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Local Scaling: a Method to Reduce Systematic Errors in Isomorphous Replacement and Anomalous Scattering Measurements

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Systematic errors between sets of isomorphous replacement and anomalous scattering data may in some cases be reduced by a technique of 'local scaling'. Methods are proposed which may be used to analyse a data set for significant systematic errors, and to determine whether or not 'local scaling' will be worth applying in a given situation. The proposed method also predicts the optimum number of reflections to be included in the determination of the local scale factors. The technique is illustrated by several examples including 'good' and 'bad' isomorphous-replacement data for α-chymotrypsin, and anomalous scattering data for the lysozyme from bacteriophage T4, and for cytochrome b_{562} from *Escherichia coli*.

Introduction

The success of the isomorphous replacement method in protein crystallography is well known. This technique, first exploited by Green, Ingram & Perutz (1954), and in some instances supplemented by the use of anomalous scattering data, has been employed in virtually every macromolecular crystal structure determination to date.

It is also well known that the successful application of both the isomorphous replacement and anomalous scattering techniques necessitates the measurement of small changes in structure amplitudes. Typically the magnitude of these differences is 5–25% of the average structure amplitude so that small systematic errors in either of the measurements being compared can lead to large relative errors in the difference between them.

The purpose of this communication is to suggest a simple method, which will be termed 'local scaling', by which systematic errors between sets of data may be detected and, insofar as possible, eliminated. This method was first developed and applied during the determination of the structure of α-chymotrypsin (Matthews, Sigler, Henderson & Blow, 1967).

Suppose that F_1 and F_2 are structure amplitudes taken from two sets of measurements which are to be compared. For example, F_1 and F_2 might correspond to structure amplitudes of a native protein and its heavy-atom isomorph or they might correspond to a Friedel-related pair of measurements for the heavy-atom derivative. We desire to obtain, as accurately as possible, the true difference between F_1 and F_2.

Now the measured values of F_1 and F_2 will differ from each other for a number of reasons which may be considered as a combination of (*a*) experimental error, both systematic and random, and (*b*) real differences, *e.g.*, due to the presence or absence of heavy atoms. We will assume for the moment that all these contributions can be combined together and considered as 'errors'.

In the case of isomorphous replacement differences, the amplitudes F_1 are measured from one crystal, F_2 from another and the two data sets are usually brought to a common scale by applying an overall scale factor and an overall exponential 'thermal factor' which is intended to compensate for different radiation damage in the crystals being compared.

For anomalous scattering measurements made from the same crystal, the two sets of measurements F_1 and F_2 will be automatically on a common scale, although in the case of separate crystals, scaling of the two sets of measurements will again be necessary.

In practice it is not uncommon to find that after applying the scale factor described above apparent systematic errors remain. For example, when measuring Friedel-related reflections from the same crystal it may be observed that in one region of reciprocal space the reflections $F(\mathbf{h})$ tend to be consistently larger than $F(\bar{\mathbf{h}})$, while in another region of reciprocal space the opposite may be true. Obviously such systematic differences could be due to a number of causes such as absorption by the crystal and capillary, variations in the diffraction profile, non-uniformity of the X-ray beam, crystal or instrumental misalignment and so on.

Having observed these apparently systematic trends during the study of several protein structures, we have attempted to minimize them by applying a 'local'

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scale factor. If it is assumed that the systematic error is constant in a region of reciprocal space, then one can eliminate the error by defining a 'local' scale factor which is determined not from all the reflections, but from only those reflections in the 'local' region. Of course the price one pays for this procedure is that the 'local' scale factor is determined from far fewer reflections than the overall scale factor, and therefore has a larger statistical uncertainty. In other words the systematic error may be reduced, but another error is introduced in its place. The larger the local area, the smaller will be the probable error in the local scale factor, but the less responsive will the scale factor be to local errors. Obviously the problem is to decide whether local scaling will be worth while in a given situation, and if so what will be the optimum size of the local area from which the scale factors are to be determined. In the following section we outline a possible approach to this problem. For 'good' data,

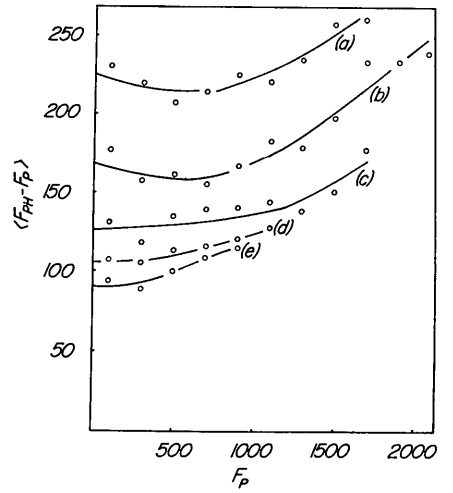

Fig. 1. Distribution of the root-mean square isomorphous differences for the chloroplatinite derivative of α-chymotrypsin. The ranges of $\sin^2\theta/\lambda^2$ are as follows: (*a*) 0–0·01, (*b*) 0·01–0·02, (*c*) 0·02–0·03, (*d*) 0·03–0·04, (*e*) 0·04–0·05.

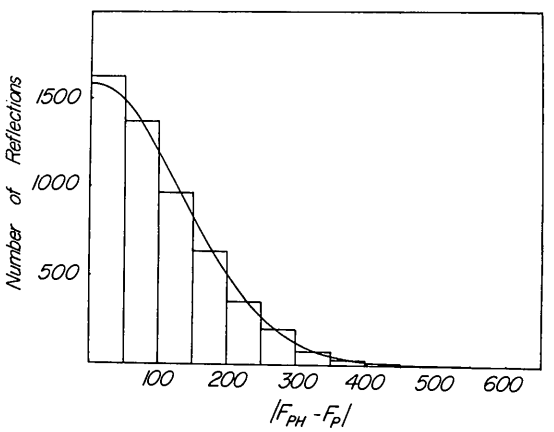

Fig. 2. Frequency distribution for the isomorphous differences plotted in Fig. 1(*c*), *i.e.*, $0{\cdot}02 \leq \sin^2\theta/\lambda^2 < 0{\cdot}03$.

i.e. data free of systematic error, the application of local scaling is unnecessary and in fact undesirable, but in situations where, for one reason or another, it is necessary to use data which contain systematic errors, the application of local scaling can reduce the consequences of these errors.

Theory

(*a*) *Determination of scale factors*

It may be noted at the outset that the determination of scaling factors between overlapping sets of data is a non-trivial problem which has been discussed with increasing sophistication by a number of authors (Kraut, 1958; Dickerson, 1959; Rollett & Sparks, 1960; Hamilton, Rollett & Sparks, 1965; Fox & Holmes, 1966). In the present context a local scale factor is desired for each *reflection* so that rapidity of calculation is essential. We have therefore adopted a somewhat simplified approach which is physically reasonable and which leads to simple (non-iterative) expressions for the local scale factors and for their probable errors. The criterion we have adopted is that the local scale factor K should minimize the sum

$$\Psi = \sum_{\mathbf{h}} w \left(K^{-1} - \frac{F_2(\mathbf{h})}{F_1(\mathbf{h})}\right)^2 \qquad (1)$$

where the summation is over the reflections in the 'local area'. In the present context $F_1(\mathbf{h})$ and $F_2(\mathbf{h})$ will be considered as structure amplitudes, although they could equally well be regarded as intensities. Hereafter we shall omit the subscripts **h**, and denote the inverse scale factor K^{-1} by Q $(=1/K)$.

Setting the derivative of (1) with respect to Q equal to zero, one immediately obtains

$$Q = \sum w \frac{F_2}{F_1} \Big/ \sum w \qquad (2)$$

or in other words Q is simply the weighted mean of (F_2/F_1).

The standard error in Q is given by

$$\sigma(Q) = \left[\frac{\Psi}{(n-1)\sum w}\right]^{1/2}. \qquad (3)$$

One can now consider the form of the expression for the local scale factor for some typical error distributions in the structure amplitudes.

Rather than considering the probable error in F_2/F_1 it is more convenient to think of the error in F_1 or F_2. Plots of the average isomorphous difference (F_2-F_1) for a number of derivatives suggest that at a given Bragg angle the average difference is approximately independent of the magnitude of F_1. This is illustrated for the chloroplatinite derivative of α-chymotrypsin in Fig. 1. The average difference tends to increase for the very strong reflections, but since these are few in number and perhaps less reliably measured, they are usually not used for scaling. The distribution of the differences is very close to Gaussian (Fig. 2).

Making the simplifying assumption that the native amplitudes F_1 are error free, and that all the error resides in F_2, then if $\sigma(F_1)=0$ and $\sigma(F_2)=c$, a constant, as suggested by Fig. 1,

$$w=\frac{1}{\sigma^2(F_2/F_1)}=\frac{F_1^2}{c^2} \tag{4}$$

which leads to

$$Q=\sum F_1F_2/\sum F_1^2 . \tag{5}$$

Also

$$\sigma(Q)=\left\{\frac{\sum(QF_1-F_2)^2}{(n-1)\sum F_1^2}\right\}^{1/2} \tag{6}$$

$$=\left\{\frac{\sum Q^2F_1^2-2Q\sum F_1F_2+\sum F_2^2}{(n-1)\sum F_1^2}\right\}^{1/2} . \tag{7}$$

Using (5) and (7), both Q and $\sigma(Q)$ can be evaluated directly from sums of the products F_1^2, F_2^2 and F_1F_2. Since Q is always close to unity, $\sigma(Q)\simeq\sigma(K)$.

Equations (5) and (7) were obtained on the assumption that the 'error' in F_2-F_1 was independent of the magnitude of F_1. If, in a given instance, this assumption is unrealistic then suitably modified weighting factors should be substituted in (2) and (3).

Two special cases may be worth comment; the first is that in which the error in (F_2-F_1) is proportional to the amplitude F_1, and the second is that in which the error is proportional to $\sqrt{F_1}$. Again assuming all the error to lie in F_2, and F_1 to be error-free, the error distribution $\sigma(F_2)\propto F_1$ leads to

$$Q=[\sum(F_2/F_1)]/n . \tag{8}$$

This scale-factor equation has a similar form to that proposed by Kraut (1958), and has the property that it gives high weight to the weak reflections. The second special case, $\sigma(F_2)\propto\sqrt{F_1}$ leads to

$$Q=\sum F_2/\sum F_1 , \tag{9}$$

an equation similar to that proposed by Dickerson (1959). In cases where the large amplitudes have large uncertainties, one might consider using (9) in preference to (5), although another alternative is to retain (5), but to omit from the respective summations the very strong structure amplitudes.

It should be emphasized that the theory presented above for the determination of scale factors is in no way intended as a substitute for the more general treatment of, for example, Hamilton, Rollett & Sparks (1965). Rather, the intent here is to outline an approximate treatment which leads to simple equations for the scale factors and their standard errors, and which takes into account in a reasonable way the errors in the data.

(*b*) *Choice of local area*

Having obtained suitable equations which allow the estimation of the local scale factors K and their probable errors $\sigma(K)$, we now consider the choice of the size of the local area, or, in other words, the number of reflections on which each local scale factor is to be based.

If two sets of data have been scaled together by an overall scale factor, and local scale factors determined subsequently, then $\langle K-1\rangle$, the r.m.s. value of $(K-1)$ for all reflections (*i.e.*, for all local scale factors), gives the average fluctuation of the local scale factor.

We will write

$$\langle\Delta K\rangle=\langle K-1\rangle . \tag{10}$$

Now $\langle\Delta K\rangle$ will differ from zero for a combination of two reasons, firstly because of *systematic* variations in K from unity, and secondly because of *errors* in K due to random errors in the structure amplitudes, and the finite number of observations on which the calculation of K is based. The *random error* for each local scale factor is given by (3) or (7), and by averaging over all the $\sigma(K)$ one can obtain an estimate of the average random error, denoted $\bar{\sigma}(K)$.

Thus if we write

$$\langle\Delta K_s\rangle=\{\langle\Delta K\rangle^2-\bar{\sigma}(K)^2\}^{1/2} \tag{11}$$

then $\langle\Delta K_s\rangle$ can be considered as the average *systematic* variation in the local scale factor.

If $\langle\Delta K_s\rangle$ exceeds $\bar{\sigma}(K)$, *i.e.*, the average *systematic* variation in the local scale factor exceeds the average error in the estimation of the local scale factor, then it can be concluded that local scaling is worth applying. Furthermore we postulate that the optimum local scaling area is that for which $\langle\Delta K_s\rangle$ exceeds $\bar{\sigma}(K)$ by the greatest amount. If $\langle\Delta K_s\rangle$ should be less than $\bar{\sigma}(K)$ for a given set of data, then it can be predicted that local scaling is not worth applying.

The tests of these ideas in some actual cases are described in the following sections.

Tests of the method

The effectiveness of local scaling has been tested in a number of different situations, three of which will be reported here. The first test uses isomorphous replacement data for a centrosymmetric projection of α-chymotrypsin, the second test is for anomalous scattering data for the lysozyme from bacteriophage T4, and the third test utilizes anomalous scattering data for cytochrome b_{562}.

(*a*) (*h*0*l*) *Projection data for α-chymotrypsin*

Reaction of crystals of the serine proteases with suitable sulfonyl fluorides has in several instances provided excellent isomorphous heavy-atom derivatives. In the case of α-chymotrypsin, space group $P2_1$, $a=49{\cdot}1$ Å, $b=67{\cdot}4$ Å, $c=65{\cdot}9$ Å, $\beta=101{\cdot}8°$, reaction with toluene sulfonyl fluoride and *p*-iodosulfonyl fluoride yielded the isomorphous pair tosyl- and pipsyl-α-chymotrypsin which differ only in the substitution of iodine for methyl at two sites per asymmetric unit (Sigler, Jeffery, Matthews & Blow, 1966). Since tosyl-

and pipsyl-α-chymotrypsin are by all the usual criteria highly isomorphous, with no secondary sites of substitution, they are very suitable as a test case.

In the present context, data from three (*h*0*l*) precession photographs ($\mu=17°$) were used. The first and second films were 'standard' photographs of tosyl- and pipsyl-α-chymotrypsin, while the third was a 'bad' film in which the layer-line screen was slightly mis-set in order to introduce a typical systematic error in the data. All films were measured with the automatic densitometer described by Arndt, Crowther & Mallett (1968).

The two pipsyl data sets were first scaled to the tosyl data in the usual way using an overall scale and temperature factor. Then the pipsyl data were scaled to the tosyl data using the local scaling procedure described in the previous section. In a series of tests the size of the 'local area' was varied, and the results were compared by calculating difference Fourier maps of pipsyl minus tosyl-α-chymotrypsin, and evaluating the peak-to-background ratio. In addition to the local scale factor, the mean correction $\langle \Delta K \rangle$, the mean scaling factor error $\sigma(K)$ and the average systematic variation in the local scale factors, $\langle \Delta K_s \rangle$ [equation (11)], were also determined. The results for both the 'good' and 'bad' pipsyl films are summarized in Table 1. The peak-to-background ratio is defined as the ratio of the mean iodine peak height to the mean of the ten highest noise peaks in the (*h*0*l*) difference map.

In the case of the 'bad' film it is clear that local scaling was worth while since the peak-to-background ratio increased from 4·47 to 4·80, a 7% improvement. Furthermore the value of 4·80 is almost as high as was obtained with a 'good' film.

For both the 'good' and 'bad' films the average error in the local scale factor decreases with the size of the local area, although for the same size area the error is only marginally less for the 'good' film. On the other hand $\langle \Delta K_s \rangle$, the average systematic component of the local scale factor, is much larger for the 'bad' than for the 'good' film. For the bad film $\langle \Delta K_s \rangle$ exceeds $\overline{\sigma(K)}$ by the greatest amount for local scale factors based on 8×8 to 15×15 boxes of reflections, and it is for the same raster sizes that the peak-to-background ratio has its highest values. In contrast, for the 'good' film $\langle \Delta K_s \rangle$ is always less than the error $\overline{\sigma(K)}$ and, as is expected, local scaling always reduces the peak-to-background ratio, although local scaling with a 15×15 raster does little harm.

In these and other tests, local scale factors for reflections toward the inner or outer edge of the film were calculated using a 'local region' centered on each reflection and including the observed reflections within this area. In cases where the number of observed reflections in the 'local area' was less than say 40% of the theoretical maximum, no local scale factor was calculated.

(*b*) *Friedel differences for T4 phage lysozyme*

The lysozyme from bacteriophage T4 crystallizes in space group $P3_221$ with cell dimensions $a=b=61{\cdot}1$ Å, $c=96{\cdot}3$ Å (Matthews, Dahlquist & Maynard, 1963; Matthews & Remington, 1974). Conventional precession photographs of the reciprocal-lattice levels *hNl* contain pairs of reflections which are symmetry-equivalent, whereas the planes of the form $(h,h-N,l)$ contain pairs of Friedel-related reflections. The latter case is the most favorable one for measurement of the small differences in intensity caused by the anomalous scattering of the heavy atoms, since most sources of error will tend to affect both reflections equally. Nevertheless an inspection of the amplitudes of Friedel-related reflections, measured using a computer-controlled drum film scanner (Matthews, Klopfenstein & Colman, 1972), suggested that a number of films seemed to be subject to significant systematic error. There were several factors which could have contributed to these apparent errors including the fact that the crystals were grown in the presence of concentrated phosphate solutions of rather high X-ray absorbance, and in order

Table 1. *Local scaling of pipsyl- vs. tosyl-α-chymotrypsin*

The peak-to-background ratio P/B and the other symbols are defined in the text.

Raster	P/B	$\langle \Delta K \rangle$ (%)	$\overline{\sigma(K)}$ (%)	$\langle \Delta K_s \rangle$ (%)	$[\langle \Delta K_s \rangle - \overline{\sigma(K)}]$ (%)
'Bad' (*h*0*l*) film					
4×4	4·28	7·9	5·6	5·7	0·1
5×5	4·62	7·0	4·4	5·4	1·0
6×6	4·74	6·7	3·9	5·4	1·5
8×8	4·79	6·2	3·1	5·3	2·2
10×10	4·80	5·7	2·6	5·1	2·5
13×13	4·79	4·9	2·1	4·5	2·4
15×15	4·80	3·9	1·6	3·5	1·9
20×20	4·75	3·6	1·5	3·3	1·8
30×30	4·68	2·5	1·1	2·2	1·1
∞	4·47	–	–	–	–
'Good' (*h*0*l*) film					
8×8	4·70	3·2	2·7	1·6	−1·1
10×10	4·68	2·7	2·3	1·4	−0·9
15×15	4·81	2·1	1·6	1·3	−0·3
∞	4·82	–	–	–	–

to obtain sufficiently intense exposures within the life of the crystal, it was necessary to use crystals about 0·6 mm in each direction. Also, in order to obtain a sufficient number of large crystals, it was on occasion necessary to use crystals which had slight cracks.

Following the experience gained in the tests described in the previous section, the Friedel-related reflections were locally scaled to each other by comparing groups of reflections in one half of a film with the corresponding group of reflections in the other half of the same film. The local scale factor K to be applied to $F(\bar{\mathbf{h}})$ was determined using equation (5), although after local scaling both $F(\mathbf{h})$ and $KF(\bar{\mathbf{h}})$ were multiplied by a constant so that their mean value did not change – in other words the isomorphous replacement difference between the derivative and the native protein was kept constant.

Local scale factors were applied to the anomalous-scattering data for two heavy-atom derivatives, the first obtained by soaking the lysozyme crystals in solutions of K_2PtCl_4 and the second by soaking in $HgCl_2$. These two derivatives subsequently sufficed for the initial determination of the structure of the protein (Matthews & Remington, 1974). The local scale factor for each reflection was derived from neighboring reflections contained in a 15×5 raster. This apparently asymmetric shape simply reflects the fact that for the $(h,h-N,l)$ planes the reflections are much more widely spaced in one direction than the other, and on the film itself the raster corresponds approximately to a box 16 mm square.

A summary of the local scaling statistics for each film is given in Table 2. It is immediately apparent that a few films, notably $(h,h-3,l)$ for the platinum derivative, and $(h,h-1,l)$ for the mercury derivative, are relatively free of systematic errors; however if the criteria described previously are any guide, then the data from most of the films can be expected to be improved by local scaling. The apparent systematic errors for the $(h,h-3,l)$ mercury film are particularly obvious, and in retrospect it could be argued that this film should have been re-taken.

In Table 2 we have also included the mean Friedel difference $\langle F_+ - F_- \rangle$ before and after local scaling. As might be expected for the 'good' films, the mean difference remains almost constant, whereas for the 'bad' films this quantity drops substantially due to partial elimination of the 'systematic' part of the difference. For comparison, local scale factors based on a 19×7 raster of reflections were also calculated. The full data summary is not given, but for comparison, the values of $[\langle \Delta K_s \rangle - \sigma(K)]$ are included in Table 2. It would be expected that local scaling with a 19×7 raster size would give results very similar to that for the 15×5 raster.

The procedure used to test the effect of locally scaling the Friedel differences was somewhat different from that employed in the previous section, and was

Table 2. *Local scaling statistics for T4 phage lysozyme Friedel differences*

Film	$\langle \Delta K \rangle$ (%) (15×5)	$\overline{\sigma(K)}$ (%) (15×5)	$\langle \Delta K_s \rangle$ (%) (15×5)	$\langle \Delta K_s \rangle - \overline{\sigma(K)}$ (%) (15×5)	$\langle \Delta K_s \rangle - \sigma(\bar{K})$ (%) (19×7)	$\langle F_+ - F_- \rangle$ Before local scaling	$\langle F_+ - F_- \rangle$ After local scaling
Platinum derivative							
$h,h-1,l$	4·8	0·9	4·7	3·8	3·8	43·5	30·5
$h,h-2,l$	5·0	1·4	4·8	3·4	3·4	45·9	38·5
$h,h-3,l$	1·9	1·3	1·4	0·1	−0·2	38·4	37·5
$h,h-4,l$	2·6	1·6	2·0	0·4	−0·1	41·2	40·1
$h,h-5,l$	4·4	1·1	4·3	3·2	3·0	36·2	32·8
Mercury derivative							
$h,h-1,l$	1·6	1·3	0·9	−0·4	−0·1	39·4	38·7
$h,h-2,l$	6·0	1·4	5·8	4·4	4·4	45·7	41·7
$h,h-3,l$	10·6	1·3	10·5	9·2	9·5	60·7	40·0
$h,h-4,l$	6·8	1·7	6·6	4·9	4·9	57·4	45·5
$h,h-5,l$	4·7	1·5	4·5	3·0	2·6	44·1	42·8

Table 3. *Tests of phase angles derived from anomalous differences before and after local scaling*

The phase angles for the 'Hg–Nat' maps are derived exclusively from the anomalous scattering differences for the platinum derivative, before and after local scaling, and conversely for the 'Pt–Nat' maps.

		Before local scaling			After local scaling		
Map	Peak	Peak height	Average highest background	P/B	Peak height	Average highest background	P/B
Hg–Nat	Hg1	67	29·7	1·67	67	26·2	2·02
	Hg2	32			39		
Pt–Nat	Pt1	90	39·3	1·38	94	34·5	1·63
	Pt2	43			47		
	Pt3	30			28		

intended to illustrate the effectiveness of the anomalous scattering data in phase determination, since this is the main purpose of measuring the Bijvoet differences. First the respective $(h,h-N,l)$ films were merged together to obtain a quasi-three-dimensional data set to a nominal resolution of 2·5 Å containing about 3400 reflections, *i.e.* almost half of a full data set. Because of the relatively high symmetry of space group $P3_221$, the data were reasonably well distributed through reciprocal space. Then, using just the Bijvoet pairs for the platinum derivative, protein phase angles were determined by what might be described as the 'single anomalous scattering method', in analogy to the single isomorphous replacement technique (Blow & Rossmann, 1961). The characteristics of the single anomalous scattering method have been discussed and illustrated previously (Matthews, 1969). The protein phase angles obtained by this procedure, although necessarily approximate, were then used to calculate a difference Fourier map with coefficients equal to the difference in structure amplitude between mercury and native phage lysozyme, and weighted by the figure of merit of the single platinum anomalous-scattering phase determination. Such a map is expected to show peaks at each of the mercury binding sites, and the peak-to-background ratio can be used as a measure of the quality of the anomalous scattering data used to determine the phase angles. In the present case two similar mercury minus native maps were calculated using the platinum phases determined before and after local scaling, and the results are summarized in Table 3. Subsequently the test was repeated in reverse, using the mercury anomalous data to determine phases, and then calculating platinum minus native difference maps. In the latter case peaks were expected at the three platinum binding sites. Again the results are summarized in Table 3.

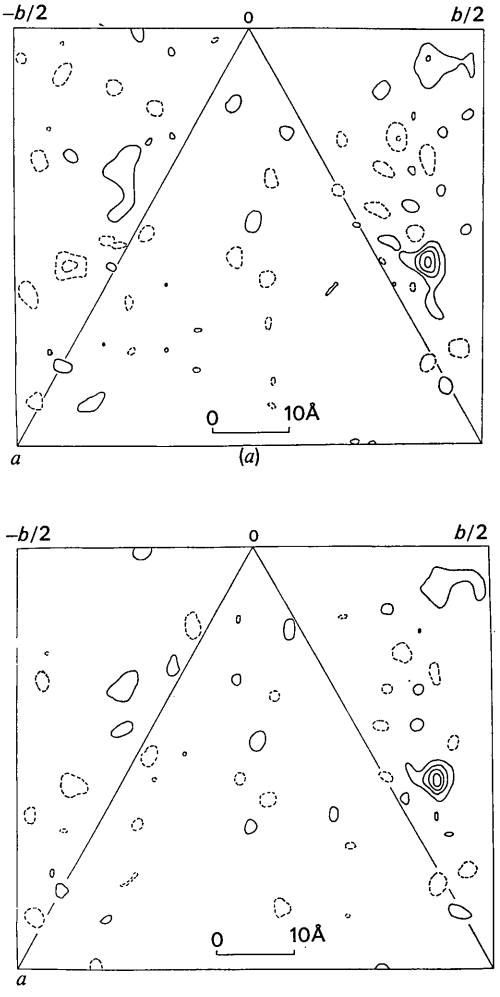

Fig. 3. Section through the major mercury site in a mercury minus native difference map using partial data to 2·5 Å resolution and phase angles determined from platinum anomalous differences. (*a*) Before local scaling of platinum data. (*b*) After local scaling.

In one comparison the peak-to-background ratio increased by 21 % and in the other by 18 % as a result of local scaling. In both cases it appears that a significant improvement in the accuracy of the Bijvoet differences has been realized. Use of local scaling resulted in a slight increase in the average peak height (about 5 %) but the main improvement manifests itself in the reduction of the highest noise peaks. A section through the major mercury site in the mercury minus native difference map is illustrated in Fig. 3. In this case the peak heights are the same, but comparison of both sections reveals that almost without exception every background feature has been reduced following the application of local scaling.

It might be noted that the above tests presuppose the correct identification of the space group as $P3_221$ rather than $P3_121$. In the enantiomorphic space group no peaks would be expected, and in fact the appearance of the expected peaks can be used as a method of resolving the space-group ambiguity (*cf* Matthews, 1966; Colman, Jansonius & Matthews, 1972).

(*c*) *Friedel differences for cytochrome* b_{562}

In the previous two sections the test data were collected photographically. It is, of course, possible to apply local scaling to data collected by other techniques, and in this section we describe tests with diffraction data collected using an automatic diffractometer.

Ferricytochrome b_{562} from *Escherichia coli* crystallizes in a triclinic unit cell, $a=33{\cdot}7$, $b=50{\cdot}5$, $c=32{\cdot}7$ Å, $\alpha=102{\cdot}8°$, $\beta=86{\cdot}6°$, $\gamma=106{\cdot}7°$ with two molecules per unit cell (Czerwinski, Mathews, Hollenberg, Drickamer & Hager, 1972). Three-dimensional data to a resolution of 4·5 Å were collected using a Picker FACS-1 automatic diffractometer. A modified Wyckoff step-scan procedure was used (Wyckoff *et al.*, 1967; Mathews *et al.*, 1972) and the data were corrected empirically for absorption, background and radiation damage (North, Phillips & Mathews, 1968; Czerwinski & Mathews, 1974).

Anomalous scattering differences were measured both for the native crystals, which contain two iron atoms per unit cell, and for a platinum derivative, and in both cases anomalous scattering difference Patterson syntheses with coefficients $(F_+ - F_-)^2$ were calculated (Rossmann, 1961). In these tests the approximately 1200 Friedel pairs were locally scaled to each other by summing F^2 for a $5\times5\times5$ block of reflections surrounding each reflection and its Friedel mate. The reflection whose 'block sum' was the smaller of the two sums was scaled upward by the ratio of the two sums (Czerwinski & Mathews, 1974). This scaling procedure is analogous to that given by equation (9), replacing the amplitudes by intensities, and corresponds to the reasonable assumption that $\sigma(I)$ is proportional to I.

The local scaling statistics and the results for the respective anomalous difference Patterson functions are summarized in Tables 4 and 5. In the case of the native data the expected iron–iron vector peak increased in height by about 10% and the background peaks decreased by about the same amount to give an improvement in the peak-to-background ratio of 15%. In the case of the platinum data, the improvement resulting from local scaling was much more dramatic, amounting to a 36% increase in peak height and an improvement in the peak-to-background ratio of 52%. The dramatic improvement for the platinum derivative is also to be anticipated from the large value of $\langle \Delta K_s \rangle - \overline{\sigma(K)}$ (Table 4).

Table 4. *Local scaling statistics for cytochrome b_{562} Friedel differences*

Derivative	$\langle \Delta K \rangle$	$\overline{\sigma(K)}$	$\langle \Delta K_s \rangle$	$\langle \Delta K_s \rangle - \overline{\sigma(K)}$
Native	0·025	0·0054	0·024	0·019
Platinum	0·245	0·0173	0·244	0·227

The success of the local scaling method for cytochrome b_{562} can be attributed in part to the fact that the crystals were small, and, in the case of the platinum data, irregular in shape. For both these reasons, and also because of the triclinic unit cell, it was very difficult to align the crystal accurately on the diffractometer, and to collect accurate data.

It might be noted that the use of a 'local region' for diffractometer-collected data is appropriate for some types of errors, but not for others. For example errors due to X-ray absorption of the crystal, adhering mother liquor and the capillary generally change fairly continuously through reciprocal space and tend to affect neighboring reflections in a similar manner. In cases such as this a 'local region' as used for cytochrome b_{562} is appropriate. On the other hand, errors due to counting statistics, to overlapping of reflections, or to radiation streaks affect neighbouring reflections in quite different ways and will not be reduced by 'local scaling'.

Discussion and conclusion

The tests described here demonstrate that in a variety of cases the application of the principle of 'local scaling' can improve the accuracy of isomorphous replacement and anomalous scattering differences. However, it must be added that in a number of other tests the use of local scaling did not significantly change the apparent quality of the data. For example, in an extension of the tests described above for α-chymotrypsin, three-dimensional difference Fourier syntheses at 2·0 Å resolution were calculated for tosyl- minus native-α-chymotrypsin, with and without the application of local scaling. In the locally scaled map the density of the prominent peak due to the sulfonyl group and the average background density were almost exactly the same as the corresponding densities in the normally scaled difference map (Sigler, Blow, Matthews & Henderson, 1968). Thus, in this instance, local scaling caused no material improvement.

In the case of α-chymotrypsin, large equi-dimensional crystals were readily available and it was possible to routinely obtain films which would be regarded as 'good' both by visual inspection and by a comparison of symmetry-related reflections (*cf.* Arndt *et al.*, 1968; Matthews *et al.*, 1972). Photographs having obvious systematic errors were retaken. In cases such as this, local scaling was found to be of marginal benefit, and not worth applying, although it may be noted that in a number of tests it was never found that local scaling significantly *decreased* the quality of the data.

On the other hand, one often has to deal with less favourable situations. For example, as was the case with bacteriophage T4 lysozyme, it was necessary to use large crystals in order to obtain sufficient intensity, yet such large crystals had severe absorption, and tended to be cracked. At the other extreme, for cytochrome b_{562} only small irregular crystals were available so that crystal alignment and accurate data collection were difficult. In other instances, crystal slippage, crystal or instrumental misalignment, non-uniformity of

Table 5. *Tests of local scaling of Friedel differences for cytochrome b_{562}*

		Before local scaling			After local scaling		
Map	Peak	Peak height	Average highest background	P/B	Peak height	Average highest background	P/B
Native $(F_+ - F_-)^2$	Fe–Fe	64·7	34·2	1·89	71·3	32·7	2·18
Platinum $(F_+ - F_-)^2$	Pt–Pt	36·9	38·6	0·96	50·0	34·4	1·45

the X-ray beam, anisotropic crystal mosaicity, radiation damage and other factors may cause systematic errors, and it is in situations such as these, where one is forced to be content with poorer data, that local scaling may be useful.

Throughout this paper we have used 'local' to denote a local region in reciprocal space, but the general principle of 'local scaling' could be applied in the scaling together of any sets of reflections which are affected by a common systematic error. For example, one could use 'local scaling' to test whether a set of derivate amplitudes differed systematically from the corresponding native amplitudes as a function of reflection intensity. In this case 'local' would refer to a (small) range of structure amplitude. In other situations, 'local' might refer to a range of Bragg angle. Also, if one were scaling sets of sequentially measured reflections, then 'local scaling' could be applied to groups of reflections measured during a limited time span in order to minimize systematic errors due to radiation damage.

Ideally one would like to be able to measure data free of all systematic errors, but in practice this is often impossible. As proteins of higher molecular weight crystallizing in larger unit cells are studied, it will become increasingly difficult to measure with accuracy the small isomorphous-replacement and anomalous-scattering differences. Fortunately, it is in just these situations, where the density of observations in reciprocal space becomes increasingly higher, that local scaling should work best.

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