that they rotate the plane of polarization in opposite directions when polarized light passes through them. It is not, however, possible to calculate the sign of optical rotation, given the exact spatial arrangement or the 'absolute configuration' of the molecule. Therefore, one cannot distinguish between the possible enantiomorphic configurations of a given asymmetric molecule from measurements of optical rotation. This is also true of molecules with chiralities generated by overall asymmetric geometry instead of the presence of asymmetric carbon atoms in them.

Normal X-ray scattering does not distinguish between enantiomers. Two structures $A\ (x_j, y_j, z_j)$ and $B\ (-x_j, -y_j, -z_j)$ $(j = 1, \ldots, N)$ obviously produce the same diffraction pattern on account of Friedel's law. The situation is, however, different when anomalous scatterers are present in the structure. The intensity difference between reflections $\mathbf{h}$ and $-\mathbf{h}$, or that between members of any Bijvoet pair, has the same magnitude, but opposite sign for structures A and B. If the atomic coordinates are known, the intensities of Bijvoet pairs can be readily calculated. The absolute configuration can then be determined, *i.e.* one can distinguish between A and B by comparing the calculated intensities with the observed ones for a few Bijvoet pairs with pronounced anomalous effects.

2.4.3.5. *Determination of phase angles*

An important application of anomalous scattering is in the determination of phase angles using Bijvoet differences (Ramachandran & Raman, 1956; Peerdeman & Bijvoet, 1956). From Figs. 2.4.3.2 and 2.4.3.3, we have

$$F_N^2(+) = F_N^2 + F_Q''^2 + 2F_N F_Q'' \cos\theta \qquad (2.4.3.3)$$

and

$$F_N^2(-) = F_N^2 + F_Q''^2 - 2F_N F_Q'' \cos\theta. \qquad (2.4.3.4)$$

Then

$$\cos\theta = \frac{F_N^2(+) - F_N^2(-)}{4F_N F_Q''}. \qquad (2.4.3.5)$$

In the above equations F_N may be approximated to $[F_N(+) + F_N(-)]/2$. Then θ can be evaluated from (2.4.3.5) except for the ambiguity in its sign. Therefore, from Fig. 2.4.3.2, we have

$$\alpha_N = \alpha_Q + 90^\circ \pm \theta. \qquad (2.4.3.6)$$

The phase angle thus has two possible values symmetrically distributed about $\mathbf{F}_Q''$. Anomalous scatterers are usually heavy atoms and their positions can most often be determined by Patterson methods. α_Q can then be calculated and the two possible values of α_N for each reflection evaluated using (2.4.3.6).

In practice, the twofold ambiguity in phase angles can often be resolved in a relatively straightforward manner. As indicated earlier, anomalous scatterers usually have relatively high atomic numbers. The 'heavy-atom' phases calculated from their positions therefore contain useful information. For any given reflection, that phase angle which is closer to the heavy-atom phase, from the two phases calculated using (2.4.3.6), may be taken as the correct phase angle. This method has been successfully used in several structure determinations including that of a derivative of vitamin B_{12} (Dale *et al.*, 1963). The same method was also employed in a probabilistic fashion in the structure solution of a small protein (Hendrickson & Teeter, 1981). A method for obtaining a unique, but approximate, solution for phase angles from (2.4.3.6) has also been suggested (Srinivasan & Chacko, 1970). An accurate unique solution for phase angles can be obtained if one collects two sets of intensity data using two different wavelengths which have different dispersion-correction terms for the anomalous scatterers in the structure. Two equations of the type (2.4.3.6) are then available for each reflection and the solution common to both is obviously the correct phase angle. Different types of Patterson and Fourier syntheses can also be employed for structure solution using intensity differences between Bijvoet equivalents (Srinivasan, 1972; Okaya & Pepinsky, 1956; Pepinsky *et al.*, 1957).

An interesting situation occurs when the replaceable atoms in a pair of isomorphous structures are anomalous scatterers. The phase angles can then be uniquely determined by combining isomorphous replacement and anomalous-scattering methods. Such situations normally occur in protein crystallography and are discussed in Section 2.4.4.5.

2.4.3.6. *Anomalous scattering without phase change*

The phase determination, and hence the structure solution, outlined above relies on the imaginary component of the dispersion correction. Variation in the real component can also be used in structure analysis. In early applications of anomalous scattering, the real component of the dispersion correction was made use of to distinguish between atoms of nearly the same atomic numbers (Mark & Szillard, 1925; Bradley & Rodgers, 1934). For example, copper and manganese, with atomic numbers 29 and 25, respectively, are not easily distinguishable under normal X-ray scattering. However, the real components of the dispersion correction for the two elements are -1.129 and -3.367, respectively, when Fe $K\alpha$ radiation is used (*IT* IV, 1974). Therefore, the difference between the scattering factors of the two elements is accentuated when this radiation is used. The difference is more pronounced at high angles as the normal scattering factor falls off comparatively rapidly with increasing scattering angle whereas the dispersion-correction term does not.

The structure determination of $KMnO_4$ provides a typical example for the use of anomalous scattering without phase change in the determination of a centrosymmetric structure (Ramaseshan *et al.*, 1957; Ramaseshan & Venkatesan, 1957). f' and f'' for manganese for Cu $K\alpha$ radiation are -0.568 and 2.808, respectively. The corresponding values for Fe $K\alpha$ radiation are -3.367 and 0.481, respectively (*IT* IV, 1974). The data sets collected using the two radiations can now be treated as those arising from two perfectly isomorphous crystals. The intensity differences between a reflection in one set and the corresponding reflection in the other are obviously caused by the differences in the dispersion-correction terms. They can, however, be considered formally as intensity differences involving data from two perfectly isomorphous crystals. They can be used, as indeed they were, to determine the position of the manganese ion through an appropriate Patterson synthesis (see Section 2.4.4.2) and then to evaluate the signs of structure factors using (2.4.2.6) when the structure is centrosymmetric. When the structure is noncentrosymmetric, a twofold ambiguity exists in the phase angles in a manner analogous to that in the isomorphous replacement method. This ambiguity can be removed if the structure contains two different subsets of atoms $Q1$ and $Q2$ which, respectively, scatter radiations λ_{Q1} and λ_{Q2} anomalously. Data sets can then be collected with λ, which is scattered normally by all atoms, λ_{Q1} and λ_{Q2}. The three sets can be formally treated as those from three perfectly isomorphous structures and the phase determination effected using (2.4.2.7) (Ramaseshan, 1963).

2.4.3.7. *Treatment of anomalous scattering in structure refinement*

The effect of anomalous scattering needs to be taken into account in the refinement of structures containing anomalous scatterers, if accurate atomic parameters are required. The effect of the real part of the dispersion correction is largely confined to the thermal parameters of anomalous scatterers. This effect can be eliminated

by simply adding f' to the normal scattering factor of the anomalous scatterers.

The effects of the imaginary component of the dispersion correction are, however, more complex. These effects could lead to serious errors in positional parameters when the space group is polar, if data in the entire diffraction sphere are not used (Ueki *et al.*, 1966; Cruickshank & McDonald, 1967). For example, accessible data in a hemisphere are normally used for X-ray analysis when the space group is $P1$. If the hemisphere has say h positive, the x coordinates of all the atoms would be in error when the structure contains anomalous scatterers. The situation in other polar space groups has been discussed by Cruickshank & McDonald (1967). In general, in the presence of anomalous scattering, it is desirable to collect data for the complete sphere, if accurate structural parameters are required (Srinivasan, 1972).

Methods have been derived to correct for dispersion effects in observed data from centrosymmetric and noncentrosymmetric crystals (Patterson, 1963). The methods are empirical and depend upon the refined parameters at the stage at which corrections are applied. This is obviously an unsatisfactory situation and it has been suggested that the measured structure factors of Bijvoet equivalents should instead be treated as independent observations in structure refinement (Ibers & Hamilton, 1964). The effect of dispersion corrections needs to be taken into account to arrive at the correct scale and temperature factors also (Wilson, 1975; Gilli & Cruickshank, 1973).

2.4.4. Isomorphous replacement and anomalous scattering in protein crystallography

2.4.4.1. *Protein heavy-atom derivatives*

Perhaps the most spectacular applications of isomorphous replacement and anomalous-scattering methods have been in the structure solution of large biological macromolecules, primarily proteins. Since its first successful application on myoglobin and haemoglobin, the isomorphous replacement method, which is often used in conjunction with the anomalous-scattering method, has been employed in the solution of scores of proteins. The application of this method involves the preparation of protein heavy-atom derivatives, *i.e.* the attachment of heavy atoms like mercury, uranium and lead, or chemical groups containing them, to protein crystals in a coherent manner without changing the conformation of the molecules and their crystal packing. This is only rarely possible in ordinary crystals as the molecules in them are closely packed. Protein crystals, however, contain large solvent regions and isomorphous derivatives can be prepared by replacing the disordered solvent molecules by heavy-atom-containing groups without disturbing the original arrangement of protein molecules.

2.4.4.2. *Determination of heavy-atom parameters*

For any given reflection, the structure factor of the native protein crystal ($\mathbf{F}_N$), that of a heavy-atom derivative ($\mathbf{F}_{NH}$), and the contribution of the heavy atoms in that derivative ($\mathbf{F}_H$) are related by the equation

$$\mathbf{F}_{NH} = \mathbf{F}_N + \mathbf{F}_H. \qquad (2.4.4.1)$$

The value of $\mathbf{F}_H$ depends not only on the positional and thermal parameters of the heavy atoms, but also on their occupancy factors, because, at a given position, the heavy atom may not often be present in all the unit cells. For example, if the heavy atom is present at a given position in only half the unit cells in the crystal, then the occupancy factor of the site is said to be 0.5.

For the successful determination of the heavy-atom parameters, as also for the subsequent phase determination, the data sets from the native and the derivative crystals should have the same relative scale. The different data sets should also have the same overall temperature factor. Different scaling procedures have been suggested (Blundell & Johnson, 1976) and, among them, the following procedure, based on Wilson's (1942) statistics, appears to be the most feasible in the early stages of structure analysis.

Assuming that the data from the native and the derivative crystals obey Wilson's statistics, we have, for any range of $\sin^2\theta/\lambda^2$,

$$\ln\left\{\frac{\sum f_{Nj}^2}{\langle F_N^2\rangle}\right\} = \ln K_N + 2B_N\frac{\sin^2\theta}{\lambda^2} \qquad (2.4.4.2)$$

and

$$\ln\left\{\frac{\sum f_{Nj}^2 + \sum f_{Hj}^2}{\langle F_{NH}^2\rangle}\right\} = \ln K_{NH} + 2B_{NH}\frac{\sin^2\theta}{\lambda^2}, \qquad (2.4.4.3)$$

where f_{Nj} and f_{Hj} refer to the atomic scattering factors of protein atoms and heavy atoms, respectively. K_N and K_{NH} are the scale factors to be applied to the intensities from the native and the derivative crystals, respectively, and B_N and B_{NH} the temperature factors of the respective structure factors. Normally one would be able to derive the absolute scale factor and the temperature factor for both the data sets from (2.4.4.2) and (2.4.4.3) using the well known Wilson plot. The data from protein crystals, however, do not follow Wilson's statistics as protein molecules contain highly non-random features. Therefore, in practice, it is difficult to fit a straight line through the points in a Wilson plot, thus rendering the parameters derived from it unreliable. (2.4.4.2) and (2.4.4.3) can, however, be used in a different way. From the two equations we obtain

$$\ln\left\{\frac{\sum f_{Nj}^2 + \sum f_{Hj}^2}{\sum f_{Nj}^2}\cdot\frac{\langle F_N^2\rangle}{\langle F_{NH}^2\rangle}\right\} = \ln\left(\frac{K_{NH}}{K_N}\right) + 2(B_{NH} - B_N)\frac{\sin^2\theta}{\lambda^2}. \qquad (2.4.4.4)$$

The effects of structural non-randomness in the crystals obviously cancel out in (2.4.4.4). When the left-hand side of (2.4.4.4) is plotted against $(\sin^2\theta)/\lambda^2$, it is called a comparison or difference Wilson plot. Such plots yield the ratio between the scales of the derivative and the native data, and the additional temperature factor of the derivative data. Initially, the number and the occupancy factors of heavy-atom sites are unknown, and are roughly estimated from intensity differences to evaluate $\sum f_{Hj}^2$. These estimates usually undergo considerable revision in the course of the determination and the refinement of heavy-atom parameters.

At first, heavy-atom positions are most often determined by Patterson syntheses of one type or another. Such syntheses are discussed in some detail elsewhere in Chapter 2.3. They are therefore discussed here only briefly.

Equation (2.4.2.6) holds when the data are centric. F_H is usually small compared to F_N and F_{NH}, and the minus sign is then relevant on the left-hand side of (2.4.2.6). Thus the difference between the magnitudes of $\mathbf{F}_{NH}$ and $\mathbf{F}_N$, which can be obtained experimentally, normally gives a correct estimate of the magnitude of $\mathbf{F}_H$ for most reflections. Then a Patterson synthesis with $(F_{NH} - F_N)^2$ as coefficients corresponds to the distribution of vectors between heavy atoms, when the data are centric. But proteins are made up of L-amino acids and hence cannot crystallize in centrosymmetric space groups. However, many proteins crystallize in space groups with centrosymmetric projections. The centric data corresponding to these projections can then be used for determining heavy-atom positions through a Patterson synthesis of the type outlined above.

references