3829.
Riesen, W. F., Braun, D. G., & Jaton, J. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2096.
Rudikoff, S., & Claflin, J. L. (1976) *J. Exp. Med. 144,* 1294.
Rudikoff, S., & Potter, M. (1974) *Biochemistry 13,* 4033.
Rudikoff, S., & Potter, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2109.
Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., & Davies, D. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4298.
Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., & Ballantyne, D. L. (1971) *Biochemistry 10,* 4912.
Summers, M. R., Smythers, G. W., & Oroszlan, S. (1973) *Anal. Biochem. 53,* 624.
Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem.* (in press).
Vrana, M., Rudikoff, S., & Potter, M. (1977) *Biochemistry 16,* 1170.
Vrana, M., Rudikoff, S., & Potter, M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Weigert, M., Cesari, M., Yonkovich, S. J., & Cohn, M. (1970) *Nature (London) 228,* 1045.
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1977) *Anal. Biochem. 77,* 569.

Location and Interactions of Phospholipid and Cholesterol in Human Low Density Lipoprotein from ^{31}P Nuclear Magnetic Resonance†

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ABSTRACT: The major phospholipids, phosphatidylcholine and sphingomyelin, of low density lipoprotein (LDL) are accessible to small amounts of Pr^{3+}, suggesting that the head groups of all mobile phospholipids are on the surface of the particle in contact with the aqueous medium. The major source of the nuclear Overhauser effect enhancement of ^{31}P resonances is the *N*-methyl proton of the choline moiety, indicating close *N*-methyl phosphate group interactions, probably similar to those found previously in phospholipid vesicles. This behavior of the phospholipid head groups in LDL is similar to that in small vesicles without cholesterol, suggesting that in LDL most of the cholesterol is not associated with mobile, surface phospholipids. In contrast to LDL, where the presence of a large protein immobilizes some phospholipid head groups, immobilization does not occur in high density lipoprotein, consistent with occurrence of smaller peptides in the latter.

Human low density lipoprotein (LDL)[1] is the major serum carrier of cholesterol, plays a role in the control of biosynthesis of cholesterol, and is implicated in the formation of atherosclerotic plaques. The overall molecular organization suggested is that of a spherical particle with a neutral lipid core surrounded by phospholipids and protein with their polar portions encountering the solvent (for a recent review, see Morrisett et al., 1975). Such molecular details have only begun to be established experimentally, however. The most recent small-angle x-ray scattering data have been interpreted as arising from a monolayer of phospholipid and protein around a core of cholesterol esters containing some triglyceride (Deckelbaum et al., 1975; Tardieu et al., 1976; Deckelbaum et al., 1977). The cholesterol esters in the core are sufficiently organized to undergo a phase transition (Atkinson et al., 1977).

In order to obtain more molecular information, we have explored the behavior of the phospholipid portion of LDL using ^{31}P NMR. Since the only significant phosphorus in the particle is in the phospholipid, no ambiguities exist concerning the location of this nonperturbing probe. Previously it was found that about one-fifth of the phospholipid of LDL is immobilized by the B protein (Yeagle et al., 1977a). In the present study, the evidence suggests that all the mobile phospholipid head groups reside on the surface of the particle, that the unesterified cholesterol may not be associated with the surface phospholipids, and that the head-group conformations of the major phospholipids are similar to their conformations in phospholipid vesicles.

Materials and Methods

Human plasma low density lipoprotein ($1.019 < d < 1.063$) was isolated from several donors and characterized as previously described (Yeagle et al., 1977a). Human high density lipoprotein ($1.063 < d < 1.21$) was isolated by flotation, washed, and further purified on a Sepharose CL-6B column. Their purity was evaluated by immunoelectrophoresis and by $NaDodSO_4$-polyacrylamide gel electrophoresis as previously described (Yeagle et al., 1977a). Egg phosphatidylcholine and brain sphingomyelin were purchased from Avanti Biochemicals and cholesterol from General Biochemical. TRTPCK

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[1] Abbreviations used are: LDL, low density lipoprotein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOEE, nuclear Overhauser effect enhancement; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate.

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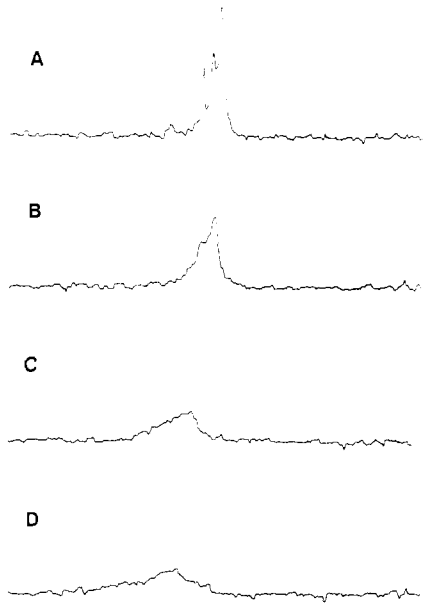

FIGURE 1: Titration of ^{31}P NMR spectrum of human LDL with Pr^{3+} in 2 mM phosphate, pH 6.8. (A) LDL (without Pr^{3+}) with the upfield peak from phosphatidylcholine, the middle peak from sphingomyelin, and the downfield (left-hand) peak from the phosphate buffer. (B) LDL plus 2 mM Pr^{3+}. (C) LDL plus 4 mM Pr^{3+}. (D) LDL plus 6 mM Pr^{3+}.

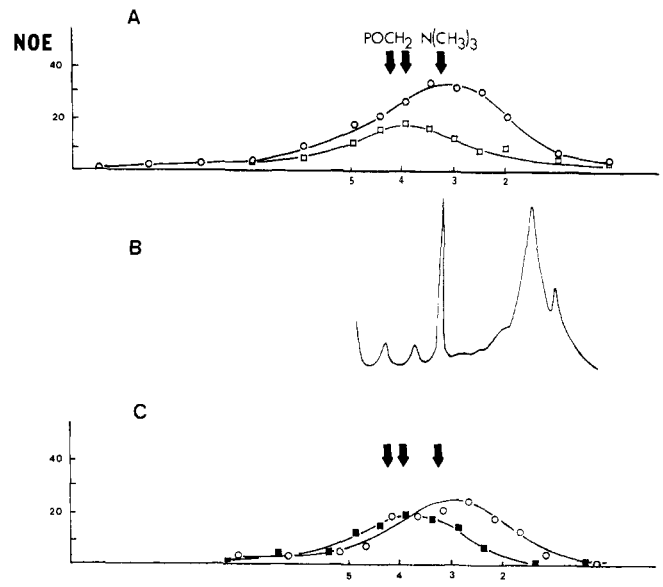

FIGURE 2: Frequency dependence of the ^{31}P [^1H]NOE. The magnitude of the [^{31}P]NOEE (percent increase in resonance intensity from proton decoupling) is plotted as a function of the portion of the proton spectrum of the phospholipids irradiated by the CW ^1H decoupler. The position of the maximum indicates the proton source of the NOE as described in the text. The scale for the proton decoupler is in ppm from tetramethylsilane. (A) (○) Egg phosphatidylcholine vesicles and (□) egg phosphatidylcholine vesicles containing 40 mol % cholesterol. (B) ^1H NMR spectrum of egg phosphatidylcholine vesicles. (C) (○) Human LDL and (■) phosphatidylcholine/sphingomyelin/cholesterol (2:2:1) vesicles. The arrow labeled $N(CH_3)_3$ represents the ^1H NMR resonance position of the *N*-methyl protons of the choline moiety, and the other two arrows represent the ^1H NMR resonance positions of the methylene protons next to the phosphate of the phospholipids.

trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical.

Vesicles were prepared by sonication with a Heat Systems W-350 sonifier until clear, in 100 mM NaCl, 10 mM EDTA, D_2O solution (Huang, 1969). Mixed lipid systems were colyophilized from benzene before sonication. LDL samples were dialyzed against 2 mM phosphate buffer, pH 6.8, 1 mM EDTA, before use. Trypsin treatment was performed as described previously (Margolis & Langdon, 1965; Yeagle et al., 1977a).

The 40.48-MHz ^{31}P NMR spectra were obtained with a JEOL PS100/EC100 Fourier transform spectrometer at 23 °C. T_1 measurements were obtained with the 180°-τ-90° pulse sequence. Intensity measurements were obtained with 90° pulses separated by four to five T_1 and the decoupler was gated to remove the NOE. The proton frequency dependence of the NOEE was determined as described previously for the vesicles (Yeagle et al., 1977b); however, for LDL samples three determinations were obtained for each point and intensities evaluated by cutting and weighing copies of the spectra. Intensity measurements for high density lipoprotein were obtained without NOE and compared with the intensity of a known amount of internal HPO_3^- standard by computer integration. This result was compared with the phosphate content of the sample determined by the method of Bartlett (1959). By this means the percent of the phospholipids in the sample contributing to the ^{31}P resonance was determined.

Results

Shift Reagents. Lanthanide shift reagents have been widely used to measure the accessibility of phospholipids from the aqueous medium. For example, single bilayer phospholipid vesicles of egg lecithin are impermeable to the lanthanide shift reagents, so that only the nuclear magnetic resonances from phospholipids residing on the exterior of the vesicle are shifted by the shift reagents. Pr^{3+} shifts the phosphorus resonances of exterior phospholipids downfield, leaving the resonances due to phospholipids on the inside of the vesicle unshifted. With the resonances thus separated and with care taken to ensure that the intensities of the resonances are proportional to the concentration of the molecules, the ratio of phospholipids on the outside of the vesicle to those on the inside of the vesicle can be determined. A value of 2.1 has been obtained for egg phosphatidylcholine vesicles (Hutton et al., 1977), which is consistent with geometrical considerations of the vesicle structure (Mason & Huang, 1978).

In a similar way, Pr^{3+} was used to measure the amount of LDL phospholipid accessible to the water soluble shift reagent. The results of a titration with Pr^{3+} of ^{31}P NMR resonances from the LDL phospholipids appear in Figure 1. Spectrum A shows the ^{31}P NMR spectrum of native LDL with a phospholipid concentration of about 30 mM. The upfield (right-hand) peak arises from phosphatidylcholine and the middle from sphingomyelin, the two major phospholipids of LDL. The downfield peak arises from the phosphate buffer. As can be seen in subsequent spectra, addition of Pr^{3+} shifts the ^{31}P resonances downfield, but in contrast to the results with vesicles, no resonances are left unshifted. Therefore all the unimmobilized phospholipids in LDL are accessible to the water soluble shift reagent. Trypsin-treated LDL was unstable to the presence of the shift reagent.

NOE Measurements. Recently a method was developed which allowed the determination of phospholipid head-group conformation in phosphatidylcholine and sphingomyelin vesicles (Yeagle et al., 1977b). The method is illustrated in Figure 2. When the protons in a sample are saturated, the phosphorus resonance can exhibit an increase in intensity, the nuclear Overhauser effect, NOE (Yeagle et al., 1975a). NOE is observed for phospholipids in vesicles (Yeagle et al., 1975b) and, since it arises from dipolar interactions between the phosphorus

and the protons, one can ask which protons in the molecule are causing the NOE. That question can be answered by measuring the magnitude of the NOE enhancement (NOEE) as a function of which protons are being saturated. In Figure 2, the nuclear Overhauser effect enhancement, which is the percent increase in resonance intensity upon irradiation of the protons, is plotted as a function of proton decoupler frequency. For phospholipid systems without cholesterol, a maximum is observed at the frequency corresponding to the *N*-methyl protons of the choline moiety.

Cholesterol in sufficiently high concentrations has been shown to disrupt the intermolecular interactions between phospholipid head groups in phospholipid vesicles (Yeagle et al., 1977b). Representative data for the frequency dependence of the NOEE for egg phosphatidylcholine vesicles containing cholesterol are graphed in Figure 2A. Addition of high cholesterol leads to a shift in the maximum of the plot from the *N*-methyl protons to the methylene protons (Yeagle et al., 1977b). As a consequence the NOE serves as an indicator of the presence of cholesterol in the phospholipid portion. That a similar effect can be observed in mixed vesicles of phosphatidylcholine and sphingomyelin can be seen in Figure 2C. In these vesicles separate resonances for each of the phospholipid classes permit simultaneous, independent observation of each. The data for the resonances arising from both phosphatidylcholine and sphingomyelin in mixed vesicles containing no cholesterol are superimposable upon the data for pure phosphatidylcholine vesicles. However, in vesicles containing cholesterol at a mole ratio of phosphatidylcholine:sphingomyelin:cholesterol of 2:2:1 the position of the maximum NOE shifts in the same way that it does when cholesterol is present in phosphatidylcholine vesicles. At a mole ratio of 1:1:1, the proton resonance lines become too broad to permit measurements of this kind.

The purpose of these model studies was to provide a background for the study of LDL by the same methods. The frequency dependence of the NOEE for LDL appears in Figure 2C. As with all other such experiments, a single smooth curve is drawn through the points since the inherent resolution of the experiment does not allow fine structure such as more than one maximum in the curve to be observed. The maximum occurs in the same place as it does for phosphatidylcholine, sphingomyelin, and mixed phosphatidylcholine/sphingomyelin vesicles without cholesterol. Trypsin treatment of the LDL had no effect on the position of the maximum NOE.

HDL. Our previous measurements of the ^{31}P NMR resonance intensities of native LDL revealed that about one-fifth of the phospholipid was not contributing to the high resolution resonance. Since trypsin cleavage of the B peptide of LDL restored the full resonance intensity, it was suggested that, in the native particle, one-fifth of the phospholipid was immobilized by interaction with the protein (Yeagle et al., 1977a). In a similar manner, intensity measurements were obtained for human high density lipoprotein ^{31}P NMR spectra. In contrast to the results with LDL, little or no immobilization of phospholipids was seen in three independent measurements on samples from three different donors. Samples from a fourth donor suggested about one-fourth of the phospholipids were immobilized. The reason for the discrepancy with the other donors is uncertain, but $NaDodSO_4$–polyacrylamide gel electrophoresis revealed an abnormal apoprotein pattern in that sample.

Discussion

The accessibility of all the phospholipids of LDL (actually four-fifths of the phospholipid observed in the ^{31}P NMR spectrum of native LDL) to the shift reagent is consistent with a monolayer of phospholipid on the outside of the lipoprotein particle. There has been some debate based on x-ray data between a bilayer and a monolayer of phospholipid in the structure of LDL. The most recent evidence favors a surface monolayer (Tardieu et al., 1976). In such a structure, all the phospholipid would be available for interaction with shift reagents, as is observed here. A compositional analysis also suggests that all the phospholipid should be on the surface (Shen et al., 1977). A bilayer structure could be consistent with the shift reagent results presented here only if the bilayer was freely permeable to metal ions. Since phospholipid vesicles without ion carriers are impermeable to metal ions, this seems improbable, and the monolayer model remains the most attractive for LDL. This is analogous to the results obtained with high density lipoprotein (Assmann et al., 1974).

A previous attempt to explore the distribution of phospholipids in LDL with lanthanides was unsuccessful (Henderson et al., 1975). They used a high pH (7.2) so that the lanthanides no longer exist as simple aquo ions, but also as hydroxo complexes. An effort in this laboratory to employ Pr^{3+} as pH 7.8 resulted in no shifting of the phosphorus resonances at low metal ion concentrations, but some precipitation at high metal ion concentrations. Henderson et al. (1975) also attempted to employ Mn^{2+}/EDTA (1:2.2) as a broadening agent at pH 7.2 to measure the accessibility of the phospholipids of LDL and only about 50% of the resonances were affected. Differences between their results and ours may be ascribed to the high molar concentrations of Mn^{2+} used (greater than that of the phosphorus, while our lanthanide to phosphorus ratio is only 0.1), and to the large size of the negatively charged Mn–EDTA chelate. Their chelate gives an anomalously low result for the outside/inside ratio of phospholipids in vesicles.

The location of cholesterol in LDL is a problem important to the structure of the particle as a whole. Currently free cholesterol is felt not to be in the core of the particle. The core is thought to consist of cholesterol esters, along with some triglyceride, which are capable of undergoing a smectic–disordered phase transition similar to that seen with pure cholesterol esters (Atkinson et al., 1977). Free cholesterol does not mix well with cholesterol esters and triglycerides (Small, 1970), and because of its polar hydroxyl group the cholesterol most likely resides in the surface monolayer of lipid and protein surrounding the cholesterol ester core.

If cholesterol is located in the surface of the LDL particle, one question which could be asked is whether the cholesterol is distributed throughout the phospholipid monolayer, or associated with the protein component. The results of this investigation suggest that most of the free cholesterol is not located among the phospholipids of LDL. The frequency dependence of the ^{31}P [^{1}H]NOEE exhibits maxima which are diagnostic of the presence of cholesterol as demonstrated by vesicle studies with phosphatidylcholine or phosphatidylcholine and sphingomyelin. The position of maximum NOEE shifts as the cholesterol content is increased so that, in mixtures containing more than about 30 mol % cholesterol, the maximum occurs at the phosphate methylene protons rather than at the resonance position of the *N*-methyl protons. Thus it is possible to distinguish between high cholesterol contents (30 mol % or greater) and low cholesterol contents (10 mol % or less). Insufficient resolution is available in the data to fully characterize intermediate cholesterol contents.

In LDL, although the mole ratio of cholesterol to phospholipid is about 0.8, the maximum in the NOEE occurs at the *N*-methyl proton position; despite the high cholesterol content, the LDL phospholipids behave as though they were in a vesicle

with low or no cholesterol. Because vesicles consist of a bilayer and LDL apparently a monolayer of phospholipid, the validity of the model system might be questioned. However, the outer half of the vesicle bilayer is a monolayer with about the same radius of curvature as the monolayer in LDL. Likewise the presence of protein might change the system in some undefined way, leading to different phospholipid head-group behavior in LDL than in the vesicle. However, the motional parameters for the head groups as measured by ^{31}P [^{1}H]NOE and T_1 are essentially the same in both systems (Yeagle et al., 1977a). Furthermore, trypsin treatment does not affect the motional or conformational properties of the phospholipid head groups.

An interesting (though not unique) explanation of these results is that most of the cholesterol is associated, not with the phospholipids, but with the protein of LDL. This conclusion is consistent with the results from a fluorescent derivative of cholesterol which suggested association between the steroid and the protein (Smith & Green, 1974), and computational analysis led some investigators to suggest a similar possibility (Shen et al., 1977).

It is possible that some cholesterol may be associated with the phospholipids and still produce these results; as much as 15–20% of the free cholesterol in LDL could be located in the phospholipid, and this technique would not be able to detect it. Furthermore, rapid exchange of the cholesterol between the protein and the phospholipid is not ruled out, as long as the cholesterol spends most of its time next to the protein. If a perturbation of the cholesterol ester core by the protein permitted the incorporation of some free cholesterol, these results could be explained by a partitioning into the core of the majority of the free cholesterol (Deckelbaum et al., 1977), rather than an association with the protein.

The major source of the NOE in LDL, and phosphatidylcholine, sphingomyelin, and mixed phosphatidylcholine–sphingomyelin vesicles, is the *N*-methyl protons which must be in close contact with the phosphate group. Evidence similar to this in other phospholipid systems led to a model of the head-group conformation of phosphatidylcholine and sphingomyelin in which the head group was predominantly parallel to the surface of the bilayer, with the positively charged *N*-methyl interacting intermolecularly with the negatively charged phosphate on a neighboring phospholipid (Yeagle et al., 1975b, 1977b). The data presented in Figure 2C suggest that the phospholipid head-group conformations are likely similar in LDL and phospholipid vesicles.

Recent data from this laboratory demonstrated that about one-fifth of the phospholipid in native LDL was immobilized by interaction with the protein (Yeagle et al., 1977a). It was possible to remove the immobilization by trypsin cleavage of the protein. As noted in the results, trypsin cleavage does not free the cholesterol to interact with the phospholipid. While it could be proposed that the phospholipid was immobilized in crevasses in the protein which were destroyed by trypsin cleavage, a similar explanation cannot be offered for cholesterol.

In contrast to LDL where one-fifth of the phospholipid is sufficiently immobilized so as not to be apparent in ^{31}P magnetic resonance, virtually all of the phospholipid is evident in HDL. These contrasting results indicate that there is a difference in the structure of the protein components of the two lipoproteins.

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References

Assmann, G., Sokoloski, E. A., & Brewer, H. B., Jr. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 549.

Atkinson, D., Deckelbaum, R. J., Small, D. M., & Shipley, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 1042.

Bartlett, G. R. (1959) *J. Biol. Chem. 234,* 466.

Deckelbaum, R. J., Shipley, G. G., Small, D. M., Lees, R. S., & George, P. K. (1975) *Science 190,* 392.

Deckelbaum, R. J., Shipley, G. G., & Small, D. M. (1977) *J. Biol. Chem. 252,* 744.

Henderson, T. O., Kruski, A. W., Davis, L. G., Glonek, T., & Scanu, A. M. (1975) *Biochemistry 14,* 1915.

Huang, C. (1969) *Biochemistry 8,* 344.

Hutton, W. C., Yeagle, P. L., & Martin, R. B. (1977) *Chem. Phys. Lipids 19,* 255.

Margolis, S., & Langdon, R. G. (1965) *J. Biol. Chem. 241,* 485.

Mason, J., & Huang, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 308.

Morrisett, J. D., Jackson, R. L., & Gotto, A. M., Jr. (1975) *Annu. Rev. Biochem. 44,* 183.

Shen, B. W., Scanu, A. M., & Kézdy, F. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 837.

Small, D. M. (1970) in *Surface Chemistry of Biological Systems* (Blank, M., Ed.) Plenum Press, New York, N.Y.

Smith, R. J. M., & Green, C. (1974) *Biochem. J. 137,* 413.

Tardieu, A., Mateu, L., Sardet, C., Weiss, B., Luzzati, V., Aggerbeck, L., & Scanu, A. M., Jr. (1976) *J. Mol. Biol. 101,* 129.

Yeagle, P. L., Hutton, W. C., & Martin, R. B. (1975a) *J. Am. Chem. Soc. 97,* 7175.

Yeagle, P. L., Hutton, W. C., Huang, C., & Martin, R. B. (1975b) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3477.

Yeagle, P. L., Langdon, R. G., & Martin, R. B. (1977a) *Biochemistry 16,* 3487.

Yeagle, P. L., Hutton, W. C., Huang, C., & Martin, R. B. (1977b) *Biochemistry 16,* 4345.