Direct determination of crystallographic phases for diffraction data from phospholipid multilamellar arrays

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ABSTRACT Direct determination of crystallographic phases based on probabilistic estimates of Σ_1 and Σ_2 "triplet" structure invariants has been found to be an effective technique for structure analysis with lamellar x-ray or electron diffraction intensity data from phospholipids. In many cases, nearly all phase values are determined, permitting a structure density (electron density for x-ray diffraction; electrostatic potential for electron diffraction) map to be calculated, which is directly interpretable in terms of known bilayer lipid structure. The major source of error is found to be due to the distortion of observed electron diffraction intensity data by incoherent multiple scattering, which can significantly affect the appearance of the electrostatic potential map, but not the success of the phase determination, as long as the observed Patterson function can be interpreted.

INTRODUCTION

Because of the difficulties experienced in growing sufficiently large single crystals (Albon, 1976), the crystal structures of only a small number of 1,2-diacyl phospholipids have been determined. These include a phosphatidylethanolamine (Elder et al., 1977) and a lecithin (Pearson and Pascher, 1979), which have similar diglyceride and headgroup conformations, as well as a phosphatidic acid (Harlos et al., 1984), a phosphatidylglycerol (Pascher et al., 1987), and an *N,N*-dimethyl phosphatidylethanolamine (Pascher and Sundell, 1986), which express different conformational possibilities. Such three-dimensional crystal structures are of interest for elucidating geometric molecular factors important for determining the lowest energy layer packing.

When crystallization of a particular lipid has proved to be difficult, some clues about the molecular conformation have been uncovered by analyzing one-dimensional diffraction intensity data from multilamellar arrays, which sometimes can be recorded to reasonably high resolution (e.g., 3 Å). However, to use these data for a structure determination, reliable means must be found to phase the intensity data so that the Fourier transform can be calculated to produce the one-dimensional structure image.

A number of inventive approaches to the crystallographic phasing problem have been pursued. Given the existence of some three-dimensional crystal structures, it is obvious that a sufficiently accurate model might be constructed to be compared with observed lamellar diffraction data from a similar compound. For example, multilamellar arrays of 1,2-dimyristoyl *rac* glycerophosphoethanolamine built up by repeated dipping through a Langmuir-Blodgett layer were shown to have a molecular packing similar to the crystal structure of the dilauroyl homologue (Hitchcock et al., 1975). Because the one-dimensional lamellar mass distribution from such stacked bilayer arrays conforms to a pseudocenter of symmetry, the possible number of phase sets for n reflections is 2^n. When a suitable conformational model is translated past an arbitrary origin in $P\bar{1}$, the solution to the structure problem can, in principle, be found by location of the minimum for the crystallographic residual R. Recently such an approach was adapted to the analysis of one-dimensional electron diffraction data from epitaxially-oriented multilamellar crystals (Fryer and Dorset, 1986), including the solid-state packings of several phosphatidylethanolamines (Dorset et al., 1981). It was soon discovered that the low precision of the crystallographic residual for limited data sets (Hamilton, 1964) sometimes made it difficult to distinguish objectively between minima of nearly equal depth.

Alternative approaches to the phasing problem have been pursued. The Patterson function calculated from the lamellar intensities may be correlated to features of the headgroup packing, as shown in an analysis of a sphingomyelin structure by Khare and Worthington (1978). The structure was shown to be similar to that of the lecithins. Also using Patterson techniques, the position of a heavy atom label, such as bromine substitution on an acyl chain, can be found (McIntosh and Halloway, 1987; Lytz et al., 1984) to provide enough initial phase information to determine the bilayer packing. Such an approach has been used with great success in neutron diffraction analyses of lipids synthetically labeled with deuterium. The exhaustive study of 1,2-dipalmitoyl phosphatidylcho-

line was effective for describing the conformation of the choline headgroup (Büldt et al., 1979) and also to locate the positions of acyl carbons for both long chains, demonstrating the nonequivalence of *sn*-1 and *sn*-2 substitutions (Zaccai et al., 1979) also found in the x-ray crystal structure. Further studies were made on 1,2-dipalmitoyl phosphatidylethanolamine (Büldt and Seelig, 1980), as well as a 1,3-dipalmitoyl phosphatidylcholine (Büldt and de Haas, 1982) and a phosphatidylglycerol (Mischel et al., 1987).

From the work of Hosemann and Bagchi (1962), it was found that if the autocorrelation function of a single bilayer (so-called "Q-function") were known, then some means could be found to deconvolute this function to determine uniquely the density profile across the bilayer. It was found that the shape transform broadening of diffraction from a stacked bilayer array of limited size would furnish some information about this function (Lesslauer and Blasie, 1972). Alternatively, the Patterson function itself might be used to uncover details about the autocorrelation function (e.g., the part due to the polar headgroups), especially if a large domain of the structural motif could be considered as a flat "solvent" region (Worthington et al., 1973). Use of the chain hydrocarbon region for this purpose allowed low-angle data to be phased and based on trial structure analyses of a phosphatidylethanolamine and a phosphatidylcholine, the deconvolution approach appeared to be successful (Worthington and Khare, 1978).

The major technique for finding the continuous intensity Fourier transform of the unit-cell autocorrelation function is found in a discussion of the Shannon sampling theorem by Sayre (1952*a*). This theorem states that the continuous modulus transform can be generated from the discrete transform of a unit cell $x = \pm a/2$, spaced at $X = 0, \pm 1/a, \pm 2/a, \ldots$, by additionally superimposing the sinc πaX function at each delta function, weighted according to the modulus $|F(X)|$. This continuous transform can be obtained experimentally by swelling structures, which can be continuously solvated whereas the lamellar motif itself remains intact, to search for zeros of the transform and, thus, define boundaries for reciprocal space domains where all reflections within an intensity envelope have the same phase sign (Worthington and McIntosh, 1973). The correctness of the structure analysis is assessed either by a crystallographic residual or a comparison of calculated and derived autocorrelations in real space. Although this approach has been employed for the structure analysis of pure lipid (Levine and Wilkins, 1971) or membrane bilayer (Caspar and Kirschner, 1971) arrays, not all lipid or membrane multilayers can be swollen in order that the continuous transform can be sampled. Moreover, the structural unit is sometimes not preserved during the swelling experiments (Torbet and Wilkins, 1976) because hydration itself can induce the formation of different lipid packing motifs.

Thus, for structures that cannot be swollen in solvent, the only recourse for structure analysis, after finding some approximation of the unit cell autocorrelation function, as discussed above, is to attempt a direct analysis such as a deconvolution by either of two techniques proposed (Worthington et al., 1973). Unfortunately, the technique can fail due to error propagation, and, for unknown structures, it is not known whether the phase set determined by this method is a unique solution. Subjective constraints are placed on the solution based upon what "looks to be correct." Recently, hopes for a unique solution have been revived with a method for correctly determining where the zeros of the continuous intensity transform occur (Burge and Fiddy, 1981).

Electron microscopy has also been suggested as a means of directly determining phase information from multilamellar arrays of phospholipids. An initial attempt (Fernandez-Moran and Finean, 1957) to use electron microscope images for elucidation of phase information from membanes failed largely due to the presence of artifacts induced by embedding procedures and also uncertainties about stain locations. More recently, low-dose phase-contrast images of phospholipid bilayer assemblies, which are neither stained nor fixed, have been shown to provide correct low-angle phase information (Dorset, 1988). With a cryomicroscope, the resolution of such direct images from a phosphatidylethanolamine has been extended from 16 to 6 Å (Zemlin, F., E. Beckmann, and D. L. Dorset, unpublished data), again establishing the correctness of the first seven low-angle phases as determined by a structure analysis based on a model. The phases of the remaining nine reflections, moreover, could be recovered by use of crystallographic direct phasing techniques based on the estimated combined phases of triplet structure invariants (Hauptman, 1972), incorporating the phase set furnished from the electron microscope image in the analysis of the electron diffraction structure factor magnitudes. A surprising result of this study is that most of the phases could be correctly determined with these structure invariant relationships after defining the origin by initially setting the phase of only one reflection, i.e., assuming that the image data do not exist. In this paper, it is shown that this approach has a general application both for x-ray and electron diffraction data from phospholipid multilayers, thus, avoiding constraints imposed by low-precision figures of merit or difficulties with determining or analyzing the continuous intensity transform of a single unit cell.

DIFFRACTION DATA

X-Ray diffraction intensities from various multilamellar phospholipid and phospholipid-like arrays were taken from published sources for which various means were used to determine the phases of the 00ℓ reflections. These are listed in Table 1 along with the lamellar spacings. For lipids that can be swollen with water, only data from samples at 0% relative humidity were considered.

Electron diffraction intensities were taken from samples, which had been epitaxially crystallized on naphthalene, as described in several papers cited in Table 1. For these data sets, the phase assignments were made from translational searches with a molecular conformational model constructed from similar crystal structures after the approximate headgroup conformation was established by interpretation of the one-dimensional Patterson function (Dorset, 1987).

TABLE 1 Diffraction data from various phospholipid lamellar systems

Compound	Lattice spacing	Reference
	Å	
X-Ray data		
1,2-Dimyristoyl *rac* glycero-phosphoethanolamine (DL-DMPE)	49.5	Hitchcock et al., 1975
1,2-Dimyristoyl *sn* glycero-phospho-ethanolamine (L-DMPE)	50.2	Suwalsky and Duk, 1987
1,2-Dipalmitoyl *sn* glycero-phosphocholine (L-DPPC)	56.6	Torbet and Wilkins, 1976
1-Oleoyl-2-n-hexadecyl-2-deoxy-glycero-3-phosphorylcholine (lecithin analogue) (LECAN)	51.4	Lesslauer et al., 1973
Beef brain sphingomyelin (SPHING)	68.5	Khare and Worthington, 1978
Electron data		
1,2-Dimyristoyl-*sn*-glycero phosphoethanolamine (L-DMPE)	51.1	Dorset (1988*b*)
1,2-Dihexadecyl-*sn*-glycero-phosphoethanolamine (L-DHPE)	55.2	Dorset et al. (1987)
1,2-Dihexadecyl-*sn*-glycero-phospho-*N*-methylethanolamine (L-DHPEM)	58.4	Dorset (1988*a*)
1,2-Dipalmitoyl-*sn*-glycero-phospho-*N*,*N*-dimethyl ethanolamine (L-DPPEM$_2$)	58.7	Dorset and Zhang (unpublished data)
1,2-Dihexadecyl-*sn*-glycero phosphocholine (L-DHPC)	59.2	Dorset (1987*b*)

DIRECT PHASING WITH STRUCTURE INVARIANTS

As is well known, the crystal structures of many diacyl phospholipids have a space group $P2_1$, when the molecule is optically active, and $P2_1/a$, when a racemic compound is being examined. Whereas it is obvious that the racemic lipids might pack in a centrosymmetric unit cell, it is also true that the 00ℓ diffraction data from chiral material also conform to a centrosymmetric phase constraint due to the twofold screw axis along *b*. Thus, for our phase determinations, we can assume that the structure crystallizes in space group $P\bar{1}$.

It can be demonstrated (Hauptman, 1972) that for space group $P\bar{1}$, the phase of a structure invariant, a linear combination of phases

$$\sum_{\vec{h}} g_{\vec{h}} \phi_{\vec{h}},$$

where the integers $g_{\vec{h}}$ satisfy

$$\sum_{\vec{h}} g_{\vec{h}} \vec{h} = 0,$$

may be predicted by probabilistic means. A three-phase invariant ("triple"), thus, is defined

$$\phi = \phi_{\vec{h}_1} + \phi_{\vec{h}_2} + \phi_{\vec{h}_3}, \tag{1}$$

where the Miller indices

$$\vec{h}_1 + \vec{h}_2 + \vec{h}_3 = 0.$$

From a diffraction experiment, one measures the structure factor magnitudes $|F_{\vec{h}}|$ which can be converted to a normalized value $|E_{\vec{h}}|$ by

$$|E_{\vec{h}}|^2 = |F_{\vec{h}}|^2 / \left(\epsilon \sum_{i=1}^{N} f_i^2 \right), \tag{2}$$

where ϵ is a multiplicity factor and f_i is the (electron or x-ray) atomic scattering factor at $(\sin\theta/\lambda)_{\vec{h}}$. There are scaled so that

$$\langle |E_{\vec{h}}|^2 \rangle = 1.00.$$

Given only the three normalized structure factor magnitudes

$$|E_{\vec{h}_1}|, \quad |E_{\vec{h}_2}| \quad \text{and} \quad |E_{\vec{h}_3}|,$$

for a general sigma 2 (Σ_2)-triple invariant where

$$\vec{h}_1 \neq \vec{h}_2 \neq \vec{h}_3,$$

one can estimate the probability of the linear combination of phases having the value $\phi = 0$ by calculating

$$A_2 = (2\sigma_3/\sigma_2^{3/2})\,|E_{\vec{h}_1}E_{\vec{h}_2}E_{\vec{h}_3}|,$$

where

$$\sigma_n = \sum_{j=1}^{N} Z_j^n.$$

N is the number of atoms in the unit cell, and Z_j is the scattering factor for atom j at $\sin\theta/\lambda = 0$. It is important that the value of A_2 be sufficiently large to allow this phase prediction to be made with enough certainty. One is left with a number of three-phase relationships as in Eq. 1. A limited number of phases can be arbitrarily specified to define the unit-cell origin (in this case, only one with an odd Miller index for the one-dimensional row of diffraction data) and, thus, new phases are determined algebraically.

Additionally, one can define a sigma 1 (Σ_1) triple where

$$\vec{h}_1 + \vec{h}_1 - 2\vec{h}_1 = 0.$$

The phase of $\phi_{2h} = \pi$ can be determined for values of

$$A_1 = (|E_{\vec{h}}|^2 - 1)|E_{2\vec{h}}|/\sqrt{N},$$

which are negative. In the following examples, we shall demonstrate how successful these relationships are for determining phases for one-dimensional diffraction data from phospholipids.

RESULTS

The procedure for phase determination can be demonstrated with the x-ray data from DL-DMPE published by Hitchcock et al. (1975), which result in the normalized structure factors listed in Table 2. The phase of the 00ℓ reflection with highest $|E|$ value is first defined, i.e., $\phi_{001} = 0$, to fix the origin. For this and all other problems considered here, the diffraction data have a common characteristic in that a region of intense low-angle reflections are separated from another intense domain at higher angle by a low-intensity node. Sigma 2 phase triples, where high-angle phases are related to the origin-defining low-angle phase, can be valid for values of $A_2 \geq 0.1$. Sigma 1 triples are reliable for determining some phases where $\phi_h = \pi$ but are much less reliable for defining $\phi_h = 0$ at positive A_1 values (although one such example is listed in Table 2). For this data set, all but one phase can be determined directly with the structure invariant relationships. Results of similar phase determinations for x-ray diffraction data and electron diffraction data are represented in Tables 3 and 4, respectively, where the directly determined values are compared with the assignments based on other phasing techniques.

After reviewing the results outlined in Tables 3 and 4, several generalizations can be made about the success of such direct phasing procedures: (*a*) As stated, the method seems to work best when the observed unit-cell transform sampled by the reciprocal lattice contain two separated regions of high intensity. When the lamellar intensities fall under a single Gaussian envelope, the phasing tech-

TABLE 2 Phase determination for D, L-DMPE using lamellar x-ray data published by Hitchcock et al. (1975)

$\phi_{00\ell} = \phi_{00\bar{\ell}} = -\phi_{00\ell}$

Scaled normalized structure factors					
ℓ	$\lvert E_\ell\rvert$	ℓ	$\lvert E_\ell\rvert$	ℓ	$\lvert E_\ell\rvert$
001	2.496	6	0.382	11	1.208
2	0.382	7	0.333	12	0.998
3	0.465	8	0.354	13	1.393
4	0.994	9	0.891	14	0.891
5	0.200	10	0.801	15	0.607

Origin definition: $\phi_{001} = 0$

Sigma 2 relationships A_2	
0.800	$\phi_{001} + \phi_{0012} + \phi_{00\bar{1}\bar{3}} = 0$
	$\phi_{0012} = \phi_{0013} = a$
0.712	$\phi_{001} + \phi_{0013} + \phi_{00\bar{1}\bar{4}} = 0$
	$\phi_{0013} = \phi_{0014} = a$
0.696	$\phi_{001} + \phi_{0011} + \phi_{00\bar{1}\bar{2}} = 0$
	$\phi_{0011} = \phi_{0012} = a$
0.557	$\phi_{001} + \phi_{0010} + \phi_{00\bar{1}\bar{1}} = 0$
	$\phi_{0010} = \phi_{0011} = a$
0.410	$\phi_{001} + \phi_{009} + \phi_{00\bar{1}\bar{0}} = 0$
	$\phi_{009} = \phi_{0010} = a$
0.312	$\phi_{001} + \phi_{0014} + \phi_{00\bar{1}\bar{3}} = 0$
	$\phi_{0014} = \phi_{0015} = a$
0.267	$\phi_{001} + \phi_{003} + \phi_{00\bar{4}} = 0$
	$\phi_{003} = \phi_{004} = b$
0.179	$\phi_{001} + \phi_{008} + \phi_{00\bar{9}} = 0$
	$\phi_{008} = \phi_{009} = a$
0.114	$\phi_{001} + \phi_{004} + \phi_{00\bar{5}} = 0$
	$\phi_{004} = \phi_{005} = b$

Sigma 1 relationships A_1	
0.23	$\phi_{001} + \phi_{001} + \phi_{00\bar{2}} = 0;\ \phi_{002} = 0$
−0.03	$\phi_{003} + \phi_{003} + \phi_{00\bar{6}} = \pi;\ \phi_{006} = \pi$
−0.10	$\phi_{006} + \phi_{006} + \phi_{00\bar{1}\bar{2}} = \pi;\ \phi_{0012} = \pi;\ a = \pi$
−0.10	$\phi_{002} + \phi_{002} + \phi_{00\bar{4}} = \pi;\ \phi_{004} = \pi;\ b = \pi$

TABLE 3 Review of direct phase determinations for published x-ray diffraction data

	DL-DMPE		L-DMPE		L-DPPC		LECAN$^+$		SPHING	
ℓ	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	—	0	0
3	π	π	0	0	0	—	0	0	0	—
4	π	π	π	—	π	π	π	π	π	π
5	π	π	π	—	*	*	*	*	π	π
6	π	π	π	π	π	π	π	π	π	π
7	π	—	π	—	*	*	*	*	π	π
8	π	π	π	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	—
11	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
14	π	π	π	π			π	π	π	π
15	π	π	π	π						

ϕ_M, previous determination; ϕ_{DP}, direct phase determination (this work); *, structure factor has zero value or not measured; +, an alternate unit-cell origin has been chosen for this phase set.

nique is less successful. As examples of the latter condition, the interdigitated polymorph of L-DHPEM (Dorset, 1988) and the α-form of L-1,2-dipalmitin (Dorset and Pangborn, 1988) were found to give unsatisfactory results. (*b*) The sigma 1 triples seem to be most reliable for finding reflections where $\phi_{00\ell} = \pi$. Those with $\phi_{00\ell} = 0$ cannot be identified with confidence, and we only include cases in Tables 3 and 4 where a comparison can be made to a known structure. (*c*) Sigma 2 triples are also unreliable for identifying phase relationships solely between low-angle data within the first intensity envelope. Relationships between reflections at low and high angle are most useful, particularly if an origin-defining reflection with high $|E|$ value is included.

It should be pointed out here that the phase determination for L-DPPEM$_2$ was a direct assignment with structure invariants made before a separate analysis with a structural model was carried out. (The translational structure search with a molecular packing model will be published elsewhere.)

TABLE 4 Review of direct phase determinations for electron diffraction data

	L-DHPE		L-DMPE		L-DHPEM		L-DHPEM$_2$		L-DHPC	
ℓ	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}	ϕ_M	ϕ_{DP}
1	0	0	0	0	0	0	0	0	0	0
2	π	π	π	π	π	—	π	—	π	—
3	0	0	0	—	0	0	0	—	0	—
4	π	π	π	—	π	—	π	π	π	—
5	π	—	π	π	0	—	π	π	π	—
6	π	π	π	—	π	—	π	π	π	π
7	π	—	π	—	π	—	π	π	π	π
8	π	π	π	—	π	—	π	π	π	π
9	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		
14	π	π	π	π						
15	π	π								
16	π	π								

ϕ_M, Previous model determination; ϕ_{dp}, direct phase determination (this work); *, structure factor value not determined.

EVALUATION OF DIRECT PHASING RESULTS

Perhaps the most appropriate evaluation that can be made of these direct phase determinations is whether or not the computed structure density map can be interpreted.

X-Ray diffraction determinations

The most complete phase determination made in this study is for the racemic phosphatidylethanolamine, DL-DMPE, and the one-dimensional electron density map in Fig. 1 *a* contains details due to the headgroup and the hydrocarbon portions of the amphiphilic molecule. The latter acyl chain region is found to be at a rather uniform density level. Because only one reflection is missing from the direct phase determination, the map is not greatly different from the one calculated with a complete phase set. A similar observation can be made for L-DPPC, even though the data resolution is not so great as that for the phosphatidylethanolamine (Fig. 1 *b*). Although only one phase is missing for the determination of the lecithin analogue (Fig. 1 *c*), the hydrocarbon region, found in the reverse Fourier transform of the incomplete data set, contains ripples that do not correspond to an actual structure.

Other distortions in the map can be found when more phases remain undetermined. For example, the sphingomyelin (Fig. 1 *d*) electron density distribution has a rippled hydrocarbon region, even if all phases are used to

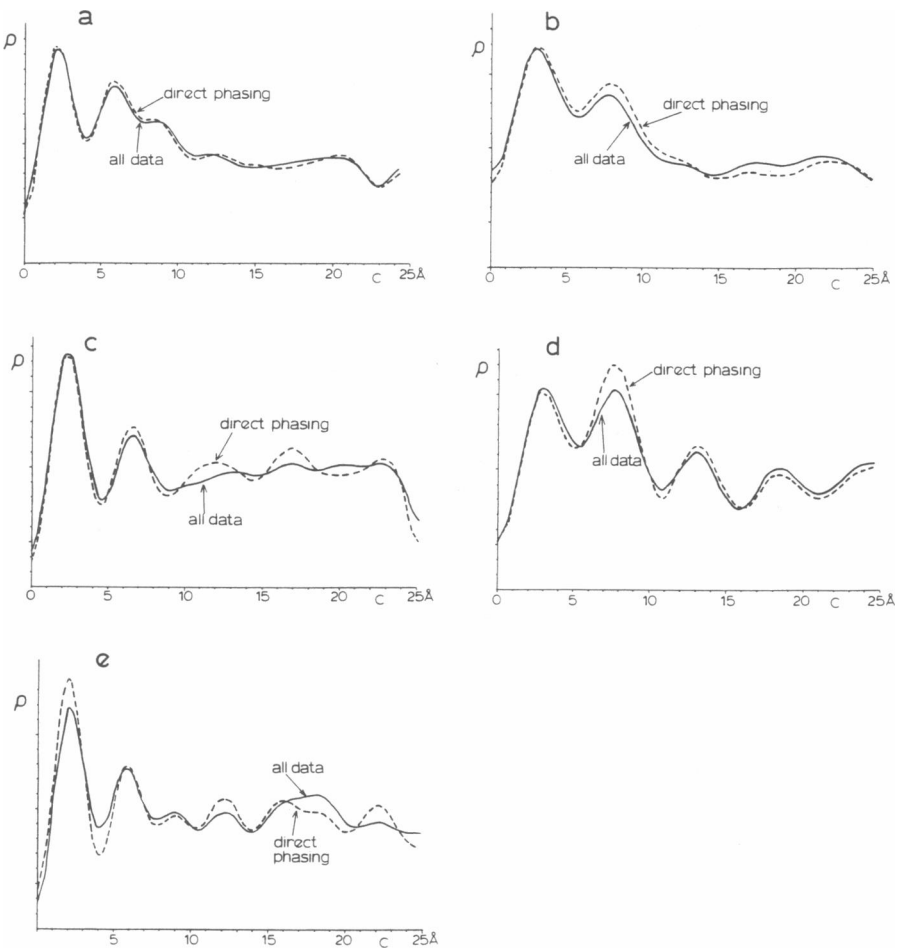

FIGURE 1 One-dimensional electron density maps of phospholipids determined from x-ray data (original phasing technique indicated parenthetically). Maps from full phase sets are compared with those calculated from direct phase determinations (Table 3) (*a*) DL-DMPE (crystal structure model). (*b*) L-DPPC (swelling). (*c*) Lecithin analogue (swelling). (*d*) Sphingomyelin (Patterson function and density strip model). (*e*) L-DMPE (model).

calculate the map. The incomplete determination raises the level of one of the two headgroup peaks but does not change its position. Other distortions of the acyl-chain regions are found when complete or incomplete phase sets used are for the reverse Fourier-transformation of the L-DMPE structure factors (Fig. 1 *e*). Again polar group peak heights can change, but their positions remain unaffected.

Electron diffraction determinations

There are greater problems in the interpretation of electrostatic potential maps computed from phased electron diffraction structure factors. For example, maps calculated for L-DHPE (Fig. 2 *a*) have recognizable polar group details when observed structure factors are used to compute the map. However, the hydrocarbon region has uneven density. There is a slight improvement in the map when ϕ_{005}, ϕ_{007}, and ϕ_{009} are left out of the calculation. This can be compared to the map calculated from phased $|F_{calc}|$ values (Fig. 2 *b*) where the hydrocarbon region is found to be flatter and more uniform, even when the phases undetermined by the use of structure invariants are left out.

Greater distortions of the computed electrostatic potential map are found in other examples. The structure of the L-DMPE (Fig. 2 *c*) hydrocarbon region is severely affected when undetermined phases are not included in the computation. The rippling of this region is extreme enough to obscure a headgroup peak contribution for

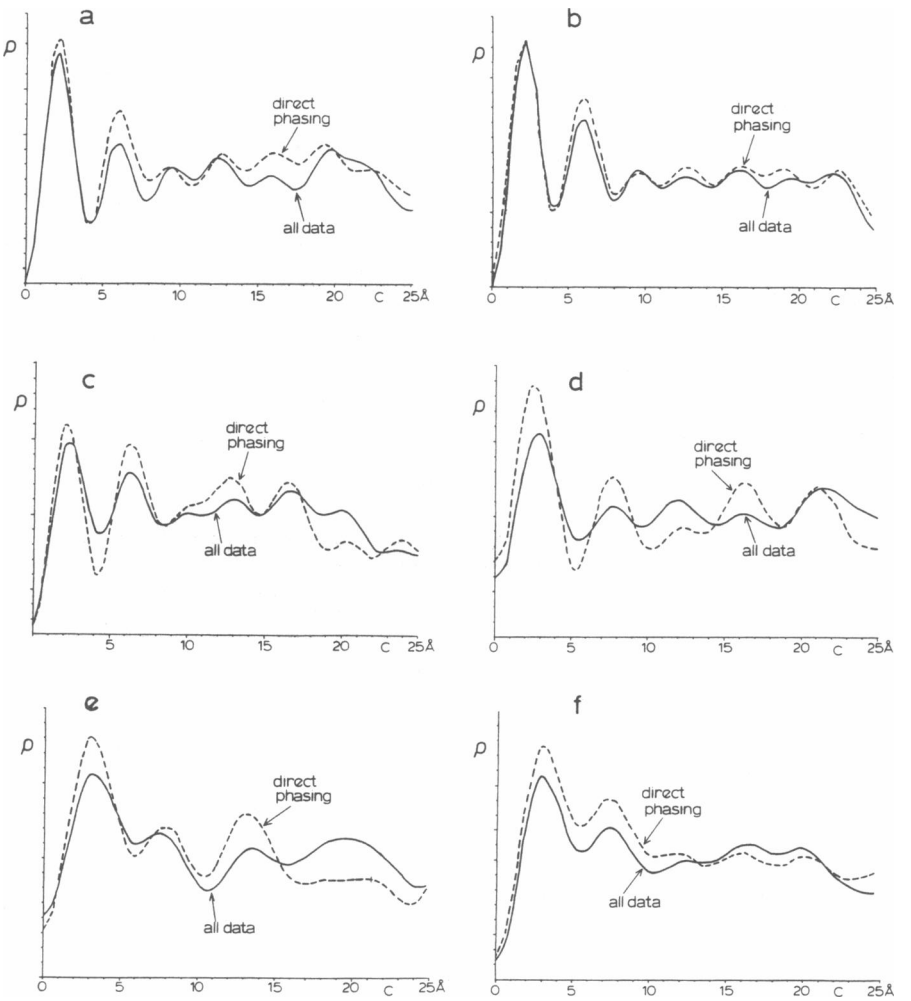

FIGURE 2 One-dimensional electrostatic potential maps of phospholipids determined from electron diffraction data. All structures were determined with conformational models, and the direct phase determinations (Table 4) are compared to these. (*a*) L-DHPE, (*b*) L-DHPE, F_{calc} data, (*c*) L-DMPE, (*d*) L-DHPEM, (*e*) L-DHPC, (*f*) L-DPPEM$_2$. If not indicated otherwise, observed structure factors were used to calculate the maps.

DHPEM (Fig. 2 *d*) and DHPC (Fig. 2 *e*). On the other hand, the density map for $DPPEM_2$ (Fig. 2 *f*) seems to be more similar to electron density maps obtained from x-ray analyses.

SOURCES OF ERROR

It is apparent that any data perturbation, which will significantly change the hierarchy of observed normalized structure factor magnitudes $|E_{\vec{h}}|$, will also adversely affect the success of the direct phase determination. For the x-ray data sets considered above, one parameter which might influence the order of relative magnitudes is the effective temperature factor applied to the scattering factor values when normalized structure factors are calculated. Because the data considered here are from the low-angle region, which is least affected by Gaussian positional disorder of the first kind (Vainshtein, 1966), such a correction was not used here, mainly because it is virtually impossible to determine *a priori* what it should be for data sets with limited resolution. It appears that such a correction would have been most appropriate for L-DPPC, although most of the phases were correctly identified by use of structure invariant relationships.

It is apparent from the maps in Fig. 2, however, that some scattering phenomenon significantly affects the electron diffraction intensity data from the phospholipids. It has already been determined that this perturbation is not due to *n*-beam dynamical scattering (Dorset et al., 1987). Recent work on epitaxially crystallized *n*-paraffins (Hu et al., 1989) and polyethylene (Hu and Dorset, 1989) revealed that the epitaxial orientation on an organic substrate itself results in a layered crystal growth (Hu and Dorset, 1989) and that this can lead to incoherent multiple scattering contributions to the intensity data.

Following the analysis of Cowley et al., (1951), the observed intensity J_ℓ is obtained if the single scattering intensity I_ℓ is combined with weighted convolution (*) products of the intensity due to the multiple scattering, i.e.,

$$J_\ell = I_\ell + m(I_\ell * I_\ell) + \cdots$$

This merely states that strongly diffracted beams from upper layers of a crystalline mat can function as primary beams for lower layers. For structures with space group forbidden reflections, this effect is recognized by observation of appreciable intensity where these extinctions should occur. For lamellar data, such as those considered here, the effect is more subtle, but weak reflections are observed to have larger magnitudes than they should have. Hence, the observed normalized structure factors are distributed over a narrower range of magnitudes.

If a correction for multiple scattering is made to electron diffraction data, there is a marked improvement in the fit between observed and calculated data sets, as shown in Table 5. (Although such an improved fit of data is also found for $DPPEM_2$, this will be published elsewhere.) In general, for structure determinations where an *R*-factor minimum was found in the region of $R = 0.30$, it is seen that a rather simple correction for a single-intensity convolution term will lower this figure of merit to $R = \sim 0.20$.

TABLE 5 Correction of electron diffraction data from phospholipids for incoherent multiple scattering $|F'_c|$

	L-DHPE			L-DMPE			L-DHPEM			L-DHPC		
ℓ	$\|F_o\|$	$\|F_c\|$	$\|F'_c\|$	$\|F_o\|$	$\|F_c\|$	$\|F'_c\|$	$\|F_o\|$	$\|F_c\|$	$\|F'_c\|$	$\|F_o\|$	$\|F_c\|$	$\|F'_c\|$
1	2.22	4.10	3.12	1.38	1.92	1.52	2.20	3.50	2.38	3.04	2.96	2.93
2	1.13	0.48	2.13	1.59	0.24	0.97	0.90	1.10	1.83	0.68	0.10	1.21
3	2.30	2.22	1.99	0.87	0.40	0.93	2.40	1.60	1.61	2.24	0.92	1.31
4	1.30	1.51	1.60	1.36	1.44	1.28	1.80	1.70	1.53	1.55	1.49	1.72
5	1.69	0.18	1.17	0.89	0.79	1.06	1.80	0.60	1.49	1.18	0.94	1.24
6	0.99	1.55	1.03	0.78	0.88	1.02	1.50	1.80	1.56	1.58	1.69	1.81
7	1.33	0.65	1.32	0.82	0.83	1.03	1.10	1.40	1.53	1.14	1.59	1.43
8	0.37	0.78	1.03	0.44	1.03	1.12	1.50	1.00	1.37	1.38	1.17	1.23
9	1.14	1.44	1.23	0.90	1.51	1.29	1.40	1.40	1.43	—	—	—
10	1.77	0.78	1.78	1.34	1.42	1.30	2.00	1.30	1.53	2.02	1.62	1.40
11	1.92	2.29	1.79	2.04	2.05	1.45	1.40	1.80	1.72	1.17	2.66	1.73
12	3.05	2.66	2.57	1.47	1.55	1.37	2.30	2.30	1.96	1.39	1.74	1.36
13	2.93	3.44	2.46	1.44	1.96	1.41	1.40	2.20	1.78			
14	2.96	3.18	2.56	1.40	0.68	0.97						
15	2.72	2.85	2.30									
16	2.20	1.92	1.94									
	R	0.31	0.20		0.31	0.22		0.30	0.21		0.30	0.21

F_o, observed structure factor; F_c, calculated structure factor.

DISCUSSION

It is rather surprising that direct phasing of lamellar diffraction data from phospholipids can be realized by applying three-phase structure invariant relationships normally used to solve molecular crystal structures. Even though the structure density map is somewhat altered by the absence of some reflections, it is nevertheless directly interpretable in terms of well-known features of bilayer phospholipid packing. Although the theoretical framework of this methodology is based on a concept of "atomicity" (Hauptman and Karle, 1953), the phasing procedure still can be applied to diffraction data extending out to a less than atomic resolution, subject to the constraints listed above. However, because the probability distributions are based on the definition of the normalized structure factor (Eq. 2), the scaling of these $|E|$'s for the low-angle data sets, as mentioned, may, in fact, underestimate their true value, so that the triple relationships defined are actually more valid than indicated by their estimated probability (H. A. Hauptman, personal communication). The phasing technique, moreover, appears to be somewhat robust, in that some perturbations to the measured intensities are tolerated, as demonstrated for the electron diffraction data affected by incoherent multiple scattering. Although the perturbations to the structure factor magnitudes are great enough to distort the electrostatic potential maps, the distortion is not so large as to change the peak position in the Patterson map (Dorset et al., 1987; Dorset, 1987). This may indeed be the reason why the phasing procedure is still successful.

As shown above, the triple structure invariants are best for interrelating high-angle and low-angle reflections. They are less useful for establishing phase relationships within one domain of the unit-cell transform, e.g., the low-angle phases themselves. This phasing of the low-angle region is a problem that can be eliminated by use of high-resolution, low-dose electron microscopy on the same unstained, unfixed samples used to obtain the electron diffraction patterns. As will be described elsewhere, the initial verification of the utility of electron microscope images at 16 Å resolution (Dorset, 1988) has been extended recently to 6 Å resolution (F. Zemlin et al., unpublished data) for L-DHPE. The phases of the first seven reflections taken from the computed Fourier transform of the L-DHPE electron microscope image were combined with the direct-phasing technique to determine the phases of the complete data set.

The advantages of this phasing approach are obvious. There are numerous phospholipids, for which the crystal structure is unknown, which cannot be swollen in a solvent so that the continuous Fourier transform of the bilayer unit can be found. Multilamellar paracrystals of these lipids can be easily obtained by epitaxial orientation on organic substrates, as demonstrated by the large catalogue of lipids already crystallized by this procedure (Dorset, 1989). From the derived one-dimensional structures, a facile comparison can be made to the various conformational possibilities found in the existing 3-D crystal structures.

It is also gratifying that the use of structure invariant relationships independently verify phase determinations for those lipids that can be swollen in water so that the phase information can be derived from the experimental transform. Examples of other structure analyses, which were based on density models, are also shown here to be correct. The direct phasing procedure, however, removes uncertainties in using the R-factor as a figure of merit when the number of refineable parameters in a model approaches the number of observable data (Hamilton, 1964). That is to say, a direct-phasing technique places no subjective prejudices on the structure determination and, in principle, does not rely on such a figure of merit for establishing the correctness of a particular model.

Obviously more work on the direct phasing procedure with such limited data sets must be carried out to understand the phenomenological observations made in this paper. Such studies will be based on known phospholipid crystal structures for which a more accurate representation of the $|E_h|$ distribution can be obtained for comparison with the estimations made in this paper. Additionally, some means must be found for completing a phase set after the reliable use of direct methods is pushed to its limit. Given n reflections which remain unphased, then 2^n phase ambiguities can be generated. One can either calculate the structure maps from these phase sets and back-transform these to compare $|F_c|$ with $|F_o|$ or one can perhaps evaluate the self-consistency of the phase set with the Sayre (1952*b*) equation

$$\sum_p F(p)F(h-p) \simeq \ell s(h)F(h).$$

The validity of this Sayre convolution is based on the near identity of scattering factors of the atomic species, nonoverlap of atoms, and the condition that most of the scattering power of the sample is expressed by the measured data. Although these criteria are not rigorously obeyed for the phospholipid structures, work in progress indicates that this method might be useful for completion of a phase set.

Finally, for the electron diffraction data, it is clear that the somewhat distorted electrostatic potential maps in Fig. 2 are due to structure factors affected by incoherent multiple scatter. Some means must be found to carry out an approximate deconvolution of the observed intensity so that a structurally more meaningful map can be calculated and, perhaps, more accurate phases estimated by direct methods, given a better set of relative $|E_h|$ values.

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