

Isolation and Characterization of Lipoprotein B from High-Density Human Serum Lipoproteins

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1. Lipoprotein B from female Lp(a)-lipoprotein-negative serum was isolated from the fraction of density 1.073-1.125 by using immunoabsorbent; 2.5 mg of freeze-dried material was obtained from 100 ml of serum from a fasting patient. 2. The hydrated density of this lipoprotein was found to be 1.084 g/cm³. A flotation rate $F_{1.200}$ of 9.4 and lipid/protein ratio 1.40 were found, similar to that of high-density (d 1.073-1.125) lipoprotein preparations. 3. From immunochemical and electrophoretic studies of the intact and totally delipidized lipoprotein B it was concluded that this lipoprotein represents a separate family within the high-density range of human serum lipoproteins. 4. The possibility that the isolated lipoprotein B is an artifact created by the isolation procedure is discussed.

The fact that lipoprotein density fractions from human serum are composed of multiple lipoprotein families has slowly gained widespread acceptance. Although there seems to exist no density range from which a single lipoprotein family can be isolated solely by ultracentrifugation, there are certain intervals characterized by the predominance of one or the other family. This is the case, for example, with LpB* in d 1.025-1.053 and with LpA in d 1.100-1.21 fractions. On the other hand, the d 1.060-1.100 fraction represents a mixture of lipoproteins with respect to their chemical, immunochemical and physicochemical properties.

The existence of LpB (previously called β_1 -lipoprotein and sometimes equated with LDL) in lipoprotein preparations isolated at a density higher than 1.063 by ultracentrifugation has been reported by several investigators (Ayrault-Jarrier *et al.*, 1963; Seegers *et al.*, 1965; Alaupovic, 1968). In addition, the lipoprotein allotype Lp(a) discovered by Berg (1963), which has been found to be related to a histocompatibility antigen (Berg, 1971) and apparently represents a complex lipoprotein (Ehnholm *et al.*, 1971; Seidel *et al.*, 1971) sharing antigenic determinants with LpB, was isolated almost exclusively

* Abbreviations: LpA, LpB and LpC, lipoprotein family A, B and C respectively, characterized by the protein moiety; Lp(a), allotype of a lipoprotein found by Berg (1963); LDL, low-density lipoproteins, d 1.006-1.063 (unless stated otherwise); HDL₁, lipoproteins isolated at d 1.063 and floating with $F_{1.063}$ 0-3S; HDL₂, high-density lipoproteins, d 1.073-1.125; HDL₃, high-density lipoproteins, d 1.125-1.21; LpB_{HDL}, lipoprotein B isolated from HDL₂ fraction; apoAI and apoAII, major apolipoproteins of LpA, corresponding to R-Thr and R-Gln respectively found by Shore & Shore (1968).

from the range d 1.063-1.100. Because of the relatively small amount of LpB present in HDL, few attempts have been successful in isolating and characterizing this LpB_{HDL}. Several groups have recently isolated lipoproteins which reacted with 'anti- β -lipoprotein' antisera from HDL₁ or HDL₂ preparations (Utermann & Wiegandt, 1969; Roelcke & Weicker, 1969) and were distinguishable from Lp(a) (Albers *et al.*, 1972). We previously described a successful isolation procedure for LpB_{HDL} (Kostner & Alaupovic, 1971a) and this lipoprotein has been partially characterized (Kostner & Alaupovic, 1972). The present paper presents further results including chemical, immunochemical and physicochemical investigations.

Materials and Methods

Blood samples

Blood samples were collected from apparently healthy female volunteers, who had been informed about the aim of the present investigation. None of the subjects was under medication. Blood was collected after at least 12 h of fasting and allowed to clot for 6-10 h at 23°C. After being centrifuged in a low-speed centrifuge, the serum was collected and 1 mg of NaN₃ and 0.1 mg of disodium EDTA were added/ml. The serum of each individual donor was investigated by lipoprotein electrophoresis in agarose gels (Kostner *et al.*, 1971), and total lipids, phospholipids, total cholesterol and triglycerides were determined as described before (Kostner & Holasek, 1970). Since no antiserum to Lp(a) lipoproteins was available, the possible presence of this lipoprotein was checked by polyacrylamide-gel electrophoresis by the method of

Garoff *et al.* (1970). Sera of subjects with abnormal lipid concentrations, abnormal lipoprotein patterns in agarose-gel electrophoresis or showing the presence of Lp(a) in polyacrylamide-gel electrophoresis were not used during this study.

Isolation of lipoproteins

The density of 300 ml of pooled serum from two or three subjects was adjusted to 1.073 by adding solid NaCl and the serum was centrifuged in the 60Ti rotor of a Beckman L4 preparative ultracentrifuge at 45000 rev./min (144000g) for 24 h at 15°C. Floating lipoproteins that accumulated in the upper third of the tube were removed by a tube-slicer. The infranatant was brought to the original volume by addition of 0.15M-NaCl (pH 7.4 adjusted with 3M-NaOH); the density was again adjusted to 1.073 with solid NaCl, the preparation centrifuged again and sliced under identical conditions. The density of the lower two-thirds of the tubes was then adjusted to 1.125 by addition of solid NaBr and the mixture was spun in the ultracentrifuge for 30 h (144000g) at 15°C with the 60Ti rotor. Floating HDL₂ was removed by tubeslicing and dialysed against 0.15M-NaCl, pH 7.4 (adjusted by addition of 3M-NaOH). HDL₃ was isolated (*d*1.23) from sedimenting proteins (Kostner & Alaupovic, 1972). All density measurements were performed with a digital densitometer from Paar Instruments, Graz, Austria, at a temperature of 15°C.

Immunoabsorption technique

Pure antibodies to LpB were isolated from horse antisera as described by Kostner & Holasek (1969) and an immunoabsorbent was prepared as described by Kostner & Holasek (1970). HDL₂ fractions were passed through a column (15 cm × 1.2 cm) packed with the immunoabsorbent specific to LpB at room temperature and eluted with 0.15M-NaCl (pH 7.4) at a flow rate of 10 ml/h. The size of the column was sufficient for adsorption of the total amount of LpB present in HDL₂ from at least 300 ml of serum. After concentration of the eluate to the original volume by vacuum dialysis, no LpB could be detected in the eluate by immunodiffusion. The column was washed with 300 ml of 0.15M-NaCl, pH 7.4, at 4°C and adsorbed LpB was eluted with 20 ml of 0.1M-β-alanine-HCl buffer, pH 3.2, followed by 20 ml of 0.1M-β-alanine-HCl, pH 2.4. The progress of desorption was monitored by photoelectric scanning of the eluate at 280 nm. Fractions were dialysed against 0.15M-NaCl, pH 7.4, and investigated by immunodiffusion, immunoelectrophoresis, polyacrylamide-gel electrophoresis and in the analytical ultracentrifuge.

For preparation of apoLpB_{HDL}, the combined β-alanine eluates from the immunoabsorbent column

was dialysed exhaustively against glass-distilled water and freeze-dried. Removal of lipid from the freeze-dried material was carried out with ethanol-diethyl ether (3:2, v/v), followed by diethyl ether extractions as described by Kostner & Holasek (1972). Polyacrylamide-gel electrophoresis, immunoelectrophoresis, immunodiffusion and lipid analysis were performed as described previously (Kostner & Holasek, 1969, 1970, 1972). Antisera to lipoprotein families, as well as to their constitutive polypeptides, were prepared by immunizing sheep, goats, rabbits and horses with highly purified lipoproteins or separated apoLp polypeptides (Kostner & Alaupovic, 1972; Kostner & Holasek, 1970, 1972; Kostner & Alaupovic, 1971b). Sedimentation and flotation experiments were performed in an analytical ultracentrifuge (Beckman model E) equipped with electronic speed control. Flotation coefficients were calculated from the formula:

$$(F_c)_{app.} = -\frac{\ln r_2 - \ln r_1}{60\omega^2(t_2 - t_1)}$$

where $(F_c)_{app.}$ is the apparent flotation coefficient at concentration c , and r_1 and r_2 are the distances of the boundaries from the rotor axis at different time-intervals (t_1 and t_2); ω is the angular velocity.

Amino acid analysis

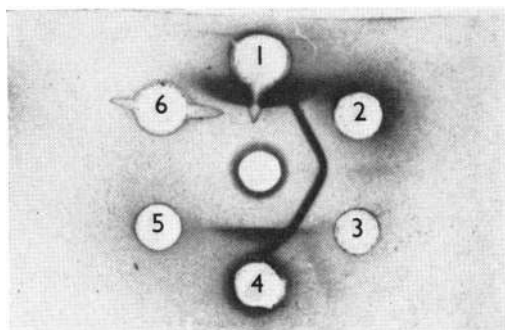
ApoLpB_{HDL} (0.2–0.4 mg) was hydrolysed in constant-boiling HCl under vacuum at 110°C for 24, 48 and 72 h. Norleucine was used as internal standard and amino acid loss was corrected after extrapolation to zero time. Cysteine was determined as cysteic acid after performic acid oxidation before hydrolysis by the method of Schram *et al.* (1954). Tryptophan was determined by the method of Gaitonde & Dovey (1970). The analysis was performed in a Bio-Cal BC200 amino acid analyser (Bio-Cal Instruments, München, Germany) with automatic sample injector by using the high-sensitivity cell. The resin Aminex A-G from Bio-Rad Laboratories (Richmond, Calif., U.S.A.) was used in a one-column programme. Amino acid analyses as well as calculation of individual amino acids were performed according to the instruction manual of the Bio-Cal BC 200 amino acid analyser.

Determination of partial specific volume (\bar{v})

The formula used for the determination was:

$$\bar{v} = \frac{1}{d_2} \left(1 - \frac{d - d_2}{c} \right)$$

where d_2 is the density of the solvent, d the density of the solution and c the concentration in g/cm³. NaCl (0.15M, pH 7.4, adjusted with 3M-NaOH) served as solvent and measurements were carried

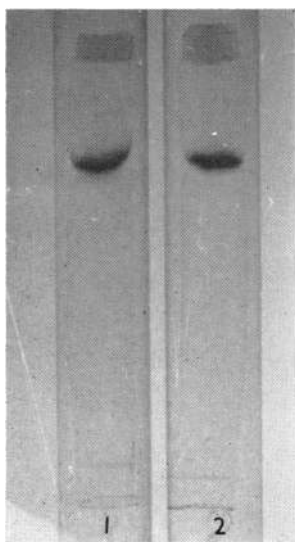


(a)

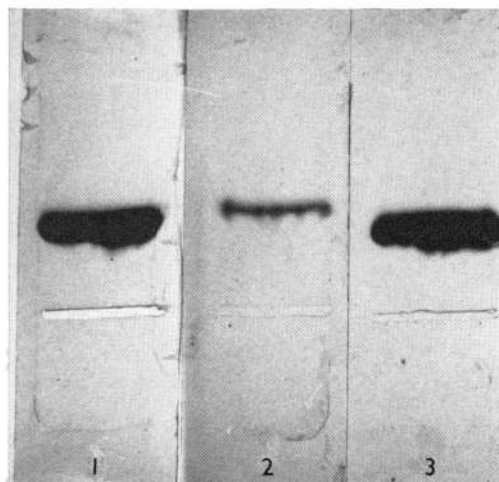
EXPLANATION OF PLATE 1(a)

Immunochemical reaction of HDL₂ (d1.073–1.125) with different antibodies

(a) The immunochemical heterogeneity of HDL₂, isolated from pooled serum of three different subjects, in 1% agarose gel after staining with Amido Black 10B. The centre hole contains HDL₂. Surrounding holes contain antisera to: 1, LpA; 2, LpB; 3, apoLpB; 4, LpC; 5, albumin (Behring Werke A.G., Marburg, Germany); 6, immunoglobulin G (Behring Werke).



(b)

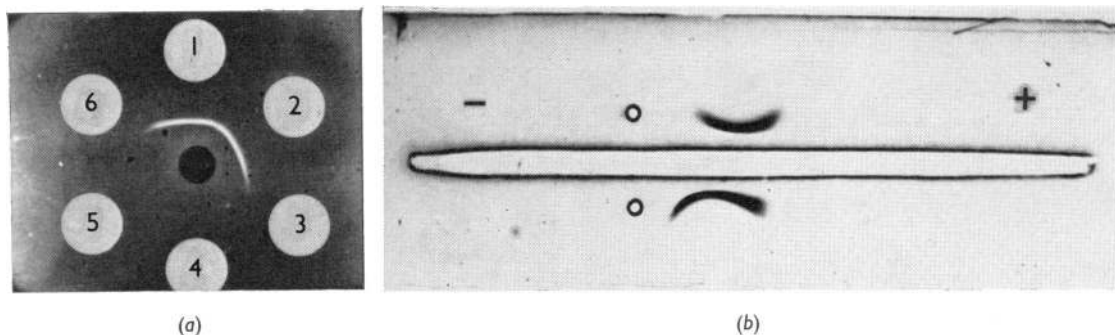


(c)

EXPLANATION OF PLATE 1(b) AND (c)

Gel electrophoresis of LpB_{HDL} compared with LDL (d 1.025–1.053)

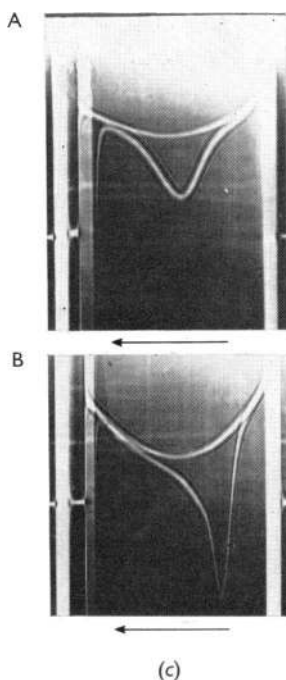
(b) Electrophoresis of 1, LpB_{HDL} and 2, LDL in 3.5% polyacrylamide gels after staining with Amido Black 10B.
 (c) Agarose-gel electrophoresis of 1, LDL, 2, LpB_{HDL} and 3, LDL plus LpB_{HDL} after staining with Amido Black 10B. For details see the Material and Methods section and the Results section.



EXPLANATION OF PLATE 2(a) AND (b)

Immunochemical examination of intact LpB_{HDL} after elution from immunoabsorbent with 0.1 M-β-alanine buffer, pH 3.2

(a) Immunodiffusion analysis of LpB_{HDL} (centre hole) with antisera to: 1, apoLpB; 2, whole human serum (Behring Werke); 3, ApoAI; 4, apoAII; 5, LpC; 6, albumin. The plate was photographed in diffuse light without staining. (b) Immunoelectrophoretic pattern of LpB_{HDL} (top hole) and LDL (bottom hole) with antiserum to LpB. Staining was performed with Sudan Black. For details see the Materials and Methods section.

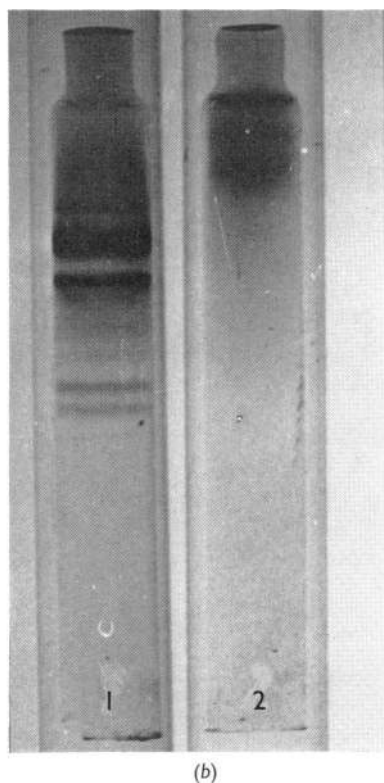
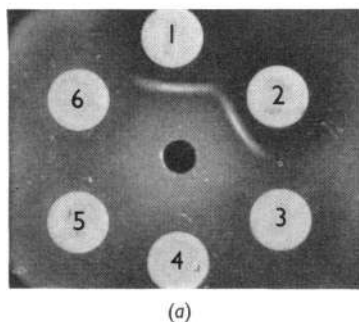


EXPLANATION OF PLATE 2(c)

Flotation pattern of LpB_{HDL}

LpB_{HDL} was dialysed against NaBr solution (*d*1.200) and examined in the analytical ultracentrifuge at 52000 rev./min and 25°C. The concentration was 16 mg/ml. A, Photograph at 20 min after reaching full speed; B, photograph at 35 min after reaching full speed. The acceleration time from zero speed to 52000 rev./min was 10 min.

G. KOSTNER



EXPLANATION OF PLATE 3

Immunodiffusion pattern and polyacrylamide-gel electrophoresis of totally delipidized apoLpB

(a) Two-dimensional immunodiffusion experiment of apoLpB_{HDL} (centre hole) with antisera to: 1, apoLpB; 2, whole human serum; 3, apoAI; 4, apoAII; 5, LpC; 6, albumin. Plates were photographed in diffuse light without staining. (b) Electrophoresis in 10% polyacrylamide gels containing 8M-urea. Gel 1, apoHDL₂ solubilized in 0.05M-tris-HCl buffer, pH 8.4, containing 8M-urea; gel 2, apoLpB_{HDL} solubilized in 0.05M-tris-HCl buffer, pH 8.4, containing 0.9% (w/v) sodium dodecyl sulphate. Staining was performed with Coomassie Blue. For details see the Materials and Methods section.

out at 25.0°C. Densities were determined with a digital densitometer (Paar Instruments, Graz, Austria) as described by Kratky *et al.* (1969). Concentrations of lipoprotein solutions were determined gravimetrically after evaporation of the solvent in a desiccator.

Results

Plate 1(a) shows the immunochemical reaction of HDL₂ with different antisera. In HDL₃ and $d > 1.23$ fractions from at least 30 different male and female subjects, no reaction with anti-LpB could be obtained at any antigen/antibody ratio tested. It was concluded that no LpB-containing lipoproteins are present in human serum with hydrated density higher than 1.125.

Fig. 1 shows the elution pattern of LpB from the immunoabsorbent column with 0.1M- β -alanine at pH 3.2 and 2.4 respectively. The total amount of freeze-dried LpB isolated from 100ml of serum from fasting patients was 2–3mg. Electrophoretic and ultracentrifugal studies of intact LpB_{HDL} were performed only on fraction I since part of fraction II was precipitated during dialysis and showed signs of 'denaturation'. For immunodiffusion studies and preparation of apoLpB_{HDL}, fractions I and II were mixed, since in previous experiments no difference in the amino acid composition between these two fractions could be detected.

In Plate 1(b,c) the electrophoretic patterns of LpB_{HDL} in polyacrylamide gel and 1% agarose gel are shown and compared with that of LpB from LDL ($d_{1.025-1.055}$). A somewhat faster migration of LpB_{HDL} was noticed but, after these two fractions were mixed, only a single band was detectable.

Plate 2(a,b) shows the immunochemical examination of LpB_{HDL} by immunodiffusion and immunoelectrophoresis. The only antiserum giving a positive immune reaction was anti-LpB.

Plate 2(c) shows the flotation pattern of LpB_{HDL} in the analytical ultracentrifuge at $d_{1.200}$. In a NaBr solution ($d_{1.200}$) the flotation coefficient of a LpB_{HDL} solution (8.5mg/ml) at 25°C was calculated to be $-9.4S$. A molecular polydispersity may be deduced from the broadening of the peak after prolonged running time. The partial specific volume from two separate preparations was calculated to be 0.924 and 0.922g/cm³ and the corresponding values for the hydrated densities were 1.082 and 1.084g/cm³ respectively. The molecular weight from one preparation was calculated as described by Archibald (1947) and an apparent value for molecular weight of 1.3×10^6 was found in a solution containing 4.5mg of LpB_{HDL}/ml.

After total removal of lipid, apoLpB_{HDL} was completely soluble in 0.05M-tris-HCl buffer, pH 8.4, containing 0.9% sodium dodecyl sulphate or sodium

decyl sulphate, or in 2M-acetic acid. The solution of LpB_{HDL} in detergents showed an immunochemical reaction of identity only for antisera to LpB or apoLpB and gave none with antisera to the two major apoLpA peptides apoAI or apoAII (Kostner & Alaupovic, 1971b) or antisera to the three major LpC polypeptides (Kostner & Holasek, 1972). In polyacrylamide-gel electrophoresis (Plate 3) most of the material migrated through the concentrating gel and came to a dead stop at the beginning of the separating gel, at monomer concentrations of 3.5, 7 or 10% acrylamide. Some diffuse broad bands stainable with Coomassie Blue were observed in the separating gel but none of those bands exhibited a migration rate

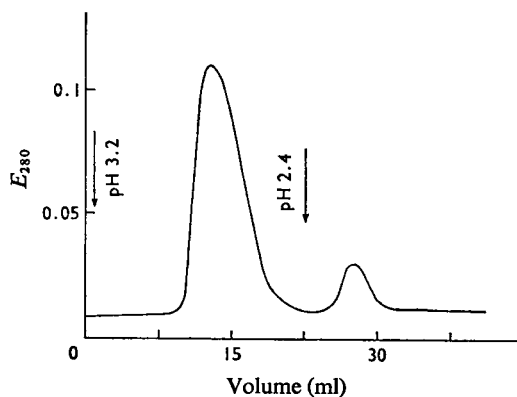


Fig. 1. Elution pattern of LpB_{HDL} from immunoabsorbent with 0.1M- β -alanine at pH 3.2 and pH 2.4

For details see the Materials and Methods section.

Table 1. Chemical composition of LpB isolated from serum HDL₂ by immunoabsorption compared with that of Lp(a) lipoprotein isolated by Ehnholm *et al.* (1971)

Values for LpB_{HDL} are means \pm S.D. for three different preparations. For details see the Materials and Methods section.

| | % of total lipoprotein mass | |
|--------------------------|-----------------------------|-------|
| | LpB _{HDL} | Lp(a) |
| Protein + carbohydrates | 41.5 \pm 1.2 | 35 |
| Glycerides | 7.1 \pm 0.9 | 2 |
| Free cholesterol | 6.9 \pm 0.4 | 9 |
| Cholesterol esters | 23.7 \pm 0.8 | 38 |
| Phosphatidylcholine | 13.2 \pm 0.9 | 9 |
| Lysophosphatidylcholine | 4.4 \pm 0.5 | 0.1 |
| Sphingomyelin | 2.1 \pm 0.3 | 5 |
| Phosphatidylethanolamine | 0.7 \pm 0.1 | 0.2 |

Table 2. *Amino acid composition of LpB from HDL₂ compared with that of Lp(a) isolated by Ehnholm et al. (1971)*

A pooled sample from eight different individuals was used. Values for serine, threonine and tyrosine were obtained by linear extrapolation of recoveries from 24–48h and 72h hydrolysis. For details see the Materials and Methods section.

| Amino acid | Content (mol/1000mol of amino acids) | |
|------------|---|-------|
| | LpB _{HDL} | Lp(a) |
| Asp | 86 | 98.9 |
| Thr | 75 | 68.0 |
| Ser | 90 | 78.2 |
| Glu | 110 | 117.8 |
| Pro | 68 | 54.1 |
| Gly | 64 | 75.1 |
| Ala | 60 | 82.6 |
| Val | 82 | 60.1 |
| Met | 11 | 15.8 |
| Ile | 46 | 43.5 |
| Leu | 81 | 84.9 |
| Tyr | 35 | 34.8 |
| Phe | 38 | 35.2 |
| Lys | 63 | 62.8 |
| His | 20 | 21.3 |
| Arg | 27 | 40.3 |
| Cys | 7 | 17.8 |
| Trp | Trace | 8.7 |

like that of the two major apoLpA or any of the apoLpC bands.

Tables 1 and 2 show the protein/lipid ratio, the distribution of individual lipids and the amino acid composition of apoLpB_{HDL} as compared with that of Lp(a) lipoprotein from Ehnholm *et al.* (1971). Values for amino acids in Table 2 were calculated from a special batch of apoLpB_{HDL}, which was pooled from eight different individuals. On summing the weights of individual amino acids after 24h hydrolysis, 86% of the original weight of apoLpB_{HDL} could be recovered.

Discussion

It has recently become evident that the lipoproteins which react with antibodies to LpB and which can be found in lipoprotein fractions isolated at a density greater than 1.063 may no longer be dismissed by attributing them to 'contaminations' or 'improper isolation procedure'. This has been verified by the isolation and characterization of Lp(a), a lipoprotein which apparently represents a complex of LpB with other known or unknown lipoproteins or

apolipoproteins (Ehnholm *et al.*, 1971; Seidel *et al.*, 1971; Utermann & Wiegandt, 1969; Utermann *et al.*, 1972). LpB has also been found in HDL₂ preparations of Lp(a)-negative subjects (Alaupovic, 1968; Roelcke & Weicker, 1969), but because the small amount present was masked by the predominant LpA and LpC (Kostner & Alaupovic, 1971*a*), its isolation and characterization were unsuccessful. Results from the present study demonstrate that, by using the procedure outlined above, it is possible to isolate a LpB fraction from human serum HDL₂ with \bar{v} 0.923 and hydrated density 1.083. Considering the flotation rate ($F_{1,200}9.4$) and the lipid/protein ratio (1.40), the LpB_{HDL} exhibits most of the characteristics found for HDL₂ preparations.

A LpB_{HDL} showing the above-mentioned physicochemical characteristics should not be interpreted as a contaminant LpB from HDL as a consequence of the isolation procedure (low pH for desorption from immunoabsorbent), since a density of 1.073 was used for removal of LDL and an additional wash of the infranatant at the same density was carried out. Ultracentrifugation at 34.5×10^5 g-h and the removal of the upper third from the tubes containing LDL by tube-slicing should also prevent contamination of the HDL fraction with LDL. In addition, it has been observed (Scanu, 1971), and could be confirmed by our own investigations, that HDL loses some lipids (mainly neutral lipids) during isolation by ultracentrifugation, which shifts it to a fraction of higher hydrated density. This phenomenon could not be observed with freshly prepared LDL (G. Kostner, unpublished work). When LDL preparations were centrifuged again at a density of 1.073 under the conditions outlined in the present paper, no material reacting with anti-LpB could be found in the sediment.

Chemical, immunochemical, physicochemical and electrophoretic properties of LpB_{HDL} differed significantly from those of Lp(a) preparations (Tables 1 and 2). It therefore does not seem likely that LpB_{HDL} prepared during the present work was contaminated with Lp(a) lipoproteins. It has been stated that apoLp(a) lipoproteins react with antisera to albumin (Utermann *et al.*, 1972) and to LpC (Seidel *et al.*, 1971); apoLpB_{HDL} gave no reaction with those antisera. The preparation of LpB_{HDL} described in the present paper cannot be compared directly with the LDL₃ isolated by Albers *et al.* (1972), since in their studies another density for isolation was used. In addition pooled sera were used, which probably contained Lp(a) lipoproteins, and it therefore cannot be excluded that part of the LDL₃ found represented split fragments from Lp(a) lipoproteins, since it has been discovered that Lp(a) disintegrates on storage or chemical treatment (Ehnholm *et al.*, 1971; Utermann *et al.*, 1972).

It has been suggested previously (Alaupovic *et al.*,

1972) that lipoprotein families with a density above a certain value exist primarily as individual entities and below that range mainly as associated complexes. This could be verified by isolating different lipoprotein families from HDL (Kostner & Alaupovic, 1971a, 1972) and by isolating LpA from chyle LDL preparations (Kostner, 1972) on the one hand and by isolation of chylomicrons as complexes of lipoprotein families (Kostner & Holasek, 1972) on the other. The present report is another example of the correctness of the 'lipoprotein family concept' proposed by Alaupovic *et al.* (1972). Intact Lp_{BHDL} reacts only with antibodies to LpB and gives no reaction with any other antiserum to LpA, LpC or to their constitutive polypeptides. After removal of lipid, the apoprotein moiety also showed the same immunochemical behaviour.

In a recent study, Scanu & Edelstein (1971) demonstrated and partially determined quantitatively the nature and amount of apolipoproteins soluble in organic solvents. When we dissolved the lipid moiety of Lp_{BHDL} in chloroform-methanol (2:1, v/v) and extracted with 0.15M-NaCl, pH7.4, according to Folch *et al.* (1957), no protein could be detected either in the aqueous phase or in the interphase. We could therefore exclude the possibility that any protein was lost during removal of lipid. Although no physicochemical studies were performed on material eluted from immunoabsorbent by β -alanine at pH7.4, it seems unlikely that there is any difference between material from peak I and peak II in Fig. 1. In one experiment, samples from peaks I and II were examined separately and no difference in the immunochemical behaviour or in the amino acid composition was detectable. Rather it is assumed that, owing to the different avidity of antibodies bound to the adsorbent, part of the LpB can be eluted only at lower pH values. Results of studies presented in the present paper clearly indicate that a single lipoprotein family exhibiting characteristics of LpB can be isolated at a density range 1.073-1.125 by immunoabsorption.

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