Structure and Motion of Phospholipids in Human Plasma Lipoproteins. A ^{31}P NMR Study†

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ABSTRACT: The structure and motion of phospholipids in human plasma lipoproteins have been studied by using ^{31}P NMR. Lateral diffusion coefficients, D_T, obtained from the viscosity dependence of the ^{31}P NMR line widths, were obtained for very low density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoproteins (HDL_2, HDL_3), and egg PC/TO microemulsions at 25 °C, for VLDL at 40 °C, and for LDL at 45 °C. At 25 °C, the rate of lateral diffusion in LDL ($D_T = 1.4 \times 10^{-9}$ cm^2/s) is an order of magnitude slower than in the HDLs ($D_T = 2 \times 10^{-8}$ cm^2/s). At 45 °C, D_T for LDL increases to 1.1×10^{-8} cm^2/s. In contrast, D_T for VLDL increases only slightly going from 25 to 40 °C. The large increase in diffusion rate observed in LDL occurs over the same temperature range as the smectic to disordered phase transition of the core cholesteryl esters, and provides evidence for direct interactions between the monolayer and core. In order to prove the orientation and/or order of the phospholipid head-group, estimates of the residual chemical shift anistropy, $\Delta\sigma$, have been obtained for all the lipoproteins and the microemulsions from the viscosity and field dependence of the ^{31}P NMR line widths. For VLDL and LDL, the anisotropy is 47–50 ppm at 25 °C, in agreement with data from phospholipid bilayers. For the HDLs, however, significantly larger values of 69–75 ppm (HDL_2) and >120 ppm (HDL_3) were obtained. These results suggest differences in the orientation and/or ordering of the head-group in the HDLs. The dynamic behavior of the phosphate moiety in LDL and HDL_3 has been obtained from the temperature dependence of the ^{31}P spin–lattice relaxation rates. Values of the correlation time for phosphate group reorientation and the activation energy for the motion are nearly identical in LDL and HDL_3 and are similar to values obtained for phospholipid bilayers. This argues against long-lived protein–lipid interactions being the source of either the slow diffusion in LDL or the altered head-group orientation in the HDLs.

The human plasma lipoproteins are key components in the regulation of lipid transport within the circulatory system, and in the development of many diseased states (Dolphin, 1985; Brown & Goldstein, 1986; Mahley & Innerarity, 1983; Lusis, 1988). Lipoproteins share a common structure in which hydrophobic lipids (cholesteryl esters, triglycerides, and some free cholesterol) are partitioned into the core of the particle, which is surrounded by a monolayer of phospholipids, cholesterol, and protein. The metabolic roles of lipoproteins are determined primarily by the apoproteins whose functions include activation of plasma enzymes and receptor binding; however, recent studies suggest that the state of the lipids is also of fundamental importance. For example, modulation of the core triglyceride content of low-density lipoprotein (LDL)[1] (Aviram et al., 1988a,b) or depletion of phospholipids (Kleinman et al., 1988) has been shown to alter the conformation of apo B on the particle surface, causing significant changes in the interaction with cell-surface LDL receptors. An understanding of how the biological roles of lipoproteins are related to their structure will require knowledge not only of the location and average orientation of the constituent proteins and lipids but also of their types and rates of motions. Of all the techniques currently available, nuclear magnetic resonance is uniquely suited to address questions of structure and motion in anisotropic environments. In the application to membranes, ^2H and ^{31}P NMR in particular have proven to be extremely powerful methods for extracting information on orientation and ordering, since anisotropic motions only partially average the quadrupolar and chemical shielding tensors, respectively (Seelig, 1977, 1978). Furthermore, the rates of motion are accessible through measurement of relaxation times.

NMR has been extensively applied to the study of the lipid components of plasma lipoproteins [for a review, see Hamilton and Morrissett (1986)]. Early studies involving ^{13}C, ^1H, and ^{31}P NMR yielded useful qualitative information regarding the location and motional freedom of the lipid molecules. Recently, our laboratory has obtained both static and dynamic information on lipids located in both the monolayer and core regions of human lipoproteins using ^2H and ^{31}P NMR. Through the use of selectively deuterated lipids, details on acyl chain ordering and spin–lattice relaxation rates have been obtained for the surfaces of VLDL (Chana et al., 1990), HDL_2 (Parmar, 1985), and HDL_3 (Parmar et al., 1985), for the surface and core regions of LDL (Treleaven et al., 1986; Chana et al., 1990), and for the surface and core of reconstituted HDL (Fenske et al., 1988; Parmar et al., 1983). These studies revealed the presence of two domains of phospholipid in VLDL and two domains of both phospholipid and cholesteryl ester in LDL in which the organization differs considerably, one

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[1] Abbreviations: VLDL, very low density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; QELS, quasi-elastic light scattering; PC, phosphatidylcholine; TO, triolein; SPM, sphingomyelin; DPPC, 1,2-dipalmitoylphosphatidylcholine; DOPC, 1,2-dioleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PE, phosphatidylethanolamine.

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domain having extremely low order (approaching isotropic) while the other posesses order higher than found in phospholipid bilayers. In contrast, HDL_2 and HDL_3 contain only a single domain of phospholipid and a single domain of cholesteryl ester, which is more ordered than in VLDL or LDL. ^2H NMR has also been used to demonstrate the presence of core–monolayer interactions in reconstituted HDL (Fenske et al., 1988). Recently, we have used ^{31}P NMR to obtain the lateral diffusion coefficients of phospholipids in VLDL, LDL, HDL_2, and HDL_3, and observed that, at 25 °C, the rate of lateral diffusion in LDL is a full order of magnitude slower than in the other lipoproteins (Cushley et al., 1987). Any model of structure–function relationships in lipoproteins must account for these differences in lipid ordering and dynamics.

In the current study, we extend the application of ^{31}P NMR to the determination of the structural and motional properties of phospholipid head-groups in plasma lipoproteins. We utilize the viscosity and field dependence of the ^{31}P NMR line widths to measure lateral diffusion and the residual chemical shift anisotropy. Further, we report the temperature dependence of the ^{31}P spin–lattice relaxation rates in LDL and HDL_3, and use the model of Seelig et al. (1981) to obtain the correlation times for phosphate group reorientation, and the activation energies associated with the motion.

Materials and Methods

Egg yolk phosphatidylcholine (egg PC) was isolated from fresh hen egg yolks as described by Singleton et al. (1965) and purified as described by Richter et al. (1977). Triolein was purchased from Sigma Chemical Co. and used without further purification. Aquacide (molecular weight 70000) was purchased from Calbiochem.

Isolation of Lipoproteins. Fresh human citrated plasma (<3 days old) obtained from the Canadian Red Cross was centrifuged in a Ti 50.2 rotor for 18–20 h at 42000 rpm and 5 °C. The top fraction was collected and stored under N_2 in the presence of NaN_3 at 5 °C. Typically, the density <1.006 g/mL fractions (from several units of plasma) were pooled and dialyzed exhaustively against 0.15 M NaCl, 0.02% NaN_3, and 2.0 mM Na_2EDTA, pH 7.4. The pooled fraction was recentrifuged for 18–20 h at 42000 rpm in order to remove any trace amounts of LDL. To remove chylomicrons, the top fraction was isolated and subjected to a further 30-min centrifugation in a Ti 60 rotor at 42000 rpm at 5 °C with the brake off during deceleration. The bottom three-fourths fraction of the VLDL sample was isolated from the centrifuge tube and subjected to a further 20-min centrifugation in order to remove large VLDL particles.

LDL (1.025–1.063 g/mL), HDL_2 (1.063–1.125 g/mL), and HDL_3 (1.125–1.21 g/mL) were isolated by sequential ultracentrifugation between the salt densities shown by the addition of solid KBr or of a saturated solution of KBr (Havel et al., 1955). For LDL and HDL_3, several units of each lipoprotein were pooled and purified by reisolation in the same density range. All centrifugations were performed on a Beckman L5-75 ultracentrifuge at 42000 rpm in a Ti 50.2 or Ti 60 rotor for 22–24 h between 4 and 5 °C.

Protein was determined by the method of Lowry et al. (1951) as modified by Kashyap et al. (1980). Phospholipid was determined by the method of Ames (1966). Cholesteryl esters and cholesterol were determined enzymatically using Boehringer Mannheim clinical test kits.

Prior to NMR experiments, VLDL was dialyzed into 0.15 M NaCl/2 mM Na_2EDTA, pH 7.4, LDL and HDL_3 into 0.15 M NaCl/0.02% Na_2EDTA, pH 7.5, and HDL_2 into deuterium-depleted water. When necessary, the lipoproteins were concentrated by ultracentrifugal flotation (VLDL and HDL_3), by treatment with Aquacide (LDL), or by use of Millipore Immersible-CX Ultrafilters (CX-30) (LDL, HDL_2). For the measurement of T_1 values of LDL and HDL_3 below 0 °C, the lipoproteins were dialzyed into 3 M NaCl/0.02% Na_2EDTA, pH 7.5.

The egg PC/TO microemulsions were prepared as described by Tajima et al. (1983). Briefly, egg PC and TO (1:2.3 w/w) were codissolved in chloroform, the solvent was removed under a stream of N_2, and the lipids were further dried under high vacuum overnight. The dry lipids were dispersed into 0.15 M NaCl in ^2H_2O at pD 7.4. The samples were sonicated under N_2 for 65 min with 5–10-min pulses followed by 1–2-min cooling periods, using a Heat Sytems W-375 sonicator operating at a power level of 65–75 W. During sonication, cold water was continuously run through the sonicating vessel jacket. The temperature (40–45 °C) was monitored by inserting a thermocouple directly into the sonication suspension. The resulting solution was centrifuged on a clinical desktop centrifuge for 15 min to remove titanium particles, followed by ultracentrifugation for 20 h at 42000 rpm at 5 °C using a Ti 75 rotor. The egg PC/TO microemulsions formed a clear gel at the top of the centrifuge tube. The gel was resuspended in 0.15 M NaCl and subjected to an additional spin of 15 min at 15000 rpm using a Ti 75 rotor, with the brake off during deceleration. The bottom 80% of the sample was isolated and examined for vesicle contamination by ^1H NMR. The choline *N*-methyl proton resonance was monitored by using the paramagnetic ions Mn^{2+}, Eu^{3+}, and Pr^{3+}. Prior to the lateral diffusion study, the microemulsions were concentrated with Aquacide.

NMR Spectroscopy. The ^{31}P NMR experiments were performed at 102.2 MHz, without proton decoupling, using a home-built spectrometer and a 5.9-T Nalorac superconducting magnet interfaced to a Nicolet BNC-12 computer. Temperatures were controlled by a solid-state temperature controller with an accuracy of ±0.25 °C built by the Simon Fraser University electronics shop. High-resolution spectra were obtained by using a one-pulse sequence with phase alternation in order to minimize base-line distortion. The HDL_2 spectra were obtained by using a spinning probe while all other spectra were obtained on a broad-band probe with a horizontally mounted coil. The samples were allowed to equilibrate for 30 min at a given temperature before data were acquired. The spectral parameters are given in the figure legends.

The ^{31}P NMR spectra of VLDL and egg PC/TO microemulsions in the presence of increasing concentrations of glycerol were analyzed by using an iterative four-parameter (rms base line, chemical shift, amplitude, and line width) least-squares fit of the PC resonance to a single Lorentzian function. The ^{31}P NMR spectra of LDL and HDL_3 in the presence of increasing concentrations of glycerol were analyzed as above using either a five-parameter fit of the PC and SPM resonances to single Lorentzian functions or a four-parameter fit of the PC resonance to a single Lorentzian function. The latter was necessary for higher viscosities where the two resonances could not be resolved. For the five-parameter fit, the chemical shift between PC and SPM was constrained at 0.6 ppm, and the line widths of the two lipids were constrained to be equal. The input intensities of PC and SPM were determined from high-resolution NMR (Cushley et al., 1987).

^{31}P spin–lattice relaxation times, T_1, were measured at 102.2 MHz by the inversion–recovery method $(180°-\tau-90°-T)_n$. T_1 were determined from an iterative least-squares fit of signal intensities, I, to an exponential of the form $I_\tau = A + B \exp$-

($-\tau/T_1$). The number of delay times (τ) varied from 7 to 16. The indicated errors are the standard deviations obtained from the least-squares fit.

The double-reciprocal plots in Figures 2 and 3 and the plots of ($\Delta\nu_{1/2} - C$) vs ν_0^2 in Figure 4 were analyzed by using a weighted least-squares routine courtesy of Dr. Ian Gay, Department of Chemistry, Simon Fraser University. The uncertainties in the values of D_T obtained from eq 6 were estimated from the standard deviations of the slope and intercept obtained from the weighted least-squares analysis.

Particle Sizes. Mean diameters of VLDL (36.2 nm), LDL (24.0 nm), and the egg PC/TO microemulsions (48.3 nm) were determined by using a Nicomp Model 270 submicrometer particle sizer from repeated measurements (10–30) on the same sample. The mean diameter for HDL_3 (8.1 nm) was determined previously by negative-staining electron microscopy using a Philips EM 300 electron microscope at 80 kV (Fenske et al., 1988). The mean diameter of HDL_2 (10 nm) was obtained from the literature (Pownall & Gotto, 1983; Scanu, 1979).

Theory

The ^{31}P NMR line width, $\Delta\nu_{1/2}$, of phospholipids in lipoproteins can be expressed in terms of the effective correlation time for phospholipid reorientation, τ_e, and the residual second moment, M_2, by (Abragam, 1961; McLaughlin et al., 1975)

$$\pi\Delta\nu_{1/2} = M_2\tau_e + C \quad (1)$$

where C is a constant (15 ± 3 Hz) indicating the τ_e-independent portion of the line width. The correlation time τ_e includes contributions from particle tumbling and phospholipid lateral diffusion, and is given by

$$1/\tau_e = 1/\tau_t + 1/\tau_d \quad (2)$$

where τ_t and τ_d are

$$\tau_t = 4\pi\eta R^3/3kT \quad (3)$$

$$\tau_d = R^2/6D_T \quad (4)$$

where η is the solvent viscosity, R is the particle radius, and D_T is the lateral diffusion coefficient for phospholipids in the lipoprotein monolayer. The residual second moment M_2 is given by (McLaughlin et al., 1975)

$$M_2 = (4/45)(2\pi\nu_0)^2(\Delta\sigma)^2 \quad (5)$$

where $\Delta\sigma$ is the residual chemical shift anisotropy and ν_0 is the ^{31}P absorption frequency. Equations 1–4 can be combined to give

$$(\Delta\nu_{1/2} - C)^{-1} = \eta^{-1}(3kT/4R^3M_2) + 6\pi D_T/R^2M_2 \quad (6)$$

from which both D_T and M_2, therefore $\Delta\sigma$, can be obtained from a plot of $(\Delta\nu_{1/2} - C)^{-1}$ vs η^{-1}. Equation 6 was listed incorrectly in our previous paper (Cushley et al., 1987), but the diffusion coefficients we reported were correct. $\Delta\sigma$ is also obtained from a plot of ($\Delta\nu_{1/2} - C$) versus ν_0^2.

^{31}P spin–lattice relaxation occurs primarily via dipole–dipole (T_{1D}) and chemical shift anisotropy (T_{1CSA}) mechanisms. In the present case, both mechanisms are significant. The total relaxation rate is given by (Seelig et al., 1981; Ghosh, 1988)

$$1/T_1 = 1/T_{1D} + 1/T_{1CSA} \quad (7)$$

with

$$1/T_{1D} = K[J(\omega_H - \omega_P) + 3J(\omega_P) + 6J(\omega_H + \omega_P)] \quad (8)$$

$$1/T_{1CSA} = \alpha(\Delta\sigma)^2\omega_P^2J(\omega_P) \quad (9)$$

where ω_H and ω_P are the ^1H and ^{31}P Larmor frequencies, respectively, and K and α are empirical constants. The spectral densities, $J(\omega)$, are given by

$$J(\omega) = \tau_c/(1 + \omega^2\tau_c^2) \quad (10)$$

where the motion responsible for relaxation is described by a single effective correlation time τ_c which is assumed to obey an Arrhenius relationship with temperature

$$\tau_c = \tau_0 \exp(E_a/RT) \quad (11)$$

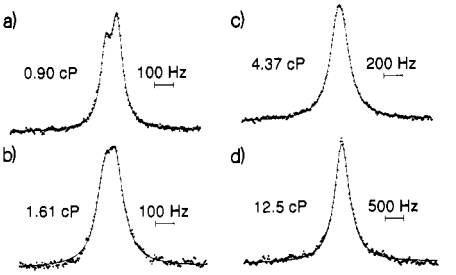

FIGURE 1: Representative ^{31}P NMR spectra of LDL in the presence of increasing concentrations of glycerol at 25 °C. The solid line represents an iterative least-squares fit of two (a–c) and one (d) Lorentzian function(s) to the data points (crosses). The concentrations of glycerol (weight percent) were: 0 (a), approximately 21 (b), 47 (c), and 65 (d). Spectral parameters: pulse width = 5 μs (60°); sweep width = 10 kHz (a, b), 20 kHz (c), 50 kHz (d); number of acquisitions = 50 000 (a), 8606 (b), 40 000 (c, d); data sets = 2K, zero-filled to 4K; delay between pulses = 1.5 s; delay before acquisition = 10 ms; LB = 3 Hz (a), 5 Hz (b), 10 Hz (c), 40 Hz (d).

Results

Phospholipid Lateral Diffusion in Lipoproteins and Microemulsions. ^{31}P NMR spectra of VLDL, LDL, HDL_2, HDL_3, and egg PC/TO microemulsions were obtained at 25 °C in the presence of increasing concentrations of glycerol. In general, solvent viscosities varied between 0.9 and 47 cP, corresponding to glycerol concentrations up to 80% by weight. Representative spectra of LDL are shown in Figure 1. Further spectra may be found in Chana (1989), Fenske (1988), and Parmar (1985). Spectra of VLDL and LDL as a function of glycerol concentration were also obtained at 40 and 45 °C, respectively (spectra not shown). In all cases, the increase in solvent viscosity caused a broadening of the phospholipid resonances and a loss of resolution of the PC and SPM peaks. In the large VLDL particles, the SPM signal is not resolved. The line widths of the PC and SPM resonances were obtained from computer fits of the spectra to Lorentzian functions (see Materials and Methods). Lateral diffusion coefficients D_T, obtained from plots of $(\Delta\nu_{1/2} - C)^{-1}$ vs η^{-1} (Figures 2 and 3), are listed in Table I. The given D_T values are for both PC and SPM, since both resonances were fit where they could be resolved. However, since PC is the dominant phospholipid, it would be difficult to determine subtle differences between the two.

In order to test whether glycerol was inducing any permanent changes in lipoprotein structure, several controls were performed. LDL was incubated in 50 wt % glycerol for 22 h at 25 °C, following which time the glycerol was removed by exhaustive dialysis. Both the ^{31}P NMR line widths and mean diameters obtained from QELS were the same within experimental error before and after glycerol treatment. Similar experiments were performed with VLDL and HDL_2. In addition, HDL_2 line widths were monitored for several samples at different viscosities, and the broadening induced by glycerol was found to be fully reversible.

Chemical Shift Anisotropy of the Phospholipid Head-Group in Lipoproteins. The residual chemical shift anisotropy of the phosphate moiety was calculated from both the viscosity

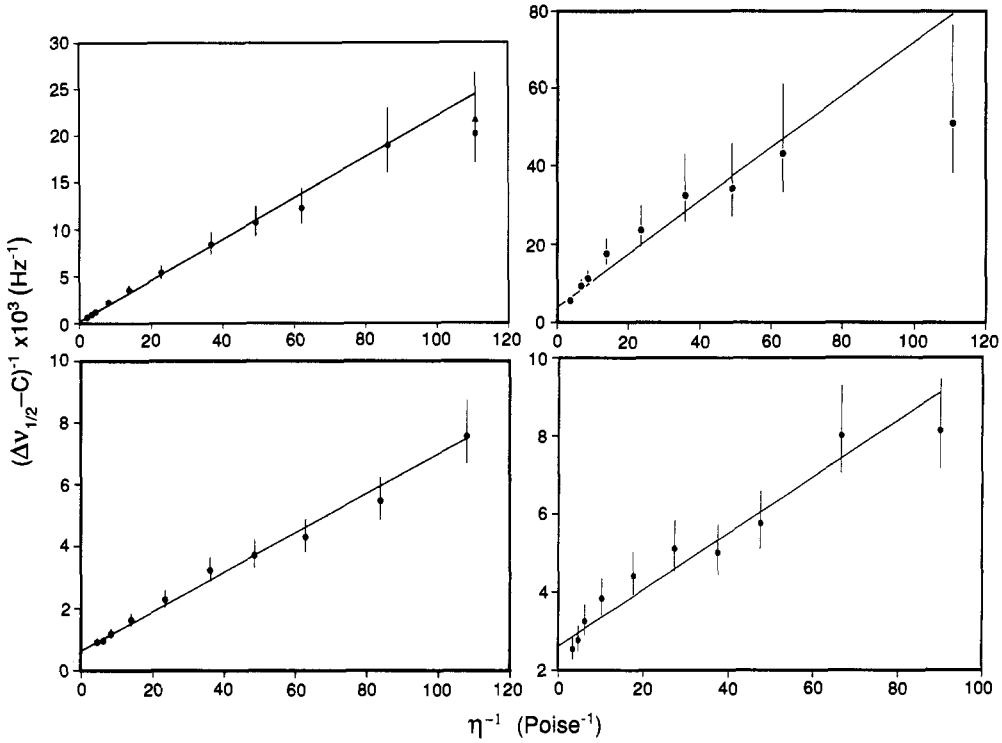

FIGURE 2: Plot of $(\Delta\nu_{1/2} - C)^{-1}$ vs η^{-1} for the ^{31}P resonances of LDL (top left), HDL_3 (top right), VLDL (bottom left), and egg PC/TO microemulsions (bottom right) at 25 °C. The straight lines are weighted least-squares fits to the data points.

Table I: Lateral Diffusion Coefficient, D_T, and Chemical Shift Anisotropy, $\Delta\sigma$, of Phospholipids in Human Plasma Lipoproteins As Determined by ^{31}P NMR

	T (°C)	D_T (cm^2/s)	$\Delta\sigma$ (ppm)[a]	
VLDL	25	$(9.1 \pm 1.0) \times 10^{-9}$	47 ± 1	46 ± 1
	40	$(1.2 \pm 0.2) \times 10^{-8}$	42 ± 1	
LDL	25	$(1.4 \pm 0.5) \times 10^{-9}$	49 ± 1	50 ± 4
	45	$(1.1 \pm 0.2) \times 10^{-8}$	52 ± 2	
HDL_2	25	$(1.8 \pm 0.3) \times 10^{-8}$	75 ± 10	69 ± 10
HDL_3	25	$(2.3 \pm 0.8) \times 10^{-8}$	136 ± 11	156 ± 30
egg PC/TO microemulsions	25	$(2.5 \pm 0.3) \times 10^{-8}$	28 ± 1	

[a] The $\Delta\sigma$ values in the left and right columns were obtained from the viscosity and field dependence of the ^{31}P NMR line widths, respectively.

and field dependence of the ^{31}P NMR line widths (Table I). Plots of $(\Delta\nu_{1/2} - C)$ vs ν_0^2 are shown in Figure 4 for spectra acquired at 40.5, 102.2, and 161.8 MHz, and the values of $\Delta\sigma$ thus obtained are also listed in Table I. The $\Delta\sigma$ values obtained by the two methods are in excellent agreement, which further suggests that glycerol does not affect head-group orientation. As an independent verification of the $\Delta\sigma$ values obtained for VLDL, LDL, and HDL_3, we have obtained ^{31}P NMR spectra of these lipoproteins in samples where particle tumbling has been reduced to the point where "powder-like" line shapes are observed (Figure 5). In the case of VLDL, flotation of a concentrated sample in the ultracentrifuge gave a gel at the tube surface, which was then examined by NMR at low temperatures (−5 °C). LDL gel, with the consistency of a viscous paste, was formed by ultracentrifugation. HDL_3 gel could not be formed by ultracentrifugation; however, the particle was concentrated from a 200 mM sucrose solution (initial concentration), until a viscous syrup was obtained. The ^{31}P spectrum for HDL_3–sucrose complexes at −20 °C is shown in Figure 5c. The spectral line shapes for VLDL (Figure 5a) and LDL (Figure 5b) were simulated assuming an axially symmetric shielding tensor motionally narrowed by isotropic Brownian diffusion, as described by Burnell et al. (1980). The spectra could only be simulated by using a value of $\Delta\sigma$ of approximately 50 ppm, in agreement with the values obtained here. The spectrum of HDL_3 (Figure 5c) has a line width >103 ppm.

Spin–Lattice Relaxation in LDL and HDL_3. ^{31}P T_1 values were obtained at 102.2 MHz, without proton decoupling, for LDL and HDL_3, as a function of temperature over the range −10 to 45 °C. The T_1 values of LDL are slightly shorter than those of HDL_3 at all temperatures. The variation of T_1 with temperature is nonlinear, and a minimum is observed in the range 3–7 °C. A semilog plot of T_1 versus $1000/T$ is shown in Figure 6. The curves were generated from eq 7–11. In cases where the PC and SPM resonances were resolved, the data points represent the values for PC; however, the values for SPM were very similar.

Discussion

^{31}P NMR has been used to study orientation and dynamics of phospholipids in human plasma lipoproteins. Motions of the phospholipid molecule as a whole have been characterized via the lateral diffusion coefficients, D_T, while information on the mean orientation and dynamics of the phosphate group derives from the chemical shift anisotropy, $\Delta\sigma$, and the temperature dependence of spin–lattice relaxation, respectively.

Phospholipid Lateral Diffusion in Lipoproteins. Inspection of Table I reveals significant differences between the various classes of lipoproteins. The rate of lateral diffusion D_T in the HDLs and in egg PC/TO microemulsions at 25 °C [$(1.8–2.5) \times 10^{-8}$ cm^2/s] is similar to that observed in egg PC unilamellar vesicles [2.6×10^{-8} cm^2/s (Cullis, 1976)]. Diffusion in VLDL is significantly slower at the same temperature (9.1×10^{-9} cm^2/s), which we attribute to the higher concentration of cholesterol in the VLDL monolayer. VLDL has 7 wt % cholesterol whereas HDL_2 has 3.6% and HDL_3 only 1.9% (Dolphin, 1985), which corresponds to mole percent values (relative to phospholipid) of 43%, 20%, and 16%, respectively, assuming that all of the cholesterol is in the monolayer. Cullis (1976) has shown that cholesterol reduces D_T in phospholipid

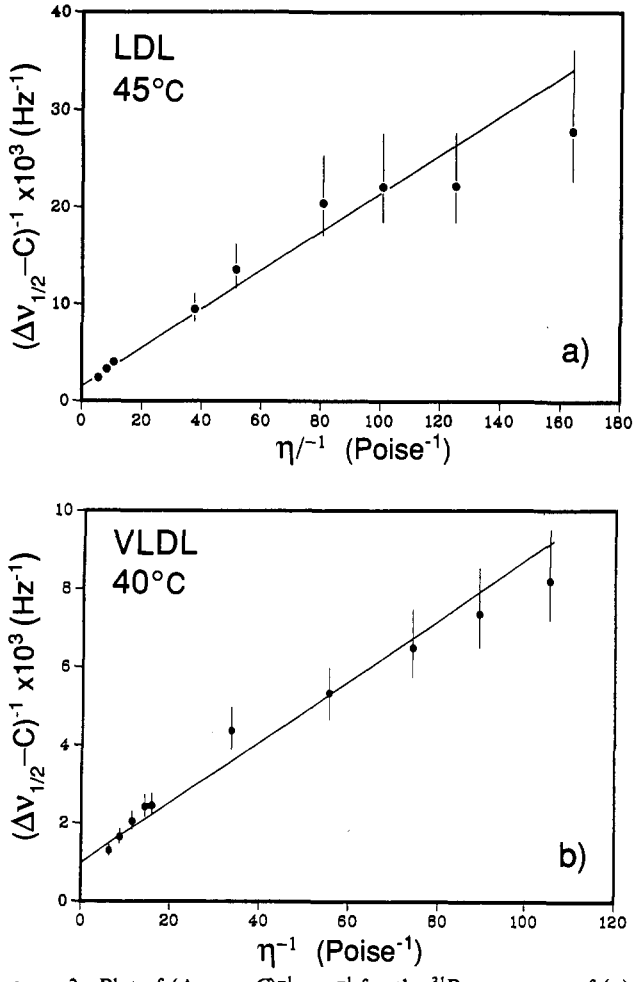

FIGURE 3: Plot of $(\Delta\nu_{1/2} - C)^{-1}$ vs η^{-1} for the ^{31}P resonances of (a) LDL at 45 °C. For ths sample of LDL, the mean radius was 11.8 nm, (b) VLDL at 40 °C. The straight lines are weighted least-squares fits to the data points.

unilamellar vesicles. Of particular interest is the value of D_T measured for LDL at 25 °C (1.4×10^{-9} cm^2/s), which is more than an order of magnitude slower than observed in the HDLs or in model systems in the liquid-crystalline phase. In a previous paper, we considered three possible explanations for the slow diffusion observed in LDL, these being (1) the higher SPM content of the LDL monolayer, (2) core–monolayer interactions, and (3) interactions of the phospholipid with apo B-100 (Cushley et al., 1987). Recently, Ibdah et al. (1989) demonstrated that LDL surface lipids form a more close-packed monolayer than those of HDL_3 due to the higher content of saturated PC and cholesterol in LDL. This effect can be discounted as the cause of the reduced diffusion in LDL since it is known that above T_m phospholipid diffusion is similar in both DPPC and egg PC bilayers (Cullis, 1976). As VLDL has a similar cholesterol and saturated fatty acid content as LDL (Scanu, 1979), its monolayer should be equally condensed; hence, further mechanisms must be responsible for the slow diffusion in LDL. Diffusion in the LDL monolayer is 6.5 times slower than in VLDL at 25 °C.

There is no precident for suggesting that phospholipid–apo B-100 interactions are responsible for the reduced diffusion in LDL. ^{2}H NMR reviews of lipid–protein interactions list no cases of immobilized head-groups in the presence of proteins (Watts, 1987). However, Yeagle et al. (1977), using ^{31}P NMR intensity measurements, found that approximately 20% of the phospholipid in LDL was immobilized, and not observable in the high-resolution spectrum. Similar results have been observed by Finer et al. (1975) and Lund-Katz and Phillips (1986) using ^{1}H and ^{13}C NMR, respectively. We have been unable to find any evidence for broad ^{31}P resonances in either VLDL or LDL over a 50-kHz sweep width, nor was there any evidence of a broad resonance in the free induction decay pattern. This is supported by spectral simulations. LDL with $\Delta\sigma$ = 150 ppm ("immobilized" phospholipid), motionally narrowed by isotropic rotation ($\tau_{rot} = 1.57 \times 10^{-6}$ s), should give a spectrum with a line width of 500 Hz, which would be easily observable. The possibility of protein–lipid interactions affecting phospholipid diffusion therefore seems remote.

On the basis of our results, the most likely source of the slow diffusion of LDL phospholipids is interactions with the core lipids. The rationale for considering core–monolayer interactions as a potential modulator of phospholipid diffusion is that in the range of 20–40 °C the core esters of LDL undergo a liquid-crystalline to liquid phase transition (Deckelbaum et al., 1977). In the more ordered state, interactions with the monolayer phospholipids would reduce the rate of lateral diffusion. Increases in phospholipid diffusion rates of an order of magnitude have been observed for the gel to liquid-crystalline phase transition of DPPC vesicles (Cullis, 1976). LDL is the only lipoprotein where the state of the core can undergo such large changes. LDL has 42.3 wt % cholesteryl ester (CE) and 5.1 wt % triglyceride (TG) in its core (Dolphin, 1985), giving a CE/TG ratio of 8.3. For VLDL, the CE/TG ratio is 0.2 (Dolphin, 1985). The core lipids of VLDL, being mostly triglycerides, are fluid at 25 °C. Both HDLs have CE/TG ratios of 7.5, but in the HDL core, there are not enough ester molecules to form a separate ordered phase (Phillips, 1977; Tall, 1980). Assuming an effect of the more ordered core on the surface organization in LDL, the rate of phospholipid diffusion should increase significantly at temperatures above the ester phase transition. This was shown in Figure 3 where values of D_T were obtained for VLDL at 40 °C and for LDL at 45 °C, giving similar values for both of approximately 1 $\times 10^{-8}$ cm^2/s (Table I). The value for VLDL is only slightly higher than at 25 °C, whereas for LDL the increase is nearly an order of magnitude. The results provide strong evidence that interactions between the core and monolayer of LDL occur and can modulate phospholipid diffusion.

Chemical Shift Anisotropy in Lipoproteins. The residual chemical shift anisotropy $\Delta\sigma$ provides a measure of the orientation and/or order of the phosphate group at the particle surface. Because $\Delta\sigma$ is dependent on two order parameters which relate the axes of the principle coordinate system to the axis of motional averaging (Seelig, 1978), it is generally used in a qualitative manner for comparison purposes. In phospholipid bilayers, values of $\Delta\sigma$ range between 40 and 50 ppm (Seelig, 1978). In lipoproteins, the shielding anisotropy is averaged by rapid particle tumbling, resulting in narrow Lorentzian lines. Nevertheless, the value of $\Delta\sigma$ can be extracted from both the viscosity and field dependence of the ^{31}P NMR line widths (see Theory). Initially, we consider the values obtained for LDL, VLDL, and egg PC/TO microemulsions (Table I). Both VLDL and LDL give similar values of 47 and 50 ppm, respectively, at 25 °C, in excellent agreement with the data from bilayers in the liquid-crystalline phase. At higher temperatures, $\Delta\sigma$ of VLDL is slightly smaller, while that of LDL remains the same. In phospholipid bilayers, $\Delta\sigma$ is insensitive to temperature in the liquid-crystalline phase (Seelig, 1978; Seelig et al., 1981; Ghosh, 1988). The results suggest that the orientation and/or order of phospholipids in VLDL and LDL is similar to that of bilayers. The $\Delta\sigma$ of egg PC/TO microemulsions at 25 °C (28 ppm) is significantly

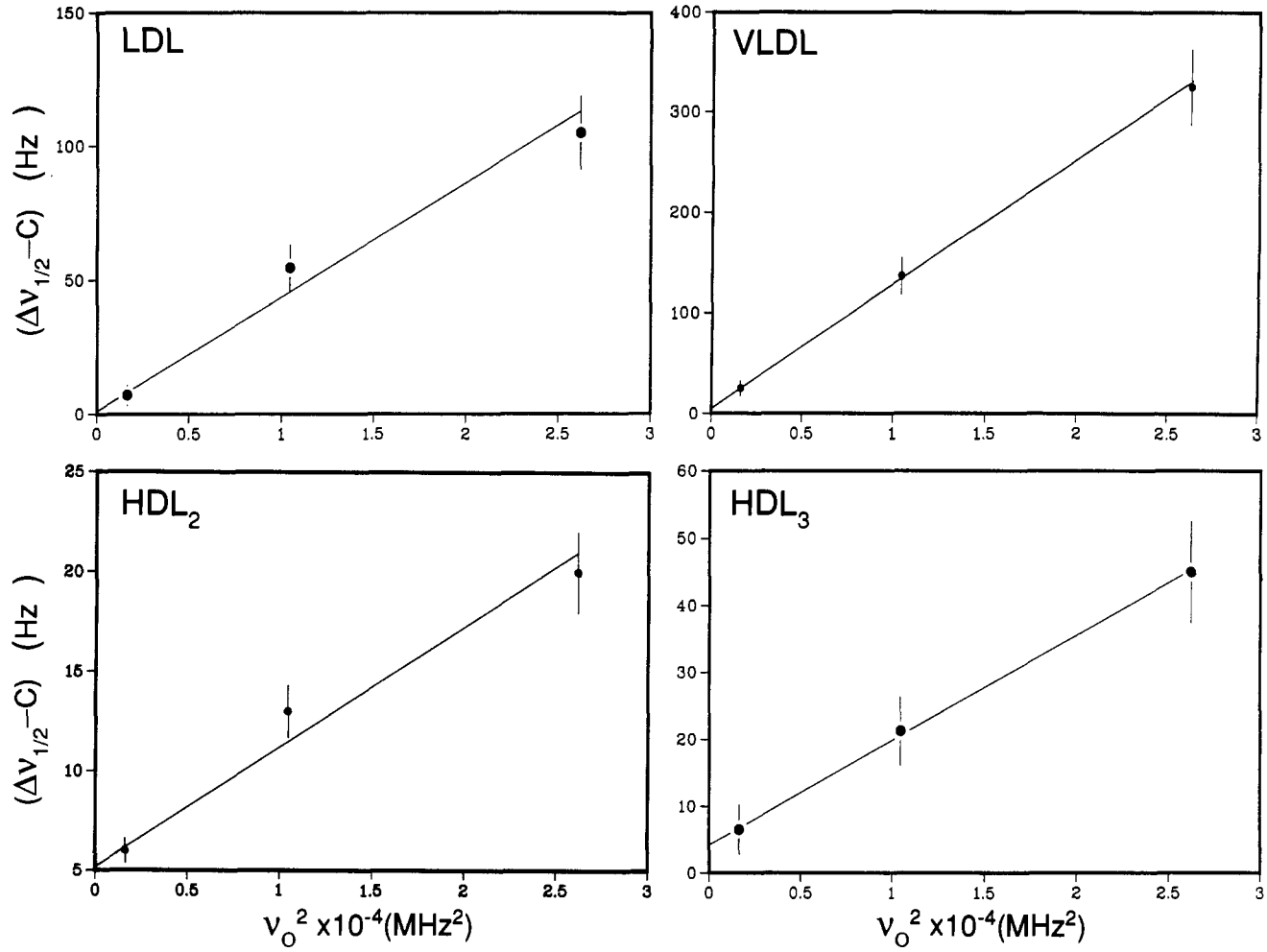

FIGURE 4: Plots of $(\Delta\nu_{1/2} - C)$ vs ν_0^2 at 25 °C for LDL (top left), VLDL (top right), HDL_2 (bottom left), and HDL_3 (bottom right). The straight lines are weighted least-squares fits to the data points.

smaller than in the lipoproteins, which is of interest as the microemulsions are better models of lipoproteins than are phospholipid bilayers, being of similar size and composition to VLDL.

As the $\Delta\sigma$ values listed in Table I have been derived from narrow-line spectra, a second estimate obtained by a different method would help ascertain the accuracy of the equations upon which our data analysis is based. It would be desirable if $\Delta\sigma$ could be observed directly, as in bilayers. We have found that both VLDL and LDL can be concentrated to viscous gels in which particle reorientation is slowed sufficiently to give "powder-like" line shapes (Figure 5), with the sign of the powder pattern and magnitude of the anisotropy characteristic of the bilayer phase. This represents the first verification of effectively axial symmetry in the lipoprotein monolayer. At low temperatures (−5 to 5 °C), the spectral width is on the order of 30 ppm, but this is certainly an underestimate of the residual $\Delta\sigma$ since lateral diffusion of the phospholipid molecules will result in averaging of the powder pattern. In order to extract the true residual $\Delta\sigma$, the spectra were simulated assuming an axially symmetric shielding tensor motionally narrowed by isotropic diffusion, as described by Burnell et al. (1980). Only values of $\Delta\sigma$ on the order of 50 ppm gave acceptable fits to the VLDL and LDL spectra, validating the use of eq 1–5 for these lipoproteins.

In contrast to the lower density lipoproteins, the shielding anisotropies in HDL_2 (75 and 69 ppm from the viscosity and field dependence, respectively) and HDL_3 (136 and 156 ppm from the viscosity and field dependence, respectively) are significantly larger than observed in most liquid-crystalline membrane systems. These larger $\Delta\sigma$'s could originate from changes in the order and/or orientation of the head-group (Thayer & Kohler, 1981; Scherer & Seelig, 1989). This is indicated by the spectrum of HDL_3–sucrose complexes shown in Figure 5c, which has a line width of >103 ppm. The spectrum narrows at higher temperatures, indicating that the phospholipids still possess significant motions in these particles. The line shape could not be simulated as described for VLDL and LDL (Burnell et al., 1980). The spectral line shape is similar to the simulations in Figure 2 of Campbell et al. (1979) wherein a (fast) diffusion axis is tilted with respect to the principal axes of the chemical shielding tensor. Such a "tilt" is consistent with our proposal of an orientational change of the head-group in HDL_3 compared to that in VLDL and LDL. Large anisotropies have been observed in at least one other system. Yeagle and Kelsey (1989), in a study of phospholipids tightly bound to human erythrocyte glycophorin in a glycolipid membrane, observed an axially symmetric powder pattern with a $\Delta\sigma$ of approximately 90 ppm, although the details of this system differ from ours in that their T_1 values were significantly shorter.

The accuracy of our calculated $\Delta\sigma$ values hinges on the reliable measurement of the particle size. For instance, the effect of the hydration shell on particle radius would be greater for the smaller HDLs, and if the hydrated radius were larger than that obtained from electron microscopy, the calculated values of $\Delta\sigma$ would be smaller. Tilley et al. (1988) recently reported that the correlation times for VLDL, LDL, and HDL

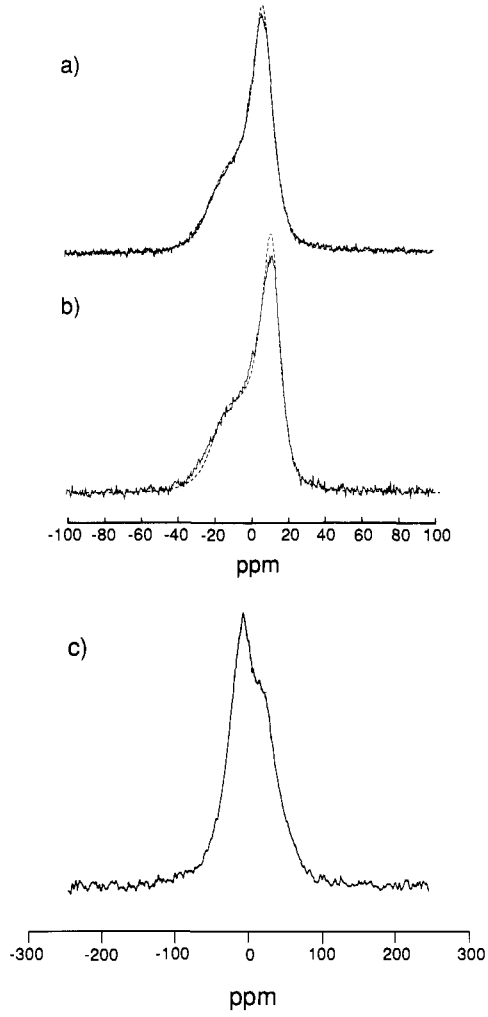

FIGURE 5: ^{31}P "powder pattern" NMR spectra of VLDL gel at −5 °C (a), LDL gel at 5 °C (b), and HDL_3 concentrated from a 200 mM sucrose solution at −20 °C (c). Further details are given in the text. The external reference was 85% H_3PO_4, and upfield is positive.

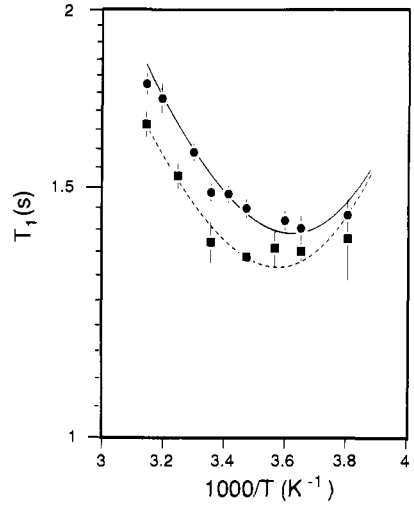

FIGURE 6: Semilog plot of T_1 vs $1000/T$ for LDL (solid squares) and HDL_3 (solid circles). The solid and dashed lines were obtained from the fitting parameters in Table II. Further details are given in the text.

particle rotation obtained from time-resolved phosphorescence anisotropy are roughly twice those estimated from the Stokes–Einstein relationship (eq 3). It was suggested that deviations from spherical symmetry or lipoprotein aggregation in solution may account for the discrepancy. With respect to the latter point, we have found that the particle sizes of VLDL and LDL obtained either by electron microscopy or by QELS are unimodal and are very similar, and LDL gives a single peak on Sepharose 6B gel exclusion chromatography, proving no aggregates are formed. In the case of HDL_3, we had a sample run courtesy of Nicomp on a QELS apparatus equipped with a 20-mW argon laser and fitted with a blue filter. The mean diameter they obtained (8.0 nm) is identical with our EM value of 8.1 nm, and any large scattering centers comprise <0.6% of the particles. Moreover, even if we increase the correlation time by a factor of 2, $\Delta\sigma$ for HDL_3 is still 96 ppm.

Table II: Best-Fit Parameters for the Temperature Dependence of Phospholipid ^{31}P Spin–Lattice Relaxation in LDL and HDL_3

	LDL	HDL_3
K (s^{-2})	1.43×10^8	1.32×10^8
$\alpha(\Delta\sigma)^2$	7.5×10^{-10}	7.5×10^{-10}
τ_0 (s)	8.75×10^{-13}	8.75×10^{-13}
E_a (kJ/mol)	17.0	16.8
T_1 minimum (°C)	7	3

The orientation giving rise to the largest anisotropies is most likely one in which the phospholipid head-group is extended normal to the membrane surface, rather than parallel to the membrane surface as in bilayers (Thayer & Kohler, 1981; Herzfeld et al., 1978; Griffin et al., 1978). Our results are consistent with a progressive change in head-group orientation, from roughly parallel to the surface in VLDL and LDL to roughly perpendicular to the surface in HDL_3. These changes could originate from packing constraints in particles as small as the HDLs, resulting from their high curvature, and/or from steric interactions with the amphipathic apoproteins (Segrest et al., 1974), which constitute 61 wt % of the HDL_3 particle.

Temperature Dependence of the ^{31}P Spin–Lattice Relaxation. The temperature dependence of T_1 for LDL and HDL_3 is plotted in Figure 6. The relationship with temperature is clearly nonlinear, and a minimum in T_1 is observed in the range of 3–7 °C. This is similar to results obtained from bilayers of DOPC (Seelig et al., 1981), egg PC (Milburn & Jeffrey, 1987), and POPC/PE mixtures (Ghosh, 1988). The curves for LDL and HDL_3 are similar, indicating that any differences in the dynamics of phosphate group motion in the two particles are small. The T_1 minimum occurs at 7 °C for LDL and at 3 °C for HDL_3. The minima observed by Seelig et al. (1981) and Milburn and Jeffrey (1987) at comparable field strengths occurred at 4 and 3 °C, respectively.

The existence of a T_1 minimum allows the direct determination of a motional correlation time, since the correlation time τ_c is on the order of the inverse of the Larmor frequency at the temperature of the minimum (1.3 ns in the present case). Parameters which were varied in the fitting routine were K, $\alpha(\Delta\sigma)^2$, and E_a (eq 7–11). The value of τ_0 was that obtained by Milburn and Jeffrey (1987). The best-fit curves are plotted in Figure 6, and the parameters are listed in Table II.

The values of K obtained for LDL and HDL_3 fall between those determined by Seelig et al. (1981) (1.65×10^8 s^{-2}) and Milburn and Jeffrey (1987) (0.9×10^8 s^{-2}). The value of $\alpha(\Delta\sigma)^2 = 7.5 \times 10^{-10}$ compares favorably with 1.5×10^{-9} obtained by Seelig et al. (1981) and Ghosh (1988). Depending on the value of α, $\Delta\sigma$ could vary between 30 and 90 ppm. As discussed by Ghosh (1988), the numerical value of α depends on the orientation and order of the phosphate segment, and thus it is possible that both α and $\Delta\sigma$ are different for LDL and HDL_3. In other words, $\Delta\sigma$ could be significantly different, as discussed in the previous section, as long as the product $\alpha(\Delta\sigma)^2$ was on the order of 7.5×10^{-10} for both lipoproteins. This may explain why the relaxation behavior is similar when

the anisotropies appear to be different. The activation energies obtained for both lipoproteins are on the order of 17 kJ/mol, in agreement with the values obtained for DOPC bilayers (17.1 kJ/mol; Seelig et al., 1981) and egg PC bilayers (16.9 kJ/mol; Milburn & Jeffrey, 1987).

The correlation times for phosphate group reorientation obtained from eq 11 are similar for both LDL and HDL_3, although small differences are observed. The T_1 minimum of LDL is 4 °C higher than that of HDL_3, indicating that the motions dominating relaxation are about 9% slower in LDL. This may result from protein effects similar to but smaller than those observed by Seelig et al. (1981), who observed a 10 °C increase in the temperature of the T_1 minimum of reconstituted sarcoplasmic reticulum membranes compared with DOPC liposomes. The correlation times we obtain are in general agreement with data from PC and PC/PE bilayers (Seelig et al., 1981; Ghosh, 1988). Thus, the dynamic behavior of the phosphate group in both LDL and HDL_3, as assessed by the correlation times and activation energies for the motion, is remarkably similar to that of phospholipids in bilayers.

Conclusions. The results of the current study demonstrate significant differences in the phospholipid monolayer regions of plasma lipoproteins. Lateral diffusion rates of phospholipid molecules vary over an order of magnitude going from LDL to VLDL to the HDLs, and in LDL seem to be largely determined by surface–core interactions. These interactions may play an important role in the development of atherosclerosis, as modulation of the core triglyceride or phospholipid content of LDL alters the conformation of apo B and its binding to receptors (Aviram et al., 1988a,b; Kleinman et al., 1988). The residual chemical shift anisotropy of the phosphate group of VLDL and LDL is similar to that of bilayers, but $\Delta\sigma$ is significantly higher in the HDLs, particularly HDL_3. This is probably due to a change in head-group orientation and possibly ordering imposed by the packing of phospholipids in small, highly curved particles with a high protein content. The correlation times and the activation energies for phosphate group reorientation are nearly identical in LDL and HDL_3, and are very similar to values obtained from phospholipid bilayers, arguing against any long-lived interactions between phospholipid and protein on the nanosecond time scale. The variation in the dynamic and orientational properties of phospholipids in lipoprotein monolayers may be important in such phenomena as receptor binding or interactions with important metabolic enzymes such as lecithin–cholesterol acyltransferase, lipoprotein lipase, and the plasma exchange proteins. Our results strongly suggest that a complete description of lipoprotein *surfaces* can only be obtained through study of the intact particles and that results from model systems such as microemulsions must be treated with caution.

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Topographical Localization of Peroxisomal Acyl-CoA Ligases: Differential Localization of Palmitoyl-CoA and Lignoceroyl-CoA Ligases†

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ABSTRACT: We found that peroxisomal lignoceroyl-CoA ligase, like palmitoyl-CoA ligase, is present in the peroxisomal membrane whereas the peroxisomal β-oxidation enzyme system is localized in the matrix. To further define the role of peroxisomal acyl-CoA ligases (membrane component) in providing acyl-CoA for peroxisomal β-oxidation, we examined the transverse topographical localization of enzymatic sites of palmitoyl-CoA and lignoceroyl-CoA ligases in the peroxisomal membranes. The disruption of peroxisomes by various techniques resulted in the release of a "latent" pool of lignoceroyl-CoA ligase activity while palmitoyl-CoA ligase activity remained the same. Proteolytic enzyme treatment inhibited palmitoyl-CoA ligase activity in intact peroxisomes but had no effect on lignoceroyl-CoA ligase activity. Lignoceroyl-CoA ligase activity was inhibited only if peroxisomes were disrupted with detergent before trypsin treatment. Antibodies to palmitoyl-CoA ligase and to peroxisomal membrane proteins (PMP) inhibited palmitoyl-CoA ligase in intact peroxisomes, and no pool of "latent" activity appeared when antibody-treated peroxisomes were disrupted with detergent. On the other hand, disruption of PMP antibody-treated peroxisomes with detergent resulted in the appearance of a "latent" pool of lignoceroyl-CoA ligase activity. These results demonstrate that the enzymatic site of palmitoyl-CoA ligase is on the cytoplasmic surface whereas that for lignoceroyl-CoA ligase is on the luminal surface of peroxisomal membranes. This implies that palmitoyl-CoA is synthesized on the cytoplasmic surface and is then transferred to the matrix through the peroxisomal membrane for β-oxidation in the matrix. Lignoceric acid, on the other hand, is first transported through the peroxisomal membrane as such and is then activated to lignoceroyl-CoA on the luminal surface of the membrane before it is oxidized by the β-oxidation system in the matrix, and implications of these findings are discussed for X-linked adrenoleukodystrophy, a disorder with deficient activity of peroxisomal lignoceroyl-CoA ligase.

Activation of fatty acids to acyl-CoA derivatives by acyl-CoA ligases is the initial and obligatory step in their metabolism (e.g., oxidation, elongation, and synthesis of complex lipids). Different acyl-CoA ligases have been shown to activate fatty acids of different chain length (Groot et al., 1976). At the subcellular level, these acyl-CoA ligases are present in mitochondria, microsomes (Groot et al., 1976), and peroxisomes (Shindo & Hashimoto, 1978). Fatty acids greater than C_{22} are primarily and possibly exclusively β-oxidized in peroxisomes (Singh et al., 1984a) by the peroxisomal β-oxidation system (Lazarow & De Duve, 1976), and fatty acids shorter then C_{10} are β-oxidized in mitochondria (Mannaerts & Debeer, 1982). Fatty acids of chain length C_{12}–C_{22} are β-oxidized in both mitochondria and peroxisomes.

An understanding of the activation, transport, and β-oxidation of very long chain (VLC) fatty acids (>C_{22}) in peroxisomes is of particular interest because pathognomic amounts of these VLC fatty acids accumulate as a result of either defect of a single enzyme (e.g., X-linked adrenoleukodystrophy) or β-oxidation enzyme pathways (e.g., Zellweger syndrome) (Singh et al., 1981, 1984a,b). The normal β-oxidation of lignoceroyl-CoA as compared to the defective β-oxidation of lignoceric acid ($C_{24:0}$) in homogenates of cultured skin fibroblast of X-linked adrenoleukodystrophy (X-ALD) suggested a defect in peroxisomal lignoceroyl-CoA ligase activity (Hashmi et al., 1986). This was later confirmed by direct demonstration of a deficiency for lignoceroyl-CoA ligase activity in peroxisomes isolated from cultured skin fibroblasts of X-ALD (Lazo et al., 1988; Wanders et al., 1988). In contrast to the defective activation and β-oxidation of lignoceric acid (Lazo et al., 1988), the activation and β-oxidation of palmitic acid in X-ALD are normal (Singh et al., 1984a; Lazo et al., 1988). By using antibodies to palmitoyl-CoA ligase, we previously demonstrated that residual activity for activation (17% of control) and β-oxidation (12% of control) of lignoceric acid in X-ALD was derived from the activation of lignoceric acid by peroxisomal palmitoyl-CoA ligase activity (Lazo et al., 1989).

Peroxisomes are single membranous organelles surrounding matrix proteins. Peroxisomal palmitoyl-CoA ligase is a con-

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