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The Theory of the Method of Isomorphous Replacement for Protein Crystals. I

BY F. H. C. CRICK*

The Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, Cambridge, England

AND BEATRICE S. MAGDOFF*

Boyce Thompson Institute for Plant Research, Yonkers 3, N.Y., U.S.A.

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The average change of intensity due to adding a heavy atom to a protein crystal is calculated for both the centric and the acentric reflections. The amount by which small shifts of the molecules change the intensities is obtained for the monoclinic and orthorhombic systems, and formulae are given for the changes caused by small translations and rotations of the molecules, by alterations of the lattice parameters, and by 'breathing' movements. The effects always increase linearly with $1/d$. It is shown that quite small molecular shifts would interfere with the isomorphous-replacement method for proteins at the higher values of $1/d$, but not at the lower values.

Introduction

The work of Perutz and his colleagues (Green, Ingram & Perutz, 1954; Bragg & Perutz, 1954) has shown that the method of isomorphous replacement can be applied to protein crystals provided a sufficiently heavy atom is used. Since other methods of attack on protein crystals have so far either failed or been inconclusive, it seems probable that no serious progress can be made unless this method is used. This series of papers deals with some of the theoretical problems which have arisen in connection with the method.

Two problems are considered in this paper. The first concerns the average change in intensity produced by adding extra atoms to the crystal, the rest of the unit cell remaining the same. The second deals with the average change of intensity produced by small shifts of the protein molecules, or by slight changes in the dimensions of the unit cell. We have considered the second problem because it has been shown for ribonuclease that such changes occur (King, Magdoff, Adelman & Harker, 1956; Magdoff & Crick, 1955) and may make it difficult to use isomorphous replacement very far out in reciprocal space.

The isomorphous replacement method

As is well known, there are two methods of using 'heavy' atoms to determine crystal structures (see, for example, Lipson & Cochran, 1953, pp. 206–20). In the *heavy atom method* proper, only a single form of the crystal is required and the phases used to calculate the first trial Fourier are those calculated for the heavy atom alone. The problem of how heavy an atom is needed has been considered by Luzzati (1953). Because protein molecules are large (molecular weights $> 10{,}000$) an impossibly heavy atom would be required for this method, and although, at some later stage, it may be possible to add a large number of heavy atoms, we shall for the moment leave this possibility on one side.

In the *isomorphous replacement method*, on the other hand, *two* forms of the crystal are required. The unit-cell dimensions and the protein molecule must be essentially the same in both, but one of them must have one or more heavy atoms at some point where the other has either none, or very much lighter atoms. The position of the extra atoms must first be found by some Patterson method. Then for any reflection the amplitude and phases of the contribution of the extra atoms can be calculated, and in favourable cases the phase of the protein contribution can be found from the observed change of intensity produced by adding the extra atoms.

The advantage of this method for proteins is that although atoms of relatively high atomic number must be used, the size required is within the bounds of possibility for the smaller proteins. For horse haemoglobin, with a molecular weight of 34,000 for the asymmetric unit, it has proved possible to determine the signs of 87 out of 94 $h0l$ reflections ($1/d < 0{\cdot}15$), using silver and mercury atoms for isomorphous replacements (Green *et al.*, 1954).

It is worth noting that for work on proteins various combinations of the two basic methods are very attractive. For example it may eventually be possible to add a large number of heavy atoms, say 20 iodines, to a protein molecule. Though it may prove difficult to locate these unambiguously from a Patterson synthesis, an isomorphous replacement of a mercury atom, for example, might enable the positions of all of them

* This work was started when both authors were members of the Protein Structure Project, Polytechnic Institute of Brooklyn, N.Y., U.S.A.

to be determined. Once these positions were found, the heavy atom method could be attempted. Alternatively, the contribution of the heavy atoms could be used to decide between the two alternative phases given for an acentric reflection by a single isomorphous replacement. These possibilities, also, we shall leave for future consideration.

Assumptions made

It is known that the intensity distribution obtained from a protein crystal does not strictly obey the statistics expected from a random distribution of atoms, which we shall refer to here as 'Wilson statistics' (Wilson, 1949; for a general account see Lipson & Cochran, 1953). Not only is the curve of $\langle I \rangle$ against $1/d$ not a monotonic one (see, for example, Perutz, 1949), but a set of intensities, all having similar values of $1/d$, does not always have the Wilson distribution (Luzzati, 1955). Nevertheless, we have developed the theory assuming that Wilson statistics are obeyed, and that the shape of the curve of $\langle I \rangle$ against $1/d$ is gaussian. This is because we wish at this stage only to obtain results of a general nature, and these are the simplest assumptions possible. For the same reason we have considered the oxygen and carbon atoms to be equivalent to nitrogen, and have ignored the hydrogen atoms. We have also ignored the solvent in the crystal. To correct for the abnormal values of $\langle I \rangle$ an 'effective' value for the number of atoms contributing to the intensities should be used in the formulae.

We have also assumed without special justification that in obtaining mean values we can simply average over all possible values of the trigonometrical functions. This will usually be sufficiently accurate if the sample of intensities is large enough, if the region near the origin of reciprocal space is avoided, and if the atomic positions are random.

Nomenclature

The usual crystallographic conventions. In addition:

$F = A+iB$ for *all* terms (i.e. $\bar{h}\bar{k}\bar{l}$ and hkl);

$I = |F|^2$;

suffixes:
- P = protein;
- E = extra atoms, i.e. the isomorphous replacement atoms;
- PE = protein plus extra atoms;
- n = the nth atom of the protein;

N, total number of effective atoms in the unit cell (not merely those in one asymmetric unit);

$\Phi\Delta I = \{\overline{(\Delta I)^2}\}^{\frac{1}{2}}/\bar{I}$, where ΔI = change in I due to the effect under consideration;

$\Phi\Delta I$ is read as 'the fractional change in intensity;

x, y, z, *fractional* coordinates of the unit cell;

x, y, z, (non-fractional) coordinates of an orthogonal frame in real space, in Å;

X, Y, Z, coordinates of an orthogonal frame in reciprocal space, reciprocal to x, y, z, in Å^{-1}.

(For the orthorhombic case x, y and z, are parallel to x, y, and z respectively. For the monoclinic case x, y and z are parallel to x, y and z^* respectively, where z^* is reciprocal to z.)

$R^2 = X^2+Y^2+Z^2$;

x_b, y_b, z_b, coordinates of the breathing point (defined on p. 904);

x_c, y_c, z_c, coordinates of the 'centre of gravity' of the protein molecule.

Symbols used only close to their place of definition are omitted here.

Intensity changes due to the extra atoms

The simplest measure to calculate is

$$\{\overline{(\Delta I)^2}\}^{\frac{1}{2}}/\bar{I}_P, \quad \text{where} \quad \Delta I = I_{PE}-I_P\,.$$

We require this so often that we propose to designate it by a special symbol, and write it $\Phi\Delta I$, to be read 'the fractional change of intensity'.

For the centric case, when the amplitudes are real, we have

$$\Delta I = 2F_PF_E+F_E^2\,,$$
$$(\Delta I)^2 = 4F_P^2F_E^2+4F_PF_E^3+F_E^4\,.$$

Now we shall assume for the moment that F_P and F_E are uncorrelated. Therefore we can write

$$\overline{F_P^2F_E^2} = \overline{F_P^2}\,\overline{F_E^2}, \quad \overline{F_PF_E^3} = 0\,.$$

We thus obtain

$$\Phi\Delta I = \frac{\{\overline{(\Delta I)^2}\}^{\frac{1}{2}}}{\bar{I}_P} = \frac{(4\overline{F_P^2}\,\overline{F_E^2}+\overline{F_E^4})^{\frac{1}{2}}}{\overline{F_P^2}} = 2\left(\frac{\overline{F_E^2}}{\overline{F_P^2}}\right)^{\frac{1}{2}}\left(1+\frac{\overline{F_E^4}}{4\overline{F_P^2}\,\overline{F_E^2}}\right)^{\frac{1}{2}} \qquad (1)$$
$$\simeq 2(\bar{I}_E/\bar{I}_P)^{\frac{1}{2}}\,.$$

For the acentric case, with complex amplitudes, we have

$$A_{PE} = A_P+A_E, \quad B_{PE} = B_P+B_E\,.$$

Whence we have

$$\Delta I = 2A_PA_E+A_E^2+2B_PB_E+B_E^2\,.$$

Assuming as before that F_P and F_E are uncorrelated, we can write

$$\overline{A_P^2 A_E^2} = \overline{A_P^2}\,\overline{A_E^2}\,, \qquad \overline{B_P B_E^2} = 0\,,$$
$$\overline{B_P^2 B_E^2} = \overline{B_P^2}\,\overline{B_E^2}\,, \qquad \overline{A_P A_E B_E^2} = 0\,,$$
$$\overline{A_P A_E^3} = 0\,, \qquad \overline{B_P B_E B_E^2} = 0\,,$$
$$\overline{A_P B_P A_E B_E} = 0\,.$$

This last relation depends upon $\overline{AB} = 0$, which is always true if our set of reflections includes $\bar{h}\bar{k}\bar{l}$ whenever hkl is included.

Writing down the expressions for $(\Delta I)^2$ and making the above substitution, we easily obtain

$$\overline{(\Delta I)^2} = 4\overline{A_P^2}\,\overline{A_E^2} + 4\overline{B_P^2}\,\overline{B_E^2} + \overline{A_E^4} + 2\overline{A_E^2 B_E^2} + \overline{B_E^4}\,.$$

Since $\overline{A_P^2} = \overline{B_P^2} = \frac{1}{2}|F_P|^2$, this becomes

$$(\Delta I)^2 = 2\,\overline{|F_P|^2}\,\overline{|F_E|^2} + \overline{|F_E|^4}$$

and so

$$\Phi\Delta I = \frac{(2\,\overline{|F_P|^2}\,\overline{|F_E|^2} + \overline{|F_E|^4})^{\frac{1}{2}}}{\overline{F_P^2}}$$
$$= \sqrt{2}\left(\frac{\overline{|F_E|^2}}{\overline{|F_P|^2}}\right)^{\frac{1}{2}}\left(1 + \frac{\overline{|F_E|^4}}{2\overline{|F_P|^2}\,\overline{|F_E|^2}}\right)^{\frac{1}{2}} \qquad (2)$$
$$\simeq \sqrt{2}\,(\bar{I}_E/\bar{I}_P)^{\frac{1}{2}}\,.$$

It can be shown that though these formulae are not exact the corrections are small compared with the main term. It should be noted that we have tacitly assumed that $\langle I \rangle$ does not change with $1/d$, and so in using the formulae the averages should strictly go over only a small range of $1/d$.

Now since proteins consist of L amino acids the only permissible symmetry elements are rotation and screw axes. For such space groups we always have (Wilson, 1949; Rogers, 1950) the statistical result

$$\langle I \rangle = \sum_{n=1}^{N} f_n^2 = Nf^2\,,$$

assuming all the atoms the same. N and I both refer to the *primitive* cell. Thus our approximate results become

$$\Phi\Delta I \simeq 2\,(N_E/N_P)^{\frac{1}{2}}(f_E/f_P)\,, \text{ centric,} \qquad (3)$$
$$\simeq \sqrt{2}\,(N_E/N_P)^{\frac{1}{2}}(f_E/f_P)\,, \text{ acentric.} \qquad (4)$$

The full formulae depend on the value of $\overline{I_E^2}$, but the correcting terms (under the square root, equations (1) and (2)) will usually be small.

As an example, consider the case of a single mercury atom added to a protein containing about 1600 atoms (other than hydrogen) and therefore having a molecular weight of about 24,000. For those reflections whose amplitude is real we obtain, using equation (3),

$$\Phi\Delta I \simeq 2\,(\tfrac{1}{1600})^{\frac{1}{2}} \times \tfrac{80}{7} = 0{\cdot}57\,,$$

so that the average change of intensity would be a little over 50%. However, in practice this value has to be corrected for three effects. First, the mercury atom may not have been added to every molecule in the crystal, and thus an 'effective' atomic number should be used. Second, the average intensity from the protein may differ from the value expected from the Wilson statistics. To allow for this an effective value of N should be used. Thus if the average intensity of the protein in the above example is only half that expected on statistical grounds, the effective number of atoms in the protein should be taken as 800. Both these effects have been found by Green *et al.* (1954) in their studies of horse haemoglobin. Third, we should strictly speaking use the ratio of (f_E/f_P) for the range of $1/d$ considered, rather than the values at $1/d = 0$.

Intensity changes due to shifts of the molecule

As in the previous case, we shall take as our measure of the change of intensity the value of $\Phi\Delta I = \{\overline{(\Delta I)^2}\}^{\frac{1}{2}}/\bar{I}$. For those interested only in the results, numerical examples are given in Table 1.

Consider first the centric case. For some particular reflection we can write $\Delta I = 2F(\Delta F)$, since we shall be considering only very small changes and thus the $(\Delta F)^2$ term can be neglected. Therefore

$$(\Delta I)^2 = 4F^2(\Delta F)^2\,.$$

Our problem therefore consists in expressing the right-hand side of this equation in terms of the usual structure-factor formulae, and then averaging.

For the acentric case we have

$$I = A^2 + B^2\,,$$
$$\Delta I = 2A(\Delta A) + 2B(\Delta B)\,,$$
$$(\Delta I)^2 = 4A^2(\Delta A)^2 + 8AB(\Delta A)(\Delta B) + 4B^2(\Delta B)^2\,,$$

and again our problem is to find the average value of the terms on the right-hand side. We shall assume that N, the total number of atoms in the unit cell, is large.

To enable us to express our results in a compact form we use the parameter γ, where $\gamma = 1$ for the centric case and $\gamma = 2$ for the acentric ones.

We have worked out the formulae for all monoclinic and orthorhombic space groups in which proteins can occur (i.e. lacking centres of symmetry, mirror planes or glide planes). We find that the three possible monoclinic space groups ($P2$, $P2_1$ and $C2$) all give the same formula, whereas the orthorhombic space groups give a slightly different formula, which is the same for all of them. Our results are:

Monoclinic:

$$\Phi\Delta I =$$
$$(4\pi/\sqrt{\gamma})\left[\overline{(h\Delta x_n + l\Delta z_n)^2} + \overline{k^2\{(\Delta y_n)^2 - (\Delta y_n)(\Delta y_{n'})\}}\right]^{\frac{1}{2}}. \qquad (5)$$

Orthorhombic:

$$\Phi\Delta I = (4\pi/\sqrt{\gamma})[\overline{h^2(\Delta x_n)^2}+\overline{k^2(\Delta y_n)^2}+\overline{l^2(\Delta z_n)^2}]^{\frac{1}{2}} . \quad (6)$$

In (5) and (6) $\gamma = 1$ for centric reflections and $\gamma = 2$ for acentric reflections.

The average values are taken over all the atoms *in one asymmetric unit* of the crystal, and then over all the reflections in the set being considered.

The difference between the term containing h and l in the two formulae reflects the fact that there is no unique choice of the a and c axes in the monoclinic case as there is in the orthorhombic.

There is no difficulty in working out the results for the other crystal classes although the algebra would be laborious. We have not done this as the great majority of protein crystals are either monoclinic or orthorhombic and our main purpose is to obtain the order of magnitude of the different effects. It is worth noting that for centric cases an alternative measure to take is

$$\{\overline{(\Delta F)^2}/\overline{I}\}^{\frac{1}{2}}$$

(which for the cases considered above is half $\Phi\Delta I$) as this can be evaluated rather more easily than $\Phi\Delta I$.

The formulae given so far are very simple and easy to grasp, but they are not in the best form for detailed application. Rather than the indices (h, k, l) and the fractional co-ordinates $(x, y, z,)$ one would prefer reciprocal co-ordinates $(X, Y, Z,)$, having dimensions Å^{-1}, and actual (not fractional) co-ordinates $(x,y,z,)$ for real space. In addition, one would wish these two frames to be orthogonal. This presents no difficulties for the orthorhombic case, but for the monoclinic cases, when $\beta \neq 90°$, it causes complications. We will therefore consider the two crystal systems separately.

The orthorhombic system

Since

$$x_n = ax_n \quad \text{and} \quad X = h/a ,$$

we can write

$$\begin{aligned} h(\Delta x_n) &= h\Delta(x_n/a) = h(\Delta x_n)/a - hx_n(\Delta a/a^2) \\ &= X(\Delta x_n) - Xx_n(\Delta a/a) , \end{aligned}$$

and similarly for

$$k(\Delta y_n) \quad \text{and} \quad l(\Delta z_n) .$$

Thus our general formula (equation (6)) becomes

$$\begin{aligned} \Phi\Delta I = (4\pi/\sqrt{\gamma})[&\overline{X^2[\Delta x_n - x_n(\Delta a/a)]^2} \\ &+ \overline{Y^2[\Delta y_n - y_n(\Delta b/b)]^2} + \overline{Z^2[\Delta z_n - z_n(\Delta c/c)]^2}]^{\frac{1}{2}} . \end{aligned} \quad (7)$$

We now consider a number of cases in detail.

(1) *Pure translations*

The molecule is translated a small distance without rotation, and the lattice parameters are unchanged. Thus $\Delta a = \Delta b = \Delta c = 0$. From equation (7) above we derive

$$\Phi\Delta I = (4\pi/\sqrt{\gamma})[\overline{X^2(\Delta x_n)^2} + \overline{Y^2(\Delta y_n)^2} + \overline{Z^2(\Delta z_n)^2}]^{\frac{1}{2}} . \quad (8)$$

Suppose we consider *all* the relevant reflections with similar values of $1/d = R$. Then for the general reflections we shall have

$$X^2 + Y^2 + Z^2 = R^2;$$

therefore

$$\overline{X^2} = \overline{Y^2} = \overline{Z^2} = \tfrac{1}{3}R^2 .$$

If we now call the shift of the molecule Δr, where

$$(\Delta r)^2 = (\Delta x_n)^2 + (\Delta y_n)^2 + (\Delta z_n)^2 \quad \text{for all } n ,$$

our formula for the average value of all the general reflections $(1/d \simeq R)$ becomes

$$\Phi\Delta I = \frac{4\pi R}{\sqrt{6}} (\Delta r) .$$

For a centric zone of reflections, say $k = 0$, we shall have

$$\overline{X^2} = \overline{Z^2} = \tfrac{1}{2}R^2$$

and if the projected shift of the molecule is Δr_p, where

$$(\Delta r_p)^2 = (\Delta x_n)^2 + (\Delta z_n)^2 \quad \text{for all } n ,$$

we obtain for this zone, for all reflections having $1/d \simeq R$,

$$\Phi\Delta I = \frac{4\pi R}{\sqrt{2}} (\Delta r_p) .$$

Notice that these two results are independent of the size or shape of either the unit cell or the protein molecule, as might be expected from an elementary argument. Notice also that they are for the *average* effects. Special areas of the reciprocal lattice may show higher local averages, especially if the translation is parallel to an axis of the cell.

(2) *Pure lattice changes*

The molecule remains fixed with respect to the origin of co-ordinates, but the dimensions of the unit cell alter. Thus,

$$\Delta x_n = \Delta y_n = \Delta z_n = 0 \quad \text{for all } n .$$

Equation (7) becomes

$$\begin{aligned} \Phi\Delta I = (4\pi/\sqrt{\gamma})[&\overline{X^2x_n^2}(\Delta a/a)^2 \\ &+ \overline{Y^2y_n^2}(\Delta b/b)^2 + \overline{Z^2z_n^2}(\Delta c/c)^2]^{\frac{1}{2}} . \end{aligned}$$

Notice that in this case the disposition of the material in the unit cell matters somewhat. If the atoms of one asymmetric unit were all grouped very near the origin then $\Phi\Delta I$ would be smaller than if they were more evenly distributed.

(3) *Breathing movements*

This term describes a movement in which the cell dimensions change, and simultaneously the molecules suffer a pure translation (without rotation) such that

one chosen point (x_b, y_b, z_b) maintains the same *fractional* co-ordinates. For this case

$$h(\Delta x_n) = h\Delta(x_n/a) = h\Delta((x_n-x_b)/a)$$

since $\Delta(x_b/a)$ is zero by definition.

Because (x_n-x_b) does not change, we obtain

$$h(\Delta x_n) = h(x_n-x_b)(-\Delta a/a^2) = X(x_n-x_b)(-\Delta a/a)\,,$$

and similar expressions for $k(\Delta y_n)$ and $l(\Delta z_n)$.

Thus equation (7) becomes

$$\Phi\Delta I = (4\pi/\sqrt{\gamma})[\overline{X^2(x_n-x_b)^2}(\Delta a/a)^2 + \overline{Y^2(y_n-y_b)^2}(\Delta b/b)^2+\overline{Z^2(z_n-z_b)^2}(\Delta c/c)^2]^{\frac{1}{2}}.$$

It can be shown that $\Phi\Delta I$ is a minimum when the breathing point coincides with the centre of gravity.

It should be clearly realized that the choice of origin in these examples is not completely arbitary. While it is true that a change of origin makes no difference to the intensities, it *does* alter the formulae used to calculate the structure factors. These are based on the assumption that only *one* asymmetric unit need be inserted in them, the symmetry taking care of the others. Thus the origin should always be that given in the *International Tables.*

(4) *Separating rotations and translations*

In this section we shall consider the lattice parameters as constant. Thus the relevant formula is that given in equation (8).

Any arbitrary movement of a body can be regarded as composed of a pure translation plus a rotation about some axis passing through its centre of gravity. It is easy to show that we can compound the effects of translations and rotations about the centre of gravity as random errors are compounded, that is, by taking the square root of the sum of their squares.

(5) *Rotations*

In this section the lattice parameters are considered to be constant. For an infinitesimal rotation of θ radians about an axis through the point (x_0, y_0, z_0), and having direction cosines $\cos\alpha_x$, $\cos\alpha_y$, $\cos\alpha_z$, it is well known that if the point (x_1, y_1, z_1) moves to $(x_1+\Delta x_1, y_1+\Delta y_1, z_1+\Delta z_1)$ then

$$\begin{aligned}\Delta x_1 &= \varepsilon_{11}(x_1-x_0)+\varepsilon_{12}(y_1-y_0)+\varepsilon_{13}(z_1-z_0)\,,\\ \Delta y_1 &= \varepsilon_{21}(x_1-x_0)+\varepsilon_{22}(y_1-y_0)+\varepsilon_{23}(z_1-z_0)\,,\\ \Delta z_1 &= \varepsilon_{31}(x_1-x_0)+\varepsilon_{32}(y_1-y_0)+\varepsilon_{33}(z_1-z_0)\,,\end{aligned}$$

where

$$\varepsilon_{11} = \varepsilon_{22} = \varepsilon_{33} = 0\,,$$

and

$$\begin{aligned}\varepsilon_{32} &= -\varepsilon_{23} = \theta\cos\alpha_x\,,\\ \varepsilon_{13} &= -\varepsilon_{31} = \theta\cos\alpha_y\,,\\ \varepsilon_{21} &= -\varepsilon_{12} = \theta\cos\alpha_z\,.\end{aligned}$$

These equations can be used both to find the movement $(\Delta x_c, \Delta y_c, \Delta z_c)$ of the centre of gravity and also to obtain $(\Delta x_n, \Delta y_n, \Delta z_n)$.

Consider as an example the case of a rotation about an axis through the centre of gravity and parallel to the b axis. Then

$$\cos\alpha_y = 1,\quad \cos\alpha_x = \cos\alpha_z = 0\,.$$

Thus we have

$$\Delta x_n = \theta(z_n-z_c),\ \Delta y_n = 0,\ \Delta z_n = -\theta(x_n-x_c)\,.$$

If we consider all the general reflections with $1/d \simeq R$, we obtain

$$\Phi\Delta I = (4\pi R/\sqrt{6})\theta[\overline{(z_n-z_c)^2}+\overline{(x_n-x_c)^2}]^{\frac{1}{2}}.$$

For the centric zone *perpendicular* to the axis of rotation (i.e. $k = 0$) we derive for all reflections having $1/d \simeq R$

$$\Phi\Delta I = (4\pi R/\sqrt{2})\theta[\overline{(z_n-z_c)^2}+\overline{(x_n-x_c)^2}]^{\frac{1}{2}},$$

and for a centric zone which is *not* perpendicular to the axis of rotation, but contains it, say the zone $h = 0$, we have

$$\Phi\Delta I = (4\pi R/\sqrt{2})\theta[\overline{(x_n-x_c)^2}]^{\frac{1}{2}}.$$

For more complicated rotations it is worth noting that there is a close analogy (for those cases where we are averaging over all the general reflections having $1/d \simeq R$) between the problem we have to consider and that of deriving the radius of gyration of a body about an arbitrary axis, which is tackled by means of the 'inertia ellipsoid' of the body, as explained in books on mechanics.

The monoclinic system

Owing to the fact that in general $\beta \neq 90°$ and that it can change, the monoclinic system is potentially more complicated than the orthorhombic. We have been unable to find a general formulation which is not algebraically cumbersome. We have thus contended ourselves with presenting only the special cases for which the solution is relatively straightforward.

(1) *Lattice parameters fixed*

We choose our orthogonal axes in real space (x,y,z) so that x is parallel to x, y to y, and z to z^* (the axis reciprocal to z). The frame (X, Y, Z) is as usual reciprocal to (x, y, z). We easily obtain

$$\left.\begin{aligned}x &= ax+cz\cos\beta\,,\\ z &= cz\sin\beta\,,\end{aligned}\right\}\qquad \left.\begin{aligned}X &= h/a\,,\\ Z &= l/c\sin\beta-h/a\tan\beta\,,\end{aligned}\right\}$$

and, therefore, $hx+lz = Xx+Zz$, as might have been expected. We also have $y = by$ and $Y = k/b$, as in the orthorhombic case. Since our axes are fixed

$$h(\Delta x)+l(\Delta z) = X(\Delta x)+Z(\Delta z)\,,$$

and thus

$$\overline{[h(\Delta x_n)+l(\Delta z_n)]^2} = \overline{X^2(\Delta x_n)^2}+\overline{Z^2(\Delta z_n)^2}+\overline{2XZ(\Delta x_n)(\Delta z_n)} . \quad (9)$$

We can also show that in the general case (axes varying)

$$\overline{k^2\{(\Delta y_n)^2-(\Delta y_n)(\Delta y_{n'})\}} = \overline{Y^2[\Delta(y_n-y_c)]^2}+\overline{Y^2(y_n-y_c)^2(\Delta b/b)^2} . \quad (10)$$

Thus, as we should except, a pure translation of the molecule, parallel to the y axis, produces no change in the intensities, since (y_n-y_c) is invariant under such a change, for all n. Without loss of generality, therefore, we can restrict ourselves to motions in which the y co-ordinates of the centre of gravity remains unchanged. Let us further arbitrarily restrict ourselves to sets of intensities for which $\overline{XZ} = 0$, for example by always including the reflection at $(-X, Z)$ whenever that at (X, Z) is included†. Then, *making these restrictions*, and substituting equation (9) and (10) into equation (5) we obtain

$$\Phi\Delta I = (4\pi/\gamma)[\overline{X^2(\Delta x_n)^2}+\overline{Y^2(\Delta y_n)^2}+\overline{Z^2(\Delta z_n)^2}]^{\frac{1}{2}},$$

and this is identical with the expression derived for fixed axes in the orthorhombic case (equation (8)). Thus the results already derived for translations and rotations of the molecules, the axes being fixed, in the orthorhombic system carry straight over into the restricted monoclinic cases. We shall therefore not discuss them further.

(2) *Breathing movements*

In the monoclinic case we cannot derive a breathing point (whose *fractional* co-ordinates do not change) in a manner independent of the choice of axes unless β is constant, and $\Delta a/a = \Delta c/c$. With this restriction it is easily shown that, the orientation of the molecules remaining constant,

$$h(\Delta x_n)+l(\Delta z_n) = -(\Delta a/a)[X(x_n-x_b)+Z(z_n-z_b)] .$$

Once again, it is found that if the averaging is such that $\overline{XZ} = 0$ we obtain, *with these restrictions*, exactly the same formulae as in the orthorhombic case, except that y_b must be taken to be equal to y_c, as might have been expected. As before, $\Phi\Delta I$ is a minimum when the breathing point coincides with the centre of gravity, or, more correctly, falls on a line, parallel to the b axis, which passes through the centre of gravity.

(3) *Change of* β

We shall deal only with the very restricted case in which the molecules remain fixed with respect to the origin, and the axes stay the same length but change their positions slightly. We shall assume the x axis to rotate† through an angle $\Delta\beta_x$ and the z axis through $\Delta\beta_z$. Since the co-ordinates of the molecule in the (stationary) x, y, z frame remain unchanged we have

$$\Delta(Xx_n+Zz_n) = x_n\Delta X+z_n\Delta Z;$$

moreover, $\Delta Y = 0$ and we thus have to obtain the values of ΔX and ΔZ in the stationary X, Y, Z frame for a lattice point which maintains the same indices (h, k, l). These changes are found to be

$$\Delta X = (\Delta\beta_x).Z ,$$
$$-\Delta Z = (\Delta\beta_z).X+Z(\Delta\beta_x-\Delta\beta_z)/\tan\beta .$$

The algebra becomes somewhat complicated, so we select as an illustration the special case $\beta = 90°$. Then

$$\Delta X = (\Delta\beta_x)Z, \quad \Delta Z = -(\Delta\beta_z)X ,$$

and so

$$\overline{[h(\Delta x_n)+l(\Delta z_n)]^2} = \overline{x_n^2Z^2}(\Delta\beta_x)^2+\overline{z_n^2X^2}(\Delta\beta_z)^2-2\overline{x_nz_nXZ}(\Delta\beta_x)(\Delta\beta_z) .$$

If we average over all the relevant values of $1/d \doteq R$, so that $\overline{XZ} = 0$, and if we have the special case $|\Delta\beta_x| = |\Delta\beta_z|$, we see that our result is the same as for the case where the axes are kept fixed and the molecule is rotated by an angle $(\Delta\beta_x)$ about an axis parallel to b and passing through the origin.

General remarks

The results we have derived are all of a statistical nature, and if exact answers were required the formulae would have to be used with caution. They should certainly not be applied to the very low orders, for example, which are influenced by the shape of the molecule and by the nature of solvent in the crystal.

Our statistical assumptions really imply that the 'lumpiness' of the protein is fairly evenly distributed within it, and is roughly the same near, say, the outside of the molecule, as near the inside. Such assumptions will occasionally break down but it is difficult to see any likely way in which our answers could be systematically wrong by a large factor.

The analogy developed above between the monoclinic and the orthorhombic cases is not complete. A simple translation of the molecule, perpendicular to the b axis, will always give, in the monoclinic system, a zone of the reciprocal lattice where the intensity changes are very small or zero. This is true for the orthorhombic case when the translation is parallel to one of the axes, but if, for example, the direction of translation is midway between two axes there is no zone for which the intensity changes are nearly zero. This is because the movement of one molecule in one

† This is not a severe restriction, because we have a choice for the x axis of the unit cell in the monoclinic case.

† Our convention for the direction of rotation is such that $\Delta\beta = \Delta\beta_x-\Delta\beta_z$.

Table 1. *Average $\Phi\Delta I$ for spacings of* 3 Å

$\Phi\Delta I \equiv \{\overline{(\Delta I)^2}\}^{\frac{1}{2}}/\bar{I}$; protein molecule assumed to be a sphere, radius 17 Å.

Nature of the shift	Centric zone ($h0l$)	General reflections
Displacement of the molecule by 0·1 Å, perpendicular to the b axis (cell dimensions and orientation of molecule unchanged)†	30 %	17 %
Change of all cell dimensions by ½ % (orientation of the molecules unchanged) — Molecule fixed with its C. of G. 12 Å from the origin in a direction perpendicular to b	24	15
Change of all cell dimensions by ½ % (orientation of the molecules unchanged) — Breathing movement‡, with the breathing point at the C. of G.	16	11
Rotation of the molecule by ½° about an axis through the C. of G. and parallel to the b axis (cell dimensions unchanged)	28	16
Rotation of the molecule by ½° about an axis through the C. of G. and perpendicular to the b axis (cell dimensions unchanged)	20	
Rotation of the molecule by ½° about an axis 12 Å from its C. of G. parallel to the b axis (cell dimensions unchanged)	42	24
Change of β of the monoclinic cell, $\beta \simeq 90°$, $\lvert\Delta\beta_x\rvert = \lvert\Delta\beta_z\rvert = ½°$ (axes constant length; molecule fixed with its C. of G. 12 Å from the origin)	42	24

† This case is independent of both the size of the molecule and the unit cell. The remainder depend on the former, but not on the latter.

‡ The meaning of this term is explained on page 904.

direction implies movements of the other molecules in different directions, which in such a case are not parallel to one another.

It may be worth pointing out that in every case we are really calculating the effective value of some length or other. It is thus not surprising that in cases of rotation, for example, we need to estimate the 'radius of gyration' of the protein molecule. For this reason the effects are not very sensitive to the molecular weight of the protein, generally increasing only as the cube root of the molecular weight. They are of course also influenced by the shape of the protein, being least, in general, for a spherical molecule.

Examples

As might be expected, all the effects increase linearly with $1/d$. As long as the changes are small, as assumed in the theory, the effects due to rotation are all proportional to the angle of rotation, and those due to breathing movements or pure translations are also proportional to the amount of change imposed. These points should be remembered in studying the examples provided here.

To simplify presentation we have calculated all the numerical examples for spacings of 3 Å ($R = \frac{1}{3}$ Å^{-1}), and for a spherical protein molecule of radius 17 Å, which corresponds to a molecular weight around 15,000. One molecule per asymmetric unit is assumed. The results are the *average* values for $\Phi\Delta I$ for all the relevant reflections having $R \simeq \frac{1}{3}$ Å. Special areas in the reciprocal lattice may have higher local averages. All the results given in Table 1 (except the last) apply to both the monoclinic and the orthorhombic cases. The other centric projections of the orthorhombic cases can be obtained by analogy.

In studying the table the results obtained in the first part of the paper for the values of $\Phi\Delta I$ produced by a heavy atom should be borne in mind. It will be possible to determine only a rather limited number of phases unless $\Phi\Delta I$ for the heavy atom is several times that due to the shifts of the molecules.

Conclusion

Our general conclusion is quite clear. Fairly small shifts of the molecules may produce sufficient changes in the intensities seriously to interfere with the isomorphous-replacement method *at the higher values of* $1/d$. That such changes can occur in certain circumstances has already been shown from an examination of the $h0l$ intensities from monoclinic ribonuclease ($P2_1$) crystallised from different solvents (Magdoff & Crick, 1955). It remains to be seen how great they will be in an isomorphous replacement and whether other proteins will show any such shifts.

On the other hand, if two unit cells have identical cell dimensions to within, say, 1 part in 500 it is unlikely that the molecule will have moved sufficiently to affect appreciably the lower orders of the diffraction pattern. It is thus a sensible precaution to measure cell dimensions as accurately as possible.

It is not yet clear exactly what is the best method for detecting a lack of strict isomorphism in an attempted isomorphous replacement, but an obvious way would be to study how the changes of intensity varied with $1/d$, collecting data from as far out in reciprocal space as possible. If $\Phi\Delta I$ increased rapidly at high $1/d$, for example, this would suggest that some shift had taken place. It should be possible in this way to estimate approximately the range of $1/d$ over which the effects were too small to matter.

Another possible method would be to examine how ΔI varied with intensities of different magnitude (but with similar values of R). The value of ΔI due to the extra atoms is on the average greater for a large intensity than for a small one. On the other hand a lack of strict isomorphism is loosely equivalent to sampling

the continuous transform of the protein at slightly different points, so that ΔI will be largest when the gradient of I in reciprocal space is greatest. Statistically this is likely to be correlated with the smaller values of I. In other words, on the average the extra atoms produce the bigger changes in the larger intensities, whereas shifts of the protein produce the bigger changes in the smaller intensities. We have not yet developed the exact theory for this approach.

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Ox Haemoglobin: Preliminary X-ray Studies

BY F. H. C. CRICK

Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, Cambridge, England

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Crystals of the carbon monoxide compound of ox haemoglobin, grown in ammonium sulphate, are orthorhombic; space group $P2_12_12_1$. The asymmetric unit consists of one haemoglobin molecule (molecular weight $\sim$ 68,000). A study of the very low order intensities shows the approximate positions of the molecules in the unit cell.

The Patterson projection along one axis resembles that of the a-axis projection of monoclinic horse haemoglobin, and suggests that the molecules of the two species may have certain broad features in common.

Introduction

This short paper presents preliminary X-ray work on ox haemoglobin. It forms part of a Ph.D. thesis accepted by the University of Cambridge (Crick, 1953). Further X-ray work on this protein is being undertaken by Dr D. W. Green, and will be reported elsewhere.

The crystals were very kindly supplied by Mr G. S. Adair. This appears to have been the first occasion on which crystals were grown successfully. The haemoglobin had been dissolved in half-saturated ammonium sulphate, and converted from oxyhaemoglobin to carbonmonoxyhaemoglobin by bubbling carbon monoxide through the solution. The saturated solution was then stored in a cold-room at 0° C. and as the solution became more concentrated, owing to slow evaporation, crystals were deposited.

1. Experimental results

In order to avoid taking X-ray pictures at 0° C. some mother liquor was first equilibriated at room temperature for a few days. As the protein is less soluble at higher temperatures, some of it precipitated and was filtered off. The crystals were then transferred to this new mother liquor. The density of the mother liquor was 1·159 g.cm.$^{-3}$, so the concentration of the ammonium sulphate was about $2\frac{1}{2}$ M.

More recently Dornberger-Schiff (1954) has obtained similar, but not identical, crystals by a method in which crystals were formed by allowing alcohol vapour to diffuse into a concentrated aqueous solution of the protein in the oxy form.

The crystals, some of which had dimensions of 1–2 mm., were tabular. Their shape and crystallographic properties are shown in Fig. 1. The most significant

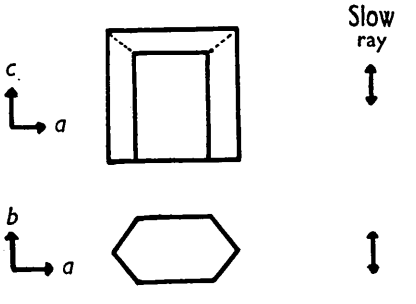

Fig. 1. Properties of crystals of ox haemoglobin.