and

$$\frac{d}{d\gamma} = \frac{e^{-2K_{20}m_{A,t}}}{1 + 2K_{20}\Sigma i^2 K_{i0}[A]^i} \frac{d}{d[A]} \quad \text{(A-53)}$$

The expansion of $m_{A,t}$ in terms of γ, using eq A-52 and A-53 assumes the form

$$m_{A,t} = \gamma + 3(K_{30} - 2K_{20}^2)\gamma^3 + \ldots \quad \text{(A-54)}$$

The coefficient of γ^2 is zero in this expansion. Upon taking the limit when γ^2 and [A] equal zero

$$\lim_{\gamma \to 0}\left[\frac{(m_{A,t}/\gamma) - 1}{3\gamma^2}\right] = K_{30} - 2K_{20}^2 \equiv \sigma'_{30} \quad \text{(A-55)}$$

This provides a relationship between K_{30} and K_{20}. From eq A-49 and A-55, we have

$$\sigma_{30} = \sigma'_{30} + 2(\sigma_{20} + (1/2)B_{AA}M_A^2)^2 - B_{AA}M_A^2(2\sigma_{20} + (1/2)B_{AA}M_A^2) \quad \text{(A-56)}$$

If σ_{30}, σ_{20}, and σ'_{30} are known with sufficient accuracy, eq A-56 may be solved for B_{AA}, which is the only unknown. Equation A-56 provides, in principle, a general method for obtaining B_{AA} which does not require any assumptions as to model. In practice data of very high accuracy would be required to obtain σ_{30}, σ_{20}, and σ'_{30} with the necessary accuracy.

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Observations on the Conformation of Human Serum High-Density Lipoproteins Using Infrared Spectroscopy, Circular Dichroism, and Electron Spin Resonance*

A. M. Gotto† and H. Kon

ABSTRACT: Human serum high-density lipoproteins (HDL) were examined before and after delipidation by the techniques of infrared spectroscopy, by circular dichroism, and by electron spin resonance. Comparisons were made with the techniques under identical conditions of the buffer system, protein concentration, and temperature. In D_2O solution the infrared band associated with the amide I resonance of HDL and apoHDL had its major absorption at 1637–1640 cm^{-1} with shoulders at about 1630 and 1650 cm^{-1}. Circular dichroic spectra were characteristic of a high helical content as previously described by others. The electron spin resonance spectrum of HDL, after spin labeling with *N*-(1-oxy-2,2,6,6-tetramethylpiperidinyl)maleimide contained signals associated with both weakly (narrow signal) and strongly (broad signal) constrained spin label. The strongly constrained signal was decreased in relative intensity at high temperatures, was increased in relative intensity at low temperatures and was practically abolished by delipidation. The change caused in the electron spin resonance spectrum by delipidation was qualitatively much greater than any detected by infrared or circular dichroism spectroscopy. It was concluded that the changes in the electron spin resonance spectrum consequent upon delipidation were more likely related to change in the local environment of the spin label rather than to large changes in the secondary structure of the molecule. The helical conformation, much of which was to be retained by apoHDL, appeared to exhibit its major infrared band associated with the amide I vibration at a significantly lower frequency than does the helical conformation in other proteins such as myoglobin.

The conformational and structural properties of human serum high-density lipoproteins (HDL)[1] have been investigated by a number of techniques, including optical rotatory dispersion (Scanu, 1965), circular dichroism (Scanu and Hirz, 1968), nuclear magnetic resonance (Steim *et al.*, 1968; Chapman *et al.*, 1969a,b), and recently by electron spin resonance (Gotto and Kon, 1969; Gotto *et al.*, 1970). The

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[1] The following abbreviations are used: HDL, human serum high-density lipoproteins of d = 1.063–1.21; apoHDL, the protein moiety of HDL after delipidation with ether–ethanol; *N*-(1-oxy-2,2,6,6-tetramethylpiperidinyl)maleimide, nitroxide radical I.

parameters of the optical rotatory dispersion and circular dichroism spectra indicated a relatively high content of helical conformation, estimated at 60–70% (Scanu, 1965; Scanu and Hirz, 1968). The lipid-free protein moiety of HDL (apoHDL) retained a large part of the native conformation, although some differences were detected by circular dichroism (Scanu and Hirz, 1968). Recombination of apoHDL with phospholipid partially reversed the changes in the circular dichroism spectrum caused by delipidation. The resistance of the circular dichroism spectrum to change in temperature was also partially restored by relipidation (Scanu, 1969).

Nuclear magnetic resonance studies have yielded relatively little information about the structure of HDL protein since the signals are derived almost entirely (>95%) from the lipid protons (Steim *et al.*, 1968; Chapman *et al.*, 1969a,b). However, it was concluded that the structure of the recombined apoHDL–phospholipid was "looser" than that of the native lipoprotein (Chapman *et al.*, 1969b).

We have recently used the technique of electron spin resonance to investigate the structure of HDL (Gotto and Kon, 1969; Gotto *et al.*, 1970). At the low levels of spin label used, no changes were detected in the circular dichroism spectrum or immunochemical activity of HDL. Delipidation caused impressive changes in the electron spin resonance spectrum.

In order to make comparisons between the results obtained by different techniques, in this communication we have examined under identical conditions HDL and apoHDL with the techniques of circular dichroism, infrared spectroscopy, and electron spin resonance. We have extended the previous electron spin resonance studies by examining the dependence on temperature and have attempted to relate our observations to those of other investigators mentioned above.

Experimental Section

Materials

N-(1-Oxy-2,2,6,6-tetramethylpiperidinyl)maleimide (referred to as nitroxide radical I) was purchased from Varian, electron paramagnetic resonance product group, Palo Alto, Calif. Human HDL was isolated ultracentrifugally from normal, fasting donors by flotation between 1.063 and 1.21 g per ml (Havel *et al.*, 1955). The HDL preparations gave a single precipitin line on immunoelectrophoresis against antisera to HDL or whole serum and was greater than 99% free of other plasma proteins as judged by immunochemical criteria. The preparations did not react to antisera prepared against human plasma low-density lipoproteins or albumin. Lecithin was purchased from Calbiochem.

HDL protein (apoHDL) was prepared by delipidation of HDL. HDL (1 ml; containing 4–6 mg of lipoprotein–protein) was extracted twice for 12-hr periods with 50 ml of ether–ethanol (3:1, v/v) at 4°. The extractions were performed in 50-ml stoppered, conical centrifuge tubes with shaking. The residues were dried under nitrogen at room temperature and were completely soluble in aqueous buffers.

Methods

Preparation of Spin-Labeled HDL. HDL was labeled with the maleimide group of nitroxide radical I by adding the spin label to the lipoprotein (10 mg of lipoprotein–protein/ml) and incubating in 20 mM sodium phosphate–0.01% EDTA (pH 8.0) for 4 hr at 25° and then for 12 hr at 4°. One milligram of spin label was added per fifty milligrams of lipoprotein–protein. Sulfhydryl (Benesch and Benesch, 1961) and the lysine ε-amino groups (Sharpless and Flavin, 1966) of proteins are known to react with the maleimide group and with nitroxide radical I (McConnell, 1969).[2] HDL does not contain free sulfhydryl groups. After the labeling procedure, unbound spin label was removed by exhaustive dialysis against the above buffer. Dialysis was continued until the dialysate contained no detectable spin label.

As previously shown (Gotto *et al.*, 1970), at the low ratio of nitroxide radical I to protein used in these labeling experiments, the circular dichroism and immunochemical reactions of HDL were not detectably altered.

Spin Label in Benzene. A rough estimation of the extent of labeling was made by comparing the peak heights of a nitroxide standard in benzene with spin-labeled apoHDL. Assuming an average molecular weight of HDL of 300,000 and a protein composition of 50%, it was estimated that there were one to two molecules of label per molecule of HDL. Antisera to HDL formed lines of identity between HDL and the nitroxide derivative of HDL.

Preparation of Spin-Labeled ApoHDL. In most instances spin-labeled apoHDL was prepared by delipidation of the nitroxide radical I derivative of HDL. The procedure used was identical with that described above for delipidation of unlabeled HDL. For purposes of comparison, a second procedure was employed in which unlabeled apoHDL was incubated with spin label as described above for HDL. There were no major qualitative differences between the electron spin resonance spectrum of this preparation and that of delipidated spin-labeled HDL. ApoHDL and its spin-labeled derivative contained between 0.5 and 1% phospholipid by chemical analysis (Stewart and Hendry, 1935). Cholesterol, cholesteryl ester, and triglyceride were not detected by thin-layer chromatography (Tschesche *et al.*, 1961). The electron spin resonance spectrum of the extracted lipids was obtained after evaporation of the solvents and suspension of the lipids in 20 mM sodium phosphate–0.01% EDTA (pH 8.0) by sonication. The lipid extract contained approximately 5% of the total quantity of spin label associated with the labeled lipoprotein. This signal was quite narrow and had no broad component. Attempts were not made to fractionate or purify this small quantity of extracted spin label. It could not be concluded that the extracted label was bound to lipid since small and variable amounts of HDL–protein were also removed by ether–ethanol extraction.

The circular dichroic spectrum and immunological reactions of apoHDL were not changed detectably by the spin-labeling procedure, as shown in earlier experiments (Gotto *et al.*, 1970). Antisera to apoHDL did not distinguish between the apolipoprotein and its nitroxide derivative.

Analytical Procedures

Circular Dichroism. Circular dichroic spectra were measured in 20 mM potassium phosphate buffer containing 0.01% EDTA, at pH 7.9 and 27° in a Cary 60 spectropolarimeter with a Model 6001 circular dichroic accessory. The machine was calibrated with D-camphorsulfonic acid in the visible range and with poly-L-glutamic acid and poly-L-lysine in the

[2] Cited by O. H. Griffith and A. S. Waggoner.

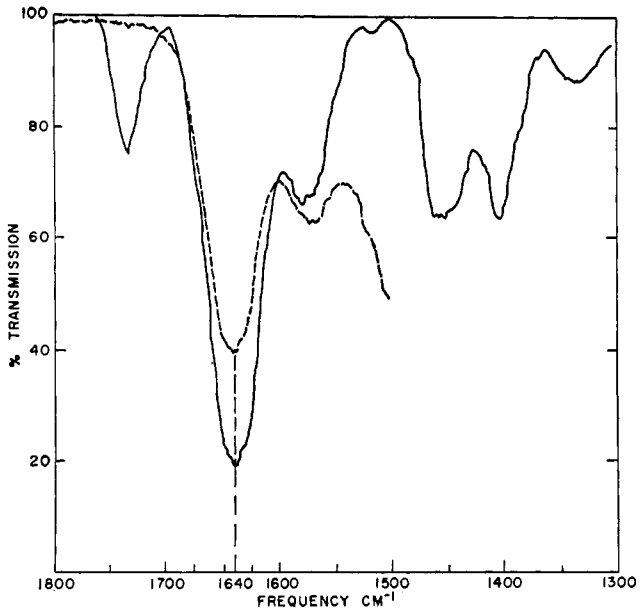

FIGURE 1: Infrared spectrum of HDL in D_2O, 6 mg of lipoprotein protein/ml (—) and of apoHDL in D_2O, 6 mg of protein/ml (– – –).

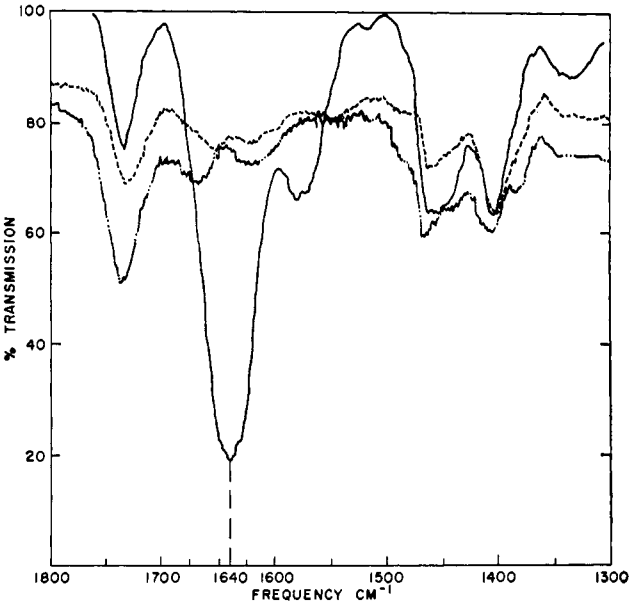

FIGURE 2: Infrared spectrum of HDL in D_2O (——), 6 mg of lipoprotein protein/ml, and of HDL–lipids as sonic dispersions in D_2O (– – –) at a concentration equivalent to 12 mg of HDL (or 6 mg of HDL–protein)/ml or as a thin film between sodium chloride plates (– · –). Lipids were obtained by chloroform–methanol (2:1, v/v) extraction of HDL and were stored in hexane at −40° after evaporation. For these experiments the hexane was evaporated and the lipids dispersed by sonication for 2 min in D_2O at 25° (– – –) or the lipids were plated directly on sodium chloride plates (– · –). These spectra are compared for qualitative purposes and do not show exact quantitative relationships owing to the differential turbidity and light scattering of the preparations at varied frequencies.

ultraviolet region. Duplicate measurements were made at two different protein concentrations (0.2–1.0 mg/ml) and at light paths of both 0.1 and 0.5 mm. For comparison with infrared spectra, a protein concentration of 5.0 mg/ml was used. Spectra were reproducible to within 5% at 210 mμ. From the ellipticity angle $\theta°$, obtained directly, mean residue ellipticity was calculated from $[\theta] = \theta°/10.\ MRW/lc$, where MRW is the mean residue weight (a value of 115 was used), l is the light path of the cell in centimeters, and c is the concentration of the solution in gram of protein per milliliter. The reduced mean residue ellipticity, $[\theta']$, in deg cm² per dmole was obtained by correction for the refractive index of the medium.

Infrared Spectroscopy. Infrared spectra were measured with both a Beckman IR-7 and a Perkin-Elmer infrared spectrometer. These instruments were calibrated against water and polystyrene. Spectra of myoglobin and of poly-L-lysine in D_2O gave the expected major bands, respectively, at 1650 cm^{-1} (Timasheff *et al.*, 1967) and at 1610 cm^{-1} (Sarkar and Doty, 1966). Calcium fluoride cells (0.1-mm path length) were used for measurements in solution and sodium chloride plates were used for measurements of films. Protein concentrations were 5–15 mg/ml. Spectra were measured at three different protein concentrations. The solvent was 20 mM potassium phosphate (containing 0.01% EDTA) at pD 7.9 in 99.8% D_2O.

Electron spin resonance spectra were measured in 20 mM potassium phosphate (with 0.01% EDTA) at pH 7.9 at protein concentrations of 5–10 mg/ml. A Varian V-4500-10 spectrometer was used at 9500 MHz. With the Hamiltonian parameters previously described (Griffith *et al.*, 1965), correlation times were calculated from the ratios of the heights of the first-derivative lines (Waggoner *et al.*, 1967).

Variable-temperature experiments were performed with a Dewar-transfer tube combination similar to the Varian V-4557 instrument. The temperature was varied by altering the current through a heater in the transfer tube. The flow rate of precooled nitrogen gas was kept constant. The temperature fluctuation during a scan was ±1°.

Results

Infrared Spectroscopy. The infrared spectrum of HDL was examined between 1300 and 1800 cm^{-1} (Figure 1). This spectrum was compared to those of HDL–lipids (Figure 2) and of HDL–protein or apoHDL (Figure 1). From these comparisons the following conclusions may be drawn. The lipid of HDL contributes significantly to the infrared bands at 1725–1745, 1450–1470, and 1370–1400 cm^{-1}. Based on infrared spectral assignments previously described (Freeman *et al.*, 1953) these bands would likely correspond, respectively, to the stretching of ester groups, to the bending of CH_2 and CH_3 groups, and to the bending of CH_3 groups. The smaller lipid bands at approximately 1625–1670 cm^{-1} may have originated from sphingomyelin (Freeman *et al.*, 1953). The lipid spectrum as a sonic dispersion in D_2O and as a thin film were qualitatively similar (Figure 2), although there was more fine structure apparent in the latter spectrum. Steim *et al.* (1968) have concluded from nuclear magnetic resonance experiments that the physical state of the lipid is similar in native lipoproteins and in protein-free, sonic dispersions in D_2O. The bands at 1570–1575 and 1637–1640 cm^{-1} appeared to be derived predominantly from the protein residue (Figure 2). In the region of the amide I band of proteins (Timasheff

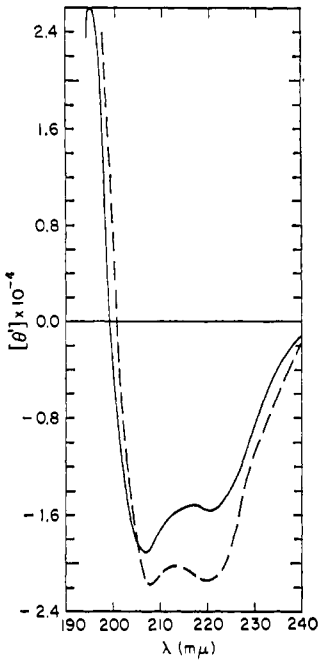

FIGURE 3: Circular dichroic spectra of HDL (- - -) and apoHDL (——).

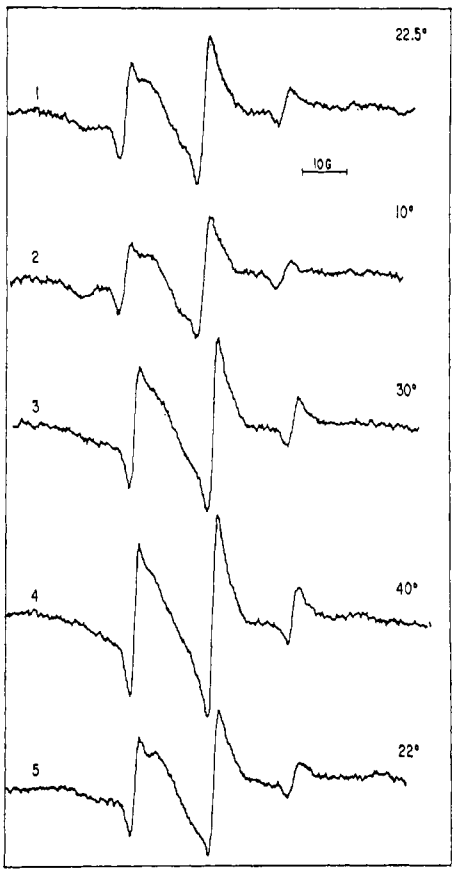

FIGURE 4: Electron spin resonance spectra of the nitroxide radical I (maleimide) derivative of HDL at varied temperatures. The temperature for each panel is given at the right of the figure. The protein concentration was 6 mg/ml. Amplification and modulation were kept consistant for each temperature used. Panels 1–5 were obtained sequentially with the same solution of spin-labeled HDL and were recorded 5 min after the indicated temperature was reached.

et al., 1967), HDL and apoHDL exhibited a major peak at 1637–1640 cm^{-1} with shoulders at about 1630 and 1650 cm^{-1}. The spectra of HDL and apoHDL were very similar in the amide I region.

Circular Dichroism. As described by others (Scanu and Hirz, 1968), the circular dichroic spectra of HDL and apoHDL had negative peaks at 222 and 208 mμ which have been characteristically found in helical protein and polypeptides (Figure 3). When samples of HDL and apoHDL with these spectra were examined under identical conditions and at the same concentrations with infrared spectroscopy, the major infrared band in the amide I region was at 1637–1640 cm^{-1} with shoulders at 1630 and 1650 cm^{-1} as described above (Figures 1 and 2).

Electron Spin Resonance Spectrum of Spin-Labeled HDL. EFFECTS OF VARIATION OF TEMPERATURE. The electron spin resonance spectrum of the nitroxide radical I derivative of HDL contained at least two types of signals (Figure 4) as previously described (Gotto and Kon, 1969; Gotto *et al.*, 1970). One signal was narrow and was characteristic of weakly immobilized spin label. The other signal was quite broad and indicated the presence of strongly immobilized label. The heterogeneity of the electron spin resonance signals could not be attributed to contamination of HDL with other plasma proteins since the lipoprotein preparations were >99% pure by immunochemical reactions.

The narrow signal was sensitive to temperature and increased in intensity with increasing temperature up to at least 70° (Figures 4 and 5). Between 10 and 40° these changes were entirely reversible (Figure 4). These temperature-dependent spectral changes of the narrow signal were not necessarily related to structural or conformational alterations within the lipoprotein, since they could be produced by varying the temperature of solutions of the unbound spin label from 6 to 60°. At 70° there was a marked decrease in the intensity of the broad signal (Figure 5) as compared to the initial spectrum at 22.5° (Figure 4).

At lower temperatures, on the other hand, there was a relative increase in the intensity of the broad signal and a decrease in the intensity of the narrow one (Figures 4 and 6). There was no trace of the broad signal in the unbound spin label at low temperatures. The broad signal could not be reproduced by incubation of spin label and lipoprotein–lipids over a wide range of temperature from 10 to 60°.

At high gain amplification an upfield splitting of the broad signal was detected at 5° (Figure 6). The distance between the outermost peaks was approximately 65 G. The electron spin resonance spectrum of spin-labeled HDL in the frozen state was reproducible (Figures 6 and 7) and was very similar to that described for dansyl nitroxide in 90% glycerol (Stryer and Griffith, 1965). A similar spectrum could be produced by freezing the spin label in the presence of lipoprotein–lipids (Figure 8). In contrast, the electron spin resonance spectrum of the unbound spin label in the frozen state was distinctly different and exhibited a single broad signal (Figure 8). This single signal may be a result of extensive aggregation of the nitroxide radical I in the frozen state. Thus, in the presence of lipids or when the spin label was covalently bound to HDL, forma-

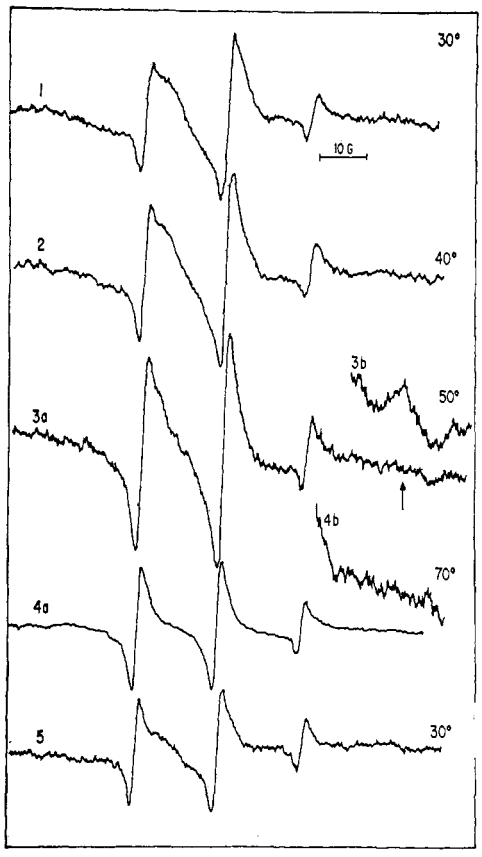

FIGURE 5: Effects of high temperatures on the electron spin resonance spectrum of the nitroxide radical I derivative of HDL. The temperature for each panel is indicated at the right of the figure. The insets, 3b and 4b show high gain amplification. The other spectra were obtained at constant amplification and modulation, except for panel 4a (70°) for which the amplification was decreased by twofold because of the increased intensity of the narrow signal. The protein concentration was 6 mg/ml. Panels 1–5 were obtained sequentially with the same solution of spin-labeled HDL and were recorded 5 min after the indicated temperature was reached.

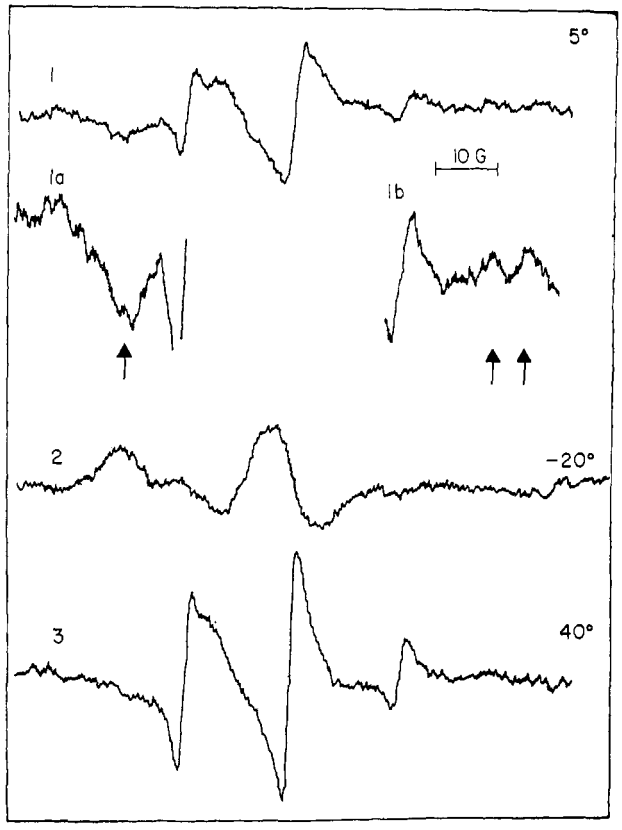

FIGURE 6: Electron spin resonance spectrum of nitroxide radical I derivative of HDL at low temperatures: reversibility of spectrum. Gain and amplification were constant. The protein concentration was 6 mg/ml. Panels 1, 2, and 3 were obtained sequentially with the same solution of spin-labeled HDl and were recorded 5 min after the indicated temperature was reached. Panels 1a and 1b show the outermost signals obtained at 5° at high gain amplification. Splitting of the upfield signal is indicated by the two arrows (1b).

tion of the lattice of aggregates may have been prevented. Freezing and thawing of spin-labeled HDL did not lead to significant qualitative changes in its electron spin resonance spectrum. After thawing and warming to 40°, the spectrum (Figure 6) was very similar to that obtained at 40° prior to freezing (Figure 5).

Electron Spin Resonance Spectrum of Spin-Labeled ApoHDL. Delipidation of spin-labeled HDL greatly reduced the relative intensity of the broad or strongly immobilized signal and increased the relative intensity of the narrow signal (Figure 9) as observed earlier (Gotto and Kon, 1969; Gotto *et al.*, 1970). The intensity of the narrow signal of spin-labeled apoHDL varied reversibly with temperature between 5 and 30° (Figure 9), the changes being similar to those occurring with spin-labeled HDL (Figure 4) or with solutions of unbound spin label. It was possible at 5° and with high gain amplification to demonstrate distinctly the presence of a small quantity of strongly immobilized signal (Figure 9, panel 3b). The spectrum of spin-labeled apoHDL in the frozen state (Figure 9) was indistinguishable from that of labeled HDL (Figures 6 and 7) or of spin label plus lipids (Figure 8). The correlation time, τ, at 30° for the nitroxide I radical derivative of apoHDL was 9.5×10^{-10} sec. This value was about 10-fold longer than for the unbound spin label in aqueous solution and reflected mainly the contribution of the weakly immobilized signal.

Discussion

It had previously been concluded from optical rotatory dispersion (Scanu, 1965) and circular dichroism (Scanu and Hirz, 1968) measurements that HDL in aqueous solution contains a high proportion of helical structure, estimated at 60–70%. A large helical complement for HDL has also been predicted from its content of certain nonhelical-forming amino acids (Gotto and Shore, 1969), as calculated by the method of Davies (1964). In the present study, when solutions of HDL were examined under identical conditions by circular dichroism and infrared, the circular dichroism spectrum was typical of those of other helical proteins while the infrared spectrum was not. This latter finding was confirmed with four different preparations of HDL, using both Beckman and Perkin-Elmer calibrated infrared spectrometers. That HDL lipids did not contribute significantly to the major infrared band at 1637–1640 cm^{-1}, was clearly shown by the infrared spectrum of apoHDL. Identical bands were present in

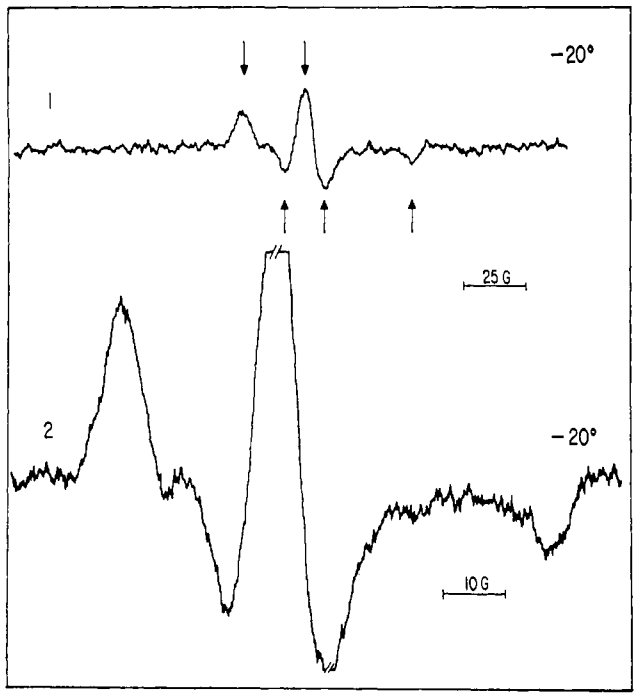

FIGURE 7: Electron spin resonance spectrum of nitroxide radical I derivative of HDL in the frozen state at −20°. Panel 2 was obtained at high gain amplification. The protein concentration was 6 mg/ml.

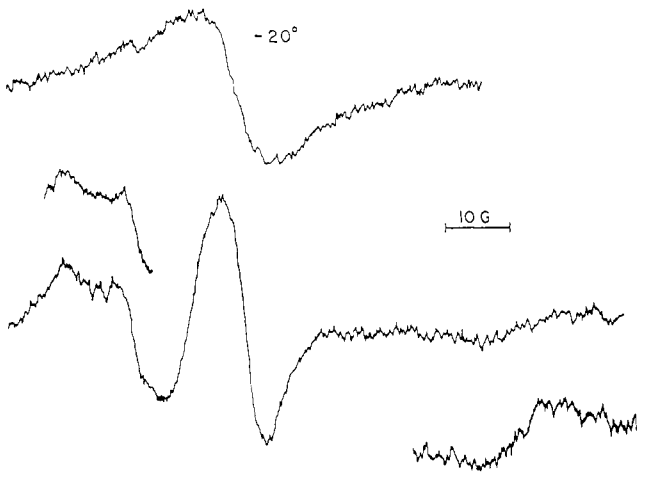

FIGURE 8: Electron spin resonance spectra of unbound nitroxide radical I in the frozen state at −20° (upper tracing) and of nitroxide radical I in the presence of sonically dispersed HDL lipids after freezing at −20° (lower tracing). The two insets accompanying the lower tracing were obtained at high gain amplification. The lipid concentration was equivalent to 12 mg of HDL/ml.

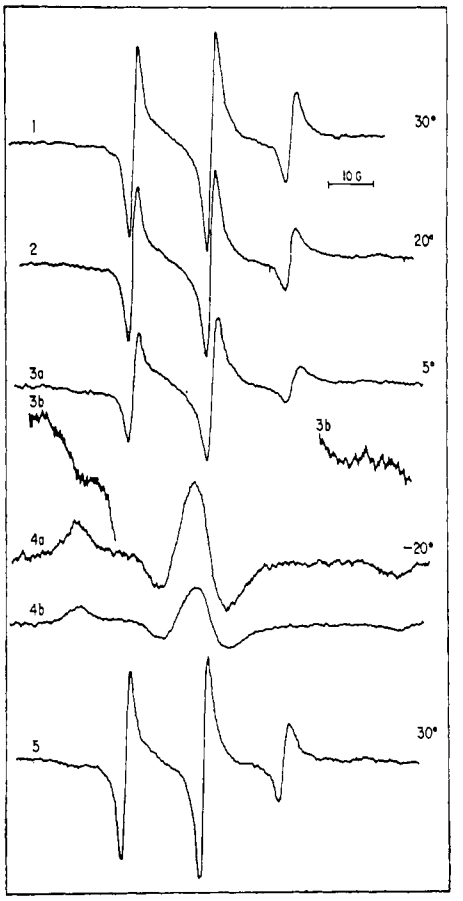

FIGURE 9: Electron spin resonance spectra of the nitroxide radical I derivative of apoHDL at varied temperature. The protein concentration was 6 mg/ml. Amplification and gain were constant except for 3b and 4a where high gain amplifiation was used. The temperatures are indicated on the right side of the figure. The spectra for panels 1–5 were obtained sequentially with the same solution of spin-labeled apoHDL and were recorded 5 min after the indicated temperature was reached.

apoHDL and HDL between 1600 and 1700 cm^{-1}. Changes consequent to delipidation were readily detected with the more sensitive technique of circular dichroism.

The location of the major infrared band associated with amide I vibration differed somewhat from the values reported by Scanu (1967) for HDL and apoHDL in the solid states which were 1650 and 1645 cm^{-1}, respectively. It appeared from our studies that the amide I structure in the helical conformation of native or delipidated HDL exhibits its major infrared band in solution in D_2O at a significantly lower frequency (1637–1640 cm^{-1}) than other helical proteins such as myoglobin (Timasheff *et al.*, 1967). The reason for this behavior is not clear, but similar results have been obtained with the helical polypeptide, polyglycine II (Suzuki *et al.*, 1966).

The lipid–protein interaction in HDL was further examined by labeling the free amino groups of the protein moiety with a maleimide nitroxide derivative. In attempting to make any comparisons between the circular dichroism, infrared, and electron spin resonance measurements it should be kept in mind that the electron spin resonance signals are sensitive to changes in the local environment of the nitroxide radical which may or may not reflect overall alterations in the conformation of the protein. In some instances changes in electron spin resonance spectra do represent overall conformational transitions; *e.g.*, when a neutral solution of spin-labeled albumin is acidified (Griffith and McConnell, 1966) or when poly-L-lysine is converted from a disordered into a helical conformation (Stone *et al.*, 1965).

The spin label covalently bound to HDL–protein was

located in at least two different environments as described in earlier communications (Gotto and Kon, 1969; Gotto *et al.*, 1970). In one of these environments the spin label was strongly immobilized (broad signal) while in the other it was weakly immobilized (narrow signal). Similar results have been described with spin-labeled albumin (Stone *et al.*, 1965), spin-labeled erythrocyte membranes (Sandberg and Piette, 1968), or spin-labeled myosin (Quinlivan *et al.*, 1969). The strongly immobilized component was reduced in relative intensity at high temperatures and was increased at low temperatures, a splitting of the upfield signal becoming apparent at about 5°. The peak to peak separation of the outermost signals of 65 G was approximately the value for powder spectra in glass of similar spin labels at very low temperatures (Griffith and McConnell, 1966). This comparison indicates the very severe immobilization of the nitroxide radical at the site or sites of HDL represented by the broad signal. That this strong immobilization was probably due to the lipid constituents of HDL was shown by the effect of delipidation, which diminished the broad and increased the narrow signal.

It may be considered whether the strongly immobilized signal might also reflect the quantity of helical secondary structure present in HDL. As mentioned above, this signal was relatively decreased in intensity at high temperatures and increased at low temperatures. Scanu (1969) has observed a shift from helical to disordered structure in HDL and apoHDL at high temperatures with the use of circular dichroism. However, the electron spin resonance changes accompanying delipidation of spin-labeled HDL were qualitatively of much greater magnitude than any changes detected by circular dichroism or infrared spectroscopy. The similarity between the infrared spectra of HDL and apoHDL corroborated other circular dichroism experiments (Scanu and Hirz, 1968), interpreted as showing that the apoprotein retains much of its secondary structure in the absence of lipids. Thus, profound reduction in the relative intensity of broad signal consequent upon delipidation suggests that this signal is dependent on different structural characteristics than those measured by circular dichroism. Somewhat analogous observations have been made after treatment of spin-labeled myosin with *p*-mercuribenzoate (Quinlivan *et al.*, 1969). A large increase in weakly immobilized signal, and presumably a major change in the conformation of the myosin was accompanied by only minimal changes in the optical rotatory dispersion spectrum, the dichotomy being suggested as representing a "partial unraveling of the two heads of myosin" (Quinlivan *et al.*, 1969). Delipidation of HDL might similarly lead to an unwinding of some of its major polypeptide constituents without abolishing much of the ordered secondary structure of the molecule.

The nuclear magnetic resonance studies of HDL (Steim *et al.*, 1968; Chapman *et al.*, 1969a,b), suggested that the lipid is in a very mobile or magnetically isotropic environment. When covalently bound to certain sites on the protein, the spin label seems to be in a magnetically anisotropic environment. The strongly constrained signal in the electron spin resonance spectrum of spin-labeled HDL was not observed in recombined complexes of phospholipid and spin-labeled apoHDL (Gotto *et al.*, 1970). It has been reported that the circular dichroism spectrum of the native lipoprotein is largely restored in such reconstituted complexes (Scanu and Hirz, 1968). However, the recombined apoHDL–phospholipid has a temperature dependence of its circular dichroism spectrum which was intermediate between those of apoHDL and HDL (Scanu, 1969) and a nuclear magnetic resonance spectrum which suggested a less rigid structure than was present in the native lipoprotein (Chapman *et al.*, 1969b).

In conclusion, the strongly immobilized electron spin resonance signal obtained with HDL appears to be derived from specific lipid–protein interactions that differ from the interactions occurring during reconstitution of the apolipoprotein with phospholipid as described by Scanu (1967). Recombination by this latter procedure has been found to induce some conformational changes. Such changes are detectable by optical measurements but not electron spin resonance. Thus the broad and narrow signals of the electron spin resonance spectrum do not seem to bear a direct relationship to the secondary structure of the HDL molecule as measured by circular dichroism, optical rotatory dispersion, or infrared spectroscopy and instead are sensitive indicators of the local environment of spin-label groups attached to the protein moiety.

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Synthesis of 11-Oxo[1,11-^{14}C]eicosanoic and 13-Oxo[1,13-^{14}C]docosanoic Acids by the Acylation of Cyclic Enamines*

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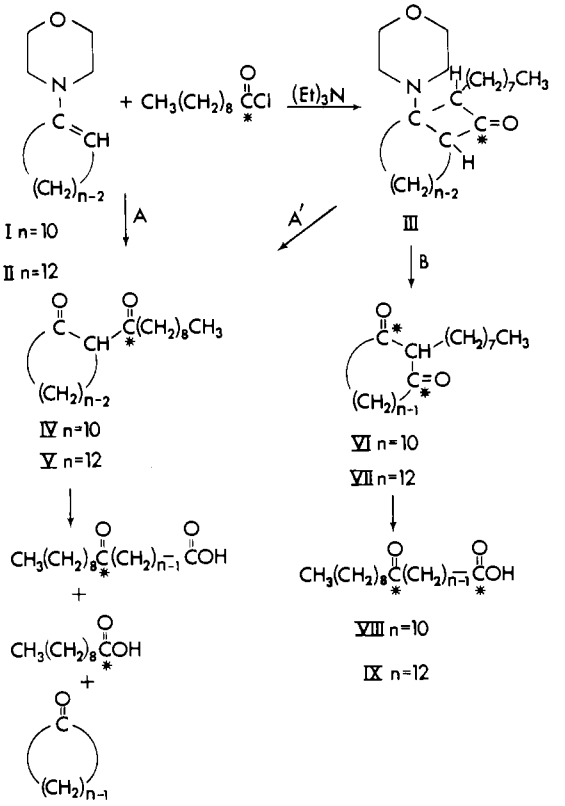
ABSTRACT: The acylation of 1-morpholino-1-cyclodecene (I) and 1-morpholino-1-cyclododecene (II) with decanoyl chloride produces predominantly the symmetrical β-diketones 2-octylcyclododecane-1,3-dione (VI) and 2-octylcyclotetradecane-1,3-dione (VII). When [1-^{14}C]decanoyl chloride is employed in the synthesis, and the β-diketones are hydrolyzed, the products are 11-oxo[1,11-^{14}C]eicosanoic acid (VIII) and 13-oxo[1,13-^{14}C]docosanoic acid (IX). The oxo acids can be reduced to the corresponding saturated fatty acids, providing a method of synthesis for long-chain fatty acid containing ^{14}C in two positions. Distribution of radioactivity in the product fatty acids and mass spectra of the intermediate β-diketones confirm the structure of the β-diketones formed in the acylation.

The enamine synthesis (Hünig *et al.*, 1967; Hünig and Buysch, 1967a) provides an attractive method for synthesis of specifically labeled long-chain oxo, hydroxy, and saturated fatty acids using commercially available [1-^{14}C]fatty acids of shorter chain length. For example, the chain length of the starting acid can be extended by *n* carbon atoms by acylating the enamine of a cyclic ketone containing *n* carbon atoms with the chloride of the starting acid (Scheme I). The intermediate keto acid produced contains an oxygen at position $n + 1$ which can be reduced to a hydroxyl group or to a methylene group. According to Optiz *et al.* (1962), acylation in the presence of triethylamine proceeds by way of the ketene which reacts with enamines to form cyclobutanones such as III. In a study of the effect of enamine ring size on reaction pathway with acetyl chloride and triethylamine, Hünig and Hoch (1966) found that the symmetrical β-diketone was produced in the case of large ring enamines where $n = 11$–15, (similar to path B, Scheme I) and hence must have involved a cyclobutanone intermediate. For smaller ring enamines where $n = 5$–8, the acetylcyclanone was the major product, and it could be formed by either path A or A′. When $n = 9$ the yield was low though both mechanisms appeared to be operative.



SCHEME I

Both reaction pathways give the same oxo acid. The mechanism becomes important, however, if the method is to be used for increasing the chain length of commercially available

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