Lipid Domains in the Crystalline Lipovitellin/Phosvitin Complex: A Phosphorus-31 and Deuterium Nuclear Magnetic Resonance Study†

Leonard J. Banaszak* and Joachim Seelig

ABSTRACT: The crystalline lipovitellin/phosvitin complex has a molecular weight of 456 000 and contains nearly 100 molecules of bound phospholipid. Earlier work using electron microscopy and three-dimensional image reconstruction methods established the symmetrical dimeric nature of this lipoprotein, but the organization of the lipid was unknown. Under conditions where the lipoprotein is in solution, the high-resolution ^{31}P nuclear magnetic resonance (NMR) spectra contain two well-resolved peaks which can be assigned to phosphoserine moieties in both lipovitellin and phosvitin and to the phospholipid microdomains. The spin–lattice relaxation times, T_1, for the phosphoserines and the phospholipid head groups are distinctly different, with the serine phosphates having faster reorientation rates. ^{31}P NMR spectra of crystalline lipoprotein contain a broad symmetric component with a chemical shielding anisotropy of about −50 ppm. By obtaining ^{31}P NMR spectra from several modified forms of the lipoprotein as well as from the extracted lipids, it is shown that the chemical shielding anisotropy is characteristic of phospholipid in a bilayer arrangement. As a further check on ^{31}P NMR results, samples of the lipovitellin/phosvitin complex containing deuterium-labeled 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine were studied by ^{2}H NMR methods. The resulting spectrum has characteristics similar to those obtained from model lipid systems in a lamellar state. The results of the ^{31}P and ^{2}H NMR experiments correlate with the low-resolution model of the crystalline lipovitellin complex obtained by diffraction studies. It is proposed that each subunit of lipovitellin contains a microdomain of phospholipid in a bilayer-like arrangement.

Lipid/protein systems can be roughly divided into two classes. The membrane proteins are characterized by their ability to insert into a lipid bilayer and are generally insoluble in aqueous solutions in the absence of detergents. The second class of lipid/protein systems, more often simply called lipoproteins, is soluble in aqueous solutions. The lipid associated with both types of lipid/protein systems is often a heterogeneous mixture. Purified forms of both membrane and soluble lipoproteins have not been easy to study by methods which can yield data about their overall molecular organization. During the last few years, crystalline or paracrystalline forms of a few membrane proteins have been prepared. In the case of soluble lipoproteins, three-dimensional crystals of one type, the yolk lipoprotein, have been found in vivo.

The microcrystalline form of the yolk lipoprotein, or as it is more frequently called, the lipovitellin/phosvitin complex, has been studied by X-ray and electron microscopic methods (Ohlendorf et al., 1975, 1978). At a resolution of about 20 Å, an electron microscopy (EM)[1] map of the crystalline lipovitellin/phosvitin complex from *Xenopus* shows that this lipoprotein is composed of two subunits related by a local 2-fold symmetry axis. The subunits aggregate in a manner which is similar to other soluble oligomeric proteins. This is a relatively important point since it implies that if the lipid is present in domains, they must obey the symmetry of the lipoprotein. Furthermore, it suggests that the lipid does not participate directly in the lipovitellin subunit aggregation but rather protein/protein interactions account for the overall oligomeric structure.

In addition to the subunit organization, the low-resolution model of the lipovitellin/phosvitin complex contained some rather crude indications of structural domains within each subunit. These domains could only be described in terms of their molecular volumes and then only by assuming that they could be approximated by ellipsoids of revolution (Ohlendorf et al., 1978). The location of the lipid in the three-dimensional structure was not known, but it was suggested that it was in a condensed form in the centralmost part of each subunit (Ohlendorf et al., 1978). This intuitively provides for maximum shielding of the hydrophobic fatty acyl chains whereas the polar groups could remain in contact with the aqueous environment.

By measurement of the X-ray data from the microcrystalline lipoprotein as a function of solvent electron density, a more definitive determination of the location of the lipid within the lipovitellin complex was obtained. The X-ray results suggested that there were two domains of low electron density within the lipovitellin/phosvitin complex which obeyed the known local symmetry of the molecule (Ross et al., 1980). In studies of model membranes, such regions of low electron density are associated generally with the CH_3 tail region of the aggregated fatty acyl chains (Luzzati & Tardieu, 1980). The position of the domains of low electron density as determined by X-ray methods coincided with the centralmost part of each lipovitellin subunit labeled as the *L* domain in the earlier structural study (Ohlendorf et al., 1978).

The dimeric lipovitellin complex from *Xenopus* contains several different polypeptide chains as well as both polar and neutral lipid. It has a molecular weight of 456 000, and it contains about 100 molecules of lipid (Ohlendorf et al., 1977).

† From the Department of Biophysical Chemistry, Biozentrum, University of Basel, Basel, Switzerland CH 4056. *Received October 19, 1981.* Supported by Swiss National Science Foundation Grant 3.746.80 and National Institutes of Health Grant GM 13925. L.J.B. is especially grateful to the Josiah Macy Foundation, New York, and the Roche Foundation, Basel, for their financial support during the studies.

* Address correspondence to this author at the Department of Biological Chemistry, Washington University Medical School, St. Louis, MO 63110.

[1] Abbreviations: T_1, spin–lattice relaxation time; τ_c, motional correlation time; EM, electron microscopy; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; LV/PV, lipovitellin/phosvitin complex; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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This number was based on the lipid composition and the fact that each subunit of the dimeric lipoprotein complex contains four polypeptide chains previously named *LV-A*, *LV-B*, *LV-C*, and phosvitin. These protein components have molecular weights of 105 000, 35 500, 32 000, and 17 000 (Ohlendorf et al., 1977). The 100 molecules of lipid (50 per subunit) in the lipovitellin complex have been shown to be present in the ratio of 70 to 30 for phospholipid to neutral lipid. The phospholipid is mainly diacylphosphatidylcholine, and more than half of the neutral lipid is in the form of triglycerides (Ohlendorf et al., 1977).

The four polypeptide components of the lipovitellin/phosvitin complex can be divided into two types. *LV-A*, *LV-B*, and *LV-C* are part of lipovitellin and in the native state cannot be easily dissociated from the lipid/protein complex. Phosvitin, on the other hand, can be removed from the lipovitellin without disrupting the lipid/protein system (Wallace, 1965). Phosvitin is a very unusual protein in that 66 out of 155 residues are serine, and most of these are thought to be phosphorylated in the native lipoprotein complex (Ohlendorf et al., 1977). In addition, both the *LV-B* and *LV-C* polypeptide chains contain a number of serine residues, 36 out of 323 and 28 out of 290, respectively (Ohlendorf et al., 1977). $NaDodSO_4$ gel staining methods indicated that these two protein components are also phosphorylated (Bergink & Wallace, 1974; Ohlendorf et al., 1977) although the extent of phosphorylation was unknown. The presence of phosvitin and phosphorylated serine residues in both the *LV-B* and *LV-C* polypeptide chains of lipovitellin is a factor which complicates the interpretation of ^{31}P NMR spectra of the lipovitellin complex.

In spite of the known lipid and protein composition of the lipovitellin/phosvitin complex, and the subunit organization derived from the EM and X-ray studies, the structural organization of the lipid within the lipoprotein is essentially unknown. Intuitively, there appears to be only two ways to envision the formation of this molecule. In the first way, the polypeptide components of lipovitellin provide unique binding sites for each lipid molecule. Clearly, 100 lipid binding sites spread throughout a complex globular protein system of M_r 380 000 seems highly unlikely. The second mode of lipid/protein organization would contain the lipid in some sort of packed form. Such a condensed lipid domain would somehow be bounded by the globular protein components of lipovitellin and is precisely what is predicted by the EM and X-ray studies. Moreover, the structural studies predict two such lipid domains, that is, 50 molecules of lipid in each subunit of lipovitellin (Ross et al., 1980). The use of magnetic resonance would provide a direct means for investigating the structural and dynamic properties of the lipids in the lipovitellin/phosvitin complex. In the present paper, we discuss the application of nuclear magnetic resonance, that is, ^{31}P and ^{2}H NMR. In the following paper, the results of spin-labeling EPR experiments are presented (Birrell et al., 1982).

The application of ^{31}P NMR methods to phospholipid/protein systems is possible in two different ways. If high-resolution spectra are obtained from the lipovitellin complex in solution, relatively narrow resonance corresponding to ^{31}P nuclei in the phospholipid or phosphoserine should be observed with any separation of the peaks in the spectra resulting from differences in their chemical shifts. Such spectra have been reported for the yolk lipoprotein system during various stages of embryological development (Colman & Gadian, 1976), and from several forms of the serum lipoproteins (Brasure et al., 1978; Assmann et al., 1974). The isotropic tumbling of the lipoprotein molecule in solution averages any anisotropic effects in the chemical shifts of the bound phospholipid. In the second method, ^{31}P spectra are obtained from immobilized forms of the lipoprotein, and the line shape of this so-called powder-type spectrum is characteristic of any chemical shift anisotropy of the phosphate group. The use of the ^{31}P line shapes from powder spectra for characterizing lipid systems has been discussed by Seelig and by Cullis and de Kruijff (Seelig, 1978; Cullis & de Kruijff, 1979).

Since the lipovitellin complex is obtainable in microcrystalline form, ^{31}P line shapes should be useful for characterizing the lipid domains of the lipovitellin complex. The NMR results can then be correlated directly with the molecular model obtained by EM and X-ray methods. Solid-state ^{31}P NMR spectra from large oocytes from *Xenopus* have been reported, but they were of poor quality due to technical limitations and were complicated by the presence of other identifiable phosphorus-containing compounds such as creatine and nucleotide phosphates (Colman & Gadian, 1976). In addition, the presence of metal ions in the oocytes alters the chemical shifts and in some instances may produce unusual line-broadening effects (Colman & Gadian, 1976). Although the purified microcrystalline form of the yolk complex is free of small phosphorus-containing compounds, ^{31}P NMR spectra of the crystalline lipovitellin/phosvitin will still be complicated by the presence of phosphoserine residues. Earlier NMR studies of phosvitin from yolk established the monoester nature of the phosphate linkage and variations in chemical shifts with pH (Ho et al., 1969). Similar results have been obtained with highly phosphorylated proteins from dentin (Lee et al., 1977; Roufosse et al., 1980). Although the phosvitin ^{31}P spectra are sensitive to pH, little effect by 8 M urea was observed (Ho et al., 1969). When NMR methods are used, the phosphoserine moiety appears to have an average pK of 6.5 (Ho et al., 1969).

In addition to identification of the state of the lipid domain from the line shape of the ^{31}P NMR spectra, information about the motional properties of the phospholipid and phosphoserine residues in the lipoprotein complex may be derived from the measurement of spin–lattice relaxation times (T_1).

Materials and Methods

Biochemical Preparations. Microcrystalline lipovitellin/phosvitin complex was prepared by centrifugation methods in sucrose solutions (Ohlendorf et al., 1977). The crystals were exhaustively dialyzed against water, lyophilized, and stored at −20 °C. The lyophilized crystals still produce X-ray diffraction patterns and can be reversibly suspended in water or dilute buffers, only producing small changes in the unit-cell dimensions. Purified lipovitellin free of phosvitin was obtained by the triethylaminoethylcellulose (TEAE) chromatography method of Wallace and was from D. H. Ohlendorf (Wallace, 1965). It was also stored as a lyophilized powder at −20 °C. Lipovitellin was also prepared by ammonium sulfate precipitation, avoiding the high pH conditions accompanying TEAE chromatography (Wallace et al., 1966). This was done by dissolving the crystalline lipoprotein complex (26 mg/mL) in a solution containing 1 M ammonium sulfate, 1 mM EDTA and thioglycerol, 0.05% NaN_3, and 10 mM Tris-HCl, pH 8.0. Trace amounts of insoluble material were removed by centrifugation, and lipovitellin was precipitated by the addition of an equal volume of saturated ammonium sulfate in the aforementioned buffer. The resultant precipitate was collected by centrifugation and reprecipitated 2 more times in the same buffer.

The microcrystalline lipoprotein could also be partially depleted of phospholipid without any overall denaturing effects

(Collins, 1977). Typically, 25 mg of lipovitellin/phosvitin crystals was extracted with 10 mL of acetone containing 5% 1 mM EDTA and 1 mM 2-mercaptoethanol, pH 7.0, in a dry ice/ethanol bath. The suspension was vortexed every 3 min for a total of 15 min. The acetone was removed by centrifugation, and the crystals were then washed 3 times with 1 mM EDTA/2-mercaptoethanol solution. If it is necessary to store the crystals, the EDTA and 2-mercaptoethanol are removed by dialysis against water. These crystals can then be lyophilized and stored at −20 °C. The amount of lipid removed under these mild conditions will be described under Results.

For preparation of samples of the lipovitellin lipid, 300 mg of the crystalline lipovitellin complex was extracted with 30 mL of a 1:2 v/v mixture of chloroform and methanol at room temperature. The extraction was carried out for 20 min with stirring. The apoproteins were then pelleted by centrifugation at 15000 rpm, and the chloroform/methanol extract was taken to dryness in vacuo. The extraction was repeated 2 times, but most of the lipid is contained in the first extract. The extracted lipid has a bright green color due to the presence of small amounts of biliverdin. It was stored dry at −20 °C until needed. In one lipid preparation, all transfers were done analytically, and the isolated lipid was weighed. The weight fraction of lipid present in the lyophilized lipoprotein crystal was 17% (w/w), in excellent agreement with earlier composition studies (Ohlendorf et al., 1977).

The denatured and lipid-free proteins resulting from the chloroform/methanol extraction were then used to prepare samples of the phosphoproteins (Hegenauer et al., 1979). The precipitate from the chloroform/methanol extraction was extracted for 24 h at 2–4 °C with 1 M KCl, pH 10.5. Insoluble protein, probably *LV-A* (Ohlendorf et al., 1977), was removed by centifugation, and the supernatant was dialyzed exhaustively against 0.01 N HCl. A white precipitate of the phosphoproteins formed during the dialysis. It was removed by centrifugation and lyophilized.

Deuterium-labeled phospholipid was introduced into the lipovitellin complex by using a phospholipid exchange protein (Jackson et al., 1978). Samples of the exchange protein were obtained from Dr. R. L. Jackson at the University of Cincinnati for the preliminary studies and Dr. Karel Wirtz at the State University of Utrecht for the exchange reactions needed in the preparative experiments. The exchange reaction and subsequent fractionation of the liposomal lipid and the lipovitellin complex were carried out in 10 mM Tris-HCl buffer containing 0.5 M NaCl, 1 mM EDTA, and 0.05% (w/v) NaN_3 at pH 8.5. Liposomes of 1,2-di[9,10-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine (DEPC) were prepared by sonication in the Tris/NaCl buffer at room temperature for about 1 h. During this sonication, heating was prevented by the occasional addition of ice to the bath surrounding the lipid sample. The sonication was stopped when the phospholipid solution became distinctly clear. The phospholipid exchange reaction was carried out for 5 h at 37–42 °C in a solution which contained 250 mg of lipovitellin complex, about 50 mg of liposomal DEPC, and 0.5 mg of the phospholipid exchange protein. Roughly this gives a liposomal to lipoprotein phospholipid ratio of 2 to 1. At the end of the incubation period, the sample was placed on a Sepharose 6B column (100 × 2.5 cm) containing the same Tris/NaCl buffer used for the exchange reaction. Figure 1 shows a typical elution pattern and indicates that the liposomes and lipoprotein complex can be purified readily by this single chromatography step. Note that the liposomes appear as a single symmetrical peak, probably because of their homogeneous size distribution. As can be seen in Figure 1, the lipovitellin complex can be judiciously pooled with no cross-contamination by liposomal lipid. It can be precipitated by dialysis against 0.01 M Tris without salt.

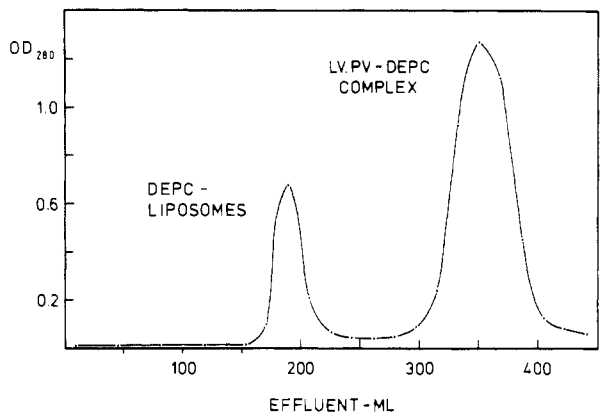

FIGURE 1: Elution profile of phospholipid liposomes and lipovitellin on Sepharose 6B. The Sepharose 6B column was about 100 cm long with a diameter of 2.5 cm. It was run at approximately 16 mL/h (2–5 °C). Separate runs were used to identify the elution points of liposomes and lipovitellin, and they are as indicated on the drawing.

Magnetic Resonance Measurements. All NMR measurements were carried out with a Bruker-Spectrospin CXP-300 spectrometer operating at ^{31}P and ^2H frequencies of 121.4 and 46.1 MHz, respectively. High-resolution ^{31}P NMR spectra of solutions were obtained with a pulse width of ~25 μs (90° pulse), a relaxation delay of 12 s, and a spectral width of 2 KHz. A broad-band proton-decoupling frequency (~10 W) was employed. Solid-state ^{31}P NMR spectra of wet pellets and crystals were recorded with pulses (90°) of ~5 μs and inverse-gated proton decoupling (~150 W). The spectral width was 31.25 kHz in this case. For the ^2H NMR spectra, the quadrupole echo technique was used (Davis et al., 1976). The 90° pulse width was ~4 μs and the separation of the echo pulse 30 μs. ^{31}P spin–lattice relaxation times (T_1) were measured by the conventional 180°–τ–90° method either without proton decoupling or under inverse-gated decoupling conditions. No differences in T_1 were observed for the two types of experiments.

Sample Preparation. For the ^{31}P high-resolution NMR experiments, the lipovitellin/phosvitin complex and its modifications were dissolved in 10 mM Tris-HCl buffer containing 0.5 M NaCl, 1 mM EDTA, and 0.05% (w/v) NaN_3 at pH 8.5. Since ^2H_2O was needed as a lock signal for the stabilization of the magnetic field, the buffer was made up from a $H_2O/^2H_2O$ mixture (50:50 v/v). A 10-mm NMR sample tube with ~1 mL of a saturated lipovitellin/phosvitin solution (~30 mg of lipoprotein/mL of buffer) was used. The same solution was also employed in some of the spin-label EPR studies to be discussed in the following paper (Birrell et al., 1982).

Solid-state NMR measurements of the lipovitellin/phosvitin complex were made by suspending the complex in essentially the same buffer as above with and without 0.5 M NaCl. After centrifugation, excess buffer was removed with a Pasteur pipet. Only the wet pellet was employed for the NMR measurements. Coarse liposomes were prepared by simply vortexing the extracted lipids with the buffer described above (with 0.5 M NaCl). The liposomes are mainly composed of phospholipids, but most, if not all, of the neutral lipid is also present in the sample.

Results

High-Resolution ^{31}P NMR. A high-resolution ^{31}P NMR spectrum of the lipovitellin/phosvitin complex in solution is shown in Figure 2A. Two well-resolved peaks are seen; the

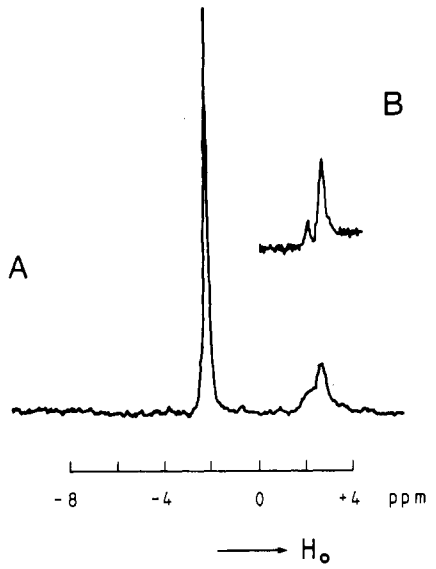

FIGURE 2: High-resolution ^{31}P NMR studies of the lipovitellin/phosvitin complex. The sample was dissolved in the standard Tris/NaCl buffer, pH 8.5, containing 50% D_2O. The concentration of lipoprotein complex was about 20 mg/mL. In one experiment, 85% H_3PO_4 was included in a capillary and used as an external standard. Measuring temperature: 20 °C. (A) Nondecoupled spectrum. (B) Phospholipid resonances under proton-decoupling conditions.

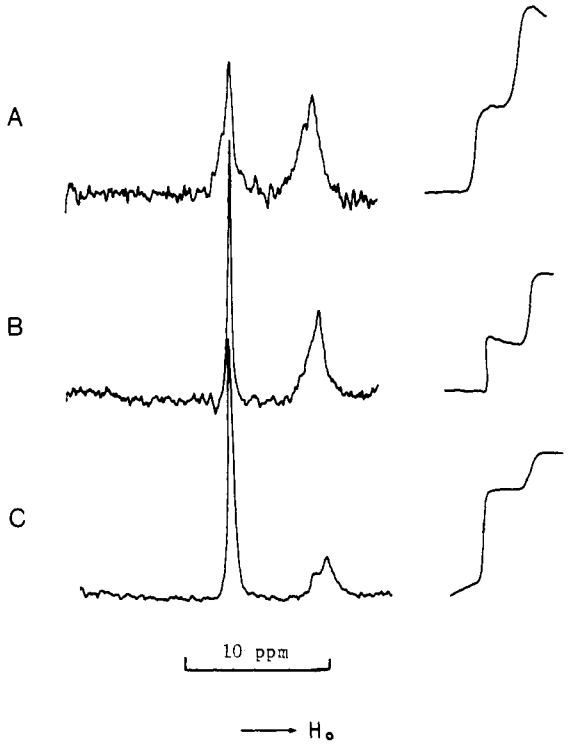

FIGURE 3: High-resolution ^{31}P NMR studies of modifications of the lipovitellin/phosvitin complex. Similar conditions as in Figure 2; no proton decoupling and no external standard. The integrated intensities are given on the right-hand side of the spectra. The small differences in chemical shifts may be due, in part, to slightly different buffer conditions. Measuring temperature: 20 °C. (A) Purified lipovitellin; prepared by TEAE chromatography. (B) Purified lipovitellin; prepared by ammonium sulfate precipitation. (C) Partially delipidated lipovitellin/phosvitin complex. Approximately 20% lipid was removed by mild acetone extraction.

larger is shifted about −2.3 ppm downfield from an external H_3PO_4 standard and corresponds to the phosphoserine residues (Colman & Gadian, 1976). By application of a proton-decoupling field, the broad resonance can be further resolved into two lines with chemical shifts of +1.6 and +2.2 ppm (Figure 2B) corresponding to the two main phospholipids present in lipovitellin, i.e., phosphatidylcholine and phosphatidylethanolamine, respectively. Evaluation of the areas of the resonances in the nondecoupled spectrum yields a phosphoserine:phospholipid molar ratio of 2.3. According to biochemical analyses, about 74 phospholipid molecules are bound per LV/PV dimer. The total number of phosphorylated serines is thus calculated to be 170.

^{31}P NMR spectra of lipovitellin free of phosvitin and of the partially delipidated LV/PV complex are shown in Figure 3. Lipovitellin was prepared either by TEAE chromatography (Figure 3A) or by ammonium sulfate precipitation (Figure 3B). Even though the spectra exhibit different line shapes for the phosphoserine residue, integration of the absorption peaks yields the same phosphoserine:phospholipid ratio of 0.76 in both cases (cf. right-hand side of Figure 3). The lipovitellin monomer contains 37 (74/2) phospholipid molecules; thus, the *LV-B* and *LV-C* chains of lipovitellin have 28 out of a combined total of 63 serine residues in a phosphorylated form. It thus follows for phosvitin that 56 out of its 66 serine residues must also be phosphorylated. These data are summarized in Table I. The phosphoserine resonance of lipovitellin prepared by TEAE chromatography is much broader than those observed in all modifications of the LV/PV complex, suggesting that the TEAE chromatography method induces some structural reorganization. In contrast, lipovitellin prepared by the ammonium sulfate precipitation gives rise to the same line shapes as observed for the intact lipovitellin/phosvitin complex.

In the spectra of the lipid-depleted LV/PV complex (Figure 3C), the intensity of the phospholipid resonance is reduced relative to that of the phosphoserine resonance. The integration of the absorption peaks shows that the low-temperature acetone extraction will remove 15–20% of the phospholipid from the lipovitellin complex. Such mild lipid removal can be done

Table I: Phosphoserine and Phospholipid Groups Present in the Lipovitellin/Phosvitin Complex

	mol/mol of lipoprotein[a]		PS:PL molar ratio[b]
	PS	PL	
LV/PV complex	170	(74)	2.3
LV (LV-B and LV-C polypeptide chains)	56	(74)	0.76
LV/PV complex (lipid depleted)	(170)	63	2.7

[a] Parentheses are used to show the form used to normalize the data. The original analytical data for the lipid components are given in Ohlendorf et al. (1977). PS is phosphoserine, and PL is phospholipid. [b] Intensity ratios as evaluated from the nondecoupled high-resolution ^{31}P NMR.

without eliminating the X-ray diffraction data from the microcrystalline lipoprotein (Collins, 1977). In addition, the lipid-depleted form of the microcrystals is readily soluble under the same chemical conditions (0.5 M NaCl) as those found for the untreated lipoprotein. The overall structure of lipovitellin thus appears to remain unchanged under conditions of mild lipid removal.

Measurements of the phosphorus spin–lattice relaxation time (T_1) shed light on the dynamics of the phosphate group motion. From the high-resolution spectra, it is possible to determine separately the T_1 relaxation times of the phosphoserine residues and the phospholipids. Figure 4 summarizes the variation of the T_1 relaxation times with temperature. The T_1 relaxation time of the phosphoserine residues in a saturated LV/PV solution is about 2 s and exhibits only a small temperature dependence. The increase of the T_1 relaxation time with

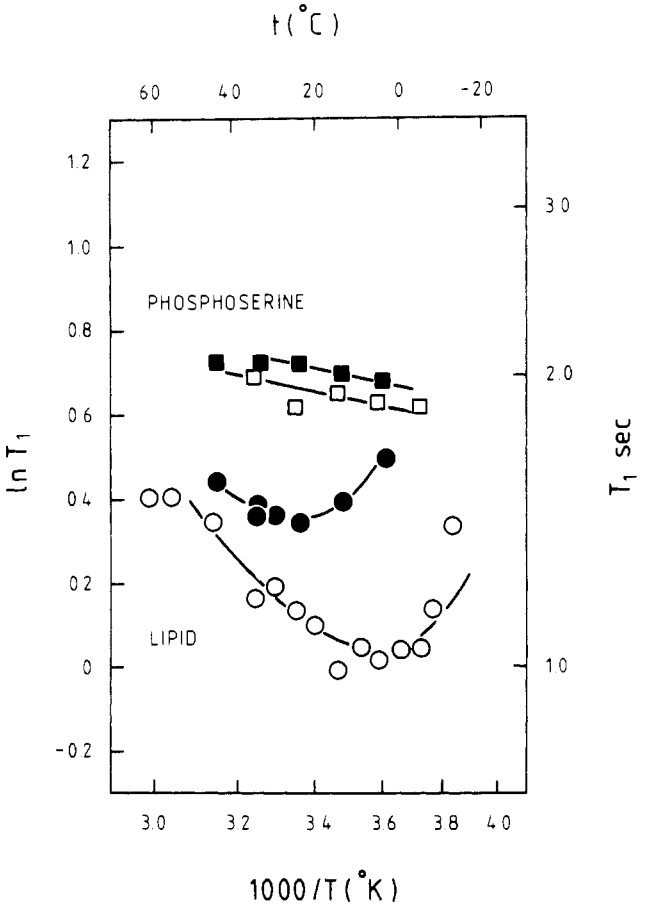

FIGURE 4: Temperature dependence of T_1 relaxation time of the lipovitellin/phosvitin complex and extracted lipids. Solid symbols represent data obtained by high-resolution ^{31}P NMR of a saturated lipovitellin/phosvitin solution in buffer containing 0.5 M NaCl: (●) phospholipid head groups; (■) phosphoserine residues. Open symbols represent data obtained on wet pellets or coarse liposomes by solid-state NMR methods: (○) coarse liposomes formed from the extracted lipovitellin lipids dispersed in buffer containing 0.5 M NaCl; (□) phosphoserine residues of a wet pellet of the LV/PV complex.

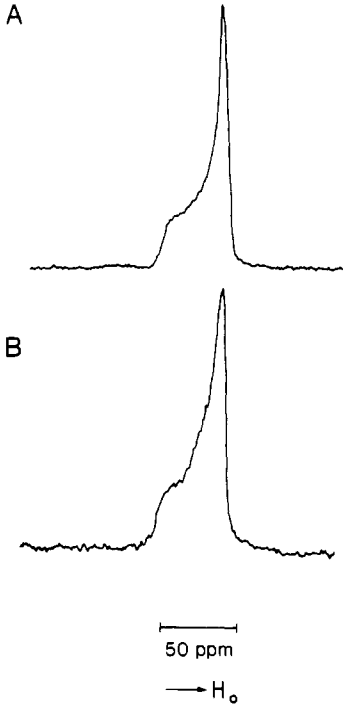

FIGURE 5: ^{31}P NMR spectra of lipovitellin lipid. The ^{31}P NMR spectra were obtained from multilamellar dispersions of lipids. Measuring temperature: 20 °C. (A) 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine dispersed in H_2O. (B) Lipids extracted from the lipovitellin/phosvitin complex and dispersed in Tris/NaCl buffer.

increasing temperature provides evidence that the rate of motion is in the so-called fast correlation time regime where $\omega_0\tau_c \ll 1$ (ω_0 = resonance frequency in radians per second; τ_c = characteristic correlation time for the phosphate group movement). A different behavior is observed for the phospholipid head groups. The T_1 relaxation time is shorter (~1.5 s), and its temperature dependence is characterized by a minimum around 23 °C. At the present level of sensitivity, it was not possible to detect any differences between phosphatidylethanolamine and phosphatidylcholine. The absolute values of the T_1 relaxation times but not the shapes of the curves are slightly dependent on the concentration of the LV/PV complex. This effect has not been studied systematically but probably arises from changes in the bulk viscosity of the solution.

Solid-State ^{31}P NMR Measurements. The results described in the section which follows have been obtained with wet pellets of the LV/PV complex and with coarse multilamellar liposomes formed from the extracted LV/PV lipids. Since proton/phosphorus dipolar couplings were removed by applying a strong proton-decoupling field, the spectra are dominated by the phosphorus chemical shielding anisotropy [cf. Seelig (1978)]. Figure 5 compares the ^{31}P NMR spectrum of bilayers of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (5A) with that of coarse dispersions of LV/PV lipids in buffer (5B). The shape of the spectra and also the size of the chemical shielding anisotropy, $\Delta\sigma$ (defined by the separation of the edges of the powder-type pattern), are almost identical. The chemical shielding anisotropy of the LV/PV lipids increases linearly from −51 ppm at −10 °C to −46 ppm at 30 °C. This behavior agrees qualitatively and quantitatively with that found for synthetic phospholipid bilayers in the liquid-crystalline state [cf. Seelig (1978)]. The line shapes of ^{31}P NMR spectra are sensitive to the bilayer or hexagonal arrangement of the phospholipids if the lateral diffusion is fast on the NMR time scale [cf. Seelig (1978) and Cullis & de Kruijff (1979)]. The spectra obtained for the lipid extract from the LV/PV complex exclusively show the bilayer line shape. Thus, it is safe to conclude that the phospholipids in the lipid extract exist only in bilayers under the conditions studied.

The ^{31}P NMR spectrum of the intact LV/PV complex (Figure 6B) consists of a superposition of a relatively sharp, symmetric peak and a broad asymmetric peak. The line width of the symmetric resonance depends critically on the buffer composition. The data shown in Figure 6B result from a suspension of the LV/PV complex in buffer containing 0.5 M NaCl; in the absence of NaCl, the line width of the more intense resonance is considerably broader, and the two spectral components are barely resolved (Figure 6A). By evaluation of the corresponding areas of the resonances in Figure 6B, the ratio of the sharp and the broad component is found to be roughly 2. This result corresponds approximately to the phosphoserine:phospholipid ratio as determined by biochemical analysis and high-resolution NMR and provides a first hint for the assignment of the two spectral components. The intense symmetric peak must be ascribed to the phosphoserine residues, whereas the broad asymmetric peak comes from the phospholipids in the LV/PV complex. Figure 6C shows a magnification of the phospholipid spectral component of the LV/PV complex (noisy line). Superimposed is the corresponding spectrum of the extracted LV/PV lipids (Figure 5B) which was artificially smoothed by a constant line broadening

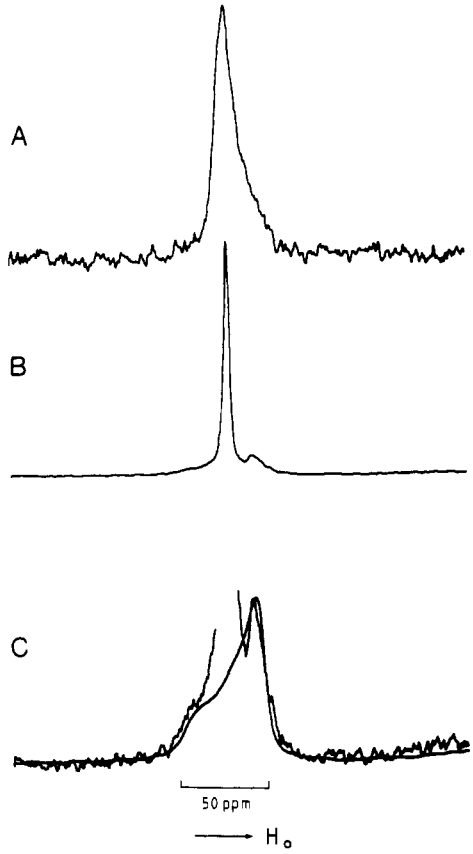

FIGURE 6: ^{31}P NMR spectra of the crystalline lipovitellin/phosvitin complex. (A) Suspension of lipoprotein complex in Tris buffer (no NaCl), pH 8.5; 20 °C. (B) Suspension of lipoprotein complex in Tris buffer containing 0.5 M NaCl; 20 °C. (C) The noisy trace is an 8-fold magnification of spectrum B. The smooth trace is the spectrum of the lipid extract recorded under exactly the same conditions. The lipid spectrum corresponds to Figure 5B but was artifically smoothed by a line broadening of 500 Hz.

of 500 Hz (smooth line of Figure 6C). The excellent match of the two spectra supports the above assignment and further suggests that the lipids in the LV/PV complex have a structural arrangement similar to a fluid lipid bilayer.

The phosvitin and the lipovitellin polypeptides were studied separately in order to further characterize the LV/PV complex. The ^{31}P NMR spectrum of a suspension of lipovitellin is shown in Figure 7A. The central phosphoserine peak is distinctly reduced in intensity since the highly phosphorylated phosvitin chain is missing. On the other hand, the asymmetric phospholipid spectrum has grown in relative intensity, allowing a more accurate comparison with the ^{31}P NMR spectrum of the pure lipid extract (dashed line). By weighing cutouts of the respective traces, it can be shown that the phospholipid accounts for 58% of the total intensity of the lipovitellin spectrum, yielding a phosphoserine:phosholipid ratio of 0.72. Within experimental error, this is identical with the result obtained by high-resolution NMR (cf. Table I).

If the lipids of the LV/PV complex are completely extracted with chloroform/methanol, the resulting apoprotein gives rise to spectrum 7B. Exactly the same spectrum is obtained for phosvitin (spectrum not shown). Taken together, these results provide most compelling evidence that the central signal in the LV/PV complex as well as in lipovitellin must be assigned to the phosphorylated serine residues.

T_1 relaxation time measurements were made of suspensions of the LV/PV complex and of coarse liposomes (Figure 4). For the LV/PV complex, the T_1 relaxation time is dominated by the phosphoserine residues and shows qualitatively and

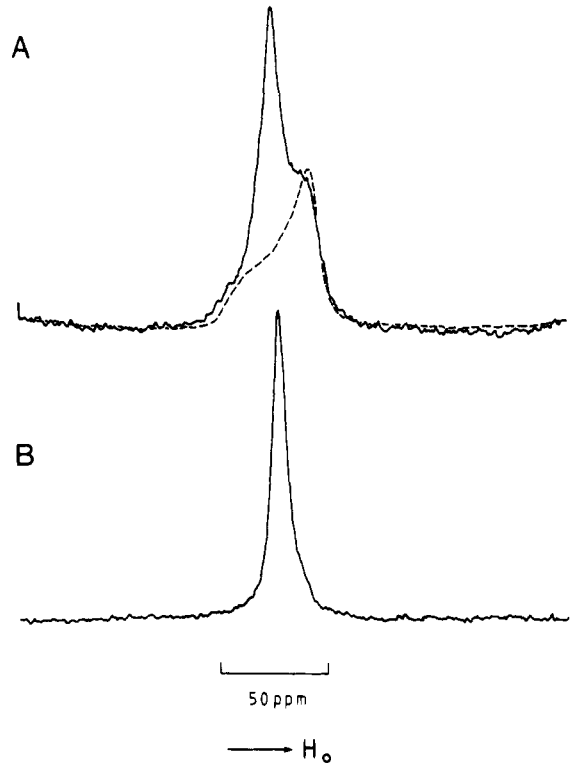

FIGURE 7: ^{31}P NMR spectra of crystalline lipovitellin and completely delipidated lipovitellin/phosvitin complex. (A) Purified lipovitellin from ammonium sulfate precipitation suspended in Tris/NaCl buffer; 20 °C. (B) Lipid-extracted lipoprotein suspended in Tris/NaCl buffer, pH 8.5; 20 °C.

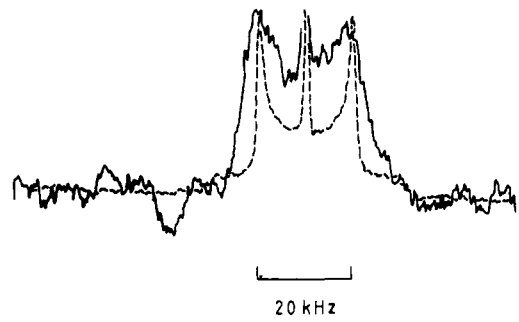

FIGURE 8: ^{2}H NMR spectra of the lipovitellin complex with bound DEPC. The ^{2}H NMR spectra (46.1 MHz) of the precipitated lipovitellin complex (noisy line) were recorded at 35 °C. The probe was DEPC labeled with deuterium at the 9 and 10 positions of the fatty acyl chain. The methods used for the exchange reaction are described in the text. The precipitate is contained in the usual Tris buffer, pH 8.5, made in this case, with D_2O-depleted water. The small amount of D_2O still present produces the isotropic centralmost peak visible in the spectrum. The dashed line corresponds to the pure lipid dispersed in water, measured again at 35 °C.

quantitatively the same behavior as observed in high-resolution NMR. In contrast, the T_1 relaxation time of the coarse liposomes formed from the extracted lipids is shorter than that of the lipids in the LV/PV complex (observable only in high-resolution NMR). Moreover, the lipid extract is characterized by a more pronounced minimum which occurs at a lower temperature (~5 °C) compared to the native complex.

^{2}H NMR. Using phospholipid exchange protein, it was possible to insert (or exchange) into the lipovitellin small amounts of 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC) which had been deuterated at the 9 and 10 positions adjacent to the trans-olefinic bond. After the transfer reaction, the lipovitellin complex can be purified from the lipid vesicles as shown in Figure 1 and precipitated. The ^{2}H NMR spectra of the resulting lipoprotein are shown in Figure 8. Although

not as prominent as is found in model lipid systems, the line shape has the double-wing feature characteristic of the deuterium probe in an anisotropic environment. The magnitude of the quadrupole splitting estimated from the separation of the symmetrical wings is qualitatively similar to the same probe in the presence of the sarcoplasmic reticulum pump protein (Seelig et al., 1981). On the basis of the number of scans needed to obtain spectra with a reasonable signal to noise ratio, only small amounts of exchange (liposome to lipovitellin) occurred. Hence, the overall composition of the lipid domain was not noticeably altered.

Discussion

Influence of Protein Rotational Diffusion on the Magnetic Resonance Spectra. The lipovitellin/phosvitin complex has dimensions of 55 × 115 × 250 Å^3 (Ohlendorf et al., 1978). For the present discussion, the lipoprotein may be approximated by a rotational ellipsoid of length $2a$ = 250 Å and thickness $2b$ = 85 Å. The calculation of the rotational diffusion constant [cf. Woessner (1962)] yields $D_{\parallel} = 10^6$ s^{-1} and $D_{\perp} = 4.5 \times 10^5$ s^{-1} for the rotation around the long and the short axis, respectively, assuming a viscosity of η = 0.01 P. These rotational rates are large compared to the anisotropies of the phosphorus chemical shielding tensor ($\Delta\sigma_{max}$ = 230 ppm, corresponding to 2 × 10^4 Hz at 7 T field strength; Griffin, 1976) but are small compared to the anisotropies of the nitroxide hyperfine tensor ($2A_{\parallel}^{max}$ = 64 G, corresponding to 9 × 10^8 Hz; Griffith et al., 1965). Thus, the molecular tumbling of the lipoprotein complex is sufficiently fast to average completely the ^{31}P chemical shielding anisotropies and to produce high-resolution ^{31}P NMR spectra in solution, even if the phospholipids were immobilized on the protein surface. On the other hand, the molecular tumbling is too slow to influence conventional spin-label EPR spectra to any appreciable extent. Any detectable averaging of the spin-label EPR anisotropies must, therefore, be ascribed to the motional properties of the microdomains in which the spin-label is dissolved.

Movement of the Phosphoserine Residues. The ^{31}P NMR spectrum of the lipovitellin/phosvitin complex in dry crystals is dominated by the anisotropies of the phosphoserine chemical shielding tensor and has a width of about 110 ppm (spectrum obtained by the cross-polarization technique). The principal elements of the shielding tensor are −43, +1, and +57 ppm and are in good agreement with those of pure phosphoserine crystals (Kohler & Klein, 1976). Upon addition of buffer, the phosphoserine resonance narrows dramatically. In wet suspensions, the width at half-height of the resonance is about 13 ppm in the absence of NaCl (Figure 6A) and 4 ppm with 0.5 M NaCl (Figure 6B). The most likely molecular origin of this effect is the onset of fast and almost isotropic reorientation movements of the phosphate moiety upon addition of buffer. Since the T_1 relaxation time falls into the fast correlation time regime, the reorientation rate of the phosphate attached at the protein is definitely faster than that of the phospholipid phosphate groups. If contributions from dipole/dipole and chemical shielding anisotropy relaxation are taken into account, a conservative estimate yields a correlation time of 0.4–0.7 ns, which is about a factor of 2–3 shorter than that of the phospholipids (cf. below). The further narrowing of the resonance upon addition of NaCl can be explained by an ionic screening of the negatively charged phosphate groups by the Na^+ counterions, entailing, perhaps, a larger motional freedom for the phosphoserine residues.

Structure and Dynamics of the Phospholipid Domains. The ^{31}P NMR spectra of the LV/PV complex (Figure 6B,C) and of lipovitellin (Figure 7A) contain a broad asymmetric component with a chemical shielding anisotropy of $\Delta\sigma$ = −50 ppm which is typical for phospholipid bilayers in the liquid-crystalline state [cf. Seelig (1978)]. The occurrence of a hexagonal phospholipid phase can be excluded. The relative intensities of the bilayer component and the phosphoserine resonance are in excellent agreement with the high-resolution ^{31}P NMR spectra, indicating that all phospholipid molecules are organized in this bilayer structure. The only notable difference between the bilayer spectra of the lipids extracted from the LV/PV complex and the spectra of the LV/PV complex itself is an increase in the intrinsic line width. The ^{2}H NMR experiments provide similar information for the fatty acyl chain region. The ^{2}H NMR spectrum of [9,10-$^2H_2$]DEPC exchanged into the lipoprotein complex is characterized by a quadrupole splitting of 19.4 kHz (at 35 °C) which is almost identical with that of pure DEPC bilayers at the same temperature (19.8 kHz; Seelig & Waespe-Sarcevic, 1978). Again, the lipoprotein complex is characterized by a considerable line broadening. The measurement of the ^{31}P T_1 relaxation times provides evidence for the highly dynamic nature of the phospholipid domains within the lipoprotein complex. The minimum which is observed in the T_1's for the phospholipids has also been found in bilayer vesicles with and without the sarcoplasmic reticulum pump protein (Seelig et al., 1981). At the minimum, the condition $\omega_0\tau_c = 1$ occurs, and the molecular correlation time τ_c is the reciprocal of the resonance frequency ω_0 (Seelig et al., 1981). The resonance frequency used in this study was 121.4 MHz (7.6 × 10^8 rad/s), and at the position of the minimum (5 °C for the phospholipid extract, 23 °C for the lipoprotein complex), τ_c is about 1.3 ns. Previously, observed minima in the spin–lattice relaxation times of sarcoplasmic reticulum membranes exchanged with dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and of pure DOPC bilayers occurred at 14 and 4 °C, respectively (Seelig et al., 1981). The reorientation rate of the phosphate groups is extremely fast and practically identical in both systems. Moreover, a comparison with pure lipid model membranes demonstrates that the head-group reorientation is slowed down by the presence of protein by less than a factor of 2.[2] At the T_1 minimum, the rotational diffusion constant of the phosphate segment is $D_{rot} = 2 \times 10^8$ s^{-1} [cf. Seelig et al. (1981)]. This is an order of magnitude lower than the reorientation rate of molecular solutions of zwitterions of about the same length. The rotational diffusion constant of the H_3N^+–$(CH_2)_3COO^-$ molecule, for instance, is 4 × 10^9 s^{-1} (Pottel et al., 1975).

General Conclusions. The nuclear magnetic resonance spectra of the lipovitellin/phosvitin complex provide compelling evidence that the phospholipid molecules are organized in fluid bilayer domains. Due to the symmetry of the LV/PV dimer, each monomeric subunit has a bilayer microdomain of about 40 phospholipids, i.e., 20 per monolayer. The characteristic NMR parameters, such as the chemical shielding anisotropy ($\Delta\sigma$), the quadrupole splitting ($\Delta\nu_Q$), and the T_1 relaxation time minimum, are little changed from the intact lipovitellin/phosvitin complex to pure lipid bilayer model membranes. Since a large part of the phospholipids [~50%, cf. Figure 8 (Birrell et al., 1982)] must be in direct contact with the protein surface, this result is quite remarkable and is indicative of

[2] The correlation time, $\tau_c = (1/6)D_{rot}$, of the phosphate group in liposomes formed from yolk lipids is ~1.3 × 10^{-9} s at 5 °C (T_1 minimum). With the assumption of an activation energy of 4 kcal/mol [cf. Seelig et al. (1981)], τ_c at 23 °C is calculated to be 0.8 × 10^{-9} s. On the other hand, the correlation time in the lipoprotein complex at 23 °C is 1.3 × 10^{-9} s (T_1 minimum). The correlation time of the lipids is thus increased by a factor of 1.63 by going from the pure lipid bilayer to the complex.

relatively weak and nonspecific phospholipid/protein interactions. Similar observations have been made for the lipid/protein interaction in reconstituted membrane systems [cf. Seelig et al. (1981)], allowing the conclusion that the structural and dynamic properties of the phospholipid microdomains of the lipovitellin/phosvitin complex are very similar to those of the bilayer domains of biological membranes.

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