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Use of Matrix Direct Methods for Low-Resolution Phase Extension for tRNA

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The interpretation of low-resolution electron-density maps for macromolecules frequently proves to be difficult and, usually, no progress can be made with phase extension by direct methods. An iterative procedure for map modification is described, which, together with consideration of the molecular model in terms of group scatterers rather than as single atoms, enables matrix methods of phase extension to be successfully applied. The results of extending phase information from 5 to 3 Å for yeast $tRNA^{Phe}$ are shown and discussed.

1. Introduction

Recently, direct methods have been used as an aid in obtaining high-resolution phase information for macromolecules. They have proved to be most useful and relatively easy to apply when there were initial phases for high-quality atomic-resolution data (Hendrickson & Karle, 1973; Sayre, 1974; de Rango, Mauguen & Tsoucaris, 1975; Carlisle, Gorinsky, Moss, Palmer, Mauguen & Tsoucaris, 1975). When only low-resolution data were available or the initial phases were poor, so that the electron-density maps became uninterpretable (as is often the case in protein crystallography), application of direct methods has been much more difficult. In fact, there are very few reports of success in refining low-resolution data (Reeke & Lipscomb, 1969; Weinzierl, Eisenberg & Dickerson, 1969).

We have been interested in developing an effective method for extending phase information from an already known set of low-resolution (5 Å) to data at medium resolution (3 Å). This range is perhaps the most critical and difficult in the structure determination of biological macromolecules, as it is often characterized by a failure of multiple isomorphous replacement to provide sufficiently good phases for building a molecular model. Furthermore, there are cases where higher-resolution data for the native macromolecule can be collected but no suitable isomorphous derivatives are available. We have previously developed a matrix procedure for medium-to-higher-resolution (3·3–2·5 Å) phase refinement and extension and have described its application to triclinic lysozyme (Podjarny, Yonath & Traub, 1976). A further step has now been taken with the use of similar methods for extending the phases of monoclinic yeast $tRNA^{Phe}$ from 5 to 3 Å resolution. Analysis of the observed data for this tRNA in terms of the quality of the electron-density MIR map as well as the applicability of matrix methods shows that, at low resolution (5 Å), the error level of the map does not allow a straightforward phase extension. We therefore suggest a simple procedure that produces a modified map suitable for matrix methods.

Direct methods relate the constraints in real space, such as non-negativity of the electron-density map and the existence of point atoms, to the observables in reciprocal space (Harker & Kasper, 1948; Karle & Hauptman, 1950; Sayre, 1952; Woolfson, 1970). We have already discussed (Podjarny & Yonath, 1975) the possibility of expressing properties of the electron-density map in terms of statistical moments $(M1,\ldots,Mi)$ of the density distribution function and of correlating them with certain structure-factor products in reciprocal space. We show below that the suitability of a map for phase extension by matrix methods can be judged from the value of $M3$, which is the measure of the skewness of the histogram of an electron-density map. In order to increase this value it is necessary to perform density modification prior to the application of the matrix methods. It is of interest that phase prediction based solely on map modification for high resolution was reported to be succesful in at least two cases (Barrett & Zwick, 1971; Collins, Cotton, Hazen, Meyer & Morimoto, 1975).

2. Influence of errors in the initial map on the quality of the predicted phases by matrix methods

Errors in matrix inversion were previously considered (Podjarny, Yonath & Traub, 1976) for a calculated model, but experimental errors were not introduced at that time. We shall now generalize our discussion to cover both types of error.

The initial data for an electron-density map consist of observed structure factor amplitudes (F_o) and phases (α MIR), usually derived by the method of multiple isomorphous replacement (MIR). Whereas in small-molecule crystallography the initial phases are usually of high quality, in the case of macromolecules the accuracy of phases is limited by factors such as the number and the quality of isomorphism of the derivatives. One of the approaches to analysing the quality of phases is to calculate the associated electron-density map and to apply to it several statistical tests. Ideally, an electron-density map should be built up of a known

number of scattering centres in the correct conformation. As a consequence, the overall electron density cannot exceed a known maximum value (ϱ_{max}), and the map is expected not to have any negative regions.

These characteristics are directly affected by any errors in the MIR phases. Moreover, since a non-negative electron-density map is highly non-random, random errors will usually result in negative regions in the map. A schematic description of this property is given in Fig. 1, which shows cross-sections of correctly and poorly phased maps and the corresponding histograms. This figure shows that the histogram of the correctly phased map is highly skewed, whereas the incorrectly phased map results in a more symmetric histogram. The skewness, which is directly related to the third moment ($M3$) of the histogram, is therefore a practical measure of the quality of the phases (see below).

It is clear that experimental phase errors strongly affect the whole process of phase prediction. The correlation between structure factors, which is the basis for phase refinement and extension by matrix methods (Castellano, Podjarny & Navaza, 1973), is derived from the assumption that the structure consists of equal positive atoms, or that the equation $Z_j^2 = kZ_j$ [where every scattering centre (j) has Z_j electrons and k is the same for all the atoms] holds for the whole contents of the unit cell (Sayre, 1952). Navaza & Silva (1974) showed that it is possible to consider unequal atoms, but the requirement for positive scattering centres is inherent in the theory. One can assign scattering centres with negative Z to the negative regions in the electron-density map. This results in terms kZ_j which are negative and therefore are no longer equal to Z_j^2; hence the approximation of the correlation of two structure factors by a third is not valid and the matrix theory fails. The extent of this failure depends on the level of negative regions in the electron-density map. It can be visualized by plotting a measure of success of self-consistency in phase prediction within a given shell, such as the mean value, $\langle\omega\rangle$, of the cosine of the difference between the initial and predicted phases (by matrix methods), *versus* $M3$, the skewness of the input electron-density map. Fig. 2 shows this relation for several examples, among them: 3 Å data for monoclinic $tRNA^{Phe}$, (Robertus *et al.*, 1974), 3·3 Å data for triclinic lysozyme (Moult *et al.*, 1976; Podjarny, Yonath & Traub, 1976), and 3 Å data for rubredoxin (Watenpaugh, Sieker, Herriot & Jensen, 1973), for which we applied the phase-prediction procedure twice, starting from the MIR phases and also from phases derived from a refined molecular model. Since the difference between the two last cases is only the quality of the phases, it is of interest to compare them in terms of map skewness. Such a comparison supports our previous hypothesis that $M3$ is an appropriate measure for phase quality, and that a preliminary analysis of the electron-density map calculated from the observed data can predict the likely success of phase extension. Moreover, if one assigns a reasonable criterion for the agreement between the predicted and original phases say $\langle\omega\rangle \geq 0{\cdot}35$, then the quality of the 3 Å MIR phases of rubredoxin [point R3(MIR) in Fig. 2] will be at the

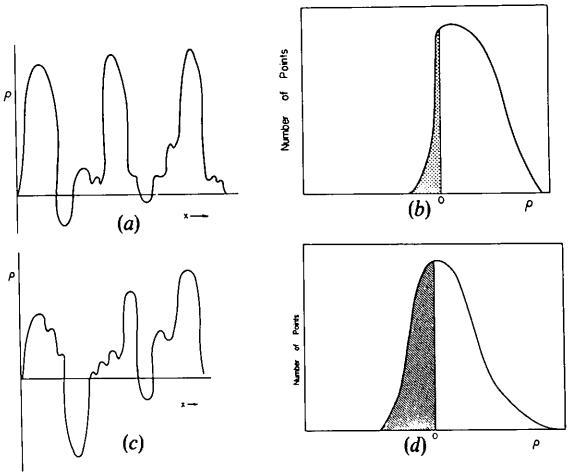

Fig. 1. Cross-section of hypothetical electron-density maps and the corresponding histograms of density distribution. (*a*) A well-phased map, suitable for point-atoms approximation and for matrix methods. (*b*) Represents the histogram of this map. (*c*) and (*d*) Cross-section and a histogram of badly phased map, which is not suitable for matrix methods.

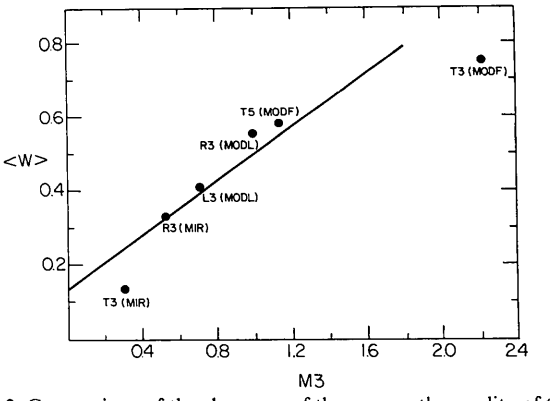

Fig. 2. Comparison of the skewness of the map *vs* the quality of the predicted phases. The ordinate (y axis) is the cosine of the average difference in the phase angle between the predicted set (based on the matrix method) and either the MIR or model phases. The abcissa (x axis) is the third moment of the electron density. Point R3(MIR) is for initial data and consists of $|F_o|$ and α (MIR) for rubredoxin out to 3 Å (Watenpaugh, Sieker, Herriot & Jensen, 1973). 483 scattering centres were considered and the optimized matrix order found to be 150. Point R3(MODL) is the same amplitudes as for R3(MIR) but with model phases. Point L3(MODL) is for 3·3 Å resolution $|F_o|$ α model data for triclinic lysozyme (Moult *et al.*, 1976). 1001 scattering centres were considered with a matrix order of 336. Point T3(MIR) is for initial 3 Å resolution data, consisting of $|F_o|$ and α MIR, for monoclinic tRNA (Robertus *et al.*, 1974). 1520 atoms (excluding residues 18–20, 55–57) were considered, and the matrix order was 226. Point T3(MODF) is the same as point T3(MIR) but with phases derived from a modified electron-density map. Point T5(MODF) is for 5 Å resolution tRNA data with $|F_o|$ and α based on a modified map. The matrix order was 226, for 228 group scatterers.

limit of applicability of the technique. It is suggested, therefore, that a careful modification of the original electron-density map, with a view to decreasing the negative regions, might, on being transferred to reciprocal space, improve the starting phases for the matrix methods.

3. Application of density modification techniques for tRNA

As shown above, the error level in the initial phases may be a limiting factor for the efficacy of the matrix method. Even phases derived from a molecular model are not perfect, and the resulting electron-density map may contain several poorly determined regions. We therefore applied a real-space modification technique to improve the map. The general connexion between real and reciprocal spaces was discussed in an earlier publication (Podjarny & Yonath, 1975). Here we describe a modification procedure based on this relation. Observed data for yeast $tRNA^{Phe}$ were kindly supplied by Dr A. Klug of the MRC, Cambridge, England. The structure determination of this tRNA, which is now known to 2·5 Å resolution by independent studies in two different laboratories (Robertus *et al.*, 1974; Kim *et al.*, 1974) as well as for another type of tRNA (Schevitz *et al.*, 1975), have met with many difficulties, particularly at the stage of interpretation of low-resolution data. It might therefore be very helpful if one could use direct methods for phase extension and refinement. However, it is clear from Fig. 2 that a straightforward phase extension by matrix methods will not be successful in this case. Therefore, the observed amplitudes and MIR phases out to 5 Å resolution were used to calculate an electron-density map and this was then modified by an iterative procedure. In each step phases were calculated from an electron-density map in which the negative regions have been attenuated by a factor of 20. The original mean value of the map is taken as the zero level. The new phases were then combined with the observed structure factors to produce a new electron-density map. The procedure was repeated until the mean square difference in phase angle between two consecutive steps was less than a predetermined limit (such as 5°). While we used a fairly simple modification function, in some instances a more sophisticated one might be needed. A flow diagram of this procedure is given in Fig. 3.

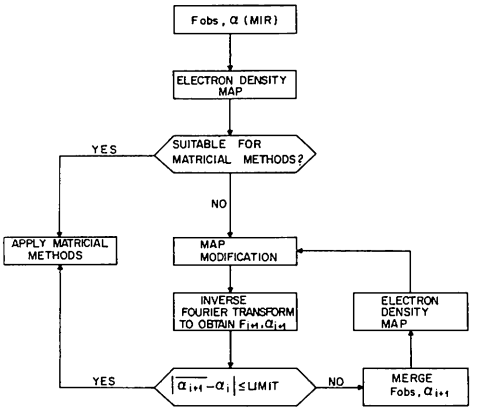

Fig. 3. Schematic representation of the map modification and phase prediction by the matrix-methods procedure.

Electron-density maps were calculated from the observed data at 3 and 5 Å resolution. The MIR 3 Å map as well as modified maps at 5 and 3 Å were subjected to matrix methods. The success in predicting phases within the shells is shown in Fig. 2. In this figure, points T3(MIR) and T3(MODF) refer to the same set of amplitudes; the difference between them is that the latter corresponds to a modified map, while the former corresponds to the map calculated from the MIR phases. It is clear that whereas phase extension for the MIR map is actually unsuccessful, the modified map can be used as an acceptable starting point.

4. Results of phase extension after map modification for tRNA from 5 to 3 Å

tRNA is a relatively small globular nucleic acid, built up of a chain of three primary groups: phosphates, riboses and bases. At low resolution each of those moieties appears in an electron-density map as a distinct group but the individual atoms are not resolved (Kim *et al.*, 1973). We therefore considered each of the above groups as a scattering centre. Thus, the molecule can be considered to be composed of 228 group scatterers, instead of approximately 1700 non-hydrogen atoms. This assumption is justified mainly by examination of the 5 Å resolution map. This map produced a suitable data set for matrix methods as shown in Fig. 2 [point T5(MODF)].

Four cycles of map modification were needed to achieve convergence, with a mean phase difference of 32° and a root-mean-square difference of 46° between the original MIR phase and the final set. The modified map has a skewness of $M3 = 1{\cdot}14$, [Fig. 2, point

Table 1. *Parameters used for 5 to 3 Å phase extension for yeast $tRNA^{Phe}$, after electron-density modification*

Number of scattering centres*	$76 \times 3 = 228$
Optimal matrix order	226
Best rt†	1·0
Occupancies of the Karle & Hauptman (1950) matrices	0·61, 0·40
Occupancies of the Karle & Hauptman (1950) U_{kn} rows	0·3, 0·1
$\langle w \rangle$ for generated reflexions	0·5
Mean standard error for generating reflexions	63°
MW‡ of predicted and MIR phases for the whole 3 Å sphere	0·85

* Group scatterers consist of phosphates, riboses and bases.

† $rt = R_g/R_k$, where R_g and R_k are the reciprocals of the smallest spacing represented by the reflexion whose phase is to be predicted (R_g) and that known *a priori* (R_k) in $Å^{-1}$.

‡ MW: mean correlation $\langle w \rangle$ weighting.

T5(MODF)] and matrix methods were consequently applied to it. Details of the phase-extension procedure are given in Table 1. In order to obtain a direct assessment of the improvement in the map due to phase extension, we analysed especially the regions of tertiary interactions, which are critical for the correct interpretation of the map. Some of them, including A9, U12, A23, U8-A14, G15-C48 and G22-M^{7}G46 are shown, before and after map modification and phase extension, in parts (*a*) and (*b*) of Figs. 4, 5, 6 and 7 respectively.

From these figures it is clear that at least in some cases the 3 Å electron-density map obtained after phases extension shows remarkable improvement over the original 5 Å map. For example, adenine 14 (Fig. 5) is not resolved at all in the 5 Å map, but lies in distinct

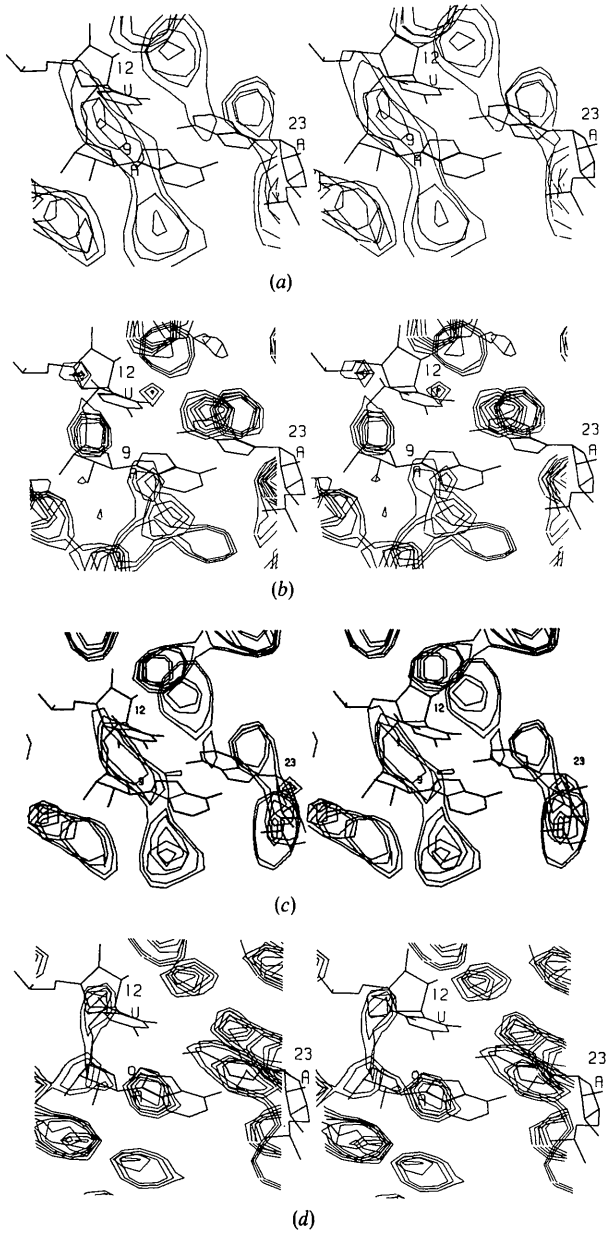

Fig. 4. Superposition of A9, U12 and A23 of the tRNA model (Ladner *et al.*, 1975) on the electron density map. (*a*) Map calculated from observed data, MIR phases out to 5 Å resolution. (*b*) Map calculated from 3 Å observed amplitudes with MIR 5 Å phases and 5–3 Å predicted phases. (*c*) Map calculated from the observed amplitudes and modified phases to 5 Å resolution. (*d*) Map calculated from 3 Å observed amplitudes and MIR phases.

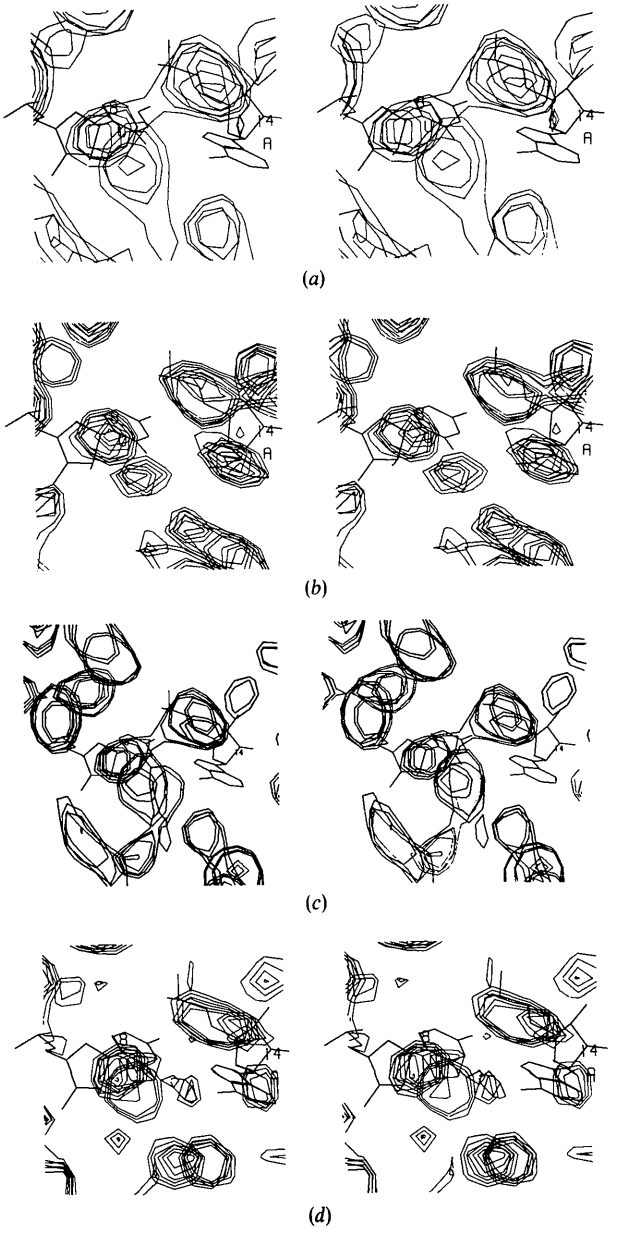

Fig. 5. Superposition of U8 and A14 of the tRNA model (Ladner *et al.*, 1975) on the electron density map. (*a*) Map calculated from observed data, MIR phases out to 5 Å resolution. (*b*) Map calculated from 3 Å observed amplitudes with MIR 5 Å phases and 5–3 Å predicted phases. (*c*) Map calculated from the observed amplitudes and modified phases to 5 Å resolution. (*d*) Map calculated from 3 Å observed amplitudes and MIR phases.

positive regions in the 3 Å phase-extended map. Other bases, such as adenine 23 (Fig. 4) and cytosine 48 (Fig. 6) which are poorly resolved in the original map, are significantly better in the 3 Å extended map. Even for the bases adenine 8 (Fig. 5), guanine 15 (Fig. 6), guanine 22 (Fig. 7) and M^7G46 (Fig. 7), which are well resolved in the 5 Å map, there is some improvement in the extended 3 Å map.

As the phase extension from 5 to 3 Å required essentially two steps, map modification and application of

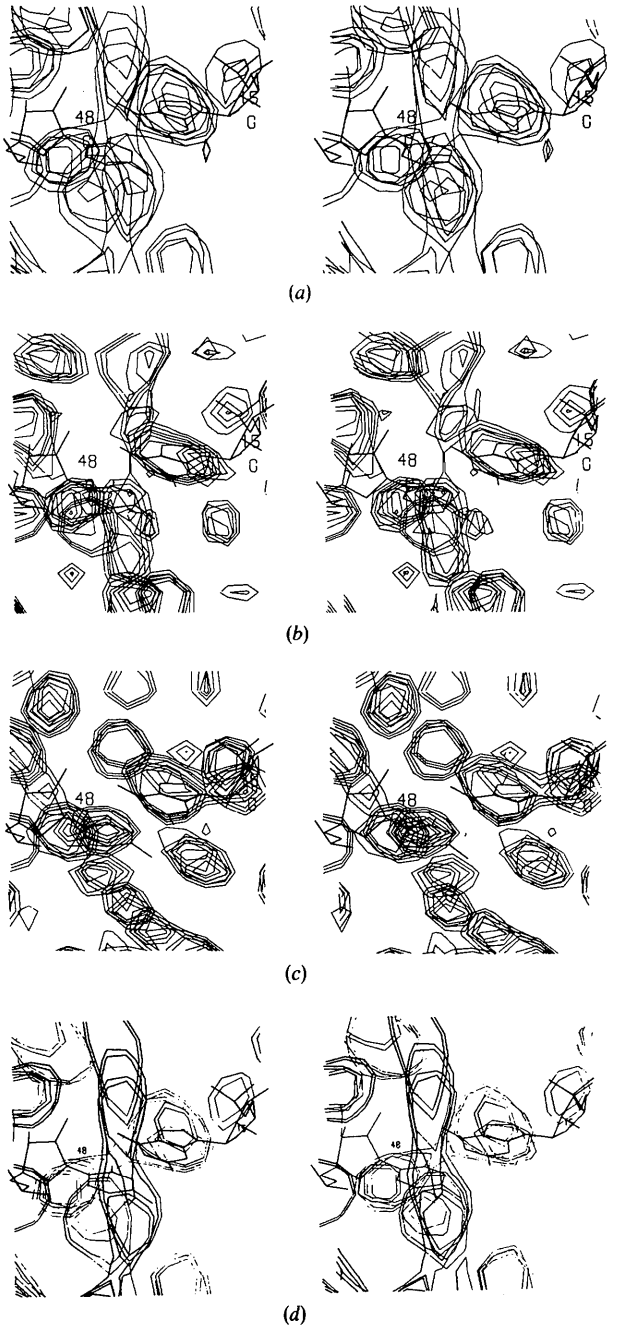

Fig. 6. Superposition of G15 and C48 of the tRNA model (Ladner *et al.*, 1975) on the electron density map. (*a*) Map calculated from observed data, MIR phases out to 5 Å resolution. (*b*) Map calculated from 3 Å observed amplitudes with MIR 5 Å phases and 5–3 Å predicted phases. (*c*) Map calculated from the observed amplitudes and modified phases to 5 Å resolution. (*d*) Map calculated from 3 Å observed amplitudes and MIR phases.

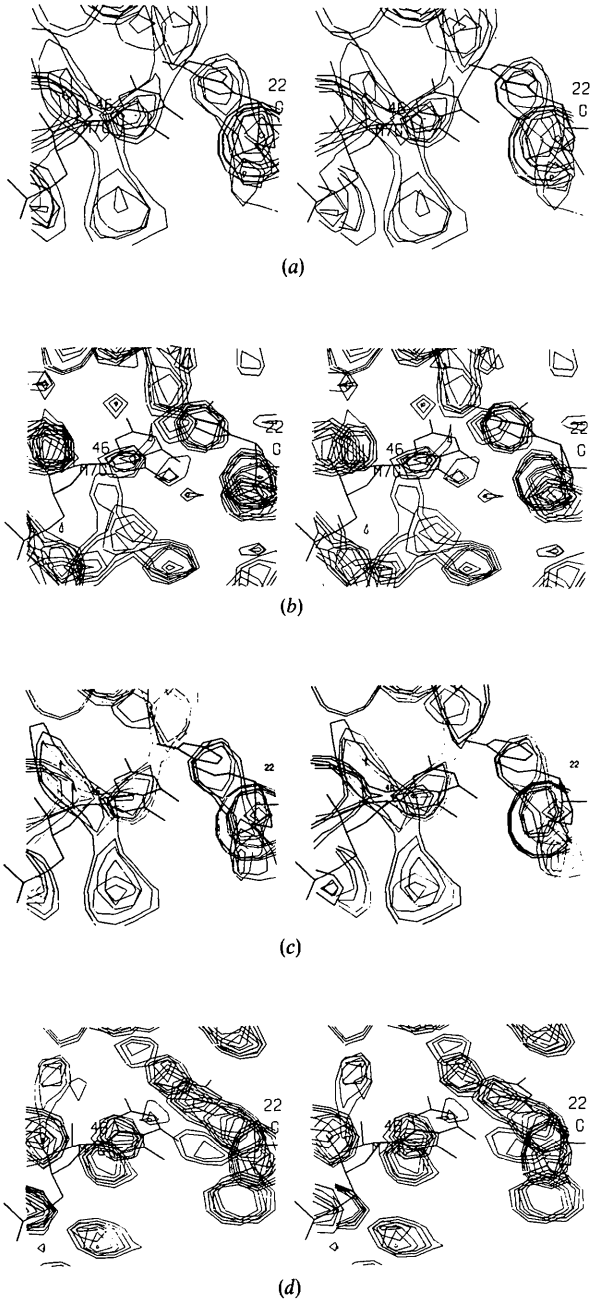

Fig. 7. Superposition of G22 and M^7G46 of the tRNA model (Ladner *et al.*, 1975) on the electron-density map. (*a*) Map calculated from observed data, MIR phases out to 5 Å resolution. (*b*) Map calculated from 3 Å observed amplitudes with MIR 5 Å phases and 5–3 Å predicted phases. (*c*) Map calculated from the observed amplitudes and modified phases to 5 Å resolution. (*d*) Map calculated from 3 Å observed amplitudes and MIR phases.

matrix methods, it is interesting to analyse the contribution of each of them. It is of particular relevance to determine to what extent map modification alone, not followed by phase extension, suffices by itself to improve the interpretability of electron-density maps. This question is of great practical importance, since matrix methods are usually time-consuming and consequently very expensive.

A way to visualize the results of each of these steps is to compare the modified map with the MIR original map and to the extended map. Sections of the 5 Å modified map which correspond to the previously mentioned regions of the molecules are shown in (*c*) of Figs. 4–7. From these figures it is clear that in most cases the modified maps are somewhat more interpretable than the original maps but significantly poorer than those obtained after phase extension.

Attempts to derive 3 Å phases directly from the 5 Å modified map were not successful, and in fact, resulted in an uninterpretable map. As mentioned above, similar studies were already reported (Barrett & Zwick, 1971; Collins *et al.*, 1975), and proved to be successful. We believe that the difference is because the other authors started with a significantly higher-resolution electron-density map and applied a more sophisticated modification technique.

We can conclude, therefore, that the phase-extension procedure added useful information to the original and to the modified electron-density maps. It emphasized the correctly located peaks already present in the 5 Å map and, for several bases, produced positive densities in places that had none at 5 Å resolution.

It is of interest to compare our predicted 3 Å map with that obtained from MIR phases at the same resolution. The previously mentioned portions of these maps are shown in (*d*) of Figs. 4–7. Obviously, there are several regions in these electron-density maps which are clearer than the corresponding regions in the 5 Å MIR and modified maps and the 3 Å predicted maps. An example of this is seen at adenine 9 (Fig. 4). There are also places in which the interpretability of the MIR and predicted 3 Å maps is very similar, and in both maps it is clearly better than the 5 Å original map, as for adenine 14 (Fig. 5). In other cases, such as adenine 23, both MIR maps, at 5 and 3 Å, show a continuous, properly shaped peak for the ribose and the base, while in the 3 Å phase-extension map there is a distinct separation between the two components. Although this separation might be a consequence of our *a priori* assumption of group scatterers for the phase-extension procedure, nevertheless it results in a correct description of the molecular model. Finally, there are cases in which all three tested maps failed to produce density for a part of the tRNA molecule, such as uridine 12 (Fig. 4).

5. Conclusions

In summary, the phase-extension procedure has produced an electron-density map of noticeably higher quality than the original 5 Å map, through not as good as a 3 Å map produced from MIR phases. However, as mentioned above, the aim of this work has been to find a solution to the phase problem in the structure determination of globular nucleic acids and proteins, using very low-resolution MIR phases, together with medium-resolution native amplitudes. By application of direct statistical methods together with real-space modification to the test case of $tRNA^{Phe}$, it has been shown that the method can provide useful structural information. It seems that the method is powerful mainly when applied to nucleic acids, because of the validity of the 'group scatterers' assumption. For proteins it is more difficult to define large-group scatterers at the same resolution, therefore some care has to be taken in application of the procedure.

Further phase refinement of the 3 Å predicted map should be easier, since the starting map is sufficiently good. It could also be possible, once in the 2 Å range, to proceed with alternative direct-methods procedures (Sayre, 1975), which might produce a reliable 2 or 1·5 Å set of phases.

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Absolute Structure Factors for D(+)-Tartaric Acid

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A method of determining absolute structure factors from large, parallel-sided crystals is applied to a sample of D(+)-tartaric acid and is shown to give a random error in the structure factors of less than 1%.

Introduction

When structure factors are measured on a relative scale, the scaling factor is usually determined with respect to a crystal model during a least-squares process. This procedure has two main defects. (1) Different atomic models will give rise to different scattering factors which will result in differing scale factors. (2) Interactions can occur during the least-squares process between the scale factor and the scattering factors and between the scale factor and the temperature factors.

A systematic error in the scale factor can have serious consequences if accurate and detailed information is to be deduced from a difference Fourier synthesis. This error has the effect of superimposing on the difference map a positive or negative electron density whose magnitude is proportional to the true electron density and to the magnitude of the error (Stevens & Coppens, 1975) and this can mask or distort the information which is being sought.

Measurements of absolute structure factors using small, single crystals can be made (Burbank, 1965) but problems in determining the volume of the crystal and in providing a strictly monochromatic beam of uniform incident energy limit their accuracy. These problems can be overcome by using a large, parallel-sided crystal whose cross-sectional area is greater than that of the main beam, a technique investigated by De Marco (1967) in determining the atomic scattering factor of aluminium. A similar method is used in this study.

For such a crystal, the integrated intensity ϱ and the reflectivity of the plane Q, is given by

$$\varrho = QA_c.$$

A_c, the transmission factor, takes into account the geometrical aspects of the scattering as well as the absorption. Since the crystal is uniform in shape, this factor can be determined exactly, (*International Tables for X-ray Crystallography*, 1967), apart from any uncertainties in the thickness of the crystal or in the absorption coefficient. The whole of the main beam is now striking the crystal surface for all reflexions and I_0 now becomes the total energy per unit time striking the crystal and thus the energy per unit area in the main beam need not be constant over the crystal surface.

The main differences between the technique used here and that of De Marco (1967) are the following. (1) The absorption coefficient has been measured with a method described by Lawrence & Mathieson (1976). (2) The thickness of the crystal was measured with a linear differential transducer. (This value of t was the average thickness over those parts of the crystal intersected by the beam during the scattering measurements. For crystals of the order of 0·1 cm thick, an accuracy of 0·1% in each measurement should be achieved. The overall accuracy of the thickness measurement will usually depend on the uniformity of the crystal thickness.) (3) Harmonics in the main beam were eliminated with the (111) planes of a Si crystal as a monochromator. (4) Four measurements of each reflexion at φ, $\varphi+180°$, $\varphi-2\theta$ and $\varphi-2\theta+180°$ were made. The measurements did not have the same integrated intensities since they had different A_c values but they were assumed to give the same structure factor. These structure factors were averaged.

The use of large crystals increases the possibility of the scattering being non-kinematic and this limits the magnitude of the structure factors which can be measured. The identification and elimination of those re-