

Direct determination of crystallographic phases for diffraction data from lipid bilayers

II. Refinement of phospholipid structures

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ABSTRACT Using a systematic approach for the acceptance of crystallographic phase assignment, based on the evaluation of triplet structure invariants, electron and x-ray diffraction data from phospholipid multilamellar arrays are analyzed by direct methods. After calculation of Fourier maps with a partial set of phased structure factor magnitudes, the structure is refined in real space by flattening of the hydrocarbon region of the bilayer and an optimal solution is sought either by the calculation of $\langle \Delta\rho^4 \rangle$ suggested by Luzzati, where ρ is the structure density or by a test of density smoothness $\langle |\partial\rho/\partial r| \rangle$, where r positions are located along the normal to the lamellar surface. Reanalyses of previously determined structures sometimes lead to new conclusions (e.g., a possible similarity of the electron density profile for DL-DMPE and L-DMPE, and a clear indication of the fatty acid adduct in the mixed L-DPPC/palmitic acid bilayer). Because of presumed secondary scattering perturbations (primarily to the least intense reflections), the refinements of the electron diffraction intensities are less easily evaluated than those carried out with x-ray diffraction data.

INTRODUCTION

In order to minimize some of the difficulties encountered in determining the structures of lipid multilamellar arrays, high resolution electron micrographs from an epitaxially-oriented phosphatidylethanolamine have been shown (Dorset et al., 1990) to be an effective device for deriving crystallographic information to at least 10 Å resolution. An attempt to extend the resolution of the phase determination to the ~ 3 -Å resolution of the electron diffraction pattern via the evaluation of three-phase structure invariant relationships (Hauptman, 1972) also proved to be quite successful (Dorset et al., 1990). As a quite unexpected result, the use of such direct phasing techniques by themselves, were also found to determine correctly many of the phases of the diffraction pattern so that the Fourier maps calculated from electron- or x-ray-diffraction data closely resembled those based on a total phase set (Dorset, 1990). However, in this initial evaluation of the method, the constraints on the use of three-phase invariants were not totally understood; i.e., while so-called sigma 2 relationships were often found to interrelate high angle data correctly, the overall reliability of the sigma 1 formula was uncertain.

As discussed in the preceding paper in this series (Dorset, 1991a), the procedure for employing these three-phase structure invariants for phase determination of lamellar structures is now better understood. Often the correct use of the sigma 1 formula can be predicted from the distribution of normalized structure factors plotted as a function of reciprocal spacing. Based on model data sets derived from 10 lipid crystal struc-

tures, the phase determination of six of these was correctly carried out with little difficulty. Because of solvation or headgroup size, placing significant density at the lamellar origins, three other structures of this set could be determined with an appropriate π -shift applied to phases found at high angle by the Σ_1 -formula. The remaining structure of a methyl cerebroside is merely a difficult problem to solve by direct methods since some errors are found in Σ_2 -triples when the A_2 values associated with them become too small. Nevertheless, a rather conservative phasing procedure, setting a threshold value for accepting these triples, based on the magnitude of A_2 , followed by refinement, arrived at an electron density profile very close to that of the model.

It is also interesting to find that a phase refinement procedure based on the density modification procedure used in protein crystallography (Wang, 1985) works quite well for these lamellar structures. As suggested originally by Worthington et al. (1973), the one region of these structures where restrictions can be placed in real space is the hydrocarbon packing, which should have a flat profile. Progress of the refinement can be monitored by a test for density flatness (Luzzati et al., 1972), which was shown in the preceding paper to be useful after an initial estimate of a partial phase set is found by direct methods. A test for density smoothness also has been shown to be successful and seems to be effective for identifying near homometric structure solutions. Most often this real space refinement requires only a single cycle to find a stable phase set. Application of these

techniques to actual data sets from phospholipid multilamellar arrays will be described in this paper.

Diffraction data

X-ray and electron diffraction data used in the analyses reported below are mostly taken from our initial paper (Dorset, 1990). To supplement the x-ray data sets, intensities from three more multilamellar arrays were obtained from a recent paper by Katsaras and Stinson (1990), for which the structures had been determined by swelling the bilayers in water to map out the continuous Fourier transform. These include another data set from 1,2-dipalmitoyl *sn* glycerophosphocholine (DPPC) and its binary adducts with ~40 mol percent of two fatty acids, viz., palmitic acid (L-DPPC:PA) and 2 bromopalmitic acid (L-DPPC:BPA). Taking their intensity data at 0% relative humidity, the corresponding lamellar spacings are, respectively, 58.0, 56.6, and 56.9 Å.

METHODS

As described in the two preceding papers (Dorset, 1990, 1991a), after the observed structure factors are normalized to $|E_h|$ so that $(|E_h|^2)_h = 1.0$, three phase invariants of the type

$$\phi = \psi_{h_1} + \psi_{h_2} + \psi_{h_3}$$

are generated where $h_1 = h_2 = -1/2h_3$, if the triple is sigma 1, and $h_1 \neq h_2 \neq h_3$, if it is sigma 2. The condition that $h_1 + h_2 + h_3 = 0$ is always satisfied. These triples then are ranked in order of values A_1 and A_2 , which are directly proportional to their probability of correctly predicting the value of ϕ . The A_i values, moreover, are functions of the $|E_h|$ magnitudes, as described before (Hauptman, 1972, Dorset, 1990), so that the larger magnitudes will correspond to the most reliable phase relationships. The resulting simultaneous equations in phase are then solved for unknown values after assigning the phase value of an origin-defining reflection, generally ϕ_{001} .

Because the value of F_{000} is generally not known for mixed bilayer systems (but see a discussion by Nagle and Wiener [1989]), it is often not used for the calculation of Fourier maps. Its effect is to set a density level for the map, since, the Fourier transform of a δ -function at the reciprocal space origin is a flat signal over all real space (Champeny, 1973). Using a test for density flatness, suggested by Luzzati et al. (1972), as a figure of merit for real space refinement, the lack of an F_{000} term is a convenience, since the mean density $\bar{\rho} = 0$. In the evaluation of a best structure, one seeks a minimum of $(\Delta\rho^4)$, where $\Delta = \rho - \bar{\rho}$ for the one-dimensional map.

RESULTS

Electron diffraction data

As stated earlier (Dorset, 1990), the measured electron diffraction intensity data may be inaccurate due to the possible influence of secondary electron scattering. Nevertheless, we can show how the methodology described in the preceding paper (Dorset, 1991a) will find crystallographic phase relationships to generate an initial

structure which can be refined by density modification. As found before (Dorset et al., 1990), direct phasing of DHPE (Fig. 1 a) determines values for 13 of 16 reflections. Numerous Σ_2 -triples involving ϕ_{001} show that the large angle reflections all have the same phase value and Σ_1 -triples specify that this value must be π . Real space refinement correctly finds the remaining phase values to result in a structural profile identical to the one based on the earlier refinement with a conformational model (Dorset, Massalski, and Fryer, 1987), as shown in Fig. 2 a. Comparison of Luzzati's moment calculation and the test of density smoothness (Table 1) for the two maps demonstrates that the model structure is at a minimum value. Another phosphatidylethanolamine, L-DMPE, is assigned phase values for 11 of 14 reflections (Fig. 1 b), one of which disagrees with the earlier determination using a conformational model (Dorset, 1988a). Refinement based on density flattening results (Fig. 2 b) in a structure that gives a significantly lower value of $(\Delta\rho^4)$ than the original model (Table 1), whereas $(|\partial\rho/\partial r|)$ favors the earlier model.

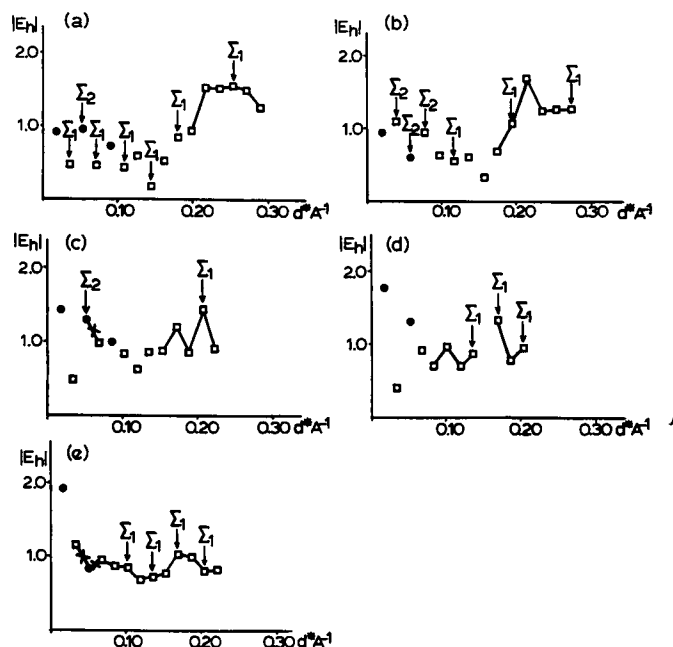


FIGURE 1 Direct phasing of electron diffraction data. Values of the normalized structure factor $|E_h|$ are plotted against d^* , and the phase sign determined from a previous structure analysis is indicated by the type of symbol used to designate this value (i.e., "●" represent phase value 0 and "□" phase value π). Reflections linked by a line are connected by Σ_2 triples via the ϕ_{001} value used to define the origin. False Σ_2 relationships are denoted by "X." Phase values of specific reflections defined by other relationships (e.g., Σ_1 -triples) are indicated. (a) L-DHPE, (b) L-DMPE, (c) L-DHPEM, (d) L-DHPC, (e) L-DPPEM₂.

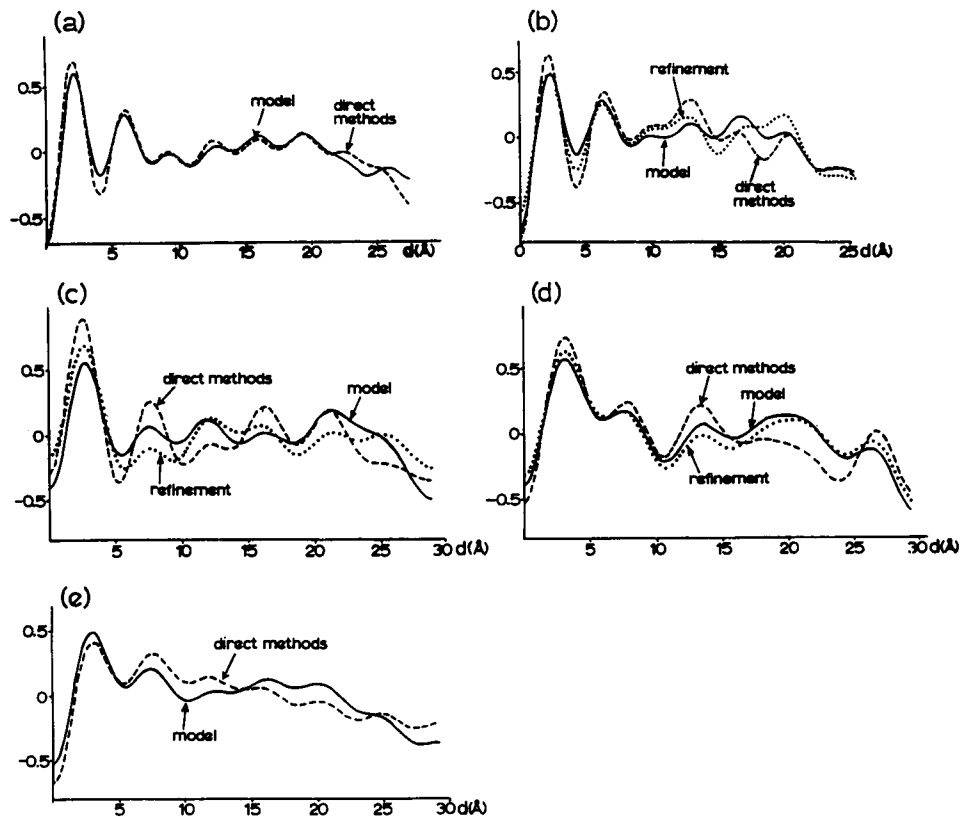


FIGURE 2 Electrostatic potential maps calculated from the phase sets. The curve marked "direct methods" is the Fourier map from the (often) incomplete phase determination schematically outlined in Fig. 1. The curve labeled "model" is a complete phase set from a previous determination. The profile labeled "refinement" is the result of the real space refinement discussed in the text. (a) L-DHPE, (b) L-DMPE, (c) L-DHPEM, (d) L-DHPC, (e) L-DPPEM₂.

Similar analyses were carried out for the other head-group classes studied earlier. For example 7 of 13 phases are found for DHPPEM (Fig. 1 c) and real space refinement evaluated by the Luzzati method is only partially successful (Fig. 2 c), as shown in Table 1 (see Dorset, 1988b). Note, however, that the density smoothness criterion indicates an improved structure. A lecithin structure, DHPC, is most successfully analyzed (Fig. 1 d) if only 8 of 11 phase values are included in the initial set.

TABLE 1 Use of $\langle \Delta\rho^4 \rangle$ (and $\langle |\partial\rho/\partial r| \rangle$) to evaluate structure refinement with electron diffraction data*

Structure	After direct phasing	After refinement	Earlier model
L-DHPE	1.18 (4.80)	0.94 (4.13)	0.94 (4.13)
L-DMPE	1.53 (5.00)	0.55 (3.89)	0.93 (3.70)
L-DHPEM	2.24 (4.52)	1.06 (3.44)	0.72 (3.58)
L-DHPC	1.87 (4.32)	1.07 (3.36)	0.97 (3.33)
L-DPPEM ₂	0.75 (2.42)	0.75 (2.42)	0.63 (2.60)

*Values multiplied by 10².

The final structural map (Fig. 2 d) corresponds to slightly higher figures of merit than found for the original model (Dorset, 1987), but both determinants agree that the model is the best structure. Finally, the structure determination of an *N,N*-dimethyl phosphatidylethanolamine, L-DPPEM₂ (Fig. 1 e) defines phase values for all observed reflections. One phase is different from the one found in the original analysis (Dorset and Zhang, 1990), and this difference from the model (Fig. 2 e) is clearly indicated by the higher value of $\langle \Delta\rho^4 \rangle$ (Table 1) but is contradicted by the test for smoothness.

Phase sets based on structural searches with conformational models are compared with those found in these direct analyses in Table 2. Except for two examples, it is clear that the two figures of merit can be contradictory in the refinement of electron diffraction data.

X-Ray diffraction data

Because measured x-ray intensities should have a much smaller multiple scattering distortion, direct structure

TABLE 2 Phase sets for electron diffraction data (based on lowest value of $\langle \Delta\rho^4 \rangle$)

<i>l</i>	L-DHPE		L-DMPE		L-DHPEM		L-DHPC		L-DPPEM ₂	
	Model	Direct analysis	Model	Direct analysis	Model	Direct analysis	Model	Direct analysis	Model	Direct analysis
1	0	0	0	0	0	0	0	0	0	0
2	π	π	π	π	π	0	π	0	π	π
3	0	0	0	0	0	0	0	0	0	π
4	π	π	π	π	π	0	π	π	π	π
5	0	0	π	π	0	0	π	π	π	π
6	π	π	π	π	π	π	π	π	π	π
7	π	π	π	π	π	π	π	π	π	π
8	π	π	π	0	π	π	π	π	π	π
9	π	π	π	π	π	π	*	*	π	π
10	π	π	π	π	π	π	π	π	π	π
11	π	π	π	π	π	π	π	π	π	π
12	π	π	π	π	π	π	π	π	π	π
13	π	π	π	π	π	π	π	π	π	π
14	π	π	π	π						
15	π	π								
16	π	π								

*Reflection missing.

analyses of such data are more successful than those carried out for electron diffraction sets. Indeed, more consistency also is found here between the two figures of merit used to assess the progress of the structure refinement (Table 3). A comparison is made for racemic and chiral dimyristoyl phosphatidylethanolamine structures based on previously published x-ray data (Hitchcock et al., 1975; Suwalsky and Duk, 1987). Analysis of the racemic compound assigns phase values for all but one reflection (Fig. 3 a). Its value is determined after real space refinement (Fig. 4 a) and the correctness of the structure is indicated by the lowest $\langle \Delta\rho^4 \rangle$ or $(|\partial\rho/\partial r|)$ values (Table 3). For the chiral material, only 11 of 15 phases are identified by direct methods (Fig. 3 b). Real space refinement finds a structure with a better Luzzati figure of merit than the one calculated with the original model (Dorset, 1988a) (Table 3), with a density profile (Fig. 4 b) now more similar to that of the racemic

bilayer. However, the test for density smoothness does not support this structure identification.

The choline-containing lipids are also analyzed. Comparing the experimental data of Torbet and Wilkins (1976) (Fig. 3 c) with those of Katsaras and Stinson (1990) (Fig. 3 d), it is found that starting phase sets include slightly different groups of reflections. Nevertheless, similar density profiles are found in the maps and real space refinement locates a minimum of both figures of merit (Table 3) for the same structure. Direct phasing finds correct values for all reflections using a data set from a lecithin analogue (Fig. 3 e and 4 e) (Lesslauer et al., 1973). As indicated in Table 3, there may be some errors in the direct phase analysis (Fig. 3 f) of a data set from sphingomyelin (Khare and Worthington, 1978) which are not removed by real space refinement (Fig. 4 f).

Finally, direct phasing, followed by real space refinement, appears to improve slightly the previous structure analysis of DPPC-fatty acid binaries, as indicated in Table 3. The effect is least apparent for the DPPC:BPA bilayer (Fig. 4 g), for which direct phasing identifies values for 11 of 12 reflections (Fig. 3 g). On the other hand, the direct analysis with triple invariants assigns phases to only 9 of 12 reflections for DPPC:PA (Fig. 3 h), and the subsequent real space refinement leads to a profile with more electron density in the second peak (Fig. 4 h) than found in the previous study, corresponding to lower values for both figures of merit.

A comparison of phase determinations based on earlier analyses with these direct determinations is given in Table 4.

TABLE 3 Use of $\langle \Delta\rho^4 \rangle$ (and $(|\partial\rho/\partial r|)$) to evaluate structure refinement with x ray diffraction data*

Structure	After direct phasing	After refinement	Previous model
DL-DMPE	1.01 (3.26)	0.94 (3.08)	0.94 (3.08)
L-DMPE	2.20 (5.07)	0.89 (4.06)	1.10 (3.96)
L-DPPC (T&W)	1.50 (2.75)	1.50 (2.75)	2.10 (2.80)
L-DPPC (K&S)	2.10 (2.76)	1.92 (2.71)	2.26 (2.71)
Lecithin analogue	1.64 (4.37)	1.57 (3.89)	1.57 (3.89)
Sphingomyelin	1.32 (4.67)	1.13 (4.39)	0.93 (4.31)
L-DPPC:BPA	2.65 (2.65)	2.41 (2.60)	2.47 (2.61)
L-DPPC:PA	1.74 (3.11)	1.35 (2.76)	1.98 (2.88)

*Values multiplied by 10².

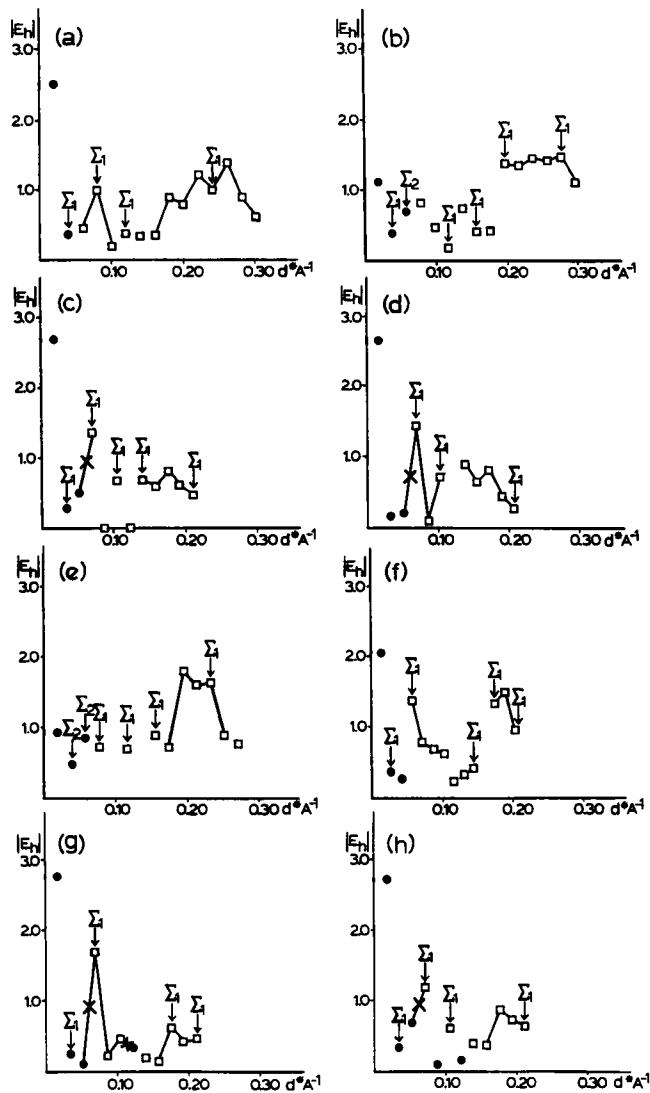


FIGURE 3 Direct phase determination of x ray diffraction data. The procedure is the same as outlined in the caption to Fig. 1. (a) DL-DPME, (b) L-DMPE, (c) L-DPPC (Torbet and Wilkins, 1976), (d) L-DPPC (Kataras and Stinson, 1990), (e) lecithin analogue, (f) sphingomyelin, (g) L-DPPC:BPA, (h) L-DPPC:PA.

DISCUSSION

Evaluation of three phase invariants followed by real space refinement appears to be a possible *ab initio* technique for the determination of lipid bilayer structures. However, it is important to speculate why this methodology should work at all, and also to explain its shortcomings.

The usual prerequisites that govern whether or not direct methods can be used to phase a diffraction data set are that the structure density (when scaled by the F_{000}

term in calculation of the Fourier transform) is positive and that a condition of atomicity can be imposed (Hauptman, 1989). For neutral molecules, the former condition is satisfied by both electron- and x-ray diffraction. Although the resolution encountered in these lamellar diffraction data clearly does not extend to atomic resolution, the condition of atomicity can still be imposed if these data are considered to be subsets of the total intensity data that would be collected from single crystals. One knows the atomic composition of the unit cell and hence a set of normalized structure factors can be calculated. However, the normalization based on the measured index range is incorrect, so that the $|E_h|$ magnitudes are somewhat underestimated (Dorset, 1990). The success of direct methods based on the probabilistic estimate of, e.g., Σ_2 -three phase invariants

$$\phi = \psi_{h_1} + \psi_{h_2} + \psi_{h_3}$$

depends on the actual magnitudes of

$$A = \frac{2}{\sqrt{N}} |E_{\vec{h}_1} E_{\vec{h}_2} E_{\vec{h}_3}|.$$

Thus, the conditional probability distribution of ϕ

$$P_{1/3} = P(\Phi | |E_{h_1}| |E_{h_2}| |E_{h_3}|)$$

based on the normalized structure factor magnitudes can be written (Hauptman, 1980)

$$P_{1/3} \approx \frac{1}{2\pi I_0(A)} \exp(A \cos \Phi),$$

where I_0 is a modified Bessel function. In all cases, the distribution of the phase values has a unique maximum at $\Phi = 0$ in the interval $-\pi$ to π ; thus the most probable value of ϕ is also zero. Nevertheless, it is the sharpness of this distribution, governed by A , that determines how reliably the phase estimates can be made. Because A is proportional to $1/\sqrt{N}$, it is easy to see why the reliability of the phase estimate decreases when the number of atoms in the unit cell increases. As evidenced by analysis of two phospholipid crystal structures (Pascher and Sundell, 1986; Pascher et al., 1987), the number of atoms in a phospholipid structure is well within the range of typical problems solved by direct methods nowadays, particularly when a constraint is placed on the phase angles to centrosymmetric values.

It should be recognized, however, that the preceding paragraph is only a partial explanation for the efficacy of triplet structure invariants in providing reliable estimates for these data. Although the correctness of Σ_2 -triples seems to correspond to the sequence of A_2 -values, as expected, some of these values seem to be very low for reliable phase estimates, even if the $|E_h|$

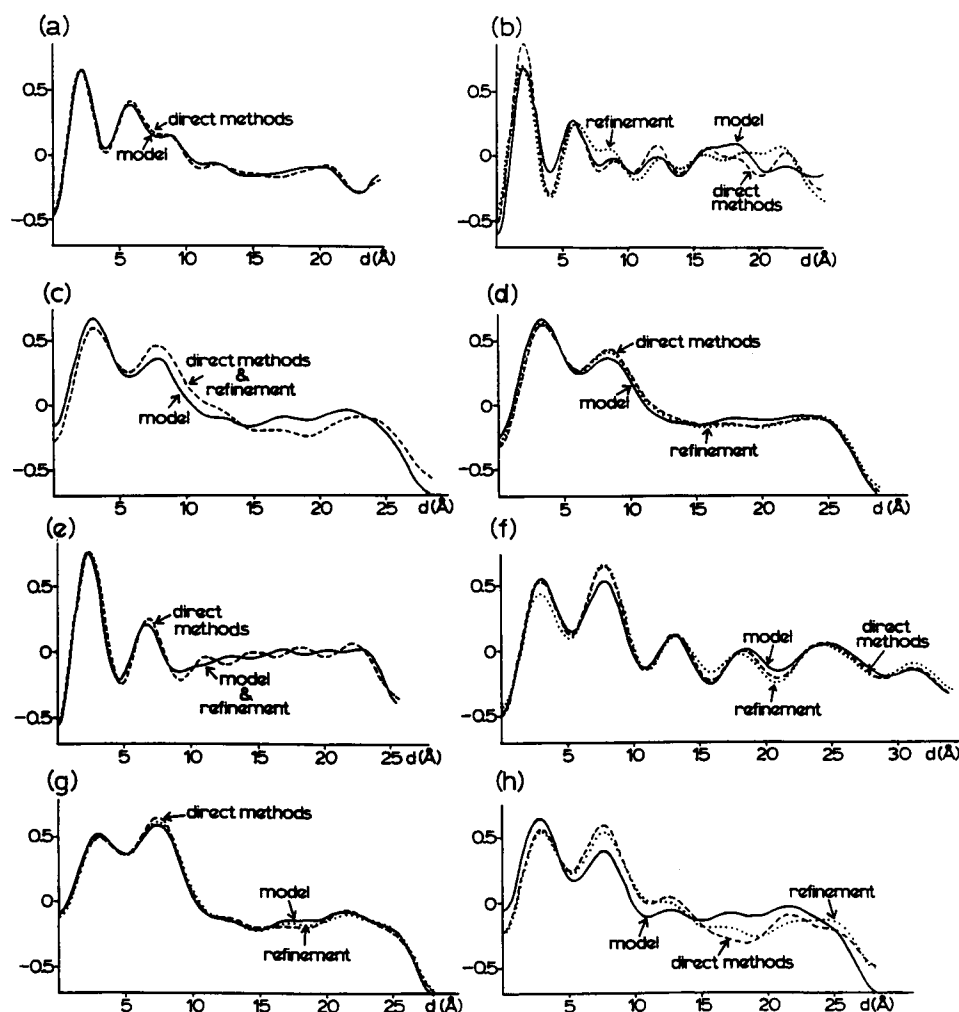


FIGURE 4 Electron density maps calculated from the phase sets determined from the analysis of x-ray data. The captions represent the same thing as specified in Fig. 2 (a) DL-DMPE, (b) L-DMPE, (c) L-DPPC (Torbet & Wilkins, 1976), (d) L-DPPC (Katsaras and Stinson, 1990), (e) lecithin analogue, (f) sphingomyelin, (g) L-DPPC:BPA, (h) L-DPPC:PA.

values are underestimated. The foregoing also does not predict the surprising reliability of Σ_1 -triples in many examples, which usually are only marginally useful in most single crystal determinations. This point is demonstrated in a review of direct phasing of 3-dimensional atomic resolution electron diffraction data from molecular crystals which has been published recently (Dorset, 1991b).

The greater difficulty in these structure analyses lies in the phase refinement. Here, one cannot use the knowledge of molecular architecture on an atomic scale to guide the progress of a refinement, and hence the analysis resembles those carried out for proteins, as pointed out by Karle (1989) in his discussion of direct methods applied to protein crystallography. How, then, can one recognize a correct solution and how can one

monitor progress of a phase refinement? As discussed in the previous paper (Dorset, 1991a), Luzzati et al. (1972, 1988) proposed a test of overall density flatness as a criterion for identifying a correct structural solution. This is very interesting, since the maximum values of this and similar moment calculations have been used to identify correct crystal structures at atomic resolution, since they test the "peakiness" of the density distribution (Stanley, 1986). Given a reasonable start to the phase assignment, it has been shown that this may be a reasonable figure of merit for refinement, but it clearly does not choose between near homometric structures. A test of density smoothness apparently works equally well, giving results that are consistent with the Luzzati calculation for most simulated and observed x-ray data sets. It may also be more useful for identifying possible

TABLE 4 Phase sets for x ray diffraction data

l	DL-DMPE		L-DMPE		L-DPPC (T&W)		L-DPPC (K&S)		Lecithin analogue		Sphingomyelin		L-DPPC:BPA		L-DPPC:PA	
	Previous model	Direct analysis	Previous model	Direct analysis	Previous model	Direct analysis	Previous model	Direct analysis	Previous model	Direct analysis	Previous model	Direct analysis	Previous model	Direct analysis	Previous model	Direct analysis
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	π	π	0	0	0	π	0	π	0	0	0	π	0	π	0	0
4	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π
5	π	π	π	0	*	*	π	π	—	—	π	π	π	π	0	π
6	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π
7	π	π	π	0	*	*	π	π	—	—	π	π	0	0	0	0
8	π	π	π	π	π	π	π	π	π	π	π	0	π	π	π	π
9	π	π	π	π	π	π	π	π	π	π	π	0	π	π	π	π
10	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π
11	π	π	π	π	π	π	π	π	π	π	**	**	π	π	π	π
12	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π	π
13	π	π	π	π					π	π	π	π				
14	π	π	π	π							π	π				
15	π	π	π	π							π	π				

*Structure factor has zero value.

**Missing reflection.

alternative structure solutions that are near homomorphs. However, it is clear from Table 1 that results from data, which appear to be seriously affected by multiple scattering contributions, are less consistently evaluated by these figures of merit. This is because the weakest intensities being refined by density modification are the ones which, on a relative scale, are most greatly changed by, e.g., secondary scattering, where a weighted self convolution of the intensities is added to the original intensity set. Even for the more ideal x-ray diffraction examples, it is still uncertain how large the relative differences in $\langle \Delta\rho^4 \rangle$ or $\langle |\partial\rho/\partial r| \rangle$ values must be to be regarded as significant for choosing between two possible models.

Given the problems with phase refinement, the results of the x-ray analyses presented here are still interesting. In the initial analysis (Dorset, 1988a) of data from L-DMPE, a density profile was obtained which is somewhat different from that of the racemic compound, implying different headgroup conformations for the two compounds. In this analysis, a solution, supported by the Luzzati figure of merit, corresponds to profiles that are more similar, with density peak positions at nearly the same place, as well as a shoulder next to the peak normally associated with superimposed glyceride atoms (Hitchcock et al., 1974). Nevertheless, there must be somewhat different hydrogen-bonding motifs for the two forms, as revealed by comparison of melting points (Tenchov et al., 1984). However, since the test of density smoothness does not find the same minimum, this solution can only be viewed as a possible alternative.

The second result which appears to make more physical sense is the analysis of the L-DPPC:PA binary.

Using phases derived in the original analysis (Katsaras and Stinson, 1990), there is very little difference between this mixed bilayer profile and the structure of the pure lipid. However, after a reanalysis by direct methods, more density is found in the second peak away from the unit cell origin, similar to the analysis of the L-DPPC:BPA bilayer. This may imply that the fatty acid is hydrogen-bonded to a carbonyl of the glycerol backbone (Koynova et al., 1987), as cholesterol does in mixed bilayers (Worcester and Franks, 1976).

Finally, it is clear that further work needs to be done with the electron diffraction method to make the phase refinement more reliable. Deleterious perturbations, possibly due to secondary scattering, can be minimized by reducing the crystal thickness and, hence, one should concentrate on collecting better data. One might also exploit a suggestion made by Moodie (1965) to test for internal phase consistency, i.e., evaluating which reflections are most affected by n-beam dynamical scattering, by obtaining patterns from the same crystals at low and high electron accelerating voltages to see if the phase set obtained after real-space refinement accounts for the observed change in intensity at low voltage. In addition, a direct comparison of the electron diffraction data to powder x-ray data could be made, providing that the same crystal packing is maintained, so that direct phase assignments based on a quasi-isomorphous replacement (here two independent sets of scattering factors for the same compound) could be made (Hauptman, 1982).

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