None of these structure determinations, including the present, are up to modern standards (counter data, anisotropic refinement, refinement of hydrogen atoms) but taken as a whole they suggest that bonds significantly longer than 1·54 Å occur in cyclobutane compounds. An explanation of long C–C bonds in cyclobutane rings has been given by Dunitz & Schomaker (1952) as arising from non-bonded carbon–carbon repulsion. In this compound only two of the four C–C bonds are lengthened; thus the non-bonded interaction is reduced without affecting the five-membered ring.

I am indebted to Professor David H. Templeton for permitting the use of his facilities and for his general encouragement of this work. I also thank Professor Philip E. Eaton for suggesting the problem and providing the crystals. The calculations were done at the Computation Center of the Massachusetts Institute of Technology, Cambridge, Massachusetts.

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Combination of Multiple Isomorphous Replacement and Anomalous Dispersion Data for Protein Structure Determination

I. Determination of Heavy-Atom Positions in Protein Derivatives

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In applying the multiple isomorphous replacement method to study the structure of proteins, it is necessary first to establish and correlate the positions occupied by the heavy atoms in the various derivatives. This paper discusses the advantages of systematic exploitation of the anomalous scattering information from the heavy atoms in these derivatives, in addition to the usual isomorphous crystal data, in establishing the heavy atom positions. It is shown that by proper combination of the isomorphous derivative and anomalous scattering information, one can remove many of the undesirable features that crop up when either of these alone is used to establish the heavy atom positions.

Introduction

As is well known, the problem of determining the phase angles of reflections for proteins is solved in the multiple isomorphous replacement method (Harker, 1956) by preparing a series of isomorphous derivatives of the protein, each one containing groups of heavy atoms attached to different, but definite, sites. The first main problem of the crystallographer is to locate the positions of these heavy atoms, and, in addition, in certain cases to correlate the positions of the heavy atom in the different derivatives. Once this stage has been successfully accomplished, the second problem is the refinement of heavy atom positions and occupancy parameters *etc.*, and the third is the use of this information to evaluate the phase angles of the protein reflections.

In our study of the protein ribonuclease, we have found that during all these stages of the structure determination, the additional information available as a result of the anomalous scattering of the heavy atom (besides the usual isomorphous data) was of great value, not only in confirming the results obtained by using isomorphous data in the usual way, but also complementing this information in a very elegant manner. Thus, for example, it has been found that by properly combining isomorphous and anomalous dispersion data, we are able to overcome many of the undesirable features that arise when either kind of data is used alone. Consequently, for all the heavy atom derivatives we studied, the experimental data for any given reflection S(*hkl*) consisted not only of the corresponding structure amplitudes $|F_P|$ and $|F_{PH}|$* of the protein and the heavy atom derivative, but also the direct and inverse reflections $|F(\mathrm{S})|$ and $|F(-\mathrm{S})|$, of the heavy atom derivative [In future, we shall, for brevity, denote $F(\mathrm{S})$ and $F(-\mathrm{S})$ simply as $F(+)$ and $F(-)$]. In this paper, we shall describe some of the techniques used in the first stage of the analysis, namely the determination of the positions of the heavy atoms in the different derivatives. In part II, the correlation of the positions of the heavy atoms in the various isomorphs is discussed, and in future communications, the refinement of the heavy atom positions and the determination of the phase angles of the protein reflections will be treated.

Various methods have been proposed and used for finding and correlating the positions of the heavy atoms (see, for a recent review, Dickerson, 1964). These methods use certain Patterson functions which yield the heavy atom–heavy atom vectors. A study of the approximate theory behind these maps indicates that, in addition to the desired vectors, the maps contain a lot of undesirable background. Also, the process of finding the heavy atom positions from the maps becomes increasingly difficult and uncertain as the number of heavy atoms in a derivative increases. We describe below some of the functions we have used for obtaining maps which contain very little undesirable background. This is achieved because the background features of the isomorphous and anomalous scattering data are, in a sense, 'anticomplementary', *i.e.* the background features tend to cancel out when the two sets of data are properly combined. This is easily seen by examining the functions used to obtain vector maps of the heavy atoms in the two cases.

Heavy atom vector map from the isomorphous data

Let us denote by $|F_P|$ and $|F_{PH}|$ the structure amplitudes of the protein and the heavy atom derivative, respectively, for a particular reflection. The difference $(F_{PH}-F_P)$ arises because of the additional scattering from the heavy atom, namely F_H (leaving aside errors due to lack of isomorphism, *etc.*). Since we measure only $|F_{PH}|$ and $|F_P|$, we have to deduce the positions of the heavy atoms from the difference of the magnitudes alone, namely $(|F_{PH}|-|F_P|)$, or some function related of this.

From Fig. 1, it is seen that the difference $(|F_{PH}|-|F_P|)$ is given by

$$\begin{aligned}(|F_{PH}|-|F_P|) &= AC-AB\\ &= AD-AB\\ &= BD\,.\end{aligned}$$

Using the relation

$$(BD/BC)=(\sin BCD/\sin BDC)$$

it is seen that

$$\begin{aligned}(|F_{PH}|-|F_P|)=|F_H|\cos(\alpha_{PH}-\alpha_H)\\ +|F_H|\sin(\alpha_{PH}-\alpha_H)\tan(\alpha_{PH}-\alpha_P)/2\,,\end{aligned}\quad(1)$$

which can be rearranged to give

$$\begin{aligned}(|F_{PH}|-|F_P|)=|F_H|\cos(\alpha_{PH}-\alpha_H)\\ -2|F_P|\sin^2(\alpha_{PH}-\alpha_P)/2\,.\end{aligned}\quad(2)$$

The heavy atom vector map could be obtained if we knew F_H, but from the measurements of $|F_{PH}|$, we get only a quantity $(|F_{PH}|-|F_P|)$, related to F_H, as shown in equations (1) and (2). We shall denote the difference $(|F_{PH}|-|F_P|)$ by $\Delta|F|_{\mathrm{iso}}$ so that we have

$$\Delta|F|_{\mathrm{iso}}=|F_H|\cos(\alpha_{PH}-\alpha_H)-2|F_P|\sin^2(\alpha_{PH}-\alpha_P)/2.\quad(3)$$

The second term on the right hand side of equation (3), $\sin^2(\alpha_{PH}-\alpha_P)/2$, will usually be small compared with unity. The only cases where the difference $(\alpha_{PH}-\alpha_P)$ can be large are when F_P is small or F_H very large. Thus, it is easily seen that the second term on the right hand side of equation (3) is almost always small compared with the first term, and hence, to a good approximation, we can write

$$\begin{aligned}\Delta|F|_{\mathrm{iso}}&=(|F_{PH}|-|F_P|)\\ &\simeq|F_H|\cos(\alpha_{PH}-\alpha_H)\,.\end{aligned}\quad(4)$$

The above relation between $\Delta|F|_{\mathrm{iso}}$ and $|F_H|$ is important, particularly since $(\Delta|F|_{\mathrm{iso}})^2$ has been used for obtaining the heavy atom vector maps (see, *e.g.* Perutz, 1956). To see this clearly, let us express the cosines in equation (4) in terms of exponentials as follows*:

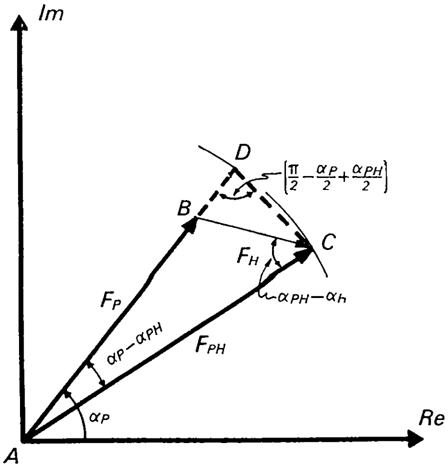

Fig. 1. Representation of structure factors F_{PH}, F_P and F_H in the complex plane: F_P, F_{PH} and F_H are the structure factors of the protein, the heavy atom derivative and the heavy atom, respectively. The α's are the corresponding phase angles. By construction $AD=AC$ in triangle ADC.

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* $|F_{PH}|$ is actually obtained as the square root of the average of $|F(\mathrm{S})|^2$ and $|F(-\mathrm{S})|^2$.

* To appreciate fully the following argument, see Ramachandran & Raman (1959) and Kartha (1961).

$$\begin{aligned} \Delta|F|_{\text{iso}} &= (\tfrac{1}{2})|F_H|\{\exp[i(\alpha_{PH}-\alpha_H)]+\exp[-i(\alpha_{PH}-\alpha_H)]\} \\ &= (\tfrac{1}{2})\{F^*_H \exp i\alpha_{PH}+F_H\exp(-i\alpha_{PH})\} \end{aligned} \tag{5}$$

Hence

$$(\Delta|F|_{\text{iso}})^2=\underset{\text{(I)}}{(\tfrac{1}{2})F^2_H}+\underset{\text{(II)}}{(\tfrac{1}{4})(F^*_H)^2\exp 2i\alpha_{PH}}+\underset{\text{(III)}}{(\tfrac{1}{4})(F_H)^2\exp(-2i\alpha_{PH})}\,. \tag{6}$$

The first term, denoted by (I), gives rise to a Patterson map of the heavy atoms with peaks of height $(\frac{1}{2})f_{Hi}f_{Hj}$ at positions $\pm(\mathbf{r}_{Hi}-\mathbf{r}_{Hj})$ (Here $\mathbf{r}_{Hi}$, $\mathbf{r}_{Hj}$ are the heavy atom sites and f_{Hi} and f_{Hj} are the corresponding scattering factors). This is the information in which we are actually interested. The second and third terms, denoted by (II) and (III), give rise to unwanted background, which tends to mask the features we are looking for. The background arising from (II) may be regarded as the convolution of the Fourier transform (F. T.) of $(F^*_H)^2$ with that of $\exp 2i\alpha_{PH}$. The F. T. of $(F^*_H)^2$ gives the Patterson function of the second kind (Patterson, 1949) for the heavy atoms and contains peaks at $(\mathbf{r}_{Hi}+\mathbf{r}_{Hj})$. By the very random nature of α_{PH}, however, the F. T. of $\exp 2i\alpha_{PH}$ is not likely to have a high concentration at any particular position. Consequently, the convolution of the F. T. of $(F^*_H)^2$ and the F. T. of $\exp 2i\alpha_{PH}$, would, to a crude approximation, result only in a fluctuating background, of strength small compared with $f_{Hi}f_{Hj}$. A similar argument holds for (III). Whether the background actually masks the peaks we are looking for depends on the details of the protein structure and also on the number and grouping of the heavy atoms. In any case, whatever the background due to (II) and (III), it can be eliminated as shown in the following sections, and hence, an elaborate discussion of the nature of the background is not attempted here.

Heavy atom vector map from anomalous dispersion data

Let us denote by $F(+)$ and $F(-)$ the structure factors of the direct and inverse reflections, namely hkl and $\bar{h}\bar{k}\bar{l}$ for the heavy atom derivative [for the free protein, there is no difference between $F(+)$ and $F(-)$]. If there were no anomalous dispersion, we could write

$$F(+)=\sum_j f_j \exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) \tag{7}$$

$$F(-)=\sum_j f_j \exp(-2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j)\,, \tag{8}$$

where the summation is over all the atoms in the unit cell. When anomalous dispersion is present, however, the scattering factor f_j is no longer real, but becomes a complex number and is given by the expression (James, 1954):

$$f_j=f^0_j+\Delta f'_j+i\Delta f''_j$$

where f^0_j is the scattering factor when there is no anomalous dispersion and $\Delta f'$ and $\Delta f''$ are the dispersion corrections. For brevity, we shall in the future denote the quantity $(f^0_j+\Delta f'_j)$ by f'_j and $\Delta f''_j$ by f''_j so that we can write f_j as equal $(f'_j+if''_j)$. Under these conditions, equations (7) and (8) take the following form:

$$F(+)=\sum_j (f'_j+if''_j)\exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) \tag{9}$$

$$F(-)=\sum_j (f'_j+if''_j)\exp(-2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j)\,. \tag{10}$$

In the case of protein derivatives, the main contribution to the anomalous scattering is due essentially to the heavy atoms (see, however, Blow, 1958; de Vries, 1960). Then equations (9) and (10) can be written

$$F(+)=\sum_j^H (f'_j+if''_j)\exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j)+\sum_j^L f^0_j\exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) \tag{11}$$

and

$$F(-)=\sum_j^H (f'_j+if''_j)\exp(-2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j)+\sum_j^L f^0_j\exp(-2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j)\,, \tag{12}$$

where the superscripts H and L over the summation signs denote that the summation has to be carried over the heavy atoms and light atoms respectively. Using the definitions

$$F'_H(+)=\sum_j^H f'_j\exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) \tag{13}$$

$$F''_H(+)=\sum_j^H f''_j\exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) \tag{14}$$

$$F_P(+)=\sum_j^L f^0_j\exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) \tag{15}$$

and the corresponding quantities for the inverse reflections, equations (11) and (12) can be compactly written

$$F(+)=F'_H(+)+iF''_H(+)+F_P(+) \tag{16}$$

$$F(-)=F'_H(-)+iF''_H(-)+F_P(-)\,. \tag{17}$$

(Note that in the definition of F'_H, the additional contribution due to the terms containing $\Delta f'$is taken into account.) From now on we shall denote F'_H simply as F_H.

Using the definition

$$F_P+F_H=F_{PH}\,,$$

equations (16) and (17) take the simple form

$$F(+)=F_{PH}(+)+iF''_H(+) \tag{18}$$

$$F(-)=F_{PH}(-)+iF''_H(-)\,. \tag{19}$$

Further, if the anomalous scatterers are of the same type, with the same value for the ratio (f''/f'), the phase angle α''_H of the vector $i\,F''_H$ will differ from the phase angle α_H of F_H by $\pi/2$ (Fig. 2). Under these conditions, it can be easily seen from Fig. 2 that (see, *e.g.*, Ramachandran & Raman, 1956)

$$|F(+)|^2=|F_{PH}|^2+|F''_H|^2 + 2|F_{PH}|\,|F''_H|\cos(\pi/2+\alpha_H-\alpha_{PH}) \quad (20)$$

$$|F(-)|^2=|F_{PH}|^2+|F''_H|^2 - 2|F_{PH}|\,|F''_H|\cos(\pi/2+\alpha_H-\alpha_{PH})\,. \quad (21)$$

In deriving the above equations, we have used the relations

$$|F_{PH}(+)|^2=|F_{PH}(-)|^2(=|F_{PH}|^2)$$

and

$$|F_H(+)|^2=|F_H(-)|^2\;(=|F_H|^2).$$

From equations (20) and (21) we have

$$|F(+)|^2-|F(-)|^2=4|F_{PH}|\,|F''_H|\sin(\alpha_{PH}-\alpha_H)\,. \quad (22)$$

To a good approximation, we have $(\frac{1}{2})[|F(+)|+|F(-)|] \simeq |F_{PH}|$, and

$$|F(+)|-|F(-)|=[|F(+)|^2-|F(-)|^2]/[|F(+)|+|F(-)|] =2|F''_H|\sin(\alpha_{PH}-\alpha_H)\,. \quad (23)$$

Assuming that the ratio (f''/f') is the same for all the anomalous scatterers in the protein derivative, it follows that

$$F''_H=\sum_j^H f''_j \exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) =\sum_j^H (f''_j/f'_j)f'_j\exp(2\pi i\,\mathbf{S}\,.\,\mathbf{r}_j) =(f''/f')\,F_H\,. \quad (24)$$

Combining equations (23) and (24), we have

$$|F(+)|-|F(-)|=2(f''/f')|F_H|\sin(\alpha_{PH}-\alpha_H)$$

or

$$\tfrac{1}{2}[|F(+)|-|F(-)|]\,(f'/f'')=|F_H|\sin(\alpha_{PH}-\alpha_H)\,. \quad (25)$$

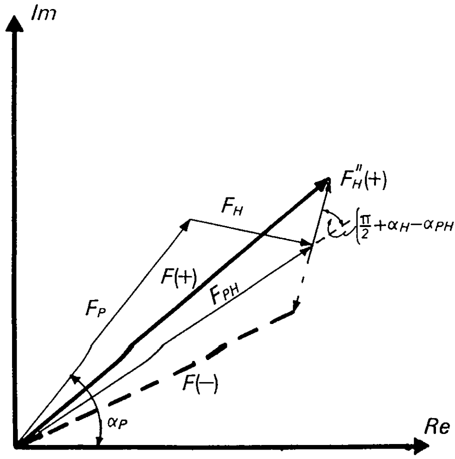

Fig. 2. Representation of the structure factors $F(+)$ and $F(-)$ in the complex plane. In this diagram $F(+)$ and $F(-)$ are the structure factors of the direct and inverse reflections, respectively, and $F_H''(+)$ and $F_H''(-)$ are the corresponding quantities for the heavy atom contribution due the 'imaginary part' of the anomalous dispersion correction. $F(-)$ and $F_H''(-)$ have been reflected about the real axis to show the difference between $|F(+)|$ and $|F(-)|$.

We shall define the anomalous difference $\Delta|F|_{\text{ano}}$ as equal to $[|F(+)|-|F(-)|]/(f'/2f'')$. Then we have

$$\Delta|F|_{\text{ano}}=|F_H|\sin(\alpha_{PH}-\alpha_H)\,. \quad (26)$$

From a comparison of equations (4) and (26), it is obvious that $\Delta|F|_{\text{ano}}$ contains the complementary features of the desired information, namely, the heavy atom positions. To show that the background features inherent in the anomalous dispersion data are 'anti-complementary' to those of isomorphous data, we express the sines in equations (26) in terms of exponentials as follows:

$$\Delta|F|_{\text{ano}} =\frac{1}{2i}|F_H|\{\exp[i(\alpha_{PH}-\alpha_H)]-\exp[-i(\alpha_{PH}-\alpha_H)]\} =\frac{1}{2i}[F_H^*\exp(i\alpha_{PH})-F_H\exp(-i\alpha_{PH})]\,. \quad (27)$$

Consequently

$$(\Delta|F|_{\text{ano}})^2=\underset{\text{(I)}}{(\tfrac{1}{2})F_H^2}-\underset{\text{(II)}}{(\tfrac{1}{4})(F_H^*)^2\exp(2i\alpha_{PH})} -\underset{\text{(III)}}{(\tfrac{1}{4})(F_H)^2\exp(-2i\alpha_{PH})}\,. \quad (28)$$

A comparison of equations (28) and (6) immediately reveals that the first term (I) is exactly the same in both the isomorphous and anomalous difference coefficients. Also, the second and third terms, (II) and (III) in equation (28) differ only in sign from the corresponding terms in equation (6), showing thereby that whenever a peak occurs in isomorphous syntheses due to (II) and (III) when $(\Delta|F|_{\text{iso}})^2$ are used as Fourier coefficients, a corresponding hole occurs in the synthesis with coefficients $(\Delta|F|_{\text{ano}})^2$, and *vice versa*. Consequently, a synthesis with coefficients $(\Delta|F|_{\text{iso}})^2+(\Delta|F|_{\text{ano}})^2$ gives just the heavy atom Patterson function alone, as the backgrounds in the two syntheses cancel out and the wanted Patterson peaks add up.

The use of $(\Delta|F|_{\text{iso}})^2$ and $(\Delta|F|_{\text{ano}})^2$ separately for determining the heavy atom position is well known (Dickerson, 1964; Rossmann, 1961). Owing to the very approximate theoretical analysis of the quantities $\Delta|F|_{\text{iso}}$ and $\Delta|F|_{\text{ano}}$, however, the inherent complementary features were not noticed by the previous workers.

Combining isomorphous and anomalous dispersion data for determining the heavy atom positions

To summarize the results of the previous sections, the two basic equations for the theory are

$$\Delta|F|_{\text{iso}}=|F_H|\cos(\alpha_{PH}-\alpha_H) \quad (4)$$

$$\Delta|F|_{\text{ano}}=|F_H|\sin(\alpha_{PH}-\alpha_H)\,. \quad (26)$$

Consequently

$$(\Delta|F|_{\text{iso}})^2+(\Delta|F|_{\text{ano}})^2=|F_H|^2\{\cos^2(\alpha_{PH}-\alpha_H) +\sin^2(\alpha_{PH}-\alpha_H)\}=|F_H|^2\,. \quad (29)$$

Thus, a synthesis with the left hand side of equation (29) as Fourier coefficients yields just the heavy atom Patterson vectors.

Equation (29) has been used for determining the heavy atom positions in the various dyed derivatives of ribonuclease with good results. The maps thus obtained by combining the isomorphous derivative and anomalous scattering information were found to give larger peak to background ratio than the maps obtained by using either of the data separately. It is to be expected that this improvement will be more marked when maps of derivatives containing more than two or three heavy atom replacements are studied at low resolution. The details of the results obtained in the different derivatives of ribonuclease will be published shortly.

The authors are grateful to Prof. David Harker for the many stimulating discussions they had with him and his keen interest in this study. We are thankful to Drs D. R. Harris and H. H. Mills and Miss Elaine De Jarnette for help in collecting and processing the data which were used in testing some of the above methods. The investigation was supported in part by the Public Health Service Research Career Development Program Award GM-K3-16737 from the National Institutes of Health to one of us (G. K.) and research grants NIH-A-3942, NSF-GB-429 and the Dean Langmuir Foundation.

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Combination of Multiple Isomorphous Replacement and Anomalous Dispersion Data for Protein Structure Determination
II. Correlation of the Heavy-Atom Positions in Different Isomorphous Protein Crystals

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As a first step in applying the multiple isomorphous series method for protein structure determination, one has to determine the positions occupied by the heavy atoms in the various heavy atom dyed crystals. Usually this part of the analysis proceeds in two steps. First, the relative arrangement of the heavy atoms in a single derivative is established from vector maps and the second step correlates these different configurations with respect to a common origin. It was shown in part I how combination of isomorphous replacement and anomalous scattering data gives very valuable information for the solution of the first stage of the analysis. In this paper we show how similar methods can be used for establishing the positions of the heavy atoms in the different heavy atom derivatives with respect to the same origin.

Introduction

In the preceding article (part I. Kartha & Parthasarathy, 1964) we have discussed a method for combining multiple isomorphous replacement and anomalous dispersion data for determining the heavy atom positions in protein derivatives. The next problem is to relate the positions of the heavy atoms in the different derivatives to a common origin. This problem of correlating the positions of the heavy atoms in various derivatives becomes serious in certain space groups; for example $P2_1$ or $P4$, where the origin of reference is not fixed by symmetry alone, but can be taken anywhere along the symmetry axis. In these cases, in order to draw the phase circles with correct orientations, we have to refer the coordinates of the heavy atoms in all the derivatives to a common origin. Various methods have been suggested for doing this (Harker, 1956; Bragg, 1958; Perutz, 1956; Blow, 1958; Rossmann, 1960), of which Rossmann's method has been particularly successful. He uses as Fourier coefficients $(|F_{PH1}|-|F_{PH2}|)^2$, where the subscripts 1 and