Protein and Carbohydrate Composition of Lp(a) Lipoprotein from Human Plasma†

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ABSTRACT: Purified Lp(a) lipoprotein from human plasma can be dissociated into three components with different mobilities in polyacrylamide gel electrophoresis. The immunological properties, electrophoretic mobility and flotation behavior of these components indicate that one contains plasma low density lipoprotein (LDL) and another albumin. The third component, which we call the Lp(a) protein, carries the Lp(a) antigenic determinant. Quantitative studies show that the protein moiety of the Lp(a) lipoprotein contains about 65% of LDL apoprotein, 20% of Lp(a) protein and less than 15% of albumin. The carbohydrate content of the Lp(a) lipoprotein is 0.26 mg/mg of protein. This value is higher than for any other plasma lipoprotein. The sialic acid content is about six times as high, the hexosamines about three times, and the hexoses about twice as high in Lp(a) lipoprotein as in plasma LDL.

The Lp(a) lipoprotein is a variant of the human β-lipoproteins (Berg, 1968). It can be purified by ultracentrifugation combined with chromatography on hydroxylapatite (Utermann and Wiegandt, 1969) or gel filtration (Ehnholm *et al.*, 1971). The Lp(a) lipoprotein resembles low density lipoprotein (LDL)[1] in some respects but differs in others. It has a higher molecular weight and different electrophoretic mobility (Simons *et al.*, 1970; Ehnholm *et al.*, 1971). The protein : lipid ratio and the density of the Lp(a) lipoprotein are higher (Utermann and Wiegandt, 1969; Simons *et al.*, 1970; Ehnholm *et al.*, 1971). In its amino acid composition the Lp(a) lipoprotein differs from LDL, whereas in its lipid composition it is similar if not identical to LDL (Simons *et al.*, 1970). The LDL antigens can also be found on the Lp(a) lipoprotein but the Lp(a) antigen cannot be detected on the LDL molecule (Utermann and Wiegandt, 1969; Simons *et al.*, 1970; Ehnholm *et al.*, 1971). The electron microscope shows that the Lp(a) lipoprotein particles are spherical and similar in appearance to LDL but have a larger diameter than LDL (Simons *et al.*, 1970). In this paper we report the carbohydrate and protein composition of Lp(a) lipoprotein (Ehnholm *et al.*, 1972).[2]

Materials and Methods

Lp(a) lipoprotein and LDL were purified as described earlier (Ehnholm *et al.*, 1971).

Electrophoresis. Disc electrophoresis in 3.75% acrylamide was performed as described by Garoff *et al.* (1970).

Immunological Methods. The anti-Lp(a) sera used have been described elsewhere (Garoff *et al.*, 1970; Ehnholm *et al.*, 1971). The anti-LDL serum was prepared by immunizing rabbits with lipoproteins of density from 1.019 to 1.060 g per ml in complete Freunds' adjuvant. Two injections 2 weeks apart were given subcutaneously. The rabbits were bled 2 weeks after the second injection.

Human albumin, anti-normal-human serum, and anti-albumin serum were from Behringwerke AG, Marburg a.d. Lahn. Double-diffusion test were performed according to Ouchterlony (1958) in 1% agarose. For immunological characterization of different bands in polyacrylamide gel electrophoresis, one gel was stained to localize the bands. The other gels were then sliced and the slices put on glass plates and covered with 1% agarose in 0.05 M barbital buffer (pH 8.6). Wells were cut in the agarose and were filled with antiserum after the antigen had diffused for 17 hr. The anti-albumin polymer was polymerized with ethyl chloroformate according to Avrameas and Ternynck (1967),

Chemical Analysis. Protein was determined according to Lowry *et al.* (1951) with bovine serum albumin as standard using 0.1% sodium dodecyl sulfate in the reaction mixture.

Amino Acid Analysis from Polyacrylamide Gel Slices. The procedure of Butler (1970) was essentially followed to determine quantitatively the separated components from polyacrylamide gels. The gels were made from acrylamide recrystallized from chloroform (Loening, 1967). Disc electrophoresis was performed as before except for the electrode buffer which was a Tris–borate buffer (0.025 M Tris–0.05 M boric acid, pH 8.0). The gels were fixed in 20% sulfosalicylic acid, stained with Coomassie Blue, and destained according to Weber and Osborn (1969). The bands were cut out and macerated in an equal volume of 12 N HCl. One crystal of phenol and 1 ml of 6 N HCl were added. The samples were hydrolyzed for 22 hr at 110°. After hydrolysis 1 ml of H_2O was added and the tube was cooled in ice. The polyacrylic acid precipitated and was removed by centrifugation. The supernatant was evaporated under vacuum, 0.5 ml of 1 N NaOH was added to liberate ammonia, and it was taken to dryness. This was repeated twice. Then 0.5 ml of 4 N HCl was added and the sample was dried. It was then dissolved in 1 ml of 0.2 M sodium citrate buffer (pH 2.2) and centrifuged. The supernatant was applied to the amino acid analyzer (Beckman-Spinco Model 120C). Half-cystine, methionine, and tryptophan could not be determined and the serine and glycine values were too high (Kyte, 1971). Therefore this

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[1] Abbreviations used are: LDL, low density lipoprotein; VLDL, very low density lipoprotein.

[2] A preliminary report of some of these findings has been presented earlier (Ehnholm *et al.*, 1972).

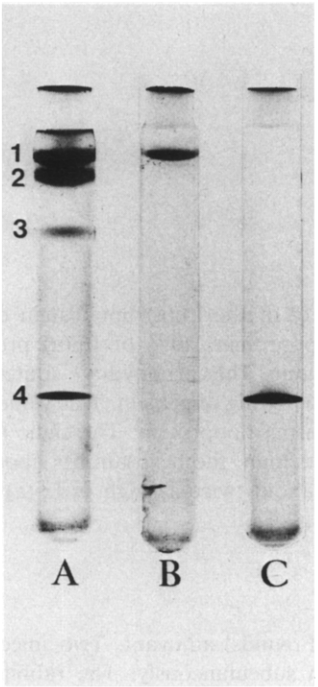

FIGURE 1: Electrophoresis in 3.75% polyacrylamide gels of: (A) stored Lp(a) lipoprotein, (B) Lp(a) lipoprotein recently prepared, and (C) albumin. Stain Coomassie Blue. Anode at the bottom.

TABLE I: Protein-Bound Carbohydrates in Lp(a) Lipoprotein and LDL.

	Hexose (μg/mg of Protein)	Hexosamine (μg/mg of Protein)	Sialic Acid (μg/mg of Protein)
1. LDL	47.4	24.7	11.7
Lp(a) lipoprotein	101.0	72.9	60.0
2. LDL	66.6	32.8	9.9
Lp(a) lipoprotein	113.0	101.0	79.0
3. LDL	49.8	27.2	9.7
Lp(a) lipoprotein	110.0	78.0	59.0
LDL mean	54.6 ± 10.5	28.2 ± 4.2	10.4 ± 1.1
Lp(a) mean	108.0 ± 6.2	84.0 ± 15.0	66.0 ± 11.3

method could not be used to determine the exact amino acid composition of these components.

Determination of Protein-Bound Carbohydrates. Samples containing 0.5–3 mg of protein were delipidated at room temperature with 1 ml of chloroform–methanol (1 : 4, v/v) before carbohydrate analysis to remove lipid-bound carbohydrate (Simons *et al.*, 1970; Tao and Sweeley, 1970) and to minimize charring during the subsequent anthrone reaction. Significant amounts of protein were not found in the lipid extracts when these were hydrolyzed and subjected to amino acid analysis. The delipidated protein was resuspended in water with 5 mg of cetyltrimethylammonium bromide. This detergent did not interfere with the carbohydrate assays. Hexoses were determined by the anthrone reaction as described by Hewitt (1958), using a 1:1 (w/w) mixture of mannose and galactose as reference standard. Hexosamines were assayed after overnight hydrolysis in 2 N HCl at 100° by the Elson–Morgan reaction as described by Gatt and Berman (1966). *N*-Acetylglucosamine was used as reference standard. The monosaccharides *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose, and galactose were identified by thin-layer chromatography, essentially according to Gal (1968). Sialic acid was assayed by the resorcinol reaction of Svennerholm as modified by Miettinen and Takki-Luukkainen (1959). *N*-Acetylneuraminic acid was used as reference standard. The carbohydrate: protein ratios were obtained by subjecting duplicate delipidated samples to protein determination by amino acid analysis.

Results

Carbohydrate Composition. We determined the protein-bound hexoses, hexosamines and sialic acid of the Lp(a) lipoprotein and LDL fractions isolated from the plasma of three persons. The results can be seen in Table I. The amount of carbohydrate is much higher in Lp(a) lipoprotein (260 μg/mg of protein) than in LDL (100 μg/mg of protein). The values for LDL are slightly higher than those reported earlier (Margolis, 1969). The sialic acid content is about six times as high, the hexosamines about three times as high and the hexoses about twice as high in Lp(a) lipoprotein as in LDL. The major hexosamines in Lp(a) lipoprotein are *N*-acetylglucosamine and *N*-acetylgalactosamine, whereas in LDL only *N*-acetylglucosamine is present in significant amounts (one experiment). The predominant hexoses in Lp(a) lipoprotein are mannose and galactose, present in about equal amounts. LDL appears to contain more mannose than galactose.

Dissociation into Protein Components. Lp(a) lipoprotein behaves as a homogeneous species in ultracentrifugation and in polyacrylamide gel electrophoresis (Simons *et al.*, 1970; Ehnholm *et al.*, 1971). In both immunodiffusion and immunoelectrophoresis only one precipitin line is obtained when Lp(a) lipoprotein is tested against anti-human serum, anti-LDL, or anti-Lp(a) serum (Simons *et al.*, 1970). However, when stored at 0° the Lp(a) lipoprotein dissociates into components and gives four bands in polyacrylamide gel electrophoresis instead of one as shown in Figure 1. Band 1 is undissociated Lp(a) lipoprotein, band 2 is in the same region as LDL (Garoff *et al.*, 1970), band 3 has an electrophoretical mobility between those of LDL and of high density lipoprotein and band 4 is in the buffer front. Figure 2 shows the immunological reactions of the different bands when cut out from the gels and tested in double diffusion against anti-LDL, anti-Lp(a), and anti-albumin sera. Band 1 reacts with both anti-Lp(a) and anti-LDL serum but not with anti-albumin serum; band 2 reacts only with anti-LDL serum. It gives a reaction of identity with band 1 indicating that they have in common the antigenic determinants characteristic of LDL. This band also gives a reaction of identity with native LDL. Band 3 does not stain with lipid stain (Sudan Black) as do bands 1 and 2. It reacts only with anti-Lp(a) serum, indicating that it contains Lp(a) antigenic determinants but not those of LDL. Band 4 also does not stain with Sudan Black. This band reacts with anti-albumin serum but not with anti-LDL or anti-Lp(a) serum. No immunological differences could be found between this band and purified serum albumin.

We also used ultracentrifugation and absorption with immunoadsorbents to separate the components from stored preparations of Lp(a) lipoprotein. The density of the lipo-

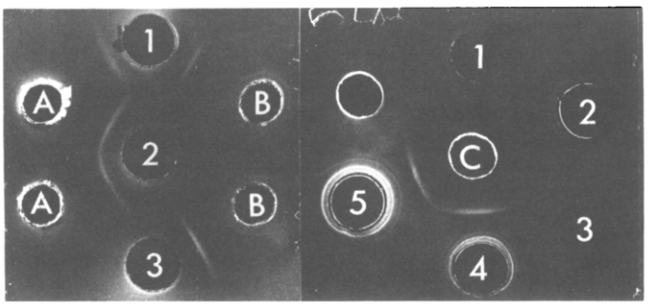

FIGURE 2: Double diffusion in 1% agarose gel. The four bands obtained by polyacrylamide gel electrophoresis of stored Lp(a) lipoprotein were sliced and embedded in agarose. The four bands are numbered as in Figure 1 from 1 to 4, 5. Albumin was electrophoresed in a separate polyacrylamide gel, sliced and embedded in agarose. (A) Anti-LDL serum, (B) anti-Lp(a) serum, and (C) anti-albumin serum.

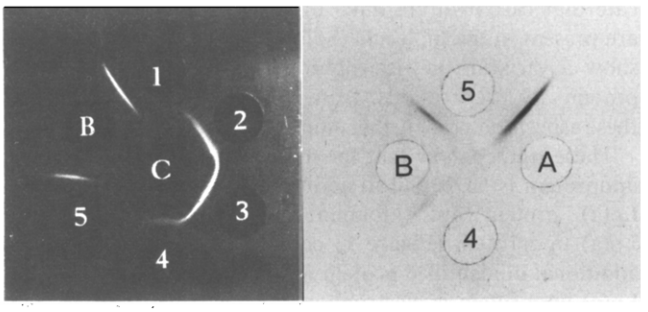

FIGURE 3: Double diffusion in 1% agarose gel. (1) Lp(a) lipoprotein, recently prepared; (2) stored Lp(a) lipoprotein; (3) albumin; (4) infranatant after ultracentrifugation of dissociated La(a) lipoprotein at a density of 1.21 g/cm^3; (5) supernatant after ultracentrifugation of dissociated Lp(a) lipoprotein at a density of 1.21 g/cm^3. (A) Anti-LDL serum, (B) anti-Lp(a) serum, and (C) anti-albumin serum.

protein preparations was adjusted to 1.21 g/cm^3 with a KBr–NaCl salt solution. They were then centrifuged at 100,000*g* for 24 hr in a Beckman Ti60 rotor. The top fraction contained the lipoprotein moiety of Lp(a) lipoprotein, and reacted with both anti-Lp(a) and anti-LDL serum but not with anti-albumin serum. Band 1 and 2 could be demonstrated in this fraction by disc electrophoresis. Band 2 was found in the top fraction also after ultracentrifugation at a density of 1.063 g/cm^3. The bottom fraction reacted with anti-Lp(a) and anti-albumin but not with anti-LDL serum, indicating that it contains the Lp(a) antigenic determinant and albumin (Figure 3). When the bottom fraction was treated with anti-albumin polymer the immunological reactivity against anti-albumin serum was abolished, but not that against anti-Lp(a) serum. When the absorbed material was eluted from the polymer with 2 M NaI and dialyzed, the eluate reacted immunologically like albumin.

Quantitation of the Components. Dissociated Lp(a) lipoprotein was run in 3.75% polyacrylamide gels. These were then stained for protein and the bands were cut out for amino acid analysis. These were used to quantitate the amount of protein in the four bands. The percentage distribution for the three components in three experiments was 66.1% (62.4–72.5) of band 2, 19.3% (15.7–23.5) of band 3, and 14.6% (11.7–18.0) of band 4. The degree of dissociation varied from preparation to preparation. In these experiments 78, 40, and 49% of the Lp(a) lipoprotein was dissociated.

When a homogeneous Lp(a) lipoprotein preparation that had been stored at 0° was rerun in disc electrophoresis it had partially dissociated into three components. All the Lp(a) lipoprotein preparations studied since have shown this dissociation. The storage time that is required for a clear dissociation varied with the preparation from 1 day to 3 weeks. The concentration of Lp(a) lipoprotein varied from 0.1–1 mg of protein/ml. Tris-HCl (0.1 M) (pH 8.7) or NaCl (0.15 M)–sodium phosphate (0.01 M) (pH 7.2) was used as buffer with or without 0.02% sodium azide as a preservative. We do not yet know how to dissociate the lipoprotein complex in a more controlled way. Attempts to dissociate the Lp(a) lipoprotein completely by freezing and thawing, high salt concentrations, neutral detergent, or urea have failed.

Discussion

The dissociation products of stored Lp(a) lipoprotein can be clearly demonstrated in disc electrophoresis as three well-separated bands. All of them migrate faster than intact Lp(a) lipoprotein. The slowest component migrates like LDL, the fastest in the buffer front, and the third component has an intermediate mobility. The latter two components stained only for protein, while the slowest one stained for both protein and lipid, which indicates that it is a lipoprotein.

Our findings indicate that the slowest component in disc electrophoresis is LDL. It resembles LDL in immunological behavior, staining properties, and migration in electrophoresis. The density of the slow component is also in the LDL range. We have earlier shown that the lipid composition of the Lp(a) lipoprotein is the same as that found in plasma LDL (Simons *et al.*, 1970).

Our results indicate that the fast-moving band in disc electrophoresis contains albumin. This component behaves immunologically like albumin both in double diffusion and in absorption–elution experiments with anti-albumin polymers. Furthermore this component is found in the infranatant after ultracentrifugation of dissociated Lp(a) lipoprotein at a density of 1.21 g/cm^3. In gel filtration, acrylamide gel electrophoresis and ultracentrifugation the undissociated Lp(a) lipoprotein behaves as a homogeneous molecule. Therefore albumin seems to be part of the lipoprotein complex and not an impurity. In addition the fact that the antigenic sites of albumin are revealed in double diffusion only after dissociation of the complex indicates that albumin is an integral part of the Lp(a) lipoprotein.

An analogous albumin–lipoprotein complex is the abnormal lipoprotein, Lp-X, found in patients with biliary obstruction (Alaupovic *et al.*, 1969). This lipoprotein is like the Lp(a) lipoprotein not precipitated by anti-albumin. The albumin can be detected after delipidation of the Lp-X complex. Otherwise these two lipoproteins are quite different. The Lp-X lipoprotein contains 95% lipid and a protein moiety consisting of a combination of approximately 40% albumin and 60% apoprotein C from VLDL.

The third component, which migrates in disc electrophoresis between LDL and albumin, reacts immunologically only with anti Lp(a) serum indicating that the Lp(a) antigenic determinant is located on this protein. We call it the Lp(a) protein. This protein is likely to be very rich in carbohydrates because undissociated Lp(a) lipoprotein contains two and one-half times as much carbohydrates as plasma LDL and the albumin part of the complex probably has no carbohydrate. The Lp(a) lipoprotein seems to contain *N*-acetylgalactosamine which is not found in LDL and may indi-

cate that carbohydrate units linked to serine and threonine are present in the molecule (Spiro, 1969). Preliminary studies show a variation in electrophoretical mobility of the Lp(a) protein in preparations from different individuals. Both these aspects deserve further study.

These studies show that the major component of the Lp(a) lipoprotein is LDL and in addition this lipoprotein contains Lp(a) protein and albumin. The albumin band from Lp(a) lipoprotein (Figure 1) contains small amounts of an additional unidentified protein which is seen when dissociated Lp(a) lipoprotein is electrophoresed in 7.5% polyacrylamide gels (our unpublished results). More work is therefore needed before the subunit structure of this complex lipoprotein is known. At present we do not know where and how the Lp(a) lipoprotein is formed from its components. Whether the Lp(a) protein is found in other structures than the Lp(a) lipoprotein is unclear. It is possible that this protein is related to components of cellular membranes (Berg *et al.*, 1968). This situation will have to be clarified before the genetics of the Lp(a) polymorphism, at present somewhat confusing (Rittner and Wichmann, 1967; Harvie and Schultz, 1970; Rittner, 1970; Utermann and Wiegandt, 1970), is understood.

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Added in Proof

After this manuscript was submitted Albers *et al.* (1972) reported the presence of a lipoprotein, LDL-a-1, in the density range 1.060–1.075 g/cm^3 of human plasma. We have exchanged antisera with Albers and both groups have found that the LDL-a-1 is identical with the Lp(a) lipoprotein antigenically.

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