

The Determination of the Position of Anomalous Scattering Heavy Atom Groups in Protein Crystals

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Methods are proposed by which isomorphous replacement and anomalous scattering measurements may be combined to locate anomalously scattering heavy atoms in protein structures. Both Patterson and Fourier methods are discussed and examples are given to illustrate the use of new methods.

The examples show how the relative coordinates of heavy atom groups in different derivatives may be determined, and how the absolute configuration of these coordinates may be established.

Introduction

The only proven method for determining the detailed structure of protein crystals is the technique of isomorphous replacement with heavy atom groups, first introduced by Green, Ingram & Perutz (1954).

In practice it is difficult to find satisfactory isomorphous derivatives and it therefore becomes important to make full use of any derivative which may be available. In particular, it may be worth while making separate measurements of the intensities of Friedel related reflexions so that the differences due to the anomalous scattering of the heavy atoms may be used to supplement the phase information obtained from the normal isomorphous replacement method (Blow, 1958; Blow & Rossmann, 1961; North, 1965; Matthews, 1966). The purpose of this communication is to show how such anomalous scattering measurements may be combined with the isomorphous replacement data to assist in the determination of the position of the heavy atom groups in the crystal (see also Kartha & Parthasarathy, 1965)*. Both Patterson and Fourier methods are discussed. Examples are given to illustrate the use of the new methods and to compare the results with those obtained by previous methods.

Theory

(a) The difference Patterson function

Let $F(\mathbf{h})$ be the observed hkl structure factor of the native protein crystal. In general, proteins consist of light atoms which give rise to very small anomalous scattering effects, and it will be assumed that such effects may be ignored. For the isomorphous derivative crystal which contains heavy atoms, the anomalous scattering effects will in general be significant and we define $F_{H+} = F_H(\mathbf{h})$ and $F_{H-} = F_H(\bar{\mathbf{h}})$ to be the observed structure amplitudes of a Friedel pair of reflexions of the substituted crystal.

Suppose that there are a total of N heavy atoms which differentiate the native and derivative structures. If the n th of these atoms has position vector \mathbf{r}_n and scattering factor

$$f_n = f'_n + if''_n$$

then for the (h, k, l) plane the structure factor of the N atoms is

$$\begin{aligned} \mathbf{f}_c(\mathbf{h}) &= \sum_{n=1}^N f'_n(\mathbf{h}) \exp(2\pi i \mathbf{h} \cdot \mathbf{r}_n) \\ &\quad + i \sum_{n=1}^N f''_n(\mathbf{h}) \exp(2\pi i \mathbf{h} \cdot \mathbf{r}_n) \quad (1) \\ &= \mathbf{f}(\mathbf{h}) + \delta(\mathbf{h}) \quad (2) \end{aligned}$$

If the N heavy atoms are 'of the same type' (Rossmann, 1961), *i.e.* the ratio $k = f''_n/f'_n$ is a constant, then

$$\mathbf{f}_c(\mathbf{h}) = \mathbf{f}(\mathbf{h}) + ik^{-1}\delta(\mathbf{h}). \quad (3)$$

Thus, in this case, \mathbf{f} and δ are orthogonal, and $\delta = k^{-1}\mathbf{f}$. Let us define \mathbf{F}_H to be the structure factor of the derivative crystal in the absence of anomalous scattering. The relationship between \mathbf{F} , \mathbf{F}_H and \mathbf{f} is shown by the simple vector triangle in Fig. 1. When there is appreciable anomalous scattering the phase diagram can be conveniently represented by Fig. 2 (Ramachandran & Raman, 1956; Rossmann, 1961). In general, δ will be small compared with F_H , and therefore to a good approximation

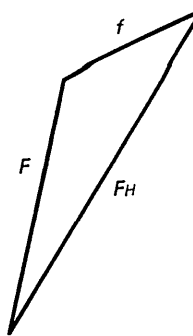


Fig. 1. Vector diagram showing the relationship between structure factors in the absence of anomalous scattering.

* The methods proposed here were formulated independently of this work.

$$F_H = \frac{1}{2}(F_{H+} + F_{H-}).$$

Several methods of determining the positions of heavy atoms in isomorphous protein structures have been suggested (see Rossmann, 1960, for a summary). Rossmann favours the use of a Patterson-like function with coefficients $(F_H - F)^2$ (Blow, 1958).

In a non-centrosymmetric structure the phases of \mathbf{F} and \mathbf{f} will, in general, not be correlated and therefore in many cases the approximation that

$$(F_H - F)^2 = f^2 \quad (4)$$

will be a poor one. However, if $(F_H - F)$ is large it follows that \mathbf{F} , \mathbf{F}_H and \mathbf{f} must be nearly collinear and in such cases the approximation will be most accurate. Therefore the largest terms in the summation are relatively error-free. In practice, if a large number of terms are included, this method can give very satisfactory results. On the other hand there will be many cases when the $(F_H - F)^2$ function is difficult to interpret, and in such cases it would clearly be desirable to have a better approximation to f^2 than that given by (4).

The location of anomalous scatterers has also been discussed by Blow (1957) and Rossmann (1961). In this case similar arguments to those given above are used to show that the positions of the anomalous scatterers can be determined by using another Patterson-like function with coefficients $(F_{H+} - F_{H-})^2$. This function will suffer from the same limitations as the $(F_H - F)^2$ function but will again have the property that the largest coefficients will tend to be most accurate.

A method will now be described by which the observed isomorphous replacement and anomalous scattering data may be combined to give an accurate estimate of f , the structure factor of the heavy atom replacement group.

Fig. 2 illustrates the relationship between \mathbf{F} , \mathbf{F}_{H+} and \mathbf{F}_{H-} and the scattering of the heavy atoms. Let α , β and γ be the angles shown. It was shown that if the heavy atoms are 'of the same type' then $\delta = k^{-1}f$ and δ and \mathbf{f} are at right angles. Since δ is generally small compared with F_H , it follows from Fig. 2 that

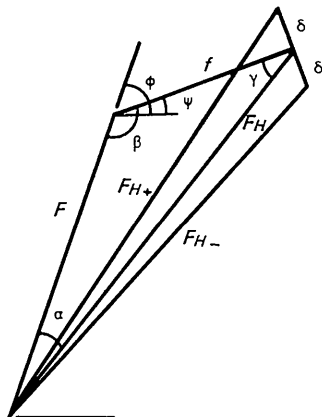


Fig. 2. Vector diagram showing the relationship between structure factors in the presence of anomalous scattering.

$$F_{H+} - F_{H-} = 2\delta \sin \gamma = 2k^{-1}f \sin \gamma. \quad (5)$$

Now

$$\sin \alpha = (f \sin \gamma) / F$$

so that

$$\begin{aligned} \sin \alpha &= f(F_{H+} - F_{H-}) / 2k^{-1}fF \\ &= k(F_{H+} - F_{H-}) / 2F. \end{aligned} \quad (6)$$

Hence

$$\begin{aligned} f^2 &= F^2 + F_H^2 - 2FF_H \cos \alpha \\ &= F^2 + F_H^2 - 2FF_H \{1 - [k(F_{H+} - F_{H-}) / 2F]^2\}^{\frac{1}{2}}. \end{aligned} \quad (7)$$

Thus, if the ratio $k = f'_n / f''_n$ is known it is in principle possible to use the observed data to calculate a difference Patterson synthesis with coefficients equal to f^2 . In practice the accuracy with which f can be determined will depend on the reliability of the various experimental measurements.

To show the relation between (7) and the result of Kartha & Parthasarathy (1965) the assumption is made that α in Fig. 2 is small. From (6) $k(F_{H+} - F_{H-}) / 2F$ will be small and so from (7)

$$\begin{aligned} f^2 &\simeq F^2 + F_H^2 - 2FF_H \{1 - \frac{1}{2}[k(F_{H+} - F_{H-}) / 2F]^2\} \\ &= (F_H - F)^2 + FF_H [k(F_{H+} - F_{H-}) / 2F]^2. \end{aligned} \quad (8)$$

Making the further approximation that $(F_H / F) = 1$, (8) leads to

$$f^2 \simeq (F_H - F)^2 + (k/2)^2 (F_{H+} - F_{H-})^2. \quad (9)$$

This is the expression used by Kartha & Parthasarathy to combine isomorphous replacement and anomalous scattering measurements. The present treatment does not rely on the assumption that α in Fig. 2 is small, but uses the observed data to estimate the value of this angle. Although the use of (7) instead of (9) would be expected to give only marginal improvement in the difference Patterson function, an estimate of α is essential for the difference Fourier method which is discussed later.

In practice, in combining anomalous scattering and isomorphous replacement measurements we have found it desirable to take into account their relative accuracies.

Generally, the $(F_H - F)$ differences are larger than the $(F_{H+} - F_{H-})$ ones and can therefore be measured with greater relative accuracy. However, as discussed by North (1965), the $(F_H - F)$ measurements are influenced by lack of isomorphism, by scaling errors, and by errors due to absorption. In favourable cases the $(F_{H+} - F_{H-})$ measurements will be free from all these sources of error and will therefore have a greater intrinsic accuracy. We have attempted to allow for the relative accuracies of the experimental measurements by using, instead of (7), the analogous expression

$$f_e^2 = F^2 + F_H^2 - 2FF_H \{1 - [wk(F_{H+} - F_{H-}) / 2F]^2\}^{\frac{1}{2}}, \quad (10)$$

where w is an estimate of the relative reliability of the $(F_{H+} - F_{H-})$ measurements compared with $(F_H - F)$, and f_e^2 is an estimate of the best coefficient to use (*i.e.* the one which will result in the highest peak to back-

ground ratio). Although this system of weighting is rather arbitrary it has been found to be satisfactory in practice. The procedure adopted has been to use the method described in the next section to estimate the value of k as a function of $\sin^2 \theta/\lambda^2$ and to assume that w is a constant. The value $w=0.75$ was found satisfactory and was used for all the calculations described here. In other cases the use of a different value of w might be more appropriate. For example, in the case of heavy atoms with relatively small anomalous scattering (k large) the observed differences between F_{H+} and F_{H-} become small and relatively inaccurate. In such cases a smaller value of w would be used.

(b) Determination of k

$k=f'/f''$ is the ratio of the real to anomalous scattering of the heavy atom groups in the derivative crystal. If the replacement groups were single heavy atoms and were all 'of the same type' then the value of k as a function of the angle of scattering could be looked up from tables. However, for many protein crystals the useful heavy atom replacement groups are complex ions which can be thought of as 'lumps' of high electron density. The $[\text{PtCl}_4]^{2-}$ derivative of α -chymotrypsin, used later as an illustration, is a typical example. In such cases it would be more difficult to predict the value of k , for the anomalous component of the scattering of the complex ion results almost entirely from the inner electrons of the platinum atom, whereas the real scattering is derived from all the electrons in the 'lump'. A simple method for determining the effective value of k in such cases will now be described. It will be assumed that the 'lumps' are of the same type and that they may be regarded as spherically symmetrical. The latter assumption will be valid unless one is working to a resolution comparable to the dimensions of the heavy atom group.

For many reflexions F and F_H are large compared with f , and for these reflexions α in Fig. 2 will be small. The only possible exceptions to this general rule are the weak reflexions, and if these are ignored, it will always be a good approximation that

$$|f \cos \gamma| = |F_H - F|. \quad (11)$$

Similarly, δ will be small compared with F_H so that

$$|\delta \sin \gamma| = \frac{1}{2}|F_{H+} - F_{H-}|,$$

or

$$|k^{-1}f \sin \gamma| = \frac{1}{2}|F_{H+} - F_{H-}|. \quad (12)$$

By averaging (11) and (12) over a large number of reflexions, we have

$$\bar{f} \cdot \overline{|\cos \gamma|} = \bar{f} \cdot (2/\pi) = \overline{|F_H - F|} \quad (13)$$

and

$$\overline{k^{-1}f} \cdot \overline{|\sin \gamma|} = \overline{k^{-1}f} \cdot (2/\pi) = \frac{1}{2}\overline{|F_{H+} - F_{H-}|}. \quad (14)$$

Since the heavy atom groups are assumed to have spherical symmetry, k will be a constant for all reflexions with the same Bragg angle. Therefore, if the average is made within a small range of scattering angles

and the weak reflexions are omitted, the value of k appropriate to that range is given by

$$k = 2\overline{|F_H - F|} / \overline{|F_{H+} - F_{H-}|}. \quad (15)$$

It would be expected that, in practice, the various types of error would increase these averages over their 'theoretical' values. In particular, non-isomorphism would increase the numerator but not the denominator. However, it has been found satisfactory to use (15) as it stands.

(c) The difference Fourier synthesis

The first section dealt with the location of heavy atom replacement groups in proteins by using difference Patterson functions. Such a method requires no prior knowledge of any of the phases of the native protein. If such information is already available, for example from other heavy atom replacements, it may be preferable to locate new heavy atom groups by difference Fourier techniques. This procedure automatically gives the coordinates of the new heavy atom sites relative to the previously chosen origin.

Let us suppose that φ is the given phase of a reflexion of the native protein and that m is an estimate of the reliability of the determination of φ . m can be defined rigorously as the 'figure of merit' of the phase determination (Blow & Crick, 1959; Dickerson, Kendrew & Strandberg, 1961).

If F and F_H are the structure amplitudes of the parent crystal and an isomorphous derivative, the Fourier synthesis used to locate the atoms differentiating the two structures has coefficients

$$m(F_H - F) \exp(-i\varphi) \quad (16)$$

(e.g. see Stryer, Kendrew & Watson, 1964). These coefficients give the vector contribution of f in the direction specified by φ , suitably weighted by m , but include no contribution perpendicular to this direction. The necessary information is not supplied by the isomorphous replacement method, but can be obtained from anomalous scattering measurements (Ramachandran & Raman, 1956). If the phase ψ of f can be determined (with figure of merit m') the corresponding 'best' difference Fourier function is defined to be one with coefficients

$$m'f \exp(-i\psi). \quad (17)$$

From Fig. 2

$$\psi = \beta + \varphi - \pi. \quad (18)$$

From this relation it follows that the reliability with which ψ may be determined is limited by the reliability of the determination of φ and it is therefore reasonable to use m in place of m' as an estimate of the reliability of ψ . The coefficients of the difference Fourier function are then given by

$$mf \exp(-i\psi) = -mf \exp(-i\beta) \exp(-i\varphi). \quad (19)$$

Also from Fig. 2

$$\sin \beta = (F_H \sin \alpha)/f$$

and

$$\cos \beta = (F^2 + f^2 - F_H^2) / 2Ff.$$

Therefore by using (6) and (7) to evaluate $\sin \alpha$ and f , and from the given values of $m \sin \varphi$ and $m \cos \varphi$, the coefficients of the 'best' difference Fourier function can be determined. In practice, the weighting system described before was again used to reduce the influence of the less accurate anomalous scattering differences.

Application to α -chymotrypsin

α -Chymotrypsin is a proteolytic enzyme of molecular weight 23000 crystallizing in the space group $P2_1$ with two molecules per asymmetric unit. The unit-cell parameters are $a = 49.6$, $b = 67.8$, $c = 66.5$ Å, $\beta = 102^\circ 10'$. This crystal form was first investigated by Bernal, Fankuchen & Perutz (1938) and has subsequently been studied by Blow, Rossmann & Jeffery (1964). Blow *et al.* prepared crystals isomorphous with α -chymotrypsin but containing the planar complex chloroplatinite $[\text{PtCl}_4]^{2-}$. Following the work of Sigler, Skinner, Coulter, Kallos, Braxton & Davies (1964) with γ -chymotrypsin, another heavy atom derivative has been made by preparing α -chymotrypsin crystals containing *p*-iodobenzenesulphonyl fluoride (pipsyl fluoride) (Sigler, Jeffery, Matthews & Blow, 1966). Pipsyl- α -chymotrypsin is closely isomorphous with the analogous derivative benzenesulphonyl- α -chymotrypsin and the two compounds are differentiated by one iodine atom per chymotrypsin molecule.

By using these derivatives it was possible to test the new method in two rather differing situations. The pipsyl and benzenesulphonyl derivatives provide an isomorphous pair of compounds differentiated by single heavy atoms at well defined sites. In contrast, the $[\text{PtCl}_4]^{2-}$ derivative is isomorphous with the native enzyme but contains heavy atom groups which are complex and which occupy several different sites with varying occupancy.

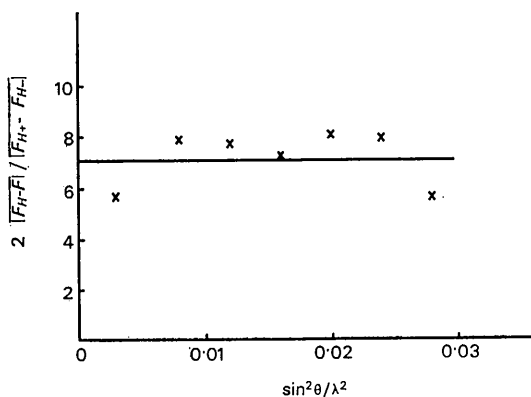


Fig. 3. Determination of k , the ratio of the real to anomalous scattering of the iodine atoms differentiating the isomorphous compounds pipsyl- α -chymotrypsin and benzenesulphonyl- α -chymotrypsin.

The non-centrosymmetric (100) projection is used to illustrate the use of the Patterson and Fourier methods. The corresponding $0kl$ spectra were recorded photographically with a Buerger precession camera using Cu $K\alpha$ radiation and a Joyce-Loebl microdensitometer was used to measure the intensities of the reflexions. The data were recorded to a resolution of 2.8 Å, this corresponding to about 900 independent $0kl$ reflexions.

(a) Difference Patterson syntheses

The difference Patterson syntheses obtained from the combined pipsyl and benzenesulphonyl data will be discussed first. As mentioned before, these two isomorphous compounds are differentiated only by single iodine atoms and it would be expected that the ratio of real to anomalous scattering of the iodine atoms would change only slightly with the small range of scattering angles used here. Using (15), k was estimated by plotting the ratio $2|F_H - F| / |F_{H+} - F_{H-}|$ as a function of $\sin^2 \theta / \lambda^2$ as shown in Fig. 3. It is noteworthy that not only does k appear to be a constant over this range of scattering angles but also that the mean value of $k = 7.1$ is equal to the value predicted from tables. Using this value of k and assuming that the weighting factor w has the constant value of 0.75, equation (10) was used to calculate the 'combined' difference Patterson coefficients. The resultant synthesis is shown in Fig. 4(d). For comparison the corresponding $(F_{H+} - F_{H-})^2$ anomalous scattering function, the $(F_H - F)^2$ isomorphous replacement function, and the sum of these two, weighted according to equation (9) (Kartha & Parthasarathy, 1965) with $k = 7.1$ are shown in Fig. 4(a), (b) and (c).

To assist in the comparison of the different results, Table 1 gives a summary of the peak and background heights in the four syntheses. In both the 'combined' and 'sum' functions the four iodine-iodine vector peaks show up clearly above the background, the contrast being slightly better for the 'combined' difference Patterson synthesis.

Turning now to the native enzyme and the isomorphous $[\text{PtCl}_4]^{2-}$ derivative, the behavior of k as a function of $\sin^2 \theta / \lambda^2$ is shown in Fig. 5. As expected, the anomalous scattering becomes relatively stronger at higher angles. Over the rather small range of scattering angles used, the change of k can be approximated equally well by either the straight line shown in Fig. 5, or the exponential approximation

$$k = 11.5 \exp(-16 \sin^2 \theta / \lambda^2).$$

The linear approximation was the one used, and by again assuming the constant value of 0.75 for w , the 'combined' (100) difference Patterson projection was calculated [Fig. 6(d)]. For comparison the $(F_{H+} - F_{H-})^2$, $(F_H - F)^2$ and 'sum' functions are shown in Fig. 6(a), (b) and (c). (In using (9) to evaluate the coefficients of the 'sum' function, k was put equal to 9.1, the expected value for platinum.) In this case the Pat-

Table 1. Comparison of difference Patterson syntheses shown in Fig. 4

(a) 'Anomalous differences' synthesis		(b) 'Isomorphous replacement' synthesis		(c) 'Sum' function		(d) 'Combined' difference Patterson function	
Peak height	Highest background	Peak	Background	Peak	Background	Peak	Background
4.0	3.6	60	33	103	49	87	40
2.8	2.2	42	33	70	45	57	38
2.6	2.1	38	31	70	40	52	32
2.5	2.1	34	27	59	40	48	31
Average peak height		(a)	(b)	(c)	(d)		
Average background		3.0	43.0	75.5	61.0		
Peak to background ratio		0.87	7.6	10.7	8.3		
Relative improvement		4.3	5.7	7.1	7.3		
		0.77	1.0	1.25	1.30		

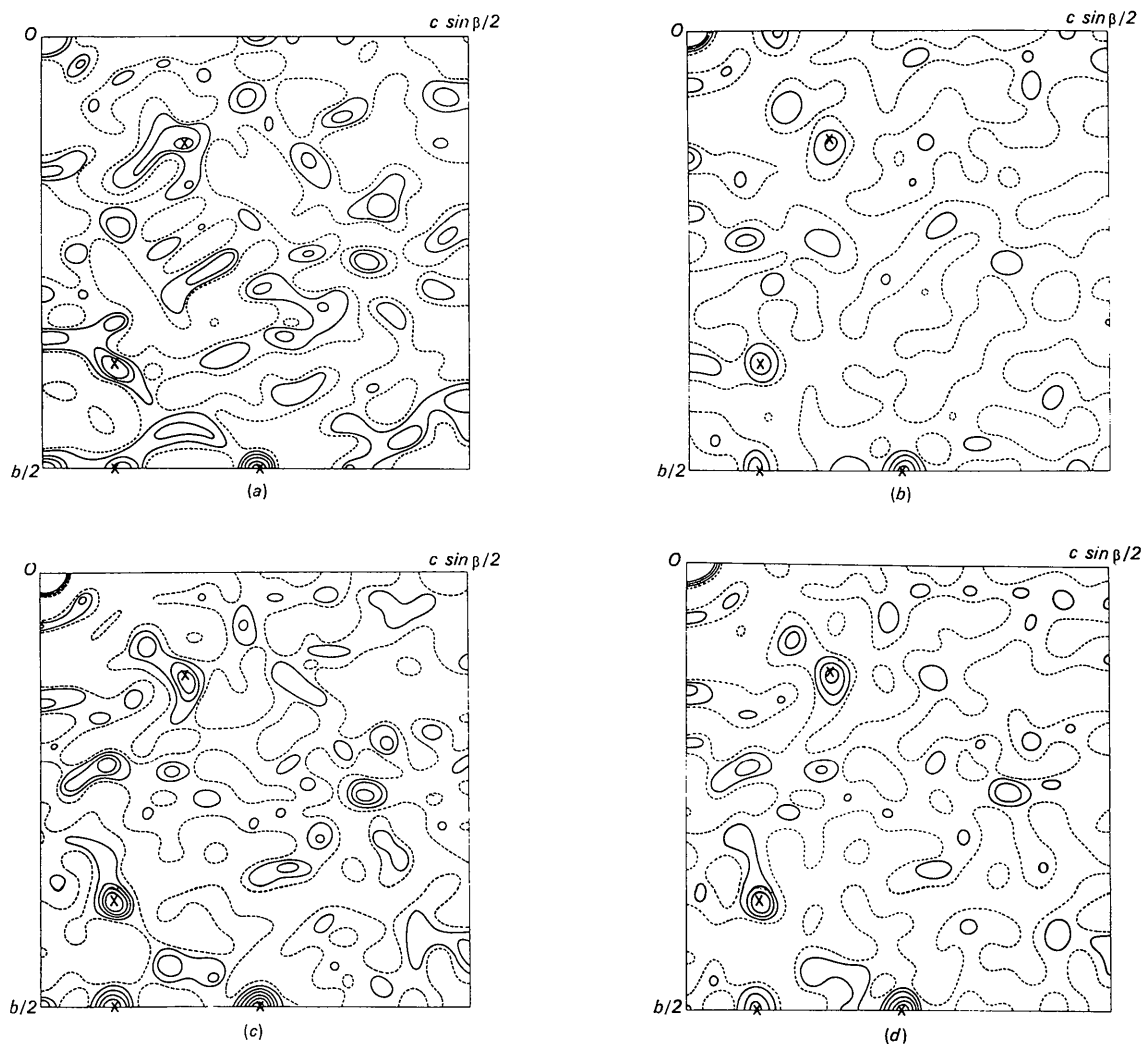


Fig. 4. Comparison of different $(0kl)$ difference Patterson syntheses used to locate the iodine atoms differentiating pipsyl- α -chymotrypsin and benzenesulphonyl- α -chymotrypsin. The crosses indicate the expected positions of the four equal-weight iodine-iodine vector interactions. In these and all succeeding diagrams the contours are drawn at arbitrary equal intervals, the zero contour being dashed. (a) 'Anomalous differences' synthesis using coefficients $(F_H^+ - F_H^-)^2$. Contours drawn at one twentieth the interval used for (b), (c) and (d). (b) 'Isomorphous replacement' synthesis using coefficients $(F_H - F)^2$. (c) 'Sum' synthesis (see text). (d) 'Combined' difference Patterson function (see text).

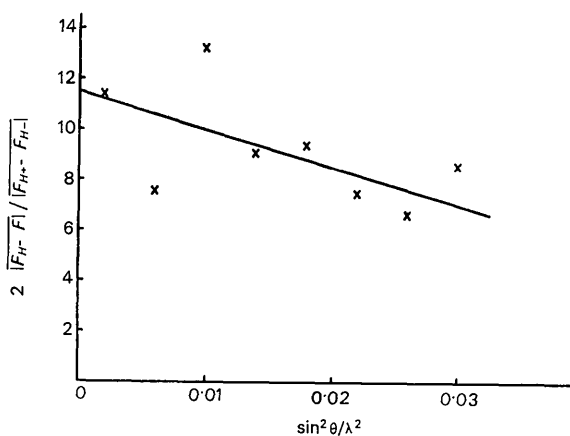


Fig. 5. Determination of the effective value of k for the chloroplatinite groups differentiating $[\text{PtCl}_4]^{2-}$ -substituted α -chymotrypsin and native α -chymotrypsin.

erson functions are more difficult to interpret as there are five sites occupied with varying occupancy by the $[\text{PtCl}_4]^{2-}$ ions. Nevertheless, from the data of Blow *et al.* (1964) and from subsequent work at higher resolution (Sigler *et al.*, 1966), the coordinates and occupancies of the various sites have been determined with some precision, and it was possible to construct the 'expected' set of weighted vector points as shown on the various maps. Because many of the vector peaks overlap it is not possible to list the individual peak heights. Instead, Table 2 gives the heights of the major composite vector peaks and the highest background peaks. It may be noted that by use of the 'combined' difference Patterson function rather than the $(F_H - F)^2$ function, the peaks are enhanced by an average of 27% and the highest background peaks are in fact reduced. In this case the 'combined' Patterson function gives appreciably better results than the 'sum' function.

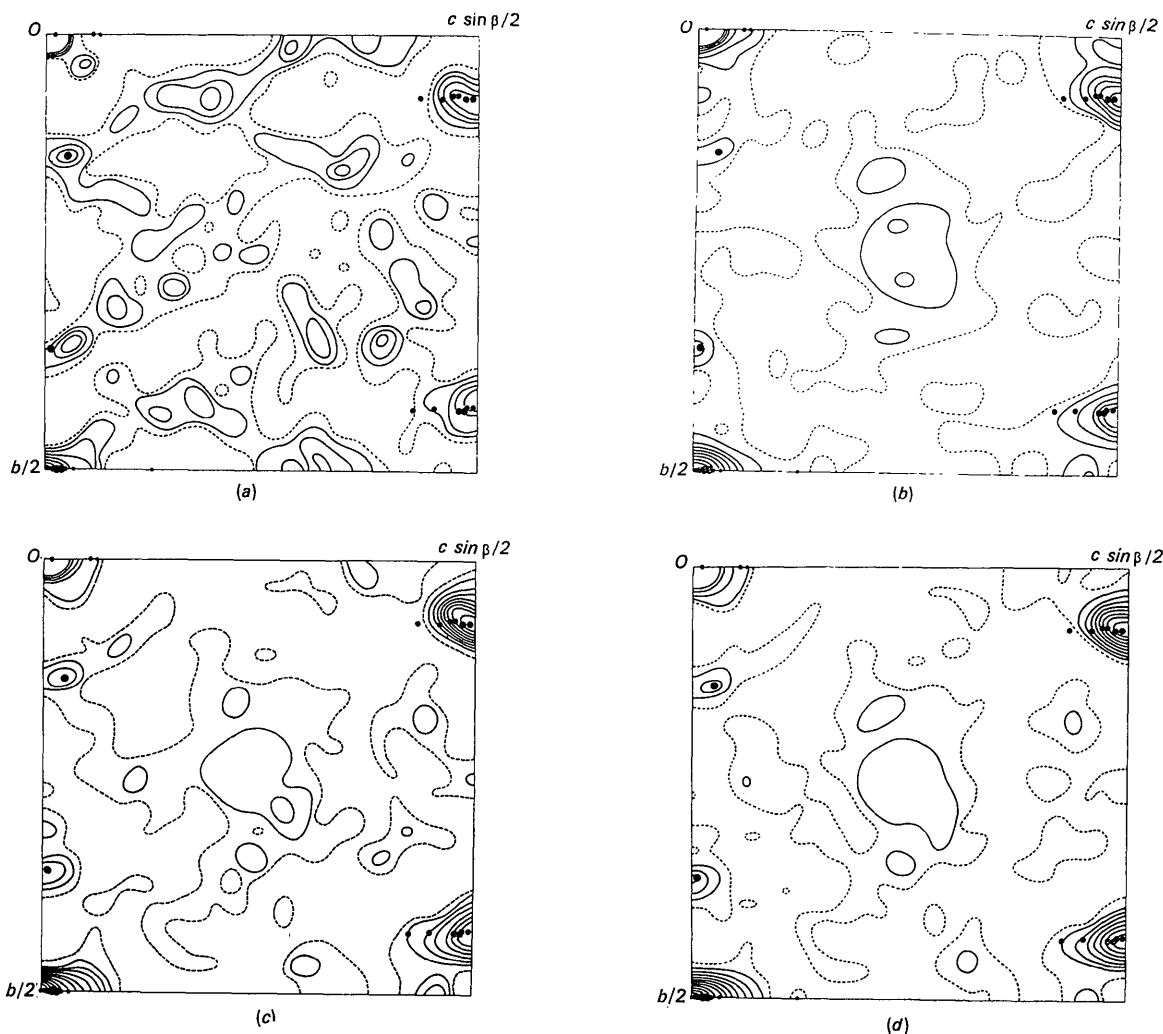


Fig. 6. Comparison of different $(0kl)$ difference Patterson syntheses used to locate the heavy atom groups in $[\text{PtCl}_4]^{2-}$ -substituted α -chymotrypsin. The position and size of the small solid circles indicate the position and approximate height of the expected vector peaks. (a) 'Anomalous differences' synthesis. Contours drawn at one twenty-fifth the interval used for (b), (c) and (d). (b) 'Isomorphous replacement' synthesis. (c) 'Sum' synthesis. (d) 'Combined' synthesis.

To compare further the relative merits of the 'sum' and 'combined' functions, several other syntheses similar to those shown in Fig. 4(c) and (d) and Fig. 6(c) and (d) were calculated with different values for k and w . The first conclusion drawn from these comparisons is that if k in equation (9) is put equal to the value of (wk) used in equation (10), the resultant syntheses are very similar, and, within our experience, give equally good results. The second conclusion is that in all cases the peak to background contrast can be improved, in some cases markedly, by allowing for the change in the ratio of the real to anomalous scattering of the heavy atom groups, and the relative reliability of the experimental measurements. In other words, the 'combined' synthesis is superior to the 'sum' synthesis mainly because of the use of the weighting factor w rather than the abandonment of the assumption that α is small.

(b) *Difference Fourier syntheses*

To test the new difference Fourier method it was necessary to have an estimate of the $0kl$ phases. In phase determination of non-centrosymmetric reflexions the conventional isomorphous replacement method leads to an ambiguous result for the phase angle (Bokhoven, Schoone & Bijvoet, 1951). However, by including anomalous scattering information in the phase determination, this ambiguity may be resolved and a unique determination made (Ramachandran & Raman, 1956). Bearing this in mind it was decided to calculate two independent sets of $0kl$ phases, the first from the isomorphous native and $[\text{PtCl}_4]^{2-}$ compounds, and the second from the pipsyl and benzenesulphonyl compounds. Since the four different crystals are almost exactly isomorphous, the 'PtCl₄' phases could then be used to calculate an 'iodine' difference Fourier synthesis, and the 'iodine' phases could be used to calculate a 'PtCl₄' difference Fourier synthesis. This procedure not only enabled the new method to be tested, but also served as a test of the effectiveness of using iodine atoms to provide phase information. Furthermore, it allowed the relative y coordinates of the

$[\text{PtCl}_4]^{2-}$ groups and the iodine atoms to be determined, and was used to establish the absolute configuration of these coordinates.

In the phase angle calculations, proper account was taken of the relative reliabilities of the isomorphous replacement and anomalous scattering measurements (North, 1965; Matthews, 1966).

The first difference Fourier synthesis used to locate the iodine atoms is shown in Fig. 7(a). The coefficients are those defined in equation (16), the observed structure amplitudes for the pipsyl and benzenesulphonyl derivatives being substituted for F_H and F respectively, and the protein phases φ being those determined by considering the native crystals and the $[\text{PtCl}_4]^{2-}$ derivative as a single isomorphous pair. In calculating the $[\text{PtCl}_4]^{2-}$ structure factors for the phase determination, the plane $y=0$ was chosen to lie midway between the $[\text{PtCl}_4]^{2-}$ sites shown in Fig. 7(a) as A and B . From the (100) Patterson projections [Fig. 6(a) to (d)] and from other evidence (Sigler *et al.*, 1966), the three other sites C , D and E also lie in or near this plane. Blow *et al.* (1964) have shown that there is a non-crystallographic twofold axis of symmetry parallel to a^* . This local symmetry axis passes through the point $x=0.22$, y_1 , $z=0.005$ where, relative to the choice of origin used here, y_1 is close to zero. (From the $P2_1$ space group symmetry there will be another local symmetry axis parallel to a^* passing through the point $(-0.22, y_1 + \frac{1}{2}, -0.005)$. Referred to these non-crystallographic axes, A and B are a pair of related sites, as are D and E , while C lies on an axis.

The 'combined' difference Fourier synthesis with coefficients defined by (19) is shown in Fig. 7(b). In both Fig. 7(a) and Fig. 7(b) the two iodine atoms I(1) and I(2) show up clearly above background at positions which are consistent with the interpretation of the (100) Patterson projections (Fig. 4) and with data for the other projections. The iodine peaks straddle the line $y=\frac{1}{2}$ and are equidistant from it, and the coordinates of the iodine atoms deduced from the Fourier maps are consistent with the non-crystallographic axis of symmetry. In the 'combined' synthesis the iodine peaks

Table 2. *Comparison of difference Patterson syntheses shown in Fig. 6*

(a) 'Anomalous differences' synthesis		(b) 'Isomorphous replacement' synthesis		(c) 'Sum' function		(d) 'Combined' difference Patterson function	
Peak height	Highest background	Peak	Background	Peak	Background	Peak	Background
10.6	5.4	670	161	849	171	851	149
6.9	5.1	496	153	657	167	648	134
6.9	4.8	441	151	506	142	539	122
5.7	4.7	187	123	207	135	229	120
5.3	4.7	126	120	197	129	173	120
		(a)	(b)	(c)	(d)		
	Average peak height	7.1	384	483	488		
	Average background	1.52	42.9	44.7	40.2		
	Peak to background ratio	4.7	9.0	10.8	12.1		
	Relative improvement	0.52	1.0	1.21	1.35		

have approximately double the height observed in the normal difference Fourier synthesis, while the average background density is only 19% higher. A more detailed comparison is given in Table 3. It is a striking feature of the 'combined' synthesis that 8 of the 11

highest 'background' peaks occur in positions which could be related by the non-crystallographic axis. The possibility that these peaks may be due to an effect such as partial substitution at secondary sites is being investigated. The average increase in the height of the

Table 3. Comparison of difference Fourier syntheses shown in Fig. 7

(a)		(b)	
Normal difference Iodine peaks	Fourier synthesis Highest background peaks*	'Combined' difference Iodine peaks	Fourier synthesis Highest background peaks*
110	60	220	83
105	59	198	72
	58		66
	58		57
	Average peak height	(a) 108	(b) 209
	Average background	14.3	17.0
	Peak to background ratio	7.5	12.3
	Relative improvement	1.0	1.63

* 'Related' background peaks omitted (see discussion).

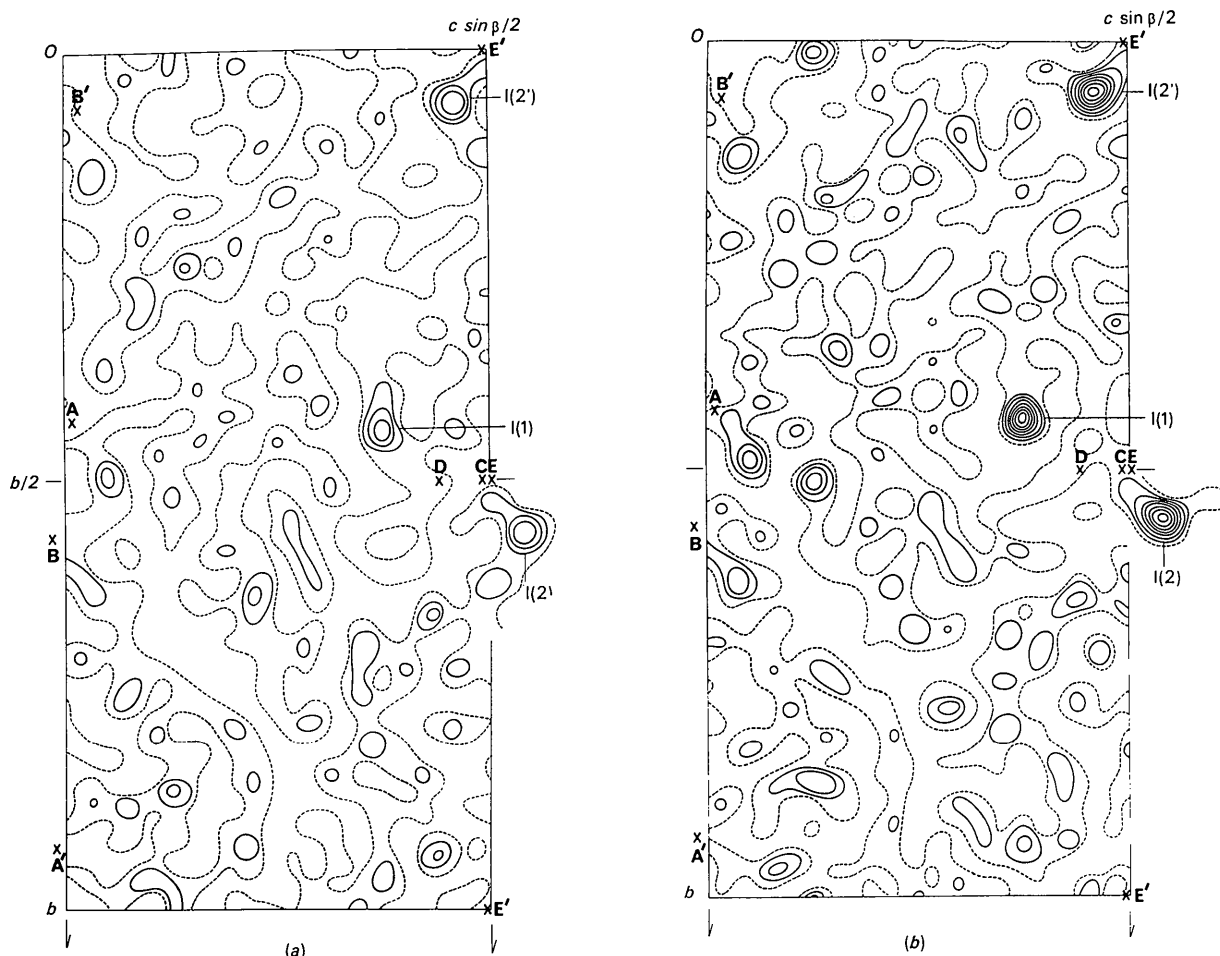


Fig. 7. Comparison of $(0kl)$ difference Fourier syntheses used to locate the iodine atoms differentiating pipsyl- α -chymotrypsin and benzenesulphonyl- α -chymotrypsin. The protein phases were determined from data observed for native α -chymotrypsin and $[\text{PtCl}_4]^{2-}$ -substituted α -chymotrypsin. In the phase determination, the coordinates of the $[\text{PtCl}_4]^{2-}$ groups used to calculate structure factors are marked by the crosses labelled A, B, C, D, E . $I(1)$ and $I(2)$ are the peaks of the difference syntheses indicating the positions of the iodine atoms. (a) Normal difference synthesis. (b) 'Combined' difference synthesis based on the same protein phases used for (a), but making use of the observed anomalous scattering of the iodine atoms in the pipsyl- α -chymotrypsin.

8 'related' peaks is 78% which considerably exceeds the average background increase of 19%.

Having shown that the phases obtained from the $[\text{PtCl}_4]^{2-}$ derivative were sufficiently good to reveal the positions of the iodine atoms unambiguously, the converse was next attempted. In this case the observed structure factors for the native and $[\text{PtCl}_4]^{2-}$ substi-

tuted crystals were substituted for F and F_H in (16), and the phases φ were those determined from the pipsyl and benzenesulphonyl derivatives. The resultant synthesis is shown in Fig. 8(a) and can be compared with the 'combined' synthesis [Fig. 8(b)] which has coefficients defined by (19). In both syntheses the $[\text{PtCl}_4]^{2-}$ groups show up clearly at their expected positions;

Table 4. Comparison of difference Fourier syntheses shown in Fig. 8

(a)		(b)	
Normal difference Fourier synthesis	Highest background peaks	'Combined' difference Fourier synthesis	Highest background peaks
$[\text{PtCl}_4]^{2-}$ peaks		$[\text{PtCl}_4]^{2-}$ peaks	
142	64	228	73
111	52	184	69
134*	51	177*	64
Average peak height		(a) 129	(b) 196
Average background		15.0	16.9
Peak to background ratio		8.6	11.6
Relative improvement		1.0	1.35

* Composite peak.

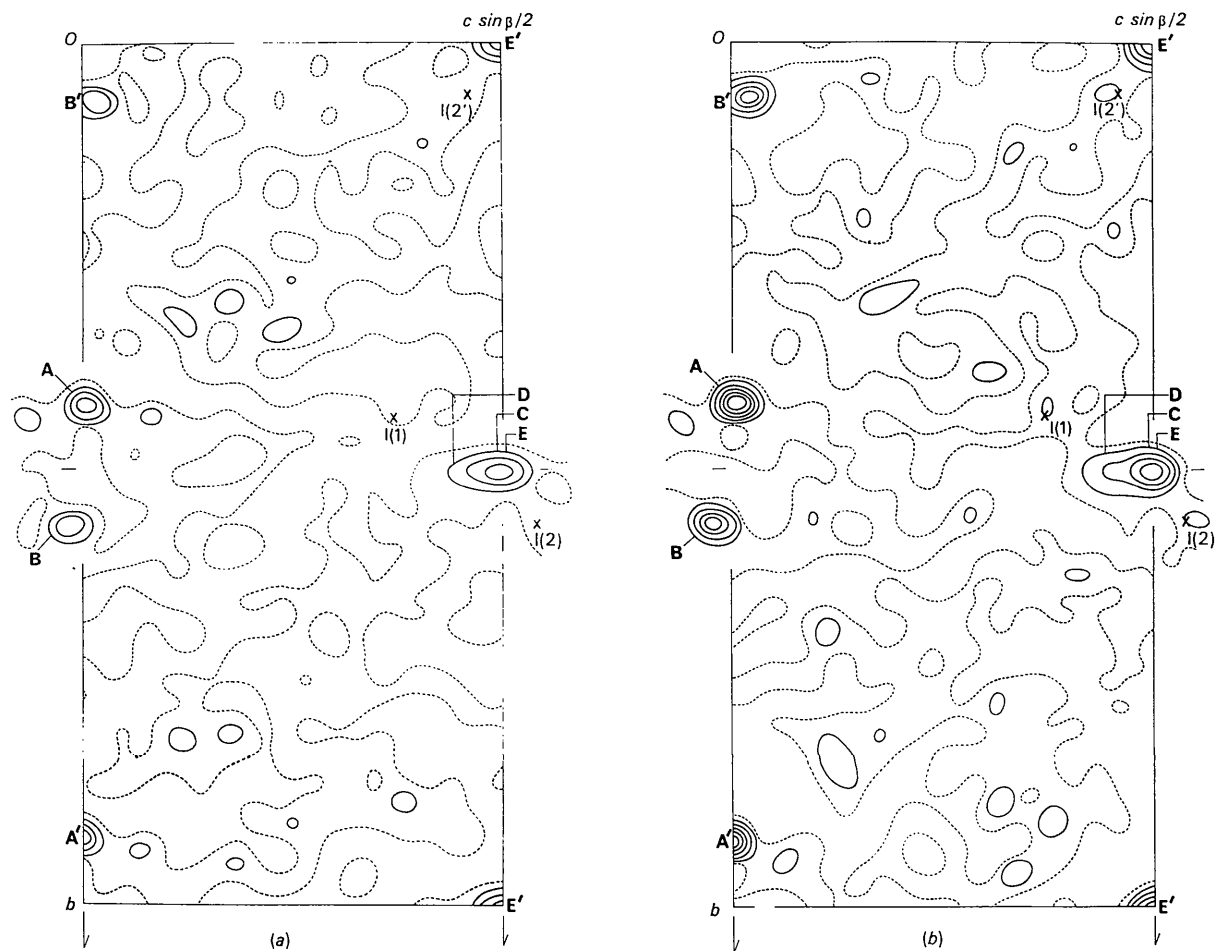


Fig. 8. Comparison of $(0kl)$ difference Fourier syntheses used to locate the chloroplatinite groups in $[\text{PtCl}_4]^{2-}$ -substituted α -chymotrypsin. The protein phases were determined from the pipsyl- and benzene sulphonyl- α -chymotrypsin data. I(1) and I(2) indicate the coordinates of the iodine atoms used to calculate structure factors for the phase determination. A, B, C, D and E indicate the sites containing $[\text{PtCl}_4]^{2-}$ as revealed by the difference syntheses. These sites are thought to have a relative occupancy of about 100:100:80:50:50. (a) Normal difference synthesis. (b) 'Combined' difference synthesis.

however, in the 'combined' synthesis the peak-to-background contrast is markedly improved. Table 4 gives a detailed comparison of the two results.

Apart from the usefulness of the 'combined' Fourier synthesis in increasing peak-to-background contrast, it also provides a powerful method of verifying that the indexing of the Friedel related reflexions F_{H+} and F_{H-} is consistent from one heavy atom derivative to the next. If a 'combined' synthesis is calculated with F_{H+} and F_{H-} in the wrong sense, then the heavy atom peaks are markedly reduced rather than being enhanced.

It may be noted that the clarity with which the heavy atom groups show up in Fig. 8(a) and (b) clearly indicates that atoms as light as iodine can be useful in helping to determine the phases of protein reflexions.

(c) The determination of absolute configuration

By including anomalous scattering measurements in the determination of protein phases, as was done here, it is possible to determine the absolute configuration

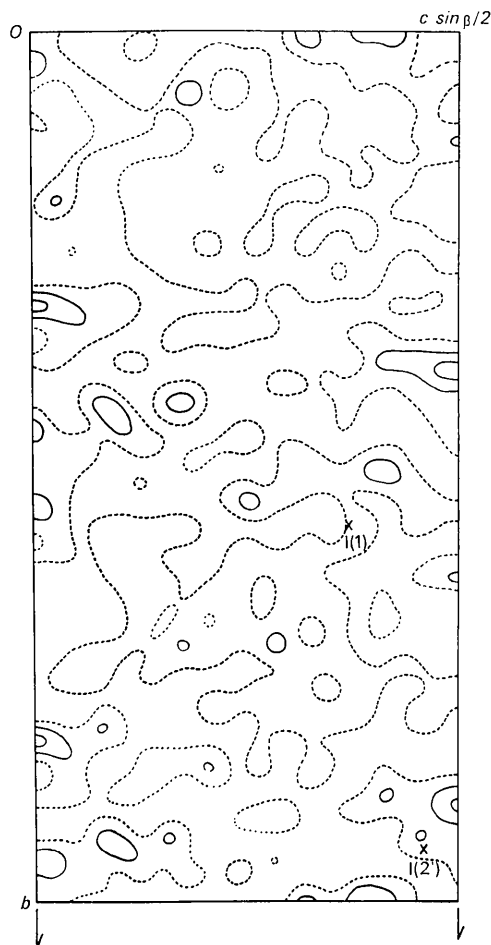


Fig. 9. Alternative difference Fourier synthesis to Fig. 8(a) resulting from the choice of the opposite hand for the coordinates of the iodine atoms I(1) and I(2). Contours at the same interval as in Fig. 8.

of the heavy atoms (and hence of the protein structure) in a simple and straightforward way.

If the wrong hand is assumed for the coordinates of the heavy atoms, then the combination of anomalous scattering and isomorphous replacement measurements leads to an unambiguous but incorrect value for the protein phase. (The correct and incorrect phases differ by an angle of 2ψ where ψ is the phase of the heavy atom vector f .) A Fourier or difference Fourier synthesis calculated with the use of these phases would lead to an 'uninterpretable' result, whereas the choice of the correct hand of the heavy atom coordinates would be expected to lead to a Fourier synthesis with reasonable features (Blow & Rossmann, 1962). Fig. 9 illustrates a difference Fourier synthesis which is in all respects similar to that shown in Fig. 8(a) except that the phases used are those obtained after changing the hand of the iodine coordinates (*i.e.* replacing y by $-y$). Clearly Fig. 8(a) is the one with 'reasonable features', indicating that the iodine coordinates used for the phase determination and the $[\text{PtCl}_4]^{2-}$ positions revealed by this map are the ones with the correct absolute configuration.

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