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THE IN VITRO TRANSFER OF [¹⁴C]DIMYRISTOYLPHOSPHATIDYLCHOLINE FROM LIPOSOMES TO SUBFRACTIONS OF HUMAN PLASMA HIGH DENSITY LIPOPROTEINS AS RESOLVED BY ISOELECTRIC FOCUSING

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1. Introduction

Both rat and human plasma are able to disrupt phosphatidylcholine liposomes *in vivo* as well as *in vitro*. The liposomal lipid becomes associated with plasma high density lipoproteins (HDL) as a result of this process [1,2]. Also isolated human plasma HDL can solubilize phosphatidylcholine liposomes [3]. Recent reports provide evidence that new particles are formed upon incubation of dimyristoylphosphatidylcholine (DMPC) liposomes with isolated human plasma HDL [4,5]. The formation of discoidal complexes from liposomal DMPC and apoprotein A-1 (apo A-1) released from the HDL was described [4]. Incorporation of liposomal DMPC into the original HDL particles and the simultaneous release of apo A-1 was found [5]. They could demonstrate the formation of the discoidal complex only after incubating HDL with excess liposomes.

We used the technique of isoelectric focusing in an attempt to isolate such a newly formed particle and to examine if any particular subfraction of HDL is involved in the interaction of the lipoprotein with liposomes. It has been shown that by preparative isoelectric focusing in a density gradient HDL can be separated into fractions with different protein and lipid composition [6,7].

Here we describe the subfractionation of human plasma HDL by isoelectric focusing in a granulated

gel before and after incubation with [¹⁴C]DMPC liposomes. The HDL could be fractionated into 5–7 subfractions. We found that major changes in the isoelectric profile as a result of incubation with liposomes only occur following uptake of relatively large amounts of DMPC. Even under those conditions, however, the specific radioactivities of total phospholipid were almost the same in all subfractions and we observed in none of the subfractions an increased phospholipid : cholesterol ratio. From these observations we concluded that under our incubation conditions most if not all liposomal DMPC which becomes lipoprotein-associated is taken up by pre-existent lipoprotein particles and blends with the endogenous phospholipids.

2. Experimental

HDL was prepared from human plasma containing 1 mg EDTA/ml obtained from normal fasting male donors by ultracentrifugation in KBr between densities 1.063–1.20 g/ml. It was washed once at 1.20 g/ml to eliminate albumin contamination and dialyzed against 0.15 M NaCl–1 mM EDTA–10 mM Tris (pH 7.4). For each experiment a sample of HDL was chromatographed on Ultrogel Aca 34. Column fractions were concentrated by means of ultrafiltration through Amicon PM-10 membranes. DMPC (Serdary Res. Labs, London, Canada) was purified by thin-layer chromatography. (*Me*-¹⁴C)Choline-DMPC was prepared according to [8]. Liposomes were prepared by sonica-

Abbreviations: DMPC, dimyristoylphosphatidylcholine; HDL, high density lipoprotein; apo A-1, apoprotein A-1

tion of 1–10 μmol [^{14}C]DMPC in 1–2 ml NaCl–EDTA–Tris buffer for 90 min at 25°C in a Branson bath sonifier. The specific activities are given in the legends of the figures.

HDL was incubated with liposomes under nitrogen at 37°C. After the incubation the remaining liposomes were separated from the lipoprotein on an Ultrogel AcA 34 column (1.5 \times 50 cm) at 4°C [2]. The HDL-containing fractions were concentrated and desalted by ultrafiltration. By varying incubation time (30–120 min) and relative amounts of liposomes and HDL in the incubation mixture we obtained 2 lipoprotein preparations which had taken up 0.04 and 1.2 mg DMPC/mg protein, respectively.

These preparations were subjected to preparative isoelectric focusing at 10°C in a flat bed of a granulated gel. Ultrodex-gel, ampholine carrier ampholytes and standard isoelectric focusing equipment were purchased from LKB Produkter AB, Bromma. To obtain a gradient ranging from pH 3.5–6.5, 4 g Ultrodex were swollen in a mixture of 95 ml distilled water, 0.5 ml ampholine (pH 2.5–4), 3.0 ml ampholine pH 4–6 and 1.0 ml ampholine pH 5–7. The gel bed was prepared as described by LKB [9]. After pre-focusing for 2–3 h at 10 W constant power the HDL sample (up to 3 ml) containing 2% ampholine pH 6–8 was applied to the gel 1 cm from the cathode wick. An overnight run was done under the same conditions. To obtain a print of the gel it was covered for 1 min with dry filter paper. The proteins thus absorbed to the paper were fixed in 10% trichloroacetic acid and stained with Coomassie blue. The gel bed was sectioned into 30 fractions with a fractionating grid and the gel from each section was transferred into a small column. For pH determinations the gels were eluted with 1.5 ml distilled water and the pH of the eluate was measured at 25°C. For quantitative recovery of the lipoproteins the gel fractions were subsequently eluted with 2.5 ml 0.15 M NaCl–1 mM EDTA–50 mM Tris (pH 8.7). The A_{280} and the radioactivity of each of the combined water and buffer eluates were determined.

Pooled peak fractions were dialyzed against distilled water and concentrated by lyophilization before SDS–polyacrylamide gel electrophoresis. Protein was determined according to [10], phospholipid by the method in [11] and total cholesterol by the method in [12].

3. Results and discussion

Isoelectric focusing of human plasma HDL in a pH gradient ranging from 3.5–6.5 resulted in a protein band pattern as presented in fig.1A. Due to the meandering of the bands resolution was partly lost upon sectioning of the gel as is shown by the A_{280} profile of the fractions (fig.1B). As a result some variation in relative peak areas was observed in similar experiments with HDL from different preparations but from the same donors. Nevertheless the profile was characterized by reproducible isoelectric points. Our results are compatible with those in [6,7] as far

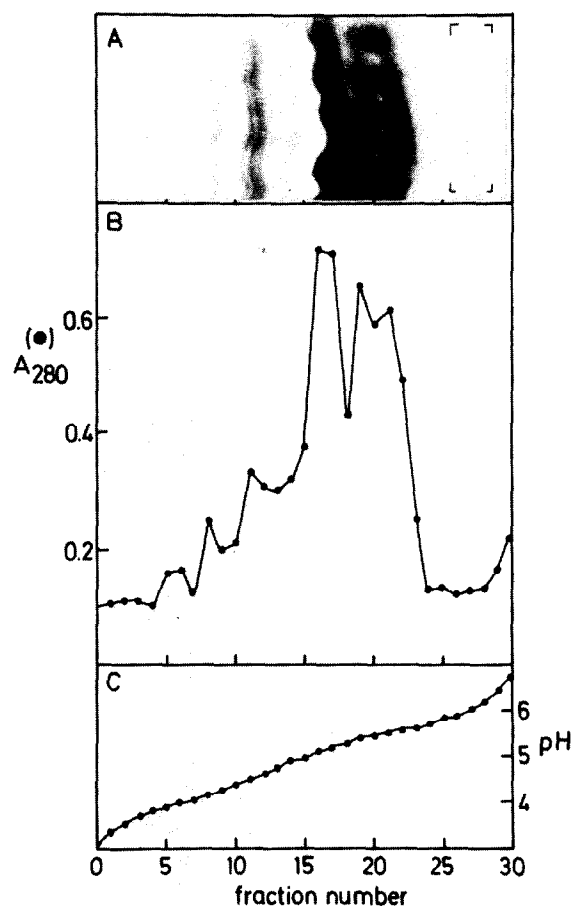


Fig.1. Isoelectric focusing of HDL in a gel bed. (A) Print of the gel stained with Coomassie blue. (B) A_{280} in the eluate after eluting the fractionated gel with distilled water and buffer. (C) pH in the eluate after eluting with distilled water.

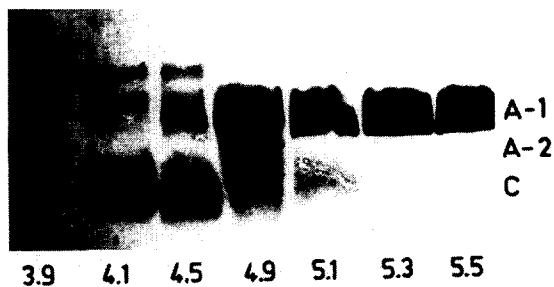


Fig.2. SDS-polyacrylamide gel electrophoresis of the peak fractions from fig.1.

as the isoelectric points of the subfractions are concerned.

The peak fractions showed different protein compositions upon SDS-polyacrylamide gel electrophoresis (fig.2). Heterogeneity of HDL has been demonstrated by using chromatographic and immunochemical techniques [13-15]. Based upon such observations the existence of HDL families was proposed [13]. Our results appear to support the HDL family concept.

HDL is frequently divided into subclasses on the basis of hydrated densities. These subclasses, HDL₂ and HDL₃, differ in size and lipid : protein ratios [16]. Since both subclasses were found to solubilize phosphatidylcholine liposomes (results not shown) and since the 2 subclasses have most of their isoelectric points in common [6,7], we did not use the separate subclasses for our electrofocusing experiments. A HDL preparation which had taken up 0.04 mg DMPC/mg HDL protein was subjected to isoelectric focusing. No major changes in the A_{280} profile were observed (fig.3B). Radiolabel was found in all major fractions. The specific radioactivity varied from ~100-150 cpm/nmol total phospholipid (fig.3C). Obviously, the liposomal [¹⁴C]DMPC (spec. radioact. 1400 cpm/nmol) became substantially diluted in all HDL subfractions and it appeared to distribute evenly among the subfractions.

When HDL was incubated with liposomes under conditions allowing a more substantial transfer of liposomal phospholipid to the lipoprotein (1.2 mg DMPC/mg HDL protein) a marked change in the isoelectric profile was observed (fig.4B). The peak around pH 5.1 was drastically reduced while a major peak

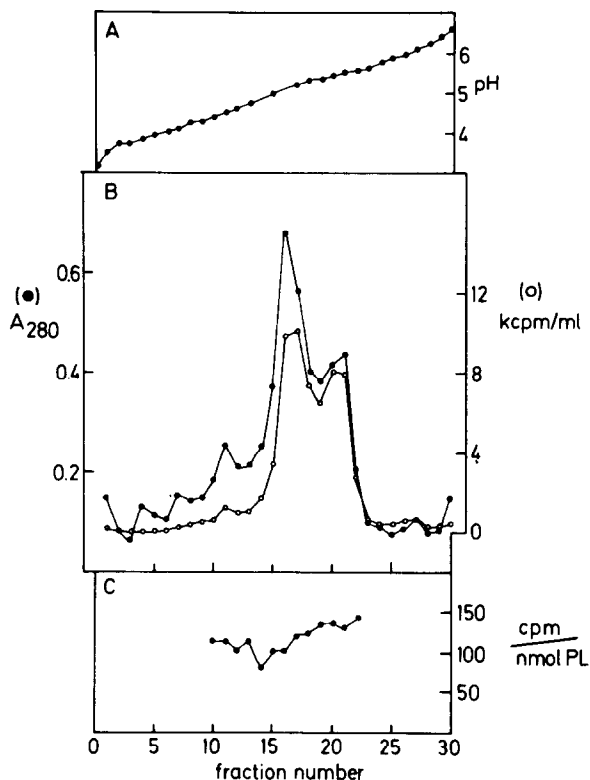


Fig.3. Isoelectric focussing of HDL after uptake of 0.04 mg DMPC/mg protein. HDL containing 10 mg protein had been incubated with sonicated [¹⁴C]DMPC liposomes (1 μ mol DMPC, 1.4×10^6 cpm) for 30 min at 37°C. (A) pH in the eluate after eluting the gel fractions with distilled water. (B) A_{280} (●) and radioactivity (○) in the eluate after eluting with distilled water and buffer. (C) Specific radioactivity of the total phospholipids.

emerged between pH 5.5 and 5.8. Half of the incorporated ¹⁴C-label was found in the new peak, but its specific radioactivity was not higher than that of the other fractions (fig.4C), which indicated that this peak fraction contained a large amount of endogenous phospholipid as well. Also most of the endogenous cholesterol was found in the same peak fraction. Relative to the other fractions the peak fractions at pH 3.5-5.8 seem to have an even somewhat higher cholesterol content as judged by the phospholipid : cholesterol ratios (fig.4D). The presence of a phospholipid-rich particle like the one formed from liposomal lipid and protein released from the HDL as described [4,5] would have resulted in a conspicuous cholesterol

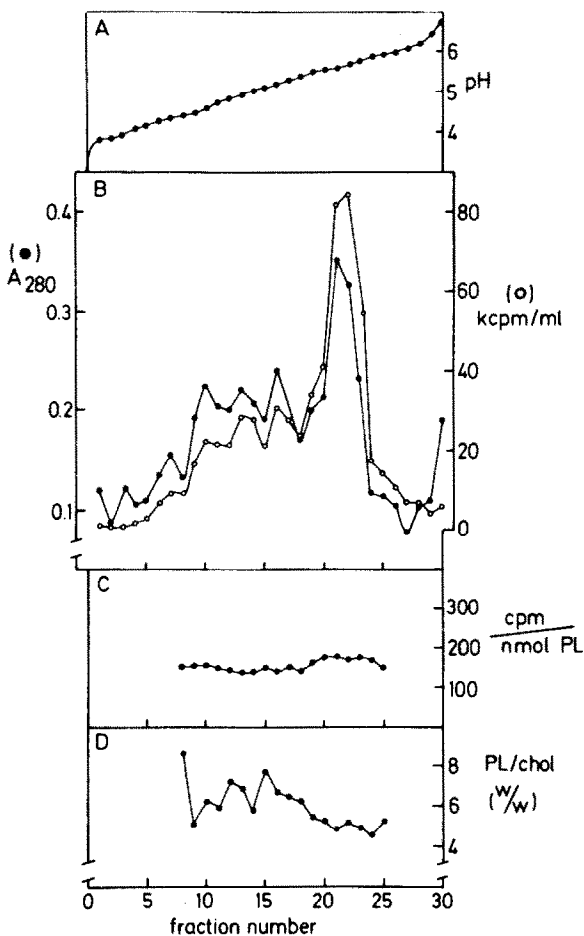


Fig.4. Isoelectric focusing of the HDL after uptake of 1.2 mg DMPC/mg protein. HDL containing 8 mg protein had been incubated with sonicated [^{14}C]DMPC liposomes ($14\ \mu\text{mol}$ DMPC, $3.7 \times 10^5\ \text{cpm}/\mu\text{mol}$) for 2 h at 37°C . (A) pH in the eluates after eluting the gel fractions with distilled water. (B) A_{280} (●) and radioactivity (○) in the eluate after eluting with distilled water and buffer. (C) Specific radioactivity of the total phospholipids. (D) Ratio phospholipid : total cholesterol (w/w).

deficit in the isoelectric focusing profile. It is unlikely that the pH 5.6 subfraction in fig.4 was formed by incorporation of cholesterol into such a phospholipid-protein complex as most of the HDL cholesterol is esterified and thus poorly transferred between isolated lipoproteins in vitro [17]. Under the conditions we used, DMPC seems to become lipoprotein-associated by incorporation into pre-existent HDL particles. The

appearance of DMPC in all subfractions could be the result of interaction between the liposomes and each subclass separately or of a redistribution by exchange of phospholipid after a preceding uptake by one particular subfraction. Phospholipids have been reported to exchange rapidly between isolated lipoprotein classes in vitro [18] and they probably will do so between HDL subclasses as well.

It is generally accepted that the phospholipid polar headgroups are located at the surface of the HDL particles [19]. It seems reasonable to assume that the incorporated liposomal phospholipids tend to occupy a similar position. Therefore, when HDL becomes 2- or 3-fold enriched in phospholipids structural changes will have to occur. The release of part of the protein from HDL as a consequence of DMPC uptake was reported [5] suggesting a substitution of surface protein by phospholipid. In addition the particles showed an increase in size which could be the result of changed lipid-protein interactions [5]. Release of protein may be a reason for altered isoelectric behaviour but there may also be a contribution by any changed lipid-protein and protein-protein interactions at the particle surface that may occur. After incorporation of a minor amount of DMPC no alteration in isoelectric behaviour could be detected. Because the incorporated DMPC probably blends with the endogenous phospholipids incubation with liposomes seems to be an appropriate method to mark the phospholipid part of HDL [20].

Acknowledgements

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