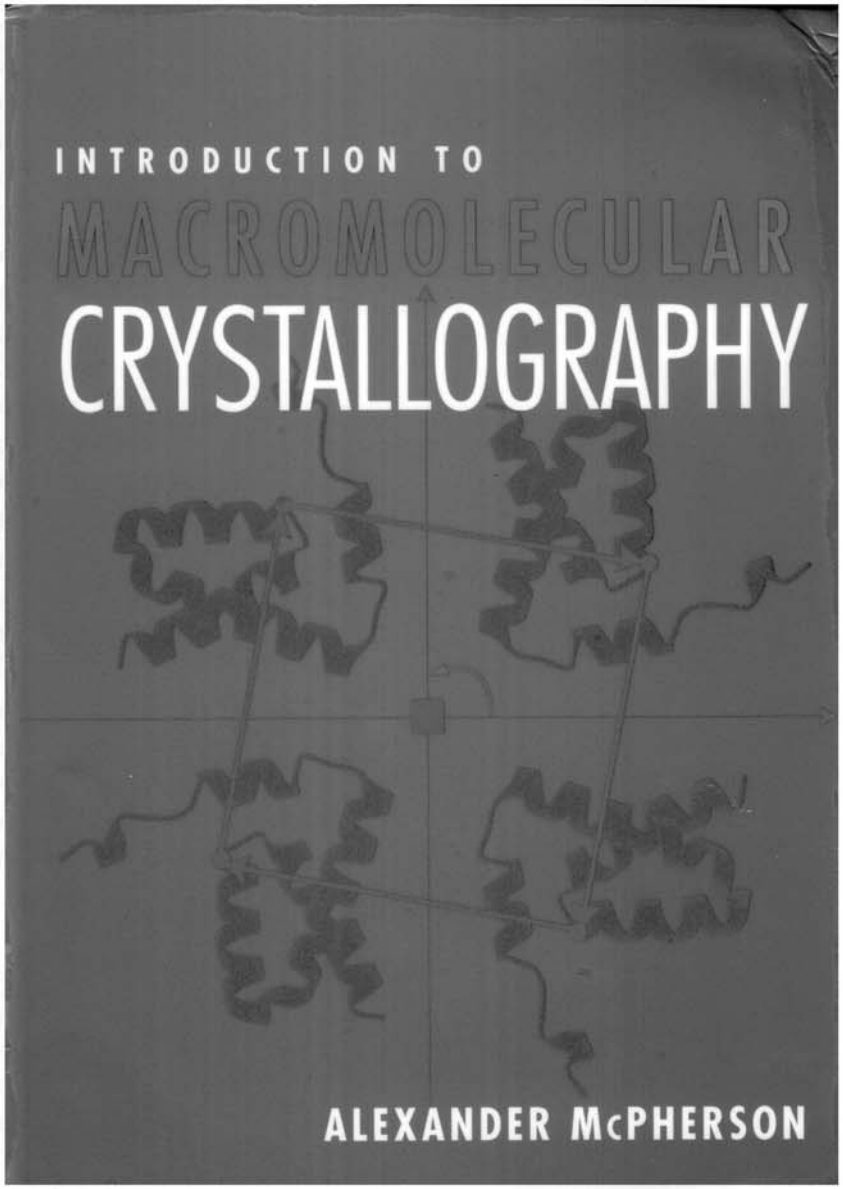


CHAPTER 5

EXAMINATION OF DIFFRACTION PATTERNS

EWALD'S SPHERE AND BRAGG'S LAW

When you have mounted a protein crystal, or any crystal, for data collection, unless you have made an exhaustive survey of the morphology of the crystal using special techniques and instruments, including polarized light, you may know with certainty very little about the orientation of planes within the crystal. Even if you have made such an optical investigation, you probably will still have only limited information, particularly for macromolecular crystals because they have such weak optical properties. Besides, protein crystallographers are seldom, if ever, trained to do such things anymore, and virtually none of them any longer know how (if you would like to be different, see the book by Wood, 1977). Let us assume then, that, in general, when you enter into data collection from a crystal, you don't know the orientation of any of the families of planes, how to bring them into diffraction position, that is, have them satisfy Bragg's law, or how to manipulate the crystal to get from one set of planes to another.

Given these unknowns, it might appear that X-ray data collection would be a very difficult process indeed. It is not, in fact. X-ray crystallographers only rarely think about planes in the crystal, or their orientation. They use instead the diffraction pattern to guide them when they orient and manipulate a crystal in the X-ray beam. Recall that the net, or lattice, on which the X-ray diffraction reflections fall is the reciprocal lattice, and that every reciprocal lattice point, or diffraction intensity, arises from a specific family of planes having unique Miller indices **h k l**.

Thus, if we expose a crystal to a collimated beam of X rays, we will observe on our film (or area detector, or image plate) a set of reflections corresponding to some families of planes, which, by chance, happen to be in diffracting position.

An example is shown in Figure 5.1. If we reorient the crystal in the beam through some rotation, other planes are brought into diffracting position and we will observe their diffraction intensities, their reciprocal lattice points, and the distribution of those reciprocal lattice points with respect to any experimental, laboratory coordinate system. We cannot discern the families of planes in the crystal, we can't see or detect them (they're imaginary anyway), but we can readily observe their reciprocal lattice vectors, or points. Remember that the orientation of the reciprocal lattice is locked to that of the real lattice, hence to the families of planes. When the crystal lattice rotates, its reciprocal lattice rotates accordingly, and so does its image on our film or detector. If we continuously reorient the crystal in a systematic way, then we can observe the appearance of entire planes of diffraction intensities as in Figures 5.2 and 5.3.

Remember further that each reciprocal lattice point represents a vector, which is normal to the particular family of planes **h k l** (and of length $1/d_{h\,k\,l}$) drawn from the origin of reciprocal space. If we can identify the position in diffraction space of a reciprocal lattice point, then, because of the defined relationship to its family of planes, the reciprocal lattice point tells us the orientation of that family. In practice, we usually ignore families of planes during data collection and use the reciprocal lattice to orient, impart motion to, and record the three-dimensional diffraction pattern from a crystal. Note, also, that if we identify the positions of only three reciprocal lattice points, that is, we can assign **h k l** indices to three reflections in diffraction space, then we have defined exactly the orientation of both the reciprocal lattice and the real space crystal lattice.

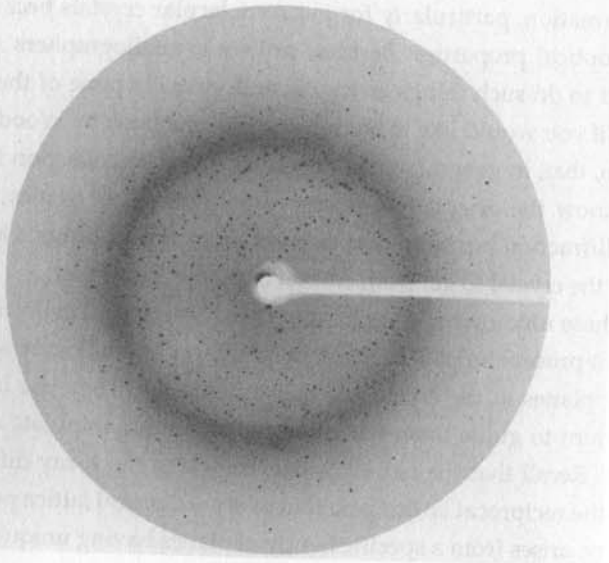


FIGURE 5.1 If a completely stationary crystal in a random orientation is subjected to a collimated beam of X rays, some families of planes will, by chance, be in diffracting position, that is, they will satisfy Bragg's law. Those families of planes will, therefore, produce diffraction intensities. A crystal of cytochrome P450 in an arbitrary orientation gives rise to the reflections seen here.

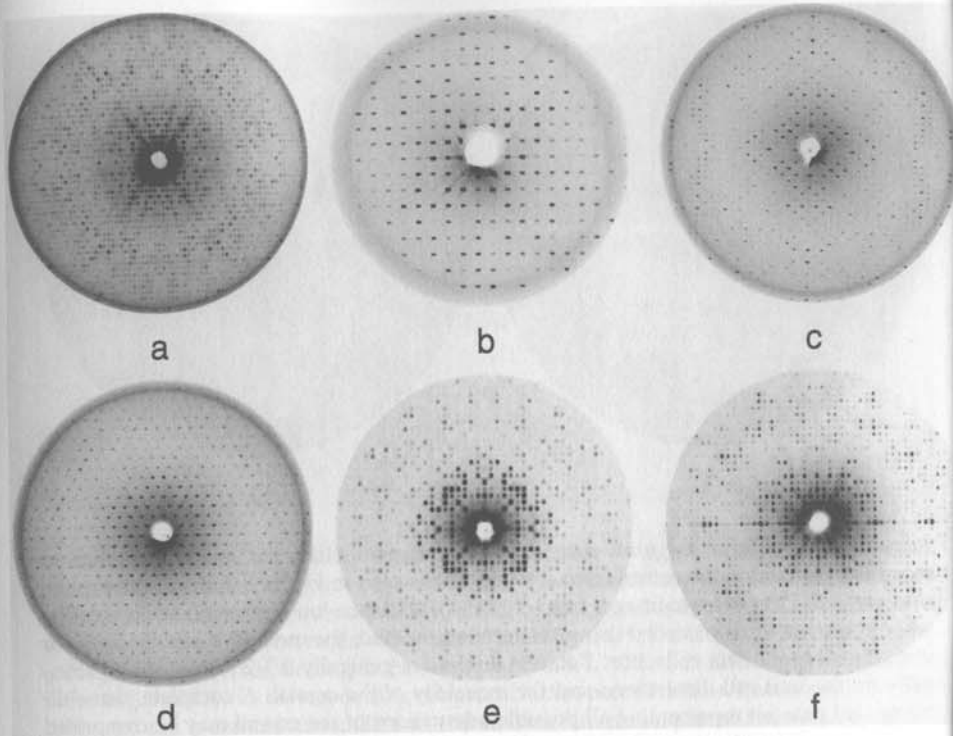


FIGURE 5.2 Diffraction photographs from a variety of protein crystals exhibiting a diversity of reciprocal lattice symmetries. With two images from a crystal, usually orthogonal to one another, the entire symmetry of the three-dimensional lattice can generally be deduced. The protein crystals and the symmetry of the diffraction patterns are *(a)* $P2_12_12_1$ porcine α-amylase, the symmetry is **mm** (mirror-mirror); *(b)* triclinic (**P**1) glycerol-3PO_4-dehydrogenase, the symmetry is $\bar{1}$; *(c)* centered orthorhombic (**C**222) fructose 1,6-PO_4 dehydrogenase, the symmetry is **mm**; *(d)* cubic crystal of phaseolin viewed along body diagonal, symmetry is 6 **mm**; *(e)* tetragonal **I**422 lactate dehydrogenase, the symmetry is 4 **mm**; *(f)* the same tetragonal crystal of lactate dehydrogenase rotated by 90°and viewed along a twofold axis, symmetry is **mm**.

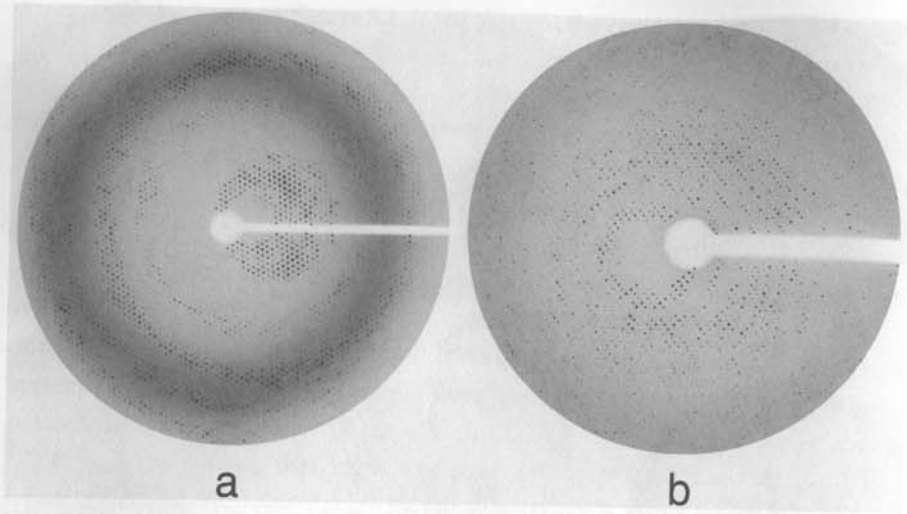


FIGURE 5.3 There are numerous experimental geometries for and approaches to recording diffraction intensities from crystals. Those seen in Figure 5.2 utilize the precession method. Here are two images from crystals of *(a)* Bence-Jones protein and *(b)* satellite tobacco mosaic virus recorded using the rotation method, the method currently used for virtually all rapid data collection. Rotation angles are generally 0.5–2°, depending principally on the unit cell dimensions and the mosaicity of the crystal. A complete, three-dimensional data set representing all possible orientations of the crystal may be comprised of up to 100 diffraction images or more, depending primarily on the symmetry of the particular crystal and the unit cell dimensions.

You may now be convinced that the diffraction pattern and its underlying reciprocal net reveal the dispositions of families of planes, but it is probably not at all clear how this relationship can be used in the laboratory. Exactly how are the reciprocal lattice vectors oriented with respect to the physical crystal? How do we get from one family to another? How do we bring a particular family of planes into diffracting position so that we can observe its reflection? To assist us in this, Ewald (1921) developed the construction shown in Figure 5.4.[1] This diagram illustrates a family of Bragg planes, its corresponding reciprocal lattice, the recording device (film), the X-ray beam, and an imaginary globe called the sphere of reflection (now more commonly called Ewald's sphere). The object of this construction is to illustrate the relationship between the components of the diffraction experiment, and to serve as a guide to the crystallographer as to how he may

[1]Actually, he had no intention of helping us, because he invented his construction before the first X-ray diffraction data was ever collected. He must have invented it for other uses, or he must have been a man of almost supernatural vision.

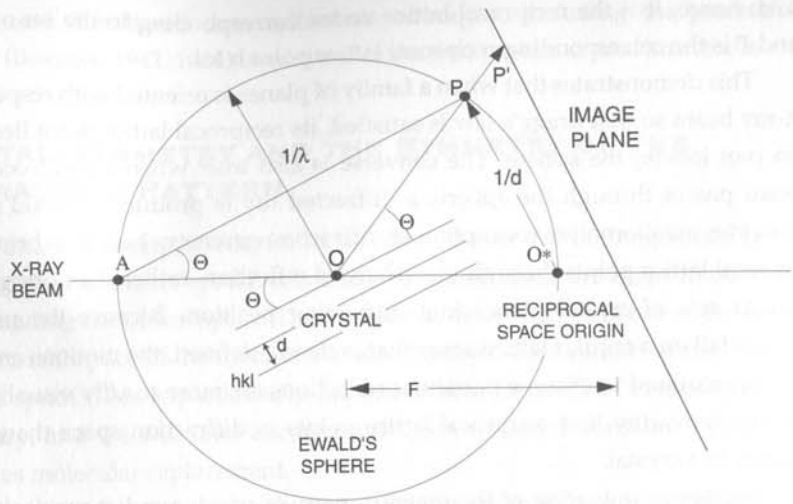


FIGURE 5.4 Ewald's sphere, a construction relating Bragg's law as it applies in real space with the reciprocal space requirement for constructive interference, and the expected location of diffraction intensities. The origin of the crystal is at point **O**, the center of a sphere of radius $1/\lambda$. The origin of reciprocal space is at **O***. The chord $\overline{\mathbf{O^*P}}$ is the reciprocal lattice vector corresponding to the set of planes **h k l**. When a family of planes **h k l** is in reflecting position, its corresponding reciprocal lattice point **P** lies on Ewald's sphere. The diffracted ray is emitted along **OP** and will strike a film plane, placed a distance **F** behind the crystal, at the point **P'**.

systematically alter the orientation of the crystal to record desired portions of the diffraction pattern.

In Figure 5.4, the **h k l** plane passing through **O** is a member of a family of planes with periodic spacing **d**, which makes an angle θ with the X-ray beam. A sphere (Ewald's sphere) is constructed of radius $1/\lambda$ centered at **O**. The origin **O** is chosen as the center of the crystal, and the point **O*** may be arbitrarily assigned as the origin of reciprocal space. Remember that although the reciprocal lattice is locked in terms of orientation to the real crystal, it bears no positional relationship, hence we can put the origin of reciprocal space anywhere we like. Ewald liked **O***. The chord $\overline{\mathbf{AP}}$ is drawn parallel with the **h k l** planes forming the triangle **APO***. Because **APO*** is a triangle within a hemisphere having one side a diameter (see Euclid, 300 B.C.), it must be a right triangle and $\overline{\mathbf{O^*P}}$ must be perpendicular to $\overline{\mathbf{AP}}$ and, therefore, to the **h k l** planes. Bragg's law states that a set of planes is in reflecting position only when $n\lambda = 2d \sin\theta$. Therefore, when the **h k l** family of planes is in diffracting position, $\sin\theta = \lambda/2d = (\overline{\mathbf{O^*P}})\,\lambda/2$, and therefore the length of $\overline{\mathbf{O^*P}} = 1/d$. Now $\overline{\mathbf{O^*P}}$ is normal to the **h k l** planes and has length

$1/\mathbf{d}$; hence, it is the reciprocal lattice vector corresponding to the set of planes, and **P** is the corresponding reciprocal lattice point **h k l.**

This demonstrates that when a family of planes is oriented with respect to the X-ray beam so that Bragg's law is satisfied, its reciprocal lattice point lies exactly on (not inside) the sphere. The converse is also true: when a reciprocal lattice point passes through the sphere, a diffracted ray is produced. Ewald's sphere provides an alternative conception of diffraction geometry based on bringing reciprocal lattice points through the sphere of reflection, rather than bringing particular sets of crystal planes into diffracting position. Because the reciprocal points fall on a regular lattice array that is clearly defined, the motions and orientations required to observe particular reflections are more readily visualized. We can more readily find reciprocal lattice points in diffraction space than we can planes in a crystal.

Further examination of the diagram permits one to predict precisely where the diffracted ray corresponding to a particular reciprocal lattice point, or family of planes, will appear. The diffracted ray may be thought of as arising from the origin of the crystal at **O.** It makes an angle of $2\,\theta$ with the X-ray beam and intersects the sphere at the position of the reciprocal lattice point **P.** If a film is placed at some distance **F** behind the sphere so that it is parallel with $\overline{\mathbf{O*P}}$, the diffracted ray will intercept the film at **P′**. The point **P′** on the film is the projection of the reciprocal lattice point **P.** A film containing a distribution of reflections is always some kind of projection of a portion of the reciprocal lattice onto a plane. It is common, though not strictly correct, to refer to a reflection on a film as a reciprocal lattice point.

Whereas the absolute distance between points in reciprocal space is a function of λ and the reciprocal lattice parameters $\mathbf{a^*}$, $\mathbf{b^*}$, $\mathbf{c^*}$, α^*, β^*, and γ^*, the absolute distances between maxima observed on the film will be expanded in proportion to **F,** which acts as a constant magnification factor.

The axes of the reciprocal lattice, remember, maintain a fixed orientation with respect to the real axes of the crystal by definition, regardless of the crystal's orientation. That is, if the crystal is rotated, the reciprocal lattice is rotated as well. If the crystal is continuously reoriented in a specific manner about its center by some constant motion, all of the points on a single reciprocal lattice plane, or region of reciprocal space, can be made to systematically pass through the sphere of reflection. If the film is maintained constantly parallel with a reciprocal lattice plane by mechanical linkage to the crystal, a magnified but otherwise undistorted replica of the reciprocal lattice plane will be recorded on the film. This principle, proposed by de Jong and Bouman (1938), was the basis for some of the more

widely used film based data collection techniques, including the precession method (Buerger, 1942, 1944) used to solve the first protein crystal structures.

CRYSTAL SYMMETRY AND THE SYMMETRY OF THE DIFFRACTION PATTERN

An important property of Fourier transforms that we did not emphasize in the previous chapter is that spatial relationships in one space are maintained in the corresponding transform space. That is, specific relationships between the orientations in real space of the members of a set of objects are carried across into reciprocal space. This is particularly important here in terms of crystallographic symmetry, and we will later encounter it again when we consider the process known as molecular replacement.

The consequence of this property of the Fourier transform is that symmetry elements, that is, space group symmetry, that characterize the arrangement of asymmetric units in the crystal, also applies to the distribution of reflections in reciprocal space. We mean by this not only that the locations of reflections in the diffraction pattern (the reciprocal lattice) mirror the unit cell symmetry, but that the distribution of the intensities also reflects the symmetry of objects in the unit cell. In a sense, then, the symmetry of the diffraction pattern represents the symmetry of the asymmetric objects in the crystal. Recall, also, that spacings between reflections, the diffraction pattern, tell us the kind of unit cell we have and the unit cell dimensions (by their reciprocals). Thus, the distribution of reflections and their intensities in diffraction space tell us everything we need to know about the crystal.

What has been said here is true, but it ignores one other fundamental property of the Fourier transform, one that complicates matters a bit, but not hopelessly so. The property is its failure to carry translational relationships from one space to another, in particular, from real space into reciprocal space. This means that the transform does not discriminate between asymmetric units based on the distances between them.

The immediate relevance of this is that a set of asymmetric units related by a screw axis symmetry operator (which has translational components) in real space is transformed into diffraction space as though it were related simply by a pure rotation axis. The translational components are lost. If our crystal has a 6_1 axis, we will see sixfold symmetry in the diffraction pattern. If we have $2_12_12_1$ symmetry in real space, the diffraction pattern will exhibit 2 2 2 symmetry.

"Now you tell us!" you might be thinking, but do not despair. It is true that

the translational components of symmetry elements are lost during transformation into reciprocal space and simply appear as the corresponding rotational symmetry element—that's the bad news. The good news is that the translational components of any symmetry element do leave cryptic evidence in the diffraction pattern of their presence in the crystal. Furthermore, we know precisely the nature and form of that evidence, it is distinct and clear to the eye of the crystallographer, and we know exactly where to look for it.

The evidence for the existence of screw axis symmetry is manifested in certain subclasses of reflections that are "systematically absent." These systematic absences, we will see, fall along axial lines (**h** 0 0, 0 **k** 0, 0 0 **l**) in reciprocal space and clearly signal not only whether an axis in real space is a screw axis or a pure rotation axis, but what kind of a screw axis it is, for example, 4_1 or 4_2, 6 or 6_3. Thus, the inherent symmetry of the diffraction pattern, plus the systematic absences, allow us to unambiguously identify (except for just a few specific cases) the space group of any crystal.

SYMMETRY AND SYSTEMATIC ABSENCES

Let us look at this question of diffraction pattern symmetry in a slightly different, but no less correct way in the hope that we may gain some insight into the sources of those "systematic absences." Reflections that appear along any reciprocal lattice line which passes through the origin of reciprocal space, are identical to those that would appear if the electron density of the crystal were projected onto the corresponding line in real space and then transformed. For example, if all of the electron density were projected onto the **a** axis in real space, and the one-dimensional crystal's diffraction pattern subsequently generated, then it would be identical to the **h** 0 0 line of reflections (along **a***) in the three-dimensional diffraction pattern. This is true of all corresponding lines in real and reciprocal space, which pass through the origin. Transform of the **h** 0 0 line of reflections, the $\bar{F}_{h\,0\,0}$ alone, would in turn yield the distribution of electron density of the unit cell projected onto the real **a** axis of the crystal.

Similarly, if all of the electron density in the unit cell were projected onto a single plane, let us say the $\bar{a} \times \bar{b}$ plane in real space, then the diffraction pattern of this two-dimensional crystal would be the **h k** 0 zone of reflections in the three-dimensional diffraction pattern. As with line projections, this theorem holds true for any and every plane passing through the origin.

This idea has some useful consequences in terms of interpreting diffraction

patterns. For example, consider the case of a twofold axis in real space, perpendicular to the $\bar{a} \times \bar{c}$ plane, as we would have in a monoclinic crystal of space group P2 or C2. The projection of all of the electron density in the unit cell, having this dyad symmetry, onto the $\bar{a} \times \bar{c}$ plane, would of course also have twofold symmetry. Because this projection has dyad symmetry, then the corresponding diffraction pattern, which is the **h** 0 **l** plane of reflections in reciprocal space, also has twofold symmetry, that is, reflection $|\bar{F}_{h\,0\,l}| \equiv |\bar{F}_{-h\,0\,-l}|$.

If, instead of a monoclinic crystal, we considered a tetragonal crystal having a fourfold axis along **c** and therefore perpendicular to the **a** × **b** plane, then the plane projection would also have fourfold symmetry. So, too, would the corresponding reflections on the **h k** 0 zone of reciprocal space, that is,

$$|\bar{F}_{h\,k\,0}| = |\bar{F}_{-h\,k\,l}| = |\bar{F}_{k\,h\,l}| = |\bar{F}_{-k\,h\,l}|$$

Similar kinds of relationships would arise among equivalent reflections for threefold or sixfold axes in the crystal as well.

This tells us that symmetry elements in real space, the crystal, may be identified by searching the appropriate zones, or planes, of reciprocal space for symmetrical patterns of diffraction spots. If we see fourfold or threefold or sixfold distributions of reflections in the diffraction patterns, then they must imply corresponding symmetry relationships in the crystal.

Consider now a 2_1 screw axis along $\bar{b}$ in real space, perpendicular to the $\bar{a} \times \bar{c}$ plane. When the contents of the unit cell are projected onto this plane, the translational component of the 2_1 operator is lost, and the projection is identical to that which would have been obtained simply from a twofold axis. Thus, we might expect the corresponding plane of reflections in reciprocal space, the **h** 0 **l** zone of reflections, to exhibit a twofold symmetrical distribution, whether a twofold axis or a twofold screw axis were present, and indeed that is the case. The same logic would apply for any rotation/screw axis choice. Because their projections in real space are identical, the Fourier transforms of those projections must be as well, and we cannot discriminate them. Thus, the presence of rotational symmetry on a plane of reflections in the diffraction pattern can tell us that an operator is present in the crystal, but not whether it is a pure rotational operator or a screw operator, for example, we cannot tell the difference between a 2 and 2_1 axis, or a 4_1 or 4_2 or 4 fold axis, etc.

In the case of symmetry operators containing translational components, that is, screw operators or centering operations (and glide planes in crystals of conventional molecules), as discussed above, there is a saving grace. The evidence of

a translation operator or component is not, in fact, lost upon transformation into reciprocal space. If a symmetry axis is present along a certain direction in real space, say along **c**, we may then consider the projection of the entire content of the unit cell onto the **c** axis as described above. If the axis is a pure rotational operator, then the projection on **c** will have an arbitrary, nonrepetitive distribution along its entire length from 0 to 1. If the symmetry axis contains a translational component, however, this will not be true. There will be a repeat within the projected density between 0 and 1. It will be a double repeat for a 2_1 axis, triple repeat for a 3_1 axis, quadruple repeat for a 4_1 axis, and so on.

The subperiodicity of the axial projection within one unit cell in real space of course has an effect in diffraction space. Recall the reciprocal relationship between the two. If a distance is halved in real space, it is doubled in reciprocal space; if a periodicity is quartered in real space, it is multiplied fourfold in reciprocal space, etc. Thus, along the corresponding reciprocal space axis, **c***, or the 0 0 **l** line of diffraction spots, reflection spacing must correspond to the increased periodicity, or smaller repeat distance in real space. In reciprocal space, reflections will appear less frequently, at increased intervals. For a 2_1 axis, the appearance of reflections will have double the normal periodic interval. For a 2_1 axis, reflections will occur along the 0 0 **l** line of reciprocal lattice points only for **l** = 2, 4, 6, 8 . . . , that is, only for reflections where **l** is an even integer. For a 4_1 screw axis, the normal interval will be quadrupled, and only reflections **l** = 4, 8, 12, 16 . . . will be nonzero, that is, the only allowed reflections will be 0 0 **l** = 4**n**.

Another way of looking at this is that, in certain projections, screw symmetry operators produce an apparent but precise subperiodicity, or subcell, within the actual unit cell. Internal destructive interference of the waves produced by the subperiodic structures with one another causes certain classes of reflections to always sum to zero. In the case of the 2_1 axis above, the class of reflections becoming zero were those for which **l** was odd. These classes of missing reflections (systematic absences), are used in inspecting the diffraction pattern of a crystal to discriminate between rotational and screw symmetry operators, between twofold and 2_1, or threefold and 3_1 axes in real space. Figure 5.5 illustrates the appearance of screw axis produced systematic absences for some real cases.

We arbitrarily chose in the example above to project the unit cell contents onto the **c** axis, but we might just as well have chosen the 2_1 axis to lie along **a** or **b**—the outcome would have been the same. Thus, if a space group contains multiple 2_1 axes, such as $P2_1\ 2_1\ 2_1$, with screw operators along all three directions, then we would expect to find systematic absences along each of the three reciprocal lattice rows **h** 0 0, 0 **k** 0, and 0 0 **l**. The presence of one screw axis is indepen-

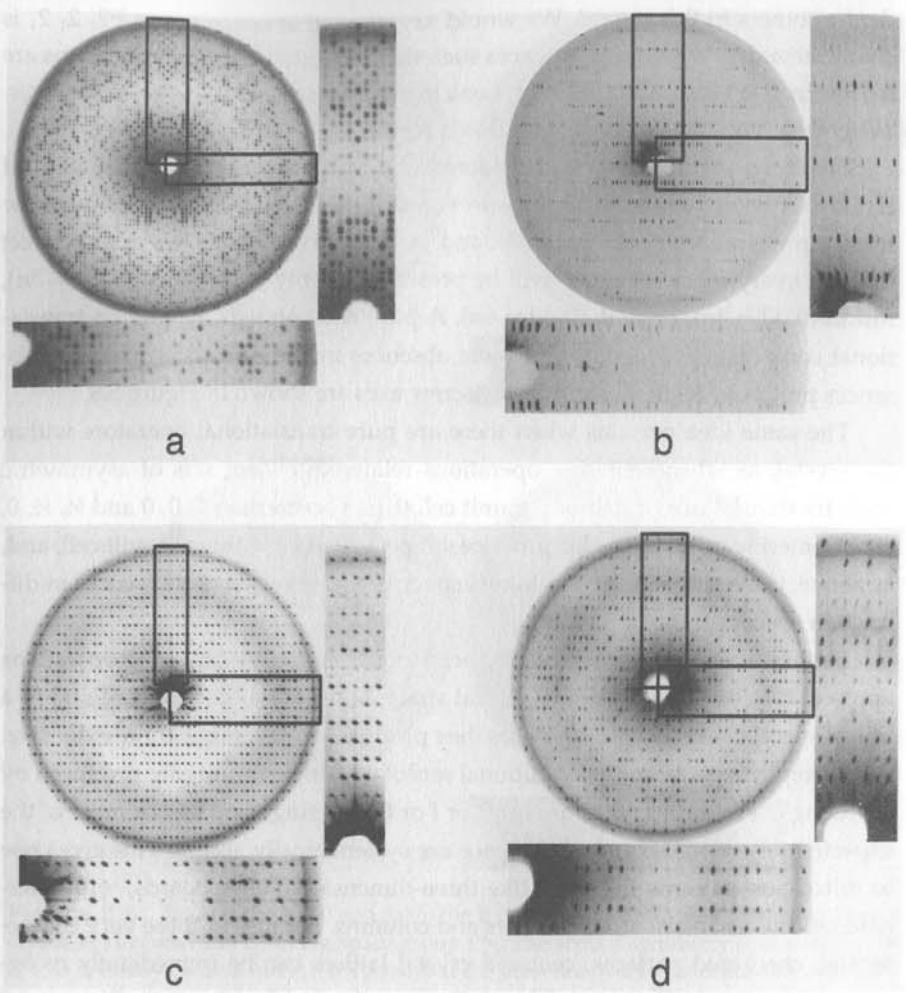


FIGURE 5.5 Shown here are planes of diffraction intensities for four different orthorhombic crystals characterized by twofold and 2_1 screw axes. Associated with each are blowups of the relevant reciprocal lattice axes where the presence of systematic absences, that is, reflections with odd indices absent, might be expected to occur. In *(a)* is the **h** 0 **l** plane from $P2_12_12_1$ α-amylase, which has 2_1 systematic absences along both the **h** 0 0 line (vertical axis) and 0 0 **l** line (horizontal axis). In *(b)* is the 0 **k** **l** plane from $P2_122_1$ yeast phenylalanine tRNA. Absences consistent with a 2_1 axis are apparent along the horizontal **0 0 l** line, but not along the vertical 0 **k** 0 line, which corresponds to a twofold axis. In *(c)* is $P2_122_1$ RNase B, the 0 **k** **l** zone, and in *(d)* the **h** 0 **l** zone from a crystal of $P2_12_12_1$ RNase A plus d(pA)4 complex. In *(c)*, alternate reflections (all having odd indices) are absent only along the 0 0 **l**, horizontal line, whereas in *(d)* they are absent along both axial lines, the **h** 0 0 and the 0 0 **l**.

dent of others in this regard. We would say, then, that space group $P2_1\ 2_1\ 2_1$ is characterized by systematic absences such that the only permitted reflections are **h** 0 0 = 2**n**, 0 **k** 0 = 2**n**, and 0 0 **l** = 2**n**. Look in the *International Tables for X-Ray Crystallography*, Vol. 1, and see whether this is correct.

Similarly, we might have considered a 3_1, a 4_3, a 6_1, or any other kind of screw axis as well as the 2_1. Note, however, that the translational components for these screw axes are not ½, but ⅓, ¼, and ⅙, respectively. Thus, we might expect that not every other reflection will be present, but only every third (0 0 **l** = 3**n**), fourth (0 0 **l** = 4**n**), or sixth (0 0 **l** = 6**n**). A pure rotation axis, having no translational component, gives no systematic absences in the diffraction pattern. Absences produced by higher symmetry screw axes are shown in Figure 5.6.

The same idea pertains when there are pure translational operators within the crystal, as when centering operations relate equivalent sets of asymmetric units by translations of half of the unit cell (e.g., **C** centering: 0, 0, 0 and ½, ½, 0, etc.) Centering operations also produce subperiodicities within the unit cell, and, as above, these subperiodicities in real space produce systematic absences in diffraction space.

Because the subperiodicities produced in projections by centering operations apply to two or three directions in real space, and are not simply confined to a line in space, the systematic absences they produce are also much more extensive. As we might expect, if entire additional sets of asymmetric units are produced by centering operations, as they are for **C** or **I** or **F** centering, then half or more of the expected reflections in reciprocal space are systematically absent. This gives rise to diffraction patterns that look like three-dimensional chessboards, with alternate reflections absent along all rows and columns. Because of these very characteristic, checkered patterns, centered crystal lattices can be immediately recognized from their diffraction patterns. Some examples are shown in Figures 5.7 and 5.8. As for the screw symmetry operators, systematic absences for all kinds of centering operations and for all space groups are contained in the *International Tables for X-Ray Crystallography*, Vol. 1.

Analysis of Diffraction Patterns

Modern computer systems and their crystallographic programs can analyze X-ray diffraction data collected on two-dimensional electronic detectors, such as image plates or charge-coupled device (CCD) arrays, and immediately provide plausible alternatives for the likely crystallographic unit cell. Generally, however, it remains to the crystallographer to refine the choices and select among them in

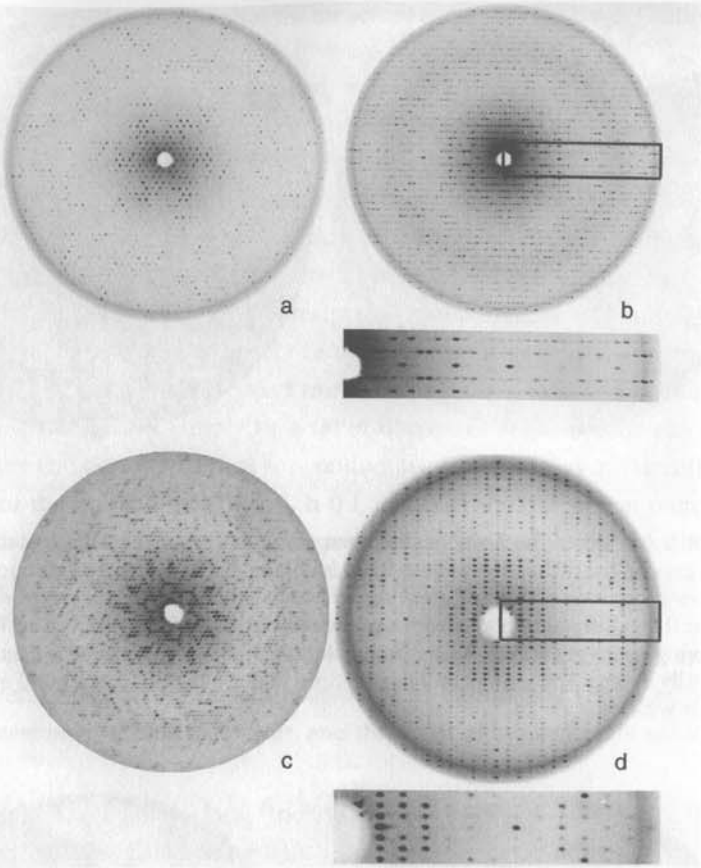


FIGURE 5.6 In *(a)* is the **h k** 0 and in *(b)* the **h** 0 **l** zones of diffraction intensities from a crystal of concanavalin B having space group $P6_1$. The sixfold symmetry produced by the 6_1 axis is clearly evident in the **h k** 0 zone, but the presence of only reflections for which **l** = 6**n** along the horizontal 0 0 **l** axis shows the order of the screw axis. In *(c)* and *(d)* are the **h k** 0 and **h** 0 **l** zones, respectively, of reflections from a $P6_3$ crystal of canavalin. Again, the sixfold symmetry is apparent in the **h k** 0, but only alternate even reflections (0 0 **l** = 2, 4, 6, 8 . . . , etc.) are present along the horizontal 0 0 **l** line. Note the difference in the systematic absence pattern for $P6_1$, concanavalin B and $P6_3$ canavalin crystals that allows discrimination of the two space groups.

order to arrive at the correct unit cell, unit cell dimensions, space group of the crystal, and other properties essential for the full three-dimensional structure determination. The machine usually cannot make all decisions independently, and, indeed, should not be allowed to do so. It must be remembered that if the unit cell dimensions are very much in error, or wrong, or the space group symmetry is

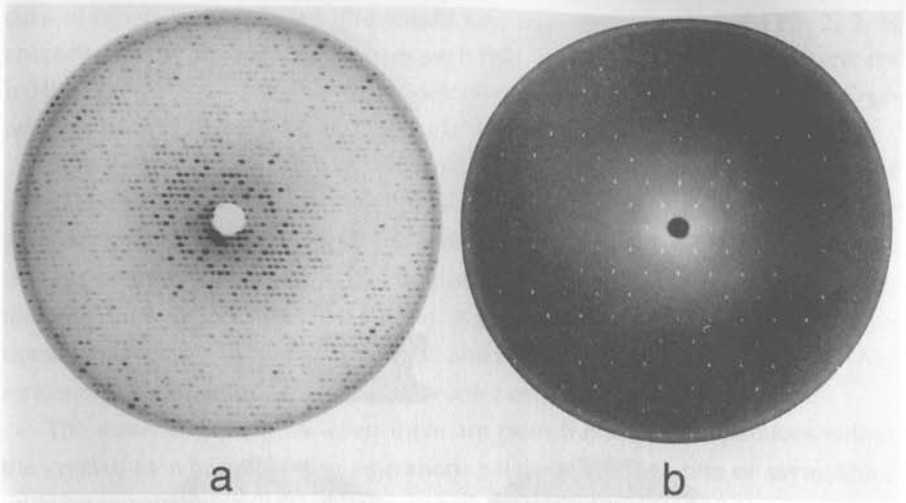


FIGURE 5.7 When the X-ray beam is perpendicular to a twofold axis of an F422 horse ferritin crystal, the diffraction pattern in *(a)* is obtained. The systematic absences due to the **F** centering produce an immediately recognizable checkerboard pattern of reflections. In *(b)* is the 0 **k l** diffraction plane from a monoclinic crystal of gene 5 DNA-unwinding protein having space group **C**2. Along both columns and rows, alternate reflections are systematically absent. The indices of the reflections that are present correspond exclusively to those for which $\mathbf{k} + \mathbf{l} = 2\mathbf{n}$.

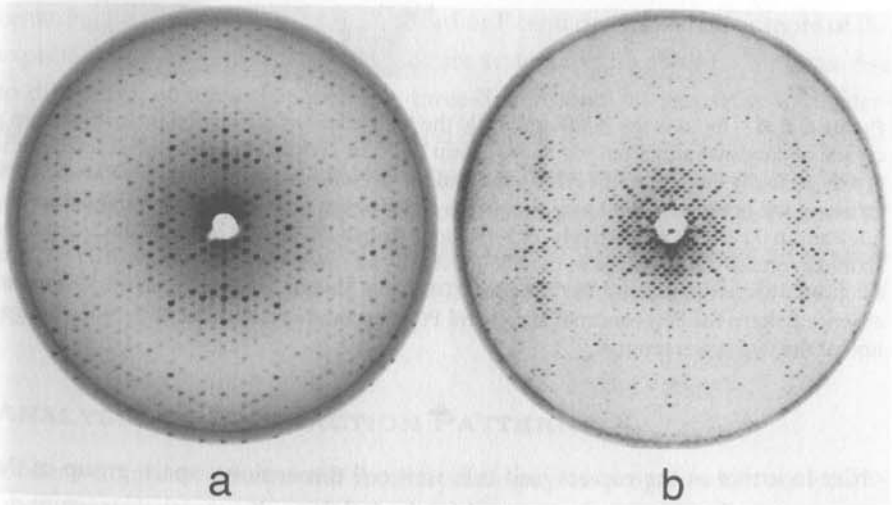


FIGURE 5.8 In *(a)* are the **h** 0 **l** diffraction intensities from a crystal of fructose 1,6-PO4 dehydrogenase from rabbit liver having space group **I**222. In *(b)* is the **h k** 0 plane of reflections from $\mathbf{C}222_1$ canavalin. In both images, the checkerboard patterns produced by systematic absences resulting from the **I** and **C** centering operations are evident.

incorrect, then there is no way that the structure can ever be solved. Getting these things initially correct is imperative.

In spite of the convenience, speed, and general precision offered by data analysis programs, it is important for the crystallographer to know what is being sought, and what considerations are being made by the various computer algorithms. That is, what features of the diffraction pattern are being investigated, how, and for what purpose? To address these questions, let us assume that we have the necessary diffraction patterns in hand, or at least certain representative zones or planes or sectors of reciprocal space that contain sufficient data for accurate judgments, particularly of symmetry relationships. This may be a complete three-dimensional data set from which certain subsets can be selected and examined, or it may simply be a set of films, from a precession camera, for example (the classical instrument for conducting a preliminary X-ray diffraction analysis), of the major zones (**h k** 0, **h** 0 **l**, 0 **k l**) of the diffraction pattern. We can pose the problem: if no computer were available to analyze the diffraction pattern, how would we go about doing this ourselves, and what might we expect to learn?

We approach this systematically by asking a series of questions, and from their answers, either fixing certain properties, or eliminating others as impossible. The questions we seek to answer, and the order of inquisition is as follows:

1. What is the crystal class (triclinic, monoclinic, orthorhombic, trigonal, tetragonal, hexagonal or cubic)?
2. Is the crystal lattice primitive or centered? That is, is it primitive **P**, **C** face centered, body centered **I**, or face centered **F**?

From (1) and (2) we determine the Bravais lattice

3. What are the unit cell dimensions? **a**, **b**, and **c** and the unit cell angles α, β, and γ in angstroms and degrees?
4. What is the symmetry of the reciprocal lattice? That is, what are the symmetry operators that relate sets of identical intensities? The symmetry group that we observe for a crystal in reciprocal space, that is, the diffraction pattern symmetry is called the Laue symmetry, or Laue group.
5. What systematic absences are present?

From (1) through (5) we obtain the crystal class, unit cell dimensions, Bravais

lattice, and space group. All of these are essential crystal properties, but the diffraction pattern also contains other useful information:

1. What is the resolution of the diffraction pattern, that is, what is the family of Bragg planes in the crystal of smallest interplanar spacing that is represented by a measurable intensity in the diffraction pattern? Definition of "measurable intensity" may here become a contentious issue.
2. What is the mosaic spread, or angular spread, of the diffraction intensities? Basically, this means, "How large is the spot on the image," and it, along with the resolution limit, serve as measures of the order of the crystal.
3. How many molecules or chemical units are there in the crystallographic asymmetric unit? This is a question that is usually easily answerable, but in ambiguous cases must be viewed with great caution, as it significantly affects other phases of the analysis.

The first determination that must be made is the net, or lattice, upon which all of the reflections in the diffraction pattern fall, that is, the reciprocal lattice. That is, we must draw three axes through the origin of reciprocal space and choose the three maximum spacings so that lines drawn parallel to the axes and separated by the assigned spacings include every reflection in the diffraction pattern. These will become the **h**, **k**, and **l** axes in reciprocal space. Don't worry about getting them mixed up in the initial assignment, you can always switch their designations later. Generally, the choice is fairly evident for diffraction patterns of high symmetry, but can be challenging for low-symmetry triclinic and, to some extent, monoclinic crystals. Sometimes the conclusions drawn at this stage must be reevaluated later after consideration of symmetry properties, which in the end supercede all others. Consider the examples presented in Figures 5.9 and 5.10.

Note that once the axes have been assigned, and knowing that their intersection at the exact center of the diffraction pattern defines the origin of reciprocal space, then every reflection in the entire diffraction pattern has a unique set of coordinates. The coordinates are always integral numbers of spacings along the three reciprocal lattice axes, and the coordinates correspond to **h k l**. Thus, we can index the diffraction pattern and assign a name, a set of indices, to every spot. As we have seen, the reflection, or diffraction intensity found at any reciprocal lattice point **h k l** represents the diffracted X-rays from the corresponding **h k l** family of planes in the crystal.

What are the angles between the axes we have chosen, and what are the spac-

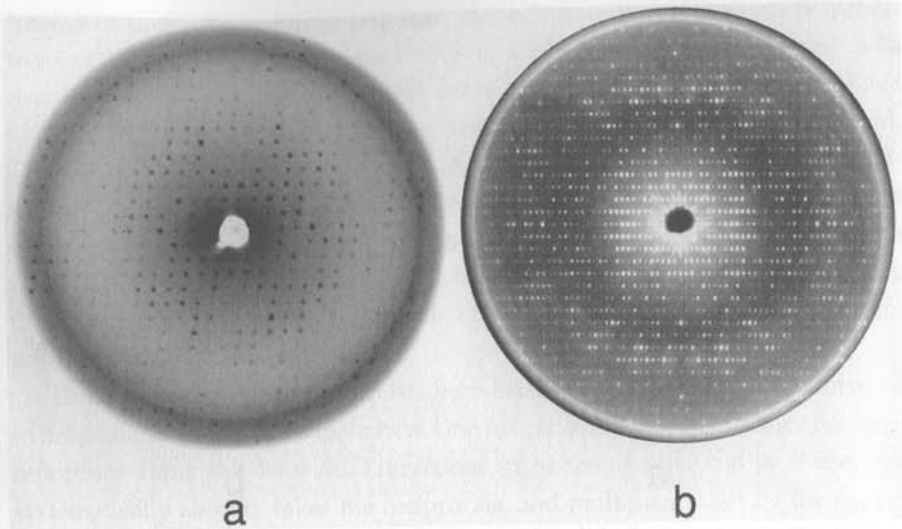


FIGURE 5.9 In the diffraction pattern from triclinic, **P**1 crystals of glycerol-3-PO_4 dehydrogenase there is no symmetry other than the center of symmetry ($\bar{1}$) produced by Friedel's law. The axes for reciprocal space may, therefore, be arbitrarily chosen. In general, the axes yielding the largest reciprocal unit cell are chosen, because this corresponds to the smallest crystallographic unit cell. In *(b)*, the photograph is from an orthorhombic crystal of RNase A+ $d(pA)_4$. The reflections fall on an orthogonal net whose axes are along the horizontal and vertical directions. More important, choice of these two axes as **a*** and **b*** preserves the inherent **mm** symmetry of the diffraction intensity distribution. Axes and unit cells are always chosen to express the highest possible symmetry of the diffraction pattern. It is more important to preserve the highest symmetry than to have the smallest possible crystallographic unit cell in terms of volume, and this may require adopting a centered unit cell.

ings between reflections along lattice rows and columns in millimeters? If the three axes are at right angles to one another, then $\alpha = \beta = \gamma = 90°$ and we must have an orthogonal system. In some cases, the best axes to choose for indexing the reflections may have one angle 120°, in which case we likely have a trigonal or hexagonal system. If axes are chosen so that two interaxial angles are 90°, but the third is not, then we must have a monoclinic system. If the axes cannot be chosen so that any angle is 90°, then the unit cell must be triclinic. If the spacings between reflections along perpendicular rows and columns are the same, then the cell may be tetragonal or cubic. If all three spacings are different but all angles are 90°, the system is probably orthorhombic.

Often one has several options for choosing the net, or reciprocal, lattice axes,

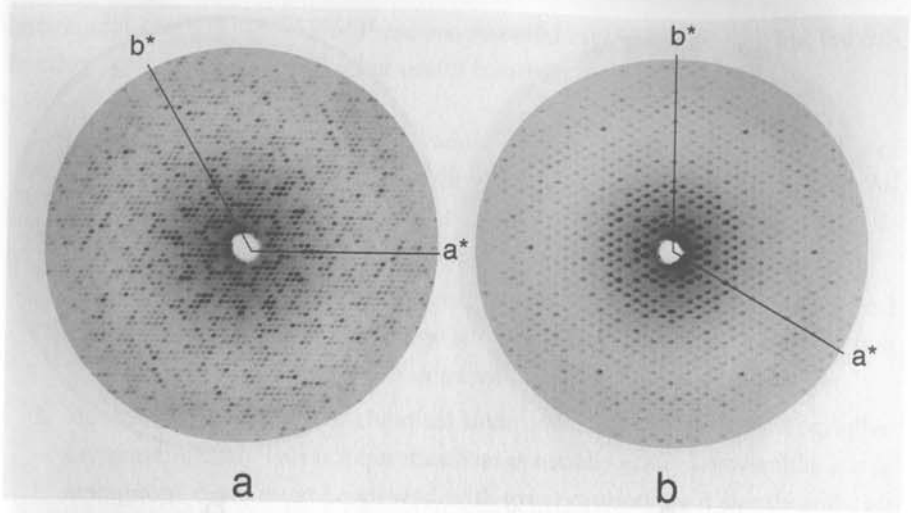


FIGURE 5.10 In *(a)* is the **h k** 0 diffraction plane of a $P6_3$ canavalin crystal having six-fold symmetry, and in *(b)* the view along the unique axis of a rhombohedral canavalin crystal that exhibits 6 **mm** symmetry. In neither case would one choose orthogonal axes on which to index the reflections. In *(a)*, the natural choice would be the **a*** and **b*** axes indicated. In *(b)*, two choices of hexagonal axes are reasonable, but those indicated are chosen as **a*** and **b*** because they correspond with the real unit cell of smallest volume.

but the rule is that an axial system is always chosen that preserves the highest symmetry of the diffraction pattern. That is, it is chosen to be consistent with the real unit cell of highest symmetry. Sometimes, before the diffraction symmetry is fully clear, incorrect axes may be chosen. The axial system, or reciprocal lattice net, can, however, always be reassigned and the **h k l** indices of the reflections re-indexed at a later time. The choice of axes determines the crystal class.

The next question is whether the reciprocal lattice arises from a primitive real lattice **(P)** or from a centered lattice, and if the latter, what kind of centering (**C, I,** or **F**)? This, in practice, is equivalent to asking whether the diffraction pattern has the appearance of a three-dimensional chessboard or not, like those in Figures 5.7 and 5.8. That is, are half or more of the reflections in the diffraction pattern systematically absent? If not, then the lattice is primitive and that is the end of that.

If it is a chessboard pattern, then some additional investigation may be required. Though usually necessary to record two zero levels (reciprocal lattice planes that pass through the origin of reciprocal space) of the diffraction pattern, sometimes an upper level (planes of reciprocal lattice points **h k l**, none of whose indices are always zero) is necessary as well. Remember, triclinic, trigonal, and

hexagonal unit cells cannot be centered. Monoclinic unit cells can only be primitive or **C** centered (centered on the **C** face, or $\bar{\mathbf{a}} \times \bar{\mathbf{b}}$ face), and tetragonal unit cells may be only primitive and **I** centered (body centered). Thus, only orthorhombic and cubic unit cells remain in question, and the latter can only be **I** or **F** centered. The reader is referred back to Figure 2.14, where these are illustrated. The specific variety of centering, if it is present, can ultimately be determined by the pattern of systematically absent reflections, the particular subsets of **h k l** indices with no measurable intensity. All of these are detailed in the *International Tables for X-Ray Crystallography*, Vol. 1, for every possible type of unit cell. From these considerations, the Bravais lattice is chosen.

Once the reciprocal lattice net has been established, then it is straightforward to determine the unit cell dimensions. One simply measures the distance between reflections along the three axial directions (or where they would be if they are systematically absent), takes the reciprocals, and multiplies them by the appropriate instrumental constants to get directly the unit cell dimensions. This is so because the distance between reflections in diffraction space are exactly related to distances in real space by the reciprocal relationships $|\mathbf{a}^*| = 1/|\mathbf{a}|$, $|\mathbf{b}^*| = 1/|\mathbf{b}|$, and $|\mathbf{c}^*| = 1/|\mathbf{c}|$ for orthogonal axes. For nonorthogonal crystal systems, the calculation is somewhat more complicated because it includes trigonometric terms specific to the crystal class, but even so they are relatively simple to calculate. Note the striking differences in the distances between reflections for crystals having large and small unit cells in Figures 5.11 and 5.12.

It must be remembered that the observed diffraction pattern on a film or image plate or CCD detector is a magnified image of the reciprocal lattice, and this magnification factor has to be taken into account. The magnification is usually just the distance between the crystal and the film. This is evident in the Ewald sphere construction in Figure 5.4. There is a simple formula that allows this to be done (Buerger, 1944) and it is:

$$\mathbf{d}(\text{real space})(\text{Å}) = \frac{\boldsymbol{\lambda}(\text{Å}) \times \mathbf{F}(\text{mm})}{\mathbf{d}_{\text{image}}(\text{mm})}$$

where $\boldsymbol{\lambda}$ (in angstroms) is the wavelength of X-rays used in obtaining the diffraction pattern, and **F** is the distance in millimeters between the crystal and the detector. Thus, by simply measuring the distance in millimeters on the detector between intensities along the three reciprocal lattice axes, $|\mathbf{a}^*|$, $|\mathbf{b}^*|$, and $|\mathbf{c}^*|$, and applying the above formula, $|\mathbf{a}|$, $|\mathbf{b}|$, and $|\mathbf{c}|$ are obtained directly in angstroms. If any angle between the reciprocal axes $\boldsymbol{\alpha}^*$, $\boldsymbol{\beta}^*$, or $\boldsymbol{\gamma}^*$ is not 90°, as for

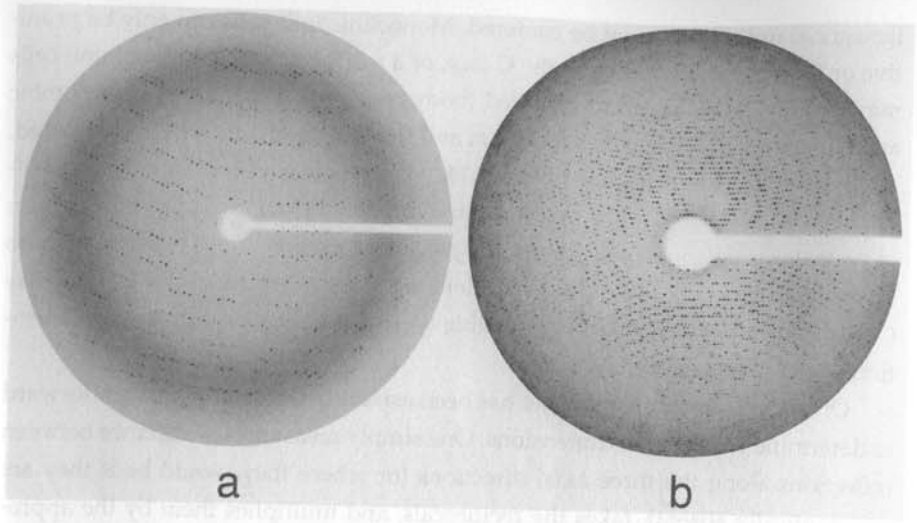


FIGURE 5.11 In *(a)* is a rotation method image of a protein crystal having moderate unit cell dimensions, a Bence-Jones protein crystal (space group $P3_121$, **a** = 153 Å, **b** = 153 Å, **c** = 94 Å. In *(b)* is a rotation method image from a crystal of brome mosaic virus (space group **R**3, **a** = 271 Å, **b** =271 Å, **c** = 646 Å. Note the relative spacings of the reflections along the reciprocal lattice lines for the crystal having small unit cell dimensions in contrast to the crystal having very large unit cell parameters.

example the β^* angle for a monoclinic lattice, then the real space angle β is simply the supplement, or 180° minus the reciprocal space angle.

The next question, with the objective of determining the space group of the crystal, is, what is the symmetry of the entire, three-dimensional diffraction pattern? This is the most demanding aspect of the analysis and deserves some care. It is greatly simplified for macromolecular crystallographers because there are only 65 permitted space groups rather than the full 230. Only those lacking inversion symmetry need be considered. In addition, detailed descriptions of all symmetry groups, their equivalent positions, associated systematic absences, and all other useful properties are contained and described, as in Figures 5.13 and 5.14, in the *International Tables for X-Ray Crystallography*, Vol. 1. The process of deducing the exact symmetry of the crystal from the symmetry of the diffraction pattern is complicated somewhat by the following:

1. Because of Friedel's law, diffraction patterns always contain a center of symmetry at the origin of reciprocal space. This means that any plane of reciprocal space that passes through the origin, in particular the **h** 0 **l**, 0 **k l**, or

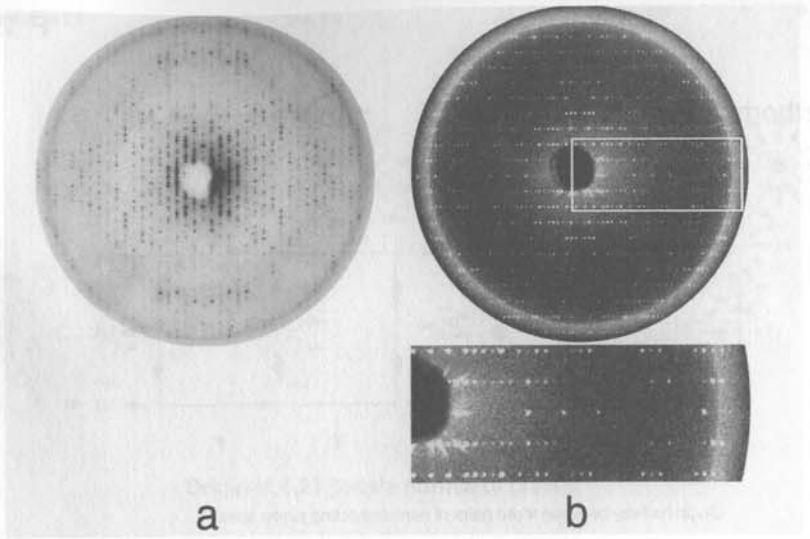


FIGURE 5.12 In the 0 **k l** diffraction image from a crystal of yeast phenylalanine tRNA ($P2_122_1$, **a** = 33 Å, **b** = 56 Å, **c** = 161 Å), note the very close spacing of reflections along the c^* direction (along the vertical) corresponding to the long **c** axis in the crystallographic unit cell, and the relatively wide spacing of reflections along the horizontal b^* direction, corresponding to the much shorter unit cell dimension. In *(b)* is the **h** 0 **l** zone of diffraction intensities from a tetragonal crystal of the complex between RNase B and $d(pA)_4$ ($P4_12_12$, **a** = 44.5 Å, **b** = 44.5 Å, **c** = 156.5 Å). Again, note the difference in the distances between reciprocal lattice points corresponding to the long real **c** axis (horizontal) and the relatively shorter **a** axis.

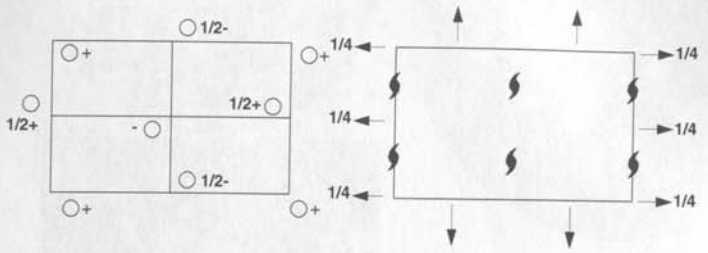
h k 0 zones, will display $\bar{1}$ (centric) symmetry. When this is integrated with the true symmetry of the crystal, it generally produces reciprocal lattice symmetry arrangements (called Laue groups) having higher symmetry than is really present in the crystal. One consequence of this is that a zero-level (a level of reciprocal space where one index is always zero) photograph of the diffraction pattern (and in some cases other zones) will always exhibit an apparent twofold axis perpendicular to that reciprocal lattice plane. This is so because projection along a twofold axis appears the same as projection of a center of symmetry-related arrangement. You cannot know, without other information, whether a twofold axis is really present or if it is simply a manifestation of Friedel's law. Additional photographs will be necessary. This knot can, however, be unraveled.

2. As noted already, symmetry elements containing translational components, such as screw axes, appear in reciprocal space as the pure rotational ele-

P $2_12_12_1$

D_2^4

Orthorhombic 222 P $2_12_12_1$ No. 19



Origin halfway between three pairs of nonintersecting screw axes

Number of positions, Wyckoff notation, and point symmetry			Coordinates of equivalent positions			Conditions limiting possible reflections
4	a	1	x,y,z;	1/2-x,y,1/2+z;	1/2+x,1/2-y,z; x,1/2+y,1/2-z.	hkl: 0kl: h0l: hk0: } No conditions h00: h=2n 0k0: k=2n 00l: l=2n

Symmetry of special projections

(001) pgg; a'=a,b'=b (100) pgg; b'=b, c'=c (010) pgg; c'=c, a'=a

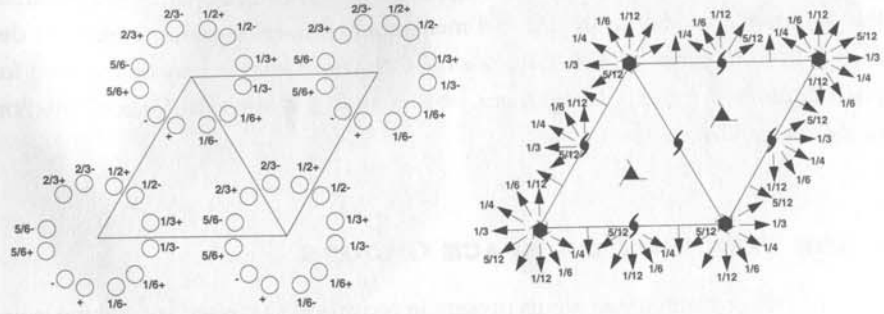
FIGURE 5.13 The diagram for space group $P2_12_12_1$ from Volume 1 of the *International Tables for X-ray Crystallography*. Notations and symbols are explained at the beginning of the volume.

ment. The translational component, if it exists, must be deduced from systematic absences. These, however, are all explicitly described in the *International Tables for X-Ray Crystallography*, Vol. 1, for each space group.

3. At least two, and occasionally more, two-dimensional planes through the diffraction pattern must be recorded and investigated in order to fix the symmetry of the three-dimensional reciprocal lattice. The nearer these are to orthogonal planes, the better. It is essential to know the spatial relationships between symmetry elements as they are identified in different images, and, therefore, it is necessary to know precisely the angular relationships between the images. The question to ask is, what unit cell, with what

Hexagonal 6 2 2 P 6_1 2 2 No. 178 P 6_1 2 2

D_6^2



Origin at 6,21 [2-axis normal to $(\bar{2}110)$]

Number of positions, Wyckoff notation, and point symmetry			Coordinates of equivalent positions	Conditions limiting possible reflection
12	c	1	x,y,z; $\bar{y}$,x-y,1/3+z; y-x,$\bar{x}$,2/3+z; $\bar{x}$,$\bar{y}$,1/2+z; y,y-x,5/6+z; xy,x,1/6+z; y,x,1/3-z; $\bar{x}$,y-x,2/3-z; x-y,$\bar{y}$,$\bar{z}$; $\bar{y}$,$\bar{x}$,5/6-z; x,x-y,1/6-z; y-x,y,1/2-z.	General: hkil: No conditions 000l: l=6n
6	b	2	x,2x,1/4; 2$\bar{x}$,$\bar{x}$,7/12; x,$\bar{x}$,11/12; $\bar{x}$,2$\bar{x}$,3/4; 2x,x,1/12; $\bar{x}$,x,5/12;	Special: as above, plus hh2$\bar{h}$l: l=6n or 6n+/1 or 6n+/2
6	a	3	x,0,0; 0,x,1/3; $\bar{x}$,$\bar{x}$,2/3; $\bar{x}$,0,1/2; 0,$\bar{x}$,5/6; x,x,1/6.	h$\bar{h}$0l: l=6n or 6n+/1 or 6n+/2

FIGURE 5.14 The diagram for space group $P6_122$ from Volume 1 of the *International Tables for X-ray Crystallography*. The diagram of symmetry relationships also contains, below, the equivalent positions and the expected systematic absences.

space group symmetry is consistent with the observed diffraction images. As one identifies features of the diffraction pattern in trying to find the correct unit cell and the correct space group, you are simultaneously eliminating those choices that are inconsistent with the evidence.

4. In a few cases, the space group cannot be determined uniquely from the symmetry of the reciprocal lattice and the systematic absences. In these cases, however, only a choice between two specific possibilities remains. The two choices are usually enantiomorphic space groups, such as $P6_1$ and $P6_5$.

In Figures 5.15, 5.16, and 5.17 are presented pairs of diffraction intensity planes for three different protein crystals. From two photographs such as these, which are usually, but not always (e.g., Figure 5.17) orthogonal to one another, the symmetry of the entire three-dimensional diffraction pattern may be deduced. In some cases, however, additional diffraction images may be required for unambiguous symmetry assignment, or they may prove useful for confirmation of the assigned symmetry.

MORE THOUGHTS ON SPACE GROUPS

In identifying symmetry elements present in reciprocal space, we are seeking to establish symmetry relationships between intensities in various parts of the three-dimensional diffraction pattern. In doing so, it is necessary to remember that a symmetry relationship observed for a single plane of the diffraction pattern, because of Friedel's law, may or may not pertain to the entire pattern, and this can only be ascertained by examining additional planes through reciprocal space.

In deducing the space group of a crystal, it cannot be overemphasized, one works by the process of elimination. If, for example, the Bravais lattice is orthogo-

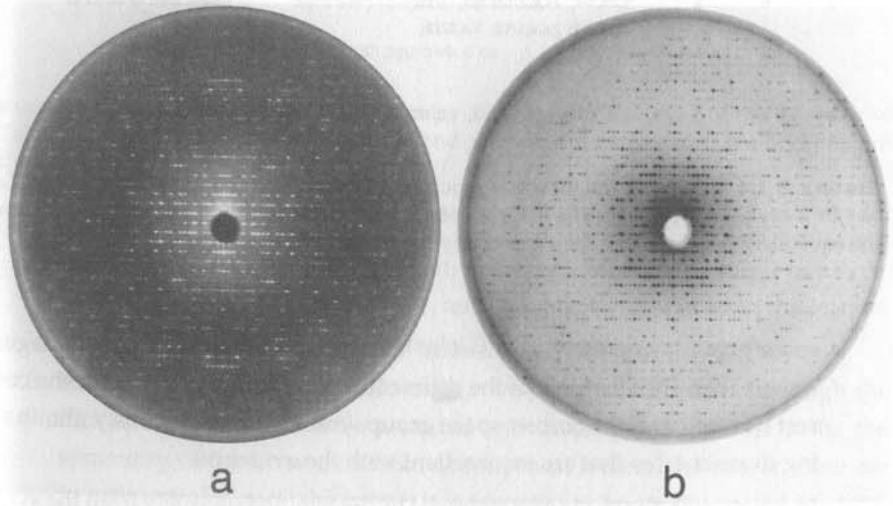


FIGURE 5.15 In *(a)*, the **h** 0 **l**, and, in *(b)*, the **h k** 0 planes of diffraction intensities from an orthorhombic crystal of canavalin (space group $C222_1$, **a** = 136.5 Å, **b** = 150.3 Å, **c** = 133.4 Å). The two photographs represent orthogonal views of the reciprocal lattice, that is, the crystal was rotated by 90° about the horizontal axis between the acquisition of *(a)* and *(b)*.

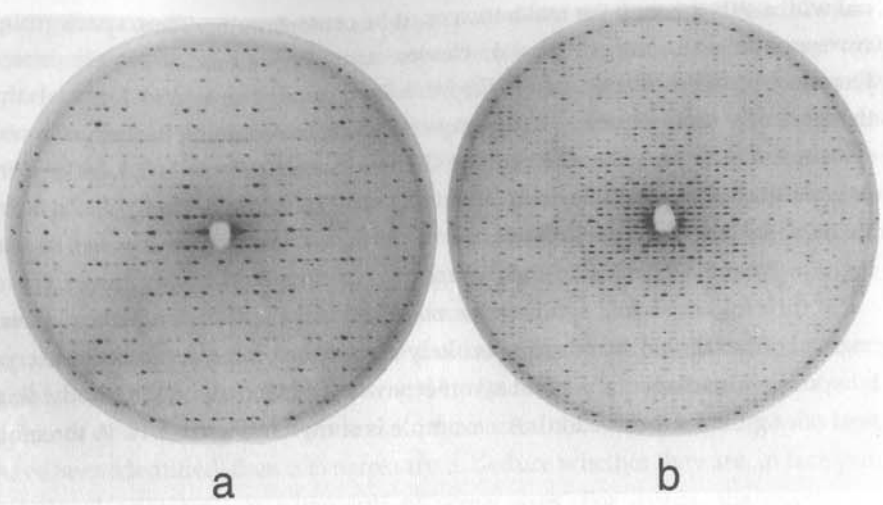


FIGURE 5.16 In *(a)* is the **h k** 0, and in *(b)* the 0 **k l** zones of reciprocal space for an orthorhombic crystal of rabbit muscle creatine kinase (space group $P2_12_12_1$, **a** = 47 Å, **b** = 86 Å, **c** = 125 Å). Both images exhibit **mm** symmetry and systematic absences characteristic of 2_1 axes along both the vertical and horizontal axes. The rotation angle of the crystal between *(a)* and *(b)* is 90°.

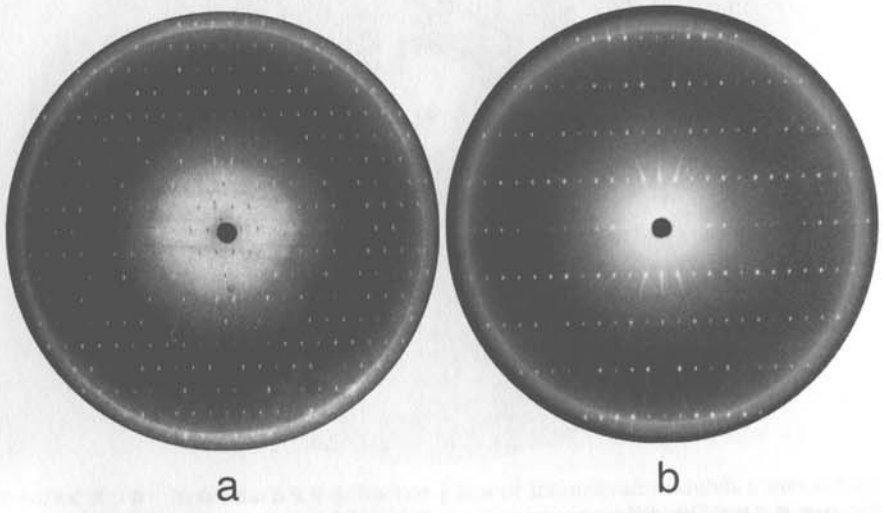


FIGURE 5.17 In *(a)* is the **h k** 0, and in *(b)* the 0 **k l** planes of diffraction intensities from a monoclinic crystal of the gene 5 DNA-unwinding protein from fd bacteriophage (space group C2, **a** = 76.5 Å, **b** = 28.0 Å, **c** = 42.5 Å, **β** = 103°). The angle of rotation of the crystal between the image in *(a)* and in *(b)* was 103°, the **β** angle.

nal with **a** ≠ **b** ≠ **c** then the real lattice must be orthorhombic. Thus, space groups corresponding to other crystal classes can be immediately eliminated. Examination of the *International Tables for X-Ray Crystallography*, Vol. 1, reveals that there are only nine possible space groups for macromolecules in the orthorhombic system and of these, four are primitive **(P)** and five are centered (**C, I,** or **F**). If the Bravais lattice is monoclinic, then the *International Tables for X-Ray Crystallography*, Vol. 1, show only three possible space groups for macromolecular crystals, two are in primitive cells, **P**2 and **P**2_1, and there is only one centered space group, **C**2.

If threefold or sixfold symmetry is observed in the diffraction pattern, then a trigonal or hexagonal space group is likely. Remember, however, that cubic crystal systems also display threefold symmetry when viewed along their body diagonal (along the **1 1 1** direction). An example is shown in Figure 5.18. A threefold

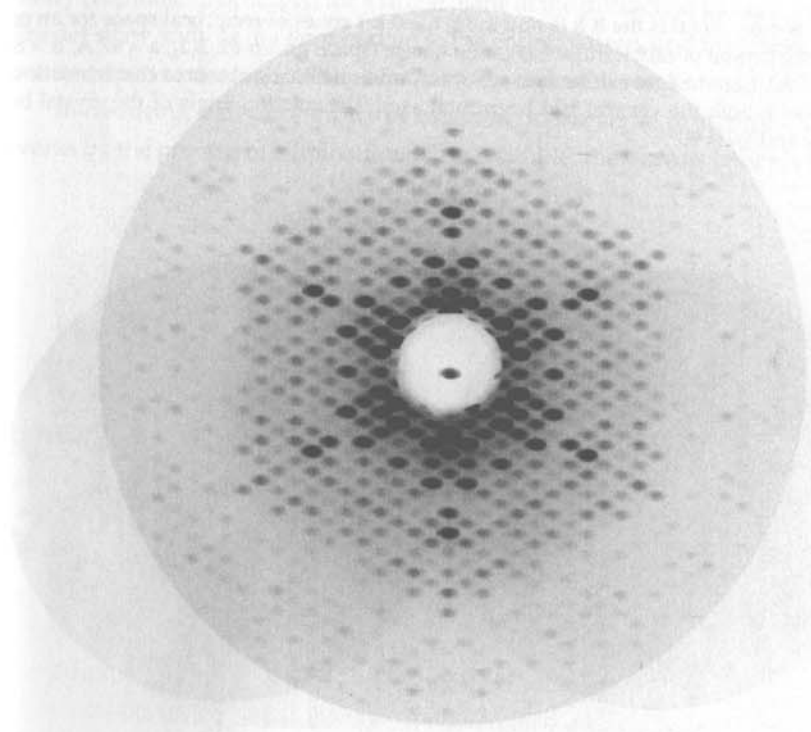


FIGURE 5.18 The diffraction image seen here exhibits exact 6 **mm** symmetry, which might initially suggest a hexagonal or trigonal Bravais lattice. Further investigation, however, would show that it corresponds to a view along the body diagonal, the threefold axis of the unit cell of a cubic tRNA crystal. The 6 **mm** symmetry is a consequence of combining the true threefold crystallographic symmetry operator with Friedel symmetry.

axis, when combined with the center of symmetry produced by Friedel's law, however, appears as sixfold symmetry when only the zero level of reciprocal space perpendicular to the axis is examined. This is because threefold symmetry becomes sixfold when a center of symmetry is added to it. Upper level images (where **h k l** and **–h –k –l** reflections do not both fall on the same plane of reciprocal space) must be recorded to determine if the sixfold axis persists or whether it is in fact a threefold axis. This is done for a crystal of rhombohedral canavalin in Figure 5.19, where a zero level photograph exhibits a sixfold axis, but the second upper layer bears only threefold symmetry.

For macromolecular crystals, the entire symmetry of the diffraction pattern (the Laue symmetry) must be generated by Friedel's law plus the rotational components of symmetry axes present in the crystal. Once the rotational elements have been identified, then it is necessary to deduce whether they are, in fact, pure rotational operators, or some sort of screw axes. For dyads, the question is whether a twofold rotation axis exists, or whether it is a 2_1 screw axis. For three-, four-, and sixfold axes, there are more extensive choices. For example, for a fourfold screw it may be 4_1, 4_2, or 4_3.

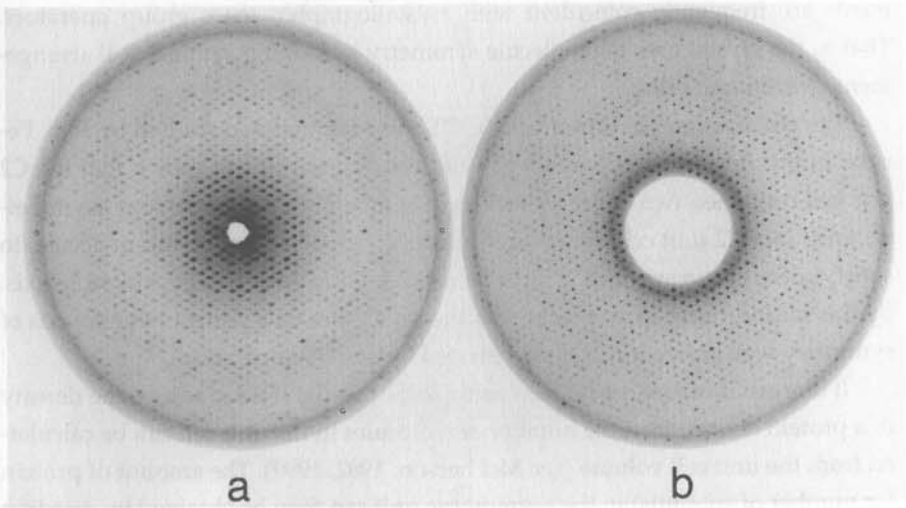


FIGURE 5.19 In *(a)*, the **h k** 0 diffraction plane of **R**3 canavalin exhibits 6 **mm** symmetry, but, because of Friedel's law, it could arise as a consequence of either a true sixfold axis, or a threefold axis plus the Friedel center of symmetry. In *(b)*, the **h k** 2 image, which is along the same direction but does not contain Friedel related reflections, exhibits only threefold symmetry. This demonstrates that the crystal does, in fact, belong to the trigonal system and not the hexagonal system.

To discriminate between the pure rotational operators and the various screw axis possibilities, the diffraction pattern must be examined for systematic absences. These are not hard to search for as they always fall along the reciprocal lattice axial lines (**h** 0 0, 0 **k** 0, 0 0 **l** lines) that correspond to the directions of the putative screw axes in real space. Allowed reflections have forms such as **h** 0 0 = 2**n** (a 2_1 axis along $\vec{a}$), 0 0 **l** = 4**n** (a 4_1 or 4_3 screw axis along $\vec{c}$), 0 0 **l** = 6**n** (a 6_1 or 6_5 screw axis along $\vec{c}$), etc. The only serious ambiguity arises in choosing between enantiomorphic screw operators such as 4_1 and 4_3, or 6_2 and 6_4. Because handedness is lost in the transformation from real space into reciprocal space (again because of Friedel's law), and enantiomorphic screw axes yield the same systematic absences, they cannot be discriminated one from the other. In those cases, the space group does remain somewhat ambiguous and the crystallographer must bear in mind that the space group could be either, for example, **P**6_1 or **P**6_5, **P**$4_1$22 or **P**$4_3$22, etc.

A feature that is generally accessible to the X-ray crystallographer from a preliminary analysis is the number of protein molecules, or protein subunits in the asymmetric unit. This is very important in the actual structure analysis, as you might expect, but it may also be useful for deducing symmetry properties of crystalline oligomeric proteins. This is possible, because molecular symmetry elements are frequently coincident with crystallographic space group operators. That is, the crystal uses the molecule symmetry in forming symmetrical arrangements within unit cells.

The classic example is monoclinic **C**2 horse hemoglobin studied by Max Perutz. In this crystal form, from density measurements it was known that the **C**2 unit cell contained two entire molecules of hemoglobin. There are four asymmetric units in a **C**2 unit cell, however. Thus, one half of a hemoglobin molecule, to satisfy space group symmetry, must be related to the other half by a twofold axis. By this means, horse hemoglobin was shown to possess a perfect twofold axis of symmetry well before it was demonstrated by any other means.

If one can measure, usually on some sort of mixed fluid gradient, the density of a protein crystal, then the number of molecules in the unit cell can be calculated from the unit cell volume (see McPherson, 1982, 1999). The amount of protein (or number of subunits) in the asymmetric unit can then be obtained by dividing by the number of asymmetric units in the cell (known from the space group).

A more convenient method that does not require direct measurement of the crystal density was introduced by Matthews (1968), though one must be cautious in its application. It has occasionally proven misleading, particularly for crystals of unusually high solvent content. Matthews pointed out (and more recent data

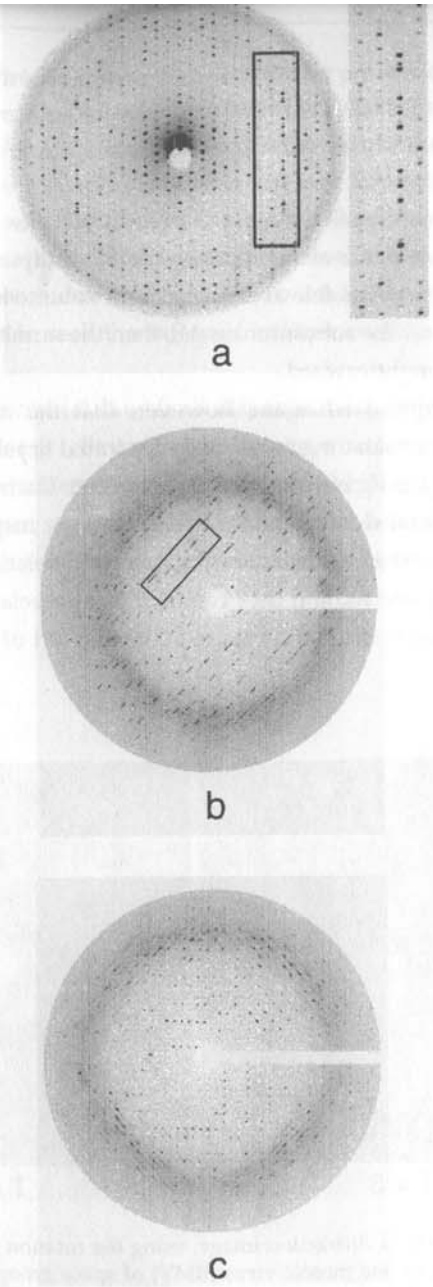


FIGURE 5.20 Diffraction images frequently reveal problems with particular crystals that are sometimes blatant, but occasionally subtle. These include disorder, multiple crystals, or twinned crystals. In *(a)*, the pattern initially appears very ordered and proper, but close inspection of the row of reflections indicated provides evidence that this monoclinic thaumatin crystal is, in fact, twinned. In *(b)*, the reflections from another Bence-Jones protein crystal fall not on a single reciprocal lattice, but multiple, interwoven lattices indicative of twinned or multiple crystals. In *(c)*, a tetragonal crystal of Bence-Jones protein is seriously disordered as evidenced by the smeared, highly mosaic reflections and high background scatter.

seem to confirm it) that the ratio between the volume of the asymmetric unit (the volume of the unit cell divided by the number of asymmetric units in the cell) and the protein mass of the asymmetric unit is about 2.5 Å^3/dalton for most crystals.

For oligomeric proteins, the mass of protein in the asymmetric unit is essentially quantized, that is, it is always some integral multiple of the subunit molecular weight. Given the calculable asymmetric unit volume in Å^3, the expected ratio of 2.5 Å^3/dalton, and the subunit mass M_r, then the number n by which M_r must be multiplied is straightforward.

It must be emphasized again, however, that the ratio, called V_m, of 2.5 Å^3/dalton may be misleading for heavily hydrated crystals containing 70–90% solvent, and for which V_m may be 3.0–5.0 Å^3/dalton. If a true density measure for the crystal can be obtained, it provides reassurance, or inspires retrospection.

There are many insights, tricks, and unexpected relationships in the symmetries of diffraction patterns, and they can only be appreciated by practice and experience. Navigation in diffraction space is the high art of X-ray crystallography,

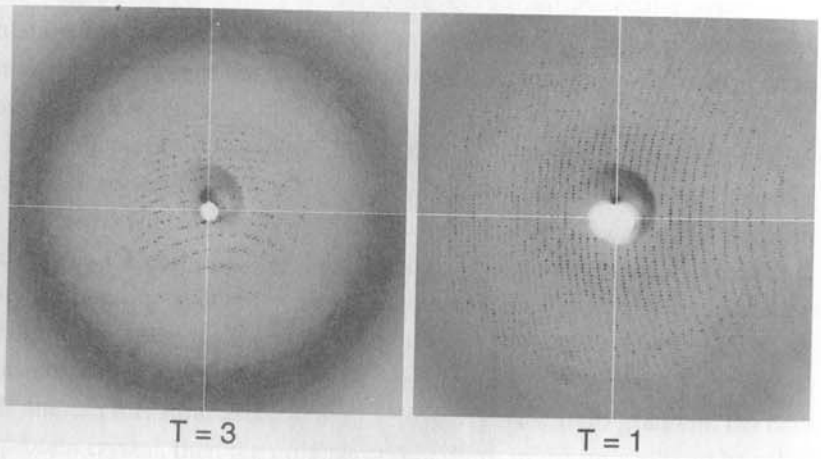


FIGURE 5.21 In *(a)* is a diffraction image, using the rotation method, of a rhombohedral crystal of, native brome mosaic virus (BMV) of space group **R**3, recorded with synchrotron radiation. In *(b)* is a corresponding image from a tetragonal crystal of reassembled T = 1 BMV particles of space group $P4_122$. Note that the maximum distance from the primary beam of the diffraction pattern in *(b)* extends much further than does the pattern in *(a)*. The most distant (highest Bragg angle) reflections in *(a)* arise from families of planes having spacings of no less than 3.4 Å. The better-ordered crystals of the T = 1 particles diffract to much higher resolution, and the most distant reflections arise from Bragg planes of spacings about 2.5 Å.

and learning it takes time and patience. For both the professional and the novice, however, there is always the *International Tables for X-Ray Crystallography*, Vol. 1, to serve as guide and reference, and it should be consulted freely.

Diffraction patterns can also yield other kinds of information that reveal the quality and physical perfection of a specific crystal. The patterns, when examined closely, can even give warnings of subtle problems such as disorder (of many kinds) or twinning. Some examples are illustrated in Figure 5.20. When these signs appear, it is best to seek the safety of another crystal, or possibly suffer a world of grief.

The resolution of a crystal, how far it diffracts into reciprocal space, is a good measure of crystal order. It tells us immediately to what limit of precision we can expect to structurally characterize the molecules that make up the crystal. This is highly variable between protein crystals, and often between different crystal forms of the same macromolecule. Consider the diffraction patterns in Figure 5.21.