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Contribution of Carotenoids to the Optical Activity of Human Serum Low-Density Lipoprotein†

G. Chi Chen and John P. Kane*

ABSTRACT: Low-density lipoprotein (LDL) (1.024–1.045 g/cm^3) was prepared by ultracentrifugal flotation from serum of subjects on diets of varying carotenoid content. Absorption, circular dichroism (CD), and optical rotatory dispersion (ORD) spectra were measured at 2, 25, and 37°. LDL from subjects receiving a β-carotene-enriched diet has a marked increase in absorbance between 350 and 550 nm attributable to carotenoids. In the same region, LDL exhibits multiple CD bands, which appear below 37° and which increase in intensity with decreasing temperature. The corresponding ORD of carotenoid-poor LDL is monotonic at all three temperatures, whereas that of carotenoid-rich LDL shows multiple Cotton effects below 37°. However, the ORD becomes monotonic after subtraction of the contribution due to carotenoids using the Kronig–Kramers transform for CD to ORD. Since the magnitudes of the CD bands increase with enrichment of β-carotene, which itself lacks optical asymmetry, the observed optical activity of carotenoids appears to be induced by environmental constraint in the lipoprotein complex.

The optical activity of human serum low-density lipoprotein (LDL)[1] has been studied extensively. Most of the published studies of optical rotatory dispersion (ORD) and circular dichroism (CD) are confined to the ultraviolet region (Scanu and Hirz, 1968; Gotto *et al.*, 1968; Dearborn and Wetlaufer, 1969). However, Gotto *et al.* (1968) reported that the plot of their data of the ORD of LDL at room temperature according to the Drude equation was nonlinear and obeyed the Moffitt–Yang equation only over a narrow wavelength interval (300–425 nm). Kobozev and Troitskii (1967) observed that LDL showed temperature-dependent optical rotations at four wavelengths (405, 435, 546, 579 nm) in the visible region. Since LDL contains carotenoids (Oncley *et al.*, 1950; Krinsky *et al.*, 1958) which absorb light between 250 and 550 nm, these compounds could contribute to optical activity in the visible and near-ultraviolet regions. In this work, we have shown that the carotenoids in LDL exhibit optical activity which is temperature dependent. This optical activity is the basis of nonlinearity of data for LDL plotted according to the Drude equation for LDL between 300 and 600 nm. This optical activity also complicates the interpretation of data using the Moffitt–Yang equation for determination of the content of helix in the protein moiety of LDL (apoLDL). The appearance of optical activity attributable to the symmetrical molecule, β-carotene, at low temperature demonstrates temperature-dependent environmental constraint upon that compound within the lipoprotein complex.

Materials and Methods

Preparation of LDL. Serum was obtained from normal fasting male subjects on diets of varying carotenoid content and

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[1] Abbreviations used are: LDL, low-density lipoprotein; ORD, optical rotatory dispersion; CD, circular dichroism; apoLDL, the protein moiety of LDL.

from two porphyric subjects who were given daily dietary supplements of 60 mg of β-carotene. 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 0.001 M EDTA and 2×10^{-3} M NaH_2PO_4 (pH 7.5). The concentration of protein was determined (Lowry *et al.*, 1951) using bovine serum albumin as a working standard, with correction for the absolute chromogenicity of apoLDL. Aliquots of the LDL preparations were extracted twice with 25 volumes of 95% ethanol–diethyl ether (3:1, v/v) to obtain the total lipid moieties for optical studies. The combined extracts were evaporated and dissolved in hexane or ethanol for the optical measurements. One preparation of LDL was succinylated at 0° (Hass, 1964).

Carotenoid Analysis. The procedures were the same as described by Krinsky *et al.* (1958) except for modification as follows. The LDL solution was denatured with 95% ethanol and extracted twice with petroleum ether (bp 30–60°). The combined extract was concentrated under nitrogen and separated on a column of alumina, 1.0 × 4.0 cm (aluminum oxide, neutral, for chromatography, J. T. Baker Chemical Co., was deactivated with 5% water by weight, ground in a mortar and then mixed into a slurry with petroleum ether, bp 30–60°). Elution, effected with stepwise increases in concentration of acetone in petroleum ether, separated the carotenoids into three colored bands which were identified as β-carotene, lycopene, and lutein, respectively (Krinsky *et al.*, 1958) by their absorption spectra. These compounds comprise essentially all the carotenoid content of human plasma. We found that 0.2% acetone in petroleum ether eluted the β-carotene band rapidly and sharply. For elution of lycopene and lutein, 4 and 40% acetone in petroleum ether respectively were used. Recovery of authentic β-carotene,[2] lycopene, and mixtures of these two carotenoids in different proportions on the alumina columns exceeded 90%. The concentrations of carotenoid were determined spectrophotometrically with the following extinction coefficients (Krinsky *et al.*, 1958): β-carotene, $\epsilon_{1cm}^{1\%}$ 2570 at 450 nm (petroleum ether); lycopene $\epsilon_{1cm}^{1\%}$ 3420 at 472 nm (4% acetone in petroleum ether); lutein, $\epsilon_{1cm}^{1\%}$ 2520 at 446 nm (40% acetone in petroleum ether).

Optical Studies. Visible and near-ultraviolet absorption spectra were measured on Zeiss PMQII and Cary 14 spectrophotometers and the data were expressed in terms of extinction coefficient, $\epsilon_{1cm}^{0.1\%}$ (indicating a protein concentration of 0.1% with a light path of 1 cm). ORD was measured with a Cary 60 spectropolarimeter and CD with a Durrum-Jasco J-5, SS-10-modified circular dichrometer, both under a constant stream of nitrogen. Specially designed thermostatted 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 solution was allowed to equilibrate at each temperature level for 30–60 min. Fused cylindrical silica cells (Pyrocell S-18-260) with path lengths of 5–50 mm were used, depending on the concentration of the LDL solution, to keep the absorbance of the solution below 2.0. Using potassium chromate in 0.06 M KOH to test for artifacts at low light intensity, less than 0.5 mdeg of ellipticity was observed at an absorbance of 2.0. The data were expressed in terms of mean residue rotation, $[m]$, and mean residue ellipticity, $[\theta]$, with dimensions in (deg cm^2)/dmol (protein residue) using a mean residue weight of 112, calculated from the amino acid composition of the protein moiety of LDL.

Analysis of Data. The ORD data were treated with the Drude equation (eq 1) and Moffitt-Yang equation (eq 2).

$$[m]\lambda^2 = \lambda_c^2[m] + k \quad (1)$$

$$[m'](\lambda^2/\lambda_0^2 - 1) = a_0 + b_0(\lambda^2/\lambda_0^2 - 1)^{-1} \quad (2)$$

By convention, the reduced mean residue rotation, $[m']$, is used in the Moffitt-Yang equation and the Lorentz correction was used to calculate $[m']$ from $[m]$, correcting for the refractive index of water at 20°.

ORD and CD share a common origin, namely optical activity. CD is an absorptive property, encountered only in the regions of the absorption of chromophores, whereas ORD is a dispersive property. The optical rotation at any wavelength is the sum of contributions from all optically active absorption bands and is influenced by electron transitions distant from the region in question. The interdependence of ORD and CD can be evaluated by the Kronig-Kramers transform (Moffitt and Moscowitz, 1959; Moscowitz, 1960). Since LDL exhibits temperature-dependent ORD, and carotenoids in LDL demonstrate temperature-dependent CD in the visible and near-ultraviolet regions, we converted the experimental CD spectra of LDL to ORD spectra using the Kronig-Kramers transform (Moscowitz, 1960) to evaluate the contribution of the carotenoids to the ORD spectrum.

$$[m(\lambda')] = (2/\pi)\mathrm{P}\int_{\lambda_1}^{\lambda_2}\{[\theta(\lambda)]\lambda/(\lambda'^2 - \lambda^2)\}d\lambda \quad (3)^3$$

where $[m(\lambda')]$ is the mean residue rotation at wavelength λ' calculated from the experimental mean residue ellipticity, $[\theta(\lambda)]$, at wavelength λ. Computations were executed on a CDC 6400 at the computer center of the University of California at Berkeley using the computer program written by Thiéry (1969). Numerical integrations were carried out over the region of 250–550 nm at intervals of 1 nm.

Results

Absorption Spectra. Visible absorption spectra of LDL over the range of 350 to 550 nm did not change with temperature between 1 and 37°. Figure 1 shows the visible and near-ultraviolet absorption spectra (room temperature) of LDL from a subject consuming a normal diet (curve 1) and after receiving a diet enriched with β-carotene for 3 weeks (60 mg/day) (curve 2). The spectra of LDL from subjects consuming normal diets are similar to that first reported by Oncley *et al.* (1950) for a preparation of LDL from normal human serum, except that the absorbance is two- to fourfold greater in our preparations (Table I). In contrast, LDL from subjects fed β-carotene shows a marked increase in absorbance between 350 and 550 nm in keeping with an increased content of β-carotene in the lipoprotein. Moreover, in these preparations the absorption maximum shifts from 458–460 to 464 nm with the appearance of a more discrete band at that wavelength.

Detailed analysis of carotenoids was made on LDL from subject 4 while on a normal diet and after consuming the β-carotene-enriched diet for 3 weeks. The contents of β-carotene, lycopene, and lutein were 0.27, 0.10, and 0.25 μg per mg of apoLDL before, and 2.02, 0.23, and 0.35 μg per mg of apoLDL, respectively, after consuming the β-carotene-enriched diet.

Lipids of the carotenoid extracts and of the ethanol–ether extracts were dissolved in hexane or petroleum ether for mea-

[2] β-Carotene and lycopene were gifts from Hoffmann La Roche, Inc.

[3] The letter P in front of the integral denotes

$$\mathrm{P}\int_{\lambda_1}^{\lambda_2} = \lim_{\delta \to 0}\left[\int_{\lambda_1}^{\lambda'-\delta} + \int_{\lambda'+\delta}^{\lambda_2}\right]$$

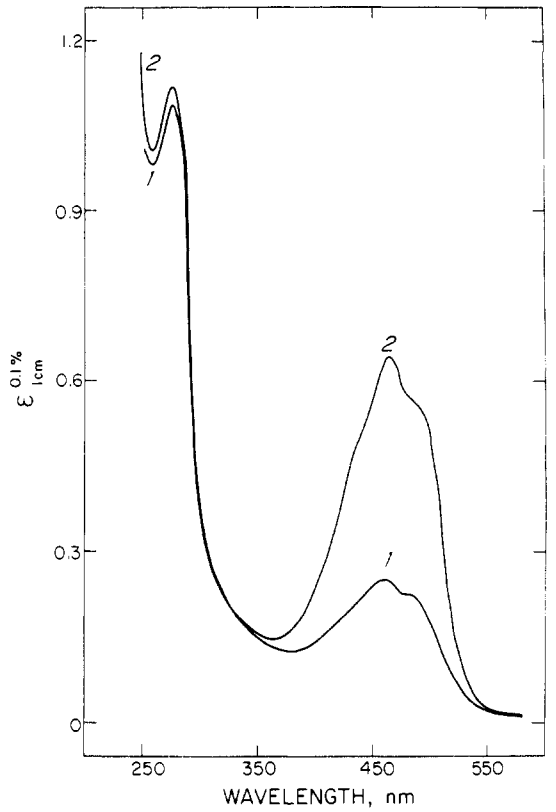

FIGURE 1: Visible and near-ultraviolet absorption spectra of LDL at room temperature from one subject on a normal diet (curve 1) and after receiving a diet enriched with β-carotene (60 mg/day) for 3 weeks (curve 2) (preparation 4A,B).

surement of absorption spectra in the visible region. These spectra were closely similar and also resemble very closely those of native LDL, indicating that the spectrum of LDL in this region is almost exclusively attributable to carotenoids.

Circular Dichroism. Figure 2 shows the temperature-dependent CD of LDL in the visible and near-ultraviolet region. The

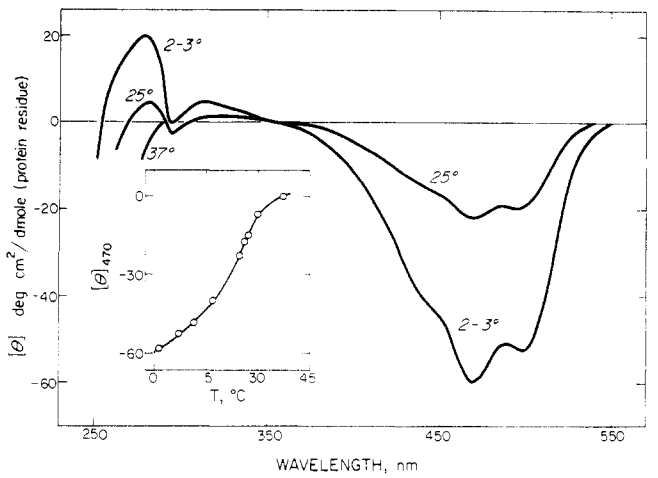

FIGURE 2: The temperature-dependent CD spectra of LDL. Inset: variations of $[\theta]_{470}$ with temperature (preparation 4B). At a wavelength greater than 290 nm the spectrum at 37° follows the $[\theta] = 0$ line.

mean residue ellipticity is expressed on the basis of protein, in the conventional fashion. If ellipticity is calculated on the basis of carotenoids instead, the magnitudes of the molar ellipticities obtained are on the order of 10^3 (deg cm²) $dmol^{-1}$. The positions of the extrema of these CD bands correspond to the maxima of the absorption bands in Figure 1. The magnitude of the multiple Cotton effects decreases upon heating the solution (inset in Figure 2). At 37°, the ellipticity is practically zero over the range of 300–550 nm and becomes negative below 290 nm. These temperature-dependent multiple Cotton effects are readily reversible.

Table I lists the CD extrema and the absorption maxima of six preparations of LDL with different contents of carotenoids. It is apparent that the ellipticity at wavelengths corresponding to absorption maxima of carotenoids varies with the absorbance at those wavelengths. Particularly, LDL from subjects fed a β-carotene-enriched diet has markedly higher ellipticity at

TABLE I: Absorbance and Temperature-Dependent CD of Human Serum Low-Density Lipoprotein.

				[θ], (deg cm²)/dmol (Protein Residue)							
				3°	25°	3°	25°	3°	25°	3°	25°
	$\epsilon_{1\,cm}^{0.1\%}$										
Preparations[a]	482–484 nm	458–460 nm	276–279 nm	486–490 nm		462–466 nm		310–320 nm		276–280 nm	280–282 nm
Normal diet											
(1)	0.13	0.14	0.9	−5.8	−2.5	−5.5	−2.3	8.6	2.3	38.4	20.6
(2)	0.13	0.13	0.83	−5.7	−1.7	−4.9	−1.3	4.2	0	19.7	2.9
(3)	0.21	0.21		−14.7	−7.4	−13.2	−6.7	6.8	2.1	26.5	11.2
(4A)[b]	0.23	0.25	1.09	−15.2	−10.8	−15.4	−11.1	10.5	4.4	39.8	29.7
	484–485 nm	464 nm	276–278 nm	496–498 nm	490–494 nm	486–470 nm	464–468 nm	312–318 nm	310–330 nm	276–278 nm	278–284 nm
β-Carotene-enriched diet											
(4B)[b]	0.56	0.64	1.12	−53.6	−34.8	−61.1	−39.3	6.9	2.4	25.6	18.1
(5)	0.62	0.72	1.12	−52.9	−20.1	−59.9	−22.6	4.8	0	20.7	4.3

[a] Numbers in parentheses denote individual subject. [b] Total carotenoid content: (4A) (0.62 μg/mg of apoLDL); (4B) (2.6 μg/mg of apoLDL).

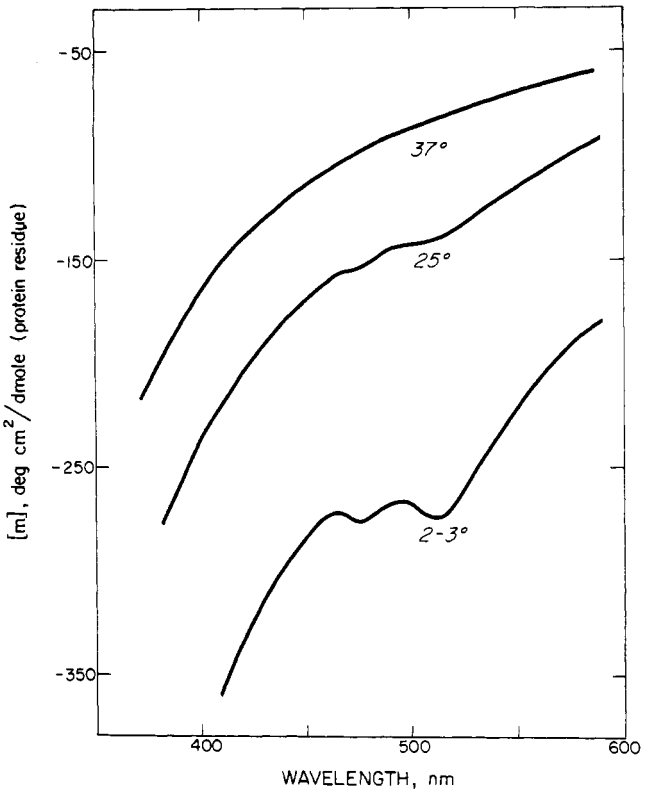

FIGURE 3: The effect of temperature on the ORD of the visible region of LDL from a subject on a β-carotene-enriched diet (preparation 4B).

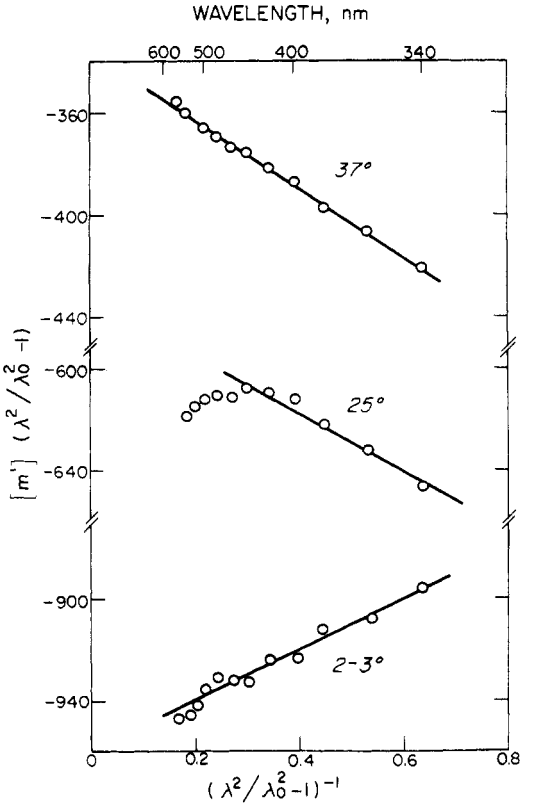

FIGURE 4: Representative plots of LDL data according to the Moffitt-Yang equation at three temperatures (subjects on normal diets).

460-500 nm. By comparing preparations 4A and 4B, it is apparent that a 4.2-fold increase in total carotenoids causes the CD magnitude between 470 and 490 nm (on the basis of protein residues) to increase by 3.7-4 times at 3°, and 3.2-3.6 times at 25°. However, if ellipticity is calculated on the basis of carotenoids, the magnitudes of the molar ellipticities obtained in this region are approximately 4.5-5 × 10^3 and 3-3.5 × 10^3 (deg cm^2) $dmol^{-1}$ at 3 and 25°, respectively, for normal and β-carotene-enriched lipoprotein. The Cotton effects between 250 and 350 nm (on the basis of protein residues) do not change with increasing content of carotenoid. The magnitude of the Cotton effects between 450 and 500 nm decreases as visible carotenoid color fades upon prolonged storage of LDL (>3 weeks at 4°).

The CD of extracts of total lipids and of carotenoids were measured at various temperatures. In all instances, no ellipticity was observed above 320 nm. For the three carotenoids separated chromatographically, changes in temperature between 2 and 37° were not associated with detectable CD above 320 nm. Below 320 nm, only the lutein fraction exhibited a small negative CD band around 260-290 nm which was not temperature dependent between 2 and 37°.

Optical Rotatory Dispersion. The optical rotation of LDL at constant temperature varies with different preparations. In addition, the ORD was found to be dependent upon both temperature and carotenoid content. Figure 3 shows the effect of temperature on the ORD in the visible region of LDL from a subject consuming a β-carotene-enriched diet. At 2-3 and 25°, LDL exhibits multiple Cotton effects over the range of 450-550 nm, whereas at 37°, it shows only a monotonic curve. Moreover, the magnitude of the levorotation decreases progressively upon heating the solution. On the other hand, LDL from subjects on a normal diet at these three temperatures shows only monotonic ORD curves in the visible region. Nevertheless, the magnitude of the levorotation also decreases with increasing temperature. This change in rotation is completely reversible over the range of temperature studied. A decrease in levorotation with increasing temperature was noted by Kobozev and Troitskii (1967) between 12 and 35° at four wavelengths (405, 435, 546, and 579 nm) which are outside the region of the multiple Cotton effects we observed.

Gotto *et al.* (1968) reported that a plot according to the Drude equation for LDL at room temperature was nonlinear. We plotted data between 320 and 580 nm at various temperatures according to the Drude equation with $[m]\lambda^2$ *vs.* $[m]$ for several preparations of LDL from subjects on a normal diet. At 37, 45, or 60°, a straight line was obtained over the region of 310-560 nm, whereas at 25° or lower, a straight line could be obtained only between 310 and 500 nm. In the case of LDL from subjects on a β-carotene-enriched diet, even at 37° a straight line was obtained only between 310 and 500 nm. From the slope and intercept of the straight lines, we calculated the values of λ_c and k and found them to vary slightly among different preparations. Furthermore, both λ_c and k decrease with decreasing temperature. The variations of λ_c and k with temperature appear in Table II along with the parameters of the Moffitt-Yang equation. The Moffitt-Yang equation was found applicable to LDL only at 37° or higher, giving b_o values from −130 to −150, which correspond to a helical content of approximately 25% (using −580 as reference value for 100% helix (Chen and Yang, 1971)). Figure 4 shows representative plots of data of LDL at different temperatures from subjects on a normal diet, according to the Moffitt-Yang equation with λ_0 = 212 nm. It is evident that at 37°, a straight line is obtained between 320 and 520 nm. At 25°, the plot is nonlinear above 500 nm and a straight line could be obtained only below 450 nm, giving b_0 values of approximately −100. At 2-3°, the plots are frequently nonlinear and have positive slopes. Similar results were obtained on a preparation of succinylated LDL at 2, 25, and 37°. Data for LDL from subjects fed a β-carotene-

TABLE II: Parameters of the Drude and Moffitt–Yang Equations for Human Serum Low-Density Lipoprotein.

	Drude			Moffitt–Yang		
T (°C)	λ (nm)	λ_c (nm)	k	λ (nm)	b_0	a_0
Normal diet						
2–3	310–500	199–204	−54 to −70		Positive	
2–3[a]	310–540	195	−72	320–520	Positive	
25	310–500	223–231	−28 to −34		Nonlinear	
37	310–560	250–256	−16 to −22	320–520	−130 to −150	−270 to −340
β-Carotene-enriched diet						
2–3[a]	310–560	193–198	−52 to −65		Positive	
25[a]	310–560	226	−27	320–460	−53	−480
37	310–500	240–245	−16 to −19	320–460	−120 to −150	−270 to −350

[a] $[m]$ is corrected by subtracting $[m]^{calcd}$ from $[m]^{expt}$.

enriched diet yield a monotonic ORD curve at 37°; however, the data do not yield a straight line above 460 nm when plotted according to the Moffitt–Yang equation.

The upper half of Figure 5 shows the computed ORD, $[m]^{calcd}$, from the CD at 2–3° between 250 and 550 nm of LDL from subjects receiving a β-carotene-enriched diet. After subtracting the $[m]^{calcd}$ from the experimental rotation, $[m]^{expt}$, the corrected visible ORD curve, $[m]^{cor}$, becomes monotonic. The magnitude of levorotation is still larger than that observed at 37° (Figure 5, lower half). This result further supports the idea that the temperature-dependent CD (Figure 2) causes the multiple Cotton effects in the visible ORD (Figure 3). We plotted the $[m]^{cor}$ according to the Drude equation at 2–3 and 25° and obtained straight lines between 310 and 560 nm. The values of λ_c and k fell in the range of those obtained on LDL from subjects on normal diets (Table II). The plot of data at

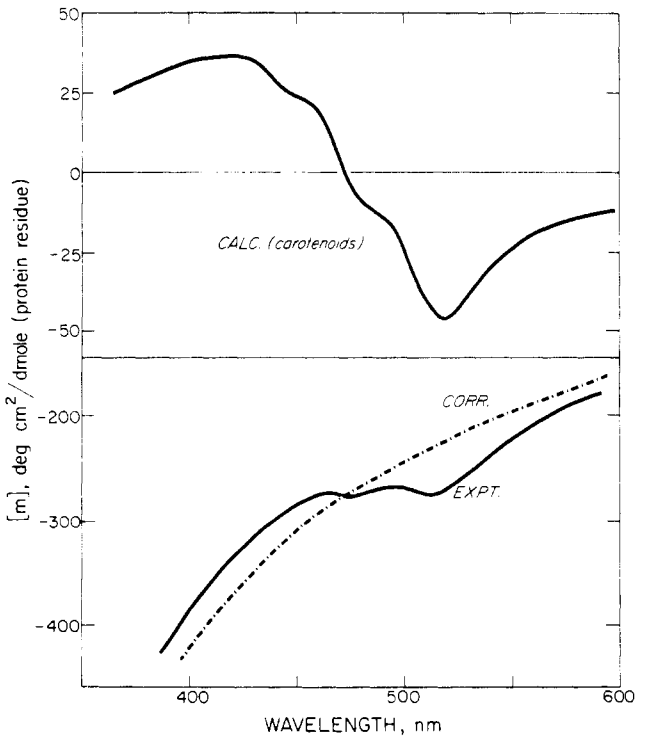

FIGURE 5: Rotatory contributions of carotenoids: EXPT, experimental results from LDL; CALC, ORD attributable to carotenoids, based on Kronig–Kramers transform of the CD spectrum between 250 and 550 nm (Figure 2); CORR, arithmetic difference of experimental and calculated curves (preparation 4B).

2–3° according to the Moffitt–Yang equation still gave a positive b_0, and at 25° a straight line could b obtained only between 320 and 460 nm. These findings indicate that the ORD of LDL in the visible region is complicated by the contribution of nonpeptide chromophores other than carotenoids.

Discussion

In this work, we have observed reversible, temperature-dependent optical activity in LDL in the visible and near-ultraviolet regions. The ORD of most proteins remain unchanged in the temperature range where these reversible effects were observed with LDL (0–37°). An effect of temperature on optical rotation of LDL at four wavelengths (405, 435, 546, and 579 nm) noted by Kobozev and Troitskii (1967) was attributed by those authors to flexibility in the structure of the protein and was compared to that of proteins such as denatured γ-globulin. They thus considered the protein in LDL to be in a "metastable condition." During the denaturation process such as induced by heating, most proteins show an increase in levorotation, with $[\alpha]_D$ ranging from −20 to − 70 ° for the native form and −80 to −120° for the completely denatured form (Yang, 1961). In the case of LDL, levorotation decreases upon increasing the temperature from 2–3 to 37°. At 37°, $[\alpha]_D$ is in the range of −50 to −70° for different LDL preparations, a value close to that of most native proteins. Therefore, we conclude that the temperature effect on optical rotation of LDL does not involve protein denaturation. However, it might be due to other rotatory contributions since $[\alpha]_D$ of LDL at 2–3° is two or three times higher than that of denatured or native proteins. Furthermore, at low temperature (Figure 3), the ORD spectra are not monotonic, showing multiple Cotton effects between 450 and 550 nm. This suggests contributions due to induced optical activity of chromophores absorbing in the visible and near-ultraviolet regions. Indeed Figure 2 clearly demonstrates that LDL exhibits temperature-dependent CD bands between 250 and 550 nm. The disappearance of the multiple Cotton effects in the visible ORD curve (Figure 5) after the $[m]^{expt}$ is corrected by $[m]^{calcd}$ from the Kronig–Kramers transform of the CD band between 250 and 550 nm further supports this conclusion.

It is customary to treat ORD in the visible region by the Drude and Moffitt–Yang equations. However, this is permissible only when it is a monotonic curve. Therefore, neither equation is applicable to data for LDL studied below 37° when the lipoprotein is rich in carotenoids. Even when the ORD curves are monotonic these equations may not apply because small Cotton effects may remain which have been overshadowed by

the rotations due to protein. For instance, LDL from subjects on a normal diet exhibits monotonic ORD spectra at 2–3 or 25°, but only at 37° or higher do the Drude and Moffitt–Yang equations become applicable (Table II and Figure 4). After correction for the contribution of the CD bands between 250 and 550 nm (attributable to carotenoids), the ORD of LDL from subjects on normal diets and diets enriched in β-carotene yield linear plots according to the Drude equation even at 2–3 and 25°. The data still do not obey the Moffitt–Yang equation, however. The corrected ORD curves at 25° or lower differ from those at 37°, indicating that temperature exerts effects on the protein moiety or upon other lipid components of LDL. (Lipids of the ethanol–ether extracts were found to exhibit temperature-dependent ORD and the data only obeyed the Drude and Moffitt–Yang equations between 320 and 420–500 nm. This perhaps explains the observation by Gotto *et al.* (1968) that the Drude and Moffitt–Yang equations were inapplicable to LDL at room temperature.) Thus, for determination of the content of helix in apoLDL using the parameter, b_0, of the Moffitt–Yang equation complications due to nonpeptide chromophores other than carotenoids must be taken into account.

The analysis of carotenoids in LDL further supports the conclusion that induced optical activities in the visible range are due to these compounds. We found that 90% of the increased carotenoid content of LDL from subjects consuming diets enriched in β-carotene were carotenoid hydrocarbons (β-carotene and lycopene), which are optically inactive. This increase in carotenoid hydrocarbons in LDL did cause multiple Cotton effects at 25° or lower in the ORD and greatly increased the magnitude of the extrema of the CD in the visible region.

While β-carotene, lycopene, and lutein are all found in each of the principal ultracentrifugal classes of lipoproteins, the major portion of each is carried in LDL (Krinsky *et al.*, 1958). This relationship is nonstoichiometric over a wide range of carotenoid content, in the case of β-carotene at least, as demonstrated by the change in the ratio of that carotenoid to LDL protein following the feeding of β-carotene. The precise locus of the carotenoids in the lipoprotein complex is unknown. However, the induced optical activity observed in these studies indicates that β-carotene at least is subject to environmental constraint at temperatures below 37°. Such constraint could arise from interaction with the protein moiety of the lipoprotein or from phase transitions in an ordered lipid system.

Acknowledgments

We thank Professor J. T. Yang for many valuable discussions on treatment of data and for use of the optical instruments employed in this study. We thank Professor N. I. Krinsky for advice on analysis of carotenoids and Dr. John H. Epstein who permitted us to study his patients. We acknowledge the generous cooperation of those two subjects who contributed their serum for this study. We are grateful to Miss Janet N. Yang for volunteer technical assistance.

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