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GEL FILTRATION OF NATIVE AND MODIFIED PIG SERUM LIPOPROTEINS*

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SUMMARY

Total lipoproteins were isolated from pig blood serum and further separated into individual classes of very low-density (VLDL), low-density (LDL) and high-density (HDL) lipoproteins by preparative centrifugation in NaBr solutions. The same classes were obtained by gel filtration of the total lipoproteins on Biogel A 50 m (agarose gel) and were eluted in the order VLDL, LDL and HDL. LDL emerged as a single broad assymmetrical peak. Its initial and final portions were enriched in the subclasses LDL_1 and LDL_2, respectively. Gel filtration separated partially delipidated LDL into three zones. The first to emerge contained large aggregates, the intermediate and final zone (which eluted in the same volume as LDL) contained several ultracentrifugally distinguishable components. Partially delipidated VLDL emerged in about the same volume as HDL.

Most of the lipids in serum are associated with proteins in the form of soluble lipoproteins. Although many investigations of the structure of lipoproteins have been made, the problem remains unsettled. Part of the difficulty lies in the instability and heterogeneity of these macromolecules and the possibility of changes during isolation. Structural analysis by degradation procedures such as partial lipid extraction of lipoproteins requires isolation of the products before interpretation of the results.

Gel filtration has been used by FRANZINI[1,2] and WERNER[3] to separate α- and β-lipoproteins (paper electrophoretic nomenclature) of human serum and by MARGOLIS[4] to estimate the molecular weight of individual lipoprotein classes previously isolated from human serum by centrifugal procedures. However, isolation of the major classes from a total serum lipoprotein sample or the preparation of distinct components from a lipoprotein class by gel filtration methods has not been reported.

Our purpose was to use agarose gel filtration methods to prepare lipoprotein classes, purify individual classes, and isolate components and degraded components for subsequent structural investigation. Pig's blood was used as a source of lipoproteins because it is available in quantity and this animal has been used for experimental cardiovascular studies[5,6]. Pig serum contains the three major lipoprotein

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classes, very low-density (VLDL), low-density (LDL) and high-density (HDL) lipoproteins[7]. There is evidence from ultracentrifugal studies that LDL contains two (LDL_1 and LDL_2) or three distinguishable components[7] although these were not demonstrated by agarose gel electrophoresis[8].

EXPERIMENTAL

Pig serum lipoprotein classes were prepared according to JANADO *et al.*[7] and KALAB AND MARTIN[8]. Partial delipidation[9] was accomplished with ethyl ether at 5°. Agarose gel (Biogel A 50 m or A 5 m, 100–200 mesh, BioRad Laboratories, Richmond, Calif.) columns (5.5 × 75 cm) were used with Tris buffer, pH 8.9 (88.89 g Tris base and 200 ml *N* HCl made to 4 l with water containing 0.02 % EDTA). Lipoprotein samples (2 to 6 % concentration) were introduced and the columns were run downwards at a flow rate of 50 ml/h (approx. 2 ml/cm²/h) at 20°. Columns were calibrated with lyophilized *Escherichia coli* (Seravac Laboratories Ltd., Maidenhead, England), bovine plasma albumin (Armour Pharmaceutical Inc., Chagrin Falls, Ohio), and $NaNO_2$ (Allied Chemical and Dye Corp., New York, N.Y.). The eluates were monitored at 280 mμ and, when required, concentrated by dialysis against solid sucrose or by filtration in a Diaflo apparatus (Amicon Corp., Cambridge, Mass.) equipped with a UM-1 membrane, and then dialyzed in Visking Nojax Castings of 10 mm diameter against appropriate solutions for futher analyses. NaBr solutions were used for analytical centrifugation of low-density components; Tris buffer, pH 8.9, for high-density components; and barbitone buffer, pH 8.6, for electrophoresis in agarose gel.

RESULTS AND DISCUSSION

In preliminary experiments with gel filtration of lipoproteins, columns filled with 4 % agar gel prepared in this laboratory were used. However, the recovery of lipoproteins was not complete and VLDL could not be eluted from the column. When VLDL and LDL samples in NaBr solution (d = 1.007) were slurried with agar gel for 3–20 h at room temperature, about 50 % of the VLDL and 7 % of the LDL was retained by the gel. Some authors suggest that sulfate groups in agar cause absorption of lipoproteins during electrophoresis[10] and this may also be true for gel filtration. In

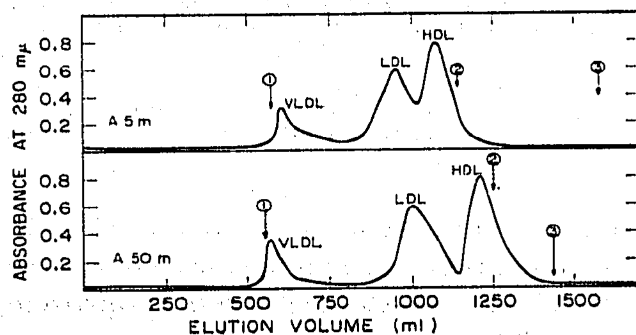

Fig. 1. Elution patterns of total pig serum lipoproteins on Biogel A 5 m and A 50 m columns. Elution volumes of *E. coli* (1), albumin (2), and cytochrome c and $NaNO_2$ (3) indicated with arrows.

contrast to the results with agar, agarose gel proved very suitable for the electrophoretic separation of lipoproteins[8] and as will be shown below, for gel filtration.

The total serum lipoproteins were separated with some overlapping into VLDL, LDL and HDL classes on Biogel A 5 m (Fig. 1) but more complete resolution occurred on A 50 m gel. The individual fractions recovered from the A 50 m column were typical of their class as shown by analytical centrifugation and electrophoresis in agarose gel. VLDL on a column of A 50 m gel had an elution volume (V_e) of 566 ml, almost equal to the void volume (V_0) of 555 ml obtained with *E. coli*. LDL (2–3×10^6 M) with a V_e of about 1000 ml was followed by HDL (0.2–$0.3 \times 10^6 M$) with V_e of 1205 ml. However, as shown in Fig. 1 the proximity of the elution volumes on A 5 m of HDL and albumin, $V_e = 1070$ and 1140 ml, respectively, would not permit the isolation of HDL from serum and such is the case also on A 50 m where V_e was 1205 ml for HDL and 1250 ml for albumin. It was also found that when serum was applied directly to the gel columns, all lipoprotein classes were contaminated by other proteins when examined by gel electrophoresis. Cytochrome c and $NaNO_2$ were eluted at V_t (1440 ml), the total solvent volume of the A 50 m column.

Attempts to separate LDL into its components, LDL_1 and LDL_2, by electrophoresis, zonal centrifugation in sucrose gradients, or repeated preparative ultracentrifugation in NaBr solutions of d = 1.20 were unsuccessful. However, the LDL class eluted from Biogel A 50 m as a broad asymmetrical peak, which was divided arbitrarily into four fractions (1 to 4) as indicated in Fig. 2. Observation in the analytical ultracentrifuge showed that LDL_1 was eluted from the gel first whereas the fraction eluted last was enriched with LDL_2. This partial separation was further confirmation that the LDL components were not ultracentrifugal artifacts.

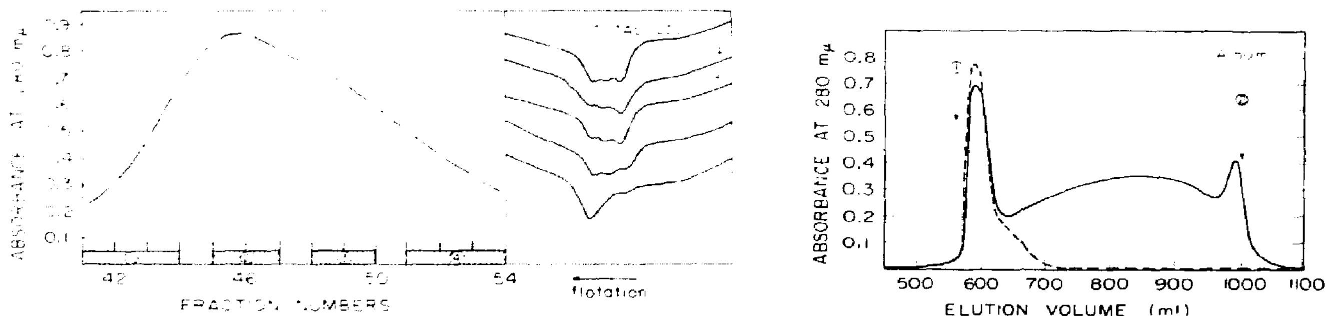

Fig. 2. Elution and ultracentrifugal patterns of low-density lipoproteins. Left hand side: Absorbance at 280 mμ of individual fractions shown by dots. Bars 1–4 indicate pooled fractions. Right hand side: Flotation patterns of pooled fractions 1–4 of total LDL 20 min after reaching 50,740 r.p.m. (NaBr solution d = 1.20).

Fig. 3. Elution of partially delipidated low-density lipoproteins. Fresh sample: solid line; sample stored four weeks: dashed line. Elution volumes of *E. coli* (1) and native LDL (2) indicated with arrows.

To obtain some information about the structure of lipoproteins, attempts were made to disassemble LDL. Although total delipidation results in an apoprotein that is insoluble in aqueous solutions, partial lipid removal permits the degraded lipoprotein residue to retain aqueous solubility. Partially delipidated LDL of human serum eluted as a single peak but in aggregated form, on gel filtration[4]. However, partially delipidated LDL from pig serum emerged from A 50 m gel in three partially resolved

zones (Fig. 3). The material in all zones was centrifugally heterogeneous in Tris buffer, pH 8.9. Aggregates eluted first as a sharp peak. Zone 2 had four components of 5.3, 9.3, 13 and 15.6 S while zone 3 contained two components which sedimented at 9.4 and 11.6 S. Sedimentation rates of the aggregates of zone 1 were not evaluated. Although the ultracentrifugal patterns of fresh partially delipidated LDL had a major component of 13 S (MARTIN AND JANADO[9]), gel filtration did not permit isolation of this component in pure form.

During storage at 5° under nitrogen the elution patterns of aliquots of the partially delipidated LDL changed. The amount of lower molecular weight material diminished and after four weeks only high molecular weight aggregates were found by gel filtration (Fig. 3). Generally, partially delipidated LDL eluted earlier than native LDL. This behaviour contrasted with that of partially delipidated VLDL, which eluted as a single peak in the same volume as HDL (V_e of 1205 ml) considerably later than native VLDL, an indication of disintegration rather than aggregation. Thus, when partially delipidated, LDL and VLDL undergo different changes.

Preparation of lipoproteins is usually based on their chemical reactivity (precipitation with dextran sulfate or similar agents), density (isolation by ultracentrifugation), or charge (electrophoretic separation). Our experimental results were reproducible when preparations were made from several different lots of pig serum. At present it does not appear that gel filtration will permit the separation of all the lipoprotein classes from whole serum since there is considerable overlap of HDL and other serum proteins. However, further fractionation of previously isolated total lipoproteins and also examination of partially delipidated lipoproteins by gel filtration is possible and in some aspects advantageous.

REFERENCES

1 C. FRANZINI, *Clin. Chim. Acta*, 14 (1966) 576.
2 C. FRANZINI, *J. Chromatog.*, 28 (1967) 37.
3 M. WERNER, *J. Chromatog.*, 28 (1967) 59.
4 S. MARGOLIS, *J. Lipid Res.*, 8 (1967) 501.
5 J. E. FRENCH, M. A. JENNINGS, J. C. F. POOLE, D. S. ROBINSON AND H. FLOREY, *Proc. Roy. Soc. London, Ser. B*, 158 (1963) 24.
6 E. A. MURPHY, H. C. ROWSELL, H. G. DOWNIE, G. A. ROBINSON AND J. F. MUSTARD, *Can. Med. Assoc. J.*, 87 (1962) 259.
7 M. JANADO, W. G. MARTIN AND W. H. COOK, *Can. J. Biochem.*, 44 (1966) 1201.
8 M. KALAB AND W. G. MARTIN, *Anal. Biochem.*, 1968 (in press).
9 W. G. MARTIN AND M. JANADO, *Intern. Congr. Biochem., 7th, Tokyo*, 1967, Abstr. IV A-23.
10 A. J. HOUTSMULLER, A. HUYSSON-HAASDIJK, A. HUYSMAN AND E. RINKEL-VAN DRIEL, *Clin. Chim. Acta*, 9 (1964) 497.