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β-Carotene as a Probe of Lipid Domains of Reconstituted Human Plasma Low-Density Lipoprotein: Induced Circular Dichroism†

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ABSTRACT: Mixtures of neutral lipids containing cholesteryl esters and β-carotene were used to reconstitute the lipid core of heptane-extracted low-density lipoproteins (LDL). The resulting preparations of reconstituted LDL, referred to as r-[cholesteryl ester + β-carotene]LDL, exhibited temperature-dependent circular dichroism (CD) in the visible region similar to that of native LDL. Since β-carotene lacks intrinsic optical asymmetry, the observed CD must be induced by environmental constraint. LDL reconstituted with cholesteryl oleate, cholesteryl linoleate, or cholesteryl linolenate in the presence of β-carotene exhibited thermotropic transitions at approximately 40, 35, and 30 °C, respectively. These temperatures are similar to the liquid-crystalline to isotropic liquid phase transition temperatures observed for pure cholesteryl ester model systems. The addition of triolein to r-[cholesteryl linoleate + β-carotene]LDL lowered the transition temperature and decreased the specific ellipticity of the visible CD bands. When triolein accounted for 70% or more of the total neutral lipid content of r-[cholesteryl linoleate + triolein + β-carotene]LDL, specific ellipticity was zero. The addition of low levels of unesterified cholesterol to r-[cholesteryl linoleate + β-carotene]LDL did not alter the transition temperature but did reduce the specific ellipticity by 40%. The current data indicate that the organization of the core of neutral lipids in reconstituted LDL resembles that of native LDL with respect to environmental constraint on the β-carotene molecule and that the helicity of the protein moiety resembles that of native LDL. In addition, the data are consistent with the possibility that the core cholesteryl esters of reconstituted LDL undergo phase transitions similar to their transitions in the free state.

The molecular organization of the lipid core of human plasma low-density lipoproteins (LDL)[1] has recently been investigated (Deckelbaum et al., 1975, 1977a,b; Sears et al., 1976; Tardieu et al., 1976; Atkinson et al., 1977; Laggner et al., 1977; Mateu et al., 1978; Hamilton et al., 1979). Data from differential scanning calorimetry and small-angle X-ray scattering studies suggest that LDL contain an apolar core of neutral lipids surrounded by a polar surface shell. The neutral lipid core, which comprises ~50% of the total mass of the particle, consists predominantly of cholesteryl esters, chiefly cholesteryl linoleate (Skipski, 1972). This cholesteryl ester rich core undergoes a reversible phase transition around body temperature associated with an ordered liquid-crystalline–liquid phase change of cholesteryl esters within the intact LDL. Cholesteryl esters isolated from LDL (Deckelbaum et al., 1975, 1977a,b) undergo similar phase changes.

Chen & Kane (1974) have shown that the carotenoids normally present in trace amounts in LDL exhibit induced optical activity between 37 and 2 °C. β-Carotene comprises ~90% of the native carotenoids of LDL. As the temperature of LDL is lowered below 37 °C, the β-carotene, which is a symmetrical molecule lacking intrinsic optical activity, begins

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[1] Abbreviations used: CD, circular dichroism; LDL, low-density lipoproteins; r-[lipid + β-carotene]LDL, heptane-extracted LDL reconstituted with the indicated lipid and β-carotene; r-LDL, reconstituted LDL; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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to show induced optical rotatory dispersion and circular dichroism (CD) at wavelengths between 300 and 550 nm. This induced asymmetry appears to reflect environmental constraint arising from the formation of ordered liquid crystals of cholesteryl esters at temperatures below 37 °C (Chen & Kane, 1974).

In order to study the effect of lipid composition upon the induced CD of β-carotene, we have taken advantage of the LDL reconstitution procedure described by Krieger et al. (1978, 1979a,b) in which >99% of the core cholesteryl esters are removed from the LDL particle by extraction with heptane. These core lipids can then be replaced with an equal amount of exogenous lipids. Hydrophobic molecules that can be used to reconstitute LDL include (1) lipids containing cis-unsaturated fatty acyl groups, such as cholesteryl esters, triglycerides, and methyl esters, and (2) lipids containing polyisoprenoid groups, such as ubiquinone-10, retinyl palmitate, and β-carotene. The reconstituted water-soluble LDL particles retain many of the chemical, physical, and biological properties of native LDL. We have used the reconstitution procedure to introduce β-carotene together with other neutral lipids (cholesteryl esters, unesterified cholesterol, and triolein) into the lipid-depleted LDL. The resultant reconstituted particle, designated r-[lipid + β-carotene]LDL, has a high content of β-carotene. Using the induced CD of β-carotene as an intrinsic probe, we have shown that the β-carotene in reconstituted LDL (r-LDL) exhibits a temperature-dependent CD which appears to be a consequence of cholesteryl ester phase transitions in the lipoprotein core. Our data suggest that the lipid composition of the core (namely, the degree of unsaturation in the fatty acyl component of the cholesteryl esters, the amount of triacylglycerols, and the presence of unesterified cholesterol) is important in determining the thermotropic properties of the reconstituted lipoproteins.

Experimental Procedures

Materials. Cholesterol, cholesteryl oleate, and cholesteryl linoleate were obtained from Applied Science Laboratories, Inc. Cholesteryl linolenate and triolein were purchased from Nu Check Prep, Inc. Samples of β-carotene were purchased from Sigma Chemical Co. or were a gift from Hoffmann-La Roche Inc. Silica gel-HR was obtained from Merck Co. Other reagents and materials were obtained as previously described (Krieger et al., 1978).

Preparation of Native LDL. Native LDL (density 1.019–1.063 g/mL) were obtained from the plasma of individual healthy subjects (Brown et al., 1974) and prepared by sequential ultracentrifugation (Havel et al., 1955).

Preparation of r-LDL Containing β-Carotene. r-LDL were prepared as described by Krieger et al. (1978, 1979a). In brief, LDL were dialyzed against 0.3 mM EDTA (pH 7), samples of 1.9 mg of LDL protein were lyophilized in the presence of potato starch, and more than 99% of the neutral lipids were removed by two extractions with heptane. The heptane-extracted LDL were then reconstituted by the addition of 30 μL of carbon tetrachloride containing 1 mg of β-carotene and 200 μL of heptane containing a total of 6 or 7 mg of other neutral lipids as indicated in Table I. After a 1-h incubation at −10 °C, the heptane was evaporated under nitrogen for 45 min at 4 °C and the r-LDL were solubilized by a 14-h incubation at 4 °C in 10 mM Tricine (pH 8.4). The r-LDL were then isolated by centrifugation and filtered through a Unipore membrane (pore size 1.0 μm). The neutral lipids used to reconstitute LDL included cholesteryl oleate, cholesteryl linoleate, cholesteryl linolenate, triolein, and mixtures of cholesteryl linoleate with triolein and unesterified cholesterol.

Measurement of Cholesteryl Esters, Cholesterol, and Triolein in r-LDL. Lipids were extracted from the r-LDL with chloroform–methanol (2:1 v/v). The content of cholesteryl esters was determined by gas–liquid chromatography as previously described (Krieger et al., 1978). Aliquots of each lipid extract were also applied to a thin-layer plate prepared from silica gel-HR, which was developed in petroleum ether (bp 30–60 °C)–diethyl ether–glacial acetic acid (85:12:3 v/v/v). The fractionated lipid spots were detected with iodine vapor (Dalal et al., 1971), and the content of unesterified cholesterol and triolein was determined by the method of Schlierf & Wood (1965).

Measurement of β-Carotene in r-LDL. Each r-LDL preparation was denatured with 95% ethanol and extracted twice with petroleum ether (bp 30–60 °C). The concentration of β-carotene was determined spectrophotometrically with the extinction coefficient, $\epsilon_{1cm}^{1\%}$, of 2570 at 450 nm for β-carotene in petroleum ether (Krinsky et al., 1958).

Other Assays. The concentration of protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Electrophoresis was carried out in agarose gel at pH 8.6 in the barbital buffer (Noble, 1968) to determine the electrophoretic mobility of each preparation of r-LDL.

Optical Measurements of r-LDL. Visible absorption spectra were measured on a Zeiss PMQ II, and the data were expressed in terms of the extinction coefficient, $\epsilon_{1cm}^{0.1\%}$ (indicating a protein concentration of 0.1% with a light path of 1 cm). CD spectra were measured with a Jasco J-500A spectropolarimeter by using a thermostated sample chamber under constant nitrogen flush. 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. The CD data above 250 nm were expressed in terms of specific ellipticity, $[\psi]$, in deg cm^2/dag based on the concentration of β-carotene and below 250 nm in terms of mean residue ellipticity, $[\Theta]$, in deg cm^2/dmol by using a mean residue weight of 115 for the protein moiety of r-LDL. In the region of 200–230 nm, a fused cylindrical silica cell with path length of 0.5 mm was used, and the measurements were taken at the most sensitive scale. Because of the high absorbance in the solution, we were unable to measure CD below 200 nm.

Results

Chemical Composition of r-LDL. After native LDL were lyophilized in the presence of starch and extracted with heptane, exogenous β-carotene was introduced along with other lipids into the starch–LDL residue. Table I lists the lipids used to reconstitute the LDL and the chemical composition of the resultant r-LDL preparations. Four aspects of the data in Table I should be noted. (1) β-Carotene did not inhibit the incorporation of cis-unsaturated fatty acyl cholesteryl esters into LDL. For preparations 1A–3, the cholesteryl ester to protein ratio of ~1.7 was similar to that of native LDL and to those reported for LDL reconstituted with cholesteryl esters in the absence of β-carotene (Krieger et al., 1978, 1979a). (2) When mixtures containing varying proportions of cholesteryl linoleate and triolein were used for the reconstitution, the resulting r-LDL contained mixtures of the two lipids, the compositions of which reflected those of the starting mixtures. When only β-carotene was added, the yield of water-soluble LDL protein was low (0.18 mg/tube) as compared with that of preparations 1A–9 (Table I), indicating that β-carotene by itself could not reconstitute completely the core of LDL. (3) Unesterified cholesterol could be incorporated into r-LDL in

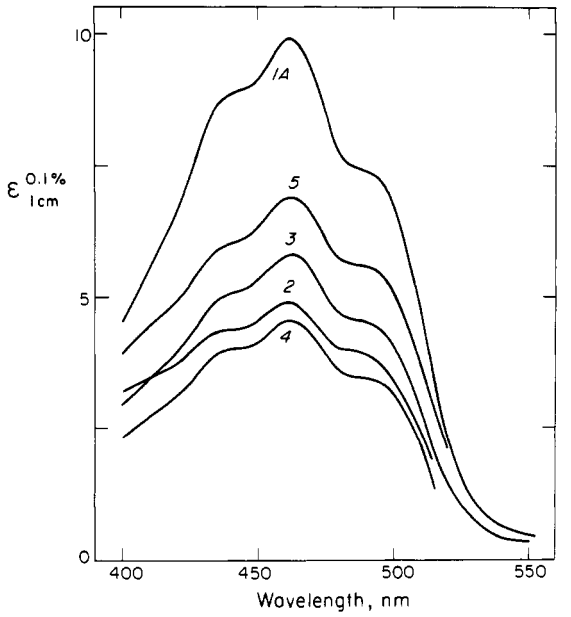

FIGURE 1: Visible absorption spectra of r-[lipid + β-carotene]LDL at room temperature. Extinction coefficient, ϵ, is expressed in terms of 0.1% concentration of LDL protein with a light path of 1 cm. r-LDL were prepared as described in Table I. Curves 1A–5 correspond to preparations 1A–5 in Table I.

combination with cholesteryl linoleate and β-carotene (preparation 4). (4) The content of β-carotene in various r-LDL preparations ranged between 14 and 55 μg/mg of LDL protein. This value is much higher than that found in native LDL, which contain <0.5 μg of β-carotene/mg of LDL protein when prepared from subjects on a normal diet and ~2 μg of β-carotene/mg of LDL protein when prepared from subjects on a β-carotene-enriched diet (60 mg/day for 3 weeks) (Chen & Kane, 1974).

The various preparations of r-LDL, hereafter referred to as r-[lipid + β-carotene]LDL, exhibited visible absorption spectra, visible CD spectra, and reversible thermal transitions comparable to those of native LDL (discussed below). Control preparations of LDL reconstituted with lipid in the absence of β-carotene did not exhibit this characteristic absorption spectrum in the wavelength region of 400–550 nm, and therefore no CD bands were observed in the visible region. All preparations of r-LDL reconstituted with or without β-carotene were subjected to agarose gel electrophoresis, and each showed the same electrophoretic mobility as that of native LDL.

Absorption Spectra of r-[Lipid + β-carotene]LDL. Figure 1 shows the visible absorption spectra between 400 and 550 nm of five preparations of r-[lipid + β-carotene]LDL at room temperature. Curves 1A–5 correspond to preparations 1A–5 in Table I. The shapes of these curves were similar to those observed for native LDL, especially LDL from subjects receiving β-carotene-enriched diets (Chen & Kane, 1974). The quantitative differences in the absorbance of various preparations are proportional to the different contents of β-carotene shown in Table I. All spectra exhibited absorption maxima at 462 nm with shoulders around 440 and 490 nm. These absorption bands differ from those of β-carotene in true solution (Vetter et al., 1971). The peak is broader, the long-wave band of β-carotene is diffused into a shoulder, and the weak inflection of the short-wave band of β-carotene becomes a marked shoulder in the r-LDL. Rather, the spectra are more like that of β-carotene-containing liposomes (Yamamoto & Bangham, 1978), except that the latter spectrum is even more degraded.

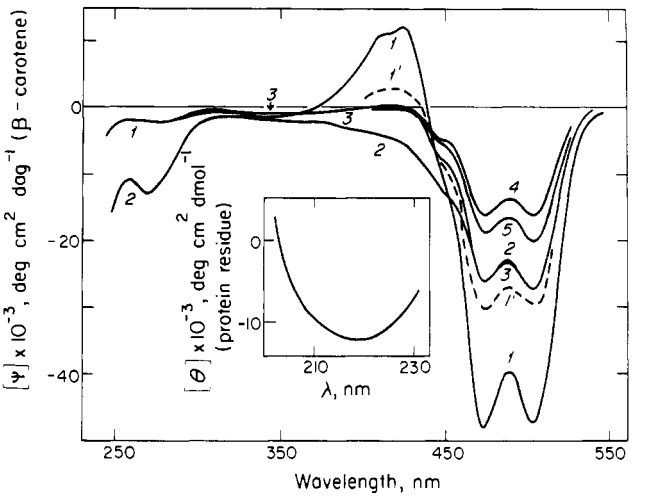

FIGURE 2: CD spectra of r-[lipid + β-carotene]LDL. (Main figure) Visible CD spectra of preparations 1A and 2–5 at 4 °C (see Table I). Curve 1′ shows the visible CD spectrum of preparation 1A at 25 °C. (Inset) UV CD spectrum of r-[cholesteryl oleate + β-carotene]LDL (preparation 1B, Table I) at 4 °C.

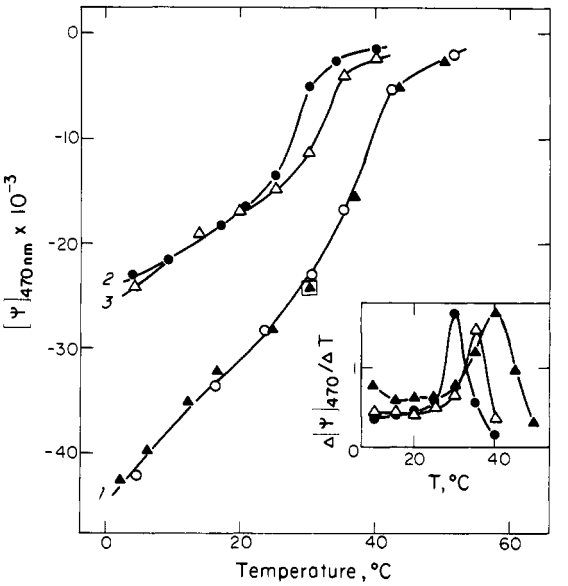

FIGURE 3: Effect of temperature on the Cotton effect at 470 nm of r-[cholesteryl ester + β-carotene]LDL. Curves 1–3 correspond to preparations 1–3 in Table I. (Inset) The data from the main figure were plotted as first derivatives to show transition temperatures. (▲) Preparation 1A, (○) 1B, (●) 2, (△) 3, and (□) 1A preheated to 50 °C and then cooled to 30 °C.

CD Spectra of r-[Cholesteryl oleate + β-carotene]LDL. Figure 2 shows the CD spectra of various r-[lipid + β-carotene]LDL preparations. The ellipticity was normalized (specific ellipticity) to account for the different amounts of β-carotene in the different preparations. The overall contours of these spectra were similar to those observed for native LDL (Chen & Kane, 1974). The positions of the extrema of the CD band correspond to the maxima of the absorption bands in Figure 1. Curve 1 (Figure 2) presents the spectrum of r-[cholesteryl oleate + β-carotene]LDL at 4 °C. Both preparations (1A and 1B, Table I) exhibited almost identical CD, showing the most pronounced multiple Cotton effect among the r-LDL preparations.

The temperature dependence of preparations 1A and 1B, which were prepared on different days with native LDL isolated from different individuals, was essentially identical (curve 1, Figure 3). The magnitude of the multiple Cotton effect

Table I: Characteristics of r-LDL Preparations[a]

prepn no.	amount added in reconstitution mixture (mg/tube)							amount recovered in r-LDL						sp ellipticity, $[\psi]_{470nm}$ (deg cm^2/dag × 10^{-3})
	LDL protein	cholesteryl ester[b]			triolein	unesterified cholesterol	β-carotene	protein (mg/tube)	cholesteryl ester (mg/mg)	mass ratio of lipid to protein for				
		C18:1	C18:2	C18:3						triolein (mg/mg)	unesterified cholesterol (mg/mg)	β-carotene (mg/mg)	
1A	1.9	6					1	1.27	1.62	0 (0%)[c]	0	0.055	−47.9
1B	1.9	6					1	1.19	1.87	ND[d] (0%)	ND	0.036	−45.6
2	1.9			6			1	1.46	1.74	0 (0%)	0	0.030	−25.2
3	1.9		6				1	0.89	1.58	0 (0%)	0	0.033	−26.2
4	1.9		6			1	1	0.73	1.41	0 (0%)	0.16	0.026	−15.9
5	1.9		5.8		0.18		1	1.17	1.64	0 (trace)[e]	0	0.040	−17.7
6	1.9		4.5		1.5		1	1.16	0.60	0.10 (14%)	0	0.020	−1.7
7	1.9		3		3		1	0.98	0.74	0.78 (50%)	0	0.017	−0.8
8	1.9		1.5		4.5		1	1.11	0.30	0.88 (70%)	0	0.019	0
9	1.9				6		1	1.18	ND	ND (100%)	ND	0.014	0

[a] Aliquots of dialyzed LDL were lyophilized in the presence of starch, the neutral lipids were extracted, and each sample was reconstituted with the indicated amount of exogenous lipid as described under Experimental Procedures. The content of protein and neutral lipid and the specific ellipticity at 4 °C for each r-LDL preparation were measured as described under Experimental Procedures. [b] The cholesteryl esters used for preparing r-LDL were cholesteryl oleate (C18:1), cholesteryl linoleate (C18:2), and cholesteryl linolenate (C18:3). [c] The values in parentheses represent the triolein content as a percentage of the total neutral lipid of the r-LDL. [d] ND, not determined. [e] No triolein was detected in preparation 5. The amount of triolein expected to have been recovered in this r-LDL was below the lower limit of sensitivity of the assay.

decreased upon heating the sample. Above 50 °C, the ellipticity of all CD bands was very small and the spectra became featureless. This temperature-dependent Cotton effect was readily reversible (designated by an open square) when the sample was cooled to 30 from 50 °C. Curve 1′ (Figure 2) shows the CD spectrum of preparation 1A at 25 °C.

The CD spectrum below 250 nm in the region containing contributions from the polypeptide moeity of r-[cholesteryl oleate + β-carotene]LDL is shown in the inset of Figure 2 and is expressed on the basis of protein concentration. The CD spectrum, which was observed at 4 °C, had a minimum around 220 nm and resembled that of native LDL (Chen & Kane, 1975) except there is no defined minimum around 210 nm. The absence of a distinct minimum between 208 and 210 nm in the CD spectrum of r-[lipid + β-carotene]LDL is consistent with increased light scattering by the reconstituted lipoprotein. This may attributable to greater heterogeneity in particle size and opacity due to high β-carotene content. Estimation of secondary structure in the protein moiety of the r-LDL based on the reference value of $[\Theta]_{222} = -30\,300\,f_H - 2340$ (Chen et al., 1972) yields a helical content of 32%, which is comparable to that of native LDL (Chen & Kane, 1975).

Effect of the Fatty Acyl Component of Cholesteryl Ester on CD Spectrum of r-[Cholesteryl ester + β-carotene]LDL. The CD spectra of r-[cholesteryl linoleate + β-carotene]LDL (curve 3) and r-[cholesteryl linolenate + β-carotene]LDL (curve 2) are shown in Figure 2. The contours of these CD spectra and that of r-[cholesteryl oleate + β-carotene]LDL (curve 1) were similar above 450 nm but obviously distinct from one another below 450 nm. At 4 °C, the magnitude of the specific ellipticity of the 470- and 500-nm bands of r-[cholesteryl oleate + β-carotene]LDL was about twice that of the cholesteryl linoleate or cholesteryl linolenate containing preparations (see Table I). In the region of 400–450 nm, where β-carotene exhibits its short-wave absorption band, the CD bands of these three r-[cholesteryl ester + β-carotene]LDL differed not only in magnitude but also in shape.

As was the case with r-[cholesteryl oleate + β-carotene]-LDL, the magnitude of the Cotton effect for the cholesteryl linoleate and cholesteryl linolenate containing r-LDL decreased with increasing temperature (curves 2 and 3, Figure 3). The sigmoidal shapes of these heating curves suggest that the spectral changes are governed by characteristic transition temperatures.

A plot of the rate of change of CD with temperature ($\Delta[\psi]/\Delta T$ vs. T)[2] (Figure 3, inset) was constructed from the experimental data in Figure 3, and the distinct peaks defined the transition temperatures of the Cotton effect to be approximately 40, 35, and 30 °C for cholesteryl oleate, cholesteryl linoleate, and cholesteryl linolenate containing r-LDL, respectively. These transition temperatures are similar to those for pure cholesteryl ester model systems undergoing liquid-crystal to isotropic phase transitions (Small, 1970).

Effect of Unesterified Cholesterol on CD Spectrum of r-[Cholesteryl linoleate + β-carotene]LDL. When 1 mg of unesterified cholesterol was added to the reconstitution mixture (1 mg of β-carotene + 6 mg of cholesteryl linoleate + starch-LDL residue), 0.16 mg of unesterified cholesterol per mg of LDL protein was incorporated into the r-LDL (Table I, preparation 4). The CD spectrum of these unesterified cholesterol containing particles at 4 °C showed multiple Cotton effects (curve 4, Figure 2) and was similar to that of the r-LDL

[2] Plot of $\Delta[\psi]/\Delta T$ vs. T_i, where $\Delta[\psi] = [\psi]^{T_i} - [\psi]^{T_i-\Delta T}$ and $\Delta T = 5$ °C.

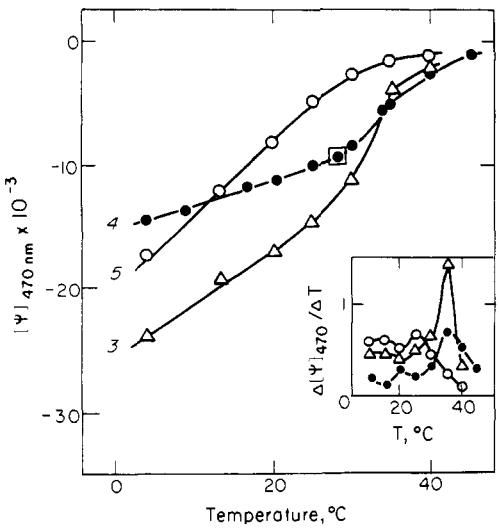

FIGURE 4: Effect of unesterified cholesterol or triolein on the thermal transition of r-[cholesteryl linoleate + β-carotene]LDL. Curves 3–5 correspond to preparations 3–5 in Table I. (Inset) The data from the main figure were plotted as first derivatives to show transition temperatures. (△) Preparation 3, (●) 4, (○) 5, and (□) 4 preheated to 45 °C and then cooled to 28 °C.

lacking unesterified cholesterol (curve 3, Figure 2), although the magnitude in the region of 450–550 nm was reduced by ~40%. The ellipticity at 470 nm gradually decreased upon heating of the sample (curve 4, Figure 4) and was readily reversible as shown by the open square when the sample was cooled to 28 from 45 °C. The plot of $\Delta[\psi]_{470}/\Delta T$ vs. T (Figure 4, inset) suggests a transition temperature of 35 °C for r-[cholesteryl linoleate + unesterified cholesterol + β-carotene]LDL, which is the same as that of r-[cholesteryl linoleate + β-carotene]LDL. However, the transition curve is very much broader for the unesterified cholesterol containing r-LDL particles.

Effect of Triolein on CD Spectrum of r-[Cholesteryl linoleate + β-carotene]LDL. To study the effect of triglyceride content on the CD of the r-LDL, we reconstituted LDL with mixtures of lipid containing varying mass ratios of triolein to cholesteryl linoleate. In preparations 5–9 (Table I), the proportions of triolein used were 3, 25, 50, 75, and 100%, respectively, so as to keep the total mass of triolein and cholesteryl linoleate at 6 mg in the reconstitution mixture. In the resultant preparations of r-[cholesteryl linoleate + triolein + β-carotene]LDL, the mass ratio of cholesteryl linoleate to protein decreased with increasing amounts of triolein (Table I, preparations 5–9).

Triglyceride had significant effects on the visible CD of r-[cholesteryl linoleate + β-carotene]LDL. A 30% decrease in the magnitude of the ellipticity was observed when small amounts of triolein were added to the reconstitution mixture (compare preparations 3 and 5, Table I). Moreover, the characteristic multiple Cotton effect disappeared when triolein accounted for 14% of the neutral lipid of the r-LDL (preparation 6, Table I), and there was a progressive decrease in the specific ellipticity and finally the complete absence of measurable CD signal as the triolein content increased (preparations 7–9, Table I). Specific ellipticities of −26 200, −17 700, −1700, −800, and 0, respectively, were observed for preparations of r-[cholesteryl linoleate + triolein + β-carotene]LDL that contained the following amounts of triolein (% of neutral lipid): none, trace, 14, 50, and 70 (preparations 3 and 5–8, Table I). Furthermore, triglyceride markedly influenced the thermal behavior of the Cotton effect. The CD spectrum of r-[cholesteryl linoleate + triolein + β-carotene]LDL containing trace amounts of triolein (preparation 5) exhibited a broad thermal transition at 25 °C (curve 5, Figure 4 and inset) in contrast to the sharp transition at 35 °C observed for preparation 3, which contained no triolein (curve 3, Figure 4 and inset).

When no cholesteryl ester was added to the reconstitution mixture (preparation 9), the resultant r-[triolein + β-carotene]LDL did not exhibit measurable visible CD even though they exhibited visible absorption bands similar to those of cholesteryl ester containing particles (data not shown). Cholesteryl ester, therefore, appears to be the component in r-LDL that is primarily responsible for inducing the visible CD of β-carotene.

Discussion

In the current study, we have incorporated mixtures of β-carotene, cholesteryl esters, triolein, and unesterified cholesterol into LDL by using a previously described reconstitution technique. The r-[lipid + β-carotene]LDL used in this work resemble previously described reconstituted LDL particles in terms of the yield of reconstituted material, the mass ratios of neutral lipid to protein, and electrophoretic behavior on agarose gels (Krieger et al., 1978, 1979a,b).

The r-[lipid + β-carotene]LDL has been used to answer several questions: First, can β-carotene be used as a spectroscopic probe to detect thermotropic transitions in the lipid core of r-LDL? Second, how do variations in the composition of core lipids affect the physical state of the core? Third, can examination of the CD spectra of LDL reconstituted with β-carotene and a variety of other neutral lipids help in interpreting the visible CD spectrum of native LDL and other lipoproteins?

The current data show that the temperature-dependent CD spectra in the visible region of the r-[lipid + β-carotene]LDL are strikingly similar to that of native LDL (Chen & Kane, 1974). Furthermore, the transition temperature of native LDL (three preparations from different donors) obtained from the plot of $\Delta[\psi]/\Delta T$ vs. T was 30 °C (data not shown), which is in the range of 26.0–36.5 °C determined by differential scanning calorimetry (Deckelbaum et al., 1977a,b). This observation, in turn, suggests that the environments that induce asymmetry in the β-carotene molecule of native and reconstituted LDL are similar.

The neutral lipid composition of r-[lipid + β-carotene]LDL had significant effects on the visible CD and its thermal behavior. The differences in fatty acyl composition of cholesteryl ester in the r-[cholesteryl ester + β-carotene]LDL influenced both the shape and the magnitude of the CD bands (curves 1–3, Figure 2). The transition temperatures of approximately 40, 35, and 30 °C (Figure 3, inset) for the cholesteryl oleate, cholesteryl linoleate, and cholesteryl linolenate containing particles, respectively, are very similar to those for pure cholesteryl ester model systems undergoing liquid-crystal to isotropic liquid phase transitions (Small, 1970). Thus, it seems likely that the thermal transitions detected by using the induced CD of β-carotene are a consequence of phase transition of the cholesteryl esters in the core of r-LDL.

The above conclusion is supported by results obtained by using two other spectroscopic techniques. In one set of experiments (Krieger et al., 1980), small amounts of 12-doxylstearate methyl ester, an apolar spin-label probe, were mixed with cholesteryl oleate, and this liquid mixture was used to reconstitute heptane-extracted LDL. Measurements of electron spin resonance (ESR) revealed a thermal transition in the r-LDL at 30 °C. This 30 °C transition for r-LDL resembled that observed for the pure lipid mixture of cho-

lesteryl oleate and 12-doxylstearate methyl ester, the latter being determined by both ESR spectroscopy and differential scanning calorimetry. In a second study conducted in collaboration with Dr. Paul Kroon of Merck Sharpe & Dohme Research Laboratories, we used nuclear magnetic resonance spectroscopy to monitor methylene and methyl protons of cholesteryl oleate and cholesteryl linoleate in r-LDL (unpublished experiments). Thermal transitions were detected by NMR spectroscopy at approximately 41 and 36 °C for r-[cholesteryl oleate]LDL and r-[cholesteryl linoleate]LDL, respectively. These transition temperatures were essentially identical with those observed in the current study. Thus, three different spectroscopic techniques have been used to detect characteristic thermal transition temperatures for r-LDL, each of which closely matches the phase transition temperature of the lipid used to reconstitute the LDL. Considered together, these observations strongly suggest that incorporation of cholesteryl esters into r-LDL does not markedly alter their ability to undergo characteristic phase transitions.

Inclusion of unesterified cholesterol (10% of the total mass of the neutral lipid) in the r-[cholesteryl linoleate + β-carotene]LDL decreased the magnitude of the induced CD bands and broadened the reversible transition without altering the transition temperature (Figure 4, curve 4). The basis of these effects is not clear. The presence of small amounts of triglyceride markedly decreased the magnitude of the CD bands and resulted in a broader transition with a transition temperature 10 °C lower than that observed in r-LDL lacking triolein. Furthermore, increasing amounts of triglyceride progressively decreased and finally abolished the induced CD bands of β-carotene (preparations 6–9, Table I). Triolein could be exerting its effects on the induced CD either directly by interacting with the β-carotene itself or indirectly by altering the structure of the core cholesteryl esters or both. It is likely that triolein indirectly modifies the CD by exerting a disordering effect on the structure of the cholesteryl esters for the following two reasons: (1) triolein can fill the core of r-LDL in a fashion similar to that of cholesteryl ester (Krieger et al., 1979), suggesting that the two lipids can exist together in the core of r-LDL, and (2) triolein's effects on the cholesteryl esters of r-LDL are in accordance with those in binary model systems of cholesteryl ester and triglyceride (Small, 1970).

The cholesteryl esters in r-[lipid + β-carotene]LDL appear to be the lipid component responsible for inducing the CD of β-carotene. Because β-carotene lacks intrinsic optical activity, the observed CD must be induced by an asymmetric environment. The cholesteryl esters in the core of r-LDL may provide an asymmetric environment when they are cooled below their characteristic liquid crystal to isotropic liquid phase transition temperature. Thus, the induced CD spectra will be sensitive to the physical state of the lipid core of r-LDL. Taken together, the current data support this hypothesis and show that β-carotene is a useful spectroscopic probe for determining thermotropic transitions in the core of r-LDL.

The general feature of the visible multiple Cotton effect observed in the r-LDL is comparable to that of native LDL, which contains a mixture of cholesteryl esters, unesterified cholesterol, and triglycerides. In fact, the magnitude of CD bands at 470 and 500 nm for native LDL prepared from subjects on normal or β-carotene-enriched diets is approximately −20 000 deg cm^2/dag at 3 °C (Chen & Kane, 1974), which is comparable with those of preparations 2–5 (−17 700 to −25 200 deg cm^2/dag). The similarity in the specific ellipticities of CD bands of r-LDL and native LDL (Chen & Kane, 1974) in which β-carotene varied over a 100-fold range strongly suggests that the molecules of β-carotene do not interact with one another in these lipoproteins. In contrast, β-carotene in a lipid bilayer system was shown to be aggregated above and below the phase transition temperature (Yamamoto & Bangham, 1978).

Although the precise locus and molecular organization of β-carotene in the r-[lipid + β-carotene]LDL is not clear, the induced CD of β-carotene observed in these reconstituted particles indicates that β-carotene is subject to conformational constraint at temperatures below the ordered liquid-crystalline–liquid phase transition temperature of cholesteryl esters. By use of the reconstitution procedure, amphipathic carotenoids such as xanthophylls could be introduced along with other lipids to probe the lipid distribution and organization of other regions in the lipoprotein complex.

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Structure of the Complex Polysaccharide C-Substance from *Streptococcus pneumoniae* Type 1†

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ABSTRACT: The complex cell-wall polysaccharide, C-substance, was isolated from *Streptococcus pneumoniae* type 1 and purified by DEAE-cellulose (HCO_3^- form) and Sephadex column chromatography. The complete structure of this antigen was obtained by the application of methylation and ^1H NMR and ^{13}C NMR spectroscopic techniques to a series of oligosaccharide fragments obtained by the selective degradation of the N-acetylated antigen. Native C-substance is composed of the following repeating unit: β-D-Glu*p*-1→3-α-AATGal*p*-1→4-α-D-GalNAc*p*-1→3-β-D-GalNH_2*p*-1→1′-ribitol-5-phosphate where AATGal is 2-acetamido-4-amino-2,4,6-trideoxygalactose. Phosphocholine substituents are situated at O(6) of the unacetylated galactosamine residues, and the repeating units are linked through a diphosphate ester from ribitol to O(6) of the β-D-glucopyranose residue. This structure has also been shown to be common to C-substances prepared from a number of other pneumococcal types based on the criterion of their identical ^{13}C NMR spectra.

Streptococcus pneumoniae produce a complex species-specific antigenic polysaccharide (PnC)[1] which was first described by Tillett et al. (1930). It is of interest as a common antigen of the various strains of the pneumococci and is a contaminant in most type-specific polysaccharide preparations. PnC contains phosphocholine (Brundish & Baddiley, 1968), and this component is responsible for several properties of the polysaccharide. It is a major antigenic determinant (Heidelberger et al., 1972), and the interactions of PnC with the acute-phase serum protein C-reactive protein (Tillett & Francis, 1930) and with certain mouse myeloma proteins (Potter & Leon, 1968) are both due to phosphocholine (Leon & Young, 1971; Volonakis & Kaplan, 1971). PnC is attached to the cell-wall mucopeptide in the organism, and the phosphocholine plays an important role in its physiology (Tomasz et al., 1975). Mucopeptide fragments are present in PnC preparations (Liu & Gotschlich, 1963; Gotschlich & Liu, 1967).

The structure of the remainder of this complex polysaccharide has been only partly determined, and its role in the above properties is not known. In addition to phosphocholine, Brundish & Baddiley (1967, 1968) identified 2-acetamido-2-deoxy-D-galactose, D-glucose, ribitol, phosphate, and an *N*-acetyldiaminotrideoxyhexose as constituents of PnC and therefore classified it as a ribitol teichoic acid. Further structural studies by Watson & Baddiley (1974) led to a partial structure being proposed. This structure was based on a trisaccharide–ribitol–phosphate repeating unit in which D-glucose was a major constituent. In later work (Poxton et al., 1978) the structure was modified essentially to a disaccharide–ribitol–phosphate repeating unit which did not include D-glucose as a major constituent, despite the fact that the presence of four anomeric signals (of approximately equal intensity) in the ^{13}C NMR spectrum of PnC strongly suggested additional hexose units.

We now report the complete structural analysis of PnC, including the identification of a glucopyranose residue and, in addition, a 2-amino-2-deoxy-D-galactose residue in its repeating unit. On the basis of chemical analyses and assignments made on the ^{13}C NMR spectra of three oligosaccharides obtained from PnC by degradation procedures, a structure for PnC is proposed. This structure is consistent with many of the structural features previously proposed for PnC by Watson & Baddiley (1974).

Experimental Procedures

Purification of PnC. For types 1, 23, and 56, crude lyophilized material, obtained by the further alcohol fractionation of the culture medium (80% ethanol) following removal of the capsular polysaccharide (Krishnamurthy et al., 1978), was kindly supplied by Dr. Thomas Stoudt of Merck Sharp & Dohme, Rahway, NJ. A similar fraction obtained from type 33 organisms was also kindly given by Dr. C. T. Bishop of our laboratories. Cells of the rough strain R36A (which derives from a type 2 organism) were extracted with trichloroacetic acid, and the extract was fractionated with acetone and ethanol as described by Brundish & Baddiley (1968). The yield from R36A cells was greatly increased by lengthening the extraction time to 2 weeks.

PnC was purified from all the above crude fractions by the method of Brundish & Baddiley (1968). Each lyophilized

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[1] Abbreviations used: PnC, C-substance or C-polysaccharide of *S. pneumoniae*; DEAE, diethylaminoethyl.

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