# Temperature Dependence of the Optical Activity of Human Serum Low Density Lipoprotein. The Role of Lipids†

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ABSTRACT: Low density lipoprotein (LDL) (1.024–1.045 g/cm$^3$) was prepared by ultracentrifugal flotation from serum of normal fasting subjects. Circular dichroism (CD) and optical rotatory dispersion (ORD) spectra in the ultraviolet region were measured at 2, 25, and 37° on LDL, lipid extracted from LDL, and on pure component lipids. All exhibit reversible, temperature-dependent optical activities. Sphingomyelin has a strong negative CD band around 195 nm. Cholesterol and cholesteryl esters have a CD minimum at 208 nm. They have positive CD bands around 201 and 198 nm which decrease sharply and become negative at 198 and 193 nm, respectively. The CD of the total lipid extract of LDL is negative and drops monotonically below 200 nm. Thus, the lipid moiety could account for the increasing negativity of the CD of LDL below 195 nm. After subtraction of the ellipticity corresponding to amounts of lipids in organic solvents equivalent to those found in LDL, the 208–210 nm trough of LDL diminishes markedly. This is accompanied by a blue-shift of the extrema from 195–196 to 193 nm and an increase in the magnitude of the positive ellipticity. The fractions of helix and of $\beta$ form in the protein, determined by the method of Y. H. Chen, J. T. Yang, and K. H. Chau ((1974), *Biochemistry 13,* 3350), in the wavelength interval of 205–240 nm, remain essentially unchanged between 2 and 37°. These observations suggest that a substantial part of the thermal change in the CD spectrum of LDL between 208 and 210 nm may be attributable to lipids.

The optical activity of human serum low density lipoprotein (LDL)[1] has been studied extensively. Circular dichroism (CD) and optical rotatory dispersion (ORD) in the ultraviolet region indicate the presence of secondary structure in the protein moiety (Gotto et al., 1968, 1973; Scanu and Hirz, 1968; Dearborn and Wetlaufer, 1969; Scanu et al., 1969). The lipid moiety, however, also contains optically active components such as cholesterol, cholesteryl esters, and phospholipids. The contribution of these elements to the ORD and CD spectra of LDL has not been explored in depth.

The CD spectrum of LDL between 190 and 250 nm at 25° is characterized by a double minimum (220 and 208 nm) and a positive maximum at 196 nm (Gotto et al., 1968, 1973; Scanu and Hirz, 1968; Dearborn and Wetlaufer, 1969; Scanu et al., 1969). Three subfractions of LDL with different proportions of protein and lipid have quite different CD and ORD spectra and show temperature dependence in the ultraviolet region (Dearborn and Wetlaufer, 1969). Scanu et al. (1969) have further found that the CD of intact LDL is less sensitive to changes in temperature than its delipidated protein moiety. These observations led to the conclusion that lipids in LDL enhanced the conformational stability of the protein moiety. Also an increase in ellipticity of the CD spectrum especially in the region of 208–210 nm at lower temperature has been attributed by both groups to a thermally dependent increase in helical content in the protein moiety.

Recently we have reported induced optical activity of carotenoids in LDL in the visible region at temperatures below 37° (Chen and Kane, 1974). Most of the other lipid components in LDL have absorption bands below 190 nm. In this report, we show that lipids extracted from LDL as well as pure cholesterol, cholesteryl esters, and phospholipids all have multiple Cotton effects in the ultraviolet region. Moreover, these Cotton effects (except those due to phosphatidylcholine) show reversible thermal dependence, becoming increasingly negative at lower temperatures just as does the spectrum of intact LDL. Rather than reflecting an increase in helix content of protein, a substantial part of the increased ellipticity seen with decreasing temperature in the region of 208–210 nm in LDL may be attributable to the contribution of lipid to the CD.

## Materials and Methods

*Materials.* Serum was obtained from normal fasting male subjects. LDL was prepared within the density interval 1.024–1.045 g/cm$^3$ by repetitive ultracentrifugation, as described previously (Kane et al., 1970). LDL was dialyzed for 48 hr at 4° against 0.1 *M* NaCl containing $1 \times 10^{-3}$ *M* EDTA and $2 \times 10^{-3}$ *M* $NaH_2PO_4$ (pH 7.5); 0.15 *M* KF was substituted for NaCl in the dialysate for optical studies of LDL in the far-ultraviolet region so that spectra could be measured at lower wavelengths. The concentration of protein was determined (Lowry et al., 1951) using hydrated bovine serum albumin as a working standard because of its ready solubility in water. Relative chromogenicity of the protein moiety of LDL to that of albumin in the Lowry method, after correction for the mass contributed by the carbohydrate moiety of LDL, was 0.92 for albumin containing 7.8% water (1.00 on the basis of anhydrous protein). Portions of LDL preparations were extracted twice with 25 volumes of 95% ethanol–ethyl ether (3:1, v/v) to obtain the total lipid moieties for optical studies. The combined lipid extracts were evaporated under $N_2$ and redissolved in trifluoroethanol–ethyl ether (3:1, v/v) to permit measurement in the far-ultraviolet region. Alternatively, the lipid residue

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[1] Abbreviation used is: LDL, low density lipoprotein.

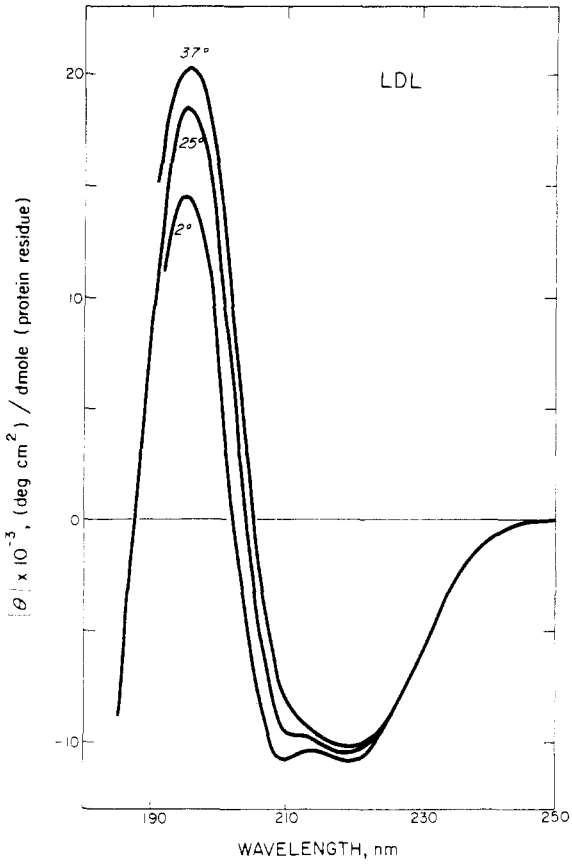


FIGURE 1: The effect of temperature on the CD spectrum of LDL.

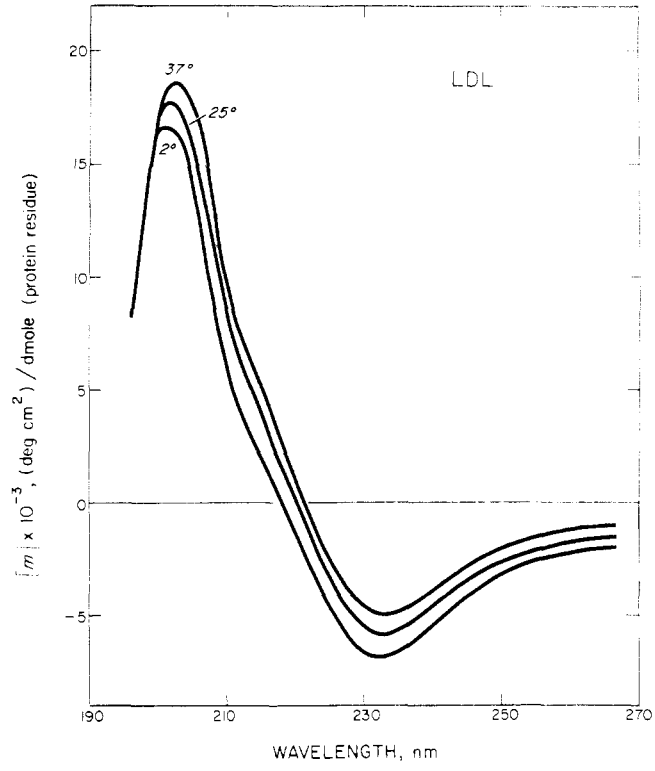


FIGURE 2: The effect of temperature on the ORD of LDL.

from the ethanol–ethyl ether solution after evaporation was extracted with trifluoroethanol, followed by hexane. Contents of free and esterified cholesterol (Sperry and Webb, 1950) and of phospholipid (Stewart and Hendry, 1935) were determined in the various extracts. Cholesterol (lot no. 721-16), cholesteryl oleate (lot no. 0542), cholesteryl linoleate (lot no. 1531), cholesteryl palmitate (lot no. 0354), phosphatidylcholine (lot no. 1668), and sphingomyelin (bovine, lot no. 1822) were purchased from Applied Science Laboratories, Inc., and were used without further purification. Identity and purity of these compounds were verified by thin-layer chromatography on silica gel G. Sphingomyelin and phosphatidylcholine were dissolved in $F_3$EtOH. The organic solvents, all of spectral quality, were obtained from Matheson Coleman and Bell, except ethyl ether which was obtained from the Eastman Kodak Company.

*Methods.* ORD was measured with a Cary 60 spectropolarimeter and CD was measured with a Durrum-Jasco J-5, SS-10-modified circular dichrometer. Both instruments employ a constant nitrogen flush. Specially designed thermostated cell holders were installed in both instruments. The temperature of the solution was monitored by a Leeds and Northrup millivolt potentiometer with a copper–constantan thermocouple. The circular dichrometer was calibrated with a solution of *d*-10-camphorsulfonic acid (Cassim and Yang, 1969). Fused cylindrical silica cells (Pyrocell S-18-260) with path lengths of 0.1–50 mm were used. Cells with path lengths under 5 mm were calibrated with a freshly prepared solution of sucrose (National Bureau of Standards). Agreement of duplicate ORD and CD measurements of LDL was within 1% at wavelengths above 210 nm and within 15% between 195 and 210 nm. All spectra presented are the averages of two or more measurements.

Data on intact LDL and on lipids extracted from LDL were expressed in terms of mean residue ellipticity, $[\theta]$, and mean residue rotation, $[m]$, in (deg cm$^2$)/dmol (protein residue) using a mean residue weight of 112. Data on pure lipids were expressed in terms of specific ellipticity, $[\psi]$, and specific rotation, $[\alpha]$, in (deg cm$^2$)/dag. Results for all lipids were reproducible within 5% above 210 nm and within 15% at lower wavelengths. No corrections were made for thermal expansion of the solvents used since measurement revealed these changes to be insignificant. The ORD data in the visible region obey the Drude equation:

$$\lambda^2[\alpha] = \lambda_c^2[\alpha] + k$$

where $[\alpha]$ is the specific rotation at wavelength $\lambda$, and $k$ and $\lambda_c$ are constants. The CD data between 205 and 240 nm of LDL were analyzed by the method of Chen et al. (1974) for estimation of the content of helix and $\beta$ form.

The CD contributions due either to lipids extracted from LDL or to mixtures of pure lipids were subtracted from the observed data of intact LDL. We have found LDL ($1.024 < d < 1.045$) to contain 22% protein, 10% free cholesterol, 44% cholesteryl esters, 21% phospholipid (6% is assumed to be sphingomyelin (Skipski et al., 1967)), and 3.1% triglyceride. At any chosen wavelength $\lambda$, the specific ellipticity, $[\psi]$, of the protein moiety can be calculated from the observed $[\psi]$ (based on protein concentration) with the following expression:

$$[\psi](\text{protein}) = [\psi](\text{obsd}) - [0.5[\psi](\text{cholesterol}) + 2.2[\psi](\text{cholesteryl esters}) + 0.75[\psi](\text{phosphatidylcholine}) + 0.3[\psi](\text{sphingomyelin})]$$

An average of the CD spectra of cholesteryl oleate and cholesteryl palmitate were used for $[\psi]$ for cholesteryl esters, because the precision of measurement of the CD spectrum of cholesteryl linoleate was somewhat less than for the other esters. It resembled them closely, however, as can be

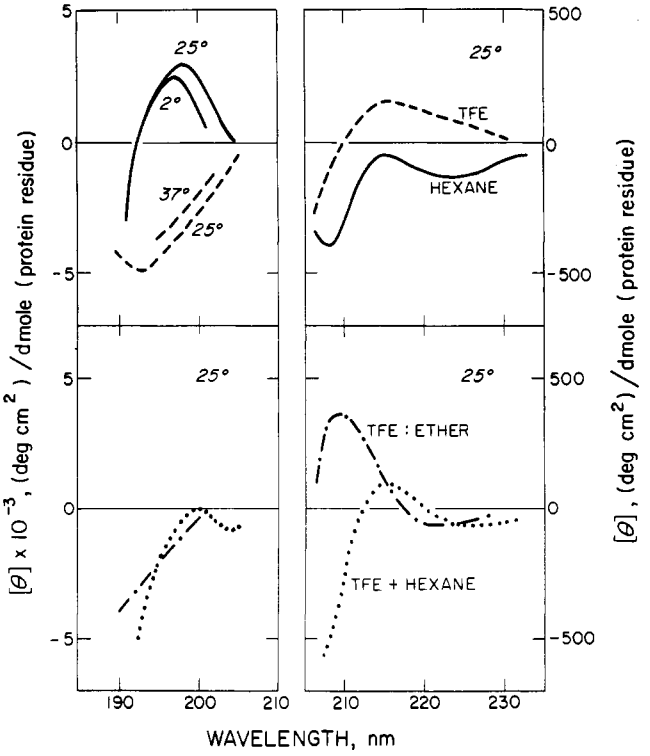


FIGURE 3: The CD spectra of lipids extracted from LDL in various solvents. TFE, trifluoroethanol; TFE:ether (3:1, v/v), experimental; TFE + hexane, calculated.

seen in Table III. Phosphatidylcholine and sphingomyelin were used in these studies since the content of other phospholipids in LDL is very small.

## Results

The CD spectra of intact LDL above 205 nm resemble the spectra of most globular proteins containing a moderate amount of helix (Figure 1). (CD spectra of LDL in 0.15 *M* NaCl were found essentially identical with those of LDL in 0.15 *M* KF, except that it was not possible to measure the former to wavelengths below 195 nm.) However, the positive CD band near 195–196 nm drops off sharply and turns to negative values below 185 nm, a characteristic not usually observed in proteins. Above 225 nm there is no detectable change in CD between 2 and 37°. Below that wavelength, the CD spectrum undergoes a negative shift when the temperature is lowered from 37 to 2°, and the minimum around 208–210 nm observed at 25° becomes more distinct at 2°. This trough is absent at 37°. A slight blue-shift of the crossover point is also noted with decreasing temperature. Both of these thermal changes in the CD spectra of LDL are completely reversible. As expected the corresponding ORD spectra in the ultraviolet region become less positive (more negative) at lower temperatures with a similar blue-shift of the crossover point (Figure 2).

Table I: Analyses of Lipid Composition of Various Solvent Extracts of LDL.

| Lipids[a] | Ethanol–Ether (3:1, v/v) | Trifluoroethanol | Hexane | Trifluoroethanol–Ether (3:1, v/v) |
|---|---|---|---|---|
| Phospholipids | 0.94 | 0.93 (99%) | <0.01 (0.7%) | 0.93 (99%) |
| Cholesterol | 0.42 | 0.38 (90%) | 0.02 (4.8%) | 0.38 (90%) |
| Cholesteryl esters | 2.00 | 0.11 (6%) | 1.73 (87%) | 1.63 (82%) |
| Total recovery as percent of lipids in ethanol–ether | | 95% | | 88% |

[a] Lipid concentration is in mg/mg of LDL protein.

The CD of both the $F_3$EtOH-soluble and hexane-soluble lipid fractions from LDL show increasing negative ellipticity at lower temperatures (Figure 3, upper portion). In the lower half of Figure 3 are shown the CD spectrum of the lipid extract in $F_3$EtOH–ethyl ether (3:1, v/v) and one calculated on the basis of the curves in the upper half. The difference between these two spectra can be attributed to differences in the lipid content of the two extracts (Table I). No measurable change in CD for the lipid extract in $F_3$EtOH–ethyl ether (3:1, v/v) was observed between 15 and 25°. Precipitation of some lipid below 10° and the loss of ethyl ether through evaporation at high temperatures prevented measurement of the CD spectrum beyond these limits.

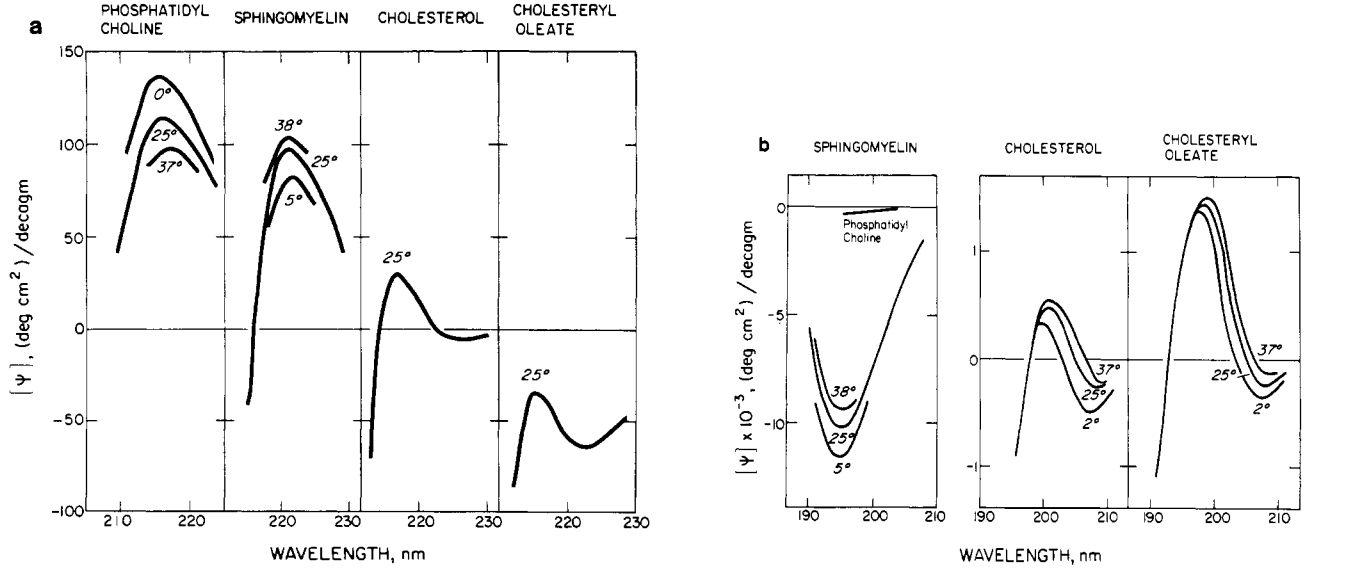


FIGURE 4: The temperature-dependent CD spectra of pure lipids. Cholesterol and cholesteryl oleate in hexane; sphingomyelin and phosphatidylcholine in trifluoroethanol (Figure 4a, 210–230 nm; Figure 4b, 190–210 nm).

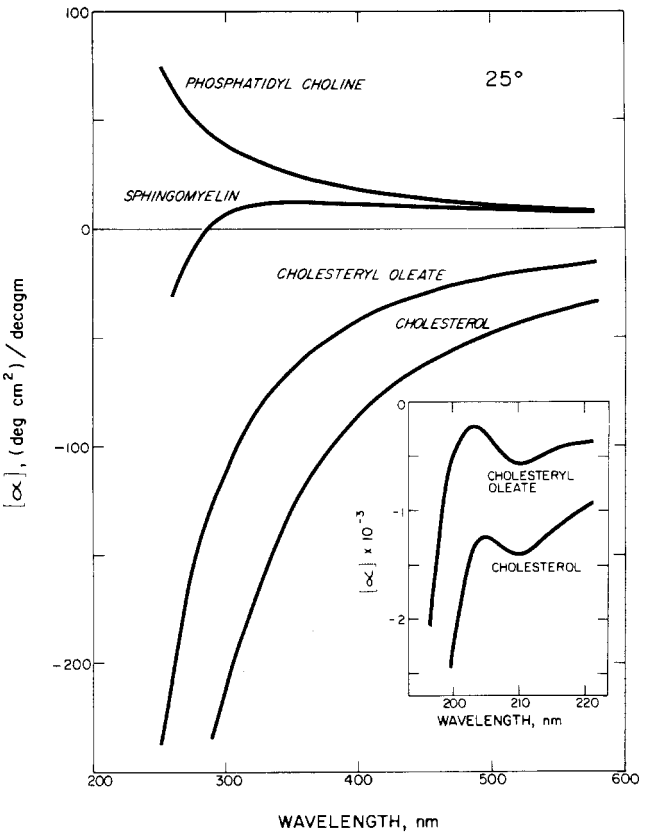


FIGURE 5: The ORD of pure lipids. Solvents are as in Figure 4.

Table I lists the chemical analyses of the lipid extracts in various solvents. The $F_3EtOH$ extract contained almost all the phospholipids and free cholesterol, while the hexane extract contained nearly all the cholesteryl esters. The mixture of $F_3EtOH$–ethyl ether (3:1, v/v) did not dissolve all the free cholesterol and cholesteryl esters (only 82% of the cholesteryl esters were recovered). There is a positive CD band around 210 nm for lipids in $F_3EtOH$–ethyl ether (3:1, v/v) in contrast to the negative band for lipids in $F_3EtOH$ plus hexane (Figure 3, lower). This difference may be due to the difference in content of cholesterol and cholesteryl esters in these solvent systems. The corresponding ORD of the lipid moieties of LDL in various solvents were all levorotatory between 200 and 600 nm with slightly enhanced magnitude at lower temperatures.

To evaluate the optical contribution of the principal classes of lipid constituents of LDL, we studied the CD and ORD of the pure lipids (Figures 4 and 5). Cholesterol and cholesteryl esters in hexane, and phosphatidylcholine and sphingomyelin in $F_3EtOH$ exhibit multiple Cotton effects in the ultraviolet region. The CD bands are small between 210 and 230 nm (Figure 4a) but become very intense below 210 nm (Figure 4b). Above that wavelength the two phospholipids display positive CD bands with opposite temperature dependence between 0 and 37°. Sphingomyelin has a very large negative CD band around 195 nm with increased ellipticity at lower temperature, whereas phosphatidylcholine shows only small negative ellipticities without any measurable temperature dependency. The CD spectra of cholesterol and cholesteryl oleate appear to be most sensitive to temperature change in the region of 200–210 nm. Lowering the temperature from 37 to 2° causes the CD spectrum to shift downward with a small blue-shift of the crossover point. These thermal changes are completely reversible. Cholesteryl palmitate and cholesteryl linoleate exhibit similar CD spectra and temperature effects to that of cholesteryl oleate in this region.

Ellipticities in hexane of the lipid extracted from LDL (Figure 3, upper) were calculated on the basis of the protein residue while those of pure cholesteryl oleate (Figure 4) were calculated on the basis of lipid mass. When converted to a common mass basis their ellipticities are of the same magnitude.

Cholesterol and cholesteryl oleate both exhibit featureless negative ORD curves between 250 and 600 nm but with different magnitudes (Figure 5). Below 220 nm both show intense, negative Cotton effects (Figure 5, inset). The effect of temperature between 2 and 37° on these compounds is the same, namely, the negative rotation is moderately increased (about 10–20%) throughout the entire range of wavelengths studied. Cholesteryl palmitate and cholesteryl linoleate show similar ORD spectra and temperature effects. On the other hand, the two phospholipids exhibit positive ORD curves of small rotation between 300 and 600 nm. Below 300 nm, the ORD spectrum of phosphatidylcholine continues to increase, reaching a plateau around 220 nm and remains positive at 205 nm. Changes in temperature between 2 and 37° do not cause any detectable changes in the ORD curve of phosphatidylcholine throughout the visible region and only very small increases in $[\alpha]$ occur with decreasing temperature below 250 nm. In contrast, sphingomyelin becomes somewhat more dextrorotatory at lower wavelengths. The curve turns downward about 330 nm with a crossover point near 290 nm. The ORD curve appears to shift downward with a slight red-shift of the crossover point when the temperature is lowered from 37 to 2°. Table II lists the constants of the Drude equation for LDL, for its total lipid extracts in ethanol–ethyl ether (3:1, v/v), for cholesterol, and for three cholesteryl esters at three temperatures. The constants, $\lambda_c$ and $k$, increase with increasing

Table II: Constants of the Drude Equation of LDL, Its Total Lipid Extract, and Some Pure Lipids.

| Materials | λ (nm) | | | $\lambda_c$ (nm) | | | $k$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2° | 25° | 37° | 2° | 25° | 37° | 2° | 25° | 37° |
| LDL[a] | 310–560 | 310–560 | 310–560 | 199–204 | 233–231 | 250–256 | −54 to −70 | −28 to −34 | −16 to −22 |
| Cholesterol | 340–540 | 340–540 | 340–540 | 211 | 216 | 215 | −10.3 | −9.7 | −9.7 |
| Cholesteryl palmitate | 330–560 | 330–560 | 330–560 | 212 | 221 | 220 | −5.8 | −5.0 | −4.8 |
| Cholesteryl oleate | 320–560 | 330–580 | 340–600 | 212 | 230 | 231 | −5.9 | −4.5 | −4.7 |
| Cholesteryl linoleate | 330–560 | 330–600 | 340–560 | 216 | 223 | 232 | −5.0 | −4.3 | −3.9 |
| Lipid extract in ethanol–ether (3:1, v/v) | 320–460 | 330–520 | 330–500 | 221 | 246 | 241 | −10.2 | −7.2 | −7.2 |

[a] Data from Chen and Kane (1974).

Table III: The CD and ORD Extrema[a] of LDL and Some Pure Lipids at 25°.

| Materials | CD Extrema | | | | | | | ORD Extrema | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\lambda_1$ | $[\theta]_1$ | $\lambda_2$ | $[\theta]_2$ | $\lambda_3$ | $[\theta]_3$ | $\lambda_4$ | $[\theta]_4$ | $\lambda_1$ | $[\alpha]_1$ | $\lambda_2$ | $[\alpha]_2$ |
| LDL[b] | 219–220 | −10.6 to −12.5 × $10^3$ | | | 211–212 | −9.7 to −11.4 × $10^3$ | 195–196 | 18.4 to 21.6 × $10^3$ | 232–233 | −5.3 to −6.3 × $10^3$ | 201–202 | −15.9 to −18.7 × $10^3$ |
| Cholesterol | 228 | −19 | 217 | 120 | 208 | −1.0 × $10^3$ | 201 | 1.8 × $10^3$ | 209–210 | −1.4 × $10^3$ | 205 | −1.2 × $10^3$ |
| Cholesteryl oleate | 220 | −540 | 215–216 | −480 | 208–209 | −1.9 × $10^3$ | 198 | 9.5 × $10^3$ | 208–210 | −7.6 × $10^3$ | 202 | −0.2 × $10^3$ |
| Cholesteryl palmitate | 223 | −430 | 215–216 | −223 | 208–209 | −1.5 × $10^3$ | 198 | 9.4 × $10^3$ | 209–210 | −0.6 × $10^3$ | 202–204 | −0.2 × $10^3$ |
| Cholesteryl linoleate | 223 | −530 | 215–216 | −250 | 208–209 | −1.7 × $10^3$ | | | 210–212 | −0.5 × $10^3$ | | |
| Phosphatidylcholine | 216 | (115)[c] | | | | | | | 225 | 0.15 | | |
| Sphingomyelin | 221 | (99) | | | | | 195 | (−10.2 × $10^3$) | | | | |

[a] $[\theta]$ is in deg $cm^2$/dmol and $[\alpha]$ in (deg $cm^2$)/dag at wavelength, $\lambda$, nm. [b] The concentration of LDL is based upon the protein residues. [c] Number in parentheses denotes specific ellipticity, $[\psi]$, instead.

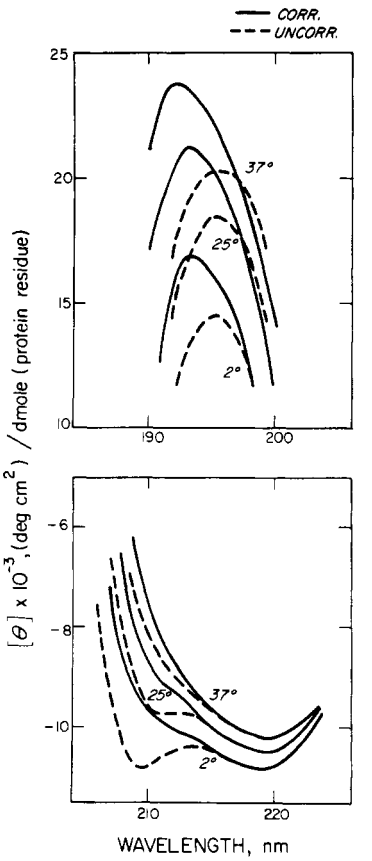


FIGURE 6: Correction for the contribution of lipid to the CD spectrum of LDL. UNCORR, experimental data; CORR, after subtraction of mass-weighted optical effect of lipid.

temperature in all cases. Table III lists the CD and ORD extrema of LDL, cholesterol, three cholesteryl esters, and two phospholipids at 25°. These lipids have their maximum ellipticities at wavelengths very close to those of the extrema of LDL. Around 220 nm, the magnitudes of the ellipticity of lipids are negligible as compared to that of LDL, whereas in the region of 195–210 nm, those of lipids could contribute significantly to the total ellipticity of the intact LDL particle. Figure 6 shows the CD spectra of LDL after subtraction of the ellipticity corresponding to its lipid composition, using specific ellipticities determined in organic solvents. Two prominent features emerge. First, the minimum at 208–210 nm diminishes markedly. After correction, the ratio of $[\theta]_{210\,nm}$ to $[\theta]_{222\,nm}$ decreases from 1.00 and 0.91 to 0.90 and 0.84 at 2 and 25°, respectively. Second, the positive ellipticity increases in the range of 190–200 nm after correction. This is accompanied by a blue-shift of the extrema from 195–196 to 193 nm at all three temperatures.

The magnitude of ellipticity of LDL varies to a small degree among preparations from different donors. For example, at 25° $[\theta]_{222\,nm}$ was found to range between 10 and 13 × $10^{-3}$ [(deg $cm^2$)/dmol (protein residue)] among eight preparations. The content of helix and $\beta$ form in one preparation has been calculated by the method of Chen et al. (1974) between 205 and 240 nm. At 2° the fractions of helix and of $\beta$ form are 33 and 23%, while at 37° they are 29 and 26%, respectively. Both changes are negligible with respect to the precision of the method. Furthermore, when the CD spectra are corrected for the contribution of lipid, as described above, either using data from lipids extracted

from LDL or mass-weighted data from pure lipids, the change in the content of helix and $\beta$ form is again negligible when calculated by the method of Chen et al. (1974).

## Discussion

The optical activity of LDL is temperature-dependent and shows complete reversibility between 0 and 37°. In this study, lowering the temperature from 37 to 2° increased the magnitude of the ellipticity in the region of 205–225 nm and decreased it below 205 nm. Dearborn and Wetlaufer (1969) and Scanu et al. (1969), however, have reported increases in ellipticity in the positive CD band below 205 nm with decreasing temperature. We have no explanation for this discrepancy, except to note that the signal-to-noise ratio is very low below 205 nm.

The lipid extracts in $F_3$EtOH–ethyl ether (3:1, v/v) show significant CD bands in the ultraviolet region. The fractions of extracted lipids soluble in $F_3$EtOH or hexane, respectively, and pure cholesterol, cholesteryl esters, and phospholipids all exhibit reversible, temperature-dependent optical activities in this region. Among the lipid moieties, sphingomyelin shows the most prominent CD spectrum. Cholesterol and cholesteryl esters, the major lipid components, also have appreciable CD bands. The large negative CD band of sphingomyelin at 195 nm and the sharp drop in ellipticity below 193–195 nm associated with cholesterol and cholesteryl ester is reflected in the marked downward curve in the lipid extracts of LDL (Figure 3, lower half). These lipids would appear to account for the increasing negativity in ellipticity observed with intact LDL below 195 nm. Thus, the contribution of the lipid moiety of LDL to the CD spectrum of the lipoprotein complex can by no means be overlooked in analysis of protein conformation.

The appearance at lower temperatures of a second minimum around 208–210 nm with only slightly larger ellipticity at 220 nm has been attributed to a thermally dependent increase in helical content in the protein moiety (Dearborn and Wetlaufer, 1969; Scanu et al., 1969). Cholesterol and cholesteryl esters, which comprise approximately half of the mass of this lipoprotein, however, also show an increasingly negative CD band between 200 and 210 nm with decreasing temperature (Figure 4). After subtraction of the ellipticity corresponding to amounts of lipids in organic solvents equivalent to those found in LDL, the minimum at 208–210 nm in the CD spectrum of LDL is found to diminish markedly (Figure 6). This is accompanied by a blue-shift of the extrema from 195–196 to 193 nm, which is then essentially identical with that of most proteins.

Correction for the absolute contribution of these lipids on the basis of studies of their optical activity in organic solvents may be complicated by potential effects of those solvents upon their CD and ORD behavior. More important still may be the ordered state of lipids in the intact lipoprotein particle. As an approach to the evaluation of this question, we prepared liposomes of sphingomyelin, phosphatidylcholine alone, and cholesterol and phosphatidylcholine in a mass ratio of 1:7. Their CD spectra showed negative ellipticity below 220 nm, as those of the pure lipids in organic solvents. None of these spectra differed substantially from those of the pure lipids in organic solvents in the region where the thermal effects on LDL are observed (200–210 nm).

While the bilayer vesicle is an imperfect model for the optical behavior of lipids in lipoproteins, this similarity suggests that the CD of organized lipids in LDL may not differ significantly from that of lipids dispersed in organic solvents. In the absence of such a difference, our data suggest that a substantial part of the thermal change observed in this region may be attributable to lipids rather than to alterations in the content of helix in the protein moiety.

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