Electron Crystallography – Accomplishments and Challenges

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Abstract

Electron crystallography is a powerful tool for the quantitative structural characterization of substances that preferentially form thin microcrystals. Because multiple-beam dynamical scattering may cause observed diffraction intensities to deviate significantly from their kinematical values, it is necessary to demonstrate that the conditions favoring *ab initio* determinations can be established. Review of similar determinations made from electron and X-ray data make clear both the strengths and weaknesses of electron crystallography. With current instrumentation, the major onus now placed on the experimentalist is to optimize specimen preparation so that the resultant diffraction data can be directly interpreted.

1. Introduction

In the 70 years since the Davisson–Germer (1927) experiment, electron diffraction has played a major role in the qualitative characterization of inorganic and organic materials of all kinds. The first attempt to solve an organic crystal structure quantitatively from electron diffraction intensities was made just over 60 years ago (Rigamonti, 1936). A major electronographic effort in Moscow was then begun, largely through the efforts of Z. G. Pinsker (1949, 1953), and vigorously developed by B. K. Vainshtein (1956, 1964), B. B. Zvyagin (1964, 1967) and others. Not only were organic structures quantitatively determined but also those containing heavy atoms. Structures were also analyzed elsewhere (Cowley, 1967) but confidence in electron diffraction structure analysis began to erode as the observed intensities were shown to deviate appreciably from their kinematical values.

Despite the prospect of obtaining high-resolution images in an electron microscope, it is easy to forget about attempting quantitative crystal structure analyses with electron beams. Because the multiple-beam dynamical scattering theory for electrons is complicated (Goodman & Moodie, 1974; Cowley, 1995), the analysis is often constrained to one direction, virtually requiring that a structural model be known before it is determined. In addition, incoherent multiple scattering can further perturb the diffraction intensities and obscure systematically absent reflections (Cowley *et al.*, 1951). Elastic crystal bending will seriously affect observed intensities if the crystal is projected down a long unit-cell axis (Cowley, 1961). Radiation damage, owing to inelastic collisions of electrons with the specimen, can introduce other difficulties, especially for the study of organic materials.

On the other hand, a good theoretical understanding of electron scattering from crystals should provide a basis for optimizing experimental design, thus ensuring that data can be collected to favor an *ab initio* structure analysis. High electron accelerating voltages are known to reduce the interaction between the electron beam and the crystal. Minimization of crystal thickness also reduces multiple-scattering effects. For organics, diffraction intensities near to a single-scattering approximation appear to be within the reach of actual experimental parameters (Dorset *et al.*, 1979; Jap & Glaeser, 1980). Probabilistic direct methods (Hauptman, 1972), long employed in X-ray crystallography for structure determination, were found to be applicable to electron diffraction data (Dorset & Hauptman, 1975).

Doug Dorset was born in southeastern Pennsylvania, some 30 miles (50 km) east of where his great-grand-father (and the Army of Northern Virginia) had been an unwelcome visitor some threescore and 19 years before. After obtaining his baccalaureate degree in Chemistry at a small college, he crossed the Mason–Dixon line to earn his PhD in Biophysics at the University of Maryland. Cooler temperatures attracted him to upstate New York (where crystallization of ambient humidity can justify the ownership of cross-country skis). After two postdoctoral positions, he started his research career in electron crystallography at the Medical Foundation of Buffalo (now the Hauptman–Woodward Institute) in 1973, enhanced by a sabbatical at the University of Basel, Switzerland, in 1980. His work in this area, strongly influenced by Herb Hauptman and the direct methodists, has recently been summarized in a monograph entitled Structural Electron Crystallography. He is currently a member of the Commission on Electron Diffraction of the International Union of Crystallography and the US National Committee for Crystallography.

Eventually, it was shown that a quasikinematical criterion (Dorset, 1995a) for solving a crystal structure from electron diffraction intensities merely requires the observed Patterson function and the actual crystal autocorrelation function to be sufficiently similar to one another. Absolute adherence to the kinematical approximation is not needed so that corrections to the intensities can be made after derivation of the initial structural model. In some cases, the parameters affecting dynamical scattering could be exploited productively to guide structure refinement (Zandbergen et al., 1997). Nowadays, it is not the lack of theoretical understanding that restricts electron crystallographic structure analyses but problems with preparing specimens that will allow collection of useable diffraction data.

2. Benchmarks for justifying quantitative electron crystallographic analyses

Talk, as they say, is cheap. Claims that structures can be solved from electron scattering data can only be justified when a favorable comparison can be made between independent analyses based on both electron and X-ray measurements.

2.1. Protein structures

Crystallographic principles were introduced into the electron microscopy of macromolecular arrays when information from repeating motifs in images of negatively stained objects was averaged to reduce the noise component of uneven stain distribution (DeRosier & Klug, 1968; Vainshtein, 1978). Eventually, these procedures were applied to low-contrast images of unstained objects (Amos et al., 1982). Thus, an important chapter in the electron crystallography of macromolecules began with a three-dimensional study (Henderson & Unwin, 1975) of the purple membrane from *Halobacterium salinarium*. Two major innovations were evident in this study. First, replacing water molecules by a saccharide to avoid desolvation in the electron microscope vacuum was very effective (Unwin & Henderson, 1975). Second, the continuous transform of the two-dimensional crystal along its surface normal could be sampled infinitely, if desired, by tomographic tilting in the electron microscope. Crystallographic phases from averaged low-contrast electron micrographs for each tilted projection immediately provided a three-dimensional view of bacteriorhodopsin after these were combined with electron diffraction amplitudes to calculate the reverse Fourier transform. The initial result at 7 Å resolution revealed the cluster of seven α-helices in the asymmetric unit (Henderson & Unwin, 1975).

Methods were then found to improve the phase accuracy from images initially averaged by Fourier filtration (Henderson et al., 1986). Residual para-

crystalline distortion of the lattice was unbent by cross-correlation techniques to provide phases for electron diffraction maxima beyond 6 Å. The influence of the objective-lens phase-contrast transfer function on phases was also tested. Observations of radiation damage in weakly illuminated objects indicated that the residual specimen motion could be minimized by spot illumination (Downing, 1988). Also, data collected in a liquid-helium-cooled cryomicroscope permitted tracing of the polypeptide chain in a potential map with 3.5 Å (diffraction) resolution in the membrane plane and 4.3 Å perpendicular to this dimension (Henderson et al., 1990). The retinal pigment could also be discerned and the structure was refined further (Grigorieff et al., 1996). This result was verified recently by a 2.5 Å-resolution three-dimensional X-ray determination (Pebay-Peyroula et al., 1997). Microcrystals used for data collection on a synchrotron source retain the layer packing of the two-dimensional arrays so that the p3 plane-group symmetry of the layer becomes a part of the $P6_3$ space group. The helical polypeptide domains determined by electron crystallography provided an accurate initial phase set for the X-ray determination that revealed details of the polypeptide connections between the helices at the layer surfaces, missing from the initial electron crystallographic study (owing to the $\pm 60°$ specimen tilt limitation). Most recently, a higher-resolution (3.0 Å) electron crystallographic determination (data collected in a 300 kV liquid-helium-cooled cryoelectron microscope with a field emission source) with flatter specimens permitted the samples to be tilted $\pm 70°$ (Kimura et al., 1997). In this new structure, the polypeptide connections between helices were also clearly visualized.

Structural results from the Gram-negative bacterial outer-membrane porins also have been compared. Electron diffraction data and images were collected to 3.0 Å resolution from reconstituted two-dimensional crystals. For one porin, Pho E, a complete three-dimensional structure was determined (Jap et al., 1991). For the other Omp F protein, the two-dimensional structure (Sass et al., 1989) revealed a close resemblance to the former phosphoporin. In both structures, the details of the β-sheet were clearly resolved. Several E. coli porin X-ray structures were solved later by molecular replacement (Pauptit et al., 1991) using intensity data from proteins crystallized in detergent. Suitable heavy-atom derivatives verified the initial model and extended it to higher resolution (Cowan et al., 1992). Again, the basic outlines of the β-sheet structure were in complete accord with the electron crystallographic results.

An extensive catalog of integral membrane proteins characterized by electron crystallography (Jap et al., 1992) continues to grow. Most often, determinations made on unstained specimens yield structures to 9 to 6 Å (diffraction) resolution. For reconstituted two-

dimensional protein crystals, highest-resolution determinations are permitted only after the specimen crystallinity is optimized.

2.2. Linear polymers

For many years, three-dimensional determinations of linear polymer chain packing have depended on the collection of good fiber X-ray data sets. Since extensive reflection overlap could restrict the accurate determination of unit-cell constants as well as space-group symmetry, single-crystal electron diffraction patterns were also used for model-based structural searches, often yielding reasonable results (Claffey et al., 1974). Later, three-dimensional electron data were collected from tilted chain-folded lamellae (Perez & Chanzy, 1989). However, owing to the restricted tilt range of the goniometer stage, the observed data excluded important information about the polymer chain repeat (Dorset & McCourt, 1993). Complete three-dimensional electron crystallographic determinations of polymer structure depended on new crystallization techniques to provide an orthogonal view onto the polymer chains, in addition to the view along the chain axes found in the untilted chain-folded lamellae (Wittmann & Lotz, 1990).

Direct phasing techniques have been employed for polymer structure analyses. For example, electron and X-ray crystallographic approaches could be compared with data from poly(ε-caprolactone), crystallizing in space group $P2_12_12_1$. The polymer was crystallized in two orthogonal orientations (Hu & Dorset, 1990). Three-dimensional single-crystal electron diffraction data (47 unique reflections) were collected by goniometry and the structure was solved by symbolic addition, relying on the centrosymmetric character of the $hk0$ and $0kl$ reflections and yielding 30 phase values linked through one algebraic term (Dorset, 1991). The three-dimensional model constructed from the initial potential maps was refined by Fourier methods. All bond distances and angles were reasonable, as were the individual isotropic temperature factors. Next, a fiber X-ray data set (Chatani et al., 1970) (108 reflections to 1.2 Å, 20 in overlapped doublets separated by equipartitioning) was analyzed using the Sayre equation to expand a small basis set (Dorset, 1997a). After two further cycles of Fourier refinement, an atomic model was derived that agreed closely with the electron diffraction model.

Favorable projections onto the chain axes sometimes can be obtained by other means, e.g. by stretching a cast film (Vainshtein & Tatarinova, 1967) or by carrying out in situ polymerizations in dilute solution to grow whiskers (Liu et al., 1992), and electron diffraction data from these preparations can be used to advantage for structure analyses (Dorset, 1995b; Liu et al., 1997). When just the chain-folded lamellae can be prepared, the orthogonal view of the chain packing can be obtained from a

microfiber. For example, $hk0$ data from poly(ethylene sulfide) lamellae were supplemented by hkl data from fibers (Hasegawa et al., 1977). Again, assuming that overlapped reflections could be initially separated by equipartitioning, the structure was solved by direct methods (Dorset & McCourt, 1997). The chain packing derived from fitting a molecular backbone to the map density profile agreed closely with an earlier fiber X-ray determination (Takahashi et al., 1968) but actually yielded better bond distances and angles.

The greatest utility of polymer electron crystallography is to study crystal polymorphs that are difficult to characterize by X-ray diffraction methods. For example, using the chain orientation techniques described above, a complete single-crystal structure was reported for form III of isotactic poly(1-butene) (Dorset et al., 1994), a polymorph that cannot be crystallized as a fiber. Similarly, the frustrated chain packing in the β-phase of isotactic polypropylene (Fig. 1) was determined from a full set of electron diffraction data (Dorset, McCourt, Kopp, Schumacher, Okihara & Lotz, 1998), despite the presence of merohedral twinning.

2.3. Small molecules

Various industrially important polymorphs of dyestuffs etc. preferentially form microcrystals while a less-desirable polymorph may grow to sizes suitable for X-ray data collection (Fryer et al., 1981). For this reason, it is often important to determine small-molecule crystal structures from data collected from these microcrystalline polymorphs. Comprehensive reviews of small-
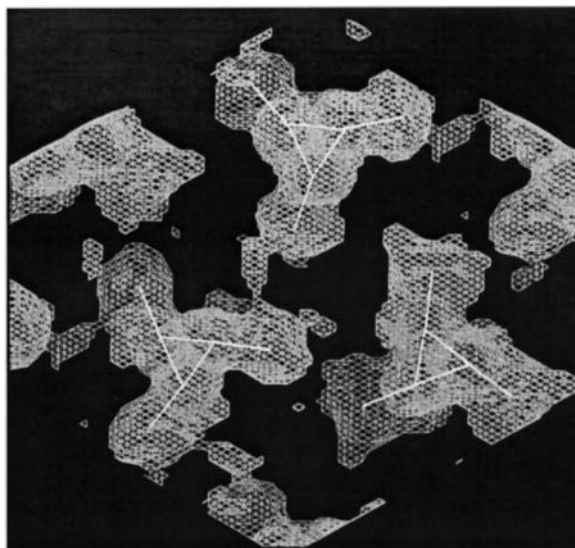

Fig. 1. Crystal structure determination of isotactic polypropylene, β-phase, based on the Fourier refinement against 88 independent hkl electron diffraction amplitudes. The three unique molecules of the unit cell (space group $P3_1$) are shown in a projection down the chain axes.

molecule determinations, employing texture electron diffraction data, have been published recently (Dorset, 1995*b*, 1996*a*). Despite dynamical perturbations to the intensity, direct methods could determine structures in reasonable agreement with X-ray crystallographic results.

Very thin crystals of small organics can be grown rather easily by evaporation of a dilute solution. Are high-voltage selected-area electron diffraction intensities from these single crystals also suitable for *ab initio* structure determinations? Experience with electron diffraction data from linear polymers has already revealed that, in order to record useful intensities, there should be a good consistency between patterns from different crystals in the same orientation (low R_{merge}) and also that the symmetry-related intensities should agree closely with one another (low R_{sym}). While, for small molecules, analysis of data from epitaxially oriented films has been encouraging, data collected from solution-crystallized samples often seem to be less tractable, mainly because of erratic crystal bends. Tests were made on electron diffraction patterns from triphenylene (Dorset, McCourt, Li & Voigt-Martin, 1998) grown by evaporation of a dilute benzene solution. Large values of R_{merge} and R_{sym} (respectively 0.22 and 0.44) indicated that the $h0l$ intensities were not very reproducible. However, when the samples were prepared by co-crystallization with naphthalene, which provides a flat substrate for nucleation, the two residuals were greatly improved, *i.e.* 0.13 and 0.15, respectively. Since some microcrystals were also epitaxially oriented,
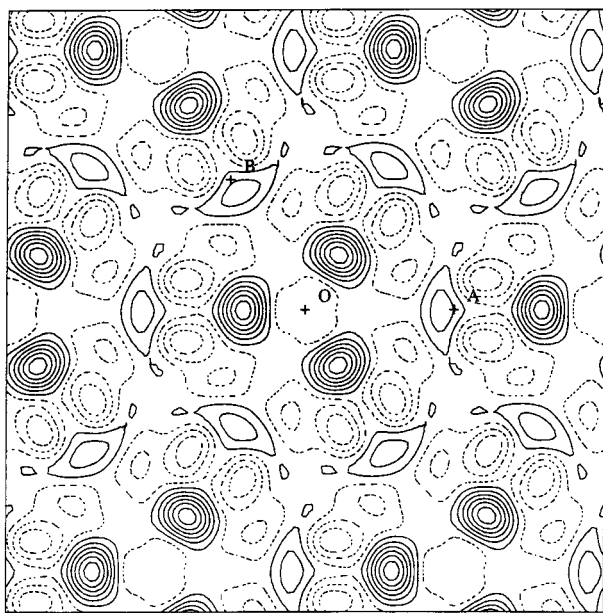

Fig. 2. Potential map after direct-methods solution of the $\sqrt{3} \times \sqrt{3}$ Au structure on Si. Largest peaks are gold sites while the elongated ones correspond to overlapped silicon positions. Axes are indicated at their half-length.

two orthogonal views of the crystal structure allowed, in principle, a complete data set to be collected by goniometry. A structural model was then found by minimization of packing energy, in good agreement with the X-ray crystal structure (Ahmed & Trotter, 1963). In another example, the ellipsoidal C_{70} molecule appeared to pack in primitive square-packed layers when crystallized from benzene (Dorset, 1996*b*). When the samples were prepared carefully by sublimation *in vacuo*, diffraction from the actual face-centered cell could be observed (Dorset & Fryer, 1997). Since the intermolecular contacts between C_{70} are statistical in nature, the layer packing is more prone to shearing as the solvent is rapidly removed by evaporation.

Recent advances in the study of small molecules by electron crystallography have been reviewed (Dorset, 1996*a*). The technique is particularly useful for characterizing multicomponent solids as single crystals, *e.g.* petroleum and mineral waxes (Dorset, 1995*c*, 1997*b*). Also, the progress of phase separation of such binary solids was followed by single-crystal structure analysis (Dorset & Snyder, 1996; Dorset, 1997*c*).

2.4. Inorganic structures

Early texture diffraction studies of inorganics, recently reanalyzed by direct methods, have been reviewed (Dorset, 1995*b*, 1996*a*). Use of higher voltages (*e.g.* 250 kV) to collect such data is advantageous. A three-dimensional set from the mineral brucite, sent to this laboratory by Professor B. B. Zvyagin, comprises 70 unique *hkl* reflections. Assuming the H atom to be positioned at one site, the final positions of O and H atoms after Fourier refinement are very close to those found in a neutron diffraction determination by Zigan & Rothbauer (1967), with $R = 0.068$. The Russian group has taken the analysis further to postulate a partial occupancy of hydrogen over three equivalent sites (Zhuklistov *et al.*, 1997).

Analyses of transmission electron micrographs and selected-area electron diffraction patterns from numerous inorganic structures has been reviewed (Dorset, 1995*b*, 1996*a*), also showing a good match to X-ray results. Of particular interest has been the study of incommensurately modulated lattices and the multidimensional direct methods used to determine their structures (Mo *et al.*, 1992). Advances have been made in the characterization of alloys. While symmetry can be determined from zero-order Laue-zone reflections in convergent-beam patterns, higher-order Laue-zone reflections are sometimes adequately near a quasikinematical limit to permit a structure analysis, as demonstrated for two AlGe alloys (Vincent & Exelby, 1991, 1993). Using a double conical beam rocking system for measurement of integrated electron diffraction intensities (Vincent & Midgley, 1994), a good three-dimensional set was collected from $Al_m Fe$ and its structure was

solved readily by Patterson techniques or direct methods (Gjønnes *et al.*, 1998). Recently, the two-dimensional structures of heavy-atom monolayers on a silicon substrate have been investigated. Transmission electron diffraction data can be edited to cull out the contribution from the overlayer. Direct methods were successful for determining the structure of the $\sqrt{3} \times \sqrt{3}$ Au layer on the (111) silicon surface based on 51 unique electron diffraction amplitudes in plane group $p3$ (Marks *et al.*, 1997). A multisolution approach *via* the Sayre–Hughes equation found two structures that satisfied a criterion of maximum peakiness for the structure, one solution having the chemically most reasonable geometry (Fig. 2). While effects of layer interference with the silicon substructure are apparent from the abnormally high resolution (0.43 Å) of the diffraction pattern (and, from a Wilson plot, an overall temperature factor near $B = 0.0$ Å^2), the intensities give good values of R_{sym} and R_{merge} (0.09 and 0.12, respectively) attesting to their selfconsistency – a better determinant than a suggested linear correlation to counting statistics (Xu *et al.*, 1994). It was necessary, however, to truncate the resolution to 1.0 Å for calculation of $|E_h|$ values useful for direct phase determination.

3. Current viewpoints

3.1. *The role of the specimen in quantitative structure determination*

There is no question, therefore, that meaningful *ab initio* structure analyses can be carried out with experimental electron diffraction data. Extensive instrumental advances for electron crystallography include accelerating voltages in the 200 to 400 kV range, which are not uncommon in electron-microscope laboratories nowadays. Objective-lens design has extended the useful resolutions of these microscopes. For radiation-sensitive materials, low-dose imaging procedures, coupled with computer software for image averaging, have been developed to investigate such structures at high resolution. Digital detectors have been evaluated for recording images and diffraction patterns.

Often, the idea that any thin crystalline particle can produce an electron diffraction pattern can be regarded as a curse, especially when intensity data are sought for quantitative structure analyses. The very ease with which electron diffraction is observed often gives the false impression that data collection should also be quite easy. Nothing could be further from the truth.

Given a suitable instrument, collection of useful electron diffraction intensities requires great care with specimen preparation, just as it does in other crystallographic measurements. Obviously, the specimen thickness should be held to a minimum, again to ensure that the deviation from the kinematical limit is not severe. Particularly when selected-area techniques are used, the diffraction intensities from a sample crystallized rapidly from dilute solution should be regarded with great suspicion. Preparations that enhance crystal flatness are to be favored over those that do not. Many of the successful structure analyses of organics in recent years have utilized data from samples prepared on flat substrates. Often this constraint was not consciously applied – the flat substrate was only used to orient a particular crystal projection by epitaxy – but the existence of the substrate also ensured that the crystals were less deformed by irregular bends than if they had been grown from dilute solution. Specimen flatness has also been shown to be very important for the quality of two-dimensional protein crystals, particularly for the collection of three-dimensional diffraction data (see above). On the other hand, the development of a precession geometry (Vincent & Midgley, 1994) for single-crystal data collection in the electron microscope may overcome some of these strictures by permitting the measurement of true integrated intensities, also of organics – a virtue of the older texture diffraction experiments (Cowley, 1967), but now without the reflection overlaps.

Great care in specimen preparation increases the likelihood of a good structure analysis. It is important to collect data from as much of the reciprocal lattice as possible, certainly the most intense parts of the unit-cell transform. When instrumental tilt limits result in missing cones of data, then, if at all possible, a second crystallization procedure should be found that will orient an orthogonal view of the molecular packing. There have also been recent developments in specimen stages permitting tilts up to $\pm 80°$ that might ease some of these constraints, but certain molecules, particularly linear ones, require a projection onto the chain axes for the most efficient collection of intensity data.

3.2. *Prospects for phase determination*

The Fourier transform of electron micrographs and/or the traditional probabilistic direct methods do not exhaust the possibilities for obtaining crystallographic phases. When convergent-beam diffraction experiments are permitted, details within individual discs of the zero-order Laue-zone reflections can be interpreted quite accurately in terms of crystallographic phases (Zuo *et al.*, 1989), since, in these patterns, Σ_2 and Σ_1 invariants are excited experimentally. Recently, dynamically derived crystallographic phases have been exploited in the structure analysis of an Al$_m$Fe alloy (Gjønnes *et al.*, 1998). Such information could further enrich basis sets for phase extension by automated direct methods or be used as an independent figure of merit for sifting through multiple solutions.

Crystallographic phases from lower-resolution images can also be important for extension to higher resolution.

This has been demonstrated already for molecular crystals (Fan *et al.*, 1991). Gilmore *et al.* (1993) have shown that a 15 Å set of phases from the electron micrograph of a protein can be extended quite accurately to beyond 3 Å by maximum entropy and likelihood. Convolutional techniques are also effective (Dorset *et al.*, 1995; Dorset, 1996*c*). Phase extension may be very useful to fill in details of the missing cone, using the hyper-resolution property of *e.g.* the Sayre equation to predict both missing amplitudes and phases (Dorset, 1998*a*).

Ab initio direct phase determinations may also be of interest at low resolution because the problem of defining the density envelope is generally important in macromolecular crystallography. Recently, the problem has been approached in X-ray crystallography by *ab initio* methods in real space (Lunin *et al.*, 1995). A reciprocal-space exploitation of the glob transform approach (Harker, 1953) has been very effective in electron crystallography for proteins where a pseudo-atomic distribution of density sites is a valid approximation (*e.g.* the projection of α-helices) (Dorset, 1997*d,f*, 1998*b*). An example is shown in Fig. 3.

Electron crystallographic determinations can also provide the initial phase set for an X-ray determination. This has already been demonstrated in the X-ray structure determination of bacteriorhodopsin (see above). In addition, the phase information from high-resolution electron micrographs of inorganic materials has been sufficient to complete the analysis with powder X-ray data (Sundberg & Lundberg, 1987; Vincent & Exelby, 1995).

3.3. Refinement procedures

At atomic resolution, Fourier refinement has been a most effective and objective technique for taking a partial structural fragment to completion. Even when heavy atoms dominate the scattering from the molecule, lighter atoms can also be found (Dorset, 1997*e*). Limited experience with unconstrained least-squares refinement has been accumulated. It was used for the improvement of a high-T_c superconductor structure (Mo *et al.*, 1992) and, recently, it has also been applied to the analysis of a titanium selenide (Weirich *et al.*, 1996). In the least-squares refinement of diketopiperazine, the shift of atoms had to be uncoupled from the refinement of thermal parameters. Because of multiple-scattering perturbations, dampening of these shift magnitudes was also necessary so that only a local minimum of the crystallographic residual would be sought rather than a global one (Dorset & McCourt, 1994). Fourier refinement is also sensitive to these intensity perturbations. After phase determination, heavy atoms are often observed in potential maps at their correct positions, whereas lighter atoms appear only near their ideal sites. In some cases, a re-scaling of the diffraction intensities,

based on a partial structure-factor calculation with the heavy-atom positions, has allowed the refinement to proceed to a geometrically reasonable structure (Huang
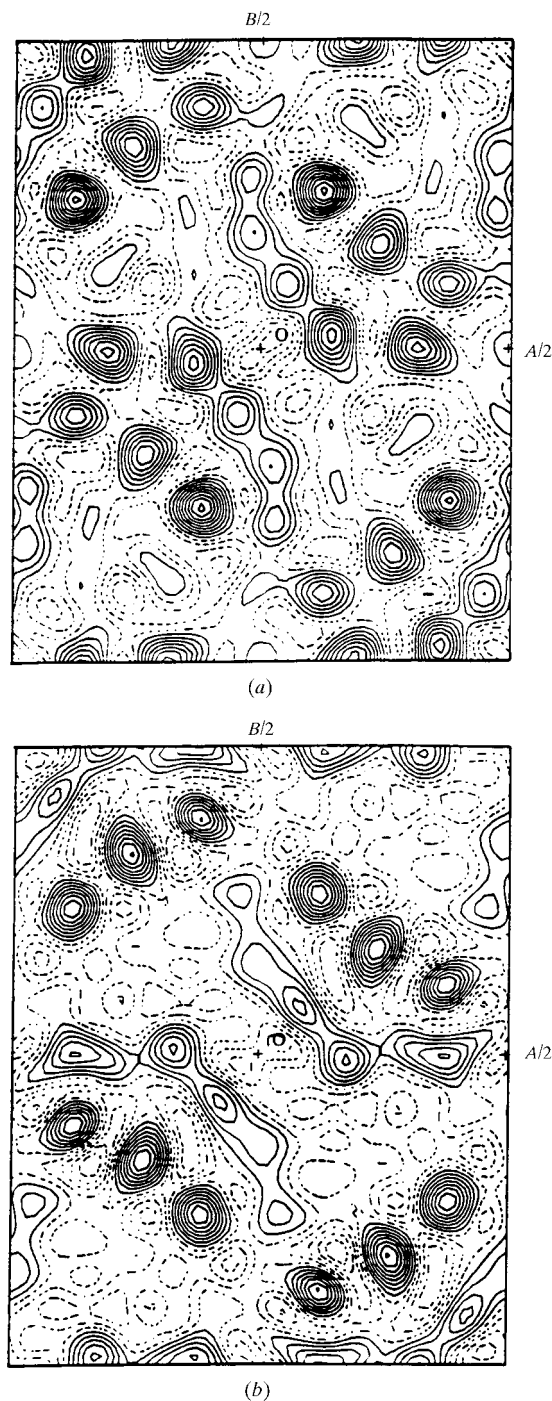

Fig. 3. Structure analysis of orthorhombic bacteriorhodopsin by direct methods. The intensity data were normalized assuming a pseudo-atomic distribution of density subunits. (*a*) Direct solution found after symbolic addition, followed by Fourier refinement. (*b*) Structure based on image-derived phases (Michel *et al.*, 1980).

et al., 1996). Although this correction was also useful for copper perchlorophthalocyanine (Dorset, 1997*e*), it was not applicable to texture diffraction data sets from thiourea. Taking advantage of a conservatism in bonding parameters for many organic materials, a rigid-body search with a geometrically optimized model, coupled with a multislice dynamical scattering correction, has permitted the justification of a chemically reasonable structure against the experimental data (Dorset, 1997*e*).

4. *Quo vadis?*

As reported in the proceedings of a recent NATO Advanced Study Institute (Dorset, Hovmöller & Zou, 1998), quantitative electron crystallography is still being developed. Nevertheless, with adequate controls of instrumental conditions and, of most recent importance, the specimen preparation, suggested by the constraints of a multiple-beam dynamical scattering model, it is clear now that valid *ab initio* structure determinations can be carried out on previously uncharacterized substances. To many spectators, valid structural results from materials composed of heavy atoms has been a particularly surprising development. It is too early yet to ascertain where future developments of the technique will take us in structural research but we have already learned by now that these developments should be made carefully!

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