Influence of Polar Polymers on the Apoprotein Region of Human Serum Lipoproteins: An Electron Paramagnetic Resonance (EPR) Study

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Abstract. Electron spin resonance spectroscopy was used for measurements of the surface potential and apoprotein structure of LDL and HDL in the presence of Ca^{2+} and dextran sulfate, heparin and chondroitin sulfate. A decrease in the absolute values of surface potential of LDL and HDL was observed after addition of Ca^{2+}. In the presence of the negatively charged macromolecules the measured surface potential was less reduced. The spectral properties of a maleimide spin label covalently attached to the apoprotein were changed under conditions of aggregation of LDL induced by dextran sulfate, chondroitin sulfate or heparin in the presence of Ca^{2+}. In the HDL system this effect was only observed for dextran sulfate.

The influence of PEG on the spectral parameters of the spin label is dependent on the molecular weight of the polymer. PEG 400 decreased the mobility of the spin-labelled apoprotein region of LDL, whereas PEGs with higher molecular weight only slightly increased the maleimide mobility. On the other hand, the maleimide-labelled apoprotein region of HDL showed a higher sensibility to all PEGs used. Addition of PEG leads to immobilization of apoprotein A.

Key words: LDL — HDL —Glycosaminoglycans — PEG — EPR

Abbreviations: Spin label, SL; Low density lipoproteins, LDL; High density lipoproteins, HDL; Electron paramagnetic resonance, EPR; chondroitinsulfate A, CSA; Dextran sulfate, DS; Poly(ethylene glycol), PEG; Apoprotein A/B, Apo A/B; Glycosaminoglycans, GAG

Introduction

Interaction of mucopolysaccharides (heparin, chondroitin sulfate, heparan sulfate

etc.) with lipoproteins of human serum was first described by Cornwall and Krueger (1961). It has been well established that low density lipoproteins (LDL) are able to form complexes with mucopolysaccharides or glycosaminoglycans (GAG). Furthermore, using proteoglycans (core protein + GAG) Vijayagopal et al. (1983) showed that in the absence of calcium proteoglycans and LDL form soluble complexes; in the presence of calcium however insoluble complexes arise. The complexes are occasionally formed by ionic interaction between the negatively charged GAG molecules and the positively charged amino acid residues of apolipoprotein B (Radhakrishnamurthy et al. 1982). This could be shown by specific modifications of amino acid residues as reported by Vijayagopal et al. (1981). In the presence of Ca^{2+} the macromolecules can bind to phospholipid headgroups in the lipoprotein surface via Ca^{2+} bridges. Electron paramagnetic resonance spectroscopy (EPR) was used to measure the effects of GAG and Ca^{2+} on the surface potential and the apoprotein mobility of lipoproteins.

Poly(ethylene glycol) is used for the precipitation and recrystallization of proteins (Atha and Ingham 1981). In a recent paper the use of PEG for sequential precipitation of human serum lipoproteins was described as a diagnostic tool in determining atherosclerotic risk factors (Kostner et al. 1985).. A possible therapeutic application (Beitz et al. 1988) would require the production of large amounts of specific lipoprotein classes. PEG could be very useful as a precipitation agent and as a component in polymer two phase systems for separation of lipoproteins (Albertsson 1971). Possible perturbation of lipoprotein structure would prevent the use of lipoproteins separated by PEG for the investigation of structural properties of lipoproteins, and the clinical application thus becomes questionable. The physical properties of PEG-induced membrane interactions have been described by different authors (MacDonald 1985; Arnold et al. 1985; 1988; Surewicz 1983). The main mechanism of the PEG action seems to be the dehydration effect of the polymer (MacDonald 1985), in addition to high osmotic pressure (Arnold et al. 1988) and change of the dielectric constant of the aqueous phase (Arnold et al. 1985).

We have studied PEG-induced aggregation of LDL and HDL under different conditions of pH and ionic strength (Arnold and Zschörnig 1988; Zschörnig et al. 1991; Arnold et al. 1989). From model membrane studies it was argued that PEG does not interact directly with membrane surfaces (Arnold et al. 1990). In the present work the influence of PEG on the protein part of lipoproteins was studied using a spin label covalently attached to the apoprotein region.

Materials and Methods

LDL and HDL were obtained by ultracentrifugation according to Havel et al. (1955). The purity of lipoproteins was checked by agarose gel electrophoresis (Rapp and Kahlke 1968) and immune turbidimetry measurements according to Winkler et al. (1978) using specific

antisera against Apo B and AI. The lipoproteins were subsequently dialyzed against appropriate buffer solutions overnight at 4 °C. Buffer I: 0.01 mol/l Tris, 0.5 mmol/l EDTA, pH 7.4; buffer II: 0.01 mol/l Tris, 0.5 mmol/l EDTA, 0.15 mol/l NaCl, pH 7.4.

The lipoprotein concentration was determined as the content of phospholipids, according to Vaskovsky et al. (1975) and as the content of proteins, according to Markwell et al. (1978).

Heparin (Gedeon Richter, Hungary), chondroitin sulfate A (Serva, FRG), and dextran sulfate MW 500,000 (Pharmacia, Sweden) were used without further purification.

For EPR investigations, a JEOL FE 2 XG spectrometer with a NM- PVT temperature controller connected to a digital set unit was used. For measurements of the surface potential, a positively charged spin probe, 7-imoxyl-octadecane-trimethylammonium methane sulfonate (SL I) (Arnold et al. 1986) was used. The spin probe/phospholipid molar ratio was about 1/20.

Table 1. Typical spectral parameters

	Maleimide	Charged spin probe
Modulation width:	10^{-4} T	6.3×10^{-5} T
Time constant:	1 s	0.3 s
Microwave power:	10 mW	10 mW
Sweep time:	16 min	8 min

The apoprotein was labelled by spin probe 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (SL II) following the procedure of Gotto and Kon (1969). Typical spectral parameters are shown in Table 1.

Fig. 1 illustrates a typical spectrum of the charged spin label. The line heights of the peaks used to calculate of changes in surface potential are indicated. The partition of spin label between lipoprotein and the aqueous phase is described by the partition coefficient λ given by the ratio h_2/h_1 derived from the spectrum:

$$\lambda \sim h_2/h_1$$

where h_1 is the amplitude of the low magnetic field component which belongs to spin probes bound to the lipoproteins, and h_2 is the amplitude of the EPR signal at high field which results from the spin probe being dissolved in the aqueous phase. A direct proportionality between signal amplitudes and the number of spin probes is assumed (Arnold et al. 1986). The calculation of the corresponding surface potentials was described by Krumbiegel et al. (1988).

EPR spectrum of the maleimide spin probe covalently attached to the apoprotein of the lipoproteins is shown in Fig. 2. The outer hyperfine splitting $2A_{II}$ of the strong immobilized label and the ratio w/s were used as parameters of measurements (Fig. 2). The accuracy of the 2 A_{II} determination was about 0.5 mT.

All experiments were done in triplicate. The figures show representative experiments.

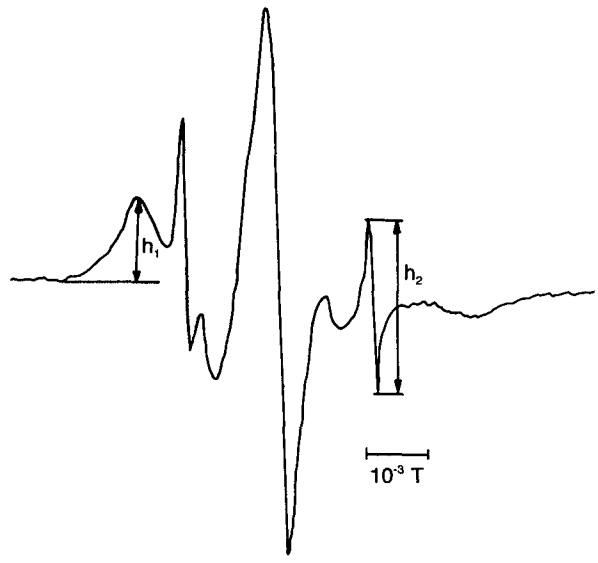

Figure 1. EPR spectrum of spin probe 7-imoxyl-octadecane-trimethylammonium methane sulfonate in the presence of 1 mg/ml LDL.

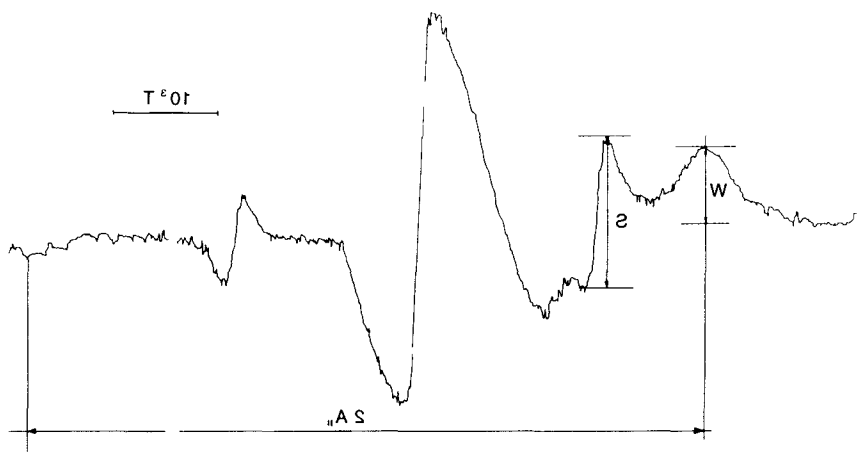

Figure 2. EPR spectrum of spin probe 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl bound to LDL (1 mg/ml protein).

Results

The positively charged spin probe was used to measure the surface potential Ψ_0 of LDL after addition of calcium The experimental results are given in Fig 3 These experiments were done in buffer I since the effect of calcium on the surface potential is stronger at low ionic strength Addition of calcium results in an increase of the partition coefficient, i e the negative surface potential of the lipoproteins is lowered Using the Boltzmann equation the calculated potential difference is about 27 mV at a Ca^{2+} concentration of 30 mmol/l If heparin (3 82 mg/ml) or CSA (4 16 mg/ml) had been present in the sample before calcium was added, the change in the measured partition coefficient was smaller compared with the sample without GAG Thus, the surface potential was changed only slightly The same alterations were observed using buffer II (i e at high ionic concentrations), but the changes of the partition coefficient were smaller

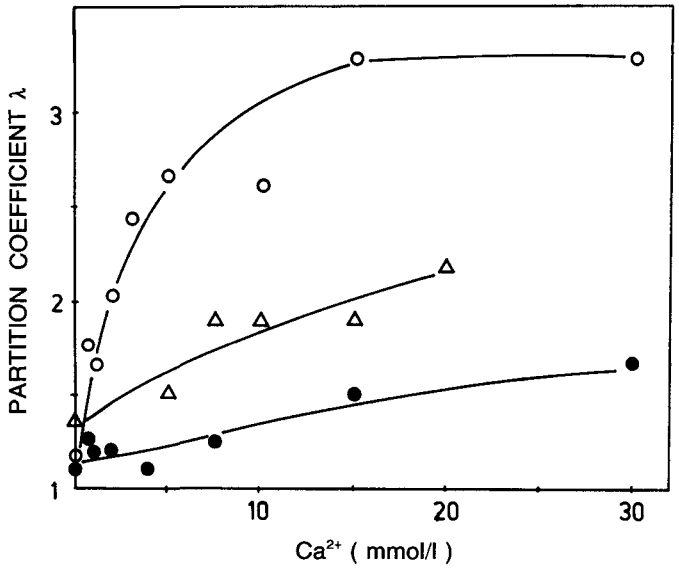

Figure 3. Partition of spin probe I in the presence of LDL (0 7 mg/ml phospholipid) without GAG (o), 3,82 mg/ml heparin (•) and 4 16 mg/ml CSA (△) 10 mmol/l Tris, pH 7 4, 37 °C

The influence of heparin and CSA on the protein region of the lipoproteins was studied using maleimide-labelled LDL The label does covalently attach to the SH-group of cysteine and the ε-amino group of lysine As positively charged amino

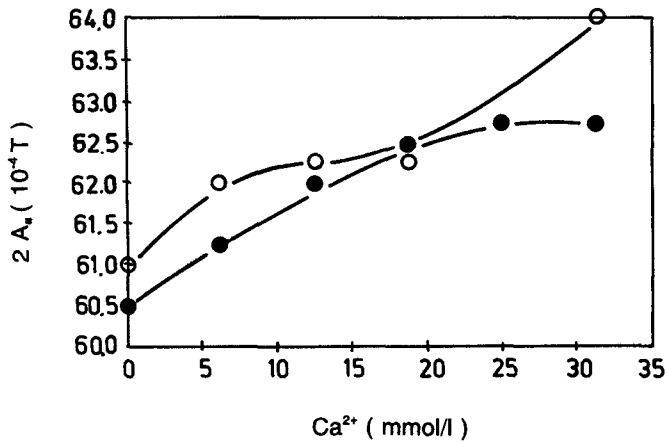

Figure 4. Outer hyperfine splitting of SL II bound to LDL (1.5 mg/ml PL) in the presence of 3.125 mg/ml heparin (○), 3.125 mg/ml CSA (●) in dependence on the calcium concentration. 10 mmol/l Tris, 0.15 mol/l NaCl, 0.5 mmol/l EDTA, pH 7.4, 37°C.

acids, such as lysine, histidine and arginine, are "potential" interaction sites for heparin the influence of calcium and GAG on the maleimide moiety was measured separately. Calcium and GAG alone did not affect the moiety of the maleimide-labelled protein region (data not shown), either at high or at low ionic strength. The results of the combined effect of calcium and GAG on the outer hyperfine splitting are shown in Fig. 4. GAG was added to the LDL suspension and then calcium was titrated. At about 20 mmol/l calcium and 3.125 mg/ml CSA the $2A_{II}$-value increased by about 2×10^{-4} T. In the presence of 3.125 mg/ml heparin and 30 mmol/l Ca^{2+} the $2A_{II}$ value changed from 6.1×10^{-3} T to 6.4×10^{-3} T. At low ionic strength the outer hyperfine splittigs are not well resolved preventing the measurement of $2A_{II}$. The reason for this behaviour lies in an increase of the signal amplitude for weakly bound maleimide under low ionic strength conditions, as described by Kanashiro et al. (1982). However, changes of $2A_{II}$ are still observable.

The influence of PEG on the outer hyperfine splitting $2A_{II}$ of maleimide-labelled LDL depends on the molecular weight (data not shown). For PEG 400 an increase of about 2×10^{-4} T was measured over a range of 0–30 wt.% PEG 400. The increase of $2A_{II}$ is monotonic, i.e. a sharp increase at a distinct PEG concentration was not observed. Addition of PEG 1500 changed $2A_{II}$ from 6.16×10^{-3} T (0 wt.% PEG 1500) to 6.275×10^{-3} T (16 wt.% PEG 1500). PEG 6000 raised $2A_{II}$ from 6.15×10^{-3} T (0 wt.% PEG 6000) to 6.35×10^{-3} T (8 wt.% PEG 6000). PEG 20,000 had no significant effect on $2A_{II}$ in a concentration range of 0–20 wt.% PEG 20,000.

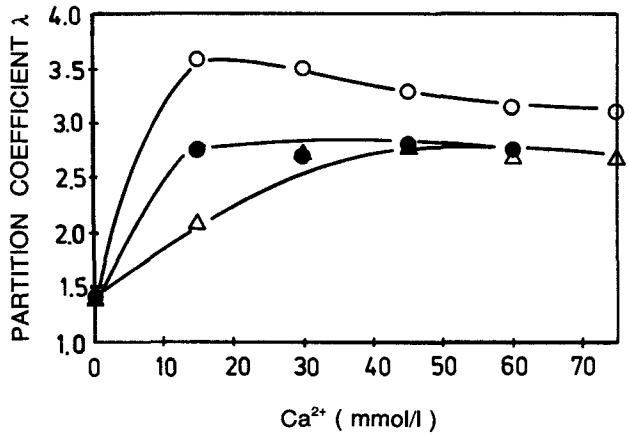

Figure 5. Partition of SL I in the presence of HDL (1 mg/ml protein) without GAG (o), 12.5 mg/ml heparin (Δ), 12.5 mg/ml CSA (•). 10 mmol/l Tris, 1 mmol/l EDTA, pH 7.4, 25 °C.

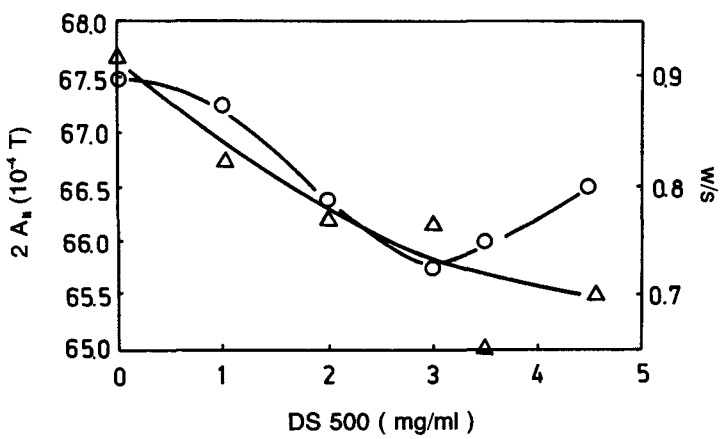

Figure 6. Outer hyperfine splitting (o) of SL II bound to HDL (1 mg/ml protein) and w/s ratio (Δ) in the presence of 100 mmol/l Ca^{2+} in dependence on the DS 500 concentration. 10 mmol/l Tris, 0.1 mol/l NaCl, 1 mmol/l EDTA, pH 7.4, 25 °C.

Ca^{2+} titration to an HDL suspension (buffer I) results in a increase of the partition coefficient λ of the charged spin probe (Fig. 5). A decrease of the negative surface potential of HDL by about 24 mV is calculated from the partition coefficient at 30 mmol/l Ca^{2+}. In the presence of CSA or heparin the change of λ is reduced

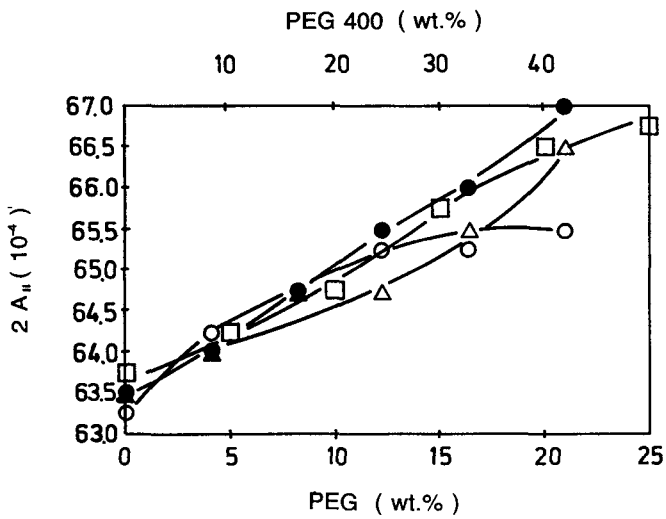

Figure 7. Influence of PEG on the outer hyperfine splitting $2A_{II}$ of maleimide labelled HDL (1.5 mg/ml protein). The lower concentration scale refers to PEG 1500 (○), 6000 (●), 20,000 (△), the upper one to PEG 400 (□). For the composition of the buffer see legend to Fig. 6.

compared to the control, and a decrease of the surface potential by 17 mV is calculated.

CSA and heparin did not influence the EPR spectra of maleimide-labelled HDL (data not shown). This indicates that these two GAG do not interact with the labelled region of the apoprotein. However, a strong decrease of $2A_{II}$ could be measured for DS 500 (Fig. 6). $2A_{II}$ changed from 6.775×10^{-3} T (0 mg/ml DS 500) to 6.55×10^{-3} T (4.5 mg/ml DS 500). The w/s ratio was also changed from 0.9 to 0.75 in this concentration range of DS 500.

In Fig. 7 the experimental results on maleimide-labelled HDL are given. PEG 400, 1500, 6000 and 20,000 influence the outer hyperfine splitting $2A_{II}$ to different extents. 2 A_{II} changed from 6.375×10^{-3} T to 6.675×10^{-3} T in the presence of 50 wt.% PEG 400. Addition of PEG 1500 (22 wt.%) led to a change of about 2.25×10^{-4} T in $2A_{II}$, whereas 22 wt.% PEG 6000 changed $2A_{II}$ from 6.35×10^{-3} to 6.7×10^{-3} T. Unlike with LDL, PEG 20,000 had an effect on $2A_{II}$, and an increase from 6.35×10^{-3} T to 6.65×10^{-3} T was observed in the presence of 22 wt.% PEG 20,000.

Discussion

Our understanding of the behaviour of the surface potential of lipoprotein particles interacting with calcium and GAG is very limited. The present findings concerning the interaction of calcium with LDL and HDL confirm the expectation that the negative surface potential of lipoproteins is reduced after addition of calcium. For LDL a similar change of the surface potential was found by Ghosh et al. (1973) who used agarose gel electrophoresis. Panasenko et al. (1985) have determined the surface potential of HDL from measurements of the partition of a spin probe. Ca^{2+} was used in these experiments to compensate for the surface charge. From our results it can be assumed that the addition of Ca^{2+} leads to a constant value of the spin probe partition coefficients. This indicates that no further change of the surface potential occurs.

After addition to LDL of GAG alone a more negative surface potential of LDL was found by Krumbiegel et al. (1988). Addition of GAG and DS molecules to HDL in the absence of calcium does not produce a more negative surface potential. The reason for this difference between HDL and LDL seem to be the more negative surface charge of the HDL particle compared to LDL and a covering of the positively charged amino acids of apoprotein A as potent reaction partners.

The combined action of Ca^{2+} and GAG does only slightly change the surface potential of LDL and HDL as measured by this particular spin probe. There are two reasons for this result: (i) Ca^{2+} does not only bind to the lipoprotein, but also to heparin, CSA or DS. As a result, the total amount of Ca^{2+} bound to the lipoprotein surface is reduced, and changes in surface potential are smaller. (ii) Ca^{2+} is bound to the lipoprotein surface and forms a bridge to negative $-SO_3^-$ groups of DS, heparin or CSA resulting in a compensation of charges of the complex. Contrary to the slightly changed potential measured at the LDL surface the whole LDL-Ca-GAG complex has a more negative surface charge, as shown by microelectrophoresis (Krumbiegel et al. 1990).

Bu using a spin label covalently attached to the apoprotein region of LDL and HDL we were not able to measure significant changes in the mobility of the spin label in dependence on the presence of calcium. This result is in agreement with literary data.

Mobility changes of the spin label after addition of GAG and Ca^{2+} in our experiments were due to electrostatic binding of $-SO_3^-$ groups of GAG to $-NH_3^+$ groups of the apoprotein amino acids.

In the concentration ranges of calcium and GAG used in our experiments aggregation of LDL and HDL occurs as shown by Burstein et al. (1970). In particular for the HDL-Ca-GAG complex it seems obvious that the mobility change of the protein region and aggregation phenomena are related. Only DS 500 is able to aggregate HDL in combination with Ca^{2+} and to cause a change in mobility

of the apoprotein region of HDL Further investigations are necessary to elucidate the exact mechanism of the formation of HDL-Ca-DS complexes and to provide answer to the question why heparin and chondroitin sulfate are not able to form complexes with HDL in the presence of Ca^{2+}

The treatment of LDL and HDL samples with PEG results in aggregation and precipitation of the lipoproteins This has been shown by several methods (Arnold and Zschornig 1988, Zschornig et al 1991) For the description of these aggregation processes the volume exclusion principle can usually be used (Atha and Ingham 1981) A good correlation of this theory with the experimental aggregation of LDL (Arnold and Zschornig 1988) and HDL (Zschornig et al 1991) has been obtained

Little is known about a direct interaction of PEG with the lipoproteins In studies with phospholipid membranes it has been suggested that there is no direct interaction of PEG with phospholipid membranes (Arnold et al 1990) On the other hand, it is known that PEG alters the physicochemical properties of the aqueous phase (Arnold et al 1985, Baran et al 1972) Herrmann et al (1983) and Surewicz (1983) who measured a $2A_{II}$ increase of maleimide labelled erythrocytes after PEG treatment interpreted their experimental results on the basis of these findings According to their conclusions a similar interpretation of the influence of PEG on LDL and HDL can be offered Increasing PEG concentrations results in a more restricted motion of the Apo B or Apo A region where the labels are located It seems that apoproteins A and B are sensitive to changes of the surrounding medium induced by PEG For LDL the largest changes in $2A_{II}$ could be obtained with PEG 400, whereas no changes in $2A_{II}$ could be measured with PEG 20,000 Obviously, the labelled sites in Apo B and Apo A have different accessibilities for PEG

It is interesting to note that the concentration ranges of PEG and GAG/Ca^{2+} used in these experiments correspond to the concentrations used in separation procedures for LDL and HDL (Kostner et al 1985, Burstein et al 1970) Changes in the apoprotein structure could become apparent under these conditions

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