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Introduction.

The use of the multiwavelength anomalous scattering method (MWM) to crystallography is of vintage origin. The present writer has been involved with developing phasing techniques using this method since 1952. The basic ideas of this method were known almost fifteen years ago, but its utility has been very limited. With the availability of intense tunable synchrotron radiation sources there is a revival and rediscovery of many of these older ideas.

Under normal conditions of scattering it is not possible to distinguish electrical or optical antipodes of non-centric crystals because of Friedel's law:

$$I(hkl) = I(\bar{h}\bar{k}\bar{l}) \quad (1)$$

However, in Zincblende using X-radiation close to the absorption edge of Zn, where the scattering factor becomes complex, it was shown [Nishikawa & Matukawa (1928); Coster et al., (1930)] that Friedel's law breaks down i.e.

$$I(hkl) \neq I(\bar{h}\bar{k}\bar{l}) \quad (2)$$

Almost 20 years later Bijvoet (1949) pointed out the profound significance of this discovery to molecular structure and crystal structure analysis, that this inequality can be used not only to determine the absolute configuration of a molecule but also the phases of reflections.

Near the absorption edge the scattering factor becomes complex.

$$f = f_0 + f' + if'' \quad (3)$$

The dispersion corrections f' and f'' vary with wavelength and the case of X-rays is illustrated in Fig. 1. f' is usually negative and f'' is scattered $\pi/2$ ahead of the phase of the real part $f_0 + f'$.

Phasing using MWM in X-rays.

From Fig. 1 it is obvious that by varying the incident wavelength one can get different values of f' and f'' and thus in effect vary the atomic scattering factor. MWM concerns itself with using different incident wavelengths for solving the phase problem. If one collects data with two incident radiations λ_1 and λ_2 for which the scattering factor of the anoma-

lous scatterer changes (say for λ_1 , $f_{an} = f_0$ and for λ_2 , $f_{an} = f_0 + f'$) it is equivalent to collecting data on two perfectly isomorphous crystals. This change in scattering factor with wavelength has been used to differentiate two atoms which are close to each other in the periodic table [Mark and Szillard (1925); Bradely and Rodgers (1934); Jones and Sykes (1937)].

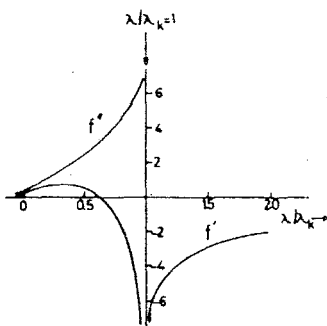


Fig. 1:-Variation of f' and f'' with wavelength near an X-ray absorption edge.

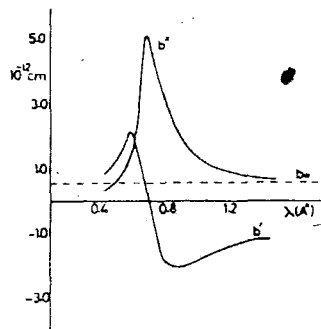


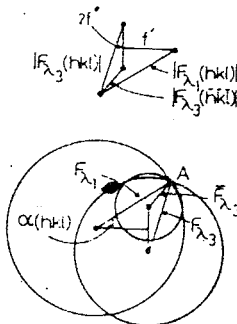
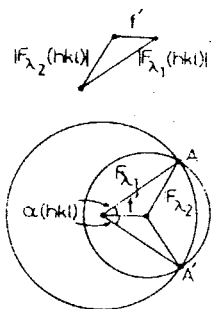
Fig. 2:-Variation of f_0 , f' and f'' for ^{113}Cd near resonance for thermal neutrons.

In early 1952 while reading the paper by Bokhoven, Schoone and Bijvoet (1951) in which the structures of strychnine sulphate and selenate were solved using the isomorphous replacement method (IRM), the potential of changing the wavelength for phase determination suggested itself to the author. The 1951 paper brings out clearly the bimodal ambiguity which exists in the phases when IRM is used. It was clear that if instead of using two isomorphous crystals one uses two wavelengths (λ_1 and λ_2) one has a similar situation, (Fig. 3), where again there is an ambiguity. If now we use Bijvoet's second suggestion and measure $I(hkl)$ and $I(\bar{h}\bar{k}\bar{l})$ then the bimodal ambiguity can also be removed (Fig. 4). It may be noticed that in the above diagrams the anomalous scatterer has been placed at the origin so that phases are determined with respect to the anomalous scatterer. Hence, apart from the actual values of f' and f'' for the different wavelengths, the position of anomalous scatterer must also be known if the structure is to be solved completely.

Position of the Anomalous Scatterer

This can be done by computing the anomalous difference Patterson (ADP) with $|F_{\lambda_n}^2 - F_{\lambda_m}^2|_{hkl}$ as coefficients, which is similar to the isomorphous difference Patterson of Buerger (1942). This contains interactions between

anomalous scatterers themselves, the anomalous scatterers and normal atoms, while those between the normal atoms get eliminated. However, another Patterson synthesis using $|F_{\lambda_m} - F_{\lambda_n}|^2_{hkl}$ which was tried out in 1954 gives only the anomalous scatterer vectors (Ramaseshan, Venkatesan and Mani 1957; also see Ramaseshan (1964) for a review and references). This synthesis was independently suggested by Rossmann and has now found great use in protein crystallography. For non-centric crystals also this synthesis gives the anomalous scatterer vectors.



Harker construction for MWM with the anomalous scatterer at the origin.

Fig.3:-Ambiguous solution: data at $\lambda_1(f_{an}=f_o)$ and $\lambda_2(f_{an}=f_o + f')$

Fig.4:-Unique solution: data at $\lambda_1(f_{an}=f_o)$ and $\lambda_3(f_{an}=f_o+f'if'')$

If one has two isomorphous crystals P and AP, the latter containing an anomalous scatterer, then from the known experimental values $|F_p(hkl)|$, $|F_{AP}(hkl)|$, $|F_{AP}(\bar{h}\bar{k}\bar{l})|$ it is possible to calculate the value of the anomalous scatterer structure factor $F_A(hkl)$ with a bimodal ambiguity [Mathews (1965); Kartha and Parthasarathy (1965); Singh and Ramaseshan (1966)]. The lower of the two values $|F_A(hkl)|_L$ is more often the correct one. Hence, a Patterson using $|F_A(hkl)_2|_L^2$ gives the vector distribution of the heavy atoms.

This method requires two isomorphous crystals and the anomalous data on one. But while considering the use of neutron anomalous scattering for phasing it was pointed out (Ramaseshan, 1966) that if data is collected with three appropriate wavelengths, the position of the anomalous scatter and the phases of reflections can be determined unambiguously.

Neutron and Gamma-ray Anomalous Scattering.

Peterson and Smith (1961) demonstrated the breakdown of Friedel's law in CdS in neutron scattering. ^{113}Cd , ^{149}Sm , ^{151}Eu and ^{157}Gd exhibit resonance absorption in the thermal neutron region. The scattering length in

neutrons in the resonance region can be represented very well by a one term Breit-Wigner formula and Fig. 2 gives the variation of f_0 , f' and f'' and with wavelength near the absorption region. In the case of X-rays the dispersion corrections are small, only about 10 to 15 per cent of f_0 while in the case of neutrons, these are large, almost 500 to 1000 per cent of f_0 . In 1964 the present writer suggested that this large anomalous dispersion can be used to solve the structures of macromolecules. The following points were made [Ramaseshan (1966) see also Singh and Ramaseshan (1968)]

1. with the proper choice of incident wavelengths one can obtain f' values of $5f_0$ to $10f_0$. This is equivalent to having a heavy atom in the structure. Hence, all the techniques associated with the heavy atom method can be used for phasing,
2. in a non-centric structure if the position of the anomalous scatterer is determined and if the intensity of Bijvoet pairs are measured, the phases of the reflections can be determined with a two-fold ambiguity,
3. if the measurements are made with two wavelengths it is equivalent to collecting data in two perfectly isomorphous crystals,
4. by collecting data using three appropriate wavelengths the position of the anomalous scatterer, and the phases of the reflections can be determined without ambiguity.

The number of structures solved using neutron anomalous scattering since then is quite small (about six). The only protein which has been successfully phased is Myoglobin (Schoenborn, 1975). The statement made by Mason in 1979 summarises the general situation "Following the suggestion of Ramaseshan in 1964 work began in Harwell on insulin crystals by Moore and Macdonald (1970) and later by Hodgkin, Willis, Fuess and Mason (1973). In the beginning it was thought that the potential power of anomalous dispersion of neutrons would contribute to the then unknown crystal structure of Insulin. But the limitations of flux did not permit this objective to be reached".

Raghavan (1961), Moon (1961) and others suggested that anomalous scattering of gamma radiation (Mossbauer Effect) can be used for phase determination. Nuclear levels being sharper, anomalous effects are greater. f' and f'' in the nuclear case are two orders greater than those in neutron scattering. There also appeared to be many advantages in using the Mossbauer Effect in phase determination: wavelengths were easily tunable to

different points on the resonance curve; gamma-ray sources are very stable etc. However, the intensity of gamma-rays are almost 4 to 5 orders lower than that of X-ray sources and 2 to 3 orders less than that of neutron sources. Although the anomalous effects increase dramatically the abysmal decrease of incident intensity makes this method not too useful at present.

In the case of electron diffraction, dynamical effects also cause the violation of Friedel's law (even in the absence of resonance) making it not very useful for phase determination.

It was clear that the multiwavelength method may not be very practical for phase determination in crystallography because of intensity problems. Hence, MWM was used for solving physical problems i.e. for the determination of static displacement, polarisation vectors etc., (for a review see Ramaseshan et al., 1975). Perhaps the most useful application of MWM so far has been to the determination of the partial structure factors of binary liquids and glasses (Ramesh and Ramaseshan, 1971).

The Synchrotron Radiation.

The picture has changed considerably since then with the advent of the intense synchrotron radiation sources as also the recent remarkable discovery (Templeton et al., 1980) of very large anomalous factors f' and f'' near the absorption edges. For example, in caesium tartrate near the L edges the observed values are as much as $f' = 26.7$ and $f'' = 16.1$. With such large changes possible in the scattering factors with wavelength it seems that MWM may be a viable alternative to IRM, [see Phillips et al., (1977); Phillips et al., (1978); Templeton et al., (1980); Lye et al., (1980); Phillips and Hodgson (1980)].

If this method is to be used systematically many questions have to be answered. To get the best phasing (i) how many wavelengths must one use?; (ii) are there any advantages in using specific wavelengths and, if so, what are the criteria for choosing them; and (iii) should the experimental time for different wavelengths be the same or different?

Phillips and Hodgson (1980) attempted to answer these questions by computer simulation. Assuming hypothetical proteins of molecular weights ranging from 12,000 to 100,000 they first chose one set of wavelengths and calculated the structure factors (with phases). Assuming random experimental errors they also calculated the r.m.s. errors. Different sets of wavelengths were chosen and from similar calculations one could select the "best" set for experimentation. While this approach is pragmatic it

consumes a great deal of computer time.

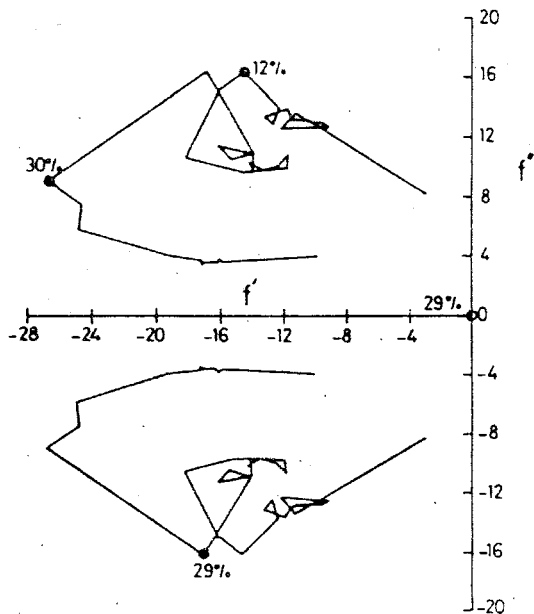


Fig.5:- Argand diagram representing f' and $\pm f''$ for calcium in calcium tartrate near L_I , L_{II} and L_{III} edges (Templeton et al., 1980).

Another approach (Narayan and Ramaseshan 1981; Ramaseshan and Narayan 1980; 1981) is to get an analytical formula for the r.m.s error $\langle \Delta\phi^2 \rangle$ in terms of f' and f'' of the atom for different wavelengths. If $f' + if''$ is represented in the complex plane as first suggested by Herzerberg and Lau (1967) then f' and f'' are the coordinates of the chosen "centres".

It is assumed that the positions of the anomalous scatterers are known. Assuming random experimental errors, structure factors F_i and r.m.s. errors Δ_i are determined by the methods of Blow and Crick (1959) and their modifications [see for example Cullis et al., (1961); North (1965); Mathews (1966)]. It must be noted that Δ_i for different wavelengths (i.e. different "centres") will be different because the source intensity and the absorption vary with wavelength. However, the measuring time can be chosen by the experimenter [for details of the procedure followed see Narayan and Ramaseshan (1981)]. If X_i and Y_i are the coordinates of a point in the complex plane then the mean square error

$$\langle \Delta\phi^2 \rangle_{\text{calc}} = 1/\sqrt{D} \quad (4a)$$

where

$$D = \left[\sum_{i=1}^n \frac{1}{\Delta_i^2} (X_i - \bar{X})^2 \right] \left[\sum_{i=1}^n \frac{1}{\Delta_i^2} (Y_i - \bar{Y})^2 \right] - \left[\sum_{i=1}^n \frac{1}{\Delta_i^2} (X_i - \bar{X})(Y_i - \bar{Y}) \right]^2 \quad (4b)$$

$$\bar{X} = \frac{\sum_{i=1}^n X_i}{\sum_{i=1}^n \frac{1}{\Delta_i^2}} \bigg/ \frac{\sum_{i=1}^n 1}{\sum_{i=1}^n \frac{1}{\Delta_i^2}} \quad (4c)$$

$$\bar{Y} = \frac{\sum_{i=1}^n Y_i}{\sum_{i=1}^n \frac{1}{\Delta_i^2}} \bigg/ \frac{\sum_{i=1}^n 1}{\sum_{i=1}^n \frac{1}{\Delta_i^2}} \quad (4d)$$

one notes that D is the determinant of the moment of inertia tensor of the two dimensional collection points (corresponding to the set of wavelengths chosen) - each point having a mass of $1/\Delta_i^2$.

Hence, to minimise the phase error $\langle \Delta\phi^2 \rangle$ one has to maximise D. For example, for three wavelengths if Δ_i 's are equal, the area of the triangle formed by the three points (X_i, Y_i) in the complex plane must be maximised.

This theory is strictly valid only when the r.m.s. error is small compared to 2π . However, a plot of the phase errors calculated using (4) against the "experimental" values determined by the computer simulation experiments of Phillips and Hodgson shows that they are monotonically related, so that if the value of $\langle \Delta\phi^2 \rangle$ is calculated from (4) is minimised then its true value will also be minimised.

Strategy to use MWM in Protein Crystallography.

- (1) Obtain a protein crystal with an anomalous scatter in it.
- (2) Measure (a) the variation with wavelength of the sources intensity and the absorption; (b) calculate $\sigma(\omega)$, the cross section and its variation with wavelength.
- (3) Calculate $f''(\omega) = m\omega\sigma(\omega)/4\pi e^2$ and $f'(\omega) = 2/\pi \int_0^\omega f'(\omega') d\omega'/\omega^2 - \omega'^2$ (Kramers Kronig relation), (this calculation is necessary since f' and f'' are different for the same atom in different environments).
- (4) Plot f'' vs f' curve and select plausible "centres" (Friedel pairs need not be chosen).
- (5) Each centre is weighted by a factor $1/\Delta_i^2$, noting that Δ_i is proportional to the number of counts; the source intensity; absorption; and time.
- (6) Calculate D (formula 4) and chose that set which corresponds to the maximum value of D.

The relative times for different wavelength can also be optimised, but this involves non-linear equations. The process is tedious but high convergence is not required as r.m.s. phase errors depend on $D^{-1/4}$.

Calculations show that between two sets of centres which "appear" to be equally good there can be as much as 20 per cent difference in efficiency.

In the case of neutrons it is best to use Friedel pairs and equal times. While in the case of X-rays it is not necessary to use Friedel pairs. Assuming a constant source intensity and constant absorption, the relative times required for centres shown in Fig. 4 are 30%, 12%, 29% and 39% of the total time available.

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