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# Electron Diffraction from Single, Fully-Hydrated, Ox-Liver Catalase Microcrystals

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High-resolution single-crystal electron diffraction intensity data from fully hydrated, flat, rectangular catalase microcrystals are subjected to various tests which support the thesis that diffraction from crystals with thicknesses up to at least 1500 Å may be treated by the kinematical theory. The ratio of the total diffracted intensity to the incident beam intensity is found to be 0·06 for a 530 Å thick crystal and 0·16 for a 1500 Å thick crystal. A plot of averaged electron-diffraction intensities *vs.* sin $\theta/\lambda$ also closely resembles molecular-transform-modulated plots of X-ray diffraction intensity data from protein crystals.

## Introduction

A significantly frustrating barrier to the crystal structure analysis of many important biomolecular structures is the difficulty in obtaining crystals of suitable size and quality for X-ray diffraction experiments. Proteins comprise a major fraction of this troublesome class of compounds.

Since atomic scattering amplitudes for electrons are at least $10^3$ greater than those for X-rays (Vainshtein, 1964), the sample size limitation would be conceivably overcome by obtaining electron diffraction data from more readily available protein microcrystals. Stabilization of protein crystals against solvent loss under high-vacuum conditions by use of cross-linking reagents and/or negative stain, however, has yielded only low-resolution electron diffraction data of dubious usefulness (*e.g. vide* Ferrier, 1969, Glaeser & Thomas, 1969; Hoppe, Langer, Knesh & Poppe, 1968; Moretz, 1973; Unwin, 1972; Wrigley, 1968). The use of differentially pumped wet specimen chambers in electron microscopes (Parsons, Matricardi, Moretz & Turner, 1974) on the other hand, has been demonstrated to efficaciously preserve unfixed, unstained, fully hydrated ox-liver catalase microcrystals from drying. These crystals, which do not give diffraction patterns below 90% relative humidity, (Moretz, 1973), will routinely give single-crystal electron diffraction data out to $3 \rightarrow 2$ Å when in the environmental chamber at 100% relative humidity (Matricardi, Moretz & Parsons, 1972).

Although single-crystal electron diffraction patterns may be routinely used to derive unit-cell constants, and sometimes for space-group determination, the most interesting problem is whether the intensity data from protein crystals may be gainfully used in crystal structure determination. This of course depends upon whether the kinematical diffraction-theory assumption often usable for X-ray and neutron diffraction intensity data can be safely applied to the electron diffraction data set.

The most rigorous description of the diffraction of any incident radiation by a crystal is a dynamical diffraction theory with which the complicated interactions of $n$ diffracted beams amongst themselves and the incident beam must be unraveled. The kinematical approximation, which assumes no significant interactions between beams (*e.g.* see Gevers, 1970), is precisely the condition to which the diffraction data must mostly conform in order to enable an *a priori* determination of an unknown crystal structure of any complexity. This is true because, for all their demonstrated utility, present formulations of $n$-beam dynamical theories themselves require an input model of the potential distribution within the crystal (from which a kinematical set of calculated structure factors can be derived). Deriving this model for a complex unknown acentric structure having many plausible conformations is often difficult enough with a kinematical intensity data set in routine crystal structure analysis.

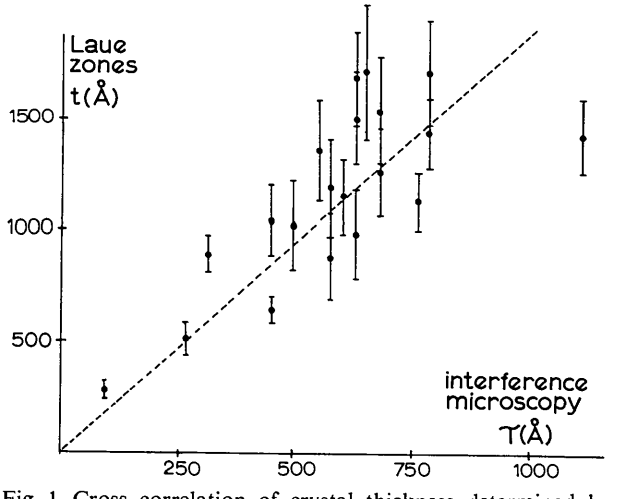


Fig. 1. Cross correlation of crystal thickness determined by Laue-zone measurements to optical phase-change retardation measurements.

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This communication evaluates single-crystal electron diffraction intensity data from wet catalase microcrystals to ascertain their suitability for future crystal and molecular structure determination.

## Materials and methods

*Catalase crystals*

Ox-liver catalase crystal suspensions were obtained from Boehringer Mannheim Corp. (N.Y., N.Y.). A one ml aliquant of the commercial suspension was solubilized at room temperature in 2·5 ml *p*H 7·4 $KH_2PO_4/Na_2HPO_4$ buffer ($\mu=0{\cdot}2$). The *p*H was then adjusted to 5·3 with saturated $KH_2PO_4$. After several days at 4°C, plate-like rectangular crystals could be harvested. Before electron diffraction experiments the crystals were washed twice with distilled water. The method of recrystallization is similar to that employed by Sumner & Dounce (1937) except that no ammonium sulfate is used to bring down the crystals.

*Electron diffraction*

Electron diffraction patterns were obtained with a JEM-200 electron microscope equipped with a two-stage, differentially pumped wet specimen chamber described in the review by Parsons, Matricardi, Moretz & Turner (1974). A drop of washed catalase suspension was placed on a grid on the side-entry sample-holder spade. One minute was allowed to elapse to ensure precipitation of the crystals from suspension onto the grid surface before excess water was blotted off and the spade inserted into the microscope. The wet specimen chamber was kept at room temperature (measured with a thermocouple) and at the corresponding water vapor pressure (measured with a Hg° manometer). The steady state was found to keep the crystals fully hydrated for at least $2\frac{1}{2}$ hours.

The microscope was always operated at 200kV ($\lambda=0{\cdot}0251$ Å). A 10$\mu$m condenser aperture was used enabling a focused spot size of $2 \rightarrow 5\mu$m diameter. After previous alignment for diffraction conditions, the inserted sample holder was translated until distinct 002 reflections were seen on the fluorescent screen. Since the typical crystal plate size is $10 \times 20$ $\mu$m, several diffraction patterns could often be obtained from a single crystal by translation to different portions of the plate; thus in most cases it is expected that the incident beam was entirely within the bounds of the crystal plate.

Catalase, like most unconjugated organics, is quite radiation sensitive. Diffraction patterns from the wet crystals were seen to disappear at beam exposures around $10^{-3}$ coulombs/cm$^2$, corroborating previous observations (Matricardi, Moretz & Parsons, 1972). Because of this radiation sensitivity the crystals were never imaged beforehand. Exposures well below the cited limit were guaranteed by use of Kodak X-ray film which has been found to be significantly more sensitive than electron image plates to electrons at several accelerating voltages (Matricardi, Wray & Parsons, 1972).

*Crystal thickness measurements*

Thicknesses of catalase crystals were measured by incident-beam attenuation through the crystal after disappearance of the diffraction pattern on the viewing screen. It is anticipated that there is some error due to mass loss, even though the remnants of the crystal lattice would tend to keep fragments 'frozen in'. Drastic mass loss, which should be detectable as significant drift in incident-beam transmission, was never observed, however. The beam current was measured with an electrically isolated viewing screen connected to a Keithley 600B electrometer. These readings were corrected to a calibration made with a Faraday cup.

Electron-beam attenuation was cross-correlated with independent phase-change retardation measurements of the catalase crystals on a Zeiss interference (optical) microscope which was also in a 100% relative humidity enclosure. The linear relationship observed indicated the applicability of a Beers-law type relationship for the electron-beam attenuation, since the phase-change retardation in an interference microscope is a linear function of sample thickness.

The parameter needed to convert phase-change retardation to thickness is the refractive-index difference between the sample and the surrounding medium (*e.g.* see Ross, 1962). Protein crystals are perfectly permeable membranes for solvents. Thus, refractive index measurements on such crystals are seldom determined although some measurements have been made on crystals stable in distilled water (Davies, 1959; Davies & Thornburg, 1960). Owing to the small dimension of our crystals, we have calibrated the thickness by measuring well defined Laue zones in diffraction patterns from tilted crystals. A cross-correlation of thickness measurements from Laue zone widths with beam attenuation and/or phase-change retardation measurements (Fig. 1) indicates that the catalase crystals have an 'isotropic' refractive index of 1·53 which is confirmed by observation of Becke line convergence (when the optical microscope objective is raised) with the wet crystals covered with a viscous immersion oil ($n_D=1{\cdot}5150$) (Bunn, 1961).

More detailed accounts of these thickness measurements are given by us in other communications (Dorset & Parsons, 1975 *a*, *b*).

*Densitometry and derivation of intensity data*

Films were scanned using a Joyce–Loebl Mk IIIC-S two-beam microdensitometer at a slit width of 26 $\mu$m and a slit height *ca.* 400 $\mu$m (corresponding to width of larger spots). Within the Wooster (1964) error the 'slit scan' represents a one-dimensional integration across the spots. The maximum possible Wooster error for this slit-scan configuration is 33% for the 002 reflections. A more typical maximum Wooster error for most of the film is about 5%. Intensities were

obtained by subtracting backgrounds from the peak height of the trace across a spot.

## Results and discussion

*Unit-cell parameters and symmetry*

A typical high-resolution electron diffraction pattern (data out to 3·2 Å) is shown in Fig. 2. This *h0l* pattern is from a *ca.* 1470 Å thick wet, unstained, unfixed catalase crystal and represents a 30 s exposure on Kodak No-Screen X-ray film at an incident-beam diameter of 5 $\mu$m and incident intensity around $19 \times 10^{-13}$ amp or a total radiation dose of $3 \times 10^{-4}$ C/cm$^2$.

In diffraction patterns from these crystals where the incident beam is nearly normal to the major crystal face, it is possible to observe systematic absences supporting *pgg* symmetry for this projection of the unit cell. Unit-cell dimensions, calibrated with a Au° powder diffraction standard are $a = 69{\cdot}7$ Å, $c = 177$ Å, with an orthorhombic unit cell assumed (Labaw, 1967), and are in good agreement with previous measurements of diffraction patterns from both wet and negatively stained catalase crystals of this habit (Ferrier, 1969; Wrigley, 1968; Matricardi, 1972; Unwin, 1972; Labaw, 1967; Moretz, 1973; Ward & Mitchell, 1972; Matricardi, Moretz & Parsons, 1972).

If Labaw's (1967) value $b = 141$ Å is used, the unit cell volume is $1{\cdot}88 \times 10^6$ Å$^3$. With a crystal density of 1·3 g cm$^{-3}$ (Sumner & Gralen, 1938) and four molecules per unit cell assumed, the fractional volume occupied by solvent in these crystals is 42%. This is compared to a 50% solvent volume in another orthorhombic polymorph of ox-liver catalase (McPherson & Rich, 1973) and a 70% solvent volume in a trigonal polymorph (Longley, 1967; Rossman & Labaw, 1967). The value for the flat orthorhombic polymorph seems to be a most typical one for protein crystals (Matthews, 1968).

*Crystal perfection*

Thin protein crystals comprised of very large globular subunits united across hydrogen-bonding surfaces would be expected to exhibit bulk mechanical properties quite different from those exhibited by thin metal foils. Protein crystals are observed to be quite soft (Boyes-Watson, Davidson & Perutz, 1954), for the intermolecular forces are very weak. Thus, tolerance of large bends along thin crystals implies strong lateral packing forces not found in globular protein crystals. Indeed, evidence in the diffraction patterns from thin catalase crystals indicates that there is no significant bending of the crystals within the area of the incident electron beam. Because of their radiation sensitivity, the catalase crystals were not imaged for the observation of bend contours *via* diffraction contrast. Rather, the presence of very well defined Laue zones from tilted crystals (Fig. 3) supports the thesis of no significant bending of these crystals (*e.g.* see Hirsch, Howie, Nicholson, Pashley & Whelan, 1965). Were there significant effects from bending, then they would be detectable in an angular dependence, *i.e.* increased width for outer Laue zones (Cowley, personal communication). This was never observed. However, as indicated by the error bars in Fig. 1, the most significant limitation to using Laue-zone measurements for crystal thickness in catalase diffraction patterns is the difficulty in defining the Laue-zone envelopes for *thicker* crystals.

Another perturbation akin to bending which should be considered is partial collapse of solvent channels in the catalase crystal (Labaw, 1967) as a result of drying. This partial collapse of the crystal would create a paracrystalline packing array which would tend to smear out the diffraction spots. Quite the opposite is seen, in fact, with diffraction spot diameters of 100 $\mu$m on the films being typical for low-intensity reflections.

Therefore it is believed that catalase crystals are free of bend distortions of the type commonly found in metal foils.

If the protein crystals are assumed to be well formed and of constant thickness within the beam area, another observation which can be made from the diffraction data is a crude estimate of their mosaicity. Vainshtein (1956) has given expressions for kinematical intensity from an ideal crystal block, *viz.*

$$I_{hkl} = J_0 S\lambda^2 \left| \frac{\phi_{hkl}}{V_{\text{cell}}} \right|^2 \frac{\sin^2 \pi t h_i}{\pi^2 h_i}$$

and from a mosaic single crystal film, *viz.*

$$I_{hkl} = J_0 S\lambda^2 \left| \frac{\phi_{hkl}}{V_{\text{cell}}} \right|^2 \frac{t \cdot d_{hkl}}{\alpha}.$$

If the crystal is assumed to be perfect, then the limit of the diffraction pattern should occur around where the curvature of the Ewald sphere (at 200 kV) intersects the first node of the ($\sin^2 x/x^2$) intensity falloff of the shape transform. The data limit of a 530 Å thick crystal was found to occur at about 0·34 Å$^{-1}$ whereas the predicted limit is at 0·40 Å$^{-1}$. For a 1520 Å thick crystal the observed data limit was found to be at 0·38 Å$^{-1}$ whereas the predicted limit is at 0·19 Å$^{-1}$. The predicted and observed data limits are very close for the 530 Å thick crystal, the discrepancy being perhaps due to error in thickness determination and/or a slight tilt of the crystal. The agreement indicates very little mosaicity. The difference between the predicted and observed data limits for the 1520 Å crystal, on the other hand, is quite large and bespeaks increasing mosaicity at greater thickness.

The type of mosaicity implied here, however, must be different from an equal variation of mosaic-block orientations with respect to the crystal axes. This is because significant increase in high-angle diffraction-spot diameter is not often seen in diffraction patterns from thick catalase crystals (*e.g.* Fig. 2) even though the discrepancy between the predicted and observed data limits is quite large. The mosaic model which

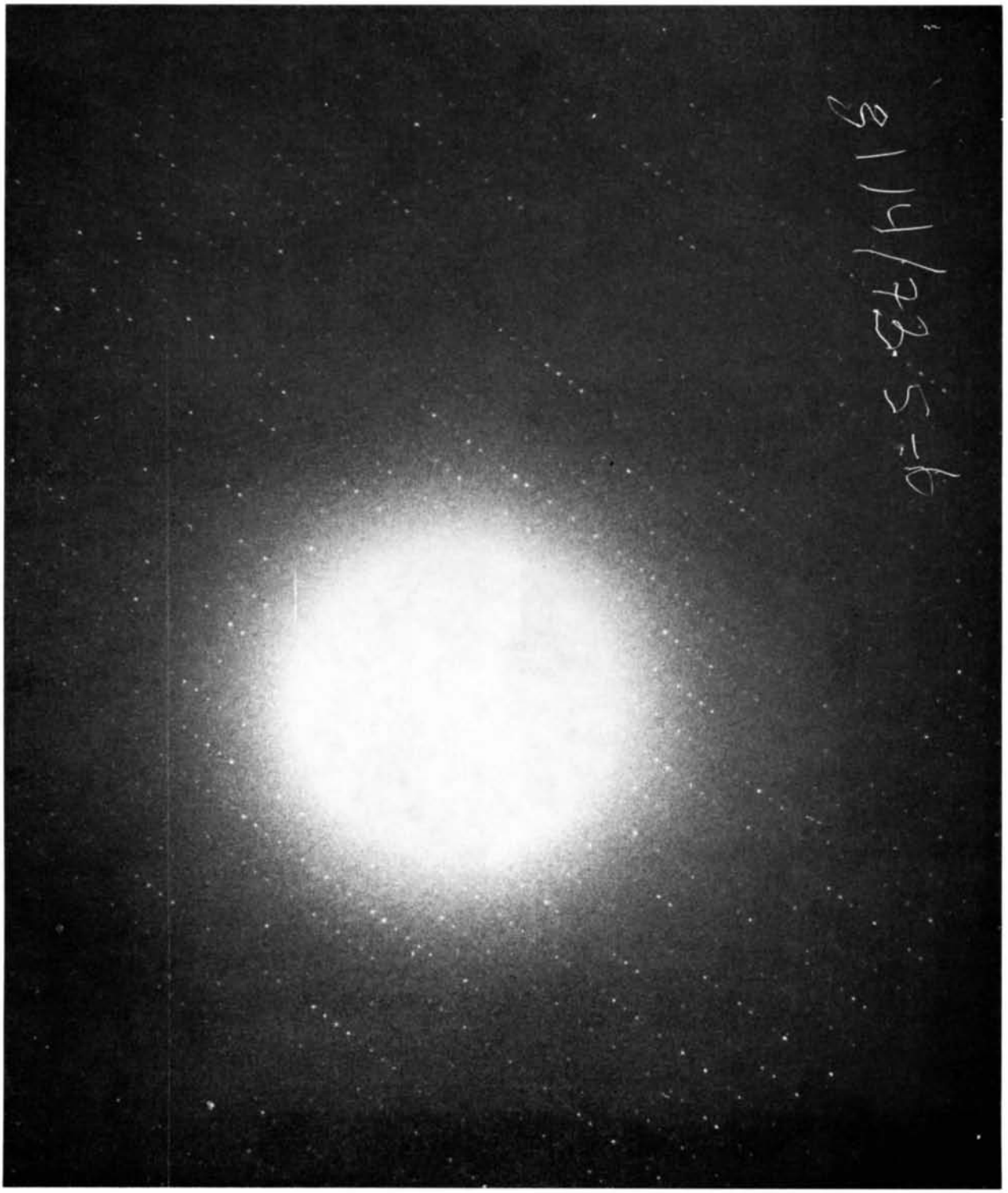
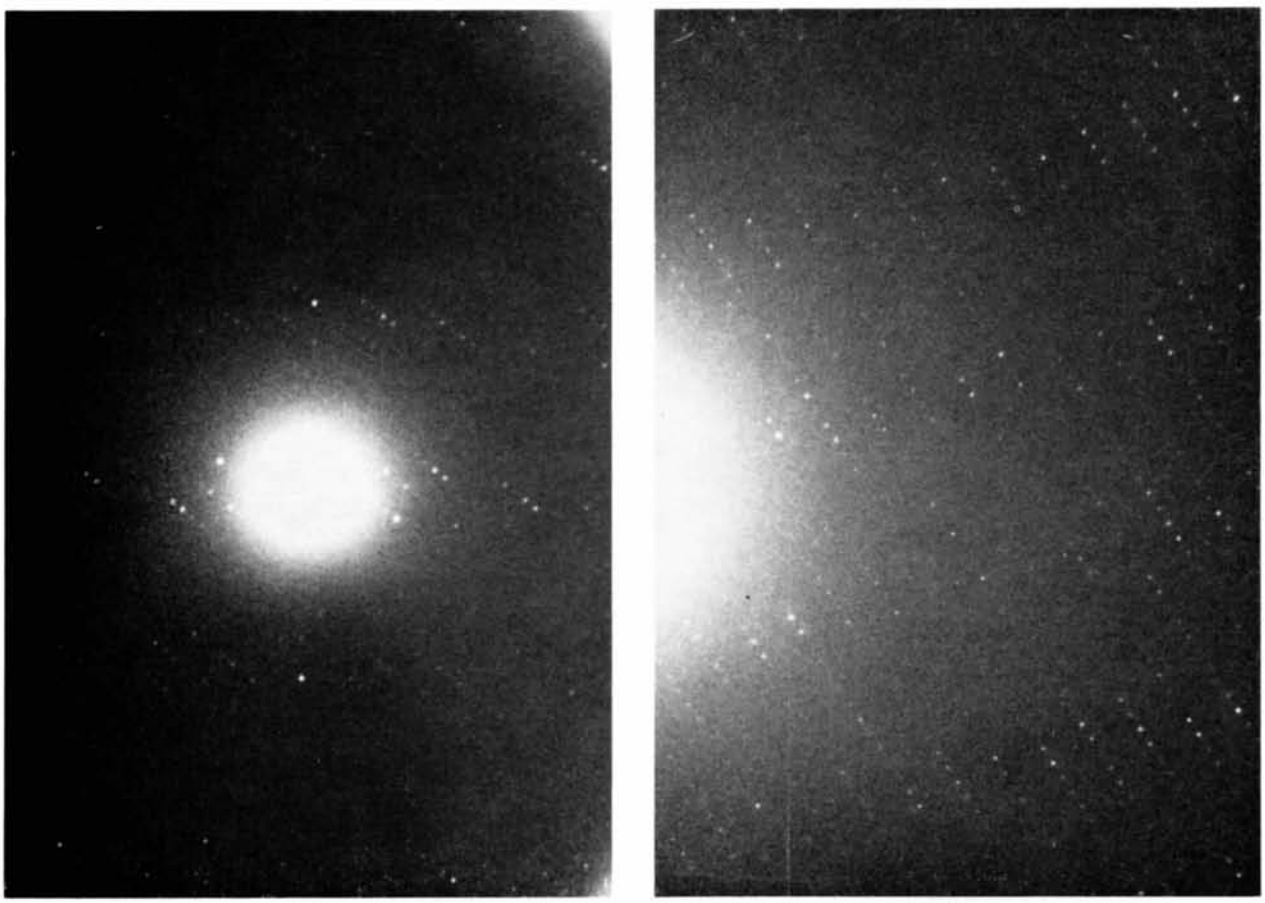


Fig. 2. Electron diffraction pattern (*h*0*l* net) from an untilted catalase crystal.

[*To face p.* 212



(*a*) (*b*)

Fig. 3. Laue zones in diffraction patterns from tilted catalase crystals.

would conform to this would be one allowing random orientation axes to lie only in the major crystal plane such that there is little random orientation around the axis parallel to the incident beam. Since a mosaic spread of this type cannot be distinguished from slight bends of the crystal by the diffraction pattern alone, an absolute resolution of this question would have to rely on an image of a thick crystal. The packing of the large catalase molecular ellipsoids however involves the creation of a well defined honeycomb of solvent channels parallel to the crystallographic *c* axis in this polymorph (Labaw, 1967). This might explain the least orientational variation of mosaic blocks about the normal to the large flat crystal surface.

*Analysis of intensity data*

A complex light-atom structure with a very large unit cell would be expected to give electron diffraction intensities conforming to the kinematical assumption

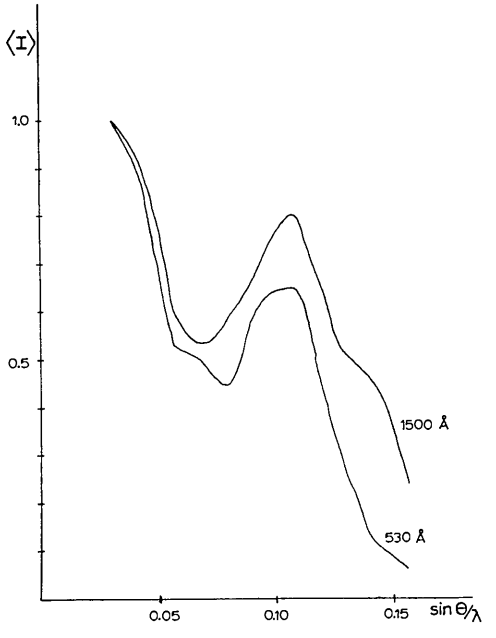



Fig. 4. Average $|I_{obs}|$ *vs.* sin $\theta/\lambda$ for two catalase crystals.

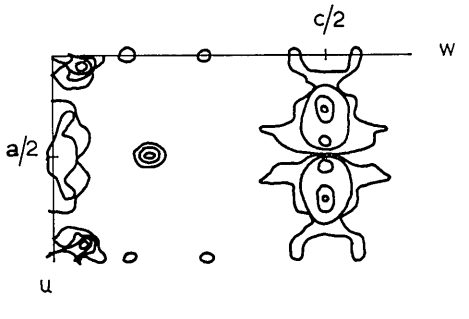



Fig. 5. Patterson map from catalase *h0l* data.

since the mean value of the structure-factor amplitudes is reduced (Vainshtein, 1956). The relative intensities of low-order reflections should be independent of crystal thickness if the kinematical approximation holds. An attempt was made to compare diffraction patterns from crystals of various thicknesses. An apparent freedom of the present specimen holder to undergo slight tilt in the hydration chamber, however, did not allow a definitive answer with this technique, although a plot of relative intensity *vs.* crystal thicknesses for low-order reflections from diffraction patterns with *mm* symmetry and no evidence of axial tilts is indicative of such an invariance.

The most compelling evidence for the validity of the kinematical assumption is a comparison of total diffracted intensity with incident intensity. According to Vainshtein (1956, 1957), a reflection intensity $I_{hkl}$ relative to the incident beam $J_0S$ received by crystal area $S$, is described for an ideal crystal by

$$\frac{I_{hkl}}{J_0S} = \lambda^2 \left| \frac{\phi_{hkl}}{V_{cell}} \right|^2 t^2 \qquad (1)$$

where $V_{cell}$ is the unit cell volume, $t$ is the thickness of the ideal crystal, $\phi_{hkl}$ is the structure factor, and $\lambda$ is the wavelength of the electron beam. For one to assume the kinematical approximation, the ratio of every $I_{hkl}/J_0S$ must be much less than one.

This formulation, derived from two-beam dynamical theory, is probably irrelevant for protein crystals with no strongly diffracting planes and is used only as a preliminary test for the strongest reflection. A more meaningful requirement for protein crystals where *n*-beam interactions might be more feasible in the densely populated reciprocal lattice (Cowley, 1967) is that $\sum I_{hkl}/J_0S$ be less than one. In order to use this criterion, the incident beam must be enclosed within the crystal area and there can be only small bend distortions within the illuminated area.

In a series of 5 s exposures at approximately the same beam currents as specified above, 106 diffraction patterns were obtained from crystals ranging in thickness from 200 to 3000 Å. With the intensities of the most intense 002 reflections monitored compared with the central-beam intensity [here the central densitometer trace through the incident-beam spot was used as a rough approximation of $J_0S$ in equation (1)], there was never an instance where the reflection intensity represented an appreciable fraction of the incident-beam intensity. The ratio of the summed diffracted intensities $\sum I_{hkl}$ over the central-beam intensity $J_0S$ was then found for two crystals with thicknesses of 530 and 1500 Å. The measured intensities were treated as a sum of OD values and compared to the central-beam intensity, measured with the isolated viewing screen coupled to an electrometer and calibrated with respect to the blacking of the X-ray film. The ratio $\sum I_{hkl}/J_0S$ is 0·06 for the 530 Å crystal and 0·16 for the 1500 Å crystal.

Another datum in support of a kinematical assumption is the appearance of the diffraction patterns from these protein crystals. A plot of averaged intensities over intervals of $\sin\theta/\lambda$ (Fig. 4) reveals a modulation of the falloff by the effect of the molecular transform superimposed on the reciprocal lattice in a way typical of protein-crystal X-ray diffraction patterns (Pauling & Corey, 1951; Rossman, Jeffery, Main & Warren, 1967). There is probably also an effect due to thickness-dependent non-coherent multiple scattering (Gjønnes, 1959).

*Phasing of diffraction data*

A $u0w$ Patterson map was calculated at 5 Å intervals with intensity data from the 530 Å crystal cited above. As can be seen in Fig. 5, the intense peaks expected for centroid–centroid vectors in plane group *pgg* are seen along the two mirror lines, corresponding to the distances $2x$ and $2z$ in the unit-cell projection (Buerger, 1959, p. 89ff). Along the mirror line parallel to $u$ there are peaks at $u=5$ Å and $u=15{\cdot}5$ Å and along the mirror line parallel to $w$ a peak at $w=60$ Å. It is throught that the first two peaks perhaps represent different parts of the protein molecule placed at $x=$ 2·5 Å and 7·8 Å and the latter vector the $z$ position at 30 Å. Using an ellipsoid approximation of the catalase molecular shape with axial lengths 70, 90 and 100 Å (Gurskaya, Lobanova & Vainshtein, 1972) and placing the molecular center of mass near the ends of the vectors suggested by the Patterson map, one can generate a packing scheme in the *ac* plane very similar in appearance to high-resolution micrographs of negatively stained catalase (Valentine, 1964; Labaw, 1967).

While interesting from the standpoint of corroboration, the Patterson map does not give any more structural information than is already known. An attempt to phase the diffraction data with the use of an algorithm of Gerchberg & Saxton (1972) is currently in progress but this technique is not expected to yield a high-resolution solution.

The best use of the high-resolution diffraction data will only come after isomorphous derivatives of these catalase crystals themselves are prepared and their electron diffraction patterns obtained. As can be ascertained from any review of protein crystal structure analysis, this is a full-time project in itself and was not within the scope of this present study.

The indication in this work that single-crystal electron diffraction intensity data from thin wet protein crystals can probably be treated with the kinematical assumption as a good first approximation will hopefully encourage further electron-diffraction structural work by protein crystallographers on proteins which only give microcrystals.

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*Acta Cryst.* (1975). A**31**, 215

# The Measurement of Anomalous Scattering factors near the Ga *K* Absorption Edge in GaP

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By the use of an SSD diffractometer and continuous X-rays, the energy dependences of $\Delta f'$ and $\Delta f''$ values of Ga have been studied on GaP with energy resolution of about several eV around the Ga *K* edge; the values of $\Delta f''$ were determined by the measurement of the absorption coefficient, and then the values of $\Delta f'$ have been obtained from the precisely measured ratio of Friedel-pair reflexions from a (111) single-crystal plate of polar GaP. Fine structures have been found in $\Delta f''$ and therefore in $\Delta f'$ corresponding to those of the absorption coefficient. The present work has shown that the measured values of $\Delta f'$ more or less reasonably agree with the curves calculated from the dispersion relation.

## 1. Introduction

The anomalous scattering factor is not only interesting from the physical point of view but also very important for determining phases, as is well known in crystallography. Generally speaking, the values of the anomalous scattering factor, especially near the absorption edge, have seldom been measured except when convenient characteristic radiations happen to have energy values nearly similar to an absorption edge of a certain atom. Therefore, hardly any systematic measurement has been carried out on anomalous scattering factors, particularly in the energy region near the edge. As for the data so far published, the agreement among measured values themselves and also between measured values and calculated values has usually been poor, as was summarized by James (1954) or more recently compared, for instance by Bonse & Materlik (1972).

However, the advent of an energy-dispersive or an SSD (solid-state detector) diffractometer has enabled us to carry out the measurements easily, even in the energy region very near the absorption edge. The energy resolution in the present work was about $\pm 2$ or 3 eV, being determined by the beam divergence of the slit system used (Fukamachi, Hosoya & Terasaki, 1973).

## 2. Intensity ratio of a Friedel pair

As was reported by Cole & Stemple (1962), the intensity ratio between Friedel-pair reflexions of a polar crystal is given by the structure factor $F$ or the intensity $I$ as

$$R_h = |F_h|^2/|F_{\bar{h}}|^2 = I_h/I_{\bar{h}}\,, \qquad (1)$$

being independent of the perfection of the specimen crystal, at least when the reflexion is in a symmetrical Bragg case. As will be separately published, this is valid when the primary extinction alone is taken into consideration, but not exactly valid when the secondary extinction is considered as well. However, at least in the energy region higher than the edge, the absorption is so heavy that the intensity is less subject to secondary extinction, which already does not much affect high-index reflexions such as are used in the present work.

Holloway (1969) confirmed the validity of this relation with characteristic radiations, and more recently the present authors have confirmed the validity with continuous radiation in the energy region very near the edge (Fukamachi, Hosoya & Okunuki, in preparation), both for nearly perfect crystals. The above ratio $R_h$ can be measured with very high accuracy because various factors common to a pair of reflexions are cancelled. It is to be noted that the deviation from uniform polarization, if any, in the white radiation is also cancelled. In the present work, the absorption correction has no effect either, because of the geometry concerning the shape of the sample and the reflexion used. As mentioned in the above, even extinction does not matter in favourable cases.