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Macromolecular crystal twinning, lattice disorders and multiple crystals

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Macromolecular crystal twinning, lattice disorders and multiple crystals¹

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Macromolecule crystal structure analyses can be severely hampered by cases of twinning or of lattice disorders or of multiple crystals. However, it is increasingly the case that twinning can readily be recognized and accounted for, or remediations found. A review of this topic is given, covering both the less-than-perfect and perfect twinning situations. Remediation of twinning cases is possible *via* alteration of crystal growth conditions including use, mainly, of chemical additives. The case of multiple crystal growths likewise can hamper crystal structure analysis and, although not necessarily associated with twinning, is a crystal growth situation where similar remediation methods are adopted. There is a nice body of case studies now in the crystallographic literature about macromolecule crystal twinning and multiple crystals situations that make it timely to write this review. The literature on other macromolecule crystal disorders is much smaller but include incommensurate superlattice effects, which are also described. This review concludes with a personal view of the future possible directions. There are new avenues to deal with multiple crystal cases using physical crystallography approaches, such as sample sprays for freezing macromolecule microcrystallites and femtosecond lasers for exactly cutting out crystal fragments. There is a considerable potential for molecular replacement to provide an efficient way for using twinned X-ray data, at least where there is a high amino acid sequence identity, and as 'protein fold space' coverage becomes much more complete.

Keywords: crystal growth; twinning; lattice disorders; multiple crystals; macromolecules; proteins; nucleic acids; synchrotron radiation

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1. Introduction

Macromolecular crystal structure analyses can be severely hampered by cases of twinning or of multiple crystals. However, it is increasingly the case that twinning can readily be recognized notably when the X-ray diffraction data are analysed in detail, especially using intensity statistics. A review of this topic is given covering both the less-than-perfect and perfect twinning situations. Remediation of twinning cases is possible via alteration of crystal growth conditions including the use, mainly, of chemical additives. The case of multiple crystal growths likewise can hamper crystal structure analysis and although not necessarily associated with twinning is a crystal growth situation where similar remediation methods are adopted, or alternatively portions of crystals ‘cut out’ or tiny portions of crystals selected through use of ‘microbeams’. There is also a nice body of case studies now in the crystallographic literature that makes it timely to write this review.

Some basic points are now described. Twinning is a crystal growth anomaly in which the specimen is composed of separate crystal domains whose orientations differ in a specific way. It is a particular case of a multiple-crystal growth disorder where some or all of the lattice directions in separate domains are parallel. Twinning can involve an imperfect macromolecule crystal growth situation, including two or more crystals clustered together, or a perfect crystal growth situation involving domains of a single crystal; the latter being where there is a coincidence of unit cell parameters (e.g. $a=b$ in orthorhombic or $\beta=90^\circ$ in monoclinic) or a coincidence of a rotational symmetry element and a coincident crystal growth direction. Basically in such a case a twinning operation can describe the effect and, whilst not an element of crystallographic symmetry, such an operation, relating two domains of a single crystal sample, for example, has the hallmarks

of such a symmetry element. The typical domain sizes within a single twinned crystal are assumed to be small enough to be undetectable by visual examination (under an optical microscope) but large enough compared with the X-ray beam coherence length that scattered waves from separate domains do not create unusual interference effects. But, whilst the vast majority of crystal habits have convex surfaces rather than re-entrant surfaces, the crystal habit showing such a re-entrant shape, can sometimes be a first clue to the existence of twinning. Diffraction data however reveals the twinning effects, if present, in detail.

2. A few historical examples

Wyckoff (1) showed an electron micrograph of a 'highly twinned virus crystal (Figure 1); he remarked that 'In spite of the twinning shown by these crystals, a study of the molecular distribution over their side faces provides a good idea of their molecular arrangement.' This figure also still offers us a vision for the future whereby it is apparent how micron-sized focused synchrotron radiation (SR) X-ray beams can be harnessed to probe different portions of such a crystal to find a suitable single crystal region, obviously within the constraint of a reasonable number of unit cells for a detailed single crystal structure analysis. Penetration of an X-ray beam through the thickness of a crystal may still encounter twinned regions however.

King (2) described the crystal morphology of ribonuclease I crystals in detail.

'Most of the crystals of ribonuclease I in any sample tube are usually intergrown [Figure 2(a)], only a minority being single [Figure 2(b)] and thus usable in X-ray diffraction studies. The intergrowths are usually composed of a series of individual crystals of ribonuclease I related by rotation about the *c*-axis by about $6^{\circ}20'$. While no simple twinning law was found to account

Figure 1. An electron micrograph ($\times 55,000$; palladium shadowing) of a highly twinned crystal of the 'Rothamsted necrosis protein'. The (100) side face is at the right. The top (111) face shows clearly the composite nature of the crystal by the frequent changes of the hexagonal net. From (1), with permission of the IUCr.

for these intergrowths, their occurrence and that of the high-index faces may be related phenomena.'

Tulinsky *et al.* (4) studied crystals of α -chymotrypsin (α -CHT), which 'grow as diamond plates, and (crystals are) chosen for X-ray examination on the basis of their morphological quality and linear dimensions (to be as similar to each other as possible, $\sim 0.8 \times 0.6 \times 0.2$ mm). Throughout our work, we have continually encountered persistent twinning of crystals... noted (earlier) by Bernal *et al.* (5) and later confirmed by Blow *et al.* (6). Although Sigler *et al.* (7) report that the inclusion of 2–4% dioxane in the crystallizing solution virtually eliminates twinning, we have found that the effect of dioxane is (in fact) apparently one of degree. ...Twin ratios of (6–7):1 or greater were generally considered suitable for further study.'

Today, in 2008, twinning is often regarded still as a serious complication in structure determination and can even lead to a project being postponed until untwinned crystals are found. As Lott *et al.* (8), for example, remark: 'Although cases have recently been reported of successful structure determination by MAD (9, 10) or MIR [e.g. (11)] using highly twinned data, in general the anomalous or isomorphous signal is sufficiently degraded by the errors introduced by detwinning so as to make structure solution impossible. This degradation increases as the twin fraction approaches 0.5, at which point the true diffraction intensities cannot be deconvoluted from the measured twinned intensities.' Likewise (12): 'Crystals of the Jel44 Fab fragment complexed with HPr diffracted X-rays to a maximum resolution of 2.5 Å... they were not characterized further owing to suspected crystal twinning.' A further example is (13): 'The majority of crystals were twinned and it was difficult to identify single crystals solely based on appearance. Typically, only one crystal in ten was suitable for data collection, which severely hampered the search for heavy-atom derivatives. All attempts to reduce the level of twinning such as the use of additives (e.g. Hampton Research Additive Screens) were unsuccessful'.

Figure 2. Ribonuclease I crystals usually grow as (a) intergrowths but sometimes (b) as a single crystal (2); the circles (a) indicate the centroids of molecules without implication as to their shapes [see also (3)], with permission of the IUCr.

Terwisscha van Scheltinga *et al.* (14) provide a range of examples, including success with MAD and MIR for structure determination. They also very usefully describe the various errors involved: ‘according to the error analysis of Fisher and Sweet (15), the standard deviation (SD) of a detwinned intensity is increased by 1.1 for a twin fraction, α , of 0.1, by 1.8 for $\alpha = 0.3$, by 5.5 for $\alpha = 0.45$ and becomes infinite when $\alpha = 0.5$.’

3. The literature on twinning and other macromolecule crystal disorders

Protein crystal twinning is a big topic: for example, a search of IUCr journals with the two keywords ‘protein’ AND ‘twinning’ brings back more than 400 articles. A more general literature search finds a significant number of further references spread across quite a lot of other journals. This combined literature forms then the basis of this review. In addition, the website of Spek and Lutz (http://www.cryst.chem.uu.nl/lutz/twin/twin_lit.html) lists a wide range of further literature, spanning mainly, but not exclusively, ‘small molecule i.e. chemical crystallography’ literature on twinning: this website also includes references besides diffraction including papers on the energetics and simulation modelling of such cases. Further references from the chemical and physical crystallography areas are given as an appendix to the references list. Also in Section 14, the physical crystallography case of diamond twinning occurrences is highlighted and whereby the robustness of diamond to examination, compared with macromolecule crystals, has allowed study by scanning electron microscopy (SEM), as well as by monochromatic and polychromatic X-ray topography (16, 17). The examination of the perfection of macromolecular crystals by topography, mosaicity and reciprocal-space mapping techniques is an evolving area [see e.g. (18, 19)]. A very clear description and introduction to twinning is given by Parsons (20). Dauter *et al.* (21) give several very nicely detailed case studies of structure determination of ‘less than perfect’ macromolecular crystals. Chandra *et al.* (22) survey how to detect and characterize twinning from an analysis of the diffraction intensity statistics and from protein crystal packing density. They also give tables of likely twinning operators in protein crystals with examples (Table 1): Figure 3 shows one such example.

The literature on other macromolecular crystal diffraction disorders is much smaller. Lovelace *et al.* (23) most recently described an incommensurate superlattice [Figure 4(a)] and, within the same team effort, Borgstahl (personal communication) is gathering examples together and these seem to number about 10 cases, some published and others ‘frustrated crystal structure analyses’ i.e. as yet uncompleted. Section 12 details some case

Table 1. Example table of possible twinning operator (22) with permission of the authors and the IUCr. A full set of look up tables are given in Chandra *et al.* (22) Table 1 to which the reader is referred for the full details.

Space-group number	Space group	Point group	Possible twin operator
75	$P4$	PG4	$k, h, -l$
76	$P4_1$	PG4	$k, h, -l$
77	$P4_2$	PG4	$k, h, -l$
78	$P4_3$	PG4	$k, h, -l$
79	I_4	PG4	$k, h, -l$
80	$I4_1$	PG4	$k, h, -l$

studies from the recent literature. Pickersgill (24) described an early example involving a crystalline ternary complex of spinach ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) with activators Mg^{2+} and CO_2 and which diffracted to high angle but showing diffraction features symptomatic of disorder. These intensity streaks were along c^* and were resolved into satellite reflections, with underlying diffuse scattering, by employing SR on SRS station 9.6 (25) with slits especially set to limit the horizontal beam convergence from the monochromator and thus giving a fine collimation [Figure 4(b)]. The disorder was described by Pickersgill (24) in terms of stacking faults between layers of molecules, with every layer randomly shifted by either $a/39$ or $-a/39$ with respect to the previous layer, giving intensity streaks for the maximally affected reflections. The observation of superlattice reflections and broadening was best accounted for by considering an approximate superlattice and by additional shifts of $\pm a/39$ leading to the diffuse features.

4. Types of twinning (guidance from the Yeates' twinning webserver and test; with permission of the author)

Yeates (26) and his website (<http://nihserver.mbi.ucla.edu/Twinning/>) has reviewed twinning in protein crystals:

'In order to know when one is at risk, it is necessary to understand the symptoms of the different types of twinning and the symmetries where they may arise. There are two

Figure 3. Self-rotation function calculated for a true space group of $P3$ but showing higher, false, symmetry due to twinning which initially suggested an erroneously high space group of the $P6/mmm$ Laue family with 12 copies of the asymmetric unit in the unit cell. The molecular-replacement translation search finally fixed the correct space group as $P3$. From (22) with permission of the authors and the IUCr.

Figure 4. (a) A profilin:actin single crystal, showing an incommensurately modulated diffraction with the b direction lattice and superlattice unit-cell dimensions shown (23) with permission of the authors and the IUCr. (b) Enlargement of part of an oscillation photograph from a Rubisco crystal (24) with permission of the author and IUCr taken around the c^* axis showing satellite reflections and diffuse scattering in the regions of h equal to 10 and 30. This photograph was taken using the Daresbury Synchrotron Radiation Source 'SRS9.6' ($\lambda = 0.88 \text{ \AA}$, 1.8 GeV, 235 mA). In order to resolve the satellite reflections, a 0.2 mm collimator was used and the horizontal beam convergence was limited to 0.6 mrad. The crystal-to-film distance was 144 mm corresponding to a resolution at the edge of the film of $2.28 \text{ \AA}$. An oscillation range of 1.5° and exposure time of 75 min were used.

fundamentally different categories of twinning, epitaxial and merohedral. In epitaxial twinning, different domains are typically oriented so that their molecular spacings match at the crystal face where they meet, but their crystal lattices are not superimposable. In other words, their crystal lattices superimpose in fewer than three dimensions. This leads to distinct, interpenetrating reciprocal lattices in the diffraction pattern. Since epitaxial twinning is recognizable in the diffraction pattern, attempts can be made to integrate reflections from a single lattice, or untwinned crystals can be sought.'

Figure 5(a) shows one such example from our own work: this crystal also shows other diffraction features such as a superlattice-type effect and diffuse streaks along layer lines

Figure 5. (a) Diffraction pattern showing two distinct lattices and is therefore indicative of a crude or 'epitaxial twin'. (b) Diffraction pattern down another direction of the crystal shows superlattice type effects and diffuse streaks. From unpublished work of N.E. Chayen, E. McKenzie and J.R. Helliwell on a 'commercially important protein'.

with the X-ray beam viewed down another orientation [Figure 5(b)]. The complicated nature of the diffraction from the crystal of this commercial protein provided a stimulus to undertake this review. Superlattice effects in protein crystals are described in (23) and references therein [Figure 4(a)].

Merohedral twinning refers to the special cases where the lattices of the different domains overlap exactly in three dimensions. Crystal domains can have superimposable lattices in cases where the rotational symmetry of the lattice exceeds the rotational symmetry of the crystal space group. This situation arises in several point symmetries: 3, 32 (hexagonal but not rhombohedral), 4, 6 and 23. Basically it is a requirement for merohedral twinning that two independent lattices overlap. Since for tetragonal, trigonal, hexagonal or cubic systems the symmetry requires that two cell axes are equal, twinning

often occurs in these systems. Possible merohedral twin operators for tetragonal, trigonal, hexagonal and cubic space groups are given as ‘look-up tables’ in (22), for an example see Table 1. The Yeates’ Twin Test webserver focusses on the most common type of merohedral twinning, hemihedral, which involves just two different domain orientations. Figure 6, from (20), nicely illustrates how additional symmetry in the diffraction intensities can arise in a monoclinic case.

As a result of hemihedral twinning, each observed diffraction intensity is then a sum of two crystallographically distinct, twin-related, reflection intensities weighted according to their respective crystal volumes, assumed to be equally bathed in the X-ray beam.¹ The observed intensity is given by Equation (1).

$$I_{\text{obs}} = \alpha I(hkl) + (1 - \alpha)I(h'k'l') \quad (1)$$

where hkl and $h'k'l'$ are twin-related indices and α is the ‘twin fraction’.

Figure 6. A simulation of twinning and its consequences on the diffraction pattern [from (20) with the permission of the author and of the IUCr]. These show the effect of twinning by a two-fold rotation about **a** on the diffraction pattern of a monoclinic crystal with $\beta = 90^\circ$. Only the $h0l$ zone is shown; the space group is $P2_1/c$. (a) The diffraction from one domain only; (b) the diffraction from another domain (c) superposition of the two domains in a twin with each domain of equal volume; (d) with the volumes reset to 80 and 20% of each domain in the twin. [The original article carries a more detailed caption to which the reader is referred if desired.]

If the twin-fraction, α , is equal or nearly equal to one-half, the observed diffraction pattern acquires an additional symmetry i.e. as imposed by the twinning operation [compare, for example, Figures 6(c) and (d)]. The diffraction data thereby possesses an erroneously high symmetry. This has been called ‘perfect twinning’. The true crystallographic intensities cannot be recovered from these measurements, but in practice it has been found that it is still possible to solve a macromolecule structure e.g. by molecular replacement, not least because the search molecule contains in effect a great deal of information independent of the diffraction measurements.

Clues that diffraction data may be from a perfectly twinned specimen include: (a) a unit cell that is too small to contain the known molecule under the apparent space group symmetry and (b) an intensity distribution that does not follow Wilson (1949) (27) statistics. The latter as a test must be performed on normalized diffraction intensity data or in thin diffraction resolution shells. Anisotropic diffraction resolution can be a complicating factor. Examples of software suites providing such statistics and their determination of twinning are discussed in Section 11.

The situation in which the hemihedral twin fraction is not one-half has been called ‘partial twinning’. The result is not higher apparent symmetry, but observed intensities that still contain contributions from distinct reflections. The example of (4) is relevant here i.e. where twin fractions of 7:1 are mentioned, basically with one dominating fraction.

True crystallographic intensities can be extracted if: (1) the twin fraction is significantly different from one-half and (2) the twin-fraction can be estimated accurately. ‘ α ’ is estimated by a statistical evaluation of the similarity between twin-related observations. Pseudo- or non-crystallographic symmetry can be a complicating factor, as it tends in special cases to mimic twinning (at least at lower resolution) by leading to similarity between crystallographically distinct reflections. Measurement errors lead to an incorrect estimation of the twin fraction, in practice, an overestimate, and so the test should be performed over a resolution range where the data are well-measured.

Merohedral twinning can only occur when the orientation of the unit cell generated by the twin operation still fits into the lattice of the original unit cell and when at the same time the orientation of the molecules in the two unit-cell orientations are no longer equivalent. This holds true for only a limited group of tetragonal, trigonal, hexagonal and cubic space groups where the space-group symmetry is lower than the lattice symmetry (26).

5. Glossary of technical terms in alphabetical order (20, 26) (with permission of the authors)

Following the coverage of the opening topics above a glossary of terms is now grouped together here.

Bizarre systematic absences can occur in cases of non-merohedral twinning and are related to the partial overlap of reciprocal lattices.

Enantiomorphic twinning: a crystal of a compound in a space group such as $P2_1$ may contain enantiomorphic domains (28). This type of twinning does not occur for an enantiopure compound and it can therefore be ruled out in protein crystallography.

Hemihedral twins: Although twins containing more than two crystal domain variants are sometimes observed, more commonly only two are present, however, and such twins are described as hemihedral twins. Perfect hemihedral twinning results in a higher order symmetry in the diffraction pattern intensities than the true symmetry of the crystal space group.

Merohedral twinning: this is the situation where lattices of two or more distinct crystal domains coincide exactly in 3 dimensions. The diffraction pattern layout reveals no obvious abnormalities. However, since each observed diffraction spot contains contributions from crystallographically unrelated intensities, the analytical (i.e. overall) distribution of spot intensities has unusual proportions of weak and strong intensities and thereby an incorrect average; this is readily tested for from the diffraction spot intensity measurements distribution.

Non-merohedral twinning: also known as epitaxial twinning, involves overlapping of the crystal lattices in fewer than 3 dimensions.

Pseudomerohedral twinning: Cases where overlap of the crystal lattices is approximately but not exactly in all 3 dimensions.

Twinning fraction: usually given the symbol $(1-\alpha)$ it is the fraction of the crystal volume in the predominant domain and ' α ' is the fraction in the other domain. This is estimated from an analysis of the diffraction spot intensities. The condition $\alpha=0$ is obviously an untwinned crystal; the condition $\alpha=0.5$ is a perfect twin (referred to as a mimetic twin); $\alpha < 0.5$ is a partial twin. In any given molecular crystal it can be observed that from one crystal specimen to the next the value of α can vary; clearly this is unwelcome if an MIR (Multiple Isomorphous Replacement) macromolecule crystal structure determination is sought! Equations for estimating α are described in Section 7. NB, having a good estimate for α is important for refining an atomic structure, since the effects of twinning must be accounted for to achieve agreement between calculated and observed reflection intensities/amplitudes. Knowing the value of α also makes it possible, as long as α is not too close to $1/2$, to detwin the observed data (i.e. to extract the true crystallographic intensities from the observed intensities) according to equations of the following form:

$$\begin{aligned} I(h_1) &= [(1-\alpha)I_{\text{obs}}(h_1) - \alpha I_{\text{obs}}(h_2)]/(1-2\alpha) \\ I(h_2) &= [(1-\alpha)I_{\text{obs}}(h_2) - \alpha I_{\text{obs}}(h_1)]/(1-2\alpha). \end{aligned} \quad (2)$$

Twinning statistical test: The diffraction data metric, $\langle F \rangle^2 / \langle F^2 \rangle$, is scrutinized and this is known as the Wilson ratio test (27). Thus for acentric data, its value is 0.88, for a perfect twin and its value is 0.785 for untwinned data. Other statistical tests, including one that is more robust to the confounding effects of diffraction anisotropy (29), have also been developed.

Twin Law: This describes mathematically, in matrix form, exactly how the twinned crystal is an aggregate of its different domains joined together. Thus the twin law is a specific symmetry operation. The diffraction patterns derived from different crystal domains can be rotated, reflected or inverted with respect to each other, depending on the nature of the relationship between the different domains, and weighted according to the volume of a particular domain present in the crystal. Thus, during structure analysis of a twinned crystal, it is important to define exactly which reflections contribute to a given diffraction spot intensity measurement: this is the role of the twin law. In order to treat twinning during structure model refinement, the twin law must obviously form part of the model. It is input into a structure model refinement computer program in the form of a 3×3 matrix. See, for example, (30).

6. Use of the optical microscope

6.1. Viewing the detailed crystal shape

Twinning, and obviously multiple crystal growths, can be revealed in an optical microscope examination, which reveals examples of poor shapes. Note that a poor shape is not always a twinned case and a good shape is not always untwinned!

Several very nice examples are given by Makino *et al.* (31) in their study of Bence-Jones protein and where, initially, crystals of a consistent though suspicious habit were observed. The suspect crystals, similar to those seen in Figure 7(a), were 'roughly cube-shaped and often grew to 0.2 mm or more in edge dimension where faces tended to be curved and the edges rather indistinct, in some cases, they were physically robust and easy

Figure 7. 'BJ-KWR' protein crystals grown at 295 K in (a) 1.5 M ammonium sulphate at pH 8.0; (b) 1.45 M ammonium sulphate at pH 8.5; (c) 1.45 M ammonium sulphate pH 8.0; (d) 2.88 M NaH_2PO_4 and 0.72 M K_2HPO_4 pH 4.0. From (31) with permission of the authors and the IUCr.

to mount for analysis. A second crystal habit formed and coexisted for some time with the first and then grew, apparently at the expense of the initial form. Frequently, this secondary crystal form nucleated on and grew from the surfaces of the cube-shaped crystals [Figures 7(b) and (c)], suggesting a possible unit-cell relationship. Occasionally, the prismatic form appeared directly without accompaniment by the first form.'

Numerous attempts were made by Makino *et al.* (31) to collect diffraction data from crystals of the cube habit with rounded faces but were unsuccessful. Often some zones clearly showed the presence of multiple misaligned reciprocal lattices. They further remark: 'Nonetheless, it was sometimes possible to index these patterns and the unit cell was found to belong to space group $P4_122$ (or $P4_322$), with unit-cell parameters $a=b=64.2\text{ \AA}$, $c=179.7\text{ \AA}$. These crystals were the subject of a detailed atomic force microscopy (AFM) study (32), which demonstrated among other things that the unique axis was 4_3 .' Subsequently, Makino *et al.* (33) reported the solved structures of several of the crystal forms.

6.2. A crystal and its optical properties under polarized light as a clue of twinning effects

Kerfeld *et al.* (34) reported the, albeit special, case of a carotenoid binding protein whose carotenoids, being aligned in a predominant direction, nicely indicated untwinned and twinned crystal cases. The crystals displayed a striking optical property (Figure 8). They remark: 'The crystals grow as elongated prisms that lie horizontally in the crystallization drop. When illuminated through a single polarizing filter, each crystal is orange or perfectly clear, depending on the orientation of the crystal relative to the direction of

Figure 8. (a) Carotenoid bound protein crystal viewed under a single polarizer and (b) after 90° rotation of the polarizer; (c) case of a twinned crystal of the same protein and which grows when 2.5% of MPD is used as an additive. From (34) with permission of the authors and the IUCr.

polarization. The maximum carotenoid absorbance (i.e. the darkest orange colour) occurs when the polarizer is approximately aligned with the long edge of the crystals, which corresponds to the crystallographic a -axis.' The crystals grew as macroscopic twins when the concentration of glycerol or MPD was increased [Figure 8(c)]. The twinning is non-merohedral or 'epitaxial', with a twinning axis along the a -axis.

7. Twinning tests including some limitations and latest developments

An early test for twinning was that of Britton (35) and was implemented by Fisher and Sweet (15), and who christened the 'Britton Plot'. Britton (35) was analysing a crystal

Figure 9. Top: the atomic model 'conventional R value' as a function of the assumed fractional size of the smaller twin fragment. Bottom: the number of pairs of reflections lying in different ranges of $I_2/(I_1 + I_2)$ vs. the ratio. The minimum in the upper curve corresponds with the cut-off point in the bottom distribution of points. From Britton (35) with permission of the author and the IUCr.

of diiodoacetylene, which was twinned and made the general observation that 'in twinned crystals, the intensity contribution from the separate individuals can be estimated from the observed intensities without any knowledge of the actual structure, even when the two reciprocal lattices are completely superimposed. Once the separate intensity contributions are known, the trial structure can be sought in the usual ways.' Figure 9 shows the experimental distribution (marked with 'x's') and so that it was concluded that the twin fraction was between ~ 0.25 and 0.30 . Also shown is the atomic model refinement R value as a function of twin fraction; and from this curve it was concluded that the twin fraction value was 0.28 , in agreement with the first method.

Fisher and Sweet (15) used this and introduced other methods, which they compared, in their successful isomorphous replacement analysis at $5 \text{ \AA}$ of merohedrally twinned beta-phycoerythrin protein crystals. Their four approaches were: '(First) the "Britton" technique, which allows estimation of the volume fraction for data from a single specimen (Figure 10). Their second and third methods provided an estimate of the approximate difference in twinning between two different(ly) twinned crystals. The fourth involved optimization of volume fractions during structure factor least-squares refinement of heavy-atom parameters.' As a variant of two differently twinned crystals of the same protein, they also considered the case of one crystal being untwinned, being used to help de-twin diffraction data from another crystal and thereby improve the data completeness. They show diffraction precession photos from crystals with twin fractions of 0.05 and 0.4 .

Rees (36) developed a general theory of X-ray intensity statistics for twins by merohedry based on a mathematical analogy of the problem to a one-dimensional random

Figure 10. The number of intensities predicted to be negative plotted as a function of the twinning fraction x for a β -phycoerythrin crystal. Fisher and Sweet (15) call this a 'Britton' plot; with permission of the authors and the IUCr.

walk. Probability distribution functions were derived (a) for intensities from a single twinned specimen, and (b) for the differences in intensities from two separate twinned specimens. Theoretical values of a discrepancy index between observed intensities from a twin, and calculated intensities from both correct and incorrect structural models were evaluated. A new method for determining volume fractions of crystals in a twin was also proposed, based on the distribution of differences in intensities between twin-related reflections. The results had implications for the treatment of isomorphic replacement data collected from twins by hemihedry. Rees also said of the perfect twin case: 'An especially striking feature of these results is the deleterious effect of perfect twinning on the R factor for a correct structure model.'

Yeates (26) introduced a twinning test such that for untwinned intensity data $\langle F \rangle^2 / \langle F^2 \rangle$, known as the Wilson ratio test (27), is 0.885 for a perfect twin and 0.785 for untwinned acentric data.

Subsequently, Padilla and Yeates (29) discussed the diagnosis of hemihedral twinning further, remarking that the statistics of local intensity differences are insensitive to the effects of anisotropic scattering and to the effects of pseudo-centring, effects which confound traditional intensity statistics. They introduced a parameter L , which they showed is more robust for discriminating between acentric and centric data and for detecting perfect twinning. They remarked that 'because this new statistic appears to perform as well as or better than traditional intensity statistics, the distribution of L should

supplement, or perhaps supplant, some of the intensity analyses in previous use.' They demonstrated the advantages gained by analysing intensity statistics by examining local differences between pairs of reflections. The difference between two intensities is divided by their sum to give a dimensionless quantity, L , defined by Equation (3):

$$L \equiv \frac{I(h_1) - I(h_2)}{I(h_1) + I(h_2)}, \quad (3)$$

where h_1 and h_2 are the component reflections and the range for L is -1 to $+1$. A similar parameter, H , had been introduced earlier (37). The robustness of their approach was demonstrated with examples using real macromolecule crystals whose diffraction data appeared aberrant by other methods of intensity analysis. The new statistic was therefore shown to be better suited than other available methods for diagnosing perfect hemihedral twinning.

Strelkov *et al.* (38) in a study of fibrin M in the unusual space group $P3$ commended the following Equation (4) so as to get a most accurate value of the twin fraction when it has a value near to 0.5 i.e. perfect twin situation:

$$E(\kappa, \alpha) \equiv \sum_{hkl} [I_{\text{twin}}(hkl) - \kappa(1 - \alpha)F_{\text{calc}}^2(hkl) - \kappa\alpha F_{\text{calc}}^2(h'k'l')]^2, \quad (4)$$

where $F_{\text{calc}}(hkl)$ and $F_{\text{calc}}(h'k'l')$ are the calculated structure amplitudes of two single crystal reflections related by the twinning operation and κ is the scale factor between the observed and calculated intensities.

Using this equation and method they obtained values for two different crystals of $\alpha = 0.49$, i.e. where the approach proved most valuable, and 0.41 (for another crystal growth mix).

Lunin *et al.* (39) have very recently developed an advanced statistical model, which is designed to estimate the twinning fraction in merohedrally (or pseudomerohedrally) twinned crystals. Their model takes experimental errors of the measured intensities into account and is adapted to the accuracy of a particular X-ray experiment through the standard deviations of the reflection intensities. The theoretical probability distributions for the improved model were calculated using a Monte-Carlo-type simulation procedure. The use of different statistical criteria (including likelihood) to estimate the optimal twinning-fraction value was discussed. Their improved model enables better agreement of the theoretical and observed cumulative distribution functions to be obtained and produces twinning-fraction estimates that are closer to the refined values in comparison to a conventional model, which disregards experimental errors. The results of the two approaches converge when applied to selected subsets of measured intensities of high accuracy.

8. Case studies

8.1. 'RMCP II' protease crystals with twin fractions $< \sim 0.25$

Reynolds *et al.* (40) described the structure determination of Rat Mast Cells Protease ('RMCP II'), where the observed data had to be analysed and corrected for twinning,

Figure 11. Twin-related reflections across the left to right of this rotation diffraction pattern, two pairs of which are arrowed. The crystal twinning fractions seen and the various data merging statistics are shown in the associated Table 2 below: both figure and table from Reynolds *et al.* (40) with permission of the authors and the IUCr. A more detailed figure caption is given by the authors in the original publication.

a phenomenon which occurred to a greater or lesser degree in all crystals of the protein. The actual solution of the structure involved a combination of the methods of single isomorphous replacement and molecular replacement. The data-collection strategy was chosen to ensure that twin-related reflections would occur in pairs on the same film (Figure 11). It was therefore possible to estimate a twinning fraction. Plots were also made of the number of reflections calculated to have negative intensities as a function of the twinning fraction (Figure 11). In the absence of experimental error, the desired twinning fraction is the largest value that predicted no negative intensities. In general, the procedure worked well for these low twin fractions (less than about 15%) and became less reliable for intermediate twinning values. The positive impact on the data merging is illustrated in the Table 2.

8.2. Detection and characterization of merohedral twinning in crystals of oxalyl-coenzyme A decarboxylase (41, 42)

Oxalyl-coenzyme A decarboxylase is a thiamin diphosphate dependent enzyme active in the catabolism of the highly toxic compound oxalate. Two crystal forms were obtained, one showing poor diffraction and the other merohedral twinning. The degree of twinning could not be reduced by chemical additives. Crystals in the former category belonged to

Table 2. Data merging statistics for the 'serine protease from rat mast cells' with and without correction for twinning. From Reynolds *et al.* (40) with permission of the authors and the IUCr.

Crystal number	Number of film packs	Initial twin fraction	Final twin fraction	R_{merge} without detwinning	R_{merge} after detwinning
Native data					
(1)	14	5%	5%	11.2	7.7
(2)	25	3	3	16.5	9.0
(3)	14	5	4.5	22.9	11.7
(4a)	8	16	17	16.1	8.4
(4b)	3	23	21	15.6	8.1
Derivative data					
(1a)	10	5	5	—	10.4
(1b)	6	9	9	—	9.4
(1c)	4	15	14	—	10.3
(1d)	4	22	21	—	11.7
(2)	25	24	24	—	11.1

the tetragonal space group $P4_22_12$. Data to 4.1 Å resolution were collected from these crystals and an incomplete low resolution structure was initially determined by molecular replacement. Crystals in the twinning category yielded diffraction data to 1.73 Å collected from one of these crystals; the data had an apparent point group 622 but in fact was $P321$ and the crystal used was determined to have a twin ratio of 0.43. With the diffraction data treated in the lower symmetry and detwinned, a molecular replacement solution was obtained using the low resolution structure as the protein molecule template. The structure revealed there was a non-crystallographic symmetry axis almost parallel to the twin axis, an arrangement that can promote twinning.

Subsequently, Berthold *et al.* (41) were able to report refined structures of the enzyme in complex with different ligands and whereby crystallization with the enzyme in a ligand complex prevented the twinning effect. The details of the new crystallizations were performed by the hanging drop vapor-diffusion method under conditions otherwise very similar to those described previously (42).

9. Finding a way around the twin situation?

9.1. Chemistry approaches

The previous case studies showed how a project can move forward in spite of twinning and case study 8.2 shows how twinning was avoided in crystals of the protein-ligand complexes. In this section varieties of chemical and physics-based ways to avoid twinning are described.

In a relatively early communication, Lin (43) described several chemical approaches including the use of additives: ammonium nitrate, sodium nitrate and dioxane. Also in cases where divalent metal ions had been used to promote protein crystal growth, the supplementary addition of calcium chloride was cited as having prevented twinning. Also reducing the protein concentration was cited as reducing twinning.

Crystallization screens are the ‘modern way’ of building further on the examples quoted by Lin (43), and which clearly offer many different possible sets of conditions. Radaev *et al.* (44) document in detail one particular protein case as an example to illustrate the power of screens of conditions to avoid twinning.

9.1.1. Case studies

9.1.1.1. *Amino-acid additives.* Przybylska *et al.* (45), in their crystallization of the haptoglobin–hemoglobin complex, found that in their early crystallization trials twinning was frequently observed, but this was prevented by including small amounts of various additives. Best results were obtained with Gly-I-Ser.

9.1.1.2. *Dioxane additive.* A reagent commonly used to overcome twinning is dioxane (46).

As remarked in Section 2, Sigler *et al.* (7) reported that with crystals of α -CHT the inclusion of 2–4% dioxane in the crystallizing solution virtually eliminated twinning. This result was qualified by Tulinsky *et al.* (4) who found that the effect of dioxane was (in fact) ‘apparently one of degree’.

Lin *et al.* (47) used dioxane in their study of a protein from *Pachyrrhizus erosus*: the droplet consisted of an equal volume of protein solution and the solution from the well. The crystals grew as octagonal plates with twinning in the direction of the *c*-axis within 10 days. Dioxane of 3% (v/v) was used to overcome the crystal twinning.

Likewise, Murakami *et al.* (48), in a study of a xylanase, obtained irregular and highly twinned crystals after 3 days in 2 mL droplets (1 mL protein solution and 1 mL reservoir solution) which contained 0.9 M potassium sodium tartrate tetrahydrate [Figure 12(a)]. Crystals suitable for X-ray diffraction were obtained using the same conditions upon addition of 1% (v/v) dioxane [Figure 12(b)].

9.1.1.3. *Use of pentaerythritol propoxylate ‘PEP426’.* Gulick *et al.* (49), during an effort to obtain high-quality crystals of several problematic proteins, used two new agents, pentaerythritol propoxylate and pentaerythritol ethoxylate, which yielded well-ordered

Figure 12. Crystals of mesophilic xylanase A from *Bacillus subtilis*: (a) in the absence and (b) in the presence of 1% (v/v) dioxane. From (48) with permission of the authors and the IUCr.

quality crystals (Figure 13) where more traditional precipitants had been unsuccessful. We quote as follows the authors' summary:

'Pentaerythritol propoxylate (PEP) and pentaerythritol ethoxylate contain a pentaerythritol backbone to which organic polymers are bound, forming a branched polymer. As such, they

Figure 13. Multiple crystal forms of PrpD protein: (a) Highly anisotropic hexagonal rods; (b) twinned rhombohedral crystals; (c) Orthorhombic crystals grown with PEP 426 as the precipitant. From (49) with permission of the authors and the IUCr.

are larger than small organic precipitants such as low molecular-weight alcohols or 2-methyl-2,4-pentanediol, but behave differently from polyethylene glycols. These compounds were used to crystallize an enzyme encoded by the *Salmonella enterica* PrpD gene that catalyzes the dehydration of 2-methylcitrate to form 2-methyl-*cis*-aconitate. While the PrpD protein crystallized readily under a number of conditions, the resultant crystals were declared unsuitable for a crystal structure determination. The new crystals obtained with $25 \pm 40\%$ pentaerythritol propoxylate belonged to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 73.2 \text{ \AA}$, $b = 216.4 \text{ \AA}$, $c = 214.3 \text{ \AA}$, and diffracted beyond $2.0 \text{ \AA}$ with SR. A further benefit of this precipitant for crystallization was its ability to function as a cryoprotectant, allowing the crystals to be transferred directly from the mother liquor to the nitrogen stream at 113 K .

9.2. Improve the macromolecule (see also Section 14)

Lagartera *et al.* (50) describe a 'Pce' esterase enzyme, whereby the first crystals of native Pce showed serious twinning problems. Their careful analysis of protein stocks by SDS $\pm$ PAGE and mass spectrometry showed a time-dependent appearance of two protein species corresponding to truncated forms of Pce (66.9 and 63.5 kDa) degraded at their C-terminal extreme, as confirmed by N-terminal sequence analysis. No further decrease in Pce molecular weight was found in protein stocks after several months. Analysis of Pce crystals by SDS $\pm$ PAGE using silver staining showed that only the 63.5 kDa species, which lacked the last 55 amino acids, crystallized. The C-terminal region seemed to be very flexible and no secondary structure element could be predicted for it. Therefore, in order to improve crystallization, the last 55 amino acids were deleted. The activity of the truncated form of Pce₅₅ was similar to that of the wild-type protein. The best crystals of the truncated Pce form were obtained in the presence of a specific detergent (*n*-dodecylphosphorylcholine). They belonged to the monoclinic space group $C2$, with unit-cell parameters $a = 173.49 \text{ \AA}$, $b = 58.12 \text{ \AA}$, $c = 68.65 \text{ \AA}$, $\beta = 108.29^\circ$.

9.3. Microseeding

This is a quite commonly used technique, which is now documented via several case studies.

9.3.1. Clustered crystals to single crystals

9.3.1.1. *Crystals of an endoglucanase enzyme.* An interesting case study is where microseeding has been used (51) to optimize conditions to achieve single crystals from rather unpromising looking, multiple interdigitating, crystals. The crystals, of an endoglucanase enzyme, 'always grew initially in layers' [Figure 14(a)] and showed multiple diffraction patterns. Their reported procedure is interesting in the details:

'Although they could be separated carefully by the use of microneedles to produce smaller crystal pieces for X-ray analysis, the overall quality of the resultant diffraction was problematic and not suitable for further structural studies. Optimization of the crystallization conditions was therefore necessary. As the crystals grew in the presence of PEG 4000, a PEG/ion combination based on the PEG/ion screen (Hampton Research, USA) was used as an optimization procedure. Crystals of identical morphology but of considerably better quality were grown using 15% (w/v) PEG 4000, 0.15M potassium acetate, 0.1M sodium acetate buffer pH 4.6. Although the new crystals displayed the same tendency to grow from a single nucleation site, they were thicker than the previous ones and could be separated more easily.

Figure 14. Single crystals of endoglucanase: (a) before optimization and (b) after optimization of crystallization conditions and using microseeding: the size of the single crystal in (b) is $\sim 0.2 \times 0.1 \times 0.05$ mm. Reproduced from (51) with permission of the authors and the IUCr.

Sodium iodide was also found to have a beneficial effect on the size of the crystals, but the diffraction of these crystals was not good. Further improvement of crystal quality and size was achieved by microseeding. In a typical microseeding experiment, two or three small crystal pieces were crushed in 10 μ L of reservoir solution (15% PEG 4000, 0.15M potassium acetate, 0.1M sodium acetate buffer pH 4.6). One microlitre of the seed-stock solution was further diluted to 50 μ L with reservoir solution. From this solution, 0.1 μ L aliquots were used to seed 2.5 μ L drops that had been equilibrated for at least 1 day against the reservoir solution at 289 K. Single crystals suitable for data collection grew within 1 to 5 days of seeding [Figure 14(b)].

9.3.1.2. *HHDD isomerase/OPET decarboxylase*. Jervis *et al.* (52) also used microseeding to obtain single crystals in their study of the enzyme HHDD isomerase/OPET decarboxylase. See Figure 15. These crystals were, in fact, not the ones the structure was solved with, since better crystals were finally grown from different conditions and the structure was reported in (53).

9.3.2. High throughput semi-automated microseeding

In the context of high throughput, Walter *et al.* (54) described semi-automated microseeding methods. A microseed stock solution was made from microcrystals using a Teflon bead. A dilution series (Figure 16) of this microseed stock was then prepared and dispensed as 100 nL droplets into 96-well crystallization plates, facilitating the incorporation of seeding into their high-throughput crystallization pipeline. Success with improving several, initially poor, crystal examples (Figure 17) is described, allowing full structure determination.

9.4. Use of gels

Sica *et al.* (55) described the use of agarose gels to slow down the process of crystallization and thereby achieved a positive impact on eliminating twinning. The method usually

Figure 15. Before (a), and after (b), use of microseeding to obtain single crystals in the study by Jervis *et al.* (52) of the enzyme HHDD isomerase/OPET decarboxylase [crystal sizes in (b) are $\sim 0.2 \times 0.3 \times 0.2$ mm]. Reproduced with permission of the authors and the IUCr.

provides for the slow diffusion of the solute or solvent into the gel, and inhibits secondary nucleation, thus lowering convective flows and sedimentation effects. As their example, they showed crystals of an alcohol dehydrogenase 'SsADH(NADH)₂', grown in agarose gels, and which lacked the clear physical twinning often observed in holoenzyme crystals grown by conventional means. This was confirmed by X-ray diffraction of a large prismatic crystal ($0.8 \times 0.4 \times 0.2$ mm), which gave a unique reciprocal lattice. Although growth in agarose gels eliminated the twinning phenomenon, unfortunately several of the crystals grown in this way completely failed to show any diffraction pattern whatsoever. Thus only crystals freshly extracted from the gelled droplet, and soaked in a stabilizing solution prior to excision from the gel and mounting, showed diffraction.

9.5. The impact of microgravity on twinning

There are rather few case studies to draw on here to make systematic conclusions but are interesting.

Figure 16. Method of microseeding in 96-well plates and progressive dilution for crystal improvement (with the number of crystals seen indicated for each well). From Walter *et al.* (54) with permission of the authors and the IUCr.

Figure 17. Example cases are shown from the Figure 16 approach. Compare equivalent cases (a) with (d), (b) with (e) and (c) with (f), illustrating the degree of optimization possible. The scale bar is 0.1 mm in each. From (54) with permission of the authors and the IUCr.

Tanaka *et al.* (56) studied the NusA protein from *Aeropyrum pernix*, purified to near-homogeneity and which was crystallized by a counter-diffusion technique in terrestrial and microgravity environments after initial terrestrial experiments using vapor diffusion failed to yield satisfactory results. The shapes of the NusA crystals

Figure 18. Crystals of archeal NusA: (a) using the counter-diffusion method on earth and (b) likewise but in microgravity. From (56) with permission of the authors and the IUCr.

grown under terrestrial gravity [Figure 18(a)] and microgravity [Figure 18(b)] counter-diffusion conditions were essentially identical to each other and the crystals belonged to the tetragonal space group $I4_1$. However, the microgravity-grown crystals were larger than the terrestrially grown crystals. Near complete datasets were collected using a single crystal from the terrestrial and microgravity experiments. It was interesting that the twinning fraction of the microgravity-grown crystals (1.0%) was reduced compared with that of the terrestrially grown crystals (18.3%). In addition to the decreased twinning, the resolution and mosaicity of the microgravity-grown crystals were also improved. Crystallization in the microgravity environment improved the maximum resolution from 3.0 to 2.29 Å and the mosaicity was improved from 2.369° (terrestrial) to 0.591° (microgravity). The reason for the detwinning in the microgravity environment is not clear: however, it is likely that the slower growth of the protein crystal in the low saturation solution under microgravity conditions made the protein molecules adopt well-ordered orientations at the growing surface of the crystal. This may have reduced the number of molecules in the twinning orientation if the positional potentials were different between the main fraction and the twinning fraction and if the slow crystal growth allowed the transition from a quasi-stable orientation to a more stable one. Tanaka *et al.* (56) further stated that more examples of the detwinning of

protein crystals under microgravity conditions would be necessary to examine the phenomenon in more detail.

Indeed an earlier study was made by McPherson and colleagues (57) who remarked specifically: 'Crystals of canavalin grown in microgravity ultimately produced the highest resolution best quality diffraction data. Those crystals, however, were no less twinned than crystals grown in laboratories on earth.' Similar results regarding the effect of microgravity on the twinning of alcohol dehydrogenase crystals were also reported by Esposito *et al.* (58) who stated that 'gravity seems to play no role in the twinning phenomenon and suggests that crystallization of macromolecules in space will not be a therapy for this problem. On the other hand, mechanical stress produced by twinning may be less in microgravity or be better accommodated and a reduced defect density may result as a consequence.'

Dao-Thi *et al.* (59) report in their protein study (of CcdB): 'In the one experiment in which crystals were produced both in space and on earth, we did observe a significant decrease of the number of twinned crystals.'

Esposito *et al.* (58) also remark further on the matter as follows: 'As far as twinning is concerned, this is the first experiment where twinned protein crystals have been grown in microgravity. Microgravity crystals of SsADH complexed to NADH remain twinned. This suggests the need for further studies on other systems of which the origin of twinning is well known, e.g. stress, secondary nucleation, etc., and may be expected to be affected under reduced gravity'. Their crystals are examples of the re-entrant surface indicative of twinning (Figure 19).

Figure 19. Microgravity-grown crystals of SsADH: NADH complex remain twinned and show 'tail-coated' ends. The larger crystal is $1.0 \times 0.3 \times 0.1$ mm. From (58) with permission of the authors and the IUCr.

9.6. Cutting out a crystal

This is probably a fairly common approach, not always reported on; the following examples are specific cases from the literature.

9.6.1. ODCase

Wu *et al.* (60) in their work on orotidine 5'-monophosphate decarboxylase (ODCase) found that whereas the apo-enzyme crystals were single [Figure 20(a)] twinning/clustering happened with the enzyme ligand complex crystals [Figure 20(b)]. The degree of twinning and the thickness of these complex crystals was very sensitive to the trisodium citrate concentration. To obtain usable crystals of the complex, larger sitting drops were used; it was then possible to cut off single chunks from the twinned crystals for diffraction analysis.

9.6.2. Shikimate dehydrogenase

In a similar approach, Schoepe *et al.* (61) in their study of a shikimate dehydrogenase proceeded via fragmenting a single crystal out using a needle. 'Crystals were grown using

Figure 20. (a) Crystals (~ 0.7 mm) of the free enzyme ODCase with 1.1 M trisodium citrate and (b) of the ODCase-6-azaUMP complex (longest single needle is ~ 0.1 mm thick). From (60) with permission of the authors and the IUCr.

Figure 21. Shikimate dehydrogenase large crystal ($\sim 1.1 \times 0.1 \times 0.1$ mm) with a smaller one beside it; a sliver from the larger crystal was taken for data collection. From (61) with permission of the authors and the IUCr.

sitting-drop vapor diffusion at 293 K... After about five months, a large rod-shaped crystal grew to dimensions of about $1.1 \times 0.1 \times 0.1$ mm amongst some smaller crystals (Figure 21). The large crystal was fragmented with a needle.'

9.6.3. *Bacteriorhodopsin*

Efremov *et al.* (62) describe in detail the splitting of hexagonal hemihedrally twinned bacteriorhodopsin (bR) crystals and which was achieved by a slow reduction of the salt concentration in the mother liquor. The split parts displayed high diffraction quality comparable to the original crystal. Checking of the split parts with respect to twinning by the Yeates' statistics and the Britton plot showed that they were free of twinning. Twinned hexagonal bR crystals consisted of several macroscopic and not twinned domains (Figure 22).

The authors describe their procedure as follows: 'At first, the attempts to separate agglutinated plates applying mechanical force were undertaken. However, the crystals cracked across the *ab*-plane before the hexagonal plates were separated. Therefore, another approach based on gradual changes of mother liquor properties was used. It was observed that whenever a bR crystal was transferred from 3 M sodium phosphate buffer to the solution with low salt concentration it cracked across the hexagonal plane into small segments and at the same time the hexagon laminated. This observation suggested that a gradual change of salt concentration can induce a crack along the surface between twin domains before the domain destruction. Indeed, it was found that a slow reduction of mother liquor salt concentration from 3 to 1M induced splitting of agglutinated plates. Some of the split crystals diffracted well enough to determine the twin ratio which in all cases was equal to zero within experimental error. The same technique was applied to well diffracting bR crystals which had no visible defects and gave normal diffraction images, i.e. without signs of the existence of distinct domains. Fifty-two crystals with twin ratios $>35\%$ (almost all crystals met this condition) diffracting beyond $2.5 \text{ \AA}$ on the rotating anode generator were selected. The crystals had the shape of single hexagon plates with sizes, 150–250 micron in diameter and 20–40 micron in thickness, as determined with optical microscopy.' See Figures 23 and 24.

Figure 22. The case of bacteriorhodopsin (bR). (a) The mutual orientation of the crystallographic axes in a hexagonal crystal of bR and (b) The domain organization and schematic packing of bR molecules on the surfaces of the crystal sample's twinning domains at their contact. From (62) with permission of the authors and the *Biophysical Journal*.

Figure 23. The case of bR. Two agglutinated hexagonal plates: longest dimension ~ 150 microns, thickness ~ 45 microns. From (62) with permission of the authors and the *Biophysical Journal*.

The nature of the twinning at the molecular level was also examined by Efremov *et al.* (62) who describe it as follows:

'The crystal twinning domains are in contact with each other whereby there are two possibilities for the adjacent proteins in the crystal layout to contact each other: by cytoplasmic to cytoplasmic (CPtoCP) or by extracellular to extracellular (ECtoEC) surfaces of bR (Figure 25). The organization of the crystals may originate from the difference in attraction of twin-domains for the contact of the ECtoEC and CPtoCP molecular surfaces. The difference in this interaction may arise due to the difference of charge distribution on the water-exposed surfaces of BR. The extracellular surface of BR is neutral, whereas the overall charge of the cytoplasmic surface is negative (Figure 25). The interaction of short loops inside

Figure 24. The case of bR continued. (a) A crystal which has no visual defects; (b) hexagonal plates together; (c) separated crystals; (d) initial stage of crystal splitting into three parts. From (62) with permission of the authors and the *Biophysical Journal*.

Figure 25. Electrostatic potential surface of the bR molecule trimer: (a) cytoplasmic surface negatively charged (i.e. predominantly 'red') and (b) the neutral extracellular surface (predominantly 'white'). Surface potentials were generated by the authors in Swiss-PDB Viewer version 3.7. From (62) with permission of the authors and the *Biophysical Journal*.

Figure 26. Bacteriorhodopsin crystals grown (at room temperature) in (a) DTPC/CHAPSO and in (b) DMPC/CHAPSO. From (63) with permission of the authors and Protein Science (<http://www.proteinscience.org/misc/terms.shtml>).

a crystal provides a relatively weak van der Waals contact between the layers: (the crystallographic structure reveals only van der Waals contacts between two amino-acid residues per molecule). Hence, even a weak electrostatic repulsion of cytoplasmic 2D crystalline surfaces may have a noticeable impact on the interdomain interaction.'

In another investigation to eliminate twinning in bR, Faham *et al.* (63) used a bicelle crystallization method, which is somewhat of a compromise between the ease of detergent crystallization and the more protein-friendly lipid cubic phase crystallization. Bicelle lipid/amphiphile mixtures tend to form small bilayer disks at low temperature, and appear to form a perforated lamellar phase at higher temperature. The DTPC/CHAPSO $P2_1$ crystals were twinned but the DMPC/CHAPSO $C222_1$ crystals were untwinned (Figure 26).

9.6.3. The enzyme 'Dicer'

MacRae and Doudna (64) worked on the crystal structure of the enzyme 'Dicer' from *Giardia intestinalis* to 3.3 Å resolution by MAD using crystals belonging to space group $P2_12_12$ (65). These crystals were derived from crystals that diffracted X-rays to 3.0 Å resolution but were described as 'refractory to structure determination because they were twinned'. It was shown by MacRae and Doudna (64) that the original Dicer crystals (Figure 27) represented an unusual case of perfect pseudo-merohedral twinning of orthorhombic crystals. Before the twinning problem was overcome (Figure 28), it was possible to calculate a low resolution electron-density map in space group $P4_1$ that was used to build a partial molecular model. Experimental phases were sufficient to identify heavy-atom sites that indicated space-group inconsistency, leading to identification of the true space group. This information guided the search for different crystallization conditions that yielded untwinned crystals and ultimately a fully interpretable electron-density map.

Manual dissection of the crystals was described by the authors as 'difficult' and the crystals were heavily damaged during the process. However, the partially dissected crystals still diffracted X-rays to 6 Å resolution, which was sufficient for crystal-system assignment. A 180° of diffraction data were measured by focussing the X-ray beam on one lobe or the

Figure 27. Original twinned crystals of 'Dicer' and a diffraction pattern showing nearly perfect overlap of spots but with some split spots observed. From (64) with permission of the authors and the IUCr.

Figure 28. The method used to dissect the twinned 'Dicer' crystals macroscopically and which could then be mounted separately for data collection. From (64) with permission of the authors and the IUCr.

other, or at the lobe junction, and symmetry R factors were compared after data reduction in tetragonal versus orthorhombic space groups.

This analysis demonstrated that data measured from either individual lobe had orthorhombic symmetry [Figures 29(a) and (b)], whereas data measured from the lobe

Figure 29. Selecting portions of a 'Dicer' crystal using a small SR X-ray beam. From (64) with permission of the authors and the IUCr.

junction, which contained overlapping contributions from both lobes, showed tetragonal symmetry analogous to the data measured from the original crystals [Figure 29(c)]. This diffraction analysis was critical to identifying the macroscopic twin domains because the Dicer crystals were not highly birefringent, and it was not possible to distinguish separate twin domains under polarized light. Furthermore, data from one lobe could be scaled reasonably well with data from the other lobe only after switching the two short axes (and inverting the long axis l).

The authors summarized as follows:

'this result suggested that the Dicer crystals were pseudomerohedrally twinned and that the underlying symmetry was a special case of orthorhombic in which the length of the a -axis is equal to that of the b -axis. In this case, unit cells can be packed into the crystal lattice in two distinct orientations, which are related to each other by a 90° rotation about the c -axis. The original crystals were likely to be composed of an equal distribution of the two unit-cell orientations such that the diffraction data contained equal contributions from each orientation. In the original crystals the twin domains were probably very small because in over 30 complete datasets collected, we never encountered crystals that did not scale well as tetragonal, even when examining small wedges of data. This would produce extra symmetry along the l -axis of the diffraction pattern and make the crystals appear to belong to the tetragonal crystal class.'

These results confirmed the authors' suspicion about the original crystals and also suggested a remedy for the twinning problem:

'By transferring Mn-grown crystals into less concentrated precipitant solutions, the macroscopically twinned crystals were partially dissolved. This treatment, similar to that used to dissect hemihedrally twinned crystals of bR by Efremov *et al.* (62), weakened the connection between each lobe, allowing the crystals to be readily dissected into their component single crystals for diffraction data collection (Figure 28). The procedure was much more gentle than the physical separation of the crystals with a cat whisker described above. The resulting crystals yielded untwinned datasets belonging to space group $P2_12_12_1$.'

9.7. The optimum way forward for structure determination if no remediation for twinning proves possible?

If none of the above remediation methods prove possible, then what to do? MIR and MAD or SAD phasing can be severely disadvantaged by twinning, working as they do

with small structure factor amplitude differences for phasing. In particular, a researcher employing MIR on a given project, working with different native and heavy atom derivative crystals, may have to face the fact that different crystals have different twinning fractions. Obviously MAD and SAD often involve use of a single crystal and is an advantage over MIR. Indeed Yang *et al.* (9) report successful MAD protein structure determination for a twinning fraction of 0.36 case, even with the twinned data. Their anomalous difference Patterson sections showed clear seleno vectors, as well as some vectors for the minor twin-fraction, and showed the correct Harker sections. Furthermore their electron density map was improved upon working with detwinned data. There can still be the headache for MAD experiments using different regions of the same crystal where the twin fraction might vary along the crystal or, more obviously, where more than one sample is required since the radiation damage sensitivity limits the amount of data per crystal sample.

Perhaps more optimistically though is the increasing chance to use molecular replacement as protein fold space coverage steadily improves. This has the advantage that it is a more robust situation to start with a reasonable molecular model for structure solution with the X-ray data from the new, twinned, crystal. One detailed case study of use of molecular replacement, described in detail, involved a twin fraction of 0.43 to solve a site-specific mutant of the N-terminal half-molecule of human lactoferrin (Breyer *et al.* 1999) (66). Obviously this is a case of very high amino acid sequence identity rather than the 'twilight zone' cases of much lower sequence identity in the 30% range and for which an instance of twinning would be a very serious complication to face as well as the low biochemical similarity of two molecules. In the case of this mutant of lactoferrin, Breyer *et al.* (1999) conclude on an optimistic viewpoint: 'Finally, even in the case of perfect hemihedral twinning, it should still be possible to solve complicated molecular-replacement problems. This can be performed by examining the behaviour of the correlation coefficient as the positioned fragments are systematically permuted among the twin domains'.

9.8. A case study with a mix of problems and solutions; the 50S ribosomal subunit

Ban *et al.* (67, 68) described a case of twinning in *H. marismortui* ribosome 50S subunit crystals.

'Merohedrally twinned $P2_1$ crystals grew under the same conditions as a $C222_1$ untwinned crystal form and which could not be distinguished by gross morphology. Other $C222_1$ crystals with a shorter c axis (540 Å) were obtained by increasing the concentration of PEG6000. Phasing at 9 Å resolution was made initially (67) using modified forms of the molecular replacement procedures implemented by the computer programs X-PLOR and CNS, whereby EM reconstructions of the 50S large ribosomal subunit were positioned in the crystal unit cell with good packing. The crystals of *Haloarcula marismortui* large ribosomal subunits used in this study were grown following procedures described by Yonath and coworkers (69). MIRAS/SAD combined phases were improved by inter-crystal and non-crystallographic averaging between three different crystal forms: the $C222_1$ form, the twinned $P2_1$ form and the $C222_1$ form with reduced c axis. The merohedrally twinned data were detwinned at the level of intensities when the twinning fraction was below 35% and by back-transforming the map when the twinning was complete. As the second method of detwinning was more accurate when the phase estimates improved, it was combined with averaging and applied every 10 cycles. Phases were yet further improved by non-crystallographic and inter-crystal averaging between four views of the

large ribosomal subunit in three crystal forms using DMMULTI and RAVE program packages.’

Moore and Steiz (70) reviewed the topic:

‘Although the *H. marismortui* 50S crystals diffracted well, they had some serious flaws. A number of their defects had been described by Yonath and coworkers (71). They included “...severe non-isomorphism, high radiation sensitivity, ...non-uniform mosaic spread, uneven reflection shape, and high fragility.” Later Harms *et al.* (72) pointed out other problems, “...the unfavorable crystal habit (plates, made of sliding layers, reaching typically up to 0.5 mm² with an average thickness of a few microns in the direction of the *c* axis), and variations in the *c* axis length (567–570 Å) as a function of irradiation.” The Yale group discovered yet another problem, crystal twinning (68). Depending on how these crystals were handled after they had grown, a 10 Å shift in the relative positions of subunits could occur and that changed their space group symmetry from orthorhombic, *C*222₁, to monoclinic, *P*2₁, with almost no alteration in unit cell dimensions, or crystal cracking, and, surprisingly, no alteration in the symmetry of the crystal’s diffraction pattern. The reason for the latter is that the direction of the packing shift varied randomly from one mosaic block to the next within a crystal, and hence crystals in which it had occurred were twinned. These crystal pathologies were all eventually overcome. Crystal to crystal isomorphism was improved, twinning was suppressed using an appropriate stabilizing solution and crystal thickness was increased to between 0.1 and 0.2 mm using a reverse extraction procedure for crystal growth that also increased crystal strength and singularity (73).’

10. Impact of small X-ray beams on twinning: synchrotrons where there is an SR micro beam diffractometer

This sort of approach involves using an X-ray beam smaller than a crystal and invariably requires use of intense and well-collimated SR. This proved a good application of 2nd generation SR sources and with even smaller beams using undulator X-radiation at 3rd generation SR sources. For an early example of use of a 2nd generation source to demonstrate the recording of good signal-to-noise diffraction from a ~20 micron gramicidin crystal, see (74) using SRS Station 9.6 (25) in ‘Laue mode’.

10.1. Hydroxylamine oxidoreductase

Igarashi *et al.* (75) performed an analysis of data collected at various positions of one crystal of the hydroxylamine oxidoreductase enzyme (Figure 30) and which suggested a heterogeneous distribution of twinning in the crystal. This approach is fairly new and has not been applied to many twinning cases as yet.

ESRF and EMBL-Grenoble originated the now so-called ‘micro-beam diffractometer’ (Figure 31) (76): i.e. for use with X-rays in micron-sized beams (typically 10 micron but now as low as 1 micron). For an example of use of a small i.e. untwinned crystal portion on this experimental set up, see (77). Xiao *et al.* (78) in their crystal structure determination of the retinoblastoma tumour suppressor protein bound to E2F targetted a crystal portion using the ESRF 10–20 microns beam and thus found a much lower crystal mosaicity suitable for data collection. The ESRF/EMBL Microdiffractometer device has been commercialized and is now to be found in SR facilities worldwide, including for example APS (several CATs such as NECAT and GM CAT) as well as at Diamond Light Source in the UK.

Figure 30. A crystal of hydroxylamine oxidoreductase with arrows showing the portions of the crystal selected using the (smaller) synchrotron X-ray beam. From (75) with permission of the authors and the IUCr.

Figure 31. A close-up view of the sample environment of the ESRF/EMBL 'Microdiffractometer'. From (76) with permission of the authors and the IUCr.

11. Webservers, software and data processing

11.1. Testing for twinning via submission of diffraction data on the world wide web

Testing for the presence of twinning through detailed scrutiny of the X-ray diffraction intensity statistics involving simple submission of individual diffraction datasets can readily be made at such websites as the UCLA 'merohedral twinning test' server, set up by T.O. Yeates (<http://nihserver.mbi.ucla.edu/Twinning/>; see also (26).

11.2. Programs

One of the most popular X-ray protein and macromolecule crystal structure analysis packages is the CCP4 package. Within this an early check/indication for twinning is the analysis of an X-ray dataset intensity statistics in the computer program TRUNCATE. An example of non-twinning and twinning intensity statistical distributions calculated by TRUNCATE (<http://www.ccp4.ac.uk/peter/documents/twinning/twinning.html>) is shown in Figure 32 below. Certain space group and cell dimensions can permit twinning and TRUNCATE issues a warning if it detects that this is the case:

```
$TEXT:Warning: $$ comment $$
WARNING: **** Beware! - Cell dimensions could permit Twinning ****
$$
```

TRUNCATE outputs both the theoretical and observed curves (Figure 32): significant deviation of the observed data from the prediction is an indication of problems. In the case of twinned data, the observed curve changes shape as there are fewer apparent 'extreme' (strong and weak) intensities and more average values.

A new version of TRUNCATE is now available, called CTRUNCATE, written by Norman Stein. Two methods are used to test for twinning, namely the Yeates' H test and the Padilla and Yeates' L test. The twinning tests, as well as the moments and cumulative intensity plots are done using data which has been corrected for anisotropy.

Programs used to diagnose and de-twin diffraction data include:

CCP4 DETWIN (see (79) as an example)

CNS (see (80) as an example)

Figure 32. Plots of cumulative intensities distributions are generated by TRUNCATE, and become increasingly sigmoidal for twinned data. From the CCP4 website with permission of CCP4.

PHENIX XTRIAGE: The xtriage software is a tool for analysing structure factor data to identify outliers, the presence of twinning and other conditions that the user should be aware of. See the PHENIX website:

<http://www.phenix-online.org/documentation/xtriage.htm>

Very recently Zwart *et al.* (81), of the PHENIX software team, describe various pathological situations such as twinning, modulated crystals and pseudo-translational or rotational symmetry.

Programs used to refine a protein model against twinned data and estimate a twin fraction include:

SHELX-97 (see (82) as an example): for details see (83, 84).

An interesting comparison can be made with chemical crystallography approaches. Flack (85) describes two algorithms implemented in their version of the XRAY76 suite of chemical crystallography programs. These use 'left coset decomposition'. The effective implementation of a treatment of merohedral twinning involves: 'at the structure refinement stage one of the algorithms generates the desired twinning operations due to (pseudo-)merohedry. Then, secondly, the volume fractions of the twin components are refined as variables.' Practical examples involving crystal structure refinements of Nb₃Si and Nb₃As are briefly described.

11.3. Case study: Careful editing of spot patterns for epitaxial twinning in nitrate reductase crystals

Lu *et al.* (86) developed data processing software (Figure 33) for the type of twin crystals where reflections from two lattices do not overlap completely. Lu *et al.* (86) encountered this problem in the crystal structure determination of a C-terminal fragment of nitrate reductase, comprising the FAD/NADH-binding domains (FDNR) of the enzyme. In their work, in spite of large amounts of recombinant protein being available, the search for single crystals failed. To solve this epitaxial twinning problem, they developed a simple method to deconvolute the diffraction dataset by directly measuring the non-overlapping reflections on oscillation images. This procedure was necessary to successfully determine the structure of FDNR (Table 3 and Figures 33 and 34).

11.4. Other data processing software and twinning cases

Szebenyi *et al.* (87) describe software experiences at a beamline:

'An integral part of the data-collection process is at least preliminary data analysis. Experienced users can assess crystal quality quite well from visual examination of snapshots, but many users are not so knowledgeable about oscillation patterns and there are some problems which only become apparent upon analysis. The data analysis package installed at CHESS is *HKL*, comprising *XdisplayF*, *denzo* and *scalepack* (Otwinowski and Minor, 1997) (88). This package can obtain unit cell parameters, crystal orientation and an estimate of mosaic spread from a single image. Display of the predicted diffraction pattern superimposed on the actual image immediately reveals twinning or satellite crystal problems, and statistics produced during refinement and integration steps using *denzo* provide information on crystal quality.'

Leslie *et al.* (89) have been pioneering the development of automation of the collection and processing of X-ray diffraction data. Initial diffraction images are examined carefully to determine the effective resolution limit, to check for the presence of disorder, twinning or multiple crystals and to obtain an approximate estimate of the mosaic spread. If the

Figure 33. An approach used for evaluation of diffraction data of twinned crystals (see also Figure 34 and Table 3). From (86) with permission of the authors and the IUCr. A more detailed caption is given by the authors in their publication.

quality of diffraction is acceptable, the images are auto-indexed to determine the cell dimensions and possible space groups. The indexing is checked by comparing the predicted diffraction patterns with the images. This provides another opportunity for detecting the presence of a second (weaker intensities) lattice.

Popov and Bourenkov (90) and Bourenkov and Popov (91) have developed a new method and a software program *BEST* for optimal planning of X-ray data collection from protein crystals using the rotation method. From one or a few initial diffraction images, *BEST* estimates the statistical characteristics of the dataset for different combinations of data-collection parameters and suggests the most optimal ones. *BEST* determines the average signal-to-noise ratio at a given resolution either in the shortest time or with the minimum total radiation dose. *BEST* has been successfully used at the macromolecule crystallography beamlines at DORIS (DESY). The software proved to be extremely useful in using the available data-collection time in the most efficient way. They state that the cases of crystals exhibiting pseudosymmetry and/or twinning are exceptions and lie outside the scope of their present considerations.

Table 3. Statistics of diffraction data for 'FDNR' with the two lattices deconvoluted and when not deconvoluted (as indicated in the column labels). See also Figures 33 and 34. From Lu *et al.* (86) with permission of the authors and the IUCr.

Name	R_{merge}			R_{merge} without deconvolution	Average multiplicity	Completeness (%)	Resolution (Å)
	Lattice 1	Lattice 2	All				
Native	0.075	0.079	0.072	0.167	2.7	82	2.8
Native (SRS) ^a			0.059	0.136	4.5	60	2.5
Merged native			0.063		5.5	81	2.5
ADP ^b	0.068	0.071	0.071	0.153	2.5	68	3.0
KAu (CN) ₂	0.067	0.073	0.066	0.152	2.5	65	2.7

Notes: ^aThis dataset was collected with oscillation films at the Synchrotron Radiation Source in Daresbury and processed by the *MOSCO* software. Three crystals with a total of six lattices were processed with internal R_{merge} values of 0.041, 0.044, 0.051, 0.053, 0.063 and 0.064. The other datasets were collected on an *R*-axis II image plate area detector and processed by *R*-axis II commercial software (Sato *et al.* 1992) (92). One crystal with two lattices was used in each case. R_{merge} values of each lattice are included in the table. The R_{merge} values of lattice 1 and 2 were calculated as $\sum_{h,i}|F(h)i - F(h)_{\text{mean}}|/\sum|F(h)_{\text{mean}}|$ by *R*-axis II commercial software, where i represents the individual reflections. The R_{merge} values of all the lattices were calculated as $\sum_{h,i}|I(h)i - I(h)_{\text{mean}}|/\sum|I(h)_{\text{mean}}|$ by the program AGROVATA in the *CCP4* package, where i represents the lattices or sources.

^bCrystal of nitrate reductase cocrystallized with ADP.

Figure 34. Electron density maps for 'FDNR': (a) MIR map and (b) solvent-flattened map, both for data without deconvolution approach, and (c) and (d) equivalent maps with deconvoluted data. All maps contoured at 2 rms. See also Figure 33 and Table 3. From (86) with permission of the authors and the IUCr.

Buts *et al.* (93) address the common situation that the only available crystals are aggregates of single crystals with different orientations, giving rise to two or more distinct but partially overlapping diffraction patterns. This situation is distinct from merohedral twinning, in which an exact symmetry relates the constituent lattices and the diffraction patterns overlap completely. Often, there are many different orientations contributing with similar intensities, yielding a very complex pattern that cannot be interpreted. In certain situations, however, it is possible to separately index two or more dominant patterns, resulting in one dataset for each sub-crystal orientation. Since 'standard' data reduction software is not aware of the presence of the other patterns' spots, which overlap with spots from another (crystal fragment) orientation, it will integrate to incorrect values for the spot intensities.

Lietzke *et al.* (94) developed methods to process diffraction data from epitaxially twinned crystals, whereby four programs for data reduction and two display programs were developed to augment the data-reduction program *XDS* (95). The programs, which the authors state can be generalized for use with other data reduction software, provide the user with a list of the reflections used to determine lattice constants and crystal orientation. One such, *LATTICE_VIEW*, generates a file containing the reciprocal-space coordinates of the strong spots found in the initial data frames. The file is visualized to identify spots that belong to the same lattice, obtains unit-cell dimensions for a lattice and assesses data quality. Their program, *VECTOR_MATCH*, is used to find additional spots belonging to a lattice. *ACCOUNT4* determines which spots have been processed by *XDS*. *COMFORT* discards reflections that are too close to a reflection in another lattice. The display programs provide useful visual information on the quality of the crystal orientations used. Data with an Rmerge of 7.1% at 2.4 Å resolution were obtained from epitaxially twinned crystals of an RNA dodecamer. The data were of sufficient quality to solve the structure with a combination of molecular replacement and single isomorphous replacement methods.

12. Examples of other diffraction disorder case studies

12.1. NTPases

Mancini *et al.* (96) studied the special case of crystal-packing arrangements and disorder of hexameric NTPases from dsRNA bacteriophages:

'Crystals of full-length "P4_8" belonged to the apparent space group $P6_322$, with unit-cell dimensions consistent with the presence of one monomer of the P4 protein in the crystallographic asymmetric unit. These crystals, however, gave rise to a rather complex diffraction pattern (Figure 35) that included a honeycomb-like effect and diffuse rods and bands in addition to the Bragg reflections. Such effects are characteristic of statistically disordered crystals, first reported in 1954 for imidazole horse methaemoglobin (97, 98) This is an unfortunate and intimate type of twinning in which crystal domains showing systematically different orientations or positions are distributed randomly throughout the crystal to produce the characteristic blurring of the Fourier transformation in a systematic way. The specific diffraction seen for the NTPase could be explained by regarding the crystals as being based upon a perfect lattice of hexamers organized in a smaller unit cell. The sheets of hexamers had well-defined structure and yielded sharp reflections. However, successive sheets were then stacked together with random fractional displacements of 1/2 in the *a* or *b*-direction. The relative displacement of the layers manifested itself as an apparent doubling of the unit-cell dimension in the *c*-direction. The reflections of this superlayer were diffuse, leading to an interesting pattern containing sharp lines and diffuse lines of various widths (Figure 35).'

Figure 35. The rather complex diffraction pattern (b) of a crystal (a) for the NTPase from dsRNA bacteriophage that included e.g. diffuse rods and bands (inset). From (96) with permission of the authors and the IUCr.

12.2. *HSIV-HSIU protein complex*

Wang *et al.* (99) faced an example of lattice-translocation defects (Figure 36), i.e. whereby two identical but translated lattices coexisted as a single coherent mosaic block in a crystal. The observed structure in such cases is a weighted sum of two identical but translated structures, one from each lattice: the observed structure factors are a weighted vector sum of the structure factors with identical unit amplitudes but shifted phases. The correction of X-ray intensities from a single crystal containing these defects of the ‘hybrid HslV-HslU complex’, which consists of *Escherichia coli* HslU and *Bacillus subtilis* HslV (also known as CodW), was reported. When diffraction intensities were not corrected, a biologically irrelevant complex (with CodW from one lattice and HslU from another) was obtained. Only upon correction did a biologically functional CodW-HslU complex structure emerge (PDB reference: 1yyf).

12.3. *The AB₅ protein hexamer*

Merritt *et al.* (100) encountered a superlattice created by multiple allowed packings of an AB₅ hexamer. The primary lattice contacts were defined entirely by the B₅ pentamer packing interactions. Considered in isolation, the A protein subunit could be oriented in any one of five settings about the pentamer’s 5-fold axis. In context, the choice of an A orientation in one cell limited the compatible choices in the adjacent cells. This led to a preference for sequential orientations of A in adjacent cells, and hence a superlattice. Merritt (pers comm.) also states that:

‘It was not clear whether this constituted a “commensurate” or “incommensurate” superlattice. The diffraction images could be indexed and integrated using various multiples

Figure 36. Observed alternating sharp-diffuse reflections from a crystal of a 'hybrid HSIV-HSIU' protein complex. In the l direction, strong reflections are observed beyond $4.0 \text{ \AA}$ and some reflections are observed at $3.6 \text{ \AA}$; in the h and k directions reflections barely pass $4.5 \text{ \AA}$. Only one-third of the reflections with index $l=3n$ (boxed) have typical Bragg spots and the remaining reflections have streaky features along the l direction. Data were processed to $4.16 \text{ \AA}$, slightly below an ice ring at $4.15 \text{ \AA}$. From (99) with permission of the authors and the IUCr.

of the minimal axis length along that one direction. The strongest correlation of adjacent cells had a repeat length of 3 along one axis, so the most evident superlattice in the diffraction images was an apparent tripling of that axis. Since the super-symmetry was stochastic, and involved a 5-fold rotational axis, you may imagine that the diffraction patterns had other interesting features as well. In the end it was decided to ignore the superlattice, and refine against only the minimal set of Bragg reflections. This produced maps in which the A subunit was multiple ordered about the 5-fold, with 3 dominate contributing orientations. But since the interest was in ligand binding by the B₅ pentamer this did not prevent interpretation of the relevant binding sites.'

13. Systematic survey of PDB depositions looking for examples of undetected twinning

Entries deposited in the Protein Data Bank as of February 2004 for which both model and X-ray data were available were analysed by Lebedev *et al.* (101) to identify cases of twinning (Table 4 and Figure 37) using such simple statistics as the *R* factor between potential twin-related reflections. Careful consideration of all identified twins showed that in quite a number of cases twinning was ignored during structure solution and refinement. Manual analysis of the models showed that twinning often occurred in association with rotational pseudo-symmetry parallel to the twinning operator.

The coexistence of these two phenomena complicated the detection and diagnostics of twinning using currently available twinning tests. It was concluded that a twinning-detection step should be incorporated in every stage of structure analysis from data acquisition to refinement and validation. Earlier Dauter (102) tabulated the cases of twinning in the literature up to that time.

14. Towards a science of twinning and untwinned crystal growth

14.1. A Chemical crystallography case study

As Lesierowitz and colleagues wrote in 1995 (103): 'Although crystals and crystallization as a process play an important role in pure and applied science, our ability to design crystals with desired properties, and our control over crystallization based on structural understanding are still limited.' They also included within their scope twinned crystals of (*R,S*)-alanine (Figure 38). The notion of designing-in twinning is of course only a 'design exercise'.

14.2. A biological crystallography case study

An example somewhat similar to this is the case of protein engineering and twinning found by Charron *et al.* (104) with their crystal contacts' engineering of aspartyl-tRNA synthetase. Two mutants were designed in order to modify the local surface hydrophobicity involved in the crystal contacts. The first one was aimed to decrease a hydrophobic contact by mutating F366 that forms such a contact with L74. By introducing the mutation F366A, the closest distance A366(C β)—L74(C γ) is 4.8 Å, thereby weakening the hydrophobic contact between AspRS-1 dimers. Crystallization experiments in the presence of sodium formate yielded twinned crystals (Figure 39). This suggests that the intermolecular contact between the two hydrophobic residues F366 and L74 is crucial for crystalline order and that disruption of this contact allows alternative interactions between dimers leading to twinning.

Table 4. Frequency of twinning in different symmetry environments (i.e. space groups). From (101). With permission of the authors and the IUCr.

Crystal symmetry or type of merohedry	No. of twins					
	Total	RPS	TNCS	RPS + TNCS	DNA	Detwinned
<i>P</i> 1	1	—	—	—	1	—
<i>P</i> 2 ₁	13	11	3	3	—	—
<i>C</i> 2	1	—	—	—	—	1
<i>P</i> 2 ₁ 2 ₁ 2	1	—	—	—	—	—
<i>P</i> 2 ₁ 2 ₁ 2 ₁	2	2	—	—	—	—
<i>C</i> 222 ₁	1	1	1	1	—	—
Pseudomerohedral total	19	14	4	4	1	1
<i>P</i> 4 ₁	4	1	1	—	—	—
<i>P</i> 4 ₃	6	4	1	1	—	—
<i>P</i> 4 ₂	1	1	1	1	—	—
<i>I</i> 4	3	2	—	—	—	—
<i>P</i> 3 ₁	7	5	—	—	—	1
<i>P</i> 3 ₂	6	4	2	1	—	—
<i>P</i> 321	2	2	—	—	—	—
<i>P</i> 3 ₁ 21	2	—	—	—	—	—
<i>P</i> 3 ₂ 21	1	1	—	—	—	—
<i>P</i> 3 ₂ 12	2	1	—	—	1	—
<i>H</i> 3	15	3	1	1	2	—
<i>P</i> 6 ₃	6	3	—	—	—	1
<i>P</i> 6 ₅	3	3	2	2	—	—
<i>I</i> 2 ₁ 3	1	—	—	—	1	—
Merohedral total	59	30	8	6	4	2
Total	78	44	12	10	5	3

Notes: RPS = rotational pseudo-symmetry; TNCS = translational non-crystallographic symmetry; DNA = deoxyribonucleic acid cases.

14.3. *A physical crystallography case study (also of industrial crystallography relevance); diamonds!*

This is a case study from physical crystallography, involving the occurrences of intergrowth twinning in crystals of diamond which illustrates the various crystal perfection techniques that can be harnessed to study twinning such as SEM along with monochromatic and polychromatic synchrotron X-ray topography. The robustness of diamond to such examination brings out various details that are largely inaccessible to the study of protein crystals. Cases of diamond twins have been studied by Machado *et al.* (16) and Yacoot *et al.* (17). The morphologies of cases of interpenetrating cubes type [Figure 40(a) and (b)] of natural diamond were studied using SEM and X-ray topography (Figures 41–44). Three specimens were twinned and one an intergrowth (specimen B). This specimen ‘B’, Figure 41(a), consisted of two cubes of unequal size: one had an edge length of 3 mm and the other an edge length of 1.8 mm. Their centres were not coincident. Their surfaces were cobbled (or hummocky) as seen in some single crystals of diamond of cubic habit. A synchrotron Laue picture of this specimen showed that the two cubes were only

Figure 37. Lebedev *et al.* (101) grouped cases of undetected twinning as their schematic plot here shows via their R factor test. RPS = Rotational Pseudo-Symmetry, with permission of the authors and the IUCr.

Figure 38. (a) Twinned crystals of (R,S) -alanine in the presence of 1% R -threonine additive. (b) Proposed structural arrangement showing the epitaxial growth of (R,S) -alanine at the (001) and $(00\bar{1})$ faces of a nucleus of (S) -alanine. From (103) with permission of the authors and the IUCr.

Figure 39. Examples of crystal contacts' engineering of aspartyl-tRNA synthetase. From (104) with permission of the authors and the IUCr.

approximately in twin orientation. It seems likely that this specimen was an intergrowth. X-ray topographs taken at separate peaks on the rocking curve mapped misorientations in the larger cube, which had an angular separation of 6 arc min. Figure 41(b), 'Howie's diamond H', is a pair of concentric interpenetrant cubes and was examined non-destructively using section X-ray topographs involving the 331 and 004 reflections, which were taken with white SR: Figure 41(c).

A macle is the French word for twin and in diamond studies a term in common usage reserved for the triangular contact twin, a flattened octahedron with one pair of opposite $\{111\}$ faces larger than all the others. Figure 42 shows a Laue photograph of a typical macle taken with SR, which illuminated the entire specimen. The incident X-ray beam was directed along the twin axis and hence the picture shows an apparent hexagonal symmetry, arising from the trigonal symmetry of each component. Each Laue 'spot' is in fact an X-ray topograph: images of defects can be seen within each triangular outline (Figure 44).

Figure 40. Drawings of a pair of interpenetrant cubes: (a) $[001]$ axis of one cube vertical, (b) the common $[111]$ axis vertical. From (17) with the permission of the authors and the IUCr.

The topograph in Figure 44 shows that this diamond crystal (Figure 43) has some dislocations emanating from the centre and some strain around the crystal's periphery. This is likely to be due to impurities, which are also responsible for the diamond's opacity and yellow colour.

As remarked in Section 3, the techniques of mosaicity analysis, topography and reciprocal space mapping are gradually being developed and applied to the study of protein

Figure 41. Scanning electron micrographs of interpenetrating diamond crystals: (a) The 'Brazilian diamond B' interpenetrant cubes and (b) 'Howie's diamond H' pair of concentric interpenetrant cubes (scale mark 2 mm). (c) Mid-section topographs of 'Howie's diamond H', involving the 331 and 004 reflections taken with white SR (image diameters 2.2 mm). It is neat to be able to image the two components of the twin separately, without molesting the crystal: i.e. non-destructively. From (17) with the permission of the authors and the IUCr.

and macromolecule crystals [Chayen, Helliwell and Snell (to be published) are compiling a book within the IUCr Book Series with Oxford University Press on these and related topics].

15. Possible avenues to deal with multiple crystal cases using physical crystallography approaches

New technology and methods are becoming available to handle the cases of multiple crystal clusters of a protein in particular. First, the clusters can be ground up into a powder and X-ray structure analysis attempted by synchrotron X-ray powder diffraction [see the recent review by Margiolaki and Wright (105)]. Secondly, as a possible future variant of the protein powder diffraction, the use of gas jets to

Figure 42. Synchrotron Laue topograph taken along the $[111]$ axis of a macle. This pair is parallel to the twin plane. From (16) with permission of the authors and of the IUCr.

Figure 43. Scanning electron micrograph of a diamond peritropic twin: i.e. diamonds may consist of two macles joined on the common (111) plane but one being rotated through 180° with respect to the other. Scale mark 2mm. From (16) with permission of the authors and of the IUCr.

produce a stream of single molecules (106) or crystallites (Spence and Shapiro *et al.*, to be published) may allow one of the principal limitations of protein powder diffraction to be overcome, namely to freeze the crystallites without undue line broadening due to increased mosaicity of the crystallites making up a powder. The big advantage of

Figure 44. Section topographs of the diamond peritropic twin of Figure 43 showing components A (left) and B (right), and the intergrowth between them, imaged by the 224 and 331 reflections. Combined image width 3.9 mm. From (16) with permission of the authors and of the IUCr.

course of frozen crystallites would be the considerably enhanced sample lifetime in the X-ray beam [for a review of single crystal cryocrystallography see e.g. Garman and Owen (107)]. Thirdly, a new processing technique to obtain an individual protein crystal from a cluster employs femtosecond laser-induced cleaving of a protein crystal in a growth vessel containing water solution. An intact protein crystal can be precisely processed without mechanical contact in its sealed growth vessel by focussing femtosecond laser pulses (108). At the IUCr Congress 2008 in Osaka Microsymposium 9 brought together these three topics (abstracts of R von Dreele, D Shapiro and Y Hosokawa to be published in *Acta Cryst A* 2009).

16. Conclusions

There is now a healthy literature on crystal twinning and it is well documented that crystal structure analyses can be severely hampered by the condition. It is increasingly the case that twinning can be diagnosed. Twinning is a situation where the macromolecular crystallographer might well step back to crystallization or the purification of a protein or in more extreme cases to systematically altering the target protein. Moreover, methods for the remediation of twinning cases, notably via alteration of crystal growth conditions including use of chemical additives, have steadily become known. The case of multiple crystal growths likewise can hamper crystal structure analysis and, although not necessarily associated with twinning, is a crystal growth situation where similar remediation methods can be adopted. Tailored apparatus and small X-ray beams have also been harnessed to work with the crystals that one has and new physical approaches still emerge, for example for preparing

protein crystallites such as using ‘jet sprays’ or of femtosecond lasers for precisely and delicately cutting up crystal clusters into individual pieces. The literature on other protein and macromolecule crystal diffraction disorders is much smaller, and is currently in the tens of publications, and these include incommensurate superlattice effects. As the throughput of macromolecular crystal structures continues to increase the likelihood of crystallographers facing twinning and/or other disorder cases also increases. Furthermore, as protein fold space coverage steadily improves the chance for harnessing an appropriate molecular replacement model also improves. Thus whilst MIR and MAD or SAD phasing can be severely disadvantaged by twinning, working as they do with small structure factor amplitude differences for phasing, the advantage of molecular replacement is that a more robust starting situation holds for structure solution with the X-ray data from a new, but twinned, crystal using an existing, precise, structure model.

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Notes on contributor

John R Helliwell is Professor of Structural Chemistry at the University of Manchester (since 1989). He has longstanding research links with Daresbury Laboratory (since 1976) and is currently a scientific adviser to the STFC CLIK's Knowledge Transfer to Industry. He was awarded a BA and a DSc in Physics from the University of York (in 1974 and in 1996 respectively), a DPhil from the University of Oxford and is a Fellow of the Institute of Physics (since 1986), of The Royal Society of Chemistry (since 1995) and of the Institute of Biology (since 1998). He is an Honorary Member of the National Institute of Chemistry, Ljubljana, Slovenia (since 1997). His research interests are in synchrotron and neutron crystallography instrumentation and methods developments based at eg the SRS, the ESRF and the Institut Laue Langevin. He has contributed to the worldwide effort to develop X-ray anomalous scattering and the so-called ‘modern X-ray and neutron Laue’ methods, which he has applied to proteins such as several enzymes, lectins and carotenoid binding proteins. He is active in serving the wider crystallographic community eg through the IUCr, the European

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Note

1. Of course for neutron protein crystallography involving a twinned crystal, Equation 1 and the diffraction statistics equations, quoted later, would apply just as they do with X-ray data. It is assumed here that a twinned crystal form for a given protein would not be studied with neutrons; this may be a false assumption and tests for the occurrence of twinned diffraction data can of course be routinely applied as a precautionary measure.

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