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Parameter Refinement in the Multiple Isomorphous-Replacement Method

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The usual procedure for parameter refinement in the multiple isomorphous-replacement method leads to a heavy bias in each round of refinement towards the previously assumed values. This leads to difficulties of convergence, which can be severe when one derivative or site is overwhelmingly powerful in phase determination. The bias arises from using the parameters of a derivative in estimating phase angles, which are then used to refine these same parameters. By omitting the derivative which is to be refined from the phase determination, this bias is avoided, and even though the phases may then be very inaccurate, convergence is rapid. This method was applied to the α -chymotrypsin structure, when the conventional procedure failed to converge correctly, and led to a greatly improved electron-density map. Correct estimation of occupancy and lack-of-closure errors remains an unsolved problem.

1. Refinement methods in the isomorphous-replacement method

Refinement of the parameters of an isomorphous substituent raises difficulties in the non-centrosymmetric case, because observed differences of structure amplitude cannot be explicitly related to the calculated structure factor of the substituent until the phase angles are known. Rossmann (1960) proposed a method based on analysis of the $(\Delta|F|)^2$ difference Patterson function, using a weighting factor which implies a statistical relationship between the amplitude difference $\Delta|F|$ and the calculated scattering factor of the substituent. This method has not been used widely with three-dimensional data, though analogous methods for two-dimensional, centrosymmetric data are satisfactory (Hart, 1961; Lundberg, 1965). It remains, however, the only least-squares method available when only a single isomorphous pair is available, unless anomalous scattering data is also used (Kartha, 1965; Adams *et al.*, 1969). The alternative method of using difference or 'residual' Fourier synthesis would appear to be a laborious method of refinement, though it provides an excellent check on the correctness of the results.

In the work on myoglobin, optimum values of the x_i and z_i coordinates, occupancy Z_i and temperature

factor B_i were found from the centrosymmetric ($h0l$) data by exploration of the residual function (Hart, 1961). (Throughout this paper the subscript i identifies a particular site of isomorphous substitution.) This left only the relative y_i coordinates of substituent atoms to be estimated from non-centrosymmetric data (Dickerson, Kendrew & Strandberg, 1961a, b).

Dickerson *et al.* (1961b) suggested that all parameters could in principle be refined simultaneously by a method similar to the one used for refining the y_i , in which rounds of least-squares refinement alternate with redetermination of the phase angles. The refinement parameter relevant to the j th derivative would be the root-mean-square lack of closure E_j (Blow &

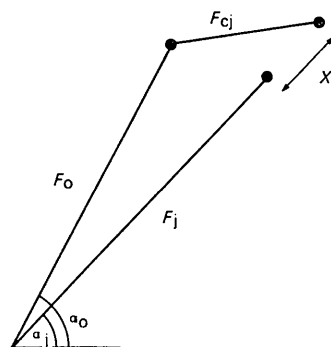


Fig. 1. The lack-of-closure vector $X_j(h)$.

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Crick, 1959), which provides an estimate of the total error from all sources in applying the isomorphous-replacement method. (The subscript j identifies a particular isomorphous derivative.) In Blow & Crick's treatment, the lack of closure error $X_j(h)$ (Fig. 1) is a real quantity given by

$$(|F_j(h)| + X_j(h)) \exp i\alpha_j(h) = F_0(h) + F_{c_j}(h)$$

and

$$E_j = \left(\sum_h X_j^2(h) / n_j \right)^{1/2}.$$

Here $j=1, \dots, N$ for the N isomorphous derivatives used, $F_j(h) = |F_j(h)| \exp i\alpha_j(h)$ being the structure factor for the j th derivative, $F_0(h)$ the structure factor of the unsubstituted or 'parent' compound, and $F_{c_j}(h)$ the calculated scattering of the substituent atoms of the j th derivative. n_j is the number of reflexions included in the summation for E_j . Blow & Crick suggested that values of $X_j(h)$, $j=1, \dots, N$, calculated for each possible value of $\alpha_0(h)$, the parent phase angle, could be

used to obtain a probability distribution for $\alpha_0(h)$. This leads to a most probable phase angle, a 'best' phase angle, and a weighting factor representing the accuracy of the phase determination. This factor was named the 'figure of merit', $m(h)$, by Dickerson *et al.* (1961a). Dickerson *et al.* (1961b) proposed that a weighted sum $\sum_h w(h) X_j^2(h)$, could be minimized with respect to the parameters used to calculate F_{c_j} to optimize the estimate of these parameters. They did not state whether this minimization should be done with respect to the values of X_j at the most probable phase or the 'best' phase. The authors recognised that inclusion of a round of phase determination in each cycle of refinement greatly increased the amount of computer time required.

As part of her work on the structure of carboxypeptidase, Muirhead wrote a Fortran program which performed the minimization proposed (Lipscomb *et al.*, 1966). The weighting factor $w(h)$ was

$$N / \sum_{j=1}^N [X_j^2(h) + 9E_j / |F_j(h)|^2].$$

In the work of Lipscomb *et al.* (1966), the refinement was based on the 'best' phase, but the program allows the choice of using the 'most probable' phase. The program proceeds by alternate rounds of phase determination by the methods of Blow & Crick (1959) or of Cullis, Muirhead, Perutz, Rossmann & North (1961), and full-matrix least-squares refinement based on the Oak Ridge program (Busing, Martin & Levy, 1962). Variants of this program have been widely used in protein structure investigations since that time.

The crucial importance of phase angles in difference Fourier syntheses is well known. Dickerson, Kopka, Varnum & Weinzierl, (1967) have demonstrated the way incorrect phases feed back incorrect detail into difference maps. The refinement method described above contains exactly the same type of feed-back loop, which heavily biases the results towards the parameters used for phase determination. This is bound to cause difficulties of convergence, and may, in extreme cases, lead to false minima. The complication of the algorithm for phase determination, and the interdependence of X_j and α_0 , makes the amount of bias hard to assess. Dr H. Muirhead, in a personal communication, has suggested that refinement will not be possible in situations where the phase distribu-

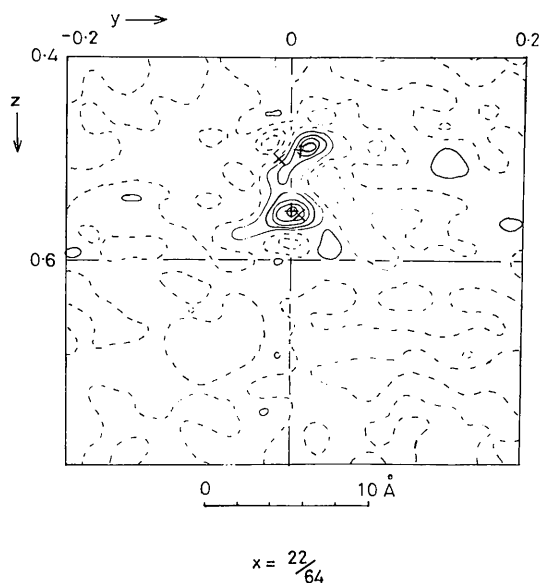


Fig. 2. Section $x=22/64$ from a 'residual' Fourier synthesis for the PtCl_4 derivative. This section includes sites D and E . The coefficients for this synthesis are given by $m(|F_j| \exp i\alpha_0 - F_{c1})$ where F_{c1} , α_0 and α_1 are derived from parameters obtained at termination of Muirhead-style refinement. \times -positions of sites D and E after Muirhead-style refinement; $+$ -final positions of sites D and E .

Table 1. *Isomorphous derivatives used in structure analysis of α -chymotrypsin*

j	Name	Substituent	Binding sites in asymmetric unit
—	Nat	—	—
0	Tos	Toluenesulphonyl, $\text{CH}_3\text{C}_6\text{H}_4\text{SO}_2^-$	2 (F, G)
(1)	PtCl_4	Chloroplatinite, PtCl_4^{2-}	2 fully occupied sites (A, B) 2 half occupied sites (C_1, C_2) 2.3 Å apart 2 half occupied sites (D, E) 4.3 Å apart
2	Pip	p -Iodophenylsulphonyl, $\text{IC}_6\text{H}_4\text{SO}_2^-$	2 (F, G)
3	PMA-Tos	Phenyl mercury acetate, $\text{HgC}_6\text{H}_4\text{CH}_2\text{COO}^-$ Toluenesulphonyl, $\text{CH}_3\text{C}_6\text{H}_4\text{SO}_2^-$	2 (A, B) 2 (F, G)

tions are predominantly bimodal. Such a situation implies that one derivative or set of substitution sites dominates the phasing, and the parameters of the relevant sites cannot be moved from the assumed positions. It appears that most workers, using full calculated shifts, have found convergence absolute, but very slow, so that 10 or more cycles have been required.

In this paper, an example is given where the procedure failed to converge. An alternative procedure is described, which avoids bias towards previously assumed parameters, and converges rapidly.

2. Application to α -chymotrypsin

Table 1 summarizes the substituents used in determining the structure of α -chymotrypsin (Matthews, Sigler, Henderson & Blow, 1967) ($P2_1$, $a=49.3$, $b=67.3$, $c=65.9$ Å, $\beta=101.8^\circ$). The PtCl_4 derivative, in which approximately three PtCl_4^- groups, each containing 146 electrons, are bound per asymmetric unit, is by far the most heavily substituted derivative, and dominates the phase determination. The Tos derivative, which only differs from the native protein by the substitution of light atoms, was included only because of its

close analogy with the Pip derivative, in which the methyl group is replaced by an iodine atom. The Pip derivative therefore gives very accurate isomorphism, but small differences (two groups of effectively 44 electrons per asymmetric unit), when compared to Tos.

We tried to exploit this precise isomorphism, by using Tos as the parent compound for isomorphous substitution. The PMA-Tos derivative followed this strategy. However, we found that tosylation of the crystals profoundly affected binding of chloroplatinite, and the PtCl_4 -Tos derivative was not worth using for isomorphous replacement.

This complication has to be mentioned, though it is barely relevant to the refinement procedure. For the purposes of refinement, a set of notional structure amplitudes was computed

$$|F_1(h)| = |F_{\text{PtCl}_4}(h)| - |F_{\text{Nat}}(h)| + |F_{\text{Tos}}(h)|.$$

$|F_1|$ therefore showed the same differences of structure amplitude from $|F_{\text{Tos}}|$ ($=|F_0|$) as $|F_{\text{PtCl}_4}|$ showed from $|F_{\text{Nat}}|$. Since the differences between Tos and Nat are small, this procedure appeared to be satisfactory for the refinement.

The parameters of the derivatives were originally

Table 2. Heavy atom parameters at various stages of refinement

D.F. — Values obtained from difference projections (Sigler *et al.*, 1966; Sigler & Blow, 1966).

1 — After Rossmann-style refinement with errors as explained in text.

2 — After Muirhead-style refinement (Matthews *et al.*, 1967). Anisotropic temperature factors were used, but have been brought to mean isotropic values in this table.

3 — After refinements described here.

PtCl ₄	Z	B	x	y	z	PtCl ₄	Z	B	x	y	z
A						B					
D.F.			0.219	-0.069	-0.008	D.F.			0.219	0.069	0.014
1	94.0	28.8	0.221	-0.072	-0.005	1	93.5	23.7	0.214	0.073	0.019
2	95.9	24.2	0.220	-0.072	-0.004	2	95.1	24.2	0.212	0.073	0.019
3	122.9	15.2	0.217	-0.075	-0.005	3	107.9	10.0	0.214	0.068	0.019
C₁						C₂					
D.F.			0.217	0.000	0.511	D.F.			} sites C ₁ and C ₂ not distinguished in these refinements		
1	80.3	51.3	0.216	-0.002	0.509	1	47.4	25.0	0.212	-0.006	0.519
2	65.2	25.0	0.218	0.010	0.497	2	55.3	21.4	0.214	-0.010	0.524
3	62.4	22.0	0.216	0.011	0.497						
D						E					
D.F.			0.355	0.000	0.557	D.F.			0.350	0.000	0.504
1	35.7	24.2	0.347	0.007	0.559	1	36.8	32.8	0.359	-0.007	0.498
2	45.0	38.1	0.350	0.006	0.558	2	37.8	38.1	0.351	-0.012	0.499
3	79.8	44.5	0.348	-0.002	0.559	3	53.9	22.8	0.352	0.009	0.494
F						G					
D.F.			0.434	0.062	0.460	D.F.			0.434	-0.062	0.626
1	42.6	16.2	0.438	0.063	0.458	1	44.7	16.9	0.438	-0.063	0.625
2	41.6	20.0	0.438	0.064	0.459	2	49.0	20.0	0.436	-0.057	0.625
3	53.0	10.0	0.435	0.061	0.457	3	66.0	10.0	0.435	-0.059	0.626
A						B					
D.F.			0.199	-0.083	-0.004	D.F.			0.199	0.080	0.014
1	59.1	14.2	0.200	-0.083	-0.004	1	44.1	41.4	0.202	0.080	0.014
2	55.9	27.8	0.200	-0.085	-0.003	2	54.8	27.8	0.195	0.080	0.012
3	73.7	16.1	0.207	-0.082	-0.004	3	92.1	21.4	0.193	0.079	0.011

estimated from difference Patterson maps and difference Fourier projections (Blow, Rossmann & Jeffery, 1964; Sigler, Jeffery & Blow, 1966; Sigler & Blow, 1966). These gave good positional parameters but no quantitative estimate of occupancy and temperature factor. Each derivative was then refined by the Rossmann (1960) procedure. These refinements were satisfactory when carried out on centrosymmetric ($h0l$) data, but were incorrect when applied to non-centrosymmetric data, due to an undetected program error affecting the y coordinate of the substituent atoms.

These parameters were next refined by the Muirhead program. All parameters were refined simultaneously, the starting values of E_j being estimated from the centrosymmetric Rossmann refinements. All subsequent refinements were done with 3000 reflexions chosen from the 25000 independent reflexions in the three-dimensional data set on the basis of (a) amplitude sufficient to be accurately measured, (b) good phasing [high $m(h)$], and (c) uniform distribution over reciprocal space.

During this refinement all positional shifts were very small. The largest overall shift was in the y coordinate of PtCl_4 atom C_1 , which moved by 0.005 (0.3 Å) (Table 2). This was not worrying, as the existence of two separate sites C_1, C_2 had not been suspected in the previous refinements by the Rossmann method, and the two sites were so close that positions estimated

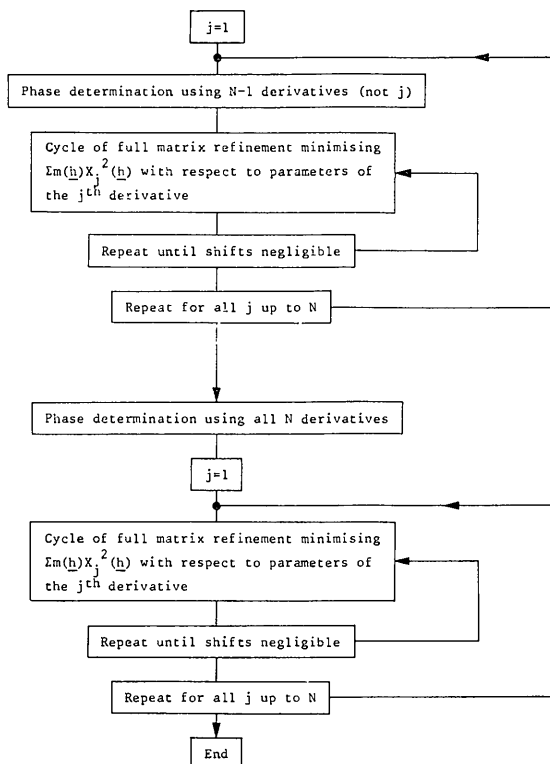


Fig. 3. Scheme for refinement.

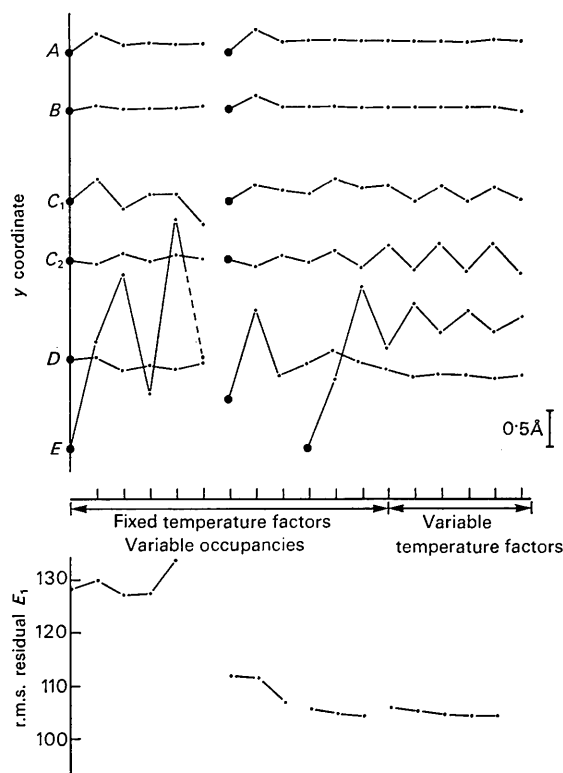


Fig. 4. Schematic diagram illustrating y -coordinate and residual changes during first refinement of PtCl_4 sites according to Fig. 3, upper part. In the first attempt large shifts in the y coordinate of PtCl_4 atom E were observed, but its occupancy went to zero. The refinement was restarted with a single atom at the mid-point of sites D and E (separated by 4.3 Å). This atom went to site D , and atom E was reintroduced at a position opposite to it. Large y -coordinate shifts again occurred, followed by small oscillations of several parameters, but the occupancy of E increased to a level similar to that of D . Inspection of Table 2 reveals the cause of the difficulty. The y coordinates of D and E were estimated as 0.000 from difference maps. The program error in the Rossmann-style refinement moved them in the wrong direction, and even though this movement was less than 0.5 Å, the Muirhead program was unable to bring them back.

from a difference map were likely to have significant errors due to overlap. A more serious problem was that the procedure showed no sign of convergence: although the shifts were extremely small, they remained about the same from cycle to cycle.

In view of the very small shifts, and the excessive computer time involved, the refinement was terminated after eleven cycles, and the resulting parameters were used to calculate a high-resolution electron-density map of α -chymotrypsin (Matthews, Sigler, Henderson & Blow, 1967). Table 2 summarizes the parameters obtained at various points.

Although this electron-density map was generally interpretable, its quality appeared worse than expected. The reason became evident when a 'residual' Fourier synthesis was calculated (Fig. 2). In spite of appearances, parameters for PtCl_4 atoms D and E were grossly

wrong. In refinement the PtCl_4 parameters were moving towards the correct values, but so slowly that hundreds of cycles of refinement would have been needed.

The 'residual' Fourier synthesis has coefficients equal to the lack-of-closure vector $X_j(h)$ (Fig. 2) weighted by the figure of merit $m(h)$. Its properties have been described by Matthews (1970). The synthesis can be regarded as the analogue in protein crystallography of the difference Fourier synthesis used in the refinement of small-molecule crystal structures. Use of the 'residual' synthesis seems to have evolved independently in a number of laboratories (Hoppe, 1959; Blake *et al.*, 1963; Sigler *et al.*, 1966; Drenth, Jansoni & Wolthers, 1967).

3. An improved refinement procedure

To avoid bias, a method of refinement was adopted which used phase angles which were not derived from the derivative being refined. A simple weighting factor $w(h) = m(h)$ was adopted. The procedure, given in the upper part of Fig. 3, gave rapid convergence.

There is always some difficulty about simultaneous refinement of occupancies Z_i and temperature factors B_i . In several trials the correlation matrix (Geller, 1961) was calculated for refinements of these quantities alone. One set of eigenvectors of the correlation matrix are approximately linear combinations of $(Z_i - B_i)$ with an eigenvalue spectrum spanning a factor of 2 or so. Another set of eigenvectors are linear combinations of $(Z_i + B_i)$, and the largest eigenvalue in these groups was never more than 1/10 of the largest eigenvalue in the $(Z_i - B_i)$ group.

In practice an initial value of B_i for all atoms in derivative j was estimated from a Wilson plot of $\log \langle |F_j| - |F_o| \rangle$ against $(\sin \theta/\lambda)^2$. Z_i were refined with B_i fixed for two or three cycles, giving a well conditioned normal matrix; then B_i were allowed to vary for three more cycles. Anisotropic temperature factors were not included.

Since the PtCl_4 parameters were most erroneous, they were refined first, even though the phases obtained only from PMA-Tos and Pip were very poor. Fig. 4 gives some of details this first PtCl_4 refinement, showing large shifts of the incorrect parameters. Fig. 5 shows the course of the total procedure outlined in Fig. 3, as one set of parameters after the other is refined. The final E_{PtCl_4} obtained from the first PtCl_4 refinement reflects the inaccuracy of phase angles derived from the other two derivatives more than errors in the PtCl_4 data. This was the main reason for the further refinement shown in the lower part of Fig. 3, where phases from all available derivatives are used for further refinement. These final refinements are much more akin to the Dickerson refinement method, but rephasing in each cycle was not considered necessary. In these refinements, the only significant changes were increases in occupancy and adjustments to temperature factor, but they establish greatly reduced values for E_j . These final parameters were used to compute an electron-density map from which an improved interpretation could be made (Birktoft & Blow, 1972).

It should be recognised that the problem of *ideal* estimates of the occupancy and E_j remains unsolved. The E_j values as estimated using phase angles from $N-1$ derivatives will be *overestimated* due to errors in

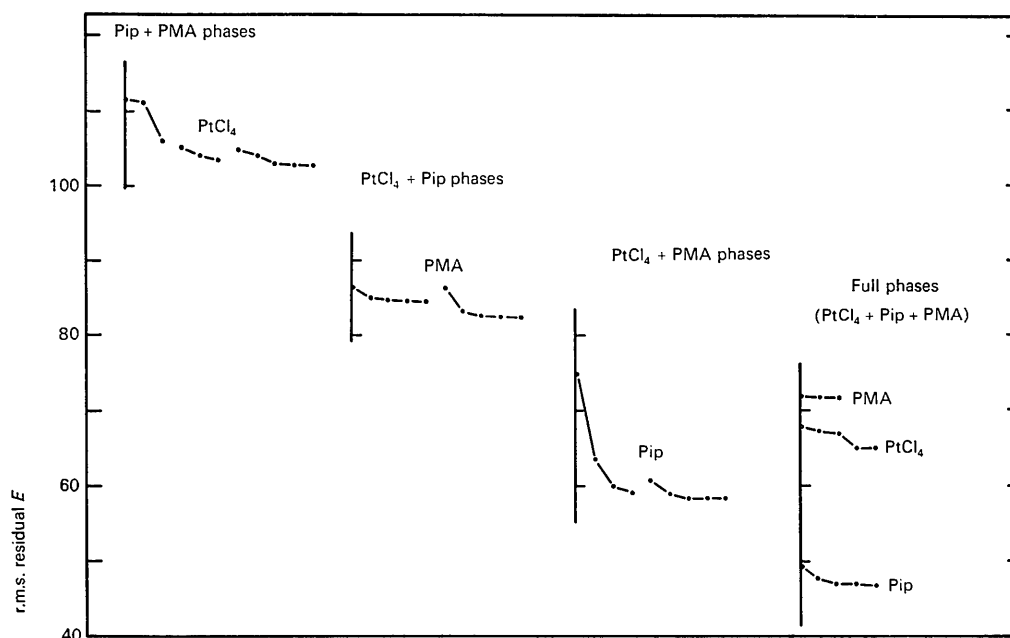


Fig. 5. Reduction of residuals E_j during the total process indicated in Fig. 3. The way in which the process is restarted when temperature factor refinement is introduced leads to a discontinuity in the residual.

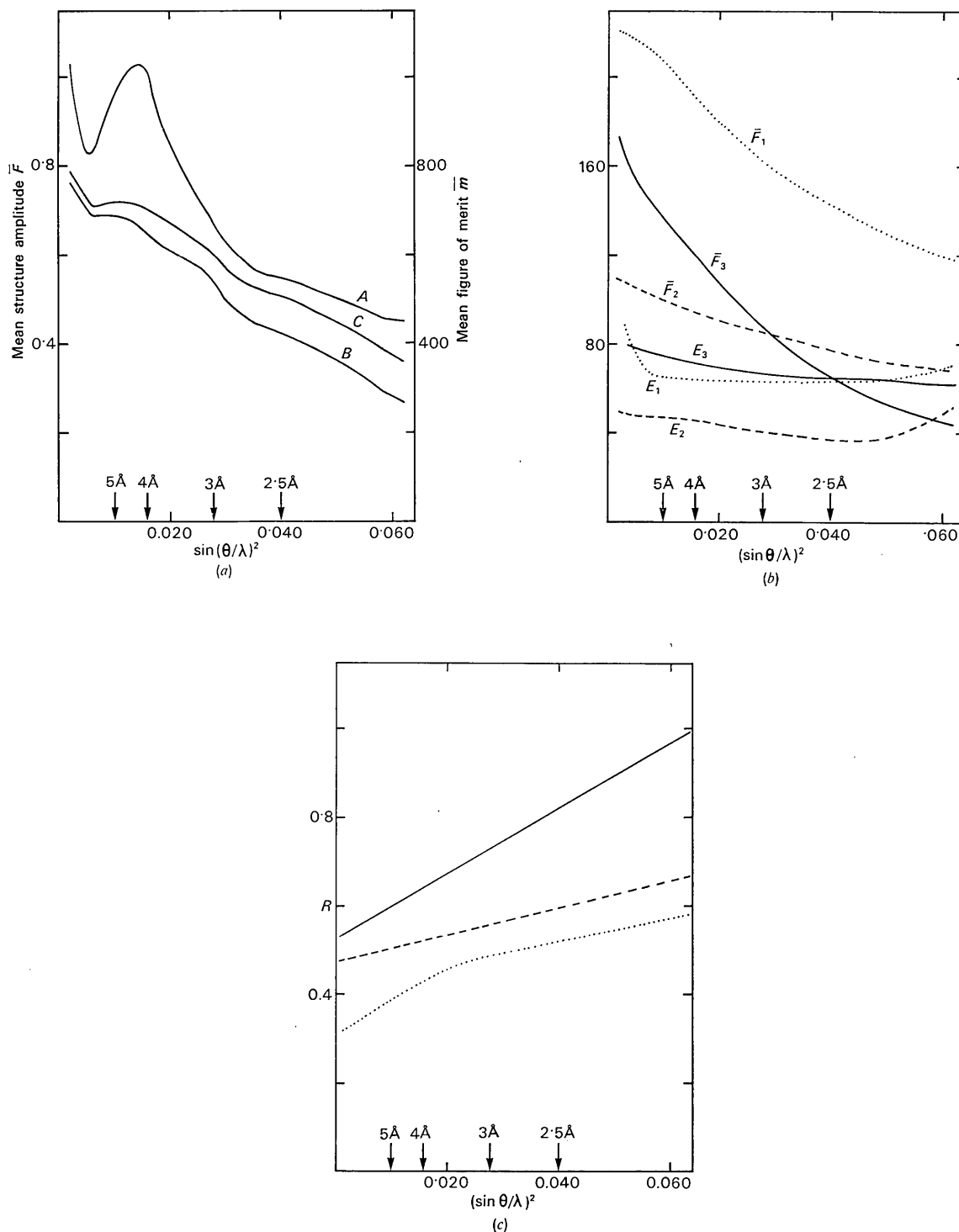


Fig. 6. (a) Curve A – mean structure amplitude \bar{F}_0 ; Curve B – mean figure of merit \bar{m} after Muirhead-style refinement; Curve C – mean figure of merit after final refinement described here. (b) Mean calculated heavy atom structure factor $\bar{F}_{c,j}$, and r.m.s. lack of closure E_j after the final refinement, for the three derivatives described in this paper. (c) The value $\frac{\sum_h X_j(h)}{\sum_h |F_j(h)|} - \frac{|F_0(h)|}{\sum_h |F_j(h)|}$ after the final refinement, for the three derivatives described in this paper. \cdots PtCl₄; $---$ Pip; $—$ PMA. The data in (a) include 37525 reflexions recorded on 31 sets of precession photographs, while those in (b) and (c) refer to 3000 selected reflexions only.

the phase angles. On the other hand, bias introduced using phase angles calculated with the help of the j th derivative will give *underestimates* of E_j and, it seems likely, corresponding *overestimates* of the occupancies.

The method of Fig. 3 can be applied using Muirhead's program, by assigning zero weight to the derivative being refined. The program, however, recomputes phase angles and refines all derivative data on each cycle of refinement, which is extremely expensive in computer time. A least-squares program was written in Fortran in which, by excluding the phase determination step from the program, only $|F_0|$, α_0 , m and $|F_j|$ need to be stored for the 3000 reflexions selected for the refinement. These quantities could be kept in core storage, and a refinement cycle took from one to five minutes on the IBM 360/44, the latter when 32 parameters were refined. A listing of this program, including one subroutine specific to the space group $P2_1$, may be obtained on request.

'Residual' Fourier syntheses calculated from the final parameters showed no interpretable features. We were therefore unable to deduce coordinates for the chlorine atoms of the PtCl_4 groups, which were treated as simple spherical groups throughout. Fig. 6 gives some statistics relating to these parameters, and the phase determinations based on them. The electron density map based on the refined parameters showed greatly improved detail, and enabled a more precise interpretation of the structure to be given (Blow, Birktoft & Hartley, 1969; Birktoft & Blow, 1972).

4. Conclusion

The obvious conclusion from this work is that the results of refinements must be checked by difference Fourier methods, such as the residual Fourier synthesis. We have not investigated whether the Muirhead program would have converged from the parameters originally estimated from difference syntheses. But it is important to emphasise that any program based on the Dickerson procedure has a restricted range of convergence, and within this range the rate of convergence is much slower than that of other possible procedures.

We thank Dr Hilary Muirhead for her comments. The referee informs us that others have used a similar

technique to the one we describe, but have not published them.

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