APPLICATION OF ELECTRON SPIN RESONANCE TO THE STUDY OF THE STRUCTURE OF HUMAN SERUM LIPOPROTEINS

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SUMMARY. Human serum high density (HDL) and low density (LDL) lipoproteins were spin labeled with isothiocyanate and maleimide derivatives of the nitroxide radical N-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidine). The ESR spectra of the HDL and LDL derivatives demonstrated at least two kinds of protein binding sites involving amino groups, one with slight and another with strong constraint on the tumbling of the nitroxide radical. There was relatively more of the strongly constrained component in labeled HDL than in LDL. The strongly immobilized signal was due to the binding of lipid as was shown by the effect of delipidation.

The technique of electron spin resonance has been recently applied to the investigation of conformational changes in a variety of systems involving lipid; e.g., mitochondrial electron transport particles (Koltover et al., 1968) and spin labeled proteins (cytochrome C, lysozyme, poly-L-lysine) mixed with phospholipid either in isooctane or sonicated aqueous dispersions (Barratt et al., 1968). This communication describes for the first time ESR studies with soluble plasma lipoproteins. We describe experiments to test the effect of the presence and absence of the lipid moiety on the molecular freedom of the nitroxide free radical when it is attached to the protein moiety of human serum low density (LDL)

and high density (HDL) lipoproteins or their lipid free apoproteins (apoLDL, apoHDL).

MATERIALS AND METHODS. The spin labels N-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidinyl)-isothiocyanate (nitroxide radical I), and N-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidinyl)-maleimide (nitroxide radical II) were purchased from Varian, EPR product group, Palo Alto, California. Lipoproteins were isolated ultracentrifugally (Havel et al., 1955) from serum of normal, fasting donors, between the densities of 1.019 to 1.063 gm/ml (LDL) and 1.063 to 1.21 gm/ml (HDL). By immunochemical criteria, the preparations were > 99% pure. Spin labeled derivatives of LDL and HDL were prepared by adding the spin label to a solution of the lipoprotein (4 to 10 mg of lipoprotein protein/ml in 20 mM sodium phosphate, 0.01% EDTA, pH 8.0) and stirring for 3 h at 25° in the dark. Spin labels were added in the ratio of 6 to 9 μmoles/100 mg of lipoprotein protein. The reaction mixture was transferred to dialysis tubing and dialyzed at 4° against several changes of the phosphate buffer until all free or unreacted spin label was removed. Spin labeled derivatives of HDL were delipidated with ether: ethanol (3:1) as previously used for succinyl-LDL (Gotto et al., 1968a) except that no detergent was required for solubilization. Spin labeled derivatives of LDL were delipidated by modification of a technique previously described from this laboratory (Gotto et al., 1968b). Delipidated derivatives of HDL or LDL contained approximately 1% phospholipid and no detectable cholesterol or triglyceride by thin layer chromatography.

ESR spectra were measured at protein concentrations of 4 to 10 mg/ml in a Varian V-4500-10 spectrometer at 9500 M cycles.

Further details are given in the legends to the figures. Correlation times (τ_C) were calculated from the ratios of heights of the first derivative lines (Waggoner et al., 1967) using both linear and quadratic terms, and employing previously described values for the Hamiltonian parameters (Griffith et al., 1965). Techniques for immunological (Levy and Fredrickson, 1965; Levy et al., 1966) and circular dichroic (Gotto et al., 1968c) measurements have been described. Assays of protein (Lowry et al., 1951), of free amino (Moore and Stein, 1948) and sulfhydryl groups (Ellman, 1959) and analysis of amino acids (Spackman et al., 1958) were performed by standard techniques.

RESULTS AND DISCUSSION. Spin Labeled Lipoproteins and Apolipoproteins. The ESR spectra of the isothiocyanate (nitroxide radical I, Figs. 1 and 2) and maleimide (nitroxide radical II) derivatives of the lipoproteins were completely comparable. Isothiocyanate reacts with amino and alcoholic hydroxyl groups and possibly to a varied extent with sulfhydryl and imidazole groups in proteins (Konigsberg). Maleimide spin labels have been shown to react with exposed sulfhydryls (Boeyens and McConnell, 1966) and with amino groups (Griffith and McConnell, 1966) in protein. Delipidation removed less than 10% of the total label of the lipoproteins. Since HDL contains no free sulfhydryl groups (Shore and Shore, 1967) and LDL contains only one sulfhydryl per 100,000 gm of protein, the spectra observed with nitroxide radical II derive predominantly from spin label covalently attached to amino groups. The labeling of amino groups by nitroxide radicals I and II was confirmed by ninhydrin assay.

At the low ratios of spin label to protein employed, the

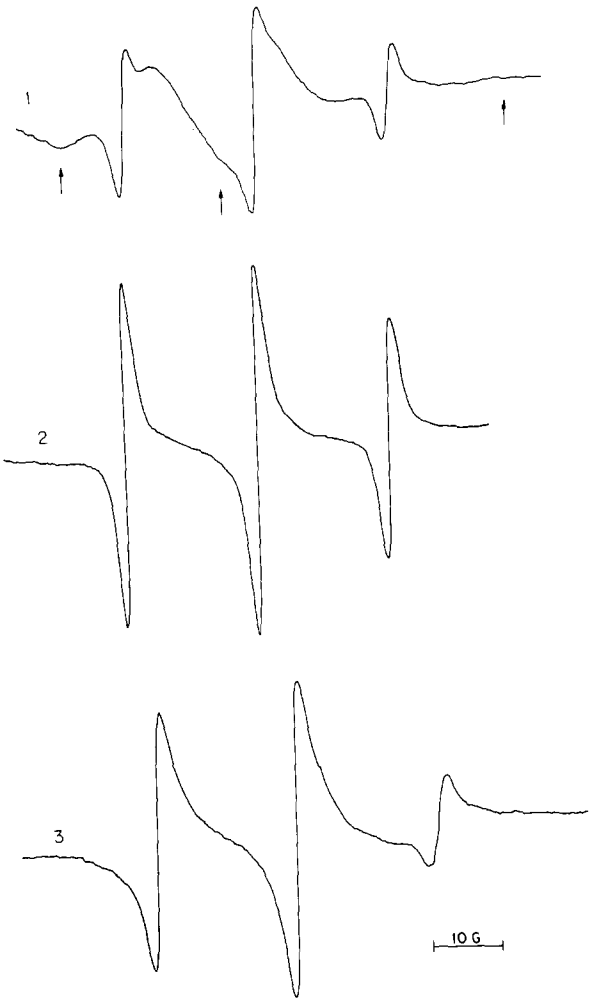

Figure 1. ESR Spectra of HDL (1) apoHDL (2) and HDL + 25 mM Sodium Decyl Sulfate (3) after Labeling with Isothiocyanate Nitroxide Radical I. Protein concentrations were 8 mg/ml in 20 mM sodium phosphate (0.01% EDTA), pH 8.0. The broad component is indicated by the arrows. The scale at the lower right is equivalent to 10 Gauss.

circular dichroism (from 200 to 300 mμ) and immunochemical reactions of the lipo- and apolipoproteins were unaffected.

ESR Spectra of Spin Labeled HDL and LDL: Effect of Delipidation. The purity of the preparations of HDL and LDL (> 99%) was based on their immunochemical reactions which excluded the presence of other plasma proteins. The ESR spectra of HDL and LDL contained at least

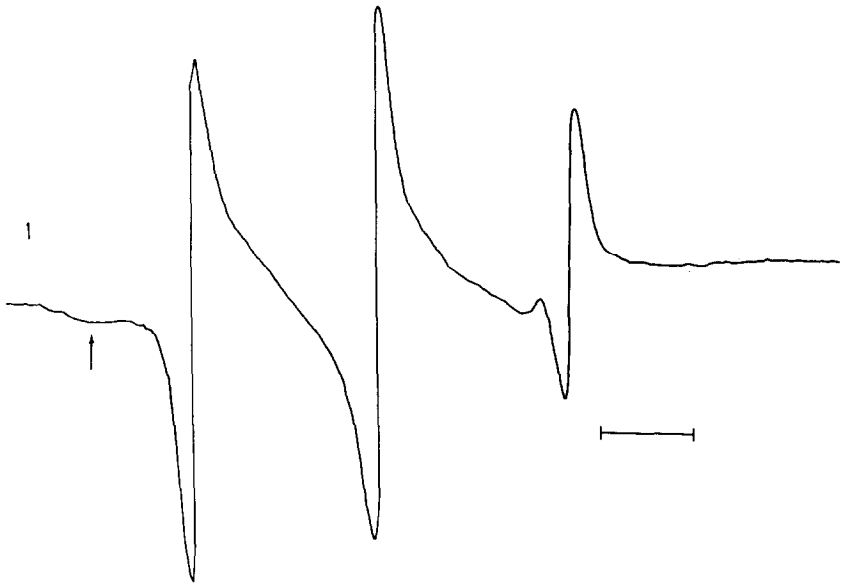

Figure 2. ESR Spectrum of LDL after Labeling with Isothiocyanate Nitroxide Radical I. Please see the legend of Figure 1 for experimental conditions and explanation of symbols.

two types of signals, a relatively sharp or narrow one and a superimposed broad one. Since the homogeneity of the preparations had been established, as indicated above, the narrow and broad signals represented spin label that was covalently bound to the protein, respectively, at weakly and strongly immobilized sites. There was relatively more of the strongly immobilized signal in HDL than in LDL. The estimated distance between the outermost lines (peak-to-peak se separation) from the spectrum of labeled HDL (Fig. 1) was 60-65 gauss. This was approximately the same as the value reported for the spectra of similar radicals at low temperature in rigid glass (Griffith and McConnell, 1966). Overlapping of the broad and narrow signals precluded the accurate calculation of correlation times for the labeled lipoproteins.

Delipidation of labeled HDL markedly reduced the contribution of the strongly immobilized signal and appeared to increase the relative intensity of the narrow signal. Addition of the detergent, sodium decyl sulfate, to labeled HDL produced a similar but less profound effect than did delipidation (Fig. 1). Addition of this detergent at high concentrations has been shown to destroy the ordered conformation (Gotto et al., 1968b, c), and partially delipidate lipoproteins (Gotto et al., 1969). The correlation time (τ) for the nitroxide radical II (maleimide) derivative of apoHDL, representing primarily the weakly immobilized component, was 9.5×10^{-10} sec., approximately an order of magnitude longer than the value for the unbound spin label in aqueous solution. While delipidation of spin labeled LDL usually reduced the contribution of the strongly immobilized component, the results and magnitude of the effect were less reproducuble than with HDL, owing to the requirement of some detergent for solubilization and to the varied extent of aggregation in different preparations.

These results indicated that the spin label was attached to at least two distinct types of substituted amino acid sites on HDL and LDL. At one of these sites the spin label was relatively weakly immobilized, while at the other it was strongly immobilized. These findings are similar to those obtained with spin labeled albumin (Stone et al., 1965) and with spin labeled protein of erythrocyte membranes (Sanberg and Piette, 1967) in which both weakly and strongly immobilized sites of attachment were also observed. It is concluded that the strongly constrained signal was probably due to a lipid-protein interaction in the native molecule which decreases the molecular motion of the spin label bound to the protein. Delipidation decreased

the intensity of this signal and increased the relative intensity of the narrow component to about the same extent as did acidification of spin labeled albumin (Stone et al., 1965).

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