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Real-time swelling-series method improves the accuracy of lamellar neutron-diffraction data

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Neutron-diffraction data were collected from stacked bilayers of 1,2-dioleoyl-*sn*-glycero-phosphocholine under conditions of increasing relative humidity at both 0 and 8.06% ^2H_2O. Over the period of data collection, the *d*-repeat of both swelling-series samples increased. Each family of structure factors, representing each of the five orders of diffraction, are shown to lie on smooth curves, allowing structure factors of intermediate *d*-repeat to be determined. In the case of the 8.06% ^2H_2O data, but not the 0% ^2H_2O data, all observed structure factors lie on a single continuous transform. 8.06% ^2H_2O has a net neutron-scattering density of zero; its use in neutron-diffraction experiments presents a novel application of the so-called 'minus fluid' approach, without mathematical manipulation. The data are used to demonstrate the increased accuracy inherent in this real-time swelling-series approach. A quantitative analysis of errors caused by differences in *d*-repeat in difference subtractions is presented.

1. Introduction

The swelling-series method has been widely utilized in X-ray diffraction measurements of lamellar phospholipid preparations as a technique for the determination of structure-factor phases. The process consists of recording structure-factor amplitudes at a range of points in reciprocal space which, when scaled to each other and plotted, trace out the continuous transform of a single bilayer. In practice, this is achieved by the use of a number of samples, each prepared to a different lamellar spacing by controlling the humidity of the atmosphere or the osmotic pressure of the solution (King & Worthington, 1971).

In an earlier paper (Bradshaw *et al.*, 1998), we described an adaptation of the swelling-series method in which oscillations of the observed diffracted intensity were caused by changing the relative humidity (rh) of a sample. This experiment was performed *in situ* by swelling dehydrated orientated stacks of phospholipid in the sample can of a neutron diffractometer whilst several consecutive θ–2θ scans were conducted. We demonstrated that this method, when applied to lamellar phospholipids, has the potential to improve both the accuracy of measurement of structure factors and their phase assignment. Such an approach is better suited to neutrons than X-rays because of the lower levels of radiation damage which allow a single sample to be scanned repeatedly. Each programmed scan gives one structure-factor value for each order of diffraction when the Bragg geometry for that order is satisfied. A number of scans are conducted as the bilayers swell by taking up water. In each scan, the position of any structure factor will have shifted slightly from the previous

scan, thereby sampling a different region of reciprocal space and having a correspondingly different amplitude. The net result is that a family of structure-factor measurements is obtained for each order.

In the previous paper, we presented data from a sample of 1,2-dioleoyl-*sn*-glycero-phosphocholine (DOPC) collected under real-time conditions of increasing rh of pure water. The term pure water means that the water contained no salts to hold the rh of the sample environment below 100%. The isotopic composition of the pure water was 100% ^1H_2O (*i.e.* no salts added, 0% ^2H_2O). We demonstrated that at this isotopic composition of water the observed structure factors did not lie on a single continuous transform. This result is to be expected because the scattering density of the unit cell changes as more water is incorporated into the sample. Incorporation of this negatively scattering water affects the bilayer structure in two ways. The mean scattering density is reduced and the neutron-scattering contrast is altered. Therefore, the observed structure factors do not lie on the same continuous transform because the scattering structure is effectively different even if the physical structure of the bilayer remains unchanged. It is common practice, however, to take calculated structure-factor values from the continuous transform and use these values in Fourier subtractions if the *d*-repeat of the samples differ by approximately 1 Å (Büldt *et al.*, 1979). Our previous paper revealed the potential errors in subtractions by doing this. In this paper, we present a swelling series of structure factors collected from DOPC at 8.06% ^2H_2O and compare them with the earlier set collected at 0% ^2H_2O.

2. Materials and methods

2.1. Sample preparation

DOPC was purchased from Avanti Polar Lipids and used without further purification. 20 mg samples were dissolved in chloroform. An artist's airbrush, using nitrogen as propellant, was used to deposit the lipid onto quartz microscope slides. The slides were placed in a vacuum over the desiccant phosphorous pentoxide (P_2O_5) for 12 h in order to remove all traces of the solvent. The slides were then hydrated before being placed back in the vacuum desiccator for a further 12 h in order to remove as much water as possible. Since some water molecules tightly bound to the phosphoryl head group may have remained, samples for measurement with 8.06% ^2H_2O were hydrated in an humid atmosphere with water at this isotopic composition before being dried again under vacuum for the second time. The samples were protected from light whenever possible in order to reduce the chance of lipid peroxidation.

2.2. Neutron data collection

Neutron-diffraction measurements were carried out on the D16 membrane diffractometer at the Institut Laue et Langevin, Grenoble, France. The sample environment was a standard aluminium can, in which temperature control is achieved by circulating water through an integral water jacket and humidity control is achieved by changing the solution in a Teflon water bath at the base of the can. Each anhydrous DOPC sample on its quartz slide was quickly transferred straight from its vacuum desiccator into the D16 can together with a bath of pure water at either 0 or 8.06% ^2H_2O. A series of continuous θ–2θ scans was immediately initiated. Each scan (from θ = 1.5 to 15.0°) took approximately 3 h to complete. The samples were run at 298 K. The mosaic spread of the second order of diffraction was determined for each sample using standard procedures.

2.3. Data analysis

The two-dimensional array of detector counts for each frame of data was corrected for variations in pixel response. The complete set of frames from each scan were then collapsed into a linear spectrum and combined to generate a pseudo θ–2θ scan. All of the analysis to this stage was carried out by the D16 instrument software. The background around each peak was fitted and subtracted using *SigmaPlot*. Gaussian distributions were then fitted to the Bragg reflections and the angular position, width and area of each peak recorded. Absorption and Lorentz corrections were applied and the square-root of the intensities taken to produce the structure-factor amplitudes.

3. Results and discussion

3.1. 0% ^2H_2O data

Structure factors for the 0% ^2H_2O samples were calculated from the observed intensities and plotted against their spatial frequencies, as shown in Fig. 1. Reference to this figure shows that the 0% ^2H_2O neutron structure factors determined at a range of spatial frequencies do not lie on the same continuous transform. Each order traces out its own curve through the observed points. A quadratic expression was used to join the observed points of each order in turn.

The constantly changing relative humidity within the sample can was reflected in a constantly changing *d*-repeat throughout the data-collection period. This meant that no two of the observed structure factors, even those within the same scan, indexed onto the same reciprocal lattice. Interpolation between the observed points of each order produced sets of structure factors for a range of *d*-repeats. These structure-factor sets were used to calculate continuous transforms $C(x)$ using

$$C(x) = \sum_{h=0}^{h_{\max}} F(h) \frac{\sin(\pi dx - \pi h)}{(\pi dx - \pi h)}, \qquad (1)$$

where $F(h)$ are the observed structure factors, d is the Bragg spacing, x is the distance along the bilayer normal to the plane and h is the order number. The resulting continuous transforms show a smooth variation in amplitude, reinforcing faith in the phase assignments (Fig. 1).

The sets of structure factors were also used to calculate a family of *trans*-bilayer coherent neutron-scattering density profiles, as shown in Fig. 2(*a*). Within this family of profiles no

scaling was necessary, since each profile was produced by the same sample. However, each family of profiles was put on a per-lipid scale using the 'relative–absolute' method (Jacobs & White, 1989; Wiener & White, 1991; Wiener *et al.*, 1991). This was achieved by scaling against previous data collected from the same lipid on the same diffractometer (Duff *et al.*, 1994; Bradshaw *et al.*, 1994; Bradshaw, 1997). This scaling process also yields the value of $F(0)$, the zeroth order, for each structure factor set, as discussed below.

3.2. 8.06% ^2H_2O data

In the case of the data collected at 8.06% ^2H_2O, all observed points were simultaneously fitted to a continuous transform using a least-squares minimization procedure. Sets of model structure factors $F(H)$, each corresponding to a d-repeat of D, were fitted against all observed data points, including the calculated $F(0)$, to satisfy

$$F(h) = \sum_{H=0}^{H_{\max}} F(H) \frac{\sin(\pi Dh/d - \pi H)}{(\pi Dh/d - \pi H)}. \qquad (2)$$

The process was repeated using a number of different values of D, since analytical continuation theory (King & Worthington, 1971) predicts that all the model structure factors should lie on the same continuous transform. The results are shown in Fig. 1(b). Sets of model structure factors resulting from this least-squares fitting procedure are shown in Table 1 and scattering-density profiles calculated from them are shown in Fig. 2 (bottom).

One distinct advantage of the swelling-series method is the potential increase in the accuracy of intensity measurement. Any change in d-repeat caused by temperature or humidity fluctuations or incomplete equilibration can result in large differences in intensity of any single order. This effect is seen at its most extreme in the first order of the 0% ^2H_2O series, where a change in d-repeat of 2.5 Å (from 50.8 to 53.2 Å) causes a 280% change in amplitude (which is equivalent to nearly an 800% change in intensity). The indexing of each structure factor to its own spatial frequency, rather than assuming that they all fit the same reciprocal lattice, removes this potential source of error. In the case of data collected at 8.06% ^2H_2O, there is the added advantage that all observed structure factors can be used to define the same continuous transform, even when they all index to different reciprocal lattices.

3.3. Zeroth order

The *trans*-bilayer distribution of coherent neutron-scattering density can be described as a one-dimensional bilayer profile which is constructed using Fourier summation. Each order of diffraction contributes a frequency component cosine function that gives a term in the summation. For centro-

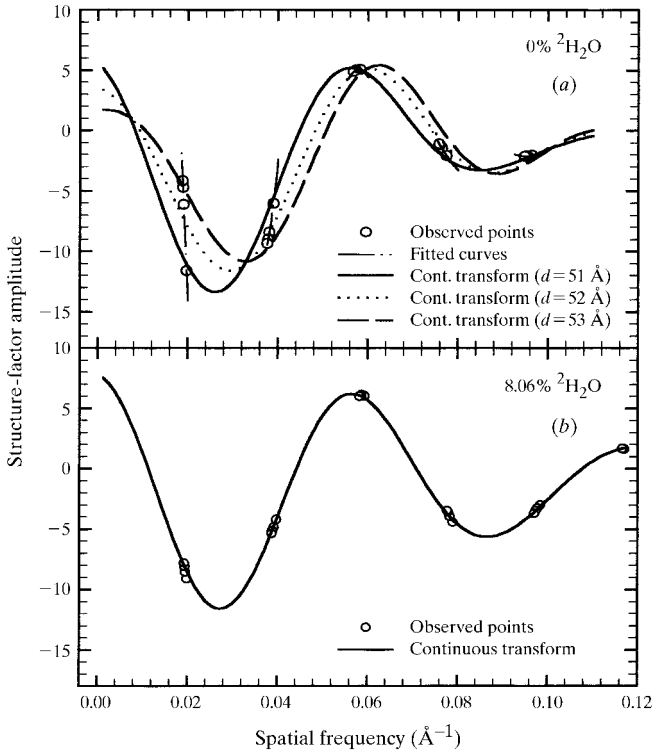

Figure 1
Plots of structure-factor amplitude *versus* spatial frequency of data points collected by real-time swelling series from highly aligned bilayers of DOPC at 298 K. The dry DOPC sample was placed in a D16 sample can along with water troughs containing water of 0% (a) or 8.06% ^2H_2O (b). Consecutive θ–2θ scans were run over a total period of 12 h (a) or 9 h (b). Also shown are continuous transforms calculated using (1).

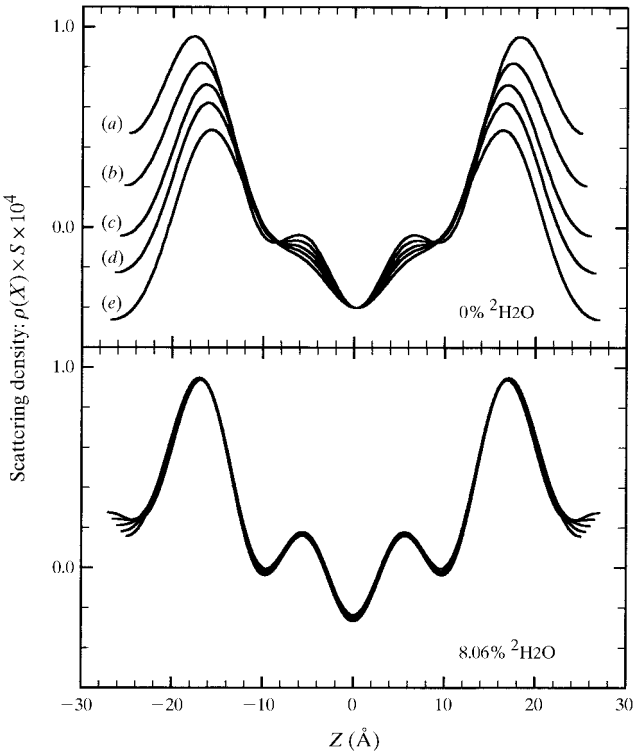

Figure 2
Swelling series of neutron-scattering density profiles of DOPC calculated by Fourier synthesis using structure factors from Table 1. (a) d = 50 Å; (b) d = 51 Å; (c) d = 52 Å; (d) d = 53 Å; (e) d = 54 Å. The 8.06% profiles, shown in the lower panel, also range from d = 50 to d = 54 Å.

Table 1
Relative absolute structure factors for DOPC hydrated to various levels with 0% ^2H_2O or 8.06% ^2H_2O, derived from the data shown in Fig. 1.

In the case of the 0% ^2H_2O data, the structure factors were determined by interpolation between observed points. For the 8.06% ^2H_2O data, the structure factors are points on the single continuous transform which best describes all observed points.

	0% ^2H_2O					8.06% ^2H_2O				
d (Å)	50	51	52	53	54	50	51	52	53	54
$F(0)$	7.28	5.56	3.53	1.69	−1.04	7.86	7.86	7.86	7.86	7.86
$F(1)$	−14.06	−10.96	−7.69	−5.13	−1.57	−8.40	−8.25	−8.09	−7.94	−7.79
$F(2)$	−2.37	−5.28	−7.63	−9.01	−10.36	−4.16	−4.76	−5.31	−5.83	−6.30
$F(3)$	4.46	4.98	5.09	4.91	4.31	5.88	6.00	6.01	5.93	5.75
$F(4)$	−3.26	−2.56	−1.71	−0.97	0.15	−4.71	−3.99	−3.19	−2.33	−1.45
$F(5)$	−1.38	−1.82	−2.05	−2.09	−1.95	−2.42	−3.26	−4.03	−4.71	−-5.28
$\sum_{h=0}^{h=5} F(h)^2/d$	5.76	4.19	3.13	2.64	2.47	4.25	4.22	4.20	4.18	4.17
$\sum_{h=1}^{h=5} F(h)^2/d$	4.72	3.58	2.89	2.58	2.45	3.01	3.00	3.01	3.02	3.03

symmetric phospholipid bilayer structures, the Fourier equation is

$$\rho(x) = \rho(0) + \sum_{h=1}^{h_{\max}} F(h)\cos(2\pi x h/d), \tag{3}$$

where $\rho(0)$ is the mean value of $\rho(x)$. The value $\rho(0)d$ is equal to the area under the bilayer profile and also equals the unobservable zeroth order, $F(0)$. Its value can be determined by summing the total coherent scattering length of each atom in the unit cell. If the unit cell is considered to contain one phospholipid molecule, then $\rho(0)d$ is equivalent to the total coherent scattering length of one phospholipid molecule plus its associated waters. The number of waters present per lipid must therefore be known from other methods.

The Fourier series of the bilayer now becomes

$$\rho(x) = \rho(0) + (2/d)\sum_{h=1}^{h_{\max}} F(h)\cos(2\pi x h/d). \tag{4}$$

Since the mean scattering density of the unit cell is related to the amplitude of $F(0)$, an alternative form of this equation, which removes the factor of two by including both sides of the diffraction pattern, is

$$\rho(x) = (1/d)\sum_{h=-h_{\max}}^{h_{\max}} F(h)\cos(2\pi x h/d). \tag{5}$$

The mean scattering density of the unit cell can be estimated by summing the coherent scattering lengths of the atoms that comprise the unit cell. Therefore,

$$\rho(0) = (2/d)(n_w b_w + b_{\rm lip}), \tag{6}$$

where n_w is the number of waters per lipid, b_w is the coherent scattering length of water and $b_{\rm lip}$ is the coherent scattering length from a single lipid molecule. The factor of two simply arises from the fact that the bilayer is composed of two monolayers.

$F(0)$ cannot be determined experimentally, yet an accurate estimate of its amplitude is essential for the construction of continuous transforms from diffraction data. The introduction of extra solvent in the swelling-series method normally also changes the mean scattering density of the unit cell and, therefore, also changes $F(0)$. This is demonstrated in Table 1. Example values of $F(0)$ for neutron and X-ray studies are given in Table 2. The change in $F(0)$ with hydration means that the structure factors from swelling-series measurements of stacked bilayers do not fit on a single continuous transform, as shown in Fig. 1. However, in the special case of neutron structure factors of stacked bilayers hydrated with 8.06% ^2H_2O, $F(0)$ does not change with hydration because water of this isotopic composition has a net neutron-scattering density of zero. With this solvent, the value of $F(0)$ determined at one hydration can be used for all other hydrations, and the fact that $F(0)$ does not change removes a large source of potential error when fitting the observed points to a single continuous transform (see Fig. 1).

3.4. Scaling

Another significant source of error in X-ray swelling series measurements comes from the scaling of the individual data sets to each other. Each point in the swelling series has to be determined from a different sample, since ionizing radiation damage precludes the reuse of samples for more than one measurement. The scaling procedure is classically based upon the formula

$$k = \sum_{h=0}^{\infty} F^2(h)/d, \tag{7}$$

where k is a constant. However, this summation must include $F(0)$ which, as we have already shown, changes with hydration. The scattering-density contrast also changes with humidity (see below). Moreover, the summation should also extend to infinity, which is clearly impractical. The result of carrying out the summation over an incomplete series and with either no zeroth order or at best an approximation is an inaccurate scaling of the data sets to each other. The errors inherent in this process are quantified in Table 1.

None of these limitations applies to the neutron adaptation of the swelling-series method when used with 8.06% ^2H_2O. The low levels of radiation damage inherent in the use of neutrons means that several swelling-series points can be obtained from the same sample. In the real-time swelling approach described here, the sample is not even disturbed

Table 2
Relative absolute $F(0)$ values for common phospholipids.

$F(0) = \sum_{n=1}^{n} b(n)$, where b is the coherent scattering-length of the nth atom and the summation includes all atoms in a pair of phospholipid molecules and associated waters. The scattering lengths used in these calculations came from (13). $F(0)$ for other hydrations can be calculated by adding 5.62 (X-rays), −0.34 (neutrons, 0% ^2H_2O) or 3.83 (neutrons, 100% ^2H_2O) per water to the value given above for five waters per lipid.

	X-rays		Neutrons 0% ^2H_2O		Neutrons 100% ^2H_2O		Neutrons 8.06% ^2H_2O†
Lipid‡	5 w/l§	10 w/l	5 w/l	10 w/l	5 w/l	10 w/l	
DOPC (18:1)	272.3	300.6	6.2	4.5	27.0	46.2	7.9
DOPG (18:1)	267.2	295.3	8.5	6.8	29.3	48.4	10.1
DOPE (18:1)	258.8	286.9	6.7	5.0	27.5	46.6	8.4
DOPS (18:1)	270.6	298.7	11.1	9.4	31.9	51.0	12.8
DPoPC (16:1)	254.3	282.4	6.9	5.2	27.7	46.8	8.5
DPPC (16:0)	256.5	284.6	3.9	2.2	24.7	43.8	5.5
DPPG (16:0)	251.4	279.5	6.1	4.4	27.0	46.1	7.8
DPPE (16:0)	243.0	271.1	4.3	2.6	25.2	44.3	6.0
DPPS (16:0)	254.8	282.9	8.8	7.1	29.6	48.7	10.4

† Water of this isotopic composition has a net neutron-scattering length of zero, so the values of $F(0)$ quoted apply to all hydration levels. ‡ DOPC: 1,2-dioleoyl-*sn*-glycero-phosphocholine; DOPG: 1,2-dioleoyl-*sn*-glycero-phosphoglycerol; DOPE: 1,2-dioleoyl-*sn*-glycero-phosphoethanolamine; DOPS: 1,2-dioleoyl-*sn*-glycero-phosphoserine; DPoPC: 1,2-dipamitoleoyl-*sn*-glycero-phosphocholine; DPPC: 1,2-dipalmitoyl-*sn*-glycero-phosphocholine; DPPG: 1,2-dipalmitoyl-*sn*-glycero-phosphoglygerol; DPPE: 1,2-dipalmitoyl-*sn*-glycero-phosphoethanolamine; DPPS: 1,2-dipalmitoyl-*sn*-glycero-phosphoserine. § Waters per lipid.

between measurements, so that the requirement for scaling between measurements in the same swelling series disappears.

3.5. Contrast

The introduction of water at any isotopic composition other than 8.06% ^2H_2O affects not just the value of $F(0)$ but also has an impact on the other structure factors. Fig. 2 compares the bilayer structure at different points on the swelling series. At 0% ^2H_2O, it is clear that water penetration into the head-group region reduces the height of the phosphate-ester peaks and shifts their centre of mass into the bilayer. This demonstrates that the effect of increasing the water content is to reduce the neutron-scattering contrast of the system. The region to which the negatively scattering water is introduced is immediately adjacent to and, indeed, partially overlaps the region of highest scattering density, namely the phosphates and ester linkages. The change in scattering contrast, the difference between the minimum and maximum scattering density or the maximum deviation from the mean scattering density $\rho(0)$, therefore has an impact on all structure factors. This effect is seen most clearly in Table 1, where $\sum F(h)^2/d$ changes as the d-repeat swells, even when $F(0)$ is included in the summation. Moreover, as the level of hydration rises, the d-repeat increases and the phosphate peaks appear to move further into the bilayer as the negatively scattering water erodes their hydrated edges. It is not possible, therefore, to use the location of these peaks as a measure of the bilayer thickness, as some authors have chosen to do, since their centre of mass shifts position with changes in hydration.

3.6. Sample disorder

The d-repeat of the 0% ^2H_2O swelling-series sample increased from 50.8 to 53.2 Å and that of the 0% ^2H_2O sample increased from 50.4 to 51.8 Å over the period of data collection. The mosaic spreads of the swelling-series samples were comparable to those from a similar sample measured under more conventional steady-state conditions. For example, the half-width at $1/e$ height ($e = 2.71828$) of the second order of the 0% ^2H_2O swelling-series sample was 0.34, 0.38, 0.38 and 0.36° for the four scans, compared with 0.38° for a steady-state sample of pure DOPC measured with identical instrument geometry, fully equilibrated to an atmosphere of approximately 100% rh.

These measurements show that there was no noticeable increase or decrease in the sample disorder throughout the swelling-series measurements. The low mosaic spread is typical for measurements of (static) unsaturated phospholipids such as DOPC, but is perhaps not expected in dynamic systems as reported here. In order to minimize disorder in the sample, two complementary factors are important in minimizing the degree of swelling during the measurement of each single order. Firstly, the sample should equilibrate with the atmosphere inside the sample can faster than the can atmosphere equilibrates with the water at the base of the can. In our measurements, this was achieved by using only one water bath, in which the surface area of the water was relatively small (approximately 5 cm^2). Secondly, the time spent in scanning each order should be the minimum consistent with good counting statistics. In this respect, the high neutron flux of the D16 instrument was advantageous to the study.

3.7. Adjusting the *d*-repeat

A standard procedure in neutron measurements is that of difference calculation, which is most powerful when both data sets in a subtraction have exactly the same d-repeat. However, this is rarely the case; typically, one has to be content with a difference of up to 2% or so. Fig. 2 shows a series of bilayer scattering profiles, calculated from points on the lines interpolated between the swelling-series points. The difference between profiles that differ by only 1.0 Å is apparent. This error is quantified in Fig. 3. The figure shows that a difference in d-repeat of 1.0 Å in a difference subtraction with 0% ^2H_2O data introduces an error of close to 20% in the result (plot *a*).

This error is approximately halved if the data are collected at 8.06% ^2H_2O (plot *b*).

It has been proposed (Büldt *et al.*, 1979) that when the two sets of structure factors to be used in a subtraction do not have exactly the same *d*-repeat, one of the sets can be recalculated using the continuous-transform method (1). Fig. 1 demonstrates that this is only possible in the unique situation where the data are collected at 8.06% ^2H_2O, since at 0% ^2H_2O the observed structure factors do not actually trace out a single continuous transform, as shown in the figure. Fig. 3(*c*) quantifies the error inherent in mistakenly using the continuous transform to adjust the *d*-repeat of data collected at 0% ^2H_2O. Up to a difference in *d*-repeats of approximately 0.6 Å the 'correction' has little effect upon the error. Above 0.6 Å difference, the 'correction' procedure actually increases the magnitude of the error.

However, the swelling-series method described here allows a degree of adjustment of the *d*-repeats of lamellar structure factors. At 8.06% ^2H_2O, the continuous-transform method can be used. Even at 0% ^2H_2O, the swelling method allows interpolation between the measured values of each structure factor in order to determine the bilayer structure at any *d*-repeat within the range covered by the measurements.

The above discussion appears to have neglected the suggestion that the bilayer structure might change during the hydration process. It has been suggested that the conformation of phosphate-containing head group of dimyristoylphosphatidylcholine in the L_α phase is dependent upon hydration level (Worcester, 1976). It is possible that the 0% ^2H_2O data are affected by this phenomenon, though it is not certain that the current resolution ($h = 5$) would be sufficient to show this. X-ray diffraction measurements have also shown that there can be structural rearrangements of the fatty-acyl chains as a function of hydration (Hristova & White, 1998). It is unlikely that neutron diffraction would be sensitive to these structural changes in undeuterated lipids. Moreover, unless any structural rearrangement occurs over a very small change in *d*-repeat (the smooth curves through each 0% order in Fig. 1 do not show this to be the case), then calculating an intermediate set of structure factors between the observed points will simply result in an intermediate structure. This view is further reinforced by the observation that the 8.06% ^2H_2O data appear to fit very closely to a single continuous transform, thereby indicating that neutron diffraction is insensitive to any lyotropic structural changes of the lipids that may have occurred during these measurements.

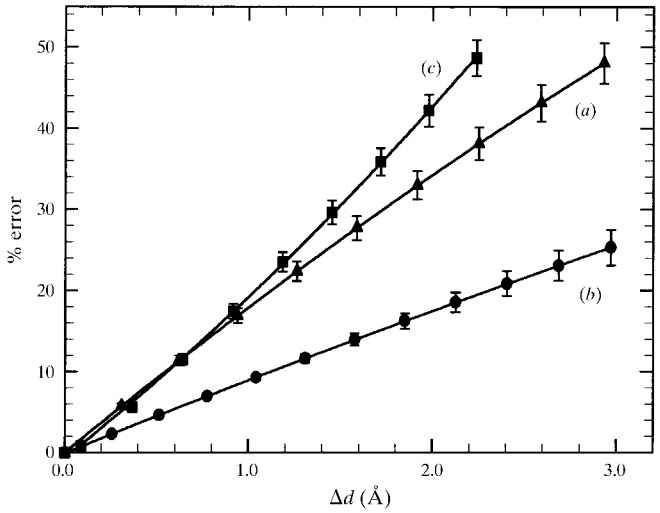

Figure 3
Determination of errors introduced into difference subtraction data by subtracting structure-factor sets that do not have the same *d*-repeat. Experimentally determined structure factors (five orders) for DOPC at various *d*-repeats were subtracted from each other and the difference expressed as a percentage. The points shown are the mean of five independent calculations with the error bars showing the maximum spread. (*a*) 0% ^2H_2O data; each data point represents the mean of five calculations of the form percentage error = $100\sum_{h=1}^{5} |[F_{(h)} - F'_{(h)}]| / 100\sum_{h=1}^{5} |F_{(h)}|$, (*b*) 8.06% ^2H_2O data; errors calculated as in (*a*). (*c*) 0% ^2H_2O data; errors calculated as in (*a*), except that the continuous-transform method (1) was used to correct one of the data sets before the subtraction. The magnitude of the error increases as the coherent neutron scattering density of water deviates from zero. In other words, the errors are lowest at 8.06% ^2H_2O, greater at 0% ^2H_2O and are therefore predicted to be greatest at 100% ^2H_2O.

4. Conclusions

We have demonstrated that it can be advantageous to sample a range of points in the reciprocal lattice during neutron-diffraction measurements of stacked phospholipid bilayers. This can be achieved in two ways: by recording several static measurements using a number of known relative humidities or, as we have demonstrated here, by changing the sample hydration during the data collection. Whichever method is used, phase information is gained. If the hydration state is slowly changed without disturbing the sample, it is not necessary to scale the different data sets to each other. Interpolation between the observed points for any order can then be used to determine structure-factor sets of any intermediate *d*-repeat, thereby reducing the errors in difference subtractions. We have demonstrated and quantified the error caused by neglecting to correct the *d*-repeat differences in subtractions and also the error introduced by mistakenly using the continuous-transform method to adjust the *d*-repeat of 0% ^2H_2O data.

We have argued (Bradshaw *et al.*, 1998) that it is valid to interpolate between observed points for any order at 0% ^2H_2O, even though these points do not lie on the continuous transform of the neutron-scattering profile. However, a more satisfactory approach is to collect the data at 8.06% ^2H_2O. At this isotopic composition the water is effectively invisible to neutrons and neutron scattering by the water is eliminated. The net result is similar to the so-called 'minus fluid model' proposed by Worthington *et al.* (1973) and developed by Hristova & White (1998), except that in the neutron method the removal of scattering from water is achieved without mathematical manipulation. Only under these conditions will all observed structure factors lie on the same continuous transform, thereby allowing the same function to be fitted simultaneously to all observed points. This approach is far more rigorous than the fitting of arbitrary functions to indi-

vidual orders of data collected at any other ^2H_2O concentration. The accuracy is improved by fitting one curve to all observed points. It also gives phase information and allows the adjustment of *d*-repeats of data sets for use in difference subtractions.

The use of 8.06% ^2H_2O has other advantages. The introduction of substances such as peptides into bilayers may affect the distribution of water within and between the stacked bilayers. This will be reflected in difference subtractions. By making the water effectively invisible to neutrons, difference subtractions at 8.06% ^2H_2O removes this contribution from the difference profile, allowing less ambiguous interpretation of the results.

Taking all these advantages together, the improved accuracy, reduced equilibration time and the ability to calculate structure-factor sets at any selected *d*-repeat (within a given range), the swelling-series method has much to recommend its adoption for neutron-diffraction studies. We therefore propose this adaptation of the swelling method, not as a replacement for the more usual neutron approach of isotopic substitution, but rather as a complimentary technique which can help to improve the accuracy of structure-factor determination whilst also providing an independent 'second opinion' for phase determination. Its added advantage of reducing the requirement for sample-equilibration time at the start of an experiment will assist in the optimal utilization of neutron beam-time allocations.

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