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HETEROGENEITY OF LIPOPROTEIN B

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SUMMARY

Combined very low and low density lipoproteins were derived from human plasma by polyanion precipitation and the low density lipoprotein fraction (density 1.027-1.050 g/ml) was isolated by sequential ultracentrifugation. When this fraction was applied to Sepharose column chromatography, three lipoproteins were eluted. The first and third peaks were minor components while the second peak represented the bulk of LDL. Further chromatographic and electrophoretic studies indicated that the component representing the second peak was heterogeneous. This component was subsequently delipidated at pH 4 in a quaternary biphasic solvent system. The apoproteins remained soluble after delipidation and were treated with various deaggregating agents. On column isoelectric focusing in the presence of 4 M urea the apoproteins banded as broad overlapping peaks between pH 3 and 7. When hexanol was added to the system, distinct apoprotein subfractions were resolved.

INTRODUCTION

Extensive studies of lipoproteins have so far failed to yield a definitive picture of lipid transport or of the interrelationship between the eight lipoprotein apoproteins identified thus far. Apoprotein B, in particular has proved difficult to study. The recent introduction of a biphasic organic solvent system for plasma delipidation without protein precipitation has opened a further avenue for the study of apoproteins (1-4). The procedure has found applications in a variety of experimental areas, including cellular lipid metabolism (5-12), plasma lipid and apoprotein metabolism (13-19), enzymatic studies (20,21), lipid-protein interactions (3,4) and diagnosis of familial hyperlipoproteinemia (22). In particular the solvent system has been successfully applied in studies

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of purified chylomicrons (23), very low density lipoprotein (VLDL) (23-25) and high density lipoprotein (HDL) (17) fractions. This communication describes the use of this method in the study of the purified low density lipoprotein (LDL) subclass d=1.027-1.050. The results indicate that this lipoprotein fraction does not represent homogeneous apoprotein B.

MATERIALS AND METHODS

Isolation Of Lipoprotein B (LpB)

Human citrate-dextrose blood was obtained from the district blood bank and plasma was recovered by low-speed centrifugation (3,000 x g). NaN_3 (0.1% w/v) and Na_2EDTA (0.05% w/v) were added and the plasma was then stored overnight in the dark at 4°C to allow chylomicrons to concentrate at the meniscus. Only plasma in which no chylomicrons were detected was used for LpB isolation. All plasma used exhibited a normal electrophorectic lipoprotein pattern. LpB was isolated from plasma by the method of Burnstein et al., (26) as modified by Kostner et al., (27). From this procedure a lipoprotein fraction of density 1.027 - 1.050 g/ml was obtained.

In the chromatographic studies 1-5 ml of lipoprotein solution containing 1-5 mg of protein per ml was applied to a 100 cm x 1.6 cm water jacketed column (4°C) packed with Sepharose 6B (Pharmacia Fine Chemicals AB). Fractions of 3 ml were collected at a flow-rate of 20 ml per hour. Absorbance at 280 nm was monitored (ISCO UV Monitor) with a flow-through cell and was continuously recorded. Fractions constituting an individual peak were pooled and when required concentrated with a Minicon concentration system (Amicon Corp, Lexington, Mass, U.S.A).

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed according to Naito et al. (28) with some modifications. 3.3% (w/v) gels were prepared containing 4 M urea. Electrophoresis was carried out at 3 mA per gel until the tracking dye (bromophenol blue) reached the bottom of the gel. Gels were stained with Coomassie Brilliant Blue G-250. After destaining the gels were scanned at 720 nm with a Unicam densitometer.

Delipidation Of Lipoproteins

Fractions of LDL were originally delipidated with butanol-diisopropyl ether (DIPE) mixtures as described earlier (1). The bulk of lipid was removed by this system. However, on thin layer chromatographic analysis it was observed that traces of phospholipid were present in the aqueous phase after 0.5 h delipidation. Extending the time of extraction to 1.5 h resulted in removal of virtually all lipids from the aqueous phase. Alternatively, the organic solvent system could be modified specifically for this particular lipoprotein fraction such that virtually all lipids were extracted in 0.5 h. The modified delipidation system consisted of chloroform-butanol-DIPE-lipoprotein solution 1:1:1:1 (v/v/v/v). When the organic phase was added to the lipoprotein solution a biphasic system resulted. After agitation LDL lipid was extracted into the organic phase, leaving the apoproteins in the aqueous phase. Delipidation was also performed at various pH values. Delipidation at pH 4.0 resulted in a less turbid apoprotein solution than at pH 7.4 or pH 8.6, suggesting that more aggregation was occurring at the higher pH values. This observation and the time required for delipidation resulted in the adoption of the quaternary biphasic solvent system in which the aqueous phase was at pH

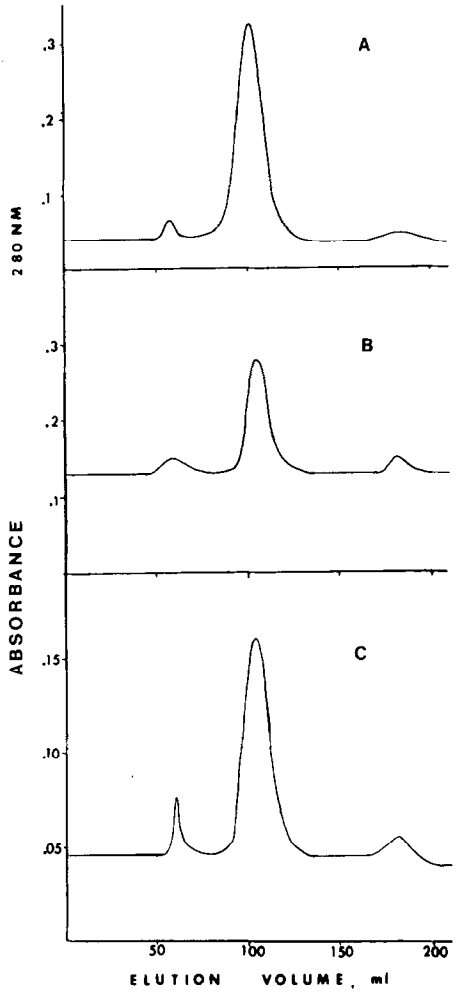

Figure 1 Elution profiles of lipoprotein fractions in the density range 1.027 to 1.050 g/ml on Sepharose 6B column chromatography. The various lipoproteins were applied and eluted from a column (1.6 cm x 100 cm) of Sepharose 6B. (A)-LDL after ultracentrifugation at d 1.027-1.050; (B)-peak II from (A) concentrated then rechromatographed; (C)-peak II from (B) concentrated then rechromatographed.

4.0 for subsequent delipidation of LDL. Thus, the lipoprotein fraction was dialysed overnight against 0.05 M citrate buffer (pH 4.0) prior to delipidation. Following dialysis, urea to a final concentration of 4 M was added to the lipoprotein solution. Lipid extractions were performed at room temperature in 11 cm x 2.5 cm glass tubes fitted with polyethylene stoppers. Fifteen ml of organic solvent consisting of DIPE-butanol-chloroform 1:1:1 (v/v/v) was added to 5ml of lipoprotein solution containing 1-2 mg protein/ml. The tubes were then fastened to a blood cell suspension rotator to provide end-over-end rotation at 28-30 rpm for 1 h. Subsequently, the organic phase containing the extracted lipids was separated from the aqueous phase by low speed centrifugation (500 x g) for 5 min. The bulk of the organic phase was carefully removed

with a Pasteur pipette. To remove the remaining traces, the delipidated aqueous solution was gently agitated with three parts of diethyl ether and partitioned by centrifugation as above. The ether layer was removed and the aqueous solution washed once more with diethyl ether. Ether remaining in the aqueous phase after the final wash was extracted under vacuum. In some experiments (see later) 0.5% (v/v) n-hexanol was added to the delipidated lipoprotein solution after ether extraction was complete. At no stage of centrifugation did a pellet form. An emulsion was visible following each centrifugation as a whitish zone some 2 to 5 mm thick immediately below the interface of the two phases. During each diethyl ether wash this band was broken up by gently agitation until clear separation of phases was achieved. Protein concentration of the lipoprotein solution was measured before and after delipidation. No detectable loss of protein in the aqueous phase resulted from delipidation.

Chemical Analysis

Protein was measured by the method of Lowry et al. (29) Quantitative lipid analyses were performed before and after delipidation by semi-automated methods for triglyceride (30), cholesterol (31) and phospholipid (32). Esterified and unesterified cholesterol were separated on Sephadex LH-20 columns (33). Qualitative lipid analyses were performed by thin layer chromatography of lipid extracts in two solvent mixtures (3).

Isoelectric Focusing

Electrofocusing was performed at 5°C using 2% (w/v) carrier ampholytes (Ampholine, LKB-Produkter AB, Stockholm), an LKB electrofocusing column (Model 8100-1) and an LKB power supply (Model 2103). Solutions and density gradients were prepared as described in LKB Instruments Instruction Manual with the cathode as the lower electrode. In some cases 0.5% (v/v) n-hexanol and 4 M urea were included in the gradient solutions. Equilibrium was usually reached within 10 h. The protein load varied from 5 to 20 mg. When focusing was complete, the column was eluted as described in the LKB Instruction Manual. The solution was pumped through a flow-through pH-electrode cell (Ionode, Brisbane, Australia) attached to a TPS-pH meter connected to a continuous recorder (Omniscribe), and then passed through a UV-monitor with a flow-through cell (Isco monitor and recorder) at a flow rate of 72 ml/h. Thus continuous elution profiles of absorbance at 280 nm and pH were recorded. Fractions of 6 ml were collected.

RESULTS

Immunoelectrophoresis

The LDL fraction isolated by polyanion precipitation followed by sequential ultracentrifugation between densities 1.027-1.050 g/ml reacted with anti-LDL, but not anti-HDL antiserum on immunoelectrophoresis.

Gel Permeation Chromatography Of LDL (d=1.027-1.050 g/ml)

When LDL (d=1.027-1.050 g/ml) was applied to a column of Sepharose 6B, three separate peaks were eluted (Fig. 1A). The peaks were designated I,

II and III in order of elution. Peak II represent the bulk of the lipoprotein fraction and appeared to represent a distinct lipoprotein species. However, when this fraction was collected, concentrated and reapplied to the same column either immediately or after storage for 10 weeks at 4°C, three peaks were resolved with the same elution volumes and profiles as found in chromatogram A (Fig. 1B). When the fractions representing peak II of chromatogram B were collected, concentrated and reapplied to the column for a third passage, three peaks were again resolved with elution profiles similar to those of A and B (Fig. 1C). In all cases the first and third peaks were minor components. Recovery of protein with this procedure was 99-101%. When peak II of chromatogram A was lyophylized, reconstituted and applied to the column, only one peak appeared on the chromatogram with an elution volume corresponding to the void volume of the column, i.e. not corresponding to either peaks I,II or III of Fig. I.

Polyacrylamide Gel Electrophoresis Of LDL (d=1.027-1.050 g/ml)

On electrophoresis in 10% polyacrylamide gels (containing 8 M urea) of either unchromatographed lipoprotein fraction or peak II of Fig. 1A or peak II of Fig. 1B, the lipoprotein did not migrate into the gel and appeared to comprise pure LpB. However, when electrophoresis was conducted in 3.3% polyacrylamide gels, unchromatographed lipoprotein (d=1.027-1.050 g/ml) was resolved into three bands (Fig. 2A). LDL (d=1.027-1.050 g/ml) peak II (Fig. 1A) also gave three bands (Fig. 2B) as did rechromatographed LDL (d=1.027-1.050 g/ml), peak II (Fig. 1B), (Fig. 2C). In all cases there were two minor and one major components.

Lipid And Protein Analyses Of Peak II (Twice Chromatographed) Of LDL (d=1.027-1.050 g/ml)

Quantitative lipid and protein analyses demonstrated that LDL (d=1.027-1.050 g/ml) (twice chromatographed, peak II of Fig. 1B) contained

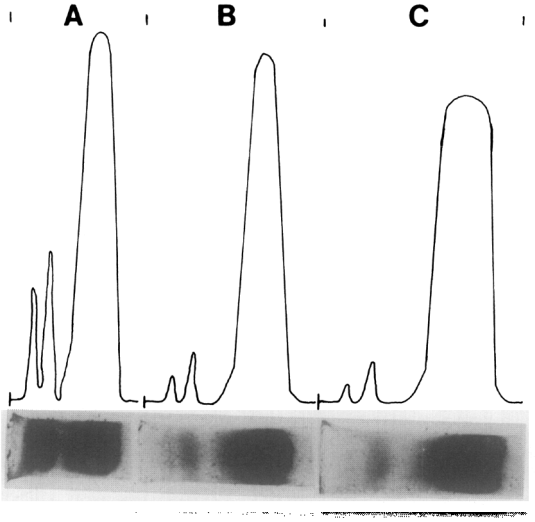

Figure 2 **Polyacrylamide gel (3.3% w/v) electrophoresis and densitometric scans of lipoprotein fractions in the density range 1.027 to 1.050 g/ml. (A)-LDL after ultracentrifugation at d 1.027-1.050; (B)-peak II lipoprotein fraction from Sepharose 6B column (peak II from Fig. 1 (A)); (C)-peak II lipoprotein fraction separated from Sepharose 6B after rechromatography (peak II from Fig. 1 (B)). Approximately 50 ug of protein was applied per gel.**

4% triglyceride, 39% esterified cholesterol, 8% unesterified cholesterol, 22% phospholipid and 27% protein (w/w). Thin layer chromatography of chloroform-methanol extracts showed that this lipoprotein exhibited qualitatively virtually the same lipid profile before and after gel chromatography. When peak II was concentrated and reapplied to the column for a third passage (Fig. 1C) the resultant peaks I, II and II were found to consist of 2.4%, 95.5% and 2.0% protein respectively.

Isoelectric Focusing Of Delipidated Peak II (Twice Chromatographed) Of LDL (d=1.027-1.050 g/ml)

When LDL (d=1.027-1.050 g/ml) was purified by two passages through Sepharose 6B, delipidated in the absence of n-hexanol and subjected to column electrofocusing (4 M urea, pH 3-9), the elution profile shown in Fig. 3 was obtained. The apoproteins formed a broad band of overlapping peaks in the range of pH 3-7. Focusing for longer periods of time did not produce better resolution and led to precipitation if continued for longer

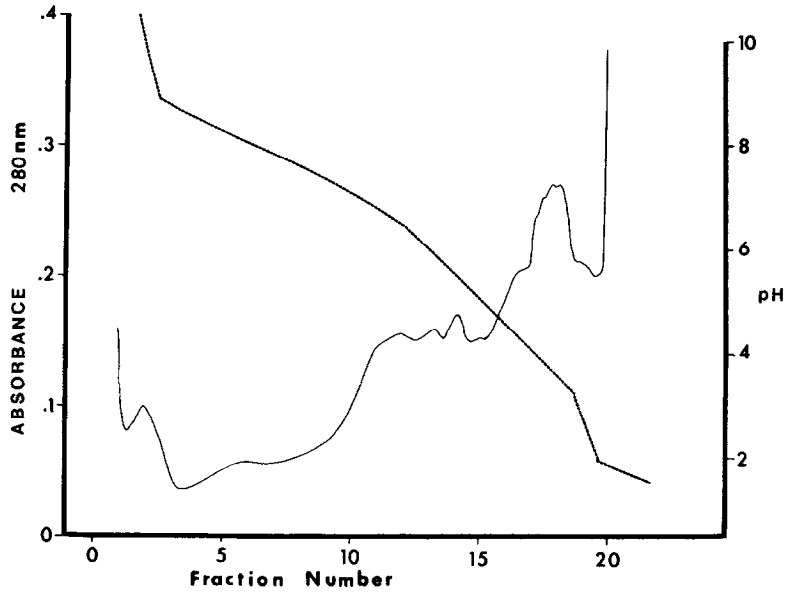

Figure 3 Column isoelectric focusing after delipidation of lipoprotein fraction in the density range 1.027 to 1.050 g/ml. Human plasma lipoprotein fraction obtained by ultracentrifugation at d 1.027-1.050 followed by Sepharose 6B chromatography (peak II of Fig. 1 B) was delipidated then subjected to isoelectric focusing at a pH range of 3-9 in a column in the presence of 4 M urea but no n-hexanol ..... pH ————protein.

than ten hours. However, when n-hexanol was included in the delipidated sample and in the isoelectric focusing gradient solution, a different banding pattern was found. Distinct apoprotein species were resolved with isoelectric points of 5.60, 5.27, 4.75 and 3.95 (Fig. 4). No lipid could be detected by thin layer chromatography in chloroform methanol extracts of delipidated samples.

DISCUSSION

The separation technique for lipoprotein B used in the present study has been regarded as yielding a 99.7% pure product, in peak II by gel permeation chromatography (27). However, the results of our further examination of this chromatographic fraction indicate that the material was not homogeneous and could be resolved into a number of species. When fractions from peak II (Fig. 1A) were re-applied to a Sepharose 6B column,

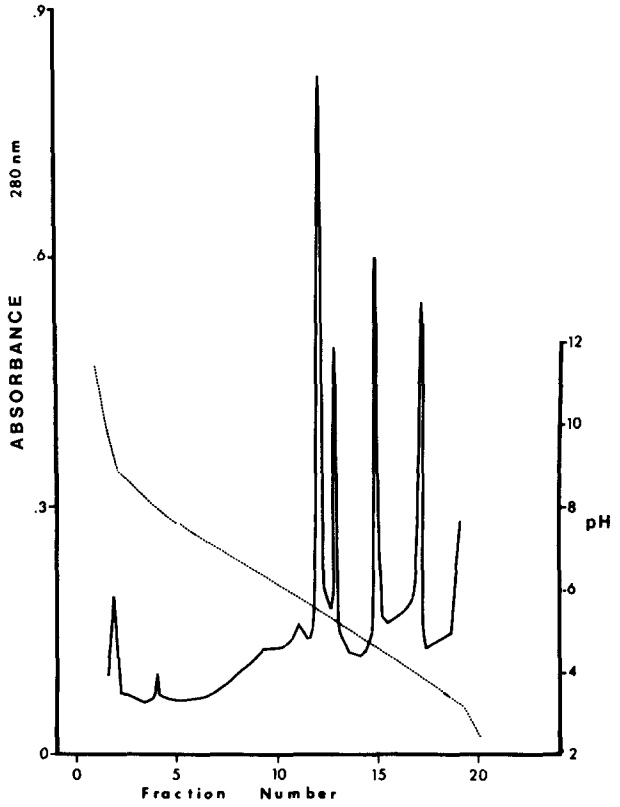

Figure 4 Column isoelectric focusing after delipidation of lipoprotein fraction in the density range 1.027 to 1.050 g/ml. Human plasma lipoprotein fraction obtained by ultracentrifugation at d 1.027-1.050 followed by Sepharose 6B chromatography (peak II of Fig. 1 B) was delipidated then subjected isoelectric focusing at a pH range 3-9 in a column in the presence of 4 M urea and 0.5% (v/v) n-hexanol .....pH ——— protein.

either on the same day or 10 weeks later, three peaks were again obtained with elution volumes identical with those of the first chromatogram. Repetition of this process with a third Sepharose column yielded identical results. This suggests that the protein in peak II dissociated rapidly and was in equilibrium with a higher molecular weight material (peak I) as well as a lower molecular weight material (peak III). Such an elution pattern would not be consistent with mere aggregation of the peak II protein after separation on the column.

When we subjected peak II fractions to electrophoresis on 10%

polyacrylamide gels containing 8 M urea, the lipoprotein did not migrate into the gel, an observation which is in agreement with the results obtained by Kostner et al. (27). However, when 3.3% polyacrylamide gels were used, peak II fractions were again resolvable into three lipoprotein species. Thus, we have been able to demonstrate by both chromatographic and electrophoretic techniques that peak II yields a heterogeneous product containing three species of protein.

We next addressed the question of the possible heterogeneity of apoprotein B. A technique for plasma delipidation previously published from this centre (1) was modified in the present investigation specifically for the delipidation of LpB at pH 4. The delipidation procedure utilized here resulted in apoproteins in solution but isoelectric focusing studies suggested that they were aggregated. However, 0.5% (v/v) n-hexanol appeared to disaggregate the apoproteins and four major components with different isoelectric points were resolved. It is suggested that n-hexanol interacted as an amphiphile with the apoproteins in such a manner as to reduce hydrophobic protein-protein interaction and to increase protein-solvent interaction. This could have resulted in disaggregation of hydrophobic protein subunits with the concomitant appearance of soluble apoproteins to which n-hexanol was hydrophobically attached. The hydroxyl group of n-hexanol would then be exposed to the hydrophilic solvent.

Recently Krishnaiah et al. (23) found that delipidation of chylomicrons and VLDL with butanol-DIPE yielded high molecular weight proteins which remained at the top of 3.5% polyacrylamide gels when subjected to electrophoresis. However, the entry of all apoproteins into polyacrylamide gels could be accomplished by performing the delipidation in the presence of 1.4% (w/v) sodium dodecyl sulphate (SDS) (final concentration in the aqueous layer). This suggests that during butanol-DIPE extraction aggregation had occurred and that SDS may have

caused disaggregation. SDS is an amphiphilic molecule and perhaps n-hexanol may act on apoproteins in a similar way. Unlike SDS, n-hexanol does not contribute to changes in net charge on the apoprotein particle (34). Kostner et al (27) delipidated fraction II with glycerol-1-hexyl ether and obtained precipitated apoproteins. Solubilization of the apoproteins with detergents followed by electrophoresis resulted in the separation of four major apoprotein components. It is interesting that the delipidation procedure used here yielded apoproteins in solution (without prior precipitation) and four apoprotein components were also obtained (Fig. 4). Kane et al.(35) have recently reported heterogeneity of apo B. These investigators indicated that there were four species of apo B with unique molecular weights and amino acid compositions. Three of these apoproteins appeared in LDL (1.024-1.050 g/ml density fraction).

Heterogeneity of the apoproteins as treated in the present study can only be claimed if there is certainty that the fraction examined was indeed apo B; absolute defined criteria for identification of apo B are lacking. So at this stage it cannot be assumed that these apoproteins represent nonidential monomers. Further studies are necessary to characterise them.

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