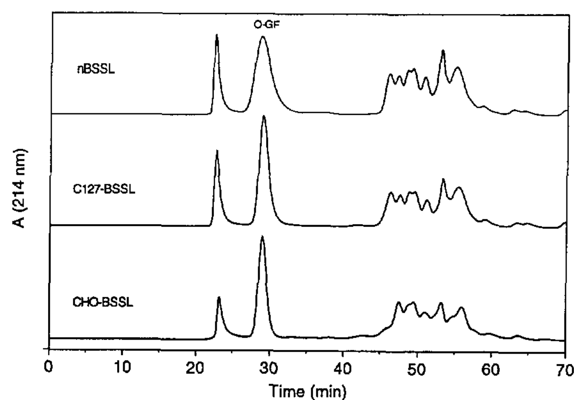

Fig. 3. Size-exclusion chromatography of BSSL cleaved with trypsin. The digest was applied to the column equilibrated with 10 mM NaH_2PO_4, 0.3 M NaCl pH 7.2. Abbreviations are as in Fig. 2. O-GF is the large O-glycosylated fragment. Approximately 40 μg of each protein digest in a volume of 100 μl was applied to the column. The peak at the void volume of the column (23 min) contains aggregated hydrophobic peptides. The material eluted after 45 min contains the "normal size" peptides. The calibrants eluted as follows under the same conditions: thyroglobulin (M_r 669) 22.6 min, ferritin (M_r 440) 26.1 min, IgG (M_r 150) 30.8 min, BSA (M_r 67) 34.1 min and ovalbumin (M_r 43) 37.9 min. Further details are given in the Experimental section.

and the two recombinant forms of BSSL (Fig. 3, Table 1). The uncleaved protein and the O-glycosylated fragment migrated as proteins larger than 300 and 200, respectively. From earlier data [5], it appears that C127 cell recombinant BSSL might be less heterogeneous than the native variants since it gives a more dense band upon SDS-PAGE. Later analysis has shown that BSSL produced by CHO cells is similar in this respect to BSSL produced by C127 cells; they comigrate and show the same band spreading (not shown). As an additional evidence for a more heterogeneous native protein, Fig. 3 shows that the peak representing the O-glycosylated fragment in native BSSL is wider than the corresponding peaks obtained with the two recombinant forms of BSSL. As a final step in the glycan analysis, the three variants of BSSL were subjected to electroblotting followed by staining with labeled lectins to obtain information on some specific glycan structures present on the glycoprotein. Also in this case, the reactions were similar; the two recombinant protein reacted exactly in the same way (Table 2). Some differences between the recombinant proteins and the native protein could however be detected (Table 2). The recombinant proteins reacted weakly with Con A and DSA, while native BSSL did not (Table 2). Native BSSL reacted more strongly with AAA and RCA indicating presence of more fucose and terminal β-galactose residues (Table 2). The reactivities with the lectins indicate that the N-glycosylation of both native and recombinant BSSL is of a complex type since Con A reacted weakly or not at all. The weak reaction of the recombinant variants with Con A was mediated through the N-glycosylation since a mutant lacking only the N-glycan did not react with this lectin (not shown). The degree of O-glycosylation is similar as judged by the chromatographic data. According to the lectin data, the native protein is certainly more fucosylated due to the very strong binding to AAA and contains also more terminal β-galactose due to the stronger binding to RCA. Similarities between the native and the recombinant forms of BSSL are that they contain no terminal mannose, they all contain α(2–3)- and α(2–6)-linked sialic acids, N-acetylgalactoseamine and that they most certainly all contain complex N-glycan structures. Another evidence for the presence of fucose-containing structures in native but not in recombinant BSSL was that the native protein reacted

Table 2
Lectin binding properties of the different forms of BSSL

Lectin	Native BSSL	C127-BSSL	CHO-BSSL
Con A	no	weak	weak
RCA	strong	weak	weak
WGA	medium	medium	medium
SNA	weak	weak	weak
AAA	strong	weak	weak
MAA	weak	weak	weak
DSA	no	no	no
GNA	no	no	no
PNA	weak	weak	weak
PHA-L	no	no	no

Further details and abbreviations are given in the Experimental section. The interactions are classified as no, weak, medium and strong

with antibodies against the Lewis^b antigen while the recombinant forms did not react with these (not shown). In summary, we could, by quite simple standard chromatographic methods, show that recombinant BSSL proteins produced by C127 and CHO cells were glycosylated to a similar extent, but were less heterogeneous than the native protein. By the lectin binding study it was established that there were some differences in the specific glycans attached to the recombinant forms of BSSL compared to those attached to native BSSL. It was also shown that the two recombinant forms of BSSL were very similar in their glycosylation despite the fact that they were produced in cells from different species, i.e. mouse and hamster. The chromatographic techniques described here do not give any detailed characterization of the glycan structures but are suitable for preparing glycopeptides for further characterization. A follow-up on this study would be to release the glycans and analyze them by high-performance anion-exchange chromatography and mass spectrometry [10] to obtain a more detailed picture of the attached carbohydrates.

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Elution of lipoprotein fractions containing apolipoproteins E and A-I in size exclusion on Superose 6 columns is sensitive to mobile phase pH and ionic strength

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Abstract

Separation of lipoproteins secreted from McA-RH7777 (rat hepatoma) cells by Superose 6 column size-exclusion chromatography, using PBS buffer (NaCl 150 m*M*, sodium phosphate 10 m*M*, pH 7.5, EDTA 1 m*M*), produced apolipoprotein (apo) E or A-I profiles that did not correlate with lipoproteins separated by density ultracentrifugation. By density ultracentrifugation, apoE and apoA-I were mostly (>90%) confined to high-density lipoproteins (HDL, $d = 1.063$–1.023 g/ml), but by chromatography apoE and apoA-I were recovered in all lipoprotein classes, including low-density lipoproteins (LDL), HDL, and post-HDL. Moreover, the elution volume of phenol red on Superose 6 greatly exceeded the total column volume. These discrepancies were attributable to pH and ionic strength effects. In low ionic strength, high pH buffer (Tris 25 m*M*, pH 8.3), elution volumes of lipoproteins, albumin, and phenol red were minimized. Elution volumes increased 25–70% when buffer pH was lowered at constant ionic strength (Tris 25 m*M*, pH 7.4) or when ionic strength was increased at constant pH (Tris 25 m*M*, pH 8.3, NaCl 500 m*M*). Altered phase partition appeared to cause the altered elution volumes, since recovery (measured as analyte peak area), resolution (measured as peak width at half height), and column void volume varied little from buffer to buffer. In Superose 6 size-exclusion chromatography with PBS buffer, then, elution volumes vary with pH and ionic strength. We propose that TBE buffer (Tris-borate 89 m*M*, pH 8.3, EDTA 2 m*M*) may produce fewer artefacts than PBS. With TBE there were (i) better correlation between size-exclusion and ultracentrifugal fractions, (ii) lower elution volumes, and (iii) less "smearing" of McA-RH7777 apoE and apoA-I containing lipoprotein bands.

1. Introduction

The most prevalent classification scheme for plasma lipoproteins is based on their hydrated density. The categories of very low density lipoproteins (VLDL, $d < 1.006$ g/ml), low density lipoproteins (LDL, $1.019 < d < 1.063$ g/ml) and high density lipoproteins (HDL, $1.063 < d < 1.23$ g/ml) have proven useful both clinically and analytically. Size-exclusion chromatography is being used increasingly as a surrogate technique for analytical fractionation of lipoproteins from plasma and from other sources, both in the clinical laboratory [1] and research settings [2–9]. The increased use of size exclusion can be

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attributed to improvements in size-exclusion technology, such as the introduction of the Superose 6 column for fast protein liquid chromatography. Plasma LDL and HDL isolated by the Superose 6 methods have lipid and apolipoprotein compositions comparable to those of the corresponding ultracentrifugal fractions [1,5], with the advantage of a 1-h separation time, compared to a day or longer for conventional size exclusion [10] or preparative ultracentrifugation [11].

The rat hepatoma cell line McA-RH7777 has been widely used to study metabolism of hepatic lipoproteins [12,13]. In the course of a study of McA-RH7777 lipoproteins, we undertook to characterize the profile of lipoproteins containing apolipoproteins E (apoE) and A-I (apoA-I) in McA-RH7777 conditioned medium by density gradient ultracentrifugation and by Superose 6 size exclusion (using phosphate buffered saline (PBS) running buffer), and observed a poor correlation between the two methods in regards to the distributions of apoE and apoA-I. When the discrepancy was investigated, we discovered that the distributions of McA-RH7777 lipoproteins containing apoE and apoA-I were highly sensitive to mobile phase pH and ionic strength during chromatography on the Superose 6 columns. We found that using Tris-borate-EDTA (TBE) buffer effectively minimized size-exclusion artefacts and generated McA-RH7777 LDL and HDL with apolipoprotein compositions which correlated well with the ultracentrifugal fractions. These observations emphasize the limitations of the size-exclusion technique as a surrogate for ultracentrifugation when analytes other than plasma are involved.

2. Methods

2.1. Cell culture

McA-RH7777 cells were grown to 60% confluence in 100-mm dishes, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 10% horse serum (Life Technologies). Conditioned medium was obtained by washing the cells twice with 5 ml of serum-free DMEM, aspirating each wash, and then adding 12 ml of fresh DMEM. After 24-h incubation, the conditioned medium was decanted into chilled 15-ml polypropylene tubes, and stock solutions of protease inhibitors were added to give final concentrations of EDTA 5 m*M*, phenylmethylsulfonylfluoride 2 m*M*, benzamidine 2 m*M*, and sodium azide 0.05% (w/v). The McA-RH7777 lipoproteins were then separated by either ultracentrifugation or by size-exclusion chromatography on Superose 6 columns.

2.2. Ultracentrifugation

For ultracentrifugation, the lipoproteins were separated by sequential flotation in a TLA 100.3 rotor using a TLA-100 table-top centrifuge (Beckman). VLDL + LDL were collected by raising the density of the conditioned medium to $d = 1.063$ g/ml by addition of solid KBr, centrifuging at 99 000 rpm (540 000*g*), 4°C, for 4 h, and aspirating the top 240 μl of each 3-ml centrifuge tube. VLDL and LDL were isolated together because McA-RH7777 cells produce negligible VLDL ($d < 1.019$ g/ml lipoproteins) under serum-free conditions [13]. The bottom 1.5 ml infranate was adjusted to $d = 1.23$ g/ml by addition of solid KBr, and centrifuged for 5 h, 99 000 rpm, 4°C. The top 240-μl sample was collected as HDL ($d = 1.063$–1.23 g/ml), while the bottom 1.5 ml was recovered as the $d > 1.23$ g/ml fraction. After ultracentrifugal isolation, the lipoproteins in each fraction were adsorbed onto fumed silica (Cab-O-Sil, Sigma [14]), and apolipoproteins were separated on 3–18% gradient SDS-polyacrylamide gels [13].

2.3. Superose 6 column chromatography

Size exclusion was performed using phosphate buffered saline (PBS: sodium phosphate 10 m*M*, pH 7.5, NaCl 150 m*M*, EDTA 1 m*M*) with the following chromatographic parameters: flowrate, 0.5 ml/min; back pressure, 1.1 MPa; monitor, mercury vapor lamp at 280 nm; recorder, analog chart. The elution volume of human

LDL or HDL standard, prepared by ultracentrifugation [11], was determined on the Superose 6 column and used for comparison with that of McA-RH7777 lipoproteins. A 12-ml volume of conditioned, serum-free McA-RH7777 medium was concentrated to 300 μl using Centricon-10 centrifugal concentrators (Amicon), 200 μl of the concentrate was loaded onto a Superose 6 column, and 30 fractions of 1 ml were collected. Apolipoproteins in each fraction were adsorbed onto fumed silica and visualized by SDS-polyacrylamide gel electrophoresis [13].

2.4. Immunoblot analysis

Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose, and visualized by immunoblotting using rabbit antisera to rat apoE, apoB, or apoA-I followed by peroxidase-labelled goat anti-rabbit secondary antibodies and enhanced chemiluminescence detection (Amersham) [12]. All the antisera used were specific to each corresponding rat apolipoprotein.

3. Results and discussion

3.1. Discrepancies of apolipoprotein profiles separated by ultracentrifugation or Superose size exclusion using PBS running buffer

The distributions of McA-RH7777 apolipoproteins in ultracentrifugal density fractions are shown in Fig. 1A. The VLDL + LDL fraction contains most of the apoB100, plus traces of apoE and apoA-I. The HDL density fraction contains the bulk of the apoE, apoA-I, and apoB48. The $d > 1.23$ g/ml infranate density fraction contains negligible apoB, traces of apoE, and somewhat more apoA-I. The Superose 6 size-exclusion distribution of McA-RH7777 apolipoproteins was in sharp contrast to this ultracentrifugal density distribution. The size-ex-

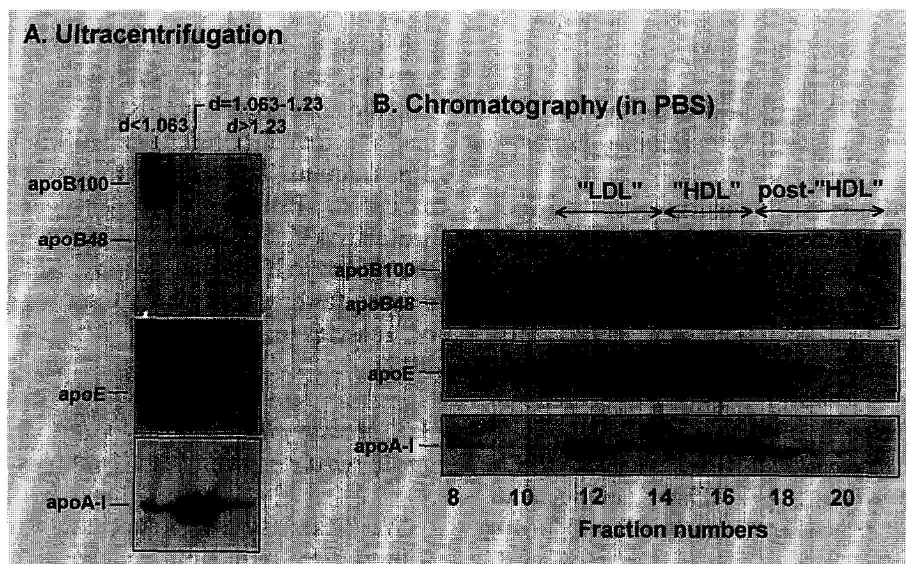

Fig. 1. Apolipoprotein composition of lipoproteins from McA-RH7777 conditioned medium isolated by density ultracentrifugation or by Superose 6 size-exclusion chromatography using PBS running buffer. (A) Distribution of apolipoprotein B (top), apoE (middle), and apoA-I (bottom) in VLDL/LDL ($d < 1.063$ g/ml), HDL ($d = 1.063$–1.23 g/ml), and $d > 1.23$ g/ml ("lipoprotein-free") infranate, isolated by sequential flotation. In the $d > 1.23$ g/ml infranate, a band which did not correspond to either apoB100 or apoB48 was observed to react with the rabbit polyclonal anti-rat-apoB antibody (top panel, right column). Its identity is unknown. (B) Distribution of apolipoproteins B, E, and A-I in Superose 6 size-exclusion chromatographic fractions. The labels "LDL" and "HDL" at the top indicate the fractions in which ultracentrifugally purified human LDL and HDL [11] eluted.

clusion distribution of McA-RH7777 apoE and apoA-I is shown in Fig. 1B. Although the peak distribution of apoE and apoA-I was in the "HDL" fractions, substantial amounts of each apolipoprotein were present in the "LDL" and the "post-HDL" fractions as well. In size exclusion apoE was particularly prominent in "LDL" and even in the early elution fractions, while apoA-I was more skewed into the "post-HDL" fractions. A manifest anomaly was also observed: phenol red (M_r 354), the dye from the McA-RH7777 medium, eluted from the Superose 6 column at a volume of 38 ml (see below), despite an absolute volume for the column of 24 ml (30 × 1 cm I.D.: $\pi r^2 h = 23.56$ cm^3). Thus, the apoE and apoA-I distributions were not consistent with centrifugal data, and the phenol red elution volume was clearly artifactual. However, the distributions of apoB100 and apoB48 after size exclusion were consistent with the ultracentrifugal data: apoB100 was confined to the "LDL" fractions, and apoB48 to the "HDL" fractions. There were no detectable degradation products of any of the medium apolipoproteins as determined by Coomassie blue staining and immunoblotting (data not shown).

3.2. *Improved apolipoprotein profiles on Superose 6 columns using TBE running buffer*

The discrepancies and artefacts observed led us to inquire whether non-ideal interactions were distorting the size-exclusion profile of McA-RH7777 apoE and apoA-I. We looked for buffer effects by repeating size exclusion using TBE buffer (Tris 89 m*M*, boric acid 89 m*M*, EDTA 2 m*M*, pH 8.3) in stead of PBS. TBE decreased the elution volume (V_e) of human LDL, human HDL, and the components of McA-RH7777 medium, as shown in Fig. 2. The V_e of purified LDL decreased from 11–13 ml in PBS to 8–10 ml in TBE (Fig. 2A), while V_e of HDL decreased from 15–17 ml to 10–12 ml (Fig. 2B). The decrease in V_e was a generalized effect of TBE buffer, since the components of McA-RH7777 medium were similarly affected, the V_e of the albumin peak shifted from 16.6 ml in PBS to 13.8 ml in TBE (Fig. 2C). These results were reproducible in each buffer; the V_e of any given analyte varied by less than 0.2 ml from run to run. The peak shapes of the analytes were not affected by the buffer change, nor were the peak areas altered, suggesting that recovery was identical. The column void volume (V_0) was essentially identical between the two buffers, suggesting that column architecture was also preserved. The V_e of phenol red (a marker of the approximate inclusion volume) decreased from 38 ml in PBS to 22.4 ml in TBE, which is appropriate for a column with a total volume of 24 ml.

When the TBE size-exclusion fractions of the medium lipoproteins were analyzed for apolipoprotein content, we found that, in contrast to our earlier results with PBS, the TBE-derived apolipoprotein distribution was consistent with the results of ultracentrifugation (Fig. 3). In TBE, the bulk of both apoE and apoA-I was confined to the "HDL" fractions, with apoE tailing into the "LDL" and apoA-I tailing more into the "post-HDL" fractions. ApoB100 and apoB48 elution profile, as before, was consistent regardless of buffer: apoB100 eluted almost exclusively in the "LDL" fractions, while apoB48 was mostly confined to the "HDL" fractions. Because McA-RH7777 apolipoproteins had improved correlation with ultracentrifugation fractions after TBE size exclusion (as compared to PBS), and because the manifestly artifactual V_e of phenol red in PBS resolved with the switch to TBE, we considered that TBE gave a more faithful representation of the distribution of analytes in McA-RH7777 medium than did PBS.

3.3. *Mobile phase pH and ionic strength affects apolipoprotein profiles on Superose 6 columns*

We next assessed the possible factors contributing to anomalous behavior on Superose 6 columns. We prepared a test solution of three standard analytes: blue dextran (marker of the void volume, V_0), bovine serum albumin, and phenol red, and adjusted their concentration in the solution so that each analyte gave an absorbance of 0.3. The V_e of these three analytes in

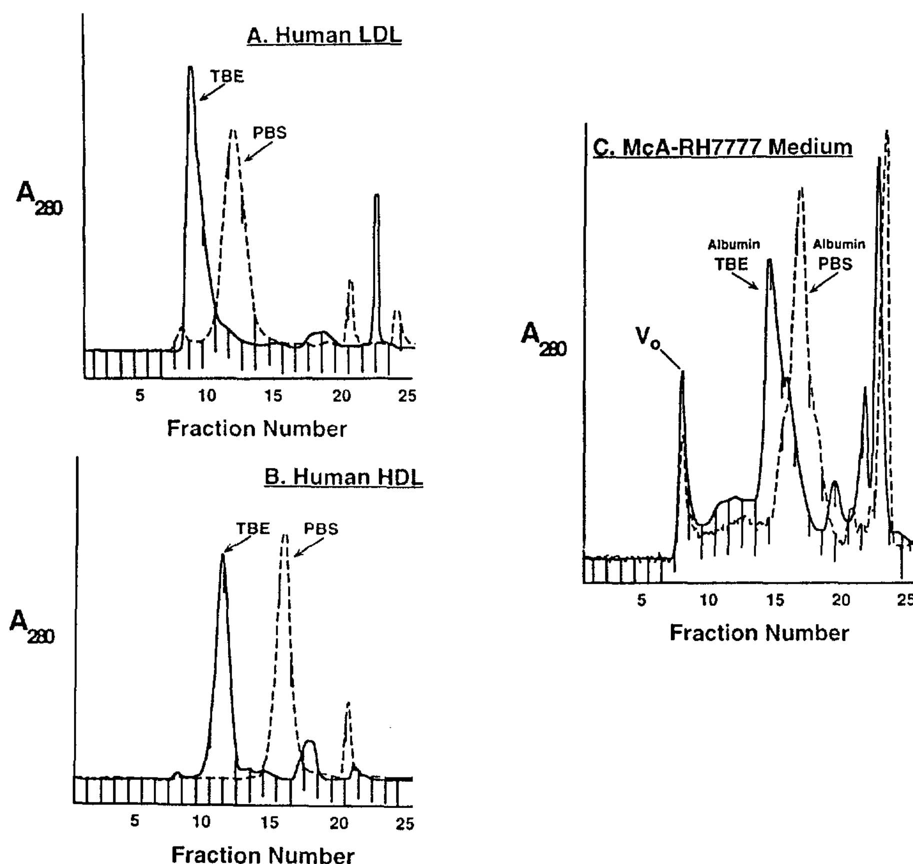

Fig. 2. Effect of PBS or TBE running buffer on Superose 6 size-exclusion profiles of control analytes. The V_e of control analytes shifted markedly when TBE running buffer (solid line) was used in place of PBS running buffer (dashed line), even though all other chromatographic parameters (see text) were held constant. The column was equilibrated with a minimum of 4 column volumes of buffer prior to chromatography, and each assay was done in duplicate. The coefficient of variance of V_0 (void volume), V_e and peak height for any given analyte was less than 2% run to run. (A) Human LDL, 250 μg protein, (B) human HDL, 250 μg protein, (C) concentrated McA-RH7777 conditioned medium, 600–800 μg protein.

running buffers of varying pH and ionic strength was then assessed. The results are shown in Table 1 and can be summarized as follows: no buffer affected the V_0 of the column to any marked degree, suggesting that column architecture (e.g. gel shrinkage, etc.) was not sensitive to buffer changes. However, the size exclusion of both albumin and phenol red were sensitive to both pH and ionic strength: at fixed ionic strength (Tris 25 m*M*), raising the pH from 7.4 to 8.3 reduced the V_e of albumin by 1.1 ml (7.4%). Likewise, at fixed pH (7.4), raising the ionic strength, from Tris 25 m*M* to Tris 25 m*M* + NaCl 500 m*M*, increased the V_e of albumin from 14.7 ml to 16.5 ml (12.2%). Phenol red was even more sensitive to pH and ionic strength than albumin; its V_e ranged from 22.8 ml in Tris 25 m*M*, pH 8.3 (cf. 22.4 ml in TBE) to 37.8 ml in Tris 25 m*M*, pH 7.4, NaCl 500 m*M* (cf. 38 ml in PBS). The addition of 100 m*M* urea had no effect on the V_e of albumin, suggesting that hydrogen bonding had little effect on the chromatographic behavior of albumin. For phenol red, though, a slight decrease in V_e was observed (Table 1). Thus Superose 6 gel filtration was remarkably sensitive to buffer conditions such as pH and ionic strength. The effect was generalized, affecting human lipoproteins, albumin, McA-RH7777 medium, and phenol red, and it could be minimized by certain buf-

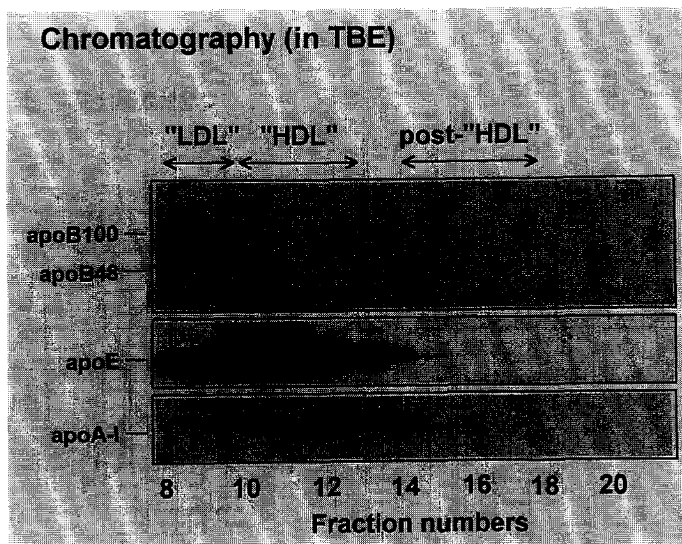

Fig. 3. Distribution of apolipoproteins from McA-RH7777 conditioned medium isolated by Superose 6 size-exclusion chromatography using TBE running buffer. This figure corresponds to Fig. 1B, with TBE used instead of PBS as running buffer. Observe the improved correlation with ultracentrifugal results (Fig. 1A).

fers. In TBE, or in Tris 25 m*M*, pH 8.3, the V_e of test analytes dropped to what appeared to be a minimum V_e value.

Our observations suggest that hydrophobic interactions artifactually increase the elution volumes of lipoproteins on Superose 6 in PBS buffer, while ion-exchange plays little role. Our data are comparable to those of Golovchenko et al. [15] who studied the non-size-exclusion interactions of the hydrophobic protein endoglucanase 1, and the hydrophilic proteins endoglucanase C and lysozyme, during chromatography on Superose 12 columns. Their data suggest that the artefacts induced by hydrophobic interactions require more careful buffer adjustment than the ion-exchange and ion-exclusion artefacts observed with hydrophilic proteins, which were readily avoided by addition of 100 m*M* $NaSO_4$ to their running buffer.

Our results demonstrate that the efficient separation of serum lipoproteins on Superose 6 columns using PBS running buffer [1,5] occurs in the presence of PBS-related elution artefacts. The efficiency of a chromatographic column, defined as the number of theoretical plates (N), is computed as: $N = 5.5\ (V_e/w_{1/2})^2$ [16], where $w_{1/2}$ is the peak width at half height. Since LDL, HDL, and albumin have an increased V_e in PBS compared to TBE, but a similar $w_{1/2}$, the number of theoretical plates is increased in PBS buffer, and column efficiency is improved. Column selectivity (α), which is related to a column's ability to resolve two analytes, is also improved by the increase in V_e for LDL and HDL. Selectivity is defined as: $\alpha = (V_{e1} - V_o)/(V_{e2} - V_o)$ [16]. Since LDL and HDL peaks tend to spread apart in PBS buffer, while V_o is unchanged, selectivity is improved. Thus the PBS buffer system has performed well for the analysis of lipoproteins in plasma, and the PBS-related Superose 6 column interactions actually improve the separation of plasma lipoproteins.

As compared to plasma lipoproteins, McA-RH7777 lipoproteins exhibit unfavorable non-size-exclusion interactions in PBS, presumably because of an altered protein conformation, a