Detection of vesicular lipoproteins in lecithin:cholesterol acyltransferase-deficient plasma by ^{1}H-NMR spectroscopy

Y. I. Parmar, D. L. Sparks, J. Frohlich, P. R. Cullis,* and P. H. Pritchard[1]

Departments of Pathology and Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada*

Summary The proton NMR spectra of the N-methyl choline region of normal and lecithin:cholesterol acyltransferase (LCAT)-deficient lipoproteins and of egg yolk phosphatidylcholine–cholesterol 55:45 (mol %) vesicle mixtures have been examined in the presence and absence of manganous sulfate as a line-broadening reagent. Manganous ions quenched all of the signal arising from normal lipoproteins and only part of the vesicle signal corresponding to the outer monolayer. There was no net loss of vesicular phospholipid when vesicles were added to normal lipoproteins and as little as 5% (or 100 μg) of the vesicular phospholipid could be detected and quantitated in the mixture of lipoproteins. Similar experiments performed on plasma lipoproteins from an LCAT-deficient patient indicated that 42% of the phospholipid was associated with vesicular lipoproteins. ▮▮ These experiments demonstrate that this technique can be used to detect and quantify small amounts of vesicular structures directly in a mixture of micellar lipoproteins.—**Parmar, Y. I., D. L. Sparks, J. Frohlich, P. R. Cullis, and P. H. Pritchard.** Detection of vesicular lipoproteins in lecithin:cholesterol acyltransferase-deficient plasma by ^{1}H-NMR spectroscopy. *J. Lipid Res.* 1989. **30:** 765–771.

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Lipoprotein X (LP-X) is an abnormal lipoprotein that accumulates in the fasting plasma of subjects with cholestasis, LCAT deficiency, and in Tangier disease following fat feeding (1, 2). All of these conditions are associated with elevated plasma levels of unesterified cholesterol which accumulates predominantly in a particle with a density similar to that of LDL and VLDL. LP-X has been purified (3–5) and is comprised of 65% phospholipid, 25% cholesterol, and 10% protein. Negative staining electron microscopy and determination of the sedimentation diffusion coefficient have indicated a diameter of 30–80 nm. X-ray diffraction and ^{31}P-NMR studies of cholestatic LP-X have shown that it is an enclosed bilayer surrounding an aqueous core (6, 7). Thus, LP-X is unlike all other lipoproteins which are micelles of phospholipid monolayers surrounding the neutral lipid core.

The phospholipid acyl chain mobility of LP-X has recently been studied by ^{1}H-NMR (7). The broad methylenes envelope observed indicates that the angular excursions of the acyl chain methylenes are severely restricted by the presence of high amounts (44 mol %) of cholesterol. In addition, it has been shown that LP-X is an extremely stable bilayer that is impermeable to sodium, praseo-

dimium, and europium ions (7, 8). In the present study, we have used this property of low permeability of phospholipid–cholesterol bilayers to cations to detect and quantitate LP-X in the presence of micellar lipoproteins. Our approach was similar to that of Brainard et al. (7) who used ^{31}P NMR of LP-X isolated from a cholestatic patient in the presence of a shift reagent, Pr(NO$_3$)$_3$, to distinguish the phosphorous signals arising from the inner and outer phospholipid monolayer. We have used a more sensitive NMR nucleus (^{1}H) to monitor the intense and narrow resonance of N-methyl choline protons. The line broadening reagent, MnSO$_4$, was used to detect the vesicular lipoproteins as the manganous ions quench all of the signal of micellar structures but only a part of the signal corresponding to outer monolayers of vesicular lipoproteins. The residual signal observed was used to quantitate the presence of enclosed bilayers in both LCAT-deficient lipoproteins and normal lipoproteins to which vesicular structures had been added.

MATERIALS AND METHODS

Materials

Deuterium oxide was purchased from MSD isotopes (Montreal). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol and MnSO$_4$ were obtained from Sigma Chemical Co. (St. Louis, MO). MnSO$_4$ was freeze-dried once in deuterium oxide to remove residual water. Sodium 2,2,dimethyl silapentane 5-sulfonate (DSS) was purchased from Stable Isotopes (Montreal, Quebec).

Isolation of plasma lipoproteins

Fresh citrated plasma of normal individuals was obtained from the Canadian Red Cross (Vancouver Branch). The EDTA plasma from a previously described patient with homozygous LCAT deficiency (9) was obtained through the Shaughnessy Hospital Lipid Clinic. The total lipoprotein fraction was isolated by ultracentrifugation at 40,000 rpm for 24 h after the addition of solid NaBr to plasma to a solution density of 1.25 g/ml. The top fraction containing all of the lipoproteins was removed by tube slicing.

Deuterium oxide exchanges were performed on both LCAT-deficient and normal lipoproteins to suppress the water signal using an Amicon ultrafiltration unit equipped with PM30 (30,000 mol wt cutoff) membranes. Lipoprotein

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Abbreviations: NMR, nuclear magnetic resonance; LCAT, lecithin:cholesterol acyltransferase; VLDL, very low density lipoproteins; LDL, low density lipoproteins; DSS, sodium 2,2-dimethyl silapentane 5-sulfonate; LP-X lipoprotein X; PC, phosphatidylcholine.

[1]To whom correspondence should be addressed at: Department of Pathology, The Research Centre, 950 West 28th Avenue, Vancouver, B.C., Canada V5Z 4H4.

fractions were concentrated to 0.5 ml, then diluted with 5 ml of deuterium oxide, and finally reconcentrated to 0.5 ml. A total of four such exchanges was performed.

Preparation of vesicles

Egg phosphatidylcholine and cholesterol (45 mol % with respect to phospholipid) were dissolved together in chloroform and, after removal of the solvent, the lipid was hydrated in deuterium oxide by vortexing. Small unilamellar vesicles were prepared by sonication using a Branson Sonifier as previously described (10). The vesicles were approximately 48 nm in diameter as determined by Nicomp submicron laser light scattering (11).

^{1}H-NMR spectroscopy

The samples (0.4 ml) were placed in a 5-mm NMR tube and the Fourier-transformed spectra were obtained with sample spinning using a Bruker WP-200 spectrometer operating at 200 MHz. The temperature was 30.0 ± 0.5°C. The NMR parameters used were the same for all of the spectra collected: sweep width = 2000 Hz; data size = 4K; pulse width = 3.5 μsec (45° flip angle); number of acquisition = 512. Line broadening was not used. The spectra were obtained with an interpulse delay of 1.02 sec. An external standard, DSS in D_2O, was used to reference the peaks. Proton peak assignments were according to Hamilton and Morrisett (12).

Other analyses

Protein was determined according to Lowry et al. modified for lipoproteins (13). Free and total cholesterol and triglycerides were determined enzymatically (14) and phospholipids were quantitated colorimetrically by the method of Rouser, Siakotos, and Fleischer (15).

RESULTS

The 200 MHz ^{1}H-NMR spectra of normal and LCAT-deficient plasma lipoproteins and of sonicated egg phosphatidylcholine–cholesterol vesicles 55:45 (mol %) in D_2O are shown in **Fig. 1**. The spectra for lipoproteins arise mainly from the lipid portion of the lipoproteins as the apoprotein resonances are too broad to be seen under these experimental conditions (12). The intense peak occurring at 4.720 ppm in all of the spectra was due to the residual water protons in the deuterium oxide-exchanged samples. The prominent lipid resonances for the normal lipoproteins (Fig. 1A) are the terminal methyls (0.86 ppm), the fatty acyl chain methylenes [$(CH_2)_n$; 1.25 ppm], the allylic protons (CH_2 C = C; 2.12 ppm), the choline N-methyl protons ($N^+(CH_3)_3$; 3.22 ppm), and the olefinic protons (CH = CH; 5.29 ppm).

The LCAT-deficient plasma lipoproteins exhibit numerous resolved peaks (Fig. 1B). The resonances are narrower as
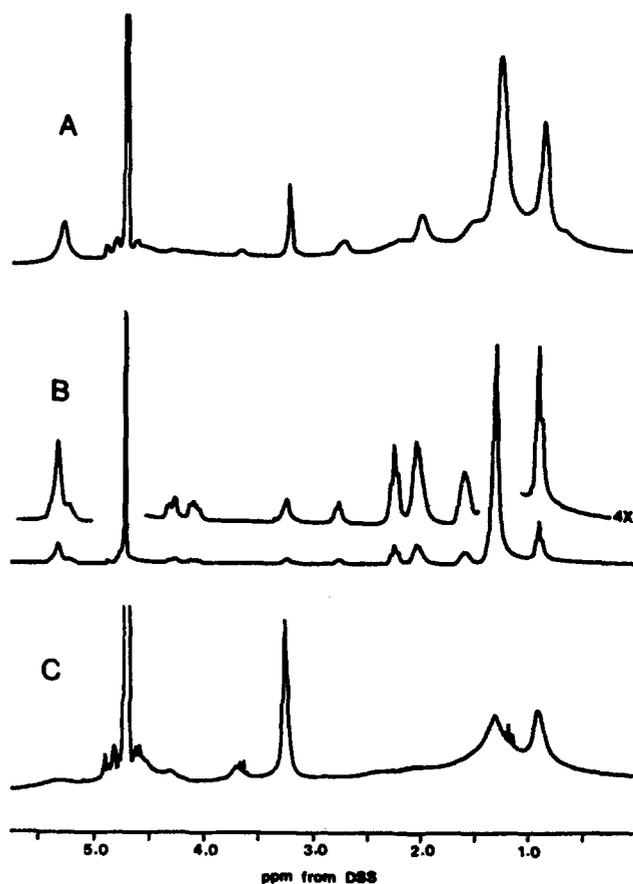

Fig. 1. Two hundred MHz ^{1}H-NMR spectra of (A) normal lipoproteins; (B) LCAT-deficient plasma lipoproteins; and (C) egg phosphatidylcholine–cholesterol 55:45 (mol %) unilamellar vesicles.

compared to normal lipoproteins and in some cases splitting can be seen. The prominent peaks are due to methylene protons [$(CH_2)_n$; 1.29 ppm] and probably arise from the high triglyceride content which makes up approximately 83% of the neutral lipid of the lipoproteins from this LCAT-deficient patient (**Table 1**). In normal plasma lipoproteins, the triglyceride content is only about 30% of the total neutral lipid (Table 1). The increases in intensity of the resonances of fatty acyl β-methylenes (1.59 ppm), the allylic methylenes (2.03 ppm), the methylene protons in between olefinic groups (2.76 ppm), and the olefinic protons (5.32 ppm), indicate the presence of core triglycerides. The sharp resonances may indicate the presence of the melted triglycerides at 30°C which undergo rapid isotropic motion within the cores of the various lipoproteins. The N-methyl choline resonance (3.24 ppm) of the LCAT-deficient plasma lipoproteins occurs as a minor peak relative to the normal plasma lipoproteins which is consistent with the low content of phospholipids. Overall, the spectrum from the LCAT-deficient lipoproteins is almost identical to the published spectrum of triglyceride-rich chylomicra (16). In both cases, the virtual absence of cholesteryl esters and the

TABLE 1. Lipid composition of normal and LCAT-deficient lipoproteins in plasma

	Normal	LCAT-Deficient
	mg/dl	
Unesterified cholesterol	48 ± 8	100
Esterified cholesterol	150 ± 25	15
Triglyceride	85 ± 24	566
Phospholipid	167 ± 62	186

Data are presented as mean ± SD (n = 6) from normal lipoproteins and as a mean of two independent preparations for the LCAT-deficient lipoproteins.

isotropic motion of melted triglycerides yield narrow and intense resonances.

For comparison purposes, we have also plotted the ^{1}H-NMR spectrum of sonicated egg phosphatidylcholine–cholesterol 55:45 (mol %) vesicles in Fig. 1C. The spectrum consists of four major lipid resonances: the acyl chain terminal methyls (0.92 ppm); fatty acyl methylenes (1.31 ppm); the choline methyl protons (3.26 ppm); and the choline methylene protons (CH_2N; 3.71 ppm). The peaks that are superimposed on the choline CH_2N and the fatty acyl methylenes are unidentified but may arise from residual

solvent impurity. The N-methyl peak is asymmetric and the high field shoulder is due to choline methyl protons of the inner monolayer (17). The vesicle spectrum bears a striking resemblance to the published spectrum for LP-X (7) as the acyl chain methylenes appear broad and featureless. This is to be expected since both particles are compositionally and structurally similar. It is well known that structures similar to LP-X accumulate in the plasma of LCAT-deficient patients (3, 4). However, ^{1}H-NMR of the total lipoproteins does not clearly indicate the presence of LP-X (Fig. 1B) as melted triglycerides dominate the spectrum. Thus, in order to detect vesicular structures (LP-X) in the LCAT-deficient plasma, we have used a Mn^{2+} quenching technique (18). Paramagnetic species such as Mn^{2+}, when present in sufficient concentration, will interact with egg phosphatidylcholine head groups to shorten the spin-spin relaxation time of the neighboring spins such that their signal is broadened out to the baseline (i.e., quenched). This has been widely used to separate ^{31}P or ^{1}H N-methyl NMR signals arising from inner or outer monolayers of small or large unilamellar vesicles (11, 18). The feasibility of the method is shown in **Fig. 2**. The N-methyl region of egg phosphatidylcholine-cholesterol 55:45 (mol %) vesicles is shown in the lower left panel of Fig. 2. The resonances at 3.26 ppm and 3.21 ppm correspond to outer and inner monolayer choline methyl
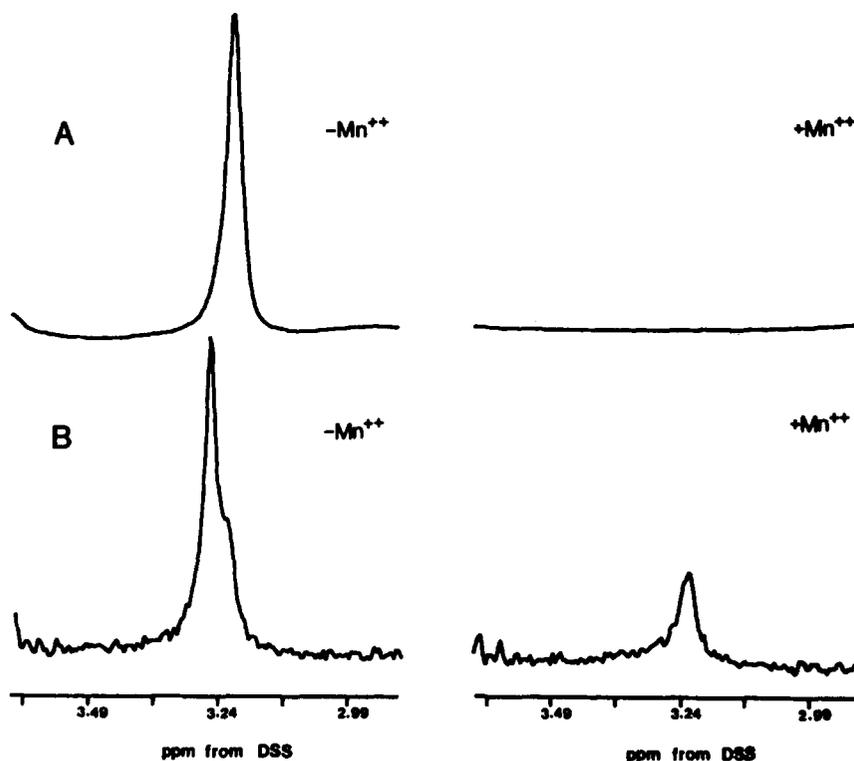

Fig. 2. Two hundred MHz ^{1}H-NMR spectrum of N-methyl choline region of (A) normal lipoproteins and (B) egg phosphatidylcholine–cholesterol vesicles. The spectra on the right are normal lipoproteins (top) and vesicles (bottom) in the presence of 1 mM $MnSO_4$. The concentration of phospholipid in normal lipoproteins and the vesicles used was 4.0 and 0.5 mg/ml, respectively.

protons. In the presence of Mn^{2+}, all of the outside resonance is broadened so that the signal is lost in the baseline, thus leaving the inaccessible choline methyl protons at 3.21 ppm (lower right). The outside/inside ratio, as calculated by weighing the peaks, was 2.65.

As a control, we have also examined the effect of manganous ions on the N-methyl region of normal lipoproteins (Fig. 2, top left). This resonance is 0.04 ppm (8 Hz) chemically shifted upfield as compared to the outer monolayer choline methyl proton of egg PC–cholesterol vesicles. The N-methyl choline region of normal plasma lipoproteins in the presence of $MnSO_4$ is shown at top right. In this case, Mn^{2+} ions broaden all of the choline methyl protons signal to the baseline since the phospholipids in normal plasma lipoproteins are arranged in a monolayer with their head groups exposed to the medium and hence the Mn^{2+} ions. Such monolayer arrangement of the phospholipids in normal LDL and HDL has been established using shift reagents $Eu(NO_3)_3$ and $Pr(NO_3)_3$ (20). The absence of any

N-methyl signal arising from normal lipoproteins is consistent with the above findings. In addition, we also show that even VLDL which is contained in the total lipoprotein fraction has phospholipid head groups that are accessible only to the medium.

In order to establish the methods for the detection and quantitation of lamellar structures in the LCAT-deficient plasma lipoproteins, we titrated vesicles into a preparation of normal plasma lipoproteins. The spectra of the N-methyl choline region in the presence of 5–50% vesicles (with respect to lipoprotein phospholipid) and 1 mM $MnSO_4$ is shown in the left column of **Fig. 3**. The right-hand column is the choline methyl proton region of an equivalent amount of vesicles in the absence of plasma lipoproteins. As can be seen from the figure, as little as 5% vesicles can be detected. Furthermore, as the spectra on the left closely match those on the right, the egg phosphatidylcholine–cholesterol vesicles and the normal lipoproteins did not interact. We have quantitated the lack of interaction by cutting out and weigh-
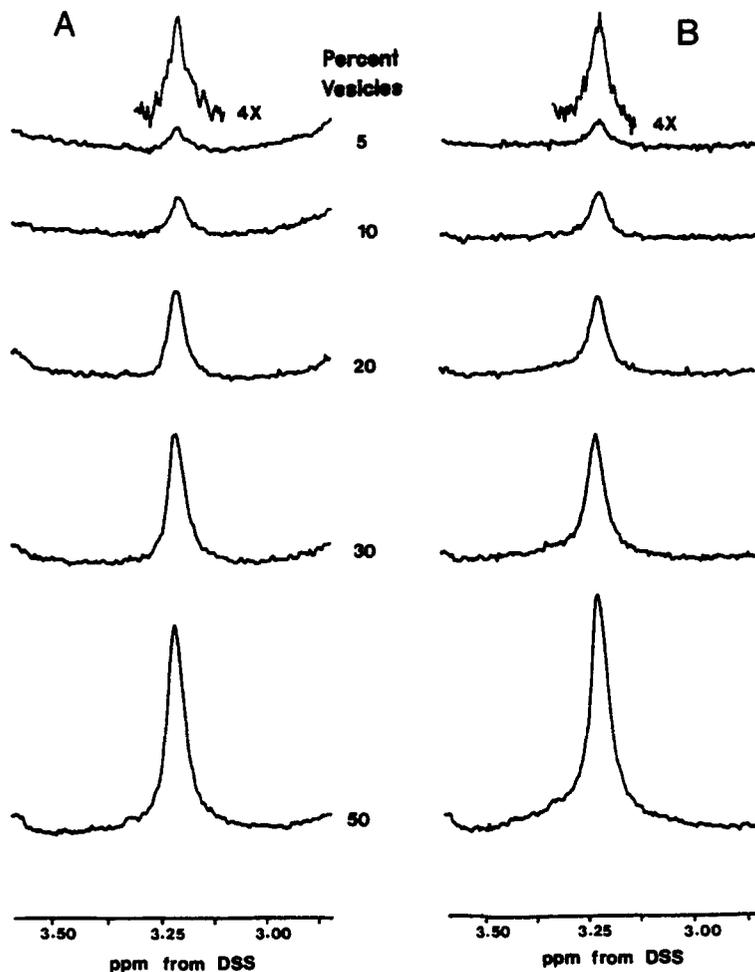

Fig. 3. Two hundred MHz ^{1}H-NMR spectra of N-methyl choline region of a mixture of egg phosphatidylcholine–cholesterol vesicles plus total lipoproteins (A) and egg phosphatidylcholine–cholesterol vesicles (B) in the presence of 1 mM $MnSO_4$. The mixture of lipoproteins contained in indicated amounts of vesicular phospholipid. (B) is the Mn^{2+}-quenched signal of equivalent amounts of vesicles in the absence of lipoproteins.

ing each peak in Fig. 3. This calculated integral was plotted against the mass of vesicular phospholipid in the NMR tube (Fig. 4). As the amount of vesicles is increased in the presence or absence of plasma lipoproteins, the choline methyl protons peak integral increased linearly. Furthermore, the increase in integral size in both systems is identical, which strongly suggests that there is no net transfer of vesicular phospholipid to the lipoproteins, and as little as 0.1 mg vesicular phospholipid in a mixture of lipoproteins can be quantitated under present experimental conditions.

The spectrum of the N-methyl region of the LCAT-deficient plasma lipoproteins is shown in **Fig. 5** (top). The strong resonance at 3.24 ppm is due to the choline methyl protons. The sharp resonance to the left is unidentified. The bottom spectrum is choline methyl protons of LCAT-deficient plasma lipoproteins in the presence of 1 mM $MnSO_4$. The peak at 3.22 ppm is due to residual choline methyl protons indicating the presence of vesicular structures in the LCAT-deficient plasma lipoproteins.

DISCUSSION

Lamellar structures accumulate in the plasma when phospholipid cannot be incorporated into the existing lipoproteins. This has been reported to occur in LCAT deficiency, cholestasis, and after intravenous infusion of Intralipid (1, 2, 21). In our experiments, we have titrated egg phosphatidylcholine–cholesterol vesicles into normal lipoproteins as a model system for the detection of vesicular structures in disease states. The marked similarity of the Mn^{2+}-quenched signal of vesicular phospholipid in the presence or absence of lipoproteins suggests that inner monolayer phospholipid is not affected by the presence of lipoproteins and that the vesicles remain impermeable to Mn^{2+}. This is to be expected since phospholipid vesicles
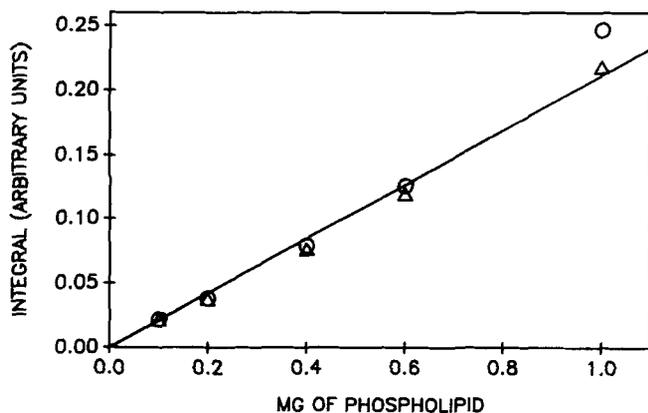

Fig. 5. Two hundred MHz ^{1}H-NMR spectra of the N-methyl choline region of 4.0 mg/ml phospholipid in the total lipoproteins isolated from an LCAT-deficient patient in the absence (top) and presence (bottom) of 1 mM $MnSO_4$.

Fig. 4. Relationship between total phospholipid and the integral of the Mn^{2+}-quenched N-methyl choline signal from Fig. 3. Data are presented for vesicles alone (O–O) and a mixture of normal lipoproteins and vesicles (△–△).

containing greater than 33 mol % cholesterol do not interact with lipoproteins (22). Nevertheless, phospholipid exchange has been shown to occur between Intralipid vesicles and LDL without the net transfer of phospholipid (23). It is conceivable that such an exchange could also occur between the outer monolayer of vesicles and lipoproteins in our system. However, exchange of phospholipids between lipoproteins and the inner monolayer of vesicles is not likely due to the slow rate of transbilayer movement of the phospholipids. Thus, the enclosed and impermeable bilayer structure of vesicles is maintained in the mixture of normal lipoproteins.

LP-X from patients with LCAT deficiency has a density similar to normal LDL and VLDL (3–5). The bilayer structure has been detected by negative staining electron microscopy and can be inferred by compositional analysis.

The results of the present study confirm the existence of vesicular structures in LCAT-deficient plasma by the inability of $MnSO_4$ to quench all of the choline methyl protons. The integral ratio of the outer/inner N-methyl resonance is 5.7. This is much higher than would be expected from phospholipid bilayers alone. The ratio of phospholipid on the outside to inside in bilayers is known to vary from 1.0 (for planar bilayers) to 2.9 (for the small unilamellar vesicles) (24). The high ratio of 2.7–2.9 is also observed for the larger (40–45 nm) egg phosphatidylcholine cholesterol (33 mol %) unilamellar vesicles (19). This possibly indicates that cholesterol has a preference for the inner monolayer and consequently more phospholipid is forced to reside in the outer monolayer. Thus, the much higher ratio observed for the LCAT-deficient plasma indicates the coexistence of vesicles and lipoproteins in the sample.

It may be argued that the proteins (5–10% particle weight) on the LP-X surface sequester some phospholipid which is then inaccessible to Mn^{2+} ions. However, normal lipoproteins also contain apoproteins but have all of their choline methyl protons signal quenched. Additional proof for the existence of bilayers in LCAT-deficient plasma is that the Mn^{2+}-quenched residual N-methyl signal is shifted upfield by approximately 4 Hz (Fig. 5). The chemical shift difference of 4–10 Hz has been reported for the outside and inside N-methyl resonance of curved bilayers (24). Similarly, we have also detected the difference of approximately 8 Hz in our egg phosphatidylcholine–cholesterol vesicle preparations. However, the same chemical shift difference is not observed for the much larger planar multilamellar vesicles. Thus, the upfield shifted resonance observed for the quenched LCAT plasma lipoproteins is probably due to the curved inner monolayer phospholipids of LP-X. The methods described in this study have enabled us to estimate the amount of vesicular phospholipid in the total lipoproteins of LCAT-deficient plasma. Assuming outside to inside phospholipid ratio of LP-X of 2.0, approximately 42% of the total phospholipid is associated with LP-X. The ratio of two was used in calculation because LP-X is relatively small (30–80 nm) having approximately 44 mol % cholesterol, which results in asymmetric distribution of phospholipid across the bilayer.

Thus, we have demonstrated that Mn^{2+} quenching of N-methyl groups can be used to detect as little as 100 μg of vesicular phospholipid in the mixture of normal or abnormal lipoproteins. ▮▮

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