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A Carbon-13 Nuclear Magnetic Resonance Study of Aortic Lesions and Cholesteryl Ester Rich Lipoproteins from Atherosclerotic Rabbits†

Paulus A. Kroon,* Daniel M. Quinn,‡ and Eugene H. Cordes

ABSTRACT: When rabbits are fed a diet supplemented with cholesterol, their plasma cholesterol levels increased markedly and they developed atherosclerosis. Most of the plasma cholesterol exists as cholesteryl esters in very low density and low-density lipoproteins. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra (at 48 °C) of lipoproteins, and of arterial lesions from cholesterol-fed rabbits, are dominated by well-resolved cholesteryl ester resonances. An analysis of their line widths shows that the cholesteryl esters are in a liquid state at this temperature. Calculations based on line widths and spin–lattice relaxation times show that the motion of the cholesteryl ester molecules is highly anisotropic; motion about the long axis of the cholesteryl moiety is 38–75 times faster than motion about the short axis. Spectra for the lipoproteins and arterial lesions show temperature-dependent line-width changes that are consistent with an order–disorder transition of the cholesteryl esters above physiological temperatures. The similarity of line widths and spin–lattice relaxation times for lipoproteins and arterial lesions indicates that their molecular organization and molecular dynamics are also similar and suggests that an appreciable fraction of the cholesteryl esters are derived from nonmetabolized lipoproteins. The phospholipid choline methyl resonance is the only one that is not the same in lipoproteins and lesions. The lipoprotein choline methyl resonance is relatively narrow (~12 Hz) at all temperatures studied, consistent with a fluid phospholipid monolayer. The same resonance for arterial lesions is 2.5 times broader. The increased line width is at least partially due to a more heterogeneous environment in the arterial lesions.

A range of biochemical and structural problems are involved in developing an understanding of disease states. For example, an understanding of atherosclerosis requires a characterization at the molecular level of normal and atherosclerotic arteries and of the structural and metabolic alterations that convert a normal artery into an atherosclerotic artery. In recent years much insight has been gained into the architecture of atherosclerotic lesions. Gross morphological differences between normal and atherosclerotic arteries of various species have been determined by electron microscopy (Bowyer et al., 1977; Veress et al., 1977; Haust, 1977; Minick et al., 1977; Gerrity et al., 1979; Weber et al., 1973). Transport of lipoproteins into the arterial wall (Hollander et al., 1977; Walton & Morris, 1977) and the abundance of arterial smooth muscle cells (Thomas et al., 1977 a,b; Stary, 1977; Holle et al., 1977) and of arterial glycosaminoglycans (Schneider et al., 1977; Toledo & Mourao, 1979) are all strongly affected by atherosclerotic complications.

Atheroslcerotic lesions are characterized by the presence of large quantities of extracellular, lipoprotein-derived cholesterol and cholesteryl esters in the intima and media of the arterial wall (Smith, 1974; VanGent & Eineis, 1977). Various investigators have studied the structure of these lipid deposits (Engelman & Hillman, 1976; Small & Shipley, 1974; Hamilton et al., 1979; Katz & Small, 1980). Engelman & Hillman (1976) have detected sharp, reversible thermotropic order–disorder transitions in human aortic samples containing fatty streak lesions. Transition temperatures ranged between 28 and 42 °C. The ordered state exhibited a sharp reflection corresponding to a Bragg spacing of about 35 Å, which is consistent with the presence of smectic[1] cholesteryl ester domains. No evidence was found for similar order–disorder transitions in normal aortae. Physical and chemical characterization of particles released from human atherosclerotic aortae by chemical methods reveal structural similarities with very low density lipoprotein (VLDL)[2] and low-density lipo-

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[1] The smectic liquid-crystalline state of cholesteryl esters is believed to consist of planar arrays stacked with a repeat distance of 36 Å. In the cholesteric liquid-crystalline state cholesteryl ester molecules are ordered in helices about an axis at right angles to the long molecular axis.

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proteins (LDL) (Hoff et al., 1979). A small number of very large particles have also been detected (Hoff et al., 1979; Hollander et al., 1979a,b). A peroxidase conjugate with anti-apoB (apoB is the major protein constituent of human serum LDL and VLDL) has been used to visualize apoB in human atherosclerotic aortae by electron microscopy (Walton & Morris, 1977). Numerous apoB-containing particles in the size range of LDL and VLDL were detected. These observations suggest that intact LDL and VLDL particles are abundant in human atherosclerotic lesions.

Animal models have been used extensively to study the genesis and progression of atherosclerosis (Clarkson, 1963). The rabbit has been particularly useful, since marked hypercholesterolemia can be induced within a few days by supplementing rabbit chow diets with cholesterol (Ross & Zilversmit, 1977; Shore et al., 1974; Camejo et al., 1973). The increase in serum cholesterol (mainly cholesteryl esters) occurs primarily in particles that float in the VLDL density range ($d \leq 1.006$ g/mL) and in the LDL density range ($1.006 \leq d \leq 1.063$ g/mL). Ross & Zilversmit (1977) have shown that these particles are primarily chylomicron remnants. The composition of these VLDL and LDL fractions differs markedly from those of normal rabbit VLDL and LDL. They are larger, enriched in cholesteryl esters, and contain less protein than their normal counterpart (Shore et al., 1974). Both VLDL and LDL from cholesterol-fed rabbits contain little triglyceride. In addition, both classes of abnormal lipoproteins are enriched in apoE relative to normal rabbit lipoproteins (Rodriguez et al., 1976). A further result of diet-induced hypercholesterolemia is the rapid formation of aortic atherosclerotic lesions. In the early stages, diet-induced aortic lesions are characterized by foam cell accumulation under an intact endothelium. With long-term cholesterol-fat feeding, fibrous material, smooth muscle cells, and both intra- and extracellular lipids are found in lesions (Clarkson, 1963).

Fourier transform nuclear magnetic resonance spectroscopy has been used extensively to probe the molecular organization and dynamics of lipid molecules in serum lipoproteins (Hamilton et al., 1979, 1973, 1974, 1976; Assman et al., 1975; Avila et al., 1978; Hamilton & Cordes, 1978; Hauser & Kostner, 1979; Henderson et al., 1975; Yeagle et al., 1978; Sears et al., 1976; Kroon, 1981; Kroon & Krieger, 1981) and in neat lipid mixtures (Sears et al., 1976; Hamilton et al., 1977). ^{13}C NMR has been particularly useful because of its high spectral resolution. The resolution is such that individual resonances can be observed for each of the major lipoprotein lipid classes: cholesterol, cholesteryl esters, triglycerides, and phospholipids. We report here the results of a ^{13}C NMR investigation of the molecular dynamics of lipids in atherosclerotic lesions and cholesteryl ester rich lipoproteins from cholesterol-fed rabbits. A previous report by Hamilton et al. (1979) described a ^{13}C NMR investigation of human atherosclerotic lesions; similarities and differences between ^{13}C NMR results for the spontaneous human lesions and experimentally induced rabbit lesions will be discussed.

Experimental Procedures

^{13}C spectra were obtained at 6.34 T (67.89 MHz) on a Bruker HX270 spectrometer that was interfaced to a Nicolet 1080 minicomputer and operated in the Fourier transform mode with quadrature detection. At temperatures above 34 °C, the probe temperature was controlled by varying the flow rate of external air through the probe and/or by using a Varian variable temperature controller. At temperatures below 32 °C, the temperature was controlled by circulating nitrogen gas from a liquid nitrogen tank through a copper coil in a Dewar filled with a mixture of salt and ice. Protons were decoupled by using a phase-modulated broad-band proton decoupler (Grutzner & Santini, 1975) at 3–4 W of power, with an effective decoupling range of about 2 ppm on either side of the center frequency. Each spectrum was obtained with decoupling centered in the aromatic and aliphatic proton regions, respectively, in order to obtain meaningful line widths. Integrated intensities were determined by weighing cut-out traces of individual resonances on an analytical balance. Line widths of selected resonances are given as the width at half-height. They contain a contribution of approximately 3 Hz from inhomogeneity and drift. Spin–lattice relaxation times (T_1's) were determined by the fast inversion recovery method (Sass & Ziessow, 1977). Peak heights were hand measured from plotted spectra, and values of T_1 were calculated by using a nonlinear least-squares fit to the three parameter exponential function $I(\tau) = A - B\exp(-\tau/T_1)$ (Kowalewski et al., 1977), in which $I(\tau)$ is the intensity for a delay time τ. The least-squares analysis uses A, B, and T_1 as adjustable parameters. Detailed accumulation conditions for various ^{13}C NMR runs are described in legends and footnotes of figures and tables throughout this paper.

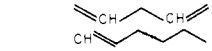
Table I: Percent Composition (w/w) of CR-VLDL and CR-LDL

	VLDL	LDL
protein	8.2	13.7
phospholipid	20.4	19.2
cholesterol	19.7	11.5
cholesteryl ester	49.3	55.1
triglyceride	2.5	0.4

Male New Zealand white rabbits were purchased from HARE (Hewitt, NJ). Experimental rabbits were fed a Purina rabbit chow diet supplemented with cholesterol (0.5% w/w) and corn oil (10% v/w). Control rabbits were fed a Purina rabbit chow. The animals were fed ad libitum. Rabbits were bled by cardiac puncture following overnight fasting. EDTA at a final concentration of 4 mM was used as an anticoagulant. Blood cells were removed by centrifugation at 2000g for 20 min. Lipoproteins were isolated by ultracentrifugal flotation with a Ti 60 rotor in a Beckman L5 centrifuge at 15 °C. VLDL ($d < 1.006$ g/mL) was isolated by centrifugation at native density for 17 h at 55000 rpm; LDL ($1.006 < d < 1.063$ g/mL) was isolated by centrifugation at $d = 1.063$ g/mL for 17 h at 60000 rpm. The plasma density was adjusted by adding an appropriate volume of a $d = 1.4$ g/mL KBr solution that contained 90 mg/mL NaCl. Each lipoprotein fraction was recentrifuged once at the same density. Fractions were collected with a Beckman tube slicer. Each of the samples was dialyzed exhaustively against a 0.15 M NaCl solution containing EDTA (0.01% w/v) and NaN_3 (0.02% w/v). Rabbit aortas were removed after sacrificing the animals in an atmosphere of CO_2. The animals had been maintained on the experimental diet for approximately 3 months. The aortic adventitia was stripped from the intima media, with a dissecting microscope. ^{13}C NMR spectra of an intact aorta at 41 °C showed sharp resonance from triglycerides, cholesteryl esters, and the choline head group of phospholipids. After the stripping, adventitial sections gave prominent resonances from triglycerides only; the stripped intima media gave essentially

[2] Abbreviations: VLDL, very low density lipoproteins; LDL, low-density lipoproteins; CR-VLDL, cholesteryl ester rich very low density lipoproteins; CR-LDL, cholesteryl ester rich low-density lipoproteins; ^{13}C NMR, carbon-13 nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

Table II: Chemical Shifts, Line Widths, and Spin-Lattice Relaxation Times of Selected ^{13}C Resonances at 42 ± 2 °C

peak no.[a]	assignment[b]	chemical shift[c] (ppm from Me_4Si)	line width (Hz)			T_1 (s)[d]		
			VLDL	LDL	aortic lesion	VLDL	LDL	aortic lesion
1	CE C18	12.0	16	19	17	0.74	0.70	0.6
2	FA CH_3	14.1	9	10	10	2.7	2.8	2.3
3		25.7				0.46	0.46	
4		27.3				0.32	0.39	0.32
5	CE C25	28.1	9	10	14	0.60	0.69	0.58
6	FA-$(CH_2)_n$-	29.5, 29.8				0.43	0.45	0.40
7	CH_2-CH_2-CH_3	32.1	10	13	13			
8	FA-CH_2-CO-	34.4	14	17	20	0.24	0.20	0.23
9	CE C9	50.1	48	50	46	0.29	0.18	0.22
10	choline $N(CH_3)_3^+$	54.2	10	12	27	0.64	0.80	0.69
11	CE C14, C17	56.8				0.23	0.21	0.19
12	choline CH_2O	59.6						
13	choline CH_2N	66.2						
14	C C3	*e*						
15	CE C3	73.1	54	50	51	0.23	0.18	0.21
16	C C6	120.9						
17	CE C6	122.3	22	21	26	0.16	0.16	0.19
18		128.0	6	12	13	0.67	0.49	0.57
19		129.7	13	16	18	0.52	0.51	0.52
20	CE C5	139.8						
21	>C=O	171.2						

[a] The peak number corresponds to that in Figure 1. [b] Abbreviations: CE, cholesteryl ester; C, cholesterol; FA, fatty acyl. The sterol numbering system is shown in Figure 1. [c] Chemical shifts for CR-VLDL, CR-LDL, and lesions are the same within experimental error. Precision of chemical shifts is ±0.1 ppm. [d] Standard errors for the acyl terminal methyl T_1 values are ±20%. Standard errors for all other resonances are ≤9%. [e] Not determined because of its large line width.

all the intensity from cholesteryl esters and phospholipids. Spectra of aortic sections and lipoproteins were run in a 10 mM phosphate–0.15 M NaCl buffer at pH 7.2, which contained 0.02% EDTA and 0.01% NaN_3. Aortic lesions from four rabbits were studied by ^{13}C NMR spectroscopy; each of these specimens gave similar results.

Lipoprotein concentrations were determined gravimetrically after exhaustive dialysis against distilled water, which was followed by lyophilization. Protein concentrations were determined by the procedure of Lowry et al. (1951). The triglyceride content of lipoproteins was analyzed with commercial reagents (Worthington Diagnostics). The lipoprotein cholesterol and cholesteryl ester content was measured by gas chromatography. For the determination of total cholesterol, 50-μL aliquots of lipoprotein were saponified in 500 μL of 0.625 N ethanolic potassium hydroxide, with heating at 80 °C for 30 min. The solution was dried down under nitrogen and the residue suspended in 500 μL of water. The sample was extracted with 2 mL of chloroform–methanol (2:1) and centrifuged to separate the aqueous and organic phases. The lower phase was washed 3 times with 1.5 mL of pure upper phase solvent (Folch et al., 1951) and evaporated under nitrogen. The residue was dissolved in chloroform for analysis by gas chromatography. For the determination of unesterified cholesterol, a 50-μL aliquot of lipoprotein was extracted with chloroform–methanol (2:1). Following the addition of 500 μL of water, the samples were centrifuged to separate the aqueous and organic phases. The bottom phase was washed once with pure upper solvent and evaporated under nitrogen. The residue was dissolved in chloroform for analysis by gas chromatography. Stigmasterol was added to the lipoprotein samples as an internal standard. A Varian Series 2100 model gas chromatograph, equipped with a flame ionization detector, and a CDS 111 integrator were used in these analyses. Sterols were separated at 260 °C on a 6-ft (2-mm i.d.) glass column packed with 3% OV-17 on 100–200-mesh Gas-Chrom Q.

For fatty acid analyses total lipids were extracted by the method of Bligh & Dyer (1959). Arterial lesions were homogenized in water with a polytron homogenizer prior to solvent extraction. Cholesteryl esters were isolated by silicic acid (Unisil, Williamsburg, PA) chromatography by using hexane with increasing (0–2%) diethyl ether as an eluant. The elution was monitored by TLC using hexane–diethyl ether–glacial acetic acid (70:30:1 v/v/v) as a solvent. The cholesteryl ester mixtures gave a single spot by TLC with the above solvent. The cholesteryl esters were transesterified in 0.5 N sodium methoxide in methanol at 80 °C for 20 min. The extracted fatty acid methyl esters were identified by gas chromatography on a 6-ft (2-mm i.d.) glass column filled with 10% SP-2330 cyanosilicone (Supelco). A linear temperature program from 160 to 260 °C at 10 °C/min was used. Methyl esters were identified by using standards obtained from Applied Science.

Results and Discussion

Comparison of ^{13}C Spectra. Male white New Zealand rabbits fed a diet supplemented with cholesterol and corn oil show a dramatic increase in total serum cholesterol levels from an average of 50 to 2000 mg/dL. The chemical compositions of the lipoprotein particles responsible for the increased total cholesterol, primarily as cholesteryl esters, are given in Table I. Figure 1 displays spectra for these cholesteryl ester rich lipoproteins and for rabbit aortic lesion-laden intima media. Table II lists assignments and chemical shifts for selected peaks in the lipoprotein and lesion spectra. All three spectra are highly resolved at the indicated temperatures and contain numerous resonances from the steroid nucleus and C17 side chain of cholesteryl esters and from fatty acyl chains of cholesteryl esters and phospholipids. Aortic intima-media sections from control rabbits give no high-resolution ^{13}C NMR spectra.[3]

Several features of the spectra are noteworthy. Two broad resonances in the LDL spectrum (peaks 14 and 16) can be assigned, on the basis of their chemical shifts (Hamilton & Cordes, 1978), to the C3 and C6 carbons, respectively, of unesterified cholesterol. A similar but less intense resonance for C6 of cholesterol can be observed in the lesion spectrum.

[3] D. Quinn, unpublished results.

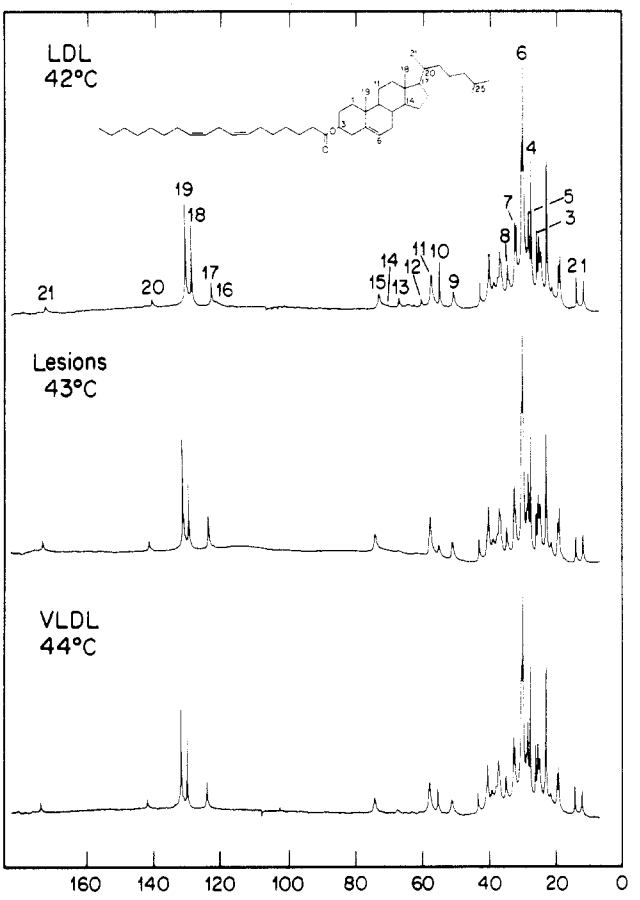


FIGURE 1: Comparison of ^{13}C NMR spectra of rabbit cholesteryl ester rich lipoproteins and aortic atherosclerotic lesions; spectra were taken at 6.34 T (67.89 MHz) with quadrature detection in 0.1 M sodium chloride and 10 mM sodium phosphate, pH 7.25, at the indicated temperatures. The lesion sample consisted of 1.4 g (blotted dry weight) of aortic intima-media strips, suspended in buffer to a total volume of 4 mL. The lipoprotein samples each contained 4 mL of VLDL (125 mg/mL) or LDL (50 mg/mL). Spectral parameters were as follows: spectral width, 13 888.89 Hz (204.6 ppm); pulse interval, 0.6 s; number of data points in the free induction decay, 8192; LDL and VLDL spectra, 16 384 accumulations; lesion spectra, 32 768 accumulations. Spectra are composites of individual spectra taken with proton decoupling centered at 2.0 ppm (to decouple carbon resonances 1–13) and at 5.0 ppm (for resonances 14–21). A digital broadening of 3 Hz was applied to each spectrum to enhance signal to noise. Peak numbers refer to the peak numbers in Table II. Peak assignments in Table II refer to the atomic positions for cholesteryl linoleate as shown in the inset.

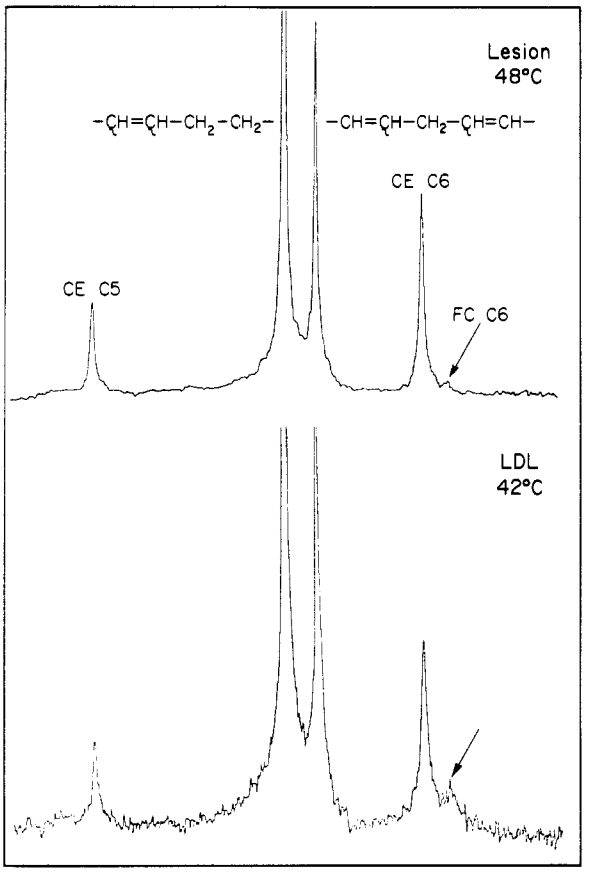


FIGURE 2: Olefinic regions of ^{13}C NMR spectra of rabbit aortic atherosclerotic lesions and cholesteryl ester rich LDL. Spectral parameters were as described in the legend of Figure 1.

Figure 2 gives an expanded view of the olefinic region of spectra from rabbit aortic lesions and cholesteryl ester rich LDL (CR-LDL) in which the C6 resonance of free cholesterol is also apparent. The widths of these resonances are consistent with cholesterol molecules that are intercalated between phospholipid molecules in the surface region of the particle. Such an arrangement has been proposed for serum lipoproteins (Shen et al., 1977).

Figure 3 compares the choline head group regions of each of the spectra. Though the line widths of cholesteryl ester resonances of each sample are essentially equal, the choline methyl resonances of lipoproteins and lesions are markedly different (refer also to Table II). The choline methyl resonance of lesions is about 2.5 times broader than that of the lipoproteins. A similar trend in the relative line widths of the choline methyl resonance of human LDL and human aortic fibrous plaques was noted by Hamilton et al. (1979). The increased width of the choline methyl resonance in spectra of the aortic lesions could be due to a greater chemical shift heterogeneity and/or to change in the mobility of the choline methyl groups. For example, phospholipid head groups may occupy different environments in the aortic lesions, giving rise to nonequivalent chemical shifts. Alternatively the motion of head groups may be lower and more restricted in lesions than in lipoproteins. London et al. (1979) have demonstrated that the choline methyl line width of sonicated phosphatidylcholine vesicles contains a contribution from ^{13}C–^{14}N coupling (J_{CN} = 4.1 Hz). While the contributions of this coupling to lipoprotein and lesion choline methyl line widths are not known, a comparison with the results for dipalmitoylphosphatidylcholine vesicles and for Chinese hamster ovary cells grown in the presence of ^{13}C-labeled choline indicates that these contributions are too small to account for the difference in line width between the lesion and lipoprotein choline methyl resonances. At 50 °C, the choline methyl line width of sonicated dipalmitoylphosphatidylcholine vesicles is 4.8 Hz with ^{1}H decoupling and 3.4 Hz with both ^{1}H and ^{14}N decoupling, a difference of 1.4 Hz (London et al., 1979). The difference for Chinese hamster ovary cells, at 20 °C, is 6 Hz (London et al., 1979). We surmise that both the lipoprotein and lesion choline methyl resonances contain a contribution from unresolved ^{13}C–^{14}N coupling but that this coupling plays a relatively minor role in the absolute difference in line width between these resonances. The possibility that particle tumbling could play a role in modulating the choline methyl line width was also considered. However, the observation by Curatolo et al. (1977) that the ^{13}C-labeled choline methyl line width of freely tumbling glycolipid containing phospholipid vesicles did not change appreciably upon lectin agglutination rules out this possibility. This does not imply that no change in the overall particle tumbling rate takes place. On the contrary, since no high-resolution ^{31}P spectrum is observed for aortic lesions while relatively sharp resonances were observed for the lipoproteins[3]

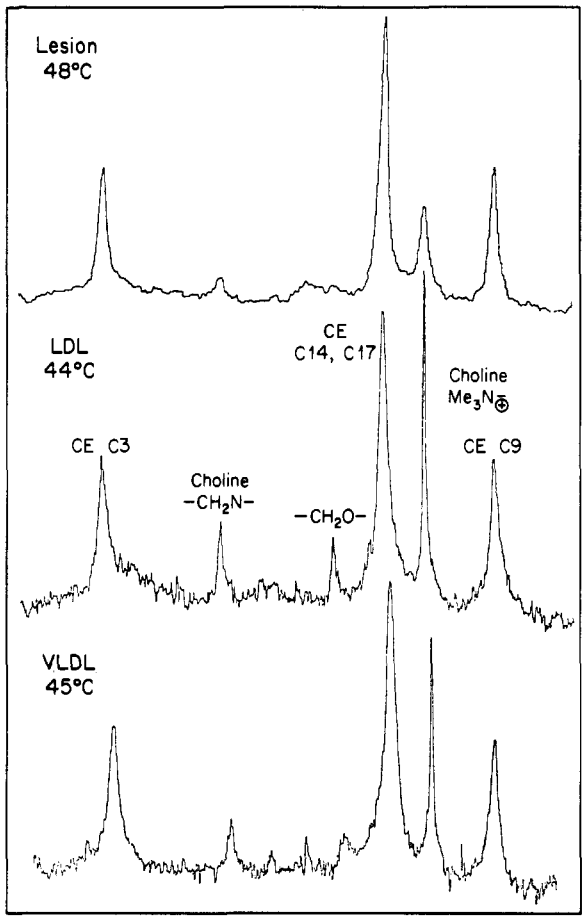


FIGURE 3: Comparison of the heteroatom regions of ^{13}C NMR spectra of rabbit cholesteryl ester rich lipoproteins and aortic atherosclerotic lesions. Spectral parameters were as described in the legend of Figure 1.

and since the ^{31}P line widths for particles of the size of lipoproteins are known to depend on tumbling rates (De Kruijff, 1978), we can conclude that the phospholipid-containing entities within the aorta tumble at a much slower rate than the lipoproteins. On the basis of the foregoing discussion, either chemical shift nonequivalence or damped local motion must be responsible for the broader choline methyl resonance. In view of the marked field dependence of the lesion choline methyl line width (10 Hz at 2.35 T and 27 Hz at 6.34 T, data not shown), we surmise that chemical shift nonequivalence is at least in part responsible for the increased choline methyl line width.

The widths of ^{13}C resonances from cholesteryl esters of rabbit aortic lesions and cholesteryl ester rich lipoproteins are very sensitive to temperature, as illustrated in Figure 4 for rabbit aortic lesions. At 48 °C resonances from the steroid nucleus of cholesteryl esters and from fatty acyl chains are sharp and well resolved. When the temperature is lowered, a progressive broadening of resonances from the steroid rings takes place, such that no steroid carbon resonances are detected at 31 °C. A similar temperature dependence is observed for cholesteryl ester rich VLDL (CR-VLDL) and CR-LDL. On the other hand, while resonances from the fatty acyl chains and the choline methyl groups also broaden as the temperature is lowered, they are still observed at the lowest temperatures studied.

Morrisett et al. (1980) reported a similar temperature dependence of ^{13}C NMR spectra at 2.35 T of CR-VLDL from cholesterol-fed rabbits, though the temperature at which their cholesteryl ester steroid resonances broaden into the base line was ~10 °C higher than the temperature we show here (cf.

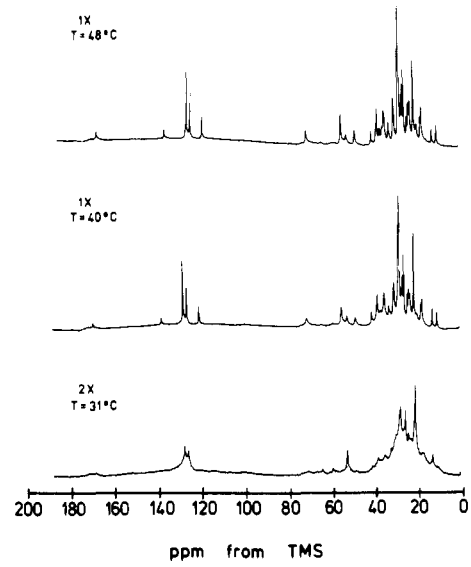


FIGURE 4: ^{13}C NMR spectra of rabbit aortic atherosclerotic lesions at 48, 40, and 31 °C. Spectral parameters were as described in the legend of Figure 1. The vertical scale expansion of the spectrum at 31 °C is twice that of the spectra at 48 and 40 °C.

Figure 5). The reason for the difference in these temperatures is not clear at present.

One question that arises in studies of order–disorder transitions by NMR is what fraction of individual resonances are actually observed at each temperature. To answer this, we obtained integrated intensities of the C3 and C6 resonances at 50 and 34 °C for lesions and CR-VLDL. The results show that at least 74% of the intensity present at 50 °C is observed at 34 °C, suggesting that the measured line widths reflect the motional state of the majority of the cholesteryl ester molecules.

Abrupt temperature-dependent changes of ^{13}C NMR spectra of neat cholesteryl esters, similar to the changes noted here, have been reported by Hamilton et al. (1977). In each case, the observed temperature dependence was consistent with the presence of a phase transition for the cholesteryl esters from a disordered liquid state at high temperature to a more ordered state as the temperature was lowered. Similar conclusions have been drawn from proton NMR studies (Kroon, 1981).[4] An analysis of the methylene spectral amplitudes as a function of temperature gave a phase transition temperature of 38–47 °C for CR-VLDL.[4] From the temperature dependence observed here for the VLDL ^{13}C resonances (Figure 5B), it appears that the most prominent ^{13}C line broadening takes place at the lower end of the phase transition as determined by proton NMR.

The fatty acyl resonances that are resolved at temperatures where most of the steroid resonances have broadened beyond detection can be attributed to fluid phospholipid fatty acyl chains. This is consistent with the continued resolution of the choline methyl resonance at low temperatures, which suggests that the phospholipids surrounding the cholesteryl ester rich core remain relatively mobile over the temperature range studied. Proton NMR results support this view.[4]

Figure 5 shows the temperature dependence of the line widths of selected carbon resonances of lesions and of CR-VLDL. These data and the line widths and spin–lattice relaxation times in Table II support the idea that in all three systems studied the molecular organization and dynamics of cholesteryl esters are qualitatively similar. This observation

[4] P. A. Kroon and J. Seidenberg, unpublished results.

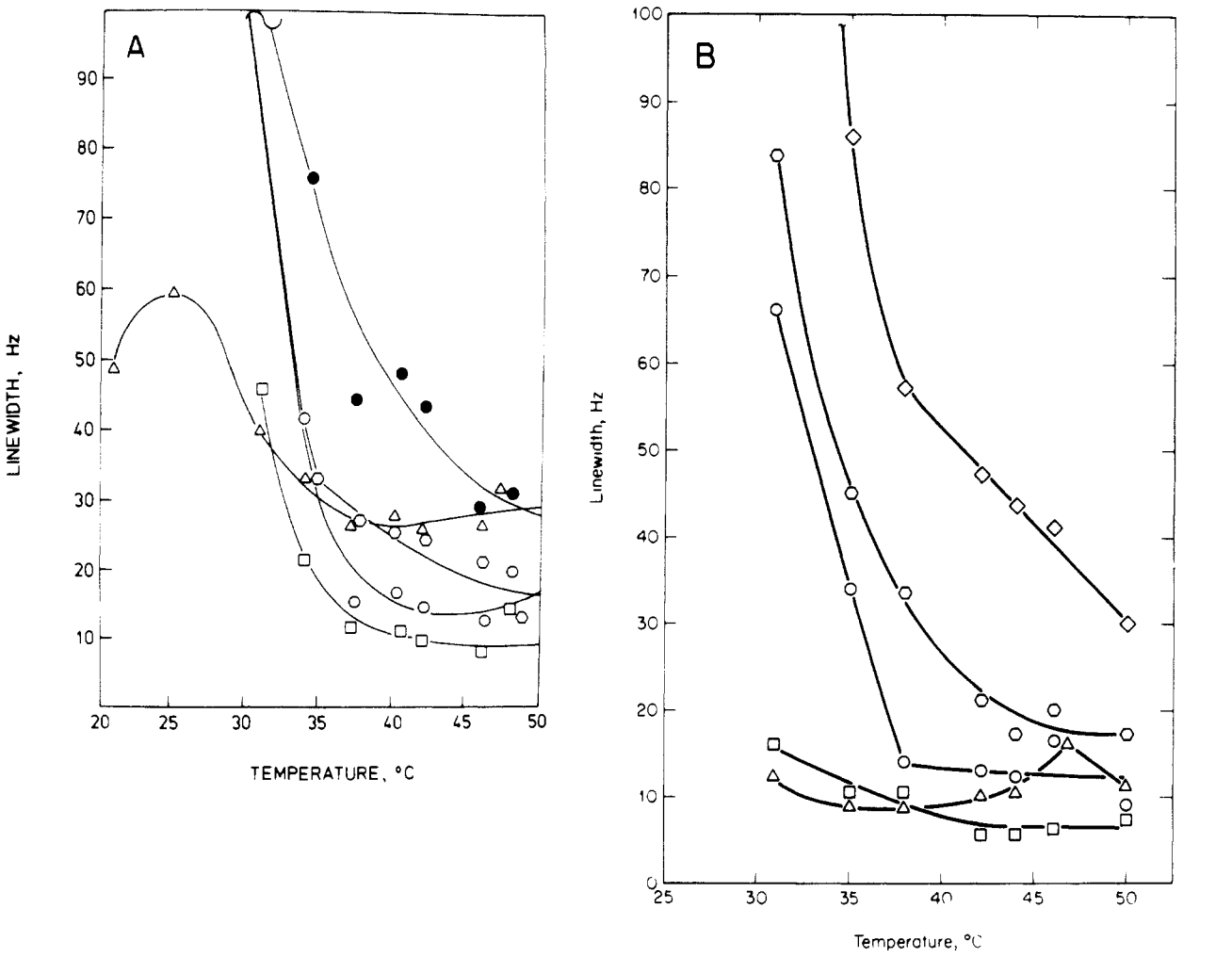


FIGURE 5: Temperature dependence of the line widths of various resonances of (A) rabbit aortic lesions and (B) CR-VLDL. For the rabbit lesions in (A), results are shown for the cholesteryl ester C6 (hexagon), C18 (○), and C9 (●), the fatty acyl terminal methyl (□), and the choline methyl resonance (△). For CR-VLDL, results are shown for the cholesteryl ester C6 (hexagon), C18 (○), and C3 (◇), the $=CHCH_2CH=$ (□), and the choline methyl resonance (△).

Table III: Fatty Acid Composition of Cholesteryl Esters[a]

ester	CR-VLDL	CR lesions	ester	CR-VLDL	CR lesions
14:0	0.5		20:1	0.5	2.5
16:0	13.0	7.0	22:0	0.3	1.0
16:1	8.0	2.0	22:1	0.5	1.8
18.0	2.5	2.0	24:0	0.5	
18:1	36.0	44.5	24:1	0.5	
18:2	35.7	32.8			

[a] Percentage of total.

suggests that an appreciable fraction of the arterial cholesteryl esters is derived from nonmetabolized lipoproteins. However, since there are quantitative differences between the CR-VLDL and lesion cholesteryl ester fatty acyl chain compositions (Table III), some metabolic modification of the cholesteryl esters does take place. This view is consistent with the recent results of Stender & Zilversmit (1981), which show that 18% of esterified cholesterol taken up by the intima media of cholesterol-fed rabbits over a 6-h period is hydrolyzed in the intima media.

Molecular Dynamics of Cholesteryl Esters. Figure 6 gives spin–lattice relaxation times (nT_1's) and line widths for various cholesteryl ester carbons of CR-VLDL. Cholesteryl oleate is used for illustrative purposes, since it is relatively abundant in lipoproteins from cholesterol-fed rabbits and in aortic lesions. There are several trends in nT_1's and line widths of Figure 6

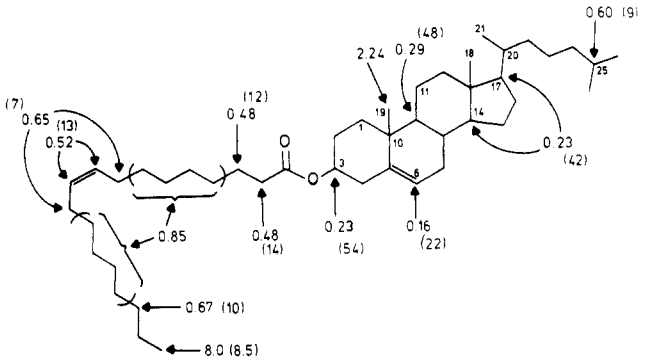


FIGURE 6: nT_1's and line widths (in parentheses) for various carbon atom resonances of cholesterol esters of CR-VLDL determined at 42 ± 2 °C.

that deserve comment. The cholesteryl ester ring carbons have broader lines and shorter spin–lattice relaxation times than carbons from the C18 methyl group, the fatty acyl chain, or the steroid C17 side chain. This is not unexpected, since the steroid ring is rigid, precluding internal motions of appreciable amplitude. The steroid C17 side chain and the fatty acyl chain, on the other hand, have a large number of accessible conformations. Nevertheless, the motion of the side and fatty acyl chain methylene group is not isotropic. Isotropic motion described by a single correlation time would predict that $T_1 = T_2$ [where $T_2 = 1/(\pi \times \text{line width})$], if T_1 increases with increasing temperature (Kainosho et al., 1978). This is not

Table IV

	line width (Hz)		C3/C6 ratio	calculated values[a]		
	C3	C6		$10^7\tau_{RX}$ (s)	$10^9\tau_{RZ}$ (s)	τ_{RX}/τ_{RZ}
CR-VLDL, 42 °C	51	19	2.7	2.4	3.2	75
CR-LDL, 42 °C	47	18	2.6	2.2	3.0	73
lesions, 42 °C	48	23	2.1	2.2	4.0	55
CR-VLDL, 38 °C	54	30	1.8	3.0	5.8	38
CR-LDL, 38 °C	65	28	2.3	3.0	5.0	60
lesions, 38 °C	58	29	2.0	2.5	5.5	45

[a] The error arising from estimated line-width uncertainties is ±8% for both τ_{RX} and τ_{RZ}.

observed here. While T_1 does increase with temperature,[3] the data in Figure 6 clearly show that $T_1 \neq T_2$.

Line widths and nT_1's are relatively constant along the fatty acyl and steroid C17 side chains. Thus the motions that contribute to T_1 and to the line widths do not vary a great deal along the chain. The extra degrees of freedom available to the side chains vs. the steroid rings must be such that all side-chain methylene C–H vectors undergo reorientation at approximately equal rates and amplitudies. One candidate to meet these stipulations is "kink diffusion", wherein the β-coupled gauche isomerizations provide the dominant T_1 relaxation mechanism for fatty acyl protons in phospholipid membranes (Gent & Prestegard, 1977; Kainosho et al., 1978). These observations are consistent with an extended conformation for the cholesteryl ester molecules, like that found for cholesteryl myristate in the liquid state (Burks & Engelman, 1981).

Quantitative Assessment of Rotational Diffusion of Cholesteryl Esters. C3 and C6 line widths have been calculated as a function of the rotational correlation times of the cholesteryl esters. Two possible conformations have been proposed for cholesteryl esters; one of these is extended and the other U-shaped. The U-shaped form is believed to predominate in bilayer phases (Gorrissen et al., 1981). An extended form has been used to explain X-ray diffraction studies of crystalline cholesteryl myristate (Craven & DeTitta, 1976) and of liquid-crystalline low-density lipoprotein cholesteryl esters (Atkinson et al., 1977). More recent neutron diffraction studies of selectively deuterated cholesteryl myristate (Burks & Engelman, 1981) have shown that cholesteryl myristate is extended in the liquid as well as in liquid-crystalline states. We have therefore used a model in which the cholesteryl ester molecules exist in an extended conformation and in which they undergo anisotropic but unrestricted motion. The extended conformation is described as a cylindrical ellipse, with correlation times τ_{RZ} about its long axis and τ_{RX} about its short axis. The long axis of the ellipse was taken to be collinear with the C3–C13 internuclear vector. Atomic coordinates of 17-bromoheptadecanoate (Abrahamsson & Dahlen, 1977) were used to define the structure. Coordinates for hydrogen atoms were calculated by using a bond length of 1.094 Å and tetrahedral geometry for aliphatic hydrogens and a bond length of 1.083 Å and trigonal geometry for olefinic hydrogens. The treatment of Woessner (1962), modified to account for heteronuclear interactions (Doddrell et al., 1972), was used to calculate relaxation times. Table IV gives line widths for C6 and C3 at 38 and 42 °C for rabbit cholesteryl ester rich lipoproteins and aortic lesions, as well as calculated values of the rotational correlation times of the steroid ring system based on these line widths. The ratio of the calculated correlation times, τ_{RX}/τ_{RZ}, is a measure of the anisotropy of rotational diffusion of the steroid rings of cholesterol esters. The calculated ratios (τ_{RX}/τ_{RZ}) shown in Table IV indicate that the rotational motion of the cholesteryl esters of all three systems is highly anisotropic at 38 and 42 °C. Similar calculations for neat cholesteryl ester samples indicate that the motion of the steroid rings is also highly anisotropic.[3] By use of calculated correlation times at 42 °C, spin–lattice relaxation times of C3 and C6 were calculated and are compared to experimental values in Table V.

Table V

	measured T_1 (s)[a]		predicted T_1 (s)[b]	
	C3	C6	C3	C6
CR-VLDL	0.23	0.16	0.23	0.28
CR-LDL	0.18	0.16	0.22	0.26
lesions	0.21	0.19	0.27	0.33

[a] Determined at 42 ± 2 °C; uncertainty in T_1 is ⩽±10%. [b] Generated from computed τ_{RX}, τ_{RZ} pairs of Table IV.

When the calculated correlation times of Table IV are used to generate predictions for spin–lattice relaxation times of C3 and C6, agreement between theory and experiment for C3 is quite good. The corresponding comparison for C6 shows agreement that is not quite as good. This may be due to the high sensitivity of projected calculated values of T_1 to τ_{RZ}, the rotational correlation time about the long axis.[3] Alternatively, the motion of the cholesteryl ester molecules may not be completely unrestricted at 42 °C. As we noted earlier, proton NMR studies show the existence of an order–disorder transition for VLDL from cholesterol-fed rabbits between 47 and 38 °C.[4] Thus at 42 °C the motion of the cholesteryl ester molecules may be such that rotations about the short axes are somewhat restricted as a result of the formation of a cholesteric or smectic-like phase. In either case, rotation about the long axis is less likely to be affected.

The high degree of rotational anisotropy of the cholesteryl ester molecule observed for CR-VLDL, CR-LDL, and aortic lesions reflects the nature of the environment of the cholesteryl ester molecules. The average C3/C6 line-width ratio for the cholesteryl ester rich lipoproteins and lesions is 2.0–2.5, whereas in human VLDL this ratio is 1.2 ± 0.1 (Hamilton et al., 1976). This differences can be attributed to the relative amounts of cholesteryl esters and triglycerides in these specimens: rabbit cholesteryl ester rich lipoproteins contain only a few percent of triglyceride (cf. Table I), while human VLDL contains more than 50% by weight of triglyceride. Similar results were found in ^{13}C NMR studies of neat cholesteryl esters and cholesteryl ester–triglyceride mixtures (Hamilton et al., 1977). In the liquid phase, within 15 °C of the isotropic–cholesteric phase transition of cholesteryl linoleate, the line-width ratio of C3/C6 at 2.35 T was ≥2.0 for cholesteryl linoleate, cholesteryl oleate, and a mixture of the two esters (Hamilton et al., 1977). However, a sample containing 58% cholesteryl linoleate, 20% cholesteryl oleate, and 22% triolein (w/w/w) gave a C3/C6 line-width ratio of ~1.3–1.6 at temperatures between 35 and 51 °C. Thus the presence of increasing amounts of triglyceride results in a decrease in the motional anisotropy for cholesteryl esters, either as neat lipids or within lipoprotein particles.

Unlike lipoproteins from cholesterol-fed rabbits, lipoproteins from normal chow-fed rabbits do not undergo order–disorder transitions between 0 and 50 °C and therefore have a relatively

fluid core at physiological temperatures.[4] Furthermore these rabbits do not develop atherosclerosis. This observation raises the possibility that the ordered CR-VLDL and CR-LDL cores are causally related to the formation of arterial lesions. There are several ways in which the physical state of the lipoprotein core may alter the ultimate fate of its constituents. For example, the conformation of the surface apoproteins may be affected by the physical state of the core, so that interaction with arterial lipoprotein receptors, leading to uptake, or with arterial surface components, leading to a deposition of intact lipoproteins, is enhanced. The ordered core cholesteryl esters may also be more resistant to degradation by the lysosomal acid lipase, following internalization by arterial cells as a result of the limited accessibility of the enzyme to the ester linkage in the smectic phase (Peters & DeDuve, 1974).

A comparison of the C3 line widths for human and rabbit lesions shows that substantial differences exist between them. At 37 °C the C3 line width for human lesions is 53 Hz (Hamilton et al., 1977), while this line width is not attained in rabbit lesions until a temperature of 42 °C is reached. Inasmuch as the C3 line width is representative of the mobility of the cholesteryl esters, these results suggest that the rabbit cholesteryl ester deposits are substantially less mobile and more ordered than the corresponding human deposits. As we noted above for lysosomal degradation, a limited accessibility of enzymes to the ester linkage in cholesteryl ester deposits in arterial lesions could severely restrict the removal of such deposits. The high degree of ordering of the rabbit cholesteryl ester deposits may therefore enhance their persistence in vivo.

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Cellular and Enzymic Synthesis of Sphingomyelin†

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ABSTRACT: The synthesis of sphingomyelin was studied in baby hamster kidney cells and in subcellular fractions derived from rat liver. During pulse–chase experiments with [^{3}H]choline in tissue culture cells, the specific radioactivity of sphingomyelin continued to increase after the specific activities of phosphocholine and cytidine 5′-diphosphate choline (CDP-choline) had declined by a factor of 10. The addition of [^{3}H]methionine to cells that were grown in 1 mM dimethylethanolamine efficiently radiolabeled phosphatidylcholine (by methylation of phosphatidyldimethylethanolamine) and sphingomyelin but not phosphocholine or CDP-choline. Thus, the proximal donor of the phosphocholine moiety of sphingomyelin was not CDP-choline but probably phosphatidylcholine. These in vivo results prompted investigation of the enzymic synthesis using phosphatidyl[^{3}H]choline or [^{3}H]ceramide as substrates. With both substrates the subcellular fraction with the highest specific enzyme activity was the plasma membrane. When phosphatidyl[^{3}H]choline was used as the substrate, phospholipid exchange proteins were included in the reaction to effect the transfer of the labeled phospholipid from liposomes into the membrane bilayer in which the enzyme resided. Under these conditions the synthesis of sphingomyelin was almost completely dependent upon the addition of phospholipid exchange proteins. When [^{3}H]ceramide was used as the substrate, the addition of detergents was necessary for sphingomyelin synthesis. The use of phospholipid exchange proteins to introduce lipid substrates to membrane-bound enzymes may have much broader applicability.

An important problem concerning the synthesis of sphingomyelin has been the identification of the proximal donor of the phosphocholine moiety of this lipid. The initial studies of sphingomyelin synthesis in cell-free systems (Sribney & Kennedy, 1958) suggested that the final step of the synthetic scheme was the transfer of phosphocholine from cytidine 5′-diphosphate choline (CDP-choline) to ceramide. The physiological relevance of this observation was difficult to evaluate because the ceramide species most active as substrate possessed the threo configuration, while ceramides and sphingolipids isolated from tissues possessed the erythro configuration (Carter et al., 1956). Although Fujino et al. (1968) reported assay conditions for the synthesis of *erythro*-sphingomyelin from CDP-choline, other investigators (Ullman & Radin, 1974; van Golde et al., 1974) have been unable to reproduce these results. A study by Diringer et al. (1972) with SV-40 transformed cells suggested that the source of the phosphocholine moiety of sphingomyelin was phosphatidylcholine. However, in these experiments neither CDP-choline nor phosphocholine levels were measured. Ullman & Radin (1974) provided enzymic evidence that the phosphocholine moiety of sphingomyelin could be derived from phosphatidylcholine, but others have reported an inability to duplicate this work (Stoffel & Melzner, 1980). In the present study both cellular and enzymic approaches were used to identify the source of the phosphocholine moiety of sphingomyelin. Precursor–product analyses of tissue culture cells labeled with [^{3}H]choline and [^{3}H]methionine provided strong evidence that CDP-choline was not the immediate source of the phosphocholine moiety of sphingomyelin. The results further implicated phosphatidylcholine as the donor in vivo. The in vivo results were coupled to measurements of enzyme activities in well-defined subcellular fractions from rat liver. The enzyme phosphatidylcholine:ceramide phosphocholinetransferase was localized in the plasma membrane.

Materials and Methods

Tissue Culture. Baby hamster kidney (BHK 21) cells (ATCC CCL10) were grown in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (100

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