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Progress with Laue Diffraction Studies on Protein and Virus Crystals†

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X-ray diffraction studies have made outstanding contributions to structural molecular biology. The resulting image of the molecule is time averaged over the period needed to make the measurements and spatially averaged over all the molecules within the volume of the crystal. Until recently, the measurements from protein crystals took days or weeks with conventional X-ray sources. Now, the ability to obtain atomic information on short-lived structures that may accumulate transiently during a reaction in the crystal is within our grasp. These new opportunities in macromolecular crystallography, which has previously been considered a static technique, require new developements for initiating and monitoring events in protein crystals.

Several approaches have demonstrated that proteins can exhibit dynamic properties in the crystal despite the constraints of the crystal lattice. Studies on catalysis have indicated that many enzymes are active in the crystal with thermodynamic properties similar to those shown in solution but often with reduced rate constants [Quiocho & Richards, 1966; reviewed by Makinen and Fink (1977)]. In the crystal, as in solution, each structural state of a protein represents a subset of closely related structures that undergo thermal fluctuations around the mean structure. Analysis of temperature factors of refined protein crystal structures [Artymiuk et al., 1979; reviewed by Petsko and Ringe (1984)] has given indications of mobility and restraints on atoms in protein molecules, and many ligand binding studies have shown the ability of proteins to respond with conformational changes in the crystal. The structural states that a molecule can adopt are restrained by lattice forces, and conformational changes incompatible with the lattice break up the crystal.

In a diffraction experiment, for a novel structural state to be detected and its structure determined, there must be a transient buildup of the intermediate before it disappears during the reaction. There is the expectation that if X-ray data collection rates can be made commensurate with the time scale of the dynamic events, then transient structural states that are simultaneously present in most of the unit cells could be analyzed. High-intensity synchrotron radiation sources and the revival of the Laue method (white X-radiation, stationary crystal) have made fast X-ray crystallographic data collection a reality. Many full diffraction data sets have been collected with overall data acquisition times ranging from seconds to milliseconds, and recent developments suggest that data collection on the picosecond time scale may be possible in the near future. The Laue method has been applied to the study of enzyme structures, ligand–enzyme interactions, viruses, and viral drug complexes (Table I). The advantages of the method have been demonstrated for small crystals (Harding et al., 1988), for radiation-sensitive crystals (Hedman et al., 1985), and for ligand binding studies where data from the native and ligand-bound structures are collected from the same crystal (Hajdu et al., 1987b). So far the Laue method has yielded acceptable diffraction data for structural studies, but full exploitation of the method for the study of dynamic events in crystals requires developments in the physics, chemistry, and biochemistry of protein crystal systems, similar to those that have been achieved for the time-resolved studies on muscle [see, e.g., Kress et al. (1986)]. The time scales of some of these dynamic events are given in Figure 1.

In this review we summarize the physical basis of Laue diffraction with synchrotron radiation and the results that have been obtained so far. We then assess the progress toward time-resolved studies with protein crystals and the problems that remain. Time-resolved macromolecular crystallography has been reviewed recently with emphasis on the biophysical principles (Moffat, 1989). Time-resolved diffraction studies on noncrystalline biological materials have also been described (Gruner, 1987; Potschka et al., 1988) and are not discussed here.

Diffraction Studies with Synchrotron Radiation

Synchrotron radiation refers to the electromagnetic radiation produced when charged particles are accelerated at relativistic

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Table I: Laue Diffraction Studies on Protein Crystals

protein	exposure time per photo	experiment	ref
(1) calcium binding protein	30 s	test photograph	Moffat et al., 1984
(2) pea lectin	45 s (single bunch, 1.8 GeV, 15 mA)	test photograph; data set leading to 6982 out of 12906 reflections subsequently collected in four such photographs with merging $R = 0.082$	Helliwell, 1984; Helliwell et al., 1988
(3) gramicidin A	50 s (20-μm^3 crystal)	demonstration of Laue diffraction from very small crystals and investigation of radiation damage	Hedman et al., 1985
(4) hen egg white lysozyme	64 ms	monitoring of time-dependent variation in intensities following thermally induced structural changes	Moffat et al., 1986
(5) glycogen phosphorylase *b*	1 s	three-dimensional data set collected in three photographs to 2.4-Å resolution; difference electron density map showed oligosaccharide bound to enzyme	Hajdu et al., 1987b
(6) xylose isomerase	1 s	three-dimensional data set collected in three photographs; difference electron density map showed Eu binding site	Faber et al., 1988
(7) insulin	3 s	demonstration of transformation of four Zn insulin crystals to two Zn insulin crystals induced by contact with vapor from two Zn crystallization buffer	Reynolds et al., 1988
(8) tomato bushy stunt virus	24 s	three-dimensional data collected for native virus and for virus in which Ca^{2+} has been removed by EDTA; data to 3.5-Å resolution contained 38720 reflections ($I > 2\sigma$) representing 31% of the data with $R_m = 0.14$ from one photograph; Ca^{2+} binding sites identified in difference electron density maps	Hajdu et al., 1989
(9) γ-chymotrypsin	1 s	three-dimensional data collected from three photographs (total exposure 3 s) to yield a data set of 5–2.5-Å resolution with $R_m = 0.078$; structure refined to $R = 0.21$ with Brookhaven Data Bank coordinates as starting model; results revealed the presence of exogenous peptide at the catalytic site	Almo et al., 1989
(10) turkey lysozyme	1 s	three-dimensional data collected from two photographs (total exposure 2 s) to yield 55% of data between 5- and 2.5-Å resolution ($I > 2\sigma$) with $R_{sym} = 0.05$; structure solved by molecular replacement and refined to $R = 0.19$	P. L. Howell et al., unpublished results

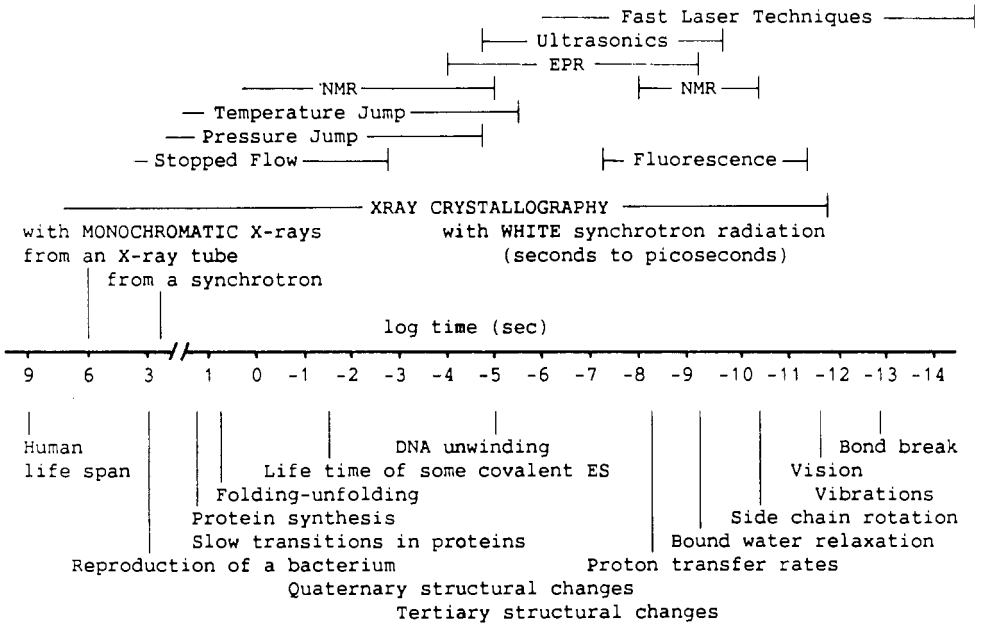

FIGURE 1: Approximate time scales for biological events (lower lines) and for physical methods for monitoring these (upper lines).

velocities and constrained to a curved trajectory by an external magnetic field. The energy the particle emits as electromagnetic radiation per unit time increases as the fourth power of the particle's energy (E^4) and decreases with the fourth power of its rest mass ($1/m^4$) (Schwinger, 1949). Hence, electrons or positrons are most useful, having low mass and being capable of acceleration to high energies. In a typical machine electrons or positrons are injected from a linear accelerator into a booster synchrotron and from there into a storage ring that is run at energies between 1 and 20 GeV with circulating currents of up to several hundred milliamps. In the storage ring, the particles are grouped into small bunches (a few millimeters in length), giving rise to short (10–500 ps long) and intense bursts of synchrotron radiation whenever they pass through the intense fields of the magnets. At relativistic speeds, the radiation is confined to a narrow cone around the instantaneous direction of flight tangential to the curved particle orbit. The radiation emitted is polychromatic, is extremely intense, has a pulsed nature, and is highly polarized. It can span a spectral range from γ-rays to radio frequencies (Figure 2). With insertion devices, such as wiggler magnets or undulator magnets, a local reduction in bending radius is achieved, and the spectral distribution of the radiation is altered (Figure 2).

The first demonstration of the applicability of synchrotron radiation to biological specimens came with the work of Ro-

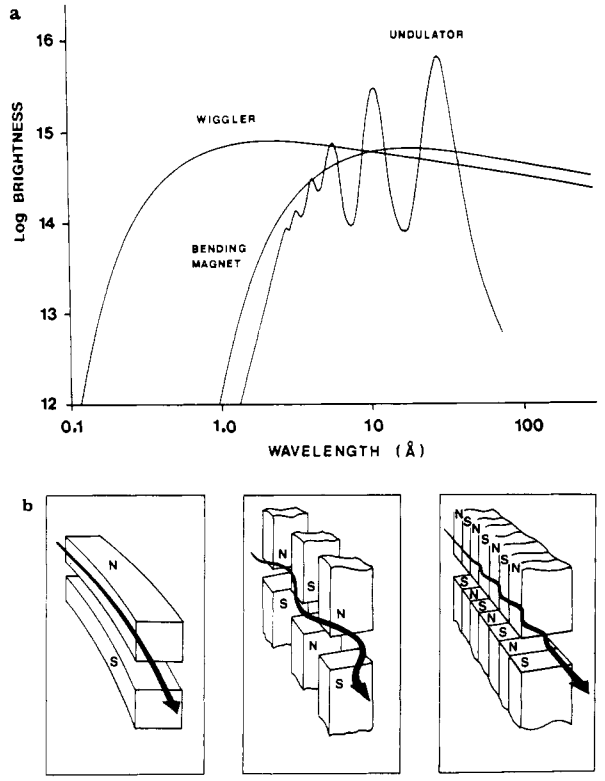

FIGURE 2: (a) Spectra of radiation emitted from the main storage ring (bending magnet) and from two special magnets, a wiggler and an undulator, at a synchrotron radiation source. The insertion of such devices into the straight sections of the storage ring allows the operation of a beam line with altered spectral properties. (b) Electron paths in the magnets. Wiggler magnets operate at very high field strength and force the circulating electron beam onto a tightly bent (and usually) periodic path on a short section. Undulators are multipole wigglers with moderate field strengths. The departure of the electron beam from a straight path is very small. The radiation produced with undulators has a spectrum with enhanced intensities at particular wavelengths produced by interference effects.

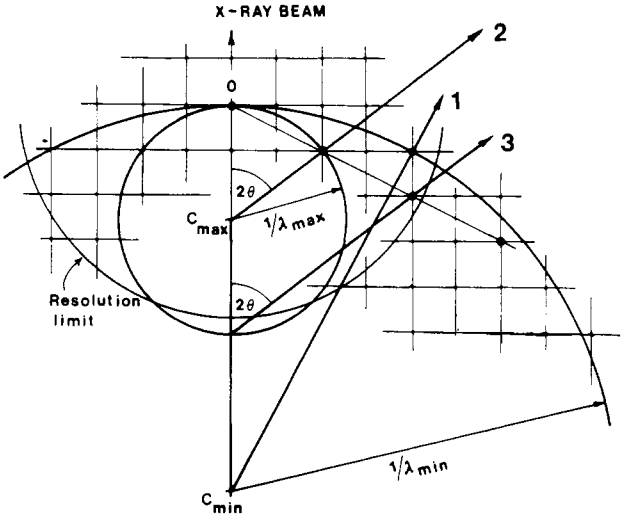

FIGURE 3: Laue geometry of diffraction. The diagram shows the Ewald construction, a geometrical representation of Bragg's law. A sphere is drawn with center C and a radius equivalent to the reciprocal of the wavelength. The reciprocal lattice is placed with origin O at the point where the straight through the beam cuts the sphere. Diffraction occurs whenever a reciprocal lattice point cuts the sphere. With the Laue method the reciprocal lattice is illuminated with a spectrum of X-rays with wavelengths continously varying from λ_{min} to λ_{max} between the two limiting spheres with radii $1/\lambda_{min}$ and $1/_{max}$. All reciprocal lattice points between the limits will lie on an appropriate sphere and will diffract (for example, rays 1, 2, and 3). Note that in reality the resolution limit and the wavelength limits are smoothly varying soft limits.

senbaum et al. (1971) on muscle. These authors used monochromatized X-rays. Throughout the 1970s protein crystallographers were parasitic users of synchrotron radiation, using sources designed for elementary particle physics. In 1981 the first synchrotron dedicated to the production of radiation came on line at SRS, Daresbury. There are now nine protein crystallographic facilities available at sources around the world: LURE, Orsay, France; DESY, Hamburg, West Germany; SRS, Daresbury, U.K.; NSLS, Brookhaven, NY; CHESS, Ithaca, NY; SPEAR and PEP, Stanford, CA; PHOTON Factory, Tsukuba, Japan. Further sources (ESRF, Grenoble, France; APS, Argonne, IL; ALS, Berkeley, CA; ELETTRA, Trieste, Italy) are also planned. The application of synchrotron radiation studies to biological macromolecules has been reviewed (Greenhough & Helliwell, 1983).

Diffraction with Monochromatic X-rays. In order to solve a protein crystal structure, an almost complete diffraction data set from a crystal to a given resolution must be measured. With monochromatic X-rays, only a small proportion of the lattice planes diffract at any particular orientation of the crystal, and the crystal has to be rocked through a small angle (1–2°) in order to record the full intensity of the reflection. To bring other planes into the diffracting position, the crystal has to be rotated to a new setting and the procedure repeated with a new film. A monochromatic data set to 2.4-Å resolution for glycogen phosphorylase *b* required 32 exposures each with a 1.5° oscillation. At the Daresbury synchrotron, the very high intensity permitted the collection of such data sets within about 20 min (in the best case). This is an increase of almost 3 orders of magnitude compared to the data acquisition rates possible with a conventional rotating-anode X-ray source in the home laboratory. Even so, a new crystal was required for each data set, and the time resolution was still relatively coarse. In spite of this, a sequence of such data sets has been used to observe a very slow catalytic reaction in tetragonal crystals of phosphorylase *b* (Hajdu et al., 1986a, 1987a).

Diffraction with Polychromatic X-rays (Laue Diffraction). Laue diffraction refers to the method used by Friedrich, Knipping, and von Laue to record the first X-ray diffraction image from a crystal of copper sulfate (Friedrich et al., 1912). The method utilizes the whole polychromatic spectrum instead of a single wavelength. It fell into disuse because conventional X-ray sources did not give a satisfactory spectrum and because of difficulties in unraveling the complicated diffraction patterns. The broad spectral range (Figure 2) and the high intensity of synchrotron radiation have been the prime driving force behind the revival of the Laue method (Wood et al., 1983) and its application to biological macromolecules (Moffat et al., 1984; Helliwell, 1984), along with new developments in computing techniques to process the data (Machin, 1985, 1987; Campbell et al., 1986, 1987; Rabinovich & Lourie, 1987). With white X-radiation, a large number of lattice planes diffract simultaneously as the Bragg condition is satisfied for each of these planes by at least one wavelength of the spectrum (Figure 3). Many reflections can thus be recorded in a short time with a single exposure. The diffracting position of a stack of lattice planes is determined by their orientation (relative to the direction of the incident beam), their spacing, and the wavelength range applied; the wider the wavelength range the greater the number of planes in the diffracting position. With crystals of high symmetry, a large proportion of the unique data set may be recorded with a single photograph. For example, under certain experimental conditions, almost 98% of the unique data set may be recorded

in a single exposure from a cubic crystal while, under similar conditions, only about 55% of the unique set is accessible from a monoclinic crystal. This requires more than one photograph to be taken in order to complete the data set in the latter case (I. A. Clifton et al., unpublished results).

White synchrotron X-radiation was first used for recording topographs from inorganic crystals by Tuomi et al. in 1974. The first biological application of this very intense radiation came in a study of collagen fibers with energy-dispersive X-ray scattering by Bordas et al. (1976). This was done at the old NINA storage ring in Daresbury (U.K.). In 1977, Steinberger et al. (1977) recorded Laue photographs from zinc sulfide crystals there, and in the same year, Bordas and Kam obtained Laue diffraction images from a crystal of lysozyme at the DESY storage ring in Hamburg, West Germany (J. Bordas and Z. Kam, unpublished results). The first successful structural work applying the broad band path Laue diffraction technique was the refinement of a known inorganic crystal structure (aluminium phosphate) from Laue diffraction data by Wood, Thompson and Matthewman at the Daresbury Laboratory in 1982 (Wood et al., 1983). Moffat et al. (1984) showed that Laue diffraction could be applied to proteins and proposed that a narrow band path of the white radiation be used in order to reduce the number of harmonic overlaps (see below). Later that year, Helliwell and colleagues (Helliwell, 1984; Helliwell et al., 1989) performed experiments in Daresbury with the complete white spectrum of radiation, which gives greater efficiency of data collection than the narrow band path technique. Our own work on Laue diffraction started that year. Various feasibility studies followed (Hedman et al., 1985; Clifton et al., 1985). The first experimental results in macromolecular crystallography (Table I) provided information on the glycogen storage site in crystals of rabbit muscle phosphorylase (Hajdu et al., 1987b), located a metal binding site in crystals of xylose isomerase (Farber et al., 1988), gave detailed structural information on the divalent cation binding sites in crystals of tomato bushy stunt virus (Hajdu et al., 1989), and led to the solution of two new protein structures: lysozyme from turkey egg white (P. L. Howell et al., unpublished results) and glyceraldehyde-3-phosphate dehydrogenase (form I) from *Trypanosoma brucei* (F. M. D. Vellieaux et al., unpublished results). These experiments were performed at station 9.7 of the Daresbury synchrotron by utilizing the full white spectrum of the wiggler magnet with a single optical element (a pinhole) in the beam. Data were processed with software written by the Computing Systems and Application Group in Daresbury under the leadership of the late Pella A. Machin.

There are several inherent problems that complicate measurements from Laue photographs:

(*1*) *Harmonic Overlaps* (*Multiplets*). The diffracted rays of polychromatic X-rays with wavelengths of λ, $\lambda/2$, $\lambda/3$, ..., and λ/n from parallel lattice planes with spacings of d, $d/2$, $d/3$, ..., and d/n (where n is a positive integer) will have the same diffracting angle (θ), according to Bragg's law ($2d \sin \theta = n\lambda$). Thus, these reflections (e.g., rays 2 and 3 in Figure 3) will produce a single spot containing more than one reflection on the film. The number of reflections buried in harmonic spots (or "multiplets") increases with increasing wavelength range, reaching an upper limit of only about 17% at an infinite wavelength range (Cruickshank et al., 1987). At realistic wavelength ranges, they form an even smaller part of the data. Harmonic reflections require separation during processing. Methods to deconvolute harmonic spots into component reflections are available (Zurek et al., 1985; Helliwell et al., 1989a,b) with some promising results for doublet spots.

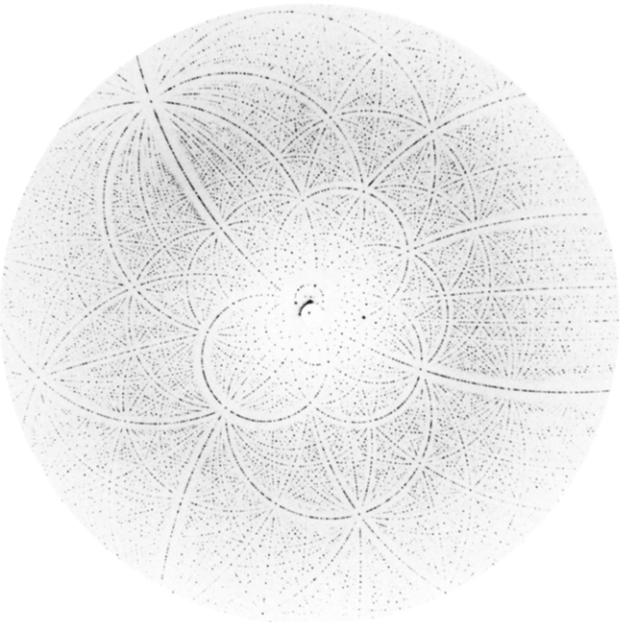

FIGURE 4: Laue photograph of ribulosebisphosphate carboxylase: space group $C222_1$, a = 157.2 Å, b = 157.2 Å, c = 201.3 Å. Wavelength range 0.2–2.1 Å; exposure time 1 s; crystal to film distance 142.3 mm; number of reflections predicted on photograph 126 270. Photograph recorded at SRS, Daresbury, U.K. (I. A. Andersson et al., unpublished results).

(*2*) *Spatial Overlaps*. As is apparent in a typical Laue photograph (Figure 4) the space on the film is crowded with spots. The photograph from a crystal of ribulosebisphosphate carboxylase [space group $C222_1$, a = 157.2 Å, b = 157.2 Å, c = 201.3 Å (Andersson & Branden, 1984)] contains 126 270 predicted reflections. In the early studies with Laue photography [e.g., Hajdu et al. (1987b)], intensities could only be obtained if the spots were separated by more than 0.2 mm. For the photograph in Figure 4 some 66 753 reflections (over 50% of the data) would be rejected as spatially overlapped by this criterion. Recently, profile-fitting procedures have been implemented that allow the spatial deconvolution of spots, and measurements of spots that are separated by more than 0.1 mm (2 raster units) are now possible (T. J. Greenhough et al., unpublished results). Thus for the photo of Figure 4, only 21 948 reflections are rejected as spatially overlapped out of 126 270 predicted on the film. This development has allowed considerably increased numbers of reflections to be measured for crystals of large unit cells including viruses.

(*3*) *Wavelength Normalization*. Techniques are needed to take into account the effects of wavelength-dependent factors on intensity measurements. The intensity of the incident radiation (Figure 2) modulated by the optical elements used to focus the beam, the interaction of the radiation with the crystal, and the interaction of the radiation with the detector all vary with wavelength. Two experimental approaches have been developed for the treatment of wavelength-dependent effects. In the first method, a wavelength normalization curve is deduced. This can be done in a number of ways. Wood et al. (1983) used a standard Si crystal (i.e., an external reference) to record the variation in intensity of a single reflection while rotating the crystal in the white X-ray beam. Another technique is based on the comparison of intensities of symmetry-related reflections stimulated by X-rays of different wavelengths (Campbell et al., 1986). The technique uses an internal reference and is fairly robust but may not be

applicable where significant anomalous scattering is present. It has been used in the determination of an unknown crystal structure of an organometallic compound (Harding et al., 1988) where only very small crystals were available. A recent comparison of structure determination of a small molecule ($C_{10}H_{11}NOClF$) has shown that a structure based on data recorded with Laue diffraction in 6.5 s is of comparable precision in atomic coordinates to a structure based on data recorded in 24 h with standard monochromatic methods (Gomez de Anderez et al., 1989). With proteins this approach was used in the location of heavy atom sites in crystals of xylose isomerase (Farber et al., 1988) and in the pea lectin data processing (Helliwell et al., 1989a).

The second method devised to compensate for the wavelength-dependent terms is designated the difference method and is used for the analysis of structural changes relative to a known starting structure. A sequence of nearly identical Laue photographs are recorded before, during, and after the initiation of the reaction or ligand binding. Data sets in the sequence are scaled to the starting native set, and the fractional difference between the structure factor amplitudes of the initial and intermediate data sets is multiplied by the appropriate structure factor amplitude from a reference data set recorded with monochromatic radiation [see Moffat et al. (1986)]. This method was used to produce the first interpretable Laue difference Fourier of a ligand bound to an enzyme, glycogen phosphorylase *b*, where the total exposure time per data set was 3 s (Hajdu et al., 1987b).

(*4*) *Low-Resolution Data.* Figure 3 reveals that the Laue geometry allows a large proportion of medium- to high-resolution reflections to be recorded but is much less efficient for low-resolution reflections. This is due to two factors: (i) the narrowing of the reciprocal space swept between the two limiting Ewald spheres at low diffraction angles (Figure 3) and (ii) the fact that many of the low-resolution reflections are buried in harmonic overlaps (Amoros et al., 1975; Cruickshank et al., 1987). In principle, some of these reflections could be retrieved through deconvolution of the harmonic overlaps. In a recent structural study on ribulosebisphosphate carboxylase (Andersson & Branden, 1984; Andersson et al., 1989; I. A. Andersson et al., unpublished results) data to 1.8-Å resolution from these orthorhombic crystals were measured with the Laue method by using four different orientations of the crystal. The low-resolution data to 5-Å resolution were recorded separately with monochromatic radiation. Such an approach allows rapid data collection from only a few crystals and is of considerable value when crystals are rare or severely radiation sensitive but would not be possible for time-resolved experiments. The low-resolution terms contribute to the definition of the boundary between protein and solvent in protein electron density maps and to the localization of less well ordered regions. In a trial study with glycogen phosphorylase (K. R. Acharya et al., unpublished results) comparison of electron density maps based on all the data to 1.9-Å resolution and that based on data from 5-Å resolution to 1.9-Å resolution showed essentially no difference in those parts of the molecule that were well localized, such as the internal cofactor pyridoxal phosphate, but those regions which were less well ordered and which exhibited high thermal factors ($B > 60$ Å^2) were significantly better defined in the maps containing all the data than those for which the low-resolution terms were ommitted, as might be expected. Lack of low-resolution terms did not affect the structure determination of a small molecule (Gomez de Anderez et al., 1989). In an experiment with Laue data the structure of a γ-chymotrypsin–tetrapeptide inhibitor complex has been analyzed. The lack of low-resolution data did not lead to any problems in the refinement of the structure, and good density for the inhibitor was observed at the catalytic site (Almo et al., 1989) in agreement with the work of Dixon and Matthews (1989). This density was also observed in the initial structure determination of native γ-chymotrypsin (Cohen et al., 1981), but since there was no reason to expect the presence of an exogenous peptide in the structure, the density was modeled by a string of water molecules. Current experience suggests that ommission of low-resolution terms does not affect structure determination by molecular replacement methods or difference electron density maps but may interfere with determination of heavy atom positions by Patterson methods (S. C. Almo et al., unpublished results).

Some Recent Examples of Structural Results from Laue Diffraction

The increasing number of applications of Laue diffraction methods to proteins and viruses is summarized in Table I. Some of the achievements are described below, and these demonstrate that the method is now proving successful as a reliable method for data collection with dramatically reduced data collection times.

Determination of the Structure of Turkey Egg White Lysozyme. Hexagonal turkey lysozyme crystals (Bott & Sarma, 1976; Sarma & Bott, 1977) are of potential interest for crystal studies on lysozyme catalysis. The catalytic site cleft may be more accessible to substrates in this crystal lattice than in the well-studied tetragonal crystals of hen egg white lysozyme (HEWL; Blake et al., 1965) where the lower part of the catalytic site (subsites E and F) is blocked by symmetry-related molecules. The structure of turkey egg white lysozyme (TEWL), which differs from HEWL in only six amino acid positions, has been solved simultaneously in two laboratories by two different methods. Parsons and Phillips (Parsons, 1989) have used monochromatic diffraction data and the technique of isomorphous replacement. P. L. Howell et al. (unpublished results) used Laue diffraction data and molecular replacement based on the structure of hen egg white lysozyme. The structures agree with each other but differ from the structure published by Sarma and Bott (1977). In the molecular replacement solution of P. L. Howell et al. (unpublished results) Laue data between 5- and to 2.5-Å resolution were used. The starting model determined by the molecular replacement method with MERLOT (Fitzgerald, 1988) gave an *R* factor of 0.39. The model was refined with the programs XPLOR (Brunger et al., 1988) and PROLSQ (Hendrickson, 1985) to a final crystallographic *R* factor of 0.18. The bound solvent was not modeled. This is the first macromolecule whose structure has been solved from Laue diffraction data alone. The results show that the structure and the positions of the key catalytic residues (Figure 5) are closely similar to those of HEWL. The packing of the molecules in the crystal lattice leaves the lower part of the catalytic cleft accessible although the upper part (subsite A and part of subsite B) is blocked by an adjacent molecule.

Tomato Bushy Stunt Virus. Laue diffraction studies have been extended to systems with very large unit cells. The structure of tomato bushy stunt virus (TBSV) ($T = 3$ icosahedral, 180 subunits in the capsid) has been solved to 2.9-Å resolution (Harrison et al., 1978). The subunits have identical sequence but are present in three different conformations in the icosahedral asymmetric unit. A reversible conformational change occurs when two structurally bound calcium ions are removed from each of the trimer interfaces. At the pH of the cell, this is followed by an expansion of the virion [see Robinson

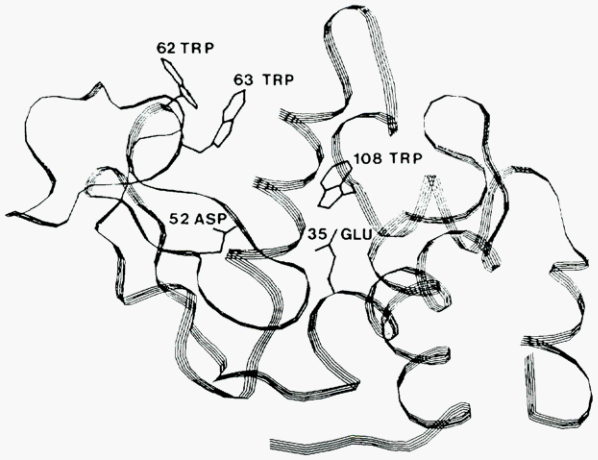

FIGURE 5: Ribbon diagram of turkey egg white lysozyme based on data from Laue diffraction. The diagram shows the chain topology and the positions of some key residues (P. L. Howell et al., unpublished results).

and Harrison (1982)], leading to uncoating.

Crystals of the virus are cubic (*I*23), allowing the recording of an almost complete data set on a single Laue photograph. A 24-s exposure was recorded from a crystal that had been soaked in EDTA to remove the bound Ca^{2+} ions. This was predicted to represent 92.7% of the unique data set to 3-Å resolution and yielded 31% of the data set with intensities greater than two standard deviations of the measurements between 6- and 3.5-Å resolution. A difference Fourier synthesis calculated with this subset of data clearly showed the three pairs of Ca^{2+} binding sites related by quasi-symmetry (Figure 6) (Hajdu et al., 1989). The extent and quality of data obtained from a single Laue photograph were sufficient to detect clearly a small alteration, i.e., the replacement of a Ca^{2+} ion by a water molecule and the conformational changes induced in the capsid. It is suggested that drug binding as well as molecular processes associated with infectivity may be studied with this technique. Following this work, Laue data on human rhino virus, canine parvo virus, mengo virus, and the ϕX174 phage as well as drug–virus complexes have been recorded (M. G. Rossmann et al., unpublished results).

Kinetic Laue Crystallography

A major limitation to the kinetic approach in X-ray crystallography is the fact that structures derived from diffraction images represent mixtures of all structural states in the crystal. There will be a time-dependent moving average of various structures in the crystal along the reaction coordinate. Even with the shortest possible data collection times (a few picoseconds), the major problem is the lack of "synchronization" among molecules in various parts of the crystal. With our present toolbox, for a novel structural state to be detected and its structure determined, there must be a transient buildup of this intermediate before it disappears during the reaction. This means a population inversion leading to one conformation largely predominant over the whole volume of the crystal (Hajdu et al., 1988). The structures observable are set by the system and not by the experimenter, and the favorable case of a single conformation (subject to some thermal motion) predominant in the crystal may not always be obtained. In general, the concentrations of some interesting intermediates may be low and masked by more predominant structures. Analysis of these mixed structures requires the development of reliable techniques to detect and to deconvolute the average into components. Further, the detailed chemical state of a bound molecule may not be identifiable even by high-resolution protein crystallography (such as the oxidation state of a cofactor, for example).

Thus, methods are needed that allow the amount and concentration of various intermediates present in the crystal at the time of the X-ray exposure to be measured. This can be accomplished if there is a suitable diagnostic such as a distinct spectral signal (fluorescence, UV or visible absorption, infrared

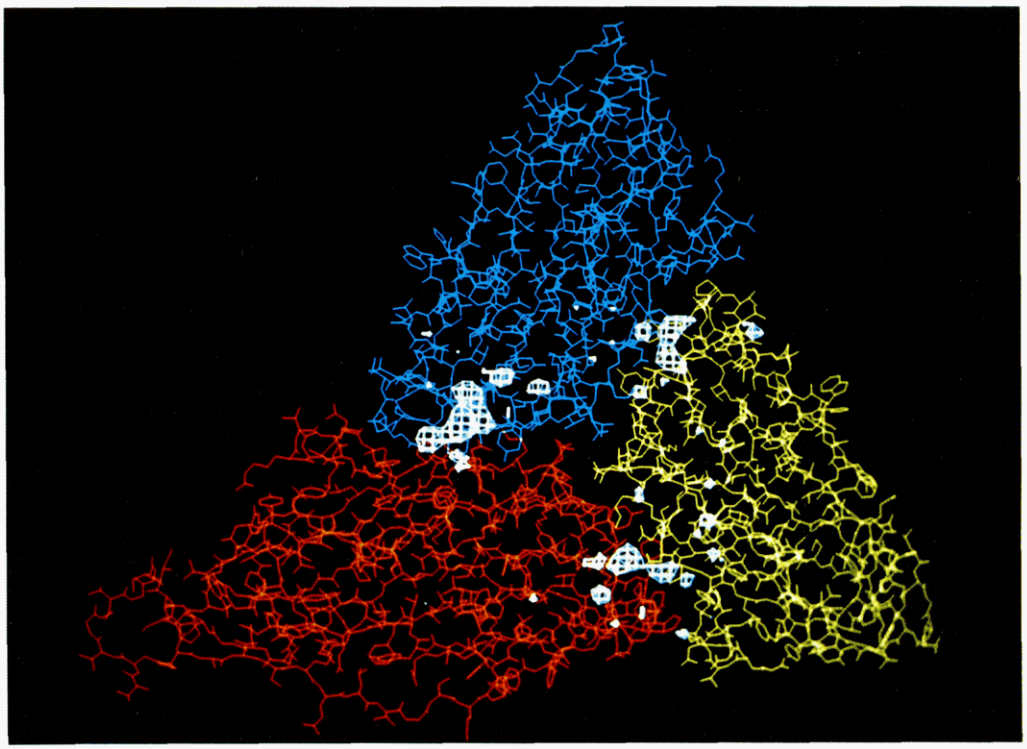

FIGURE 6: Difference Fourier map of tomato bushy stunt virus (*I*23, a = 383 Å) based on monochromatic data for the virus in the presence of Ca^{2+} and Laue data for the Ca^{2+} free virus. The location of the Ca^{2+} ions at each of the trimer interfaces is apparent (Hajdu et al., 1989).

absorption) associated with at least some of the components. In the past, spectroscopic methods have been used to establish the nature of the components in protein crystals (e.g., the semiquinone form of flavodoxin in the crystal; Eaton et al., 1975) and to follow reactions such as the transamination reaction catalyzed by aspartate aminotransferase (Eichele et al., 1978; Mozzarelli et al., 1979), formation of an acyl-enzyme intermediate with glyceraldehyde-3-phosphate dehydrogenase (Mozzarelli et al., 1982), and electron transfer between flavocytochrome b_2 and cytochrome c (Tegoni et al., 1983). With alcohol dehydrogenase, single-crystal microspectroscopic measurements were used to detect NADH present and to test for NADH/NAD conversion in crystals of complexes used for X-ray data collection (Bignetti et al., 1979). With tryptophan synthase chromophoric intermediates formed between the pyridoxal phosphate and substrates at the catalytic site of the β subunits and the effects of ligands bound to the α subunits have been monitored by polarized absorption spectroscopy for this fascinating bifunctional enzyme (Mozzarelli et al., 1989). Fourier transform infrared (FTIR) spectroscopy has been applied to crystals of the photosynthetic reaction center (Gerwert et al., 1988). The crystals were grown on the CaF_2 windows to dimensions of 1.0 mm × 0.5 mm × 6.5 μm, and the difference spectra between the dark and steady-illumination states showed that the intramolecular processes which took place in the chromophores, protein side chains, and protein backbone on light illumination in the crystal were similar to those observed for the reaction center in reconstituted lipid vesicles. These studies indicate the ease with which formation of intermediates can be monitored in the crystal with spectroscopic measurements although the need for very thin samples in the direction of the radiation beam means that FTIR may not be easily applicable to X-ray-size protein crystals.

In order to coordinate X-ray diffraction and spectroscopic studies, a diode array microspectrophotometer is being developed for use at the X-ray station. The hardware is nearing completion, and the software is at present being developed in collaboration with the Daresbury Synchrotron Laboratory (A. Hadfield et al., unpublished results). The instrument utilizes optical fibers to relocate the beam of light in the spectrophotometer onto the crystal in the confined space available in the Laue camera. A reflecting objective is then used to focus the light onto the crystal and another one to collect the light after its passage through the crystal. In addition to the advantage of being able to record spectra during X-ray exposures, it is hoped that the instrument might also be used to monitor a reacting system so that the X-ray camera shutter can be triggered at the right moment as determined by changes in the spectrum.

Initiation of Reactions in Enzyme Crystals. Protein crystals contain a large amount of water. Typically between 30 and 80% of the volume of a protein crystal may be solvent of crystallization. This is fortunate for it means that the environment of the protein in the crystal is similar to that in solution and that ligand binding studies may readily be accomplished by diffusion of ligands into preformed crystals. Many such studies have been carried out, and information on binding sites, intermolecular interactions, and conformational responses have been determined. The diffusion method is not appropriate where large conformational changes are likely to be hindered by the lattice forces of the crystal, although, surprisingly, recent studies suggest that aspartate carbamoyltransferase can accomplish a significant part of the T to R allosteric response in the crystal (Gouaux & Lipscomb, 1989).

In the time-resolved studies on the conversion of substrate to product in phosphorylase crystals using monochromatic data collection methods (Hajdu et al., 1986a, 1987a), the flow cell technique (Wyckoff et al., 1967; Hajdu et al., 1986b) was used to initiate the reaction. Solutions containing the substrate were flowed past the crystal, and the reaction was started by diffusion of the substrate into the enzyme crystal. Such an approach is possible where the diffusion and binding are not rate limiting and the reaction is slower than the time resolution of the experiment (in this instance 30 min). Diffusion of a ligand such as glucose 1-phosphate into a phosphorylase crystal of dimensions of 0.4 mm × 0.4 mm × 1.6 mm had a mean half-saturation binding time of 1.7 min, a time that is consistent with approximate calculations based on knowledge of the free diffusion coefficient of the ligand, the pore radii in the crystal, the dimensions of the crystal, and the external concentration and the dissociation constant of the ligand (Johnson & Hajdu, 1989). Laue photographs of phosphorylase crystals show an unexpected order–disorder–reorder phenomenon as substrates are diffused into the crystal. The diffraction pattern of a native crystal shows sharp spots; 40 s after the start of diffusion of glucose 1-phosphate into the crystals, the pattern exhibits disorder; 20 min later the crystal has reordered, and the pattern is sharp again. Similar effects were observed on conversion of the Zn insulin crystals (Reynolds et al., 1988). The transient disorder means that the diffraction pattern is lost just at the time it would be most desirable to monitor the structure. Thus for Laue diffraction studies with time resolution of the order of seconds or milliseconds, it is necessary to prediffuse the ligand into the crystal under inactive conditions and to initiate the reaction by (say) temperature jump, pH jump, pressure jump, or photodissociation. Indeed, photodissociation was used in one of the earliest time-resolved studies in protein crystallography. The changes in certain reflection intensities were measured following laser illumination of a CO–myoglobin complex with a time resolution of 0.5 ms (Bartunik et al., 1981). Temperature jump has been used (Moffat et al., 1986) with lysozyme crystals, and the changes in intensities in the Laue diffraction pattern were measured with a streak camera on a 0.2–6-s time scale. The interpretation of the results was complicated by crystal movement, changes in unit cell, and nonuniform heating.

Caged compounds (Kaplan et al., 1978) offer one of the most promising approaches for the synchronization of the start of the reaction with the start of data collection. The substrate is made biologically inert through covalent attachment of a photolabile protecting group, most commonly a nitrophenyl ester. Subsequent illumination by a laser or xenon flash lamp results in photodissociation of the protecting group and liberation of the substrate. The nitrophenyl group has the advantage that it absorbs strongly in the near-UV (300–360 nm) in a region where many biological molecules are optically transparent. The physiological and the chemical and physical aspects of the use of caged compounds have been reviewed (Gurney & Lester, 1987; McCray & Trentham, 1989). A number of relevent caged biologically important molecules have been synthesized: caged ATP (Kaplan et al., 1978; McCray et al., 1980; Hibberd et al., 1985); caged cyclic nucleotides (Karpen et al., 1988); caged *myo*-inositol triphosphate (Walker et al., 1987); caged calcium (Adams et al., 1988; Kaplan & Ellis-Davies, 1988); caged protons (McCray & Trentham, 1985); caged neurotransmitters such as carbamoylchloride (Milburn et al., 1989). Rate constants for the liberation of the cage vary from 100 to 100 000 s^{-1} depending on the precise chemical nature of the cage, substrate, and

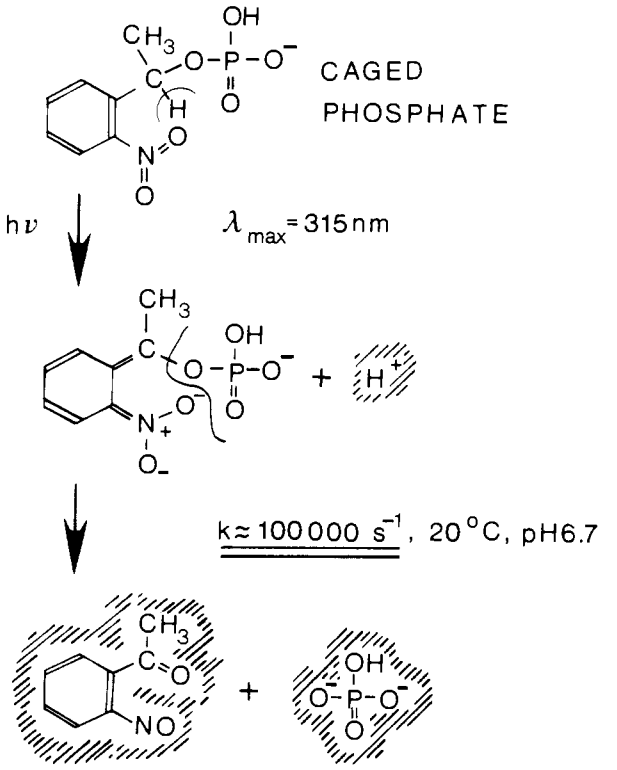

FIGURE 7: Scheme for the release of phosphate from caged 2-nitrophenyl phosphate (McCray & Trentham, 1989).

external conditions. Liberation of the cage results in an almost instantaneous change in the concentration of the biologically active form of the substrate. McCray and Trentham (1989) report that 20 mJ of 347-nm irradiation is sufficient to liberate 2 mM ATP from 5 mM caged ATP (the P^3-[(2-nitrophenyl)ethyl] ester of ATP) over an area of 10 mm^2 and 0.1 mm thickness within a period of a few milliseconds. A scheme for the liberation of phosphate from caged phosphate is shown in Figure 7.

Crystallographic experiments with tetragonal phosphorylase *b* crystals were performed in 1984 (J. Hajdu, D. R. Trentham, D. I. Stuart, K. R. Acharya, and L. N. Johnson, unpublished results), utilizing "caged" phosphate compounds similar to those that have been developed in the study of ATP utilization in muscle contraction (Hibberd et al., 1985). Full monochromatic data sets to 2.8-Å resolution were collected before and after the photolysis of 25 mM caged phosphate in the crystal. The binding sites for the intact caged phosphate have been established, and the structure of the photolyzed products has been determined. These results revealed a recognized problem (Kaplan et al., 1978; Goldman et al., 1984) with the use of saturating concentrations of 2-nitrobenzyl caged substrates. The photolabel when released (a nitroso ketone in this case) reacts with thiol and other nucleophilic groups, extensively modifying the enzyme. These modifications were observed in the electron density difference maps, and chemical modification may lead to an inhibition of the enzyme reaction. The complication of the nitroso ketone reaction is usually overcome by inclusion of equimolar amounts of scavenging thiols. Phosphorylase requires a high concentration of substrate phosphate for reaction when the enzyme is in the T state, and the crystals do not tolerate high concentrations of thiol reagents. As an alternative route to alleviate this problem, it is planned to cage the enzyme. Glycogen phosphorylase contains the essential cofactor, pyridoxal phosphate, which plays an obligatory role in catalysis through its phosphate

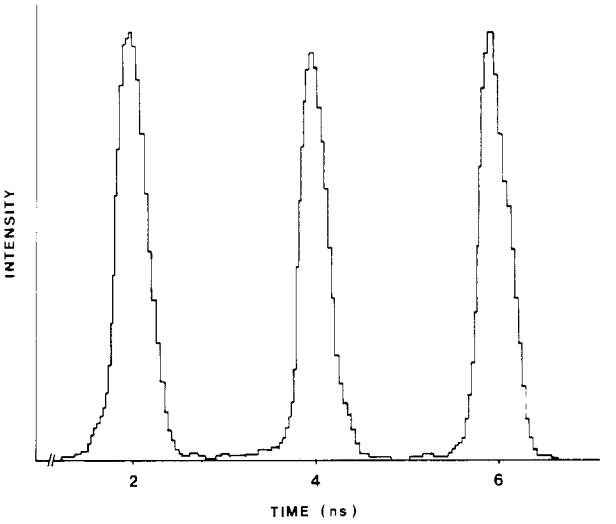

FIGURE 8: Time characteristics of pulsed radiation from the Daresbury Synchrotron Radiation Source run in the multibunch mode.

group. The enzyme concentration in the crystal is about 7 mM, so lower concentrations of cage will be liberated that can be scavenged by suitable concentration of thiols. Caged pyridoxal phosphate has been synthesized, and kinetic, reconstitution, and crystallization studies are underway. Caged ATP has been used in a recent study with hexokinase (Bartunik et al., unpublished results) and caged GTP with the ras oncogene P21 protein (Schlichting et al., 1989).

Finally, there is a need to consider the detector technology. Photographic film has served well, but there are indications that Fuji image plates (Amemiya et al., 1987) and Kodak storage phosphor plates (Whiting et al., 1988) may have advantages in terms of increased absorption efficiency and increased dynamic range, thus partly removing the need to record data with multifilm packs. In a recent development at the Photon Factory a rapid image plate exchanger has been constructed so that 40 images with minimum exposures of 0.1 s and intervals of 0.2 s can be recorded (Amemiya et al., 1989). The use of charge coupled devices is being explored with some promising results, but the small geometric size of these systems is a limitation (Strauss et al., 1987; Allinson et al., 1989).

Picosecond Laue Diffraction at High-Energy Storage Rings. Exposure times of 1 s to 1 ms are exciting to a protein crystallographer, but to a kineticist these times are still very slow. What is the shortest exposure that might be achieved with Laue diffraction? Calculations suggest that about 10^{13} photons are needed to produce an interpretable Laue photograph. In the Daresbury synchrotron a 1-s exposure is sufficient to produce such a picture. Due to the bunch structure of the radiation, 5×10^8 X-ray pulses (each about 120 ps long) will pass through the sample during that time. The source brilliance of high-energy storage rings like the PEP synchrotron in Stanford, CA, may be 8–10 orders of magnitude than that in Daresbury (Wiedemann, 1987) and under similar geometric conditions offers the unique possibility of recording a full Laue diffraction pattern from a crystal by a single X-ray pulse from a single electron bunch (Figure 8). This would mean an overall exposure time on the picosecond scale. Some work toward this goal has already been achieved. In the summer of 1988 a group at Cornell university obtained a 100-ps photograph of a lysozyme crystal using an X-ray station on the undulator magnet at CHESS, a remarkable achievement based on careful calculations of flux and ingenious instrumentation [Szebenyi et al., 1988; also reported by Poole (1988)]. The photograph was recorded with a single bunch

with flux of about 10^6 photons and showed 50–200 spots. Only a narrow radiation band-pass was possible with the undulator (compare Figure 2). This meant that a limited number of lattice planes diffracted, a limitation that can be overcome by using the broad-band Laue technique possible with wiggler magnets at synchrotron radiation sources. Single-bunch exposure experiments in the picosecond time scale using broad-band radiation may open up a completely new era in crystallography and structural chemistry. The very short (picosecond) exposure times could result in increased data quality since chemical processes associated with radiation damage may not have enough time to take place, although, due to these processes and the absorbed radiation energy, the crystal may explode in a few microseconds following the exposure. It will be interesting to see whether or not a nearly complet lack of radiation damage (on the photograph only!) could be achieved this way.

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REFERENCES

Adams, S. R., Kao, J. P. Y., Grynkiewicz, G., Minta, A., & Tsien, R. Y. (1988) *J. Am. Chem. Soc. 110*, 3212–3220.

Allinson, N. M., Brammer, R., Helliwell, J. R., Harrop, S., Magorrian, B. G., & Wan, T. (1989) *J. X-ray Sci. Technol.* (in press).

Almo, S. C., Howell, P. L., Petsko, G. A., & Hajdu, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* (submitted for publication).

Amemiya, Y., Wakabayashi, K., Tanaka, H., Ueno, Y., & Miyahara, J. (1987) *Science 237*, 164–168.

Amemiya, Y., Matsushita, T., Nakagawa, A., Kishimoto, S., Ando, M., Chikawa, J., Wakabayashi, K., Iwamoto, H., & Kobayashi, T. (1989) in *Biophysics and Synchrotron Radiation* (Hasnain, S., Ed.) Ellis Horwood, Chichester (in press).

Amoros, J. L., Buerger, M. J., & Canut de Amoros, M. (1975) *The Laue Method*, Academic Press, New York.

Andersson, I. A., & Branden, C.-I. (1984) *J. Mol. Biol. 172*, 363–366.

Andersson, I. A., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Branden, C.-I., & Lorimer, G. H. (1989) *Nature 337*, 229–234.

Artymiuk, P. J., Blake, C. C. F., Grace, D. E. P., Oatley, S. J. Phillips, D. C., & Sternberg, M. J. E. (1979) *Nature 280*, 563–568.

Bartunik, H. D., Jerzembek, E., Press, D., Huber, G., & Watson, H. C. (1981) *Acta Crystallogr. A37*, C-51.

Bignetti, E., Rossi, G. L., & Zeppezauer, E. (1979) *FEBS Lett. 100*, 17–22.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1965) *Nature 206*, 757–763.

Bordas, J., Munro, I. H., & Glazer, A. M. (1976) *Nature 262*, 541–545.

Bott, R., & Sarma, R. (1976) *J. Mol. Biol. 106*, 1037–1046.

Brunger, A. T., Karplus, M., & Petsko, G. A. (1989) *Acta Crystallogr. A45*, 50–61.

Campbell, J. W., Habash, J., Helliwell, J. R., & Moffat, K. (1986) *Inf. Q. Protein Crystallogr., Daresbury Lab. No. 18*, 23–31.

Campbell, J. W., Clifton, I. J., Elder, M., Machin, P. A., Zurek, S., Helliwell, J. R., Habash, J., Hajdu, J., & Harding, M. M. (1987) in *Springer Series in Biophysics*, Vol. 2, Biophysics and Synchrotron Radiation (Bianconi, A., & Congiu Castellano, A. Eds.) pp 52–60, Springer-Verlag, Berlin, Heidelberg, New York, London, Paris, and Tokyo.

Clifton, I. A., Cruickshank, D. W. J., Diakun, G., Elder, M., Habash, J., Helliwell, J. R., Liddington, R. C., Machin, P. A., & Papiz, M. Z. (1985) *J. Appl. Crystallogr. 18*, 296–300.

Cohen, G. H., Silverton, E. W., & Davies, D. R. (1981) *J. Mol. Biol. 148*, 449–479.

Cruickshank, D. W. J., Helliwell, J. R., & Moffat, K. (1987) *Acta Crystallogr. A43*, 656–674.

Dixon, M. M., & Matthews, B. W. (1989) *Biochemistry 28*, 7033–7038.

Eaton, W. W., Hofrichter, J., Makinen, M. W., Anderson, R. D., & Ludwig, M. L. (1975) *Biochemistry 14*, 2146–2151.

Eichele, G., Karabelnik, D., Halobrenner, R., Jansonius, J. N., & Christen, P. (1978) *J. Biol. Chem. 253*, 5239–5242.

Farber, G. K., Machin, P. A., Almo, S. C., Petsko, G. A., & Hajdu, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 112–115.

Fitzgerald, P. M. (1988) *J. Appl. Crystallogr. 21*, 274–278.

Friedrich, W., Knipping, P., & von Laue, M. (1912) *Sitzungsber. Math.—Phys. Kl. Bayer. Akad. Wiss. Muenchen*, 303–322.

Gerwert, K., Hess, B., Michel, H., & Buchanan, S. (1988) *FEBS Lett. 232*, 303–307.

Goldman, Y. E., Hibberd, M. G., & Trentham, D. R. (1984) *J. Physiol. 354*, 577–604.

Gomez de Anderez, D., Helliwell, M., Habash, J., Dodson, E. J., Helliwell, J. R., Bailey, P. D., & Gammon, R. E. (1989) *Acta Crystallogr. B45*, 482–488.

Gouaux, J. E., & Lipscomb, W. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 845–848.

Greenhough, T. J., & Helliwell, J. R. (1983) *Prog. Biophys. Mol. Biol. 41*, 67–164.

Gruner, S. M. (1987) *Science 238*, 305–312.

Gurney, A. M., & Lester, H. A. (1987) *Physiol. Rev. 67*, 583–617.

Hajdu, J., Acharya, K. R., Stuart, D. I., McLaughlin, P. J., Barford, D., Klein, H., & Johnson, L. N. (1986a) *Biochem. Soc. Trans. 14*, 538–541.

Hajdu, J., McLaughlin, P. J., Helliwell, J. R., Shelden, J., & Thompson, A. W. (1986b) *J. Appl. Crystallogr. 18*, 528–532.

Hajdu, J., Acharya, K. R., Stuart, D. I., McLaughlin, P. J., Barford, D., Klein, H. W., Oikonomakos, N. G., & Johnson, L. N. (1987a) *EMBO J. 6*, 539–546.

Hajdu, J., Machin, P. A., Campbell, J. W., Greenhough, T. J., Clifton, I. J., Zurek, S., Gover, S., Johnson, L. N., & Elder, M. (1987b) *Nature 329*, 115–116.

Hajdu, J., Acharya, K. R., Stuart, D. I., & Johnson, L. N. (1988) *Trends Biochem. Sci. 13*, 104–109.

Hajdu, J., Greenhough, T. J., Clifton, I. J., Campbell, J. W., Shrive, A. K., Harrison, S. C., & Liddington, R. C. (1989) in *Synchrotron Radiation in Structural Biology* (Sweet, R. M., Ed.) pp 331–339, Plenum Press, New York.

Harding, M. M., Maginn, S. J., Campbell, J. W., Clifton, I., & Machin, P. A. (1988) *Acta Crystallogr. B44*, 142–146.

Harrison, S. C., Olson, A. J., Schutt, C. E., Winkler, F. K., & Bricogne, G. (1978) *Nature 276*, 368–373.

Hedman, B., Hodgson, K., Helliwell, J. R., Liddington, R. C., & Papiz, M. Z. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7604–7606.

Helliwell, J. R. (1984) *Rep. Prog. Phys. 47*, 1403–1497.

Helliwell, J. R., Habash, J., Cruickshank, D. W. J., Harding, M. M., Greenhough, T. J., Campbell, J. W., Clifton, I. J., Elder, M., Machin, P. A., Papiz, M. Z., & Zurek, S. (1989a) *J. Appl. Crystallogr. 22*, 483–497.

Helliwell, J. R., Harrop, S., Habash, J., Magorrian, B. G., Allinson, N. M., Gomez, D., Helliwell, M., Derewenda, Z., & Cruickshank, D. W. J. (1989b) *Rev. Sci. Instrum. 60*, 1531–1536.

Hendrickson, W. A. (1985) *Methods Enzymol. 115*, 252–270.

Hibberd, M. G., Dantzig, J. A., Trentham, D. R., & Goldman, T. E. (1985) *Science 228*, 1317–1319.

Johnson, L. N., & Hajdu, J. (1989) in *Biophysics and Synchrotron Radiation* (Hasnain, S., Ed.) Ellis Horwood, Chickester (in press).

Kaplan, J. H., & Ellis-Davies, G. C. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6571–6575.

Kaplan, J. H., Forbush, B., & Hoffman, J. F. (1978) *Biochemistry 17*, 1929–1935.

Karpen, J. W., Zimmerman, A. L., Stryer, L., & Baylor, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1287–1291.

Kress, M., Huxley, H. E., Faruqi, A. R., & Hendrix, J. (1986) *J. Mol. Biol. 188*, 325–342.

Machin, P. A. (1985) *Inf. Q. Protein Crystallogr., Daresbury Lab. No. 15*, 1–16.

Machin, P. A. (1987) in *Computational Aspects of Protein Crystal Data Analysis* (Helliwell, J. R., Machin, P. A., & Papiz, M. Z., Eds.) DL/SCI/R25, pp 75–83, Daresbury Laboratory, Daresbury, U.K.

Makinen, M. W., & Fink, A. L. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 301–342.

McCray, J. A., & Trentham, D. R. (1985) *Biophys. J. 47*, 406a.

McCray, J. A., & Trentham, D. R. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 239–270.

McCray, J. D., Herbette, L., Kihara, T., & Trentham, D. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7237–7241.

Milburn, T., Matsubara, N., Billington, A. P., Udgaonkar, J. B., Walker, J. W., Carpenter, B. K., Webb, W. W., Marque, J., Denk, W., McCray, J. A., & Hess, G. P. (1989) *Biochemistry 28*, 49–55.

Moffat, K. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 309–332.

Moffat, K., Szebenyi, D. M. E., & Bilderback, D. H. (1984) *Science 223*, 1423–1425.

Moffat, K., Bilderback, D., Schildkamp, W., & Volz, K. (1986) *Nucl. Instrum. Methods A246*, 627–635.

Mozzarelli, A., Ottonello, S., Rossi, G. L., & Fasella, P. (1979) *Eur. J. Biochem. 98*, 173–179.

Mozzarelli, A., Berni, R., Rossi, G. L., Vas, M., Bartha, F., & Keleti, T. (1982) *J. Biol. Chem. 257*, 6739–6744.

Mozzarelli, A., Peracchi, A., Rossi, G. L., Ahmed, S. A., & Miles, E. W. (1989) *J. Biol. Chem. 264*, 15774–15780.

Parsons, M. (1989) Ph.D. Thesis, University of Leeds.

Petsko, G. A., & Ringe, D. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 331–371.

Poole, R. (1988) *Science 241*, 295.

Potschka, M., Kock, M. H. J., Adams, M. L., & Schuster, T. M. (1988) *Biochemistry 27*, 8481–8491.

Quiocho, F. A., & Richards, F. M. (1966) *Biochemistry 5*, 4062–4076.

Rabinovich, D., & Lourie, B. (1987) *Acta Crystallogr. A43*, 774–780.

Reynolds, C. D., Stowell, B., Joshi, K. K., Harding, M. M., Maginn, S. J., & Dodson, G. G. (1988) *Acta Crystallogr. B44*, 512–515.

Robinson, I. K., & Harrison, S. C. (1982) *Nature 297*, 563–568.

Rosenbaum, G., Holmes, K. C., & Witz, J. (1971) *Nature 230*, 129–131.

Sarma, R., & Bott, R. (1977) *J. Mol. Biol. 113*, 555–565.

Schlichting, I., Rapp, G., John, J., Wittinghofer, A., Pai, E. F., & Goody, R. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7687–7690.

Schwinger, J. (1949) *Phys. Rev. 75*, 1912–1925.

Steinberger, I. T., Bordas, J., & Kalman, Z. H. (1977) *Philos. Mag. 35*, 1257–1267.

Strauss, M. G., Naday, I., Sherman, M. R., Westbrook, E. M., & Zaluzec, N. J. (1987) *Nucl. Instrum. Methods A266*, 563.

Szebenyi, D. M. E., Bilderback, D., LeGrand, A., Moffat, K., Schildkamp, W., & Teng, T.-Y. (1988) *Trans. Am. Crystallogr. Assoc. 24*, 167–172.

Tegoni, M., Mozzarelli, A., Rossi, G. L., & Labeyrie, F. (1983) *J. Biol. Chem. 258*, 5424–5427.

Tuomi, T., Naukkarinen, K., & Rabe, P. (1974) *Phys. Status Solidi A25*, 93–98.

Walker, J. W., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P., & Trentham, D. R. (1987) *Nature 327*, 249–252.

Whiting, B. R., Owen, J. F., & Rubin, B. H. (1988) *Nucl. Instrum. Methods A266*, 628.

Wiedemann, H. (1987) in *Proceedings of the Workshop on PEP as a Synchrotron Radiation Source*, Oct 20–21, Stanford CT, pp 18–38.

Wood, I. G., Thompson, P., & Matthewman, J. C., (1983) *Acta Cryst B39*, 543–547.

Wyckoff, H. W., Doscher, M., Tsernoglou, D., Inagami, T., Johnson, L. N., Hardman, K. D., Allewell, N. N., Kelly, D. M., & Richards, F. M. (1967) *J. Mol. Biol. 27*, 5372–5382.

Zurek, S., Papiz, M. Z., Machin, P. A., & Helliwell, J. R. (1985) *Inf. Q. Protein Crystallogr., Daresbury Lab. No. 16*, 37–40.