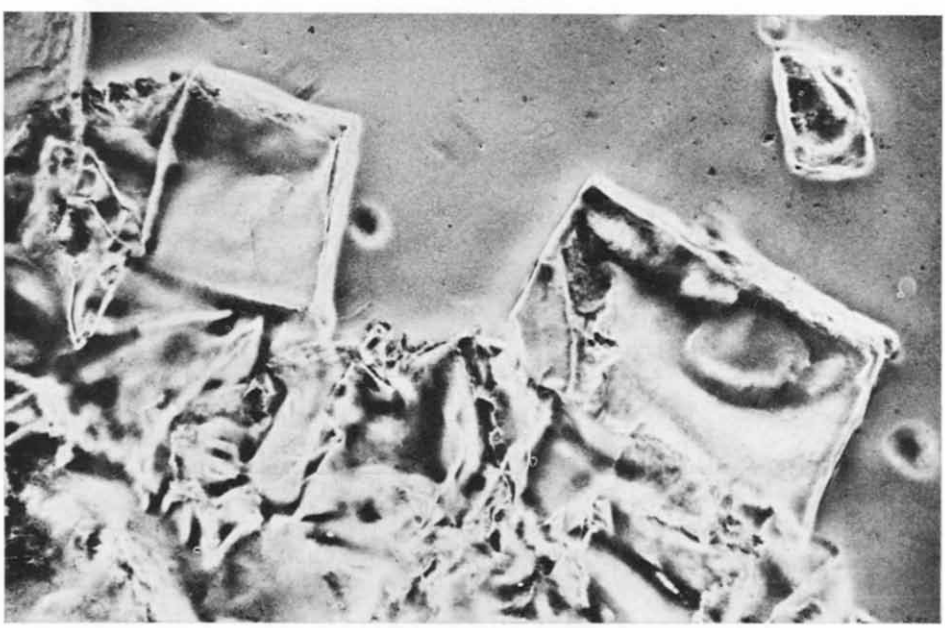


Fig. 3. Phase contrast micrograph of isolated pellicle lipoprotein, magnified 64 ×.

of the fractured areas. In experiments where the lipid to protein ratios were determined it was essential to remove all this pellicle before analyses. The lipid to protein ratio, 3.1, of the pellicle resulted from large amounts of lipid.

The pellicle lipoprotein was insoluble in all salt solutions tested, and was completely solubilized by only 10 sec of ultrasonic irradiation. When studied in the analytical ultracentrifuge at $d = 1.17$ g/ml, the typical lipoprotein schlieren boundary was seen, Fig. 2-IV. It had a high s_f (1.17) = 40, uncorrected for JOHNSTON–OGSTON[28] effects and the self-slowing of flotation rates when the concentration of lipoprotein was increased.

When the insoluble protein, $d > 1.125$ g/ml, was solubilized and studied in the analytical ultracentrifuge as illustrated in Fig. 2-II, it had an $s = 2.66$ in phosphate buffer. The soluble "lipoprotein", $d > 1.21$ g/ml, Fig. 2-VI, had a similar sedimentation coefficient under the same conditions, 2.67 (Table II).

All proteins and lipoproteins were excluded by the Sephadex and Bio-gels used, suggesting molecular weights over 200000 for the intact molecules.

When the solution containing the residual soluble protein was dialyzed to remove the KBr the protein appeared as a single boundary in moving boundary electrophoresis in phosphate–NaCl buffer at pH 7.5, I 0.2. The mobility was $+4.87 \cdot 10^{-5}$ cm$^2 \cdot$V$^{-1} \cdot$sec^{-1}. This was a very basic protein, with lysine comprising 25% of its amino acids (see below).

When four membrane preparations of comparable protein content were sonicated for different periods of time, 1.5, 3.0, 6.0 and 9.0 min and then adjusted to a solution density of 1.21 g/ml and centrifuged for 19.5 h at 50000 rev./min, all resulting amounts of lipoproteins, shapes of boundaries and s_f rates after analytical ultracentrifugation were essentially the same in all samples.

Sonication of different membrane suspensions for either 1.5 or 3 min, followed by sequential adjustment and preparative centrifugation according to the procedure outlined in Fig. 1 gave comparable values and s_f rates for lipoproteins A, B and C regardless of sonication times.

A crucial point in evaluating the effects of different sonication times on the lipoproteins was the presence or absence of the insoluble pellicle in the separated lipoprotein fractions. In an experiment to illustrate this, 10 membrane preparations were sonicated 1.5 min and the lipoproteins separated according to Fig. 1. The top 1 ml was removed from each of the three 50-rotor tubes at each stage of the separation. These samples were studied in the analytical ultracentrifuge. The separated lipoprotein fractions were then re-sonicated for 10 sec and re-studied in the analytical ultracentrifuge. When pellicle lipoprotein was present, as in lipoprotein B which can be associated with large amounts of pellicle, the pellicle was re-solubilized by the sonication and contributed to the size and movement of the boundary in lipoprotein B. However, if all the pellicle had been removed from the solutions of lipoproteins, there were no changes in the sizes, shapes nor s_f properties after re-sonication.

Ultrasonic irradiation of the lipoproteins in distilled water resulted in flocculation and denaturation. A solution of 0.25 M sucrose also was an unsatisfactory solvent. Phosphate buffers prepared in ^2H_2O were satisfactory thus permitting studies with nuclear magnetic resonance if desired.

When the separated lipoproteins from membrane preparations, other than those used for studies on the effects of ultrasonic irradiation, were collected and re-cen-

trifuged for an additional 19.5 h, neither the size, shape nor the s_f rate of the lipoprotein boundaries was affected in any way.

If sucrose were used to prepare the gradients in separations such as those in Fig. 1, floating lipoproteins were obtained. These were comparable to those obtained when KBr was used. After dialysis of the sucrose solutions against KBr solutions of the same density the lipoprotein boundaries were similar in size and shape to those obtained when KBr was used for the separations.

Freezing membrane preparations had a deleterious effect upon the lipoproteins. In the frozen samples there was 66% less lipoprotein and the s_f rates were lower than in the unfrozen samples with the same protein concentration. Isolated membranes intended for the study of their lipoprotein components should not be stored in phosphate buffer or in water, nor should they be frozen. Furthermore, they should not be treated with detergents, especially if lipoprotein studies are planned.

Amino acid analyses of lipoproteins. Fig. 4 shows the logarithms of the mole fractions (percent) for the amino acids, determined with the Beckman–Spinco Amino

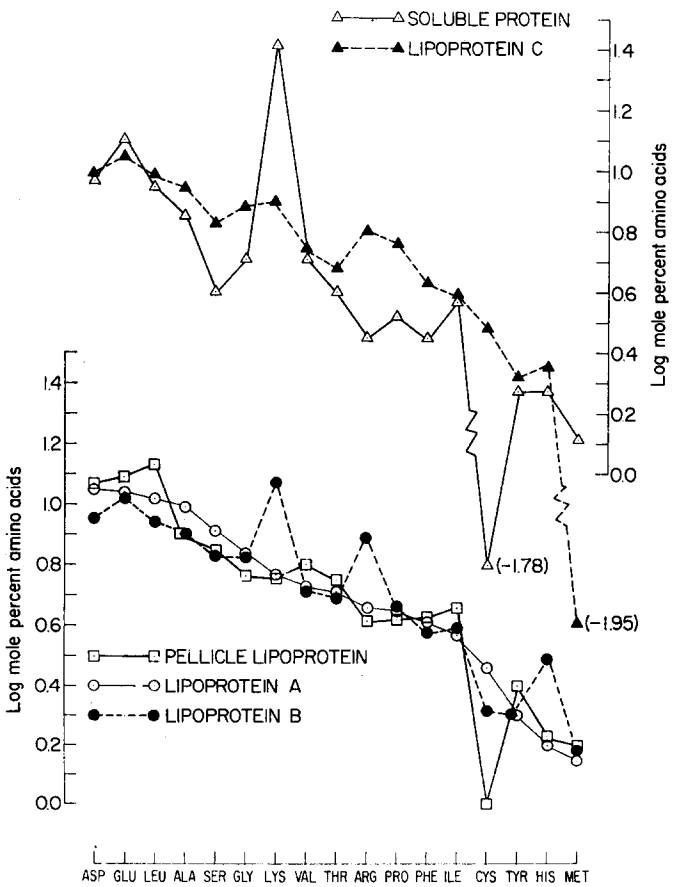


Fig. 4. Logarithmic plots of the amino acids in lipoprotein A (○—○) in descending order of values, lipoprotein B (●---●) and the pellicle lipoprotein (□—□) on the left scale (lower). On the right scale (upper) are lipoprotein C (▲---▲) and the residual soluble protein (△—△).

Acid Analyzer, of five of the fractions of lipoproteins and proteins. These were plotted according to the procedure of COOK *et al.*[29] and COOK AND MARTIN[30]. Several differences between these fractions were obvious. The values for A (open circles) were plotted in descending order, and the corresponding plots for both lipoproteins B (closed circles, lower scale) and lipoprotein C (closed triangles, upper scale) were compared with those of lipoprotein A. This comparison showed that lipoprotein B had the greatest quantities of all three basic amino acids, lysine, arginine and histidine, followed by lipoprotein C and then by lipoprotein A. The pellicle lipoprotein (open squares), which separated out at the same solution density as lipoprotein B, had more leucine, tyrosine and slightly more isoleucine than either lipoproteins A or B. The pellicle had somewhat less half-cystine and much less lysine, arginine and histidine than lipoprotein B. When lipoprotein C was plotted against the soluble protein (open triangles, upper scale) remaining in the infranatant fluid upon which lipoprotein C floated at solution density 1.21 g/ml, it was obvious that the residual soluble protein had significantly more lysine. This protein had more lysine than any of the components, conferring more polarity and basicity to it. The soluble protein had lower quantities of several amino acids compared with the other lipoproteins.

The slopes of the lines and amounts of amino acids were most similar in lipoproteins A and C, and to some extent also, the pellicle lipoprotein.

DISCUSSION

In order to separate lipoprotein subunits or subfractions from plasma membranes it was necessary to obtain an adequate quantity of membranes that were as pure as possible. The isolation procedures employed supplied this need. Because the age and weight of the rat also affected the proportional quantities of the lipoproteins obtained, as well as the specific activity of ATPase in particular (M. BARCLAY, V. P. SKIPSKI AND O. TEREBUS-KEKISH, unpublished), it was important to select rats that were sexually mature but not too old.

In the course of isolating the membranes care was taken to avoid the viscous layer beneath the fluffy membrane layer. This may have accounted for the negative results when the whole plasma membranes were tested for RNA. Tests for RNA have been positive in several laboratories, EMMELOT AND BOS[10], DAVIDOVA AND SHAPOT[31], and RAY[32] to cite a few, and incidentally, all used $CaCl_2$ in the medium. DAVIDOVA AND SHAPOT[31] observed no RNA in the saline-soluble portion of total plasma membranes but did observe evidence for RNA in the saline-insoluble portion. This comprises 75 % of the total membrane protein and our experiments with the analytical ultracentrifuge have shown this to indeed be lipoprotein. Since DAVIDOVA AND SHAPOT[31] state that at least 0.5 mg of membrane protein (saline-insoluble membrane protein) was necessary to detect RNA, and we tested 3–5 mg, apparently an adequate quantity of the whole membrane was used in our experiments even though the sample may have been "diluted out" by the 25 % saline-soluble.

If RNA is indeed an integral part of the lipoprotein(s) of the plasma membrane as DAVIDOVA AND SHAPOT[31] believe, our current studies to test the lipoprotein subunits should be helpful. The different lipoproteins are concentrated by the technique in Fig. 1; it remains to be seen whether the other aspects of the technique (sonication, high salt concentrations and dialysis) will affect the "liporibonucleoprotein complex" if present.

Increasing the sonication time and sonicating briefly even the isolated lipoproteins did not increase nor decrease the amount of lipoprotein or change its flotational (s_f) properties. This treatment, as utilized, certainly did not cause disassociation such as occurs with sodium dodecyl sulfate or deoxycholate, and aggregation seems not to have occurred since physical properties remained the same. Although KBr was usually used to prepare the density gradients both NaCl and sucrose permitted separation of the lipoproteins, in quantities obtained with KBr.

The pellicle lipoprotein requires more attention. It was present in quite large quantities, usually associated with lipoprotein B at a density of 1.170 g/ml, but it could also appear at a lower density, 1.125 g/ml. This may be a less aggregated form or it may have a different lipid to protein ratio. When old animals were studied, there was more pellicle associated with lipoprotein A. The pellicles were always quite insoluble in aqueous media, and although undoubtedly heterogeneous (from the wide, skewed boundary in the analytical ultracentrifuge and appearance under the phase microscope) they may play important structural roles in the membranes. These lipoproteins may resemble the paracrystalline lipoprotein layer cited by FERNÁNDEZ-MORÁN[5]. The material, as isolated, also had enzyme activity suggesting a functional role as well.

The particulate subfractions with densities of 1.12 and 1.21 g/ml obtained by EVANS[33,34] may have been related to the lipoproteins A and C in the work reported herein. Lipoprotein A had a lipid to protein ratio (2.2) 2-fold greater than lipoprotein C (1.02). This difference was of the same order as subfractions A and B of Evans. The ratios of EVANS' subfractions were derived from phospholipid divided by protein values, but may be comparable with those of the lipoproteins since of the total lipid in membranes from which they were derived there were significantly more phospholipids than "neutral" lipids[23]. Except for the pellicle, most lipid was associated with the three lipoproteins. From the differences in the densities of EVANS' particulate subfractions and the lipoproteins described in this report, higher ratios of lipid to protein should be expected in the less dense fractions.

Since all the lipoproteins obtained from sonicated membranes (Fig. 1) had been exposed to KBr and were dialyzed before enzyme assays were carried out, some loss of activity could have occurred. Nevertheless, the activities of the enzymes in the lipoprotein subunits could be compared with each other because they received the same treatment, except for the differences in concentrations of KBr. However, the activities observed for the lipoproteins could not be compared precisely with those in the original whole membrane suspension or sonicated membranes. The activity of the ATPase, especially, could not be accurately related between the sonicated whole membrane preparations and the lipoprotein subunits.

Lipoprotein A, of the three different lipoprotein subunits, had the greatest specific activities for both 5'-nucleotidase and ATPase. If this lipoprotein subunit can be reasonably compared with EVANS' subfraction A, Evans also noted: "...5'-nucleotidase... found in subfraction A at approximately fourfold the activity of... subfraction B". Evans also noted higher values for the activity of subfraction B (according to density properties, possibly comparable with lipoprotein C) than in his unfractionated membranes, but we did not observe this. Lipoprotein B, however, did have notable specific activities for both enzymes, but not so great as lipoprotein A.

Although EVANS' subfractions and the lipoprotein subunits described here were

obtained by different means, both experiments showed that the two enzymes defined as "membrane-localized" were probably associated more specifically with certain components, particularly those with the lowest density (1.12 g/ml) and rather high lipid to protein ratios. SONG *et al.*[35] also observed greater 5'-nucleotidase activity in the lighter (d = 1.16 g/ml) of the two plasma membrane fractions of rat liver homogenates separated by sucrose gradients. DE DUVE[36] has reported recently that the 5'-nucleotidase and other enzymes in plasma membranes subfractionated by treatment in an Ultra-Turrax blender, segregated with fragments of different density. He states that "this suggests that the plasma membrane is composed of biochemically distinct areas", a suggestion with which we agree.

The relative quantities of amino acids in EVANS' light subfraction[34] were remarkably similar to those in lipoprotein A. In both, glutamic acid, aspartic acid, leucine, glycine and alanine were present in the greatest quantities.

Three of the lipoprotein subunits were present routinely in predictable proportional quantities (B>A>C), but it must be emphasized that this occurred only in plasma membranes isolated from livers of young rats weighing 250–350 g. This could indicate firstly, that these lipoprotein subunits might indeed be integral components of the membrane, and secondly, that they might be regularly arranged in the organized membrane structure according to their chemical and physical properties, which are different.

The proportional amounts of lipoproteins A, B and C were altered when animals weighed somewhat more (550–675 g and over) and were older. Preliminary observations by the authors indicated that a shift toward greater quantities of lipoprotein A occurred. This was accompanied by a greatly diminished quantity of lipoprotein B (even when 20 preparations were pooled) and little or no lipoprotein C. Thus the shift was in the direction of an accumulation of lipoproteins with lighter densities resulting probably from increased lipid.

Although the three high-density lipoproteins were separated (after solubilization) by procedures similar to those used with blood serum, these three lipoproteins were profoundly different from the soluble lipoproteins of plasma. Whereas the high-density lipoproteins in serum have been characterized by a higher content of protein and less lipid (L/P <1.0) (SKIPSKI *et al.*[37]), lipoproteins A, B and C, as well as the pellicle lipoprotein from membranes had lipid to protein ratios greater than 1.0. Therefore, although the chemical composition, suggested by these ratios, may have resembled the low-density lipoproteins of serum, their hydrated densities were such that they floated only after the solution density was raised to greater than 1.063 g/ml, and thus they resembled the high-density lipoproteins. Detailed lipid compositions of the lipoprotein subunits will be presented subsequently.

The protein moieties of lipoproteins A, B, C and the pellicle lipoprotein had somewhat higher amounts of the neutral amino acids than, for example, the residual soluble lipoprotein with the least lipid. Since neutral amino acids have the greatest tendency to form complexes with lipids, the presence of neutral amino acids in high quantities in these lipoproteins would be expected.

The residual soluble lipoprotein which had the greatest amount of protein, least lipid and was highly basic, could be part of the saline-soluble portion of the membrane. Both had the same sedimentation coefficient, 2.6. It was doubtful that a definite structural role could be attributed to it, but it did have some ATPase activity.

Because of its solubility it may have played a more important role in the permeability of the membrane and also imparted some flexibility to a necessarily more rigid structure composed principally of quite insoluble lipoprotein entities.

Observance of regular proportional quantities of the three high-density lipoprotein subunits and the large amount of more insoluble pellicle with its lamellar appearance in plasma membranes, presented chemical and physical evidence that the plasma membrane could be constructed from a combination of the bimolecular leaflet and globular subunit types of structures as WOLMAN[4] suggested. The pellicle lipoprotein may serve as the "keystone" in the structure, imparting the essential stability. Lipoproteins A, B and C are probably present in regular arrays, sets or groups of similar but not identical lipid–protein complexes. These lipid–protein complexes which may comprise part of the substructures insoluble in 0.15 M NaCl could provide both structure and function (in their enzyme relationships). The exchange of aqueous-soluble metabolites across the cell membrane and the flexibility of the membrane could reside in the more soluble lipoprotein component with its large complement of protein and smaller complement of lipid.

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