2.5. ELECTRON DIFFRACTION AND ELECTRON MICROSCOPY IN STRUCTURE DETERMINATION

1990*a*). Similarly, lamellar electron-diffraction data to *ca* 3 Å resolution from epitaxially oriented phospholipids have been phased by analysis of Σ_1 and Σ_2-triplet invariants (Dorset, 1990*b*, 1991*e*,*f*), in one case combined with values from a 6 Å resolution electron-microscope image (Dorset *et al.*, 1990, 1993). Most recently, such data have been used to determine the layer packing of a phospholipid binary solid solution (Dorset, 1994*d*).

An *ab initio* direct phase analysis was carried out with zonal electron-diffraction data from copper perchlorophthalocyanine. Using intensities from a *ca* 100 Å thick sample collected at 1.2 MeV, the best map from a phase set with symbolic unknowns retrieves the positions of all the heavy atoms, equivalent to the results of the best images (Uyeda *et al.*, 1978–1979). Using these positions to calculate an initial phase set, the positions of the remaining light C, N atoms were found by Fourier refinement so that the final bond distances and angles were in good agreement with those from X-ray structures of similar compounds (Dorset *et al.*, 1991). A similar analysis has been carried out for the perbromo analogue (Dorset *et al.*, 1992). Although dynamical scattering and secondary scattering significantly perturb the observed intensity data, the total molecular structure can be visualized after a Fourier refinement. Most recently, a three-dimensional structure determination was reported for C_{60} buckminsterfullerene based on symbolic addition with results most in accord with a rotationally disordered molecular packing (Dorset & McCourt, 1994*b*).

2.5.8.4. The tangent formula

Given a triple phase relationship

$$\phi_{\mathbf{h}} \simeq \phi_{\mathbf{k}} + \phi_{\mathbf{h}-\mathbf{k}},$$

where $\mathbf{h}, \mathbf{k}$ and $\mathbf{h} - \mathbf{k}$ form a vector sum, it is often possible to find a more reliable estimate of $\phi_{\mathbf{h}}$ when all the possible vectorial contributions to it within the observed data set $\mathbf{k_r}$ are considered as an average, *viz:*

$$\phi_{\mathbf{h}} \simeq \langle \phi_{\mathbf{k}} + \phi_{\mathbf{h}-\mathbf{k}} \rangle_{\mathbf{k_r}}.$$

For actual phase determination, this can be formalized as follows. After calculating normalized structure-factor magnitudes $|E_{\mathbf{h}}|$ from the observed $|F_{\mathbf{h}}|$ to generate all possible phase triples within a reasonably high $A_{\mathbf{h}}$ threshold, new phase values can be estimated after origin definition by use of the tangent formula (Karle & Hauptman, 1956):

$$\tan \phi_{\mathbf{h}} = \frac{\sum_{\mathbf{k_r}} W_{\mathbf{h}}|E_{\mathbf{k}}||E_{\mathbf{h}-\mathbf{k}}| \sin(\phi_{\mathbf{k}} + \phi_{\mathbf{h}-\mathbf{k}})}{\sum_{\mathbf{k_r}} W_{\mathbf{h}}|E_{\mathbf{k}}||E_{\mathbf{h}-\mathbf{k}}| \cos(\phi_{\mathbf{k}} + \phi_{\mathbf{h}-\mathbf{k}})}.$$

The reliability of the phase estimate depends on the variance $V(\phi_{\mathbf{h}})$, which is directly related to the magnitude of $\alpha_{\mathbf{h}}$, *i.e.*

$$\alpha_{\mathbf{h}}^2 = \left[\sum_{\mathbf{k_r}} A_{\mathbf{h},\mathbf{k}} \cos(\phi_{\mathbf{k}} + \phi_{\mathbf{h}-\mathbf{k}}) \right]^2 + \left[\sum_{\mathbf{k_r}} A_{\mathbf{h},\mathbf{k}} \sin(\phi_{\mathbf{k}} + \phi_{\mathbf{h}-\mathbf{k}}) \right]^2;$$

$A_{\mathbf{h},\mathbf{k}}$ is identical to the A value defined in the previous section. In the initial stages of phase determination $\alpha_{\mathbf{h}}$ is replaced by an expectation value α_E until enough phases are available to permit its calculation.

The phase solutions indicated by the tangent formula can thus be ranked according to the phase variance and the determination of phases can be made symbolically from the most probable triple-product relationships. This procedure is equivalent to the one described above for the evaluation of phase-invariant sums

by symbolic addition. This procedure may allow determination of a large enough basis phase set to produce an interpretable map.

An alternative procedure is to use an automatic version of the tangent formula in a multisolution process. This procedure is described in Chapter 2.2. After origin definition, enough algebraic unknowns are defined (two values if centrosymmetric and four values, cycling through phase quadrants, if noncentrosymmetric) to access as many of the unknown phases as possible. These are used to generate a number of trial phase sets and the likelihood of identifying the correct solution is based on the use of some figure of merit.

Multisolution approaches employing the tangent formula include *MULTAN* (Germain *et al.*, 1971), *QTAN* (Langs & DeTitta, 1975) and *RANTAN* (Yao, 1981). *RANTAN* is a version of *MULTAN* that allows for a larger initial random phase set (with suitable control of weights in the tangent formula). *QTAN* utilizes the $\alpha_{\mathbf{h}_{est}}$ definition, where

$$\alpha_{\mathbf{h}_{est}} = \left\{ \sum_{\mathbf{k}} A_{\mathbf{h},\mathbf{k}}^2 + 2 \sum_{\mathbf{k} \neq \mathbf{k'}} \sum A_{\mathbf{h},\mathbf{k}} A'_{\mathbf{h},\mathbf{k}} \frac{I_1(A_{\mathbf{h},\mathbf{k}})I_1(A'_{\mathbf{h},\mathbf{k}})}{I_0(A_{\mathbf{h},\mathbf{k}})I_0(A'_{\mathbf{h},\mathbf{k}})} \right\}^{1/2},$$

for evaluating the phase variance. (Here I_0, I_1 are modified Bessel functions.) After multiple solutions are generated, it is desirable to locate the structurally most relevant phase sets by some figure of merit. There are many that have been suggested (Chapter 2.2). The most useful figure of merit in *QTAN* has been the NQEST (De Titta *et al.*, 1975) estimate of negative quartet invariants (see Chapter 2.2). More recently, this has been superseded by the minimal function (Hauptman, 1993):

$$R(\phi) = \frac{\sum_{\mathbf{h},\mathbf{k}} A_{\mathbf{h},\mathbf{k}}(\cos \phi_{\mathbf{h},\mathbf{k}} - t_{\mathbf{h},\mathbf{k}})^2}{\sum_{\mathbf{h},\mathbf{k}} A_{\mathbf{h},\mathbf{k}}},$$

where $t_{\mathbf{h},\mathbf{k}} = I_1(A_{\mathbf{h},\mathbf{k}})/I_0(A_{\mathbf{h},\mathbf{k}})$ and $\phi_{\mathbf{h},\mathbf{k}} = \phi_{\mathbf{h}} + \phi_{\mathbf{k}} + \phi_{-\mathbf{h}-\mathbf{k}}$.

In the first application (Dorset *et al.*, 1979) of multisolution phasing to electron-diffraction data (using the program *QTAN*), *n*-beam dynamical structure factors generated for cytosine and disodium 4-oxypyrimidine-2-sulfinate were used to assess the effect of increasing crystal thickness and electron accelerating voltage on the success of the structure determination. At 100 kV samples at least 80 Å thick were usable for data collection and at 1000 kV this sample thickness limit could be pushed to 300 Å – or, perhaps, 610 Å if a partial structure were accepted for later Fourier refinement. NQEST identified the best phase solutions. Later *QTAN* was used to evaluate the effect of elastic crystal bend on the structure analysis of cytosine (Moss & Dorset, 1982).

In actual experimental applications, two forms of thiourea were investigated with *QTAN* (Dorset, 1992*d*), using published three-dimensional electron-diffraction intensities (Dvoryankin & Vainshtein, 1960, 1962). Analysis of the centrosymmetric para-electric structure yielded results equivalent to those found earlier by symbolic addition (Dorset, 1991*b*). Analysis of the noncentrosymmetric ferroelectric form was also quite successful (Dorset, 1992*d*). In both cases, the correct structure was found at the lowest value of NQEST. Re-analysis of the diketopiperazine structure with *QTAN* also found the correct structure (Dorset & McCourt, 1994*a*) within the four lowest values of NQEST, but not the one at the lowest value. The effectiveness of this figure of merit became more questionable when *QTAN* was used to solve the noncentrosymmetric crystal structure of a polymer (Dorset, McCourt, Kopp *et al.*, 1994). The solution could not be found readily when NQEST was used but was easily identified when the minimal function $R(\phi)$ was employed instead.

MULTAN has been used to phase simulated data from copper perchlorophthalocyanine (Fan *et al.*, 1985). Within the 2 Å

resolution of the electron-microscope image, if one seeks phases for diffraction data in reciprocal-space regions where the objective lens phase contrast transfer function $|C(s)| \leq 0.2$, the method proves to be successful. The method is also quite effective for phase extension from 2 Å to 1 Å diffraction resolution, where the low-angle data serve as a large initial phase set for the tangent formula. However, no useful results were found from an *ab initio* phase determination carried out solely with the electron-diffraction structure-factor magnitudes. Similar results were obtained when *RANTAN* was used to phase experimental data from this compound (Fan *et al.*, 1991), *i.e.* the multisolution approach worked well for phase extension but not for *ab initio* phase determination. Additional tests were subsequently carried out with *QTAN* on an experimental *hk*0 electron-diffraction data set collected at 1200 kV (Dorset, McCourt, Fryer *et al.*, 1994). Again, *ab initio* phase determination is not possible by this technique. However, if a basis set was constructed from the Fourier transform of a 2.4 Å image, a correct solution could be found, but not at the lowest value of NQEST. This figure of merit was useful, however, when the basis set was taken from the symbolic addition determination mentioned in the previous section.

2.5.8.5. Density modification

Another method of phase determination, which is best suited to refining or extending a partial phase set, is the Hoppe–Gassmann density modification procedure (Hoppe & Gassmann, 1968; Gassmann & Zechmeister, 1972; Gassmann, 1976). The procedure is very simple but also very computer-intensive. Starting with a small set of (phased) $F_{\mathbf{h}}$, an initial potential map $\varphi(\mathbf{r})$ is calculated by Fourier transformation. This map is then modified by some real-space function, which restricts peak sizes to a maximum value and removes all negative density regions. The modified map $\varphi'(\mathbf{r})$ is then Fourier-transformed to produce a set of phased structure factors. Phase values are accepted *via* another modification function in reciprocal space, *e.g.* $E_{\text{calc}}/E_{\text{obs}} \geq p$, where p is a threshold quantity. The new set is then transformed to obtain a new $\varphi(\mathbf{r})$ and the phase refinement continues iteratively until the phase solution converges (judged by lower crystallographic R values).

The application of density modification procedures to electron-crystallographic problems was assessed by Ishizuka *et al.* (1982), who used simulated data from copper perchlorophthalocyanine within the resolution of the electron-microscope image. The method was useful for finding phase values in reciprocal-space regions where the transfer function $|C(s)| \leq 0.2$. As a technique for phase extension, density modification was acceptable for test cases where the resolution was extended from 1.67 to 1.0 Å, or 2.01 to 1.21 Å, but it was not very satisfactory for a resolution enhancement from 2.5 to 1.67 Å. There appear to have been no tests of this method yet with experimental data. However, the philosophy of this technique will be met again below in the description of the maximum entropy and likelihood procedure.

2.5.8.6. Convolution techniques

One of the first relationships ever derived for phase determination is the Sayre (1952) equation:

$$F_{\mathbf{h}} = \frac{\theta}{V} \sum_{\mathbf{k}} F_{\mathbf{k}} F_{\mathbf{h}-\mathbf{k}},$$

which is a simple convolution of phased structure factors multiplied by a function of the atomic scattering factors. For structures with nonoverlapping atoms, consisting of one atomic species, it is an exact expression. Although the convolution term resembles part of the tangent formula above, no statistical averaging is

implied (Sayre, 1980). In X-ray crystallography this relationship has not been used very often, despite its accuracy. Part of the reason for this is that it requires relatively high resolution data for it to be useful. It can also fail for structures comprised of different atomic species.

Since, relative to X-ray scattering factors, electron scattering factors span a narrower range of magnitudes at $\sin\theta/\lambda = 0$, it might be thought that the Sayre equation would be particularly useful in electron crystallography. In fact, Liu *et al.* (1988) were able to extend phases for simulated data from copper perchlorophthalocyanine starting at the image resolution of 2 Å and reaching the 1 Å limit of an electron-diffraction data set. This analysis has been improved with a 2.4 Å basis set obtained from the Fourier transform of an electron micrograph of this material at 500 kV and extended to the 1.0 Å limit of a 1200 kV electron-diffraction pattern (Dorset *et al.*, 1995). Using the partial phase sets for zonal diffraction data from several polymers by symbolic addition (see above), the Sayre equation has been useful for extending into the whole *hk*0 set, often with great accuracy. The size of the basis set is critical but the connectivity to access all reflections is more so. Fan and co-workers have had considerable success with the analysis of incommensurately modulated structures. The average structure (basis set) is found by high-resolution electron microscopy and the 'superlattice' reflections, corresponding to the incommensurate modulation, are assigned phases in hyperspace by the Sayre convolution. Examples include a high T_c superconductor (Mo *et al.*, 1992) and the mineral ankangite (Xiang *et al.*, 1990). Phases of regular inorganic crystals have also been extended from the electron micrograph to the electron-diffraction resolution by this technique (Hu *et al.*, 1992).

In an investigation of how direct methods might be used for phase extension in protein electron crystallography, low-resolution phases from two proteins, bacteriorhodopsin (Henderson *et al.*, 1986) and halorhodopsin (Havelka *et al.*, 1993) were extended to higher resolution with the Sayre equation (Dorset *et al.*, 1995). For the noncentrosymmetric bacteriorhodopsin *hk*0 projection a 10 Å basis set was used, whereas a 15 Å set was accepted for the centrosymmetric halorhodopsin projection. In both cases, extensions to 6 Å resolution were reasonably successful. For bacteriorhodopsin, for which data were available to 3.5 Å, problems with the extension were encountered near 5 Å, corresponding to a minimum in a plot of average intensity *versus* resolution. Suggestions were made on how a multisolution procedure might be successful beyond this point.

2.5.8.7. Maximum entropy and likelihood

Maximum entropy has been applied to electron crystallography in several ways. In the sense that images are optimized, the entropy term

$$S = -\sum_i P_i \ln P_i,$$

where $P_i = p_i / \sum_i p_i$ and p_i is a pixel density, has been evaluated for various test electron-microscope images. For crystals, the true projected potential distribution function is thought to have the maximum value of *S*. If the phase contrast transfer function used to obtain a micrograph is unknown, test images (*i.e.* trial potential maps) can be calculated for different values of Δf_{trial}. The value that corresponds to the maximum entropy would be near the true defocus. In this way, the actual objective lens transfer function can be found for a single image (Li, 1991) in addition to the other techniques suggested by this group.

Another use of the maximum-entropy concept is to guide the progress of a direct phase determination (Bricogne & Gilmore, 1990; Gilmore *et al.*, 1990). Suppose that there is a small set *H* of known phases $\phi_{\mathbf{h} \in H}$ (corresponding either to origin