brief communication

High-resolution electron density profiles reveal influence of fatty acids on bilayer structure

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ABSTRACT Small-angle x-ray diffraction studies were performed on gel phase-oriented bilayers of dipalmitoylphosphatidylcholine (DPPC) and DPPC containing 40 mol% of either palmitic acid (PA) or palmitic acid brominated at the 2-position (BPA). Oriented samples were prepared using a method developed by us, which is as simple as powder sample preparations while offering all the advantages of oriented samples made by traditional methods. Phases were determined using swelling experiments with structure factors plotted in reciprocal space, creating a relatively smooth curve as the amount of water between the bilayers was changed. Continuous Fourier transforms were also calculated to further test the consistency of the phase assignments. The diffraction data were used to calculate absolute electron density profiles for different bilayers to a resolution of 5–6 Å. Analysis indicates the following: (*a*) The electron density profiles for the three preparations are virtually identical in the hydrocarbon chain region. (*b*) There is a decrease in the electron density of the glycerol backbone-headgroup region and d-space in DPPC-PA compared to DPPC. (*c*) The bromine of fatty-acid brominated at the 2-position is in the vicinity of the glycerol backbone. (*d*) The bilayer thickness of DPPC containing either brominated or unbrominated fatty acid remains relatively constant with increased levels of hydration, unlike DPPC bilayers.

INTRODUCTION

Significant variations in the resolution of published electron density profiles of lipid bilayers are primarily due to the method of sample preparation employed. Low- to medium-resolution electron density profiles are constructed using small-angle x-ray diffraction patterns obtained from dispersions of biomolecular layers (1, 2). Samples are usually prepared in this fashion because it may be important to work with a highly hydrated system or because stacking of arrays may alter membrane structure (3). Medium- to high-resolution profiles are usually determined with highly ordered arrays of parallel membranes which can be placed in an optimum orientation in the x-ray beam. Such oriented samples are frequently produced by the evaporation of the solvent from a concentrated solution of the lipid on a curved glass substrate (4) or by use of a Langmuir-Blodgett trough (5). However, these last methods can present technical difficulties. Alternatively, well-oriented preparations of natural membranes can be obtained by a process of simultaneous centrifugation and drying (6). Here we describe a different, simple, and effective method for the preparation of oriented bilayers. With these oriented bilayers, diffraction patterns containing up to 12 well-resolved reflections can be quickly obtained.

Free fatty acids are normally a minor constituent of biological membranes. Nevertheless, they are responsible for numerous physiological effects (7). There has also been some concern about the reliability of fatty acids as nonperturbing probes of membrane structure. There have been numerous studies on lipid bilayers containing free fatty acids. Techniques used have included differential scanning calorimetry (DSC), differential thermal analysis, nuclear magnetic resonance, and x-ray diffraction (7–11). We present here electron density distributions showing the influence of fatty acid incorporation on bilayer structure.

Phosphatidylethanolamines in excess water have gel-to-liquid crystal phase transitions between 20 and 30°C higher than corresponding phosphatidylcholines (12). In 1974, Chapman et al. (13) proposed that the bulkier headgroups of the phosphatidylcholines prevent the hydrocarbon chains from packing effectively, thus preventing full utilization of the van der Waals forces as occurs with phosphatidylethanolamines. Incorporation of a significant amount of free fatty acid into DPPC bilayers might be expected to cause a reduction in the relative head group area and an upward shift in the transition temperature. Addition of palmitic acid (PA) to DPPC bilayers in a 2:1 mole ratio brings about a sharp gel-to-liquid crystal phase transition at 61.5°C, just 2.3°C below that of phosphatidylethanolamine (8). In support of the explanation put forward by Chapman et al. (13), we present here absolute electron density profiles at ~5 Å resolution of gel phase bilayers of DPPC and of DPPC containing 40 mol% PA, showing that the latter has a lower electron density in the backbone and headgroup

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regions compared with DPPC, but with no significant differences in the hydrocarbon tail region.

We also present evidence that the bromine atom of a PA analogue brominated at the 2-position is located near the glycerol backbone, thus implying that the carboxyl group of the fatty acid is also in the glycerol backbone–headgroup vicinity.

Finally, we show that bilayer thickness remains relatively constant with increased levels of hydration when DPPC bilayers contain 40 mol% of PA or brominated PA. This is in contrast to the well-known decrease in bilayer thickness due to changing molecular tilt in pure DPPC bilayers associated with changes in hydration (4, 14) and is similar to the behavior of oriented samples of phosphatidylethanolamine (3).

MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and used as supplied. Palmitic acid (GC reference) was obtained from Fluka AG (Buchs, Switzerland) and 2-bromohexadecanoic acid from Aldrich Chemical Co., Inc. (Milwaukee, WI). Lipid purity was confirmed using differential scanning calorimetry (DSC) and thin-layer chromatography (TLC, $CHCL_3/CH_3OH/H_2O/NH_4OH$, 58:35:5.4:1.6). DSC scans of fully hydrated liposomes revealed the expected main transition peak at 41.1°C having a full-width half-maximum of 0.4°C. DSC scans were obtained with a model MC-1 calorimeter (MicroCal, Inc., Amherst, MA) at a scan rate of 14.4°C/h.

Oriented bilayers were produced by a simple method developed in our lab. Dry DPPC powder from solvent was placed on the outside of a 30-ml Pyrex beaker (No. 1000) and pressed into a thin film with the aid of a stainless steel spatula. The beaker was then placed in a 100% relative humidity (RH) environment for 24 h. Once it was hydrated, anhydrous $CaSO_4$ was added to the beaker, which was placed under vacuum for at least 24 h after which the diffraction experiments were performed. Using this simple method, we consistently obtained diffraction patterns containing 10–12 orders.

A line source of copper K alpha radiation was employed and monochromation was achieved with a Franks' camera (15). Diffraction patterns were obtained with a B-OED-50S linear position sensitive detector system (M. Braun GmbH, Munich, FRG) connected to a personal computer-based multichannel analyzer card (model MP2/WIN 4K, Aptec Engineering Ltd., Downsview, Canada). Calibration was confirmed by using the diffraction pattern of potassium hydrogen pthalate as a reference.

The sample holder (volume $\simeq$250 cm^3) was designed to move the sample in two dimensions (horizontal and vertical with respect to the beam) and permit the relative humidity (RH) and temperature to be adjusted and monitored. The sample holder movement was achieved by the use of stepping motors (Superior Electric Co., Bristol, CT) interfaced through a computer. The holder was positioned so that the vertical line of the x-ray beam passed through the sample tangent to the vertical curved side of the beaker. The RH was adjusted by varying the flow rate of He gas or He gas through water in a Fisher-Milligan gas washer before passage through the sample holder. Finally, the RH was monitored by a digital hygrometer (HI 8565 Stick Hygrometer, Hanna Instruments Inc., Woonsocket, RI), which has a resolution of 0.1% RH and accuracy of ±2% RH. The temperature was controlled by a Haake F_3 water bath set (Berlin, FRG) at 20°C.

DPPC samples containing either 40 mol% palmitic acid or 40 mol% brominated palmitic acid were mixed in a 250-ml round-bottom flask containing ethanol. The solution was dried using a rotary evaporator (Buchi Laboratoriums-Technik AG, Flawil, Switzerland) at 35°C and then placed under vacuum for 24 h at room temperature. We then oriented the powder mixture as in the case of DPPC.

Intensities of the various diffraction peaks were determined after background subtraction. Measured intensities are corrected by the square of the order number (h) for a powder pattern and by the order number for a completely ordered sample. Comparison of relative intensities of various orders from our samples with those from powder samples revealed a high degree of orientation, requiring a correction factor of h raised to the power of 1.0 for DPPC samples and to the power 1.2 for mixtures. Patterns were collected in 1,000 s. When conditions were changed, data were not used until two consecutive sets were identical. The wide-angle reflections are quite weak due to the combination of collection geometry (e.g., linear position sensitive detector) and the highly oriented samples.

Unit cell volumes were determined from the small and wide angle reflections and overall electron densities calculated in the manner of Wiener et al. (16). The average electron density was then used with the known density of the saturated chain region (16) to put electron densities on an absolute scale. Phases of the various reflections were determined by the conventional swelling method (17, 18) and confirmed by superposition of continuous transforms calculated by use of the sampling theorem (19).

RESULTS AND DISCUSSIONS

For the various systems studied, DPPC and DPPC with 40 mol% PA or BPA, the x-ray diffraction patterns (Fig. 1) consisted of a lamellar series of 10–12 Bragg reflec-

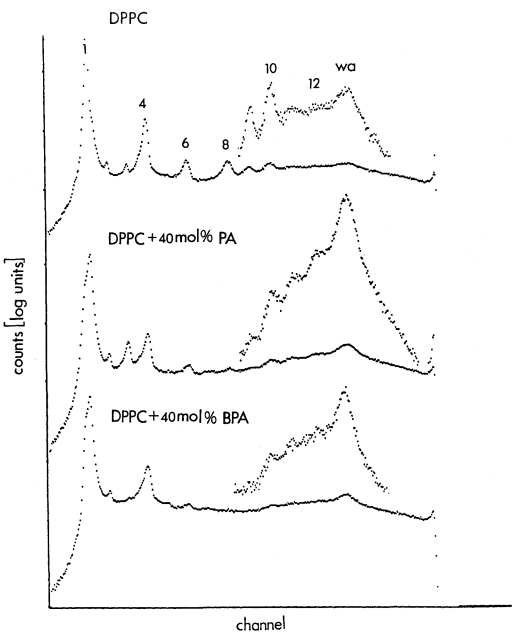

FIGURE 1 0% RH x-ray diffraction patterns of DPPC, DPPC + 40 mol% PA, and DPPC + 40 mol% BPA at 20°C.

tions with weak but sharp wide-angle reflections centered at ~4.2 Å, indicative of the gel phase (20, 21). With a linear detector and a perfectly oriented sample, one can only detect diffraction resulting from structure either parallel or perpendicular to the bilayer plane, not both. DSC scans of fully hydrated liposomes for the latter two preparations showed single gel-to-liquid crystal phase transitions for each. The phase transition began at 50.7°C and ended at 56.6°C for samples with PA. Corresponding figures were 42.3°C and 55.2°C for samples with BPA. Throughout the study, there was never an instance of a diffraction pattern indicating the coexistence of two lipid phases. In addition, the phase diagram for a DPPC-D_2O system indicated that at 25°C (phase diagram temperature range, 25–60°C) and 4–15 mol of D_2O/mol of DPPC the sample was in the L_β' or L_β' + D_2O phase (22). Also, support for the nonexistence of a phase change in the various samples is supplied by the continuous Fourier transform curves (Fig. 3) which have the same shape throughout the various hydration levels.

In Fig. 2, the structure factors obtained in a series of swelling experiments for the various systems are presented. Because at each swelling state a different d-space is observed corresponding to the sampling of the continuous Fourier transform at different points h/d, there are several sets of structure factors for each lipid system. The phases were deduced by mapping out the transform in such a manner that the orders followed a smooth curve with changes in hydration (17) and by the calculation of continuous transforms (4, 19) for various data sets (Fig. 3).

In Fig. 2, the structure factors for the various hydration levels of DPPC are presented and are in agreement in magnitude and phase with those obtained by Torbet and Wilkins (4). There are small differences (<2%) in d-space for a particular RH between the two studies. This can be attributed to some combination of the differences in sample preparation and the difficulties of making precise RH measurements. X-Ray studies on oriented samples of dimyristoyl lecithin produced different types of diffraction patterns dependent on the method of preparation of the sample (23). Also, methods of hydration differ vastly and, if achieved by the use of saturated salt solutions, the samples can take up to a few weeks to equilibrate.

The structure factors of DPPC bilayers containing either 40 mol% of BPA or PA are also presented in Fig. 2. Phase determination for the reflections obtained from the DPPC + 40 mol% BPA presented little difficulty except for the seventh order. A phase of π was finally selected for the following reasons. Electron density distributions calculated with a phase of zero for the seventh order showed impossibly large density fluctuations in the hydrocarbon tail region, including densities in some regions which were

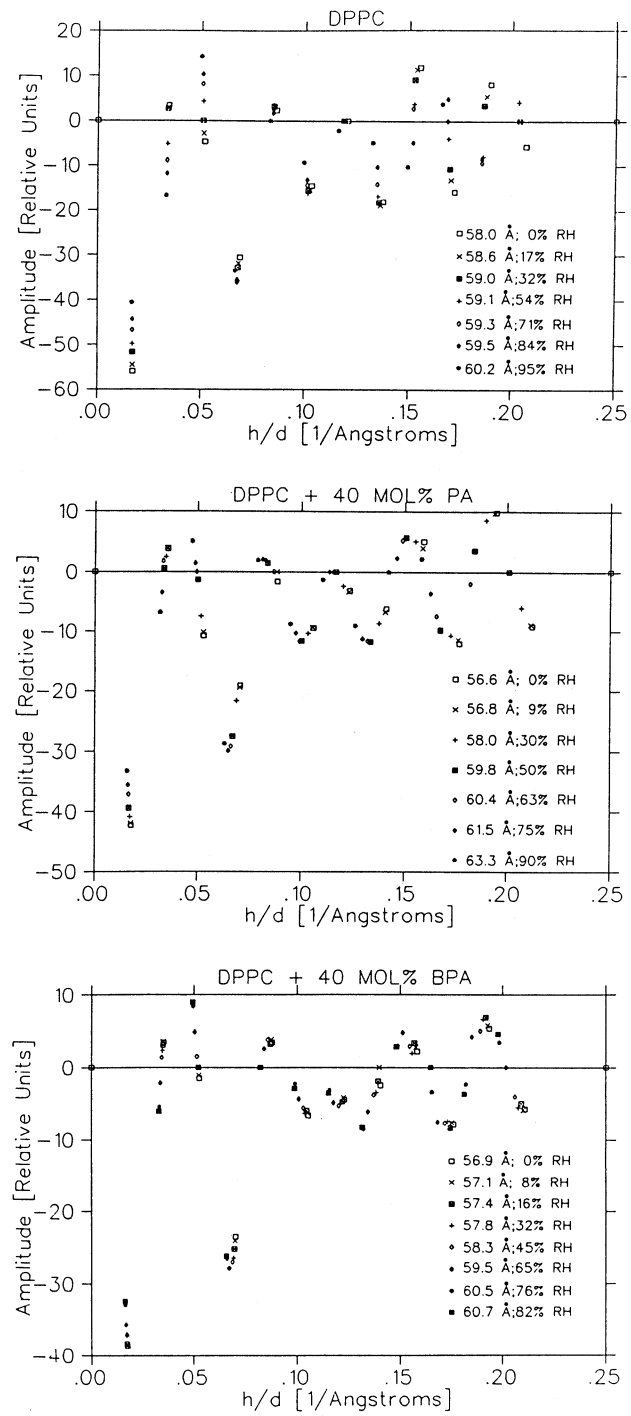

FIGURE 2 X-Ray swelling series for oriented multilayers of DPPC, DPPC + 40 mol% PA, and DPPC + 40 mol% BPA at 20°C.

significantly greater than could possibly result from the tightest possible packing of the methylene groups. In addition, a choice of zero for the seventh order resulted in some extreme shifts in the glycerol backbone and headgroup peaks with small changes in hydration. On the

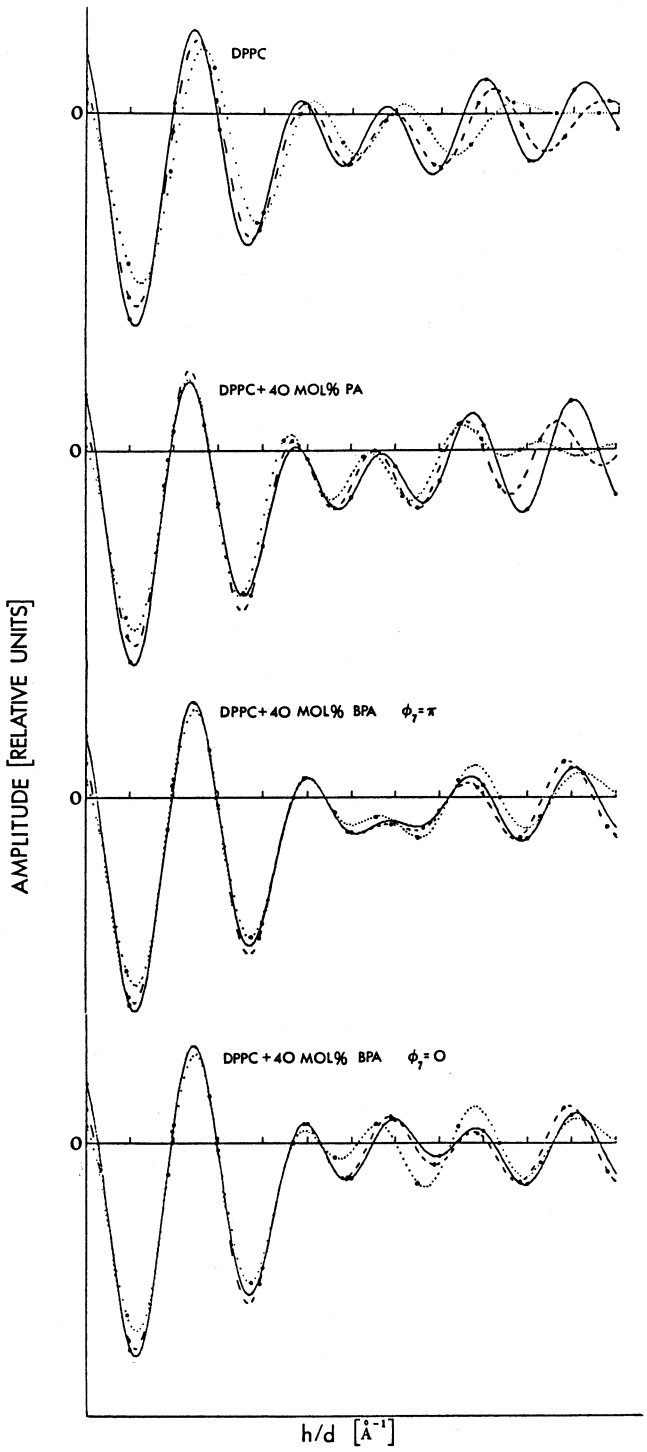

FIGURE 3 Continuous Fourier transforms of DPPC (—0% RH; --- 54% RH; ··· 95% RH), DPPC + 40 mol% PA (—0%; --- 63% RH; ··· 90% RH), and DPPC + 40 mol% BPA (—0% RH; --- 32% RH; ··· 82% RH) with $\phi_7 = \pi$ or 0.

other hand, a choice of π for the seventh order phase resulted in electron density distributions which were within reasonable limits and which were not dissimilar from those of the DPPC + 40 mol% PA in the hydrocarbon tail region. Finally, continuous Fourier transforms (Fig. 3) reconstructed by use of the sampling theorem (19) differ somewhat when the phase of π was used for the seventh order but considerably more so when a phase of zero was used. The π phase assignment corresponds to a minimum change in bilayer thickness with swelling.

In Fig. 4, electron density distributions on an absolute scale are compared for 0% RH samples. The main features are (*a*) the hydrocarbon tail regions are very similar; (*b*) there is an overall decrease in electron density of the hydrophilic region when fatty acid molecules are incorporated; and (*c*) the bromine is located in the vicinity of the glycerol backbone.

These profiles were calculated with the known mean electron density over the methylene group region of the hydrocarbon tails which is 0.317 ± 0.003 electrons/Å^3

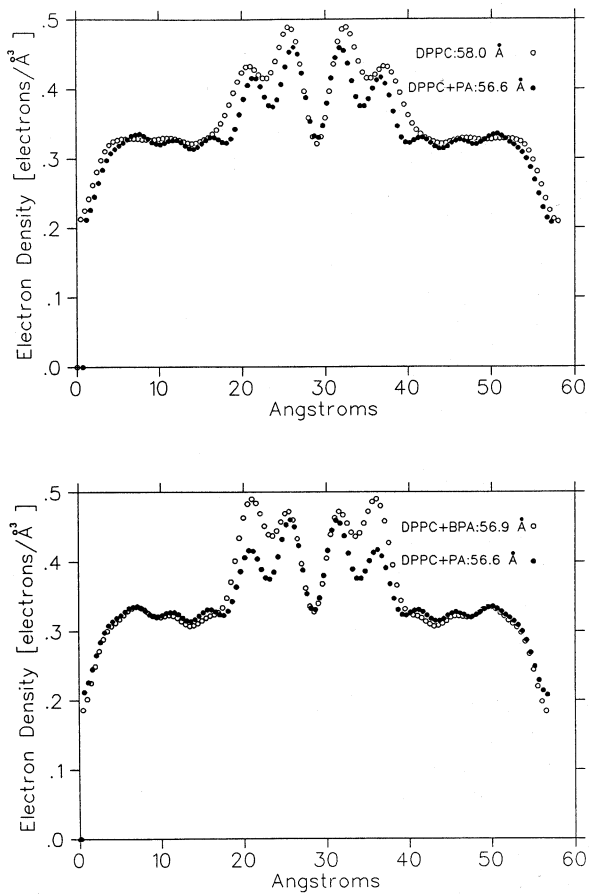

FIGURE 4 Absolute electron density distributions for dry samples showing differences between (*a*) DPPC and DPPC + 40 mol% PA and (*b*) DPPC + 40 mol% BPA and DPPC + 40 mol% PA.

(16) and the mean electron density for the various bilayers which we calculated as 0.355, 0.342, and 0.351 electrons/Å^3 for the DPPC, DPPC + 40 mol% PA, and DPPC + 40 mol% BPA, respectively. In calculating these last values, it was assumed that two water molecules were adsorbed to each lipid molecule (24) and one water molecule per fatty acid molecule. In support of this method, we note the following points. First, maximum density in the phosphate headgroup for the DPPC profile is virtually identical to that of Wiener et al. (16). Secondly, when the DPPC and DPPC + 40 mol% PA are compared, the tail regions are very similar and the decrease in density in the hydrophilic region of the sample with PA is quantitatively consistent with the decreased ratio of head groups to hydrocarbon chains. Finally, the area of the bromine peak obtained by subtraction of the profile of DPPC + 40 mol% PA from that of DPPC + 40 mol% BPA permits us to calculate an increase of 33.1 electrons for each bromine, which is very close to the correct value of 34 electrons. The similar electron densities in the hydrocarbon tail regions are consistent with the very similar wide angle reflections from the three preparations, i.e., 4.23, 4.21, and 4.23 Å from the DPPC, DPPC + 40 mol% PA, and DPPC + 40 mol% BPA, respectively.

Comparison of the electron density profiles shows that the bromine and thus the 2-position of the fatty acid is in the region of the glycerol backbone. While there are small differences in the d-spacings for particular relative humidities as seen in Fig. 2 and in the transition temperatures, the profiles suggest that the brominated fatty acid is a good analogue which can be a reliable probe of bilayer structure.

The increased average headgroup separation caused by the addition of the fatty acid molecules may then allow a molecular packing similar to that of the phosphatidylethanolamines. This is supported by the significant elevation of the gel-to-liquid crystal phase transition temperature in the preparations with PA to a value close to that of the phosphatidylethanolamines (12). Also, as will be seen in Fig. 5, the actual bilayer thickness experiences minimum change when samples containing PA or BPA are hydrated. This is consistent with observations on phosphatidylethanolamine bilayers (3) and in contrast to the decrease in bilayer thickness with hydration for pure DPPC which we observe and which has been noted by others, e.g., references 4 and 14.

In Fig. 5, the least and most hydrated electron density distributions are contrasted for the various preparations. The pure DPPC bilayers decrease in thickness, when measured peak to peak, with increasing hydration, while those with brominated or unbrominated PA show only slight changes. All three preparations have approximately the same bilayer thickness at 0% RH. The behavior of the

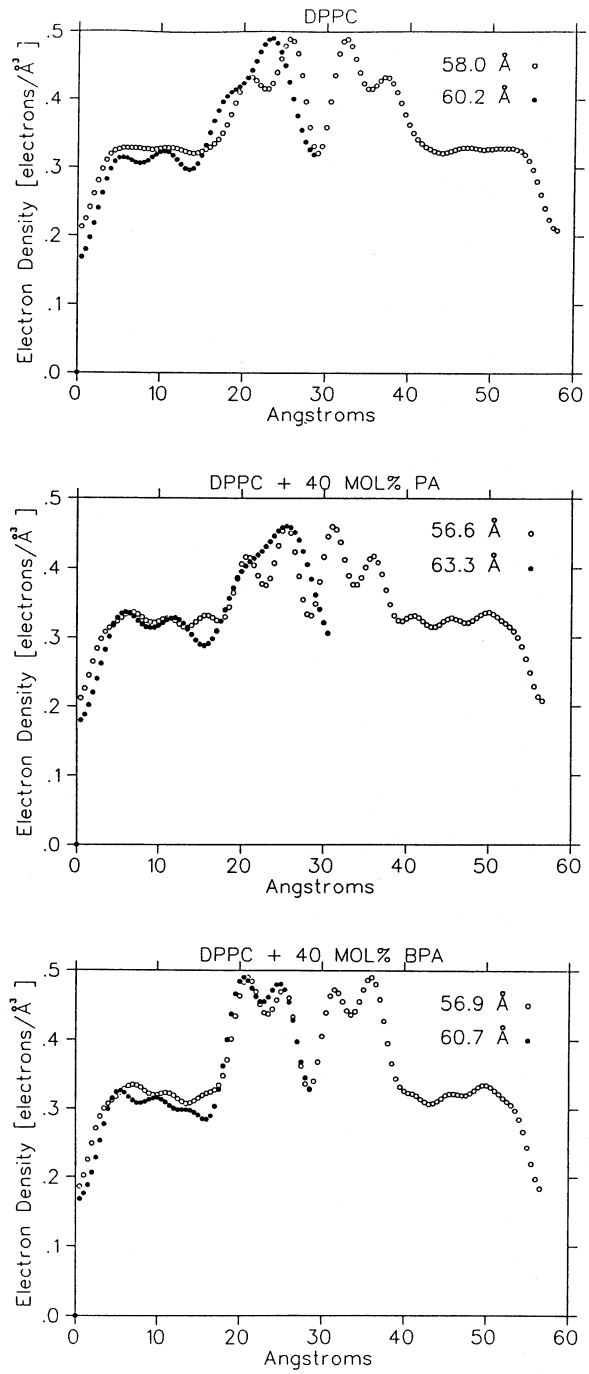

FIGURE 5 Comparison of differences in changes in bilayer thickness for dry vs. highly hydrated bilayers of DPPC, DPPC + 40 mol% PA, and DPPC + 40 mol% BPA.

pure DPPC bilayers is consistent with that reported by others. At 5% or less water content, the bilayers have no chain tilt (20) and would be expected to have maximum thickness. However, with increased hydration, the hydrocarbon chains become tilted with respect to the bilayer

normal (20, 25), the tilt increasing as a function of water content. This would lead to the observed decrease in bilayer thickness.

The hydrocarbon chains in fully hydrated dipalmitoylphosphatidylethanolamine (DPPE) are approximately normal to the bilayer plane. Chain tilt in fully hydrated DPPC bilayers can be reduced by the addition of tetradecane to the bilayers (25). The relatively constant bilayer thickness seen in Fig. 5 when samples containing PA or BPA are increasingly hydrated is probably due to the elimination of chain tilt as the increased average headgroup separation leads to a simulation of a DPPE type of structure.

Preliminary wide-angle diffraction data on lyophilized and fully hydrated multilamellar vesicles indicate that no chain tilt exists in lyophilized samples. However, in fully hydrated samples there exists a pronounced chain tilt in DPPC, a small chain tilt in DPPC + 40 mol% BPA, and no chain tilt at all for DPPC + 40 mol% PA (unpublished data). These results are in agreement with the observations of the oriented samples.

CONCLUSIONS

Very good diffraction patterns containing 10–12 orders were routinely obtained due to a simple new method of sample preparation.

High-resolution electron density profiles of pure DPPC bilayers in the gel phase were consistent with the results of other investigators, e.g., Torbet and Wilkins (4), and showed the expected decrease in bilayer thickness, due to changes in the tilt of the hydrocarbon chains, as the level of hydration was increased.

Addition of 40 mol% PA or BPA to the DPPC bilayers induced no significant change in electron density in the hydrophobic region of the bilayer. The incorporation of such fatty acids increased the transition temperatures towards those of phosphatidylethanolamine bilayers and also decreased the electron density of the hydrophilic regions.

The DPPC bilayers with PA or BPA when at 0% RH were the same thickness as pure DPPC bilayers. The thickness of the bilayers containing fatty acids did not change significantly as hydration was increased, thus indicating that hydrocarbon chain tilt is negligible. This behavior also resembled that of the phosphatidylethanolamines.

Finally, the bromine attached to the 2-position of the fatty acid was observed to be in the vicinity of the glycerol backbone of the DPPC. The influence on the DPPC bilayer structure and behavior was very similar for either BPA or PA, suggesting that one is as good a probe as the other, although either can induce some changes when present in high concentration.

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