³¹P Nuclear Magnetic Resonance Studies on Serum Low and High Density Lipoproteins: Effect of Paramagnetic Ion†

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ABSTRACT: A paramagnetic quenching reagent, $Mn^{2+}/$ EDTA (1:2.2), was developed for the purpose of investigating the phospholipid phosphate groupings of human serum low and high density lipoproteins through the quenching effect of the reagent on the ³¹P nuclear magnetic resonance signals from these complexes. Systems investigated included native low and high density serum lipoproteins (LDL, HDL_2, and HDL_3), egg phosphatidylcholine vesicles together with appropriate phosphodiester model systems, diethyl phosphate in aqueous buffer, and phosphatidylcholine and sphingomyelin both in anhydrous methanol. The results of these studies indicated that ca. 50% of the phospholipid-phosphorus signal of LDL is quenched upon titration as compared to an 80–85% figure observed for HDL_2 and HDL_3. In all cases the spectral effects were totally reversible upon removal of the paramagnetic ion by dialysis. The results of the titration studies indicated a similar but not an identical behavior between HDL_2 and HDL_3. The results are consistent with model structures of HDL and LDL particles derived from low angle X-ray diffraction.

Serum lipoproteins represent a common transport system of lipids in human and other animal species (Scanu, 1972a,b; Scanu and Ritter, 1973; Scanu and Wisdom, 1972). These water-soluble lipid–protein complexes are generally isolated by cumulative ultracentrifugal flotation (Scanu and Ritter, 1973) into major classes which comprise chylomicrons, very low density lipoproteins, low density lipoproteins (LDL),[1] high density lipoproteins (HDL), and very high density lipoproteins, each distinguishable in physical and chemical terms. Human HDL on a weight average contains about 50% protein, 25% phospholipid, 4% cholesterol, 14% cholesterol ester, and 4% triglycerides with the phospholipids comprised of 75% phosphatidylcholine (PC), 14% sphingomyelin (SPH), 5% phosphatidylethanolamine and phosphatidylinositol, and traces of others (Skipski et al., 1967). The two HDL subclasses, HDL_2 and HDL_3, differ from each other in protein–lipid distribution, average hydrated densities, dimensions, and molecular weight: HDL_2, 380,000; HDL_3, 175,000 (Scanu and Kruski, 1973).

The results of small angle X-ray diffraction studies have been interpreted to indicate that both HDL_2 and HDL_3 are

symmetrical particles with an electron poor core and an electron rich outer shell (Shipley et al., 1972). Further, electron spin resonance (ESR) (Gotto and Kon, 1969) and proton magnetic resonance (PMR) studies (Chapman et al., 1969) suggest that the lipids and proteins in HDL interact and that some choline and amino acid residues are in unrestrained motion. Recently, models for HDL have been proposed (based on ³¹P and ¹³C NMR) which suggest that the proteins are in a matrix of lipids with hydrophobic interaction occurring between the lipids, mainly PL, and the amphipathic or hydrophobic regions of the proteins (Assman and Brewer, 1974; Stoffel et al., 1974; Assman et al., 1974).

By weight the LDL contain 79% lipid and 21% protein, with the lipids consisting of 47% cholesterol esters, 28% PL, 10% unesterified cholesterol, and 14% triglycerides (Scanu and Kruski, 1973). The molecular weight has been determined to be 2.3×10^6 (Scanu and Kruski, 1973), and electron microscopic examination (Gotto et al., 1968) indicates a spherical structure with a diameter of 220 Å. PMR studies (Leslie et al., 1969; Steim et al., 1968) have suggested that the protein has little influence on the overall particle structure, whereas the reported ESR data (Gotto et al., 1969) appear to indicate that, to some extent, protein–lipid interactions do occur. Small angle X-ray diffraction studies on LDL (Mateu et al., 1972) suggest that this particle contains a spherical (170–270 Å diameter) lipid bilayer. The X-ray data were also taken to indicate that an outer network and possibly an inner core of protein was interacting with the cholesterol esters leaving the polar heads of the phospholipids on the outer face exposed to the solvent.

We recently reported data on the ³¹P nuclear magnetic resonance (³¹P NMR) chemical shifts of phospholipids in organic solvents (Henderson et al., 1974a) and we and others (Glonek et al., 1973, 1974; Assman et al., 1974; Henderson et al., 1974c) described the application of this technique to the study of the structure of human serum lipoproteins. The narrow band width of the PC resonance suggested that the polar head groups of PC are in relatively rapid motion (Glonek et al., 1974; Assman et al., 1974). Studies of HDL with the paramagnetic europium ion (Assman et
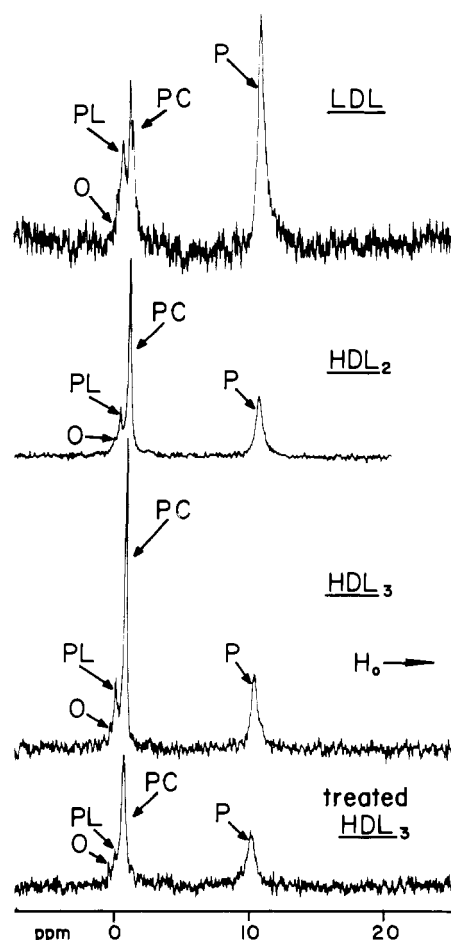
FIGURE 1: ^{31}P NMR spectra obtained from lipoprotein preparations: LDL, low density lipoproteins; HDL$_2$, high density lipoprotein two; HDL$_3$, high density lipoprotein three; and treated HDL$_3$, HDL$_3$ to which the Mn^{2+}/EDTA (1:2.2) reagent was added (Mn^{2+}/P = 0.25). Chemical shifts are given relative to external 85% H$_3$PO$_4$ as the zero (0) ppm reference. The bottom spectrum was obtained after addition of the reagent to the sample giving rise to the HDL$_3$ spectrum of the figure. The signals proceeding upfield are: O, the signal from the inorganic orthophosphate impurity in the pyrophosphate intensity reference capillary (−0.3 ppm); PL, the signal from lipoprotein phospholipids other than phosphatidylcholine, principally sphingomyelin (0.3 ppm); PC, lipoprotein phosphatidycholine (0.9 ppm); and P, the signal from the inorganic pyrophosphate intensity reference capillary (10.4 ppm). In aged or degraded preparations, a lipoprotein signal from lyso-PC is sometimes observed between that from SPH and PC (C.F.) (Glonek et al., 1974; Assman et al., 1974). The SPH resonance is shifted upfield with the addition of the paramagnetic Mn^{2+} EDTA reagent. The spectra were obtained through the use of Fourier transform NMR techniques: spectra width employed, 2100 Hz; acquisition time, 238 sec (4 K data points per signal-averaged free induction decay); cycling time, 1 sec. The sample volumes used ranged from 2 to 4 ml (ca. 6 mol of P/ml, 4.65 mg of PL/ml) and were contained in 10-mm spinning sample tubes, mounted in the center of which was the melting point capillary containing the pyrophosphate references solution; about 4 K accumulations were averaged per spectrum (total signal-averaging time ca. 1 hr); temperature, 28°. Deuterium field-frequency stabilization was employed through use of the HDO resonance from the sample (10% D$_2$O added), and proton broad-band decoupling was used to eliminate proton-phosphorus coupling.

al., 1974) led to peak broadening and chemical shifts which were taken to indicate that all of the PL phosphates of HDL are accessible to interaction and are probably located at the outer surface.

In the present investigation, we applied the techniques of ^{31}P NMR spectroscopy to the study of intact LDL, HDL$_2$, and HDL$_3$ in the presence of increasing concentrations of the paramagnetic divalent cation manganese chelated with ethylenediaminetetraacetate (EDTA). The study was extended to include egg PC vesicles of uniform size (Huang, 1969). In parallel experiments, an analysis was conducted on aqueous diethyl phosphate, egg PC in methanol, and SPH in methanol to determine the spectra response of these model systems to the chelated reagent, Mn^{2+}/EDTA (1/2.2).

Materials and Methods

Human serum lipoproteins LDL, HDL$_2$, and HDL$_3$ were prepared and purified by ultracentrifugal flotation as previously described (Scanu and Ritter, 1973; Scanu et al., 1974). Sonicated egg PC was fractioned by gel filtration (Sepharose 4B, 2 × 100 cm column at 4°, eluting buffer 0.1 M NaCl, 0.01 M Tris-HCl adjusted to pH 8.5) into vesicles which have been found to consist of a continuous PL bilayer surrounding the solvent and having an average diameter of 250 Å and vesicle weight of 2.1 × 10^6 (Huang, 1969). Circular dichroic studies were performed as previously described (Scanu and Hirz, 1968) in a Cary Model 60 spectropolarimeter provided with a Model 6001 circular dichroism attachment (Cary Instruments, Monrovia, Cal.). All preparations were dialyzed extensively against 0.15 M NaCl and 0.05% EDTA (pH 7.2) before either circular dichroic or ^{31}P NMR analysis. All titration studies were conducted against the chelated paramagnetic ion, Mn^{2+}/EDTA (1:2.2).

The various titration reagents were prepared by dissolving appropriate amounts of the metal salts (Mn^{2+} as the chloride, lanthanides as the nitrates) and the free acids of the chelating agents in water and by adjusting the pH to 7.2 with NaOH. The titration reagent chosen for this study, Mn^{2+}/EDTA (1:2.2), was also prepared in organic solvents, but in this case the neutralizing base was tetra-n-butylammonium hydroxide. After the reagent was adjusted, in water, to an appropriate pH, the solution was evaporated in a rotary evaporator at 30° and rendered anhydrous by five successive evaporations of 10 volumes of 1:1 absolute ethanol–benzene, followed by two evaporations of anhydrous benzene, and finally repeated evaporations from anhydrous methanol. The reagent was then soluble in most organic solvents to concentrations at least 0.1 M in Mn^{2+}.

The ^{31}P NMR spectra were obtained on a Bruker HFX-5 spectrometer (Glonek et al., 1971; Henderson et al., 1972) operating at 36.43 MHz for ^{31}P (^{1}H field equivalent to 90 MHz) and equipped for all modes of heteronuclear decoupling and the processing (Glonek et al., 1974; Henderson et al., 1974b) of ^{31}P Fourier transform spectra. Deuterium field-frequency stabilization was employed using a ^{2}H signal derived from 10% D$_2$O added to the sample. As is customary (Crutchfield et al., 1967) with ^{31}P NMR spectroscopy, positive chemical shifts are associated with increasing field strengths; the zero ppm reference compound is 85% orthophosphoric acid (Glonek et al., 1970). Further details can be found in the legend to Figure 1.

Signal intensity measurements were calculated with respect to the pyrophosphate signal from a reference melting point capillary coaxially mounted in the same tube and containing about 0.1 M sodium pyrophosphate in water (initial pH 6.8) (Michaelson et al., 1973). This capillary gave a signal of suitable intensity at 10.4 ppm which, being isolated from the sample, was not affected by the paramagnetic reagents used in the titrations. The pyrophosphate sample contained about 5% inorganic orthophosphate which came

Table I: The Effects of Various Paramagnetic Ion Reagents on the Phospholipid ³¹P Resonance Signals from the Purified Serum Lipoproteins in Aqueous Buffer.

Reagent[a]	Lipoprotein[b]	Effect[c]
$Mn^{2+}Cl_2$	HDL_3, HDL_2, LDL	Efficient complete quenching
$Pr^{3+}(NO_3)_3$	HDL_3	Rapid precipitate formation
$Sm^{3+}(NO_3)_3$	HDL_3	Slow precipitate formation
$Eu^{3+}(NO_3)_3$	HDL_3, LDL	Rapid precipitate formation
$Mn^{2+}NTA$	HDL_3	Efficient complete quenching
$Eu^{3+}NTA$	HDL_3	Rapid precipitate formation
$Mn^{2+}(NTA)_{2.2}$	HDL_3	Efficient complete quenching
$Pr^{3+}(NTA)_{2.2}$	HDL_3	No effect
$Eu^{3+}(NTA)_{2.2}$	HDL_3	No effect
$(Mn^{2+})_2EDTA$	HDL_3, HDL_2	Efficient complete quenching
$(Pr^{3+})_2EDTA$	HDL_3	Rapid precipitate formation
$(Eu^{3+})_2EDTA$	HDL_3, LDL	Rapid precipitate formation
$Mn^{2+}EDTA$	HDL_3	Partial quenching
$Pr^{3+}EDTA$	HDL_3	No effect
$Eu^{3+}EDTA$	HDL_3	No effect
$Mn^{2+}(EDTA)_{2.2}$	HDL_3, HDL_2, LDL	Partial selective quenching
$Pr^{3+}(EDTA)_{2.2}$	HDL_3	No effect
$Sm^{3+}(EDTA)_{2.2}$	HDL_3	No effect
$Eu^{3+}(EDTA)_{2.2}$	HDL_3, LDL	No effect

[a] NTA, nitrilotriacetate; EDTA, ethylenediaminetetraacetate.
[b] LDL, low density lipoprotein; HDL, high density lipoproteins.
[c] Appearance of the ³¹P signal at a metal ion/phosphorus ratio of 4:1 (titrant 0.1 M in metal ion). (Precipitation quite often occurred at much lower ratios.)

FIGURE 2: ³¹P NMR paramagnetic titration curves of phosphorus containing compounds. The titration reagent was 0.1 M Mn^{2+} as Mn^{2+}/EDTA (1:2.2) in either aqueous buffer (LDL, HDL_2, HDL_3, PC vesicles, and diethyl phosphate) or anhydrous methanol (PC in methanol and SPH in methanol). The counter cation in the aqueous systems was Na^+, in the methanol systems, tetra-n-butylammonium$^+$. (...) The quenching curve of either LDL, HDL_2, or HDL_3 titrated with unchelated 0.1 M Mn^{2+} ($Mn(Cl)_2$).

into resonance 12 Hz to lower field than the lowest signals from the samples of this study; however, its presence caused no particular problem. In the titrations the ratio of the sample signal was compared to the pyrophosphate signal from the reference capillary. The value obtained with no additions of titrant was assigned an intensity of 100% (Figure 1).

Because the same reference capillary could serve for a number of samples before pyrophosphate hydrolysis significantly diminished and shifted the signal (upfield with the increasing acidity of the solvent), it was possible to cross correlate the intensities from several lipoprotein preparations. In all cases, there was complete agreement (within ±2%) in the samples, as determined by colorimetric analysis, indicating a uniformity of the nature of the various preparations.

Representative ³¹P spectra of the serum lipoproteins are shown in Figure 1. These were recorded according to the conditions described in the figure. The PL samples dispersed in methanol were PC (egg) and SPH (bovine brain, Serdary Research Labs. Inc., London, Ontario, Canada).

Results and Interpretations

Quenching Reagents. Table I presents some general observations which were made with a variety of shift and quenching reagents using principally HDL_3 as the test phosphate. In general, the simple hydrated transition metal ions (as chlorides and nitrates) were effective in totally suppressing the phosphorus resonances from the lipoproteins (also see Figure 2). No selective action could be discerned, and there was no measurable difference in the overall behavior of any of the tested ions, although very early in the titration of HDL_3 with Eu^{3+} ion, where the metal ion to P ratio was 0.16, a shifted resonance at 40 ppm exhibiting a signal width at half-height of 42 Hz could be detected. Titration with the hydrated lanthanide ions always led quickly
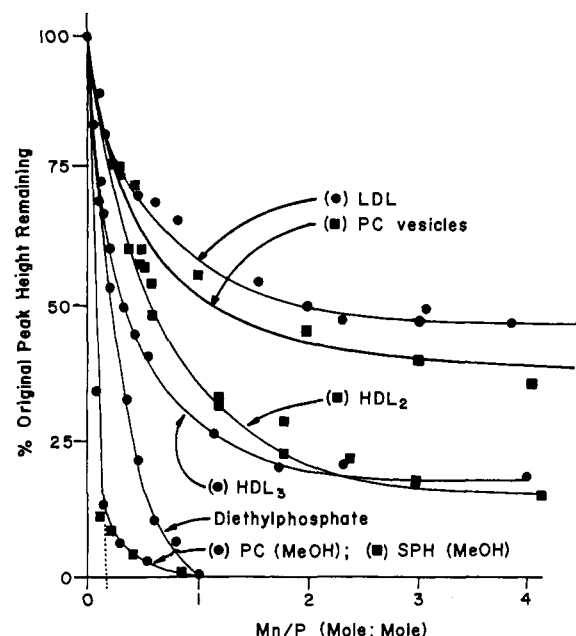
to the formation of insoluble precipitates. No such precipitates were obtained with Mn^{2+} ion until the metal ion to P ratio become large (≥ 10), and, even then, the precipitates took considerable time to form (about 8–10 hr).

Water-soluble EDTA and nitrilotriacetate chelates of the lanthanide elements containing at least a mole of chelator per mole of ion were totally ineffective as either shift or quenching reagents and, further, did not give rise to precipitates. Since the chelated Mn^{2+} ion did show a quenching effect, it is assumed that the lanthanide complexes could approach the phosphates sufficiently close to give rise to some measurable spectroscopic effect if interaction actually occurred between the phosphate and metal ion. Apparently the phosphate groups in the lipoproteins cannot effectively compete with the chelating agents in coordinating the chelated lanthanide metal ions. Only EDTA complexes of Mn^{2+} ion possessed quenching properties usable in the study of the circulating lipoproteins (Table I and Figure 2). At the Mn^{2+} concentration employed, no significant protein structural changes occurred as determined by circular dichroic studies. For each substance studied, the action of the Mn^{2+}/EDTA (1:2.2) reagent was reproducible from sample to sample, and in the cases of the circulating lipoproteins, fully reversible upon dialysis against the original buffer.

Paramagnetic Titrations. Since the action of the Mn^{2+}-EDTA reagent was unknown, it was necessary to establish its general behavior with a number of model systems (Figure 2). For example, the quenching of the phosphorus resonance from diethyl phosphate in aqueous solution was studied to test the action of the reagent on a typical orthophosphate diester. The reagent quenches the signal effectively with complete suppression occurring at a Mn/P ratio of 1 suggesting formation of equimolar complexes at the end point. During the titration, the phosphorus resonance

signal underwent progressive broadening, and this may account for the shape of the titration curve which was based upon intensity (amplitude) measurements and not signal areas. Insofar as could be determined in this and the other titrations described, the observed signal areas remained constant throughout the titration experiment. All available evidence indicates that diethyl phosphate exists as discrete hydrated ions in dilute neutral (Na^+ countercation) aqueous solutions (Van Wazer, 1958) so that the phenomenon can be considered as arising from the simple dipolar interaction of the paramagnetic spin of the metal ion with the spin of the phosphorus atom. Since, with diethyl phosphate, complete quenching of the signal occurred at a Mn^{2+}/P ratio of 1 it is suggested that the quenching phenomenon results from coordination of the phosphoryl grouping to the Mn^{2+} ion through displacement of either water or of a coordinating functional group of the chelating EDTA ion.

The two major phospholipids of the serum lipoproteins, PC and SPH, were also tested. Because of solubility requirements the titrations were carried out in anhydrous methanol with an appropriately modified reagent, (n-Bu)$_4$N$^+$/Mn^{2+}/EDTA (4.4:1:2.2). In anhydrous methanol these phospholipids occur as the monodisperse species (Wells, M.A., University of Arizona, personal communication) and, as anticipated, they interact with the reagent in a manner analogous to that observed for diethyl phosphate in water (Figure 2).

Egg PC vesicles in the aqueous buffer were also tested for their response to the Mn^{2+}–EDTA reagent. This sample, unlike those above, is composed of associated phospholipid molecules. The vesicles consist of a phospholipid bilayer, where one of the polar surfaces of the bilayer is exposed to the external aqueous environment while the other is exposed to an internal environment and is, therefore, sequestered from the bulk solvent (Huang, 1969). In these vesicles, which are about 250 Å in diameter, the ratio of external to internal phospholipid groupings approaches unity.

The intensity of the PC signal from these vesicles established a plateau at 50% intensity when the Mn^{2+}/P ratio was about 2. The value decreased to ca. 40% at an Mn^{2+} ratio of 4:1 (Figure 2). We interpret this titration curve to indicate that the portion of the signal intensity which is lost corresponds to those phosphate groups exposed to the bulk water and that which remains corresponds to those groupings on the interior surface of the phospholipid bilayer of the vesicles (see also Michaelson et al., 1973; Bystrov et al., 1971).

For the serum lipoproteins, two fundamentally different curves were obtained represented by that from HDL$_3$ and LDL. The curve from LDL approximates, within experimental error, that obtained from egg PC vesicles, i.e., a plateau was established corresponding to 50% of the original LDL–PC peak height. Further, the shapes of the curves were indistinguishable. The curve from HDL$_3$, in contrast, has a plateau at a value corresponding to 20% of the original peak height.

These data are interpreted to indicate that in LDL only 50% of the PL phosphate groupings are accessible to the paramagnetic reagent whereas in HDL$_3$ about 80% are accessible. Significantly, in HDL$_3$, 20% of these groupings are not accessible.

The curve for HDL$_2$ was similar to that from HDL$_3$ with the exception that the plateau was established at somewhat higher concentrations of the reagent and the residual signal at the plateau corresponded to somewhat lower percentages

(17%) than for HDL$_3$. The observed differences between the curves of HDL$_3$ and HDL$_2$ are experimentally significant.

All of the curves were reproducible regardless of whether a given lipoprotein was obtained from the same or a different normal male donor, and, further, the spectral changes induced by the quenching reagent were fully reversible upon its removal by exhaustive dialysis against the original buffer. If the samples of PC vesicles, LDL, or HDL$_3$ were subjected to sonication (a process which facilitates the equilibration between the internal and external aqueous phases) (Branson Sonifier Model 1850, Plainview, L.I., N.Y., 5-sec sonication at 75 W with a microtip probe placed in the sample containing 0.05% Titron X-100) at the point where the Mn^{2+}/P ratio was 4, the residual signal was totally extinguished.

Consistent with earlier ^{31}P observations with the phospholipids (Henderson et al., 1974a), a selective upfield shifting (6–8 Hz for LDL, 4–6 Hz for HDL at Mn^{2+}/P = 0.5) and broadening of the small low-field signal of the serum lipoproteins (arising from lipoprotein phospholipids other than PC, principally SPH) were observed during the titrations when the Mn^{2+}/P ratio was less than 1. Beyond this ratio the signals were too broad to be resolved. The extent of the shift depends on the lipoprotein and was less for HDL$_2$ and HDL$_3$ than it was for LDL. The significance of these results is not apparent at this time; however, in light of previous observations (Henderson et al., 1974a) it is likely that the phenomenon reflects the different affinities of the various phospholipids for the EDTA chelated Mn^{2+} ion rather than the accessibility of these different phospholipid-phosphate groupings for the reagent.

The effects described above appeared to be concentration independent since a tenfold dilution of either the samples or the reagent or both had no effect on the nature of the curves of Figure 2.

In all cases, the observed quenching resulting from any increment of reagent showed no detectable time dependence over periods of time extending from that of the initial measurement (as early as 15 min) to 2 weeks (storage at 4°).

The total signal area, relative to the known concentration of the pyrophosphate standard, of all of the various preparations described agreed, within experimental error ($\pm 2.5\%$), to that expected from total phosphate determinations on these preparations, and it is, therefore, concluded that all of the phospholipid–phosphates contributed to the total signal area; i.e., there were no unobservable phospholipid–phosphates.

Discussion

The interaction of an unpaired electron with the nuclear spin of a spin $\frac{1}{2}$ nuclide will, in general, affect the high-resolution NMR spectrum of the perturbed nucleus in one of two ways. If the interaction is such that the transverse relaxation time, T_2, of the perturbed nucleus is substantially shortened the resultant NMR signal is broadened to the extent that it can no longer be differentiated from the base line; the signal, in effect, disappears. This is the case when the Mn^{2+} ion interacts with the phosphorus atoms of phosphoryl groupings in ^{31}P NMR spectroscopy (Van Wazer and Glonek, 1972). The magnitude of the effect depends on the concentrations of the various constituents in the sample and the lifetime of the interactions (Michaelson et al., 1973); the effect is not readily assessed beforehand.

If the interaction is such that the T_2 relaxation time re-

mains essentially unchanged the resultant NMR signal will only reflect changes in the chemical shielding of the perturbed nuclide, and the spectra will show well-resolved signals from the affected atoms shifted either upfield or downfield from their normal position in the spectrum (Hinckley, 1969). This is the effect observed upon treatment of a sample with the well known lanthanide shift reagents Eu(fod)$_3$ and Pr(fod)$_3$ as well as other similar lanthanide reagents (Hinckley, 1969). The magnitude of these shift changes may be quite large.

Because of their chemistry, the usual lanthanide chelate shift reagents have not been widely applied in studies involving phosphorus magnetic resonance. Their low solubility in water currently precludes their use in the study of aqueous systems of phosphates. Further, lanthanide phosphates are insoluble substances as a rule (Van Wazer, 1958) and treatment of a phosphate-containing sample with a lanthanide ion frequently results in the formation of insoluble precipitates of lanthanide phosphates. They may, however, prove quite useful in studies involving organic solutions of phosphorous-containing molecules.

Quenching Reagents. For aqueous systems the hydrated europium ion has been employed with some success (Assman et al., 1974; Michaelson et al., 1973; Bystrov et al., 1971) as a shift reagent for phosphates. Europium^{3+} ion (Eu(NO$_3$)$_3$) causes phosphate signals to be shifted 40–50 ppm to higher fields with about fivefold broadening (partial quenching) (Michaelson et al., 1973). However, when titration of HDL$_3$ with Eu^{3+} was attempted using conditions similar to Assman et al. (1974), lipoprotein precipitation occurred, even after addition of small amounts of titrant (Table I). Thus, it was desirable to formulate a reagent which was selective in its action with respect to the observed phosphate resonances and which induced no detectable permanent changes in the structure of these complexes.

With aqueous systems of phosphates it appears that the manganese–EDTA reagent herein described has properties which may prove useful in determining the accessibility of phosphates in macromolecular structures to the bulk medium.

Paramagnetic Titrations. The paramagnetic titration data may be interpreted in a number of ways. The obvious interpretation, and the one which appears to us most likely, is that the broadening of the ^{31}P NMR PL resonance and the concomitant loss of signal amplitude reflect the relative accessibility of the phosphate groups to the relaxation effects of the paramagnetic Mn^{2+} ion in the EDTA chelate. In the case of the sonicated egg PC vesicles, which contain a core of aqueous medium separated and isolated from the bulk solvent, it seems most reasonable to explain the effect in terms of PL phosphate groups which are inside and hence isolated, as compared to those which are outside and susceptible to the reagent. In the case of LDL, a similar interpretation is plausible, although other explanations are possible. Some of the PL phosphate groups could be bound or covered by protein and this would render them inaccessible to the paramagnetic relaxation effect. This is an alternative interpretation for the quenching of the LDL signal and is the more probable explanation for the quenching observed for HDL$_2$ and HDL$_3$.

Alternatively, the quenching effect may reflect a selective affinity of some of the PL phosphate groups for the chelated Mn^{2+} ion. A selective affinity may be exhibited by the different PL classes or a different, special accessibility of some PL's of the same PL class. This is an unlikely explanation in the case of the egg PC vesicles since all of the PL molecules are similar, although possible differences in fatty acid composition may cause selective affinity. Even in this instance, however, it is difficult to conceive of any type of interaction, due only to different fatty acid composition of the egg PC molecules, which would render half of them insensitive to the paramagnetic relaxation effect (Figure 2).

In the case of the serum lipoproteins, the protein moiety may provide a selective affinity factor (other than that of covering the phosphates) which may reduce the susceptibility of the PL phosphates to the paramagnetic relaxation effect of the EDTA chelated Mn^{2+} ion. The present data do not exclude such a possibility.

Assuming that the differential paramagnetic quenching effect reflects differences in accessibility of the PL phosphate groups to the reagent, our data indicate that there is a great difference in accessibility between the two major classes of native lipoproteins, HDL and LDL (Figure 2). X-Ray diffraction studies (Mateu et al., 1972) have indicated that LDL may contain a concentric PL bilayer similar to those known to exist in sonicated vesicles. Indeed, the responses of both LDL and PL vesicles to the Mn^{2+} reagent were indistinguishable (Figure 2).

Currently, there is no evidence to indicate that a bilayer structure is present in the HDL. Indeed, Assman et al. (1974) concluded from ^{31}P data using europium ion that all of the PL phosphates of HDL were accessible to the medium. However, we were unable to duplicate their results either using their published procedure with dilute 0.01 M tris(hydroxymethyl)aminomethane buffer (Assman and Brewer, 1974) or our more concentrated 0.15 M NaCl–EDTA buffer. Our data with Mn^{2+}/EDTA (1:2.2) indicate that the bulk (80%) of the PL in the HDL are exposed to the medium. Nonetheless, there exists a consistent percentage of PL phosphates (20%) which do not respond to the chelated Mn^{2+} ion. It is suggested that this amount corresponds to PL whose polar head groups are prevented from interacting with the chelated Mn^{2+} ion, possibly through their interaction with the HDL apoproteins.

The different quenching of PL phosphate resonances observed between the EDTA chelated, as compared to the hydrated Mn^{2+} ion, should be emphasized (Figure 2). Transition metal–phosphate interactions are known to be strong (Van Wazer, 1958) and could disrupt associations between the proteins and the PL phosphate head groups. The EDTA chelated transition metal ions interact with phosphates to a far lesser extent (Van Wazer and Callis, 1958); furthermore, their size may prevent their penetration into the lipoprotein particles. The smaller hydrated Mn^{2+} ion, on the other hand, may be able to penetrate the lipoprotein particle (perhaps through substitution of the Mn^{2+}-coordinated water groups by phosphate or peptide functional groups), causing complete PL phosphate signal quenching. Thus, the Mn^{2+}/EDTA (1:2.2) chelate reagent appears to represent a useful probe in structural studies on serum lipoproteins. This reagent, unlike the unchelated Mn^{2+} ion or any of the lanthanide ions commonly used in NMR signal quenching experiments, does not affect the stability of lipoproteins and interacts with them in a reversible manner.

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