PARTICIPANT SUMMARY of:

First International Workshop on X-ray Damage to Crystalline Biological Samples.
ESRF, 9th-10th June 1999

This Workshop was hosted by Dr. Peter Lindley and attended by around 30 scientists from Europe and the U.S.A. The purpose of the workshop was to consider beam induced radiation damage and heating in macromolecular crystals used for structure determination by X-ray crystallography.

With the advent of extremely intense X-ray beams from third generation synchrotron sources, observation of damage to cryo-cooled macromolecular crystals is becoming more common. In order to fully utilise the X-ray beams now available, some understanding of the processes involved in radiation damage and beam heating is required so that, if possible, evasive action to slow the damage rate can be taken.

At present, there is insufficient knowledge or understanding about production and diffusion of radicals in protein crystals in vitreously frozen solutions. More information is also required concerning radiation damage and beam heating and their relationship to incident dose, dose rate and incident wavelength. The workshop was convened to air all these issues.

The workshop was informal and loosely structured to encourage maximum interchange of information and ideas. There were two main sessions, the first of which was to gather, collate and exchange information and to separate anecdotal evidence from fact. The second session was aimed at deciding on the most important questions to be answered experimentally, and then to apportion possible experiments to the various participating synchrotrons and laboratories on a complementary rather than competitive basis, since large amounts of beam time and effort are needed to obtain solid answers to the many open questions.

In the first session, the first two talks reported separate systematic studies on the effects of radiation decay in protein crystals.

1) RAIMOND RAVELLI (EMBL, France)
`Structural changes in biological samples at cryogenic temperature upon Radiation Damage.'

Systematic studies were performed at the ESRF beamline ID14-EH4 to investigate electron density changes that occur when a crystal is exposed to increasing dose. Multiple data sets were collected on the same crystal in an identical manner, and models were refined against each data set within a series. Three different proteins were studied in this way: acetylcholinesterase, lysozyme and winged bean chymotrypsin inhibitor. The approximate flux that was used in these experiments was 10^12 photons/s, typical exposure times were 1 sec/degree, and frames were recorded on a 2x2 ADSC detector, allowing collection of complete data sets in less than 10 minutes.

For all three proteins, an increase in cell volume was observed. The increase of B-factors, obtained from the Wilson curve, as a function of the dose exposed to the crystal, proved not always to be a good indicator of radiation damage. A better indicator, like

the cell volume, was the R merge(iso). A table was shown for all R merge values for the 9 data sets collected on acetylcholinesterase: they ranged from 6.5% between succesive data sets up to 16.5% between the first and the last data set. Some of this increase can be accounted for by superimposing the molecules resulting from different data sets and translating and rotating them up to 0.3A. However, there seemed to be a significant part left, due to some other factor.

A graph was shown were the Rmerge(iso) was plotted as function of time, with the timepoints at which the crystal was exposed to X-rays clearly indicated. There seemed to be a linear relation between damage, as expressed by R merge, and dose, although there was also some, much reduced, rate of degradation in `resting' periods when no beam was hitting the crystal.

Electron density maps showed that the S-S bond breaks first, but did so at rates which reflected the position of the S-S bond in the molecule. i.e. not all S-S bonds were equally sensitive to radiation damage (there was some correlation with solvent accessibility, but not 100%). Apart from cysteine, Asp and Glu also proved to be more sensitive than other amino acids.

Different modes of data collections were tried to mitigate the damage as judged by the breakage of the disulfide bonds. Data series were collected with the synchrotron running in multi bunch, 16 bunch and single bunch, and at 40 and 100K, all on lysozyme and with a wavelength of 0.93 Ang. Unfortunately, no clear lessening of damage was observed for these different irradiation regimes or cooling methods. Other researchers contributing to this work are: Sean McSweeney, Martin Weik, Gitay Kryger, Jan Kroon, Israel Silman and Joel Sussman.

2) WIM P. BURMEISTER (ESRF, France)
`Structural changes in cryo-cooled protein crystals due to radiation damage.'

A second systematic study of electron density changes with increasing total dose, performed on EH3 at the ESRF, by taking multiple data sets alternating with an exposure of the crystal with a defined dose. A difference Fourier synthesis has been analysed comparing the datasets collected after irradiation with the dataset collected prior to irradiation. Furthermore, occupancies of labile groups have been refined against these individual data sets. Used myrosinase, 0.15x0.15mm^2 beam, 100K, xtal 0.1x0.3x0.1mm^3, 50x10^9 photons/s, wavelength 0.9475A, 0.155x10^15 photons/dataset, 92 passes of 1 deg, each frame being 3 passes of approx 0.7s each. Irradiations were with 9x10^15 photons/mm^2 and 27x10^15 photons/mm^2. Flux was measured with a pin diode which gives 0.27Amps/W of absorbed power and monitored using an ion chamber.

Crystal diffracts to 1.2A, took data to 2.0A. Has a 1.2A native set for phases. Observed: S-S bonds breaking, decarboxylation of aspartates, then loss of some hydroxyl groups of tyrosine for example in active site, and damage starts to obliterate methanethio group of methionines. Dose: 0 to 60 x 10^15 photons/mm^2.

In glutamate see progressive disordering of Cbeta and Cgamma. The asp that was stable under beam damage coordinates with a zinc.

Talks followed which covered:

3) MARTIN CAFFREY (The Ohio State University, Columbus, U.S.A.)

`X-ray Damage in of Lipid Membranes and Mesophases.' or `Biophysics & Synchrotron Radiation. When the Marriage Fails.'

The damaging effects of synchrotron-derived x-rays on aqueous phospholipid dispersions have been evaluated. The effect of degree of lipid hydration, phospholipid chemical structure, mesophase identity, aqueous medium composition, and incident flux on the severity and progress of damage was quantified using time-resolved low- and wide-angle x-ray diffraction and chromatographic analysis of damage products. Electron spin resonance measurements of spin-trapped intermediates generated during irradiation suggest a free-radical mediated process. Interestingly, radiation effects revealed by x-ray diffraction were imperceptible when the lipid was prepared at less than full hydration despite the fact that x-ray-induced chemical breakdown of the lipid occurred regardless of hydration level. Of the fully hydrated lipid systems studied, saturated diacyl-phosphatidylcholines (PC) were most sensitive to radiation damage compared to the ester- or ether-linked phosphatidylethanolamines or the ether-linked PCs. The inclusion of HEPES, Tris/HCl, or phosphate buffer or indeed sodium chloride in the aqueous dispersing medium had only a minor effect in reducing x-ray damage development. A small inverse dose-rate effect was found when the x-ray beam intensity was changed 15-fold.

These results contribute to our understanding of the mechanism of radiation damage, to our appreciation of the importance of monitoring structure and composition when evaluating biomaterials radiation sensitivity and to the development of strategies for eliminating or reducing the severity of damage due to synchrotron x-radiation. Since damage is shown to be free radical mediated, these results also have a bearing on age-related accumulation of free radicals in cells and how these might compromise membrane integrity culminating in cell death.

References to this work:

1) X-radiation damage of hydrated lecithin membranes detected by real-time X-ray diffraction using wiggler-enhaned synchrotron radiation as the ionising radiation source.' Nuc. Inst. Meth, (1984) 222,329-338. M. Caffrey.
2) `X-rays destroy the lamellar structure of model mebranes.' J.Mol.Biol. (1993) 229, 291-294. A. Cheng, J. Hogan and M. Caffrey.
3) `Free radical mediated X-ray damage of model mebranes.' Biophys. J. (1996) 70, 2212-2222. A. Cheng and M. Caffrey.

4) GERD ROSENBAUM (ANL)
`Contradictory observations on dose rate vs. overall dose effects and estimates of the temperature increase in a crystal during exposure.'

Right from the beginning, it was observed at the SBC that when the full flux of 10^13 ph/s was concentrated on a sample crystal, the crystal would die within 20 or so frames. When the flux density was reduced to about 2 x 10^11 ph/s through the collimator slits of typical 0.2 mm x 0.2mm size, which is approximately equal to the intensity at 2nd generation wiggler sources, the crystals survived two or more data sets without much decay. This is about the same survival in number of exposures as usually observed at 2nd generation wiggler sources. There is no specific crystal killing property of 3rd generation sources as has been claimed by a prominent crystallographer. Another observation of a dose rate effect occurred with HSP70 (Andrzej Joachimiak). At first a

good, medium-resolution data set without noticeable loss of high resolution spots was acquired at the "standard" intensity. Then the intensity was increased 3 - 5 x for the acquisition of high resolution data. Noticeable damage was observed after about 20 frames.

The hypothesis to explain the dose rate effect was that the absorbed beam would increase the temperature in the crystal to a level where the mobility of the radicals produced was markedly increased. Also, at 140 K is a known phase transition in water glass which might damage the crystal lattice. To test this hypothesis, Steve Ginell, SBC, determined the B-factor of lysozyme crystals as a function of deposited dose for the following exposure sequence:
flux density = 5x10^12 ph/s through 0.2 mm x 0.2 mm collimator;
photon energy = 12 keV;
10 degree-wedge of 100 frames of 0.1 degree, 0.1 s exposure; repeated 10-times;
continuous exposure for 10 s ; another 10 degree-wedge, conditions as above;
continuous exposure for 60 s; another 10 degree-wedge, conditions as above.
Plotting the B-factors determined for each 10 degree-wedge against the accumulated dose gave an essentially straight line. This disproved the temperature hypothesis. (But not the dose rate effect.)

To support these findings, G.R. reported a simple calculation of the adiabatic temperature increase (i.e. without any cooling) in a typical crystal due to the heat absorbed for a typical dose per exposure of 1-2 x 10^12ph at 12 keV. It is only 4-8 K (in agreement with the detailed calculations presented by the next speaker) and thus not sufficient to greatly increase the mobility of radicals.

Also, the hope that smaller crystals will reduce the heating problem is unfounded. For equivalent exposure times, the flux density on the crystal has to be increased inversely to the crystal volume. The same amount of absorbed heat has then to spread out from a smaller source size to about the same surface area for removal by the cold stream. This will result in a higher temperature gradient for smaller crystals.

G.R. also presented data obtained by N. Duke, SBC, and Z. Otwinowski, U-Texas, on the increase in lattice constant of KSI with accumulated dose, an effect that has been observed regularly: repeated 10 degree-wedges on the same volume of the crystal showed a linear increase in the 500A cell dimension. When exposing an adjacent, hitherto unexposed volume, they found the original lattice constant. Further exposure of this volume for a total of 100 degrees rotation resulted in another about linear increase in lattice constant. The increase in lattice constant with exposure appears to be local and not spreading far beyond the exposed crystal volume.

5) JAMES NICHOLSON (Daresbury, U.K.).
`Modelling of the temperature rise in protein crystal during irradiation by finite element analysis.'

Model calculations using finite element analysis and performed by Barry Fell of Daresbury Laboratory were presented. Two different cases for crystals held at 100K were being modelled:

Case 1: X-ray beam smaller than crystal.
Sample and environment parameters (wrong??! - from literature):
Specific heat of crystal - 0.7 Joules/cc/K
Thermal conductivity - 0.4 watts/m/K

Expansion coefficient - 0.01%/K
Nitrogen gas flow at - 5 litres/min through a 9mm diameter nozzle at 100K
Crystal size - 0.1 mm^3
Beam parameters:
X-ray beam size - 0.05mm diameter
Power absorbed - 1.02 watts/mm^3 along beam path only (from Helliwell).

For this case, the plot for a) the corners of the crystal (assuming the beam was exactly in the centre of the crystal) showed a temperature rise from 100K to 103.5K in the first 0.1 sec but levelled off to a constant temperature of 104K with further irradiation. The plot for b) the centre of the crystal (again assuming the beam was exactly in the centre of the crystal) showed a temperature rise from 100K to 105.5K in the first 0.1 sec but again levelled off to a constant temperature of 105.75K with further irradiation.

Case 2: Crystal bathed in X-ray beam.
Sample and environment parameters same as Case 1.
Beam parameters:
X-ray beam size - 0.2mm diameter (significantly greater than the crystal).
Power absorbed - 0.2 watts/mm^3.
Results for this case were not yet available.

Note that these values should not be regarded as accurate since the sample and environment parameters from the literature are only approximates for the case of a protein crystal in vitreously frozen buffer. However, the trend is clear: equilibrium is swiftly reached and sample heating is not a significant contributor to thermal migration of radiation damage products. Further calculations are being carried out to explore the parameter space.

6) DAVE STUART (Oxford, U.K.).
'Factors to be considered when investigating radiation damage, and observations of damage to Blue Tongue Virus (BTV) crystals under different conditions.'
- Need to consider Secondary damage. Adding 35% CsCl, which dramatically increases X-ray absorption in the liquor for the crystals, reduced the crystal lifetime by a factor of 4 (at approx 8 deg C).
- The lattice stability is proportional to size of molecule. (i.e. much lower for virus crystals than for protein crystals).
- What actually happens when crystals are cooled? Is the variation in unit cell dimensions often seen between cooled crystals mirrored to a smaller extent by variation within a crystal? Is this part of the effect normally described by an inflated mosaic spread? This factor is a disaster for big cells.
- Flash cooling doesn't work for many virus crystals.
- His group have used a variety of cryoprotectants for virus crystals, often at surprisingly high concentrations (e.g. 50% glycerol).
- Have cooled down to -70C in suitable solvents, and lattice has remained intact. Then below -70C there is a change and lifetime in the beam increases while disorder increases.
- Blue Tongue Virus:
  From room temperature down to -70C get change in lifetime in the beam.

From -70C to 100K get physical change. Lifetime increases by factor 100. Disorder increases.
The cryoprotection they use takes them down to 100K without problems; they take 2 hours to cool slowly down to 100K, and it looks fine.

- Foot and Mouth Disease Virus crystals have a mosaicity of 0.2 deg when frozen optimally (using a slow cooling protocol or annealing), a huge increase on the room temperature value.
- General considerations:
  Using total dose limit from APS of 10^13 photons/(0.1mm^2)/sec, this is approx 1 photon/A^2/sec. interaction probablility is <10% in 0.1mm, so about 10^7A required for an interaction in 1A cross-section. Consider a unit cell with 100A edge, would get approx 1 photo-electron made/unit cell/sec. Thus two photon events seem very unlikely.

7) DENNIS WEISS (Gottingen, Germany)
'X-ray Damage to cryogenic biological samples and its influence in in X-ray microtomography.' D. Weiss, G. Schneider, B. Niemann, P. Guttmann, D. Rudolph, G. Schmahl.

Theoretical and experimental results concerning the required dose for computed tomography based on X-ray microscopic images, and the effect on biological specimens were presented. Fourier optics were used to compute the photon density and corresponding specimen dose necessary to acquire X-ray microscopic images with a given resolution and signal-to-noise ratio. For amplitude contrast - required for computed tomography of the absorption coefficient -, the dose for a single image was 10^6 Gy to image 30 nm protein fibres in ice with a SNR of 3. Accounting for the 10% diffraction efficiency of the optic and acquiring ~100 images for tomography gives a total dose of ~10^9 Gy.

To evaluate the effect of dose on the specimen, a theoretical model for radiation-induced kinetics (including diffusion) was proposed, where an inhomogeneous protein concentration is degraded in a first-order process, with the rate constant given by the product of quantum yield, photoelectric cross section, and photon flux. The concentration is simultaneously propagated according to Fick's Second Law, with the diffusion constant given by the Einstein-Stokes law for spherical particles. Calculations for several X-ray sources (SR, plasma) and exposure times were presented. For fixated room temperature protein, diffusion was neglected, with a significant loss of protein occurring at doses of 10^6 - 10^7 Gy. Similar results were obtained experimentally with cotton fibres in water [1]. At 73 K, water shows significant loss of H2O molecules at 10^8 - 10^9 Gy. This imposes an upper limit for the dose tolerance under cryo conditions. Cryo microscopy is the only method capable of providing the dose tolerance necessary for X-ray microtomography.

The predicted dose limit was applied to a cryogenic biological specimen, which sustained a dose of ~10^10 Gy without showing radiation damage at the microscopic resolution (~30 nm). However, subsequent tomographic experiments showed signs of radiation damage at doses of approx. 10^8 - 10^9 Gy: gaseous radiation products and degradation of protein membranes. As of yet, there are no results concerning dose rate effects.

References:
1. R.O. Bolt and J.G. Carroll, Radiation Effects on Organic Materials, Academic Press, NY 1963.

8) CHRIS JACOBSEN (Stony Brook).
`Summary of some literature and experience in electron and X-ray microscopy'.

All the overheads from this talk can be found on the www site:
http://xray1.physics.sunysb.edu/~jacobsen/esrf_damage_jun99.pdf

X-rays are fundamentally more damaging for atomic resolution imaging than electrons (refs 1,2). The success of x-ray protein crystallography is therefore based on dividing the damage AND structural information amongst many identical copies of a macromolecule. In electron and x-ray microscopy, one seeks instead to obtain high resolution information from structures with few or no identical copies, and in this case radiation damage increases rapidly as one must obtain more and more information from a smaller sample area. Electron microscopy doses of ~5 electrons/nm^2 produce significant changes in infrared spectra of organic bonds, and 1 nm^(-1) diffraction spots show significant reduction at doses of ~500 electrons/nm^2 at 100 keV (where 1 electron/nm^2 corresponds to a dose of ~3 x 10^4 Gray where 1 Gray=1 J/kg=100 rad). These damage limits are for specimens at liquid nitrogen temperature, where samples show greatly increased radiation damage tolerance due to 1) a slight reduction in the G factor of the number of bonds broken per 100 eV of ionizing radiation and especially 2) a cessation of the diffusion of free radicals created by radiolysis of water (see e.g., refs 3,4). At doses of ~5000 electrons/nm^2 or ~10^8 Gray, severe bubbling of frozen hydrated specimens is observed as H^+ bubbles are formed (ref 5).

X-ray microscopy offers advantages for imaging several micrometer thick, hydrated biological specimens (refs 6-8). One can also obtain maps of the chemical bonding state of several low-Z elements, especially carbon (ref 9), and obtain 3D reconstructions of hydrated biological specimens (refs 10,11). By using soft x-rays in the energy range between the carbon and oxygen K absorption edges, one can obtain good contrast for organic materials in water or ice and proportionally less dose is delivered to water molecules. At room temperature, living specimens show immediate, severe radiation damage at radiation doses of 10^4 Gray (refs 8,12), and even aldehyde-fixed specimens show significant radiation damage (ref 13). However, cryo specimens in x-ray microscopy can tolerate radiation doses of up to about 10^10 Gray with no observable structural damage at the 40 nm resolution level (refs 14,15). Radiation damage can be seen in the reduction of x-ray absorption near-edge resonances at room temperature at doses of ~10^6 Gray (ref 16), and experiments are now underway to elucidate the protection that cryo microscopy offers for near-edge absorption spectroscopy.

References:
1. J. R. Breedlove Jr. and G. T. Trammel, `Molecular microscopy: fundamental limitations.' Science 170, 1310-1313 (1970).
2. R. Henderson, `The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules.' Quarterly Reviews of Biophysics 28(2), 171-193 (1995).

3. R. A. Steinbrecht and K. Zierold, `Cryotechniques in Biological Electron Microscopy.' (Springer-Verlag, Berlin, 1987).
4. P. Echlin, `Low-Temperature Microscopy and Analysis.' (Plenum Publishing, New York, 1992).
5. R. D. Leapman and S. Sun, `Cryo-electron energy loss spectroscopy: observations on vitrified hydrated specimens and radiation damage.' Ultramicroscopy 59, 71-79, (1995).
6. D. Sayre, J. Kirz, R. Feder et al., `Transmission microscopy of unmodified biological materials: comparative radiation dosages with electrons and ultrasoft x-ray photons.' Ultramicroscopy 2, 337-341 (1977).
7. G. Schmahl, D. Rudolph, B. Niemann et al., `Zone-plate X-ray microscopy.' Quarterly Reviews of Biophysics. 13, 297-315 (1980).
8. J. Kirz, C. Jacobsen, and M. Howells, `Soft x-ray microscopes and their biological applications.' Quarterly Reviews of Biophysics 28(1), 33-130 (1995).
9. H. Ade, X. Zhang, S. Cameron et al., `Chemical contrast in x-ray microscopy and spatially resolved XANES spectroscopy of organic specimens.' Science 258, 972-975 (1992).
10. Y. Wang, C. Jacobsen, J. Maser et al., `Soft x-ray microscopy with a cryo STXM: II. Tomography.' Journal of Microscopy (1999).
11. D. Weiss and G. Schneider, `X-ray tomography of an algae.' presented at the X-ray Microscopy 1999 meeting, Berkeley, USA, 1999 (unpublished).
12. P. M. Bennett, G. F. Foster, C. J. Buckley et al., `The effect of soft X -radiation on myofibrils.' Journal of Microscopy 172, 109-119 (1993).
13. S. Williams, X. Zhang, C. Jacobsen et al., `Measurements of wet metaphase chromosomes in the scanning transmission x-ray microscope,' Journal of Microscopy 170, 155-165 (1993).
14. G. Schneider, `Cryo x-ray microscopy with high spatial resolution in amplitude and phase contrast.' Ultramicroscopy 75, 85-104 (1998).
15. J. Maser, A. Osanna, Y. Wang et al., `Soft x-ray microscopy with a cryo STXM: I. Instrumentation, imaging, and spectroscopy.' Journal of Microscopy (1999).
16. X. Zhang, C. Jacobsen, S. Lindaas et al., `Exposure strategies for PMMA from in situ XANES spectroscopy.' Journal of Vacuum Science and Technology B 13(4), 1477-1483 (1995).

9) ELSPETH GARMAN (Oxford, U.K.).
'Consideration of whether lowering the cryo-temperature to around 40K using helium as the cryogen could be expected to alleviate radiation damage effects.'

EG considered whether any gain in going to lower cryogen temperature might outweigh the disadvantages (mainly cost) and some of the factors which might be relevant were presented.

i) Thermal conductivity, k and specific heat, C_p of helium compared to those of nitrogen. Both the thermal conductivity and the specific heat of the cryogen affect, along with many other parameters, the heat exchange coefficient. The flow regime of open-flow cryostats is laminar: any turbulence around the crystal position will assist heat transfer. The thermal conductivity, k, of helium at 30 K is 0.35 mW/cm/K, whereas for nitrogen at 100 K, k is 0.1 mW/cm/K. The specific heat of helium is also a little higher than that of nitrogen (5.2 J gm^(-1) K^(-1) for helium compared to 1.25 J gm^(-1) K^(-1) for nitrogen: mass ratio He:N is 4:14) so more heat can be carried away by the same unit mass (at 30K the gas is denser, so care must be taken in comparing flow rates, velocities,

masses and volumes of helium and nitrogen at different temperatures). Helium at 30K should therefore be a more efficient cryogen than nitrogen gas at 100K.

ii) Reduction of thermal vibration, and thus atomic B-factor at lower temperature. As the temperature of a protein molecule is decreased from 293 K, the dynamic disorder decreases, until below around 150K, all that remains is the zero point motion (theoretical limit at 0K), the Debye-solid harmonics, and the static disorder. Of these, only the Debye-solid harmonics will decrease as the temperature is lowered further, and so the reduction of atomic B-factors, in going from 100K to 30K, is likely to be fairly small.

Two examples of B factor reduction with temperature were presented for proteins where experimental B-values are known for room temperature and 100K structures: Ribonuclease A, a 124 amino acid predominantly beta sheet protein and cytochrome cd_1 nitrite reductase, which has 2 x 567$ amino acids in the asymmetric unit.

iii) Temperature dependence of the thermal conductivity, k, of protein crystals: has not been measured for protein crystals in vitreous ice but the thermal conductivity may become very low at low temperatures. For glasses, and also for clathrate hydrates (max 6A diameter molecules trapped in a matrix of ice) where measurements are available down to 100K, k proportional to T. For perfect crystalline solids, k proportional to 1/T. Protein crystals are likely to mimic the clathrates rather than perfect crystals, and will thus have k proportional to T. If k becomes very small, the heat transfer rate (dQ/dt) from the centre of the sample to the cryogen, also decreases.

iv) Temperature dependence of specific heat capacity, C_p of crystals. This parameter is similarly not yet measured for protein crystals, but there is reason to think it may also become very low at low temperatures. Measurements for vitreous glass and various different vitrified salt solutions all show a decrease in C_p with decreasing temperature. Thus at lower temperature, less energy is needed to heat the crystal and the thermal conductivity may also be lower, so it will be harder to conduct the heat away by cooling. Although counter-intuitive, the effect of this will be that the centre of the crystal will heat up faster and be harder to cool when held at 30K compared to 100 K.

v) Temperature dependence of primary radiation damage: will depend on the (as yet unknown) temperature dependence of the density of colour centres, crystal defects, shrinkage and crystal contact changes due to cell shrinkage with decreasing temperature. This list is not exhaustive and because the mechanism of radiation damage itself is poorly understood, there may be additional factors which will affect the balance of advantage/disadvantage in going to lower cryogen temperature.

vi) Cooling regime. For the actual flash-cooling procedure, there is advantage in cooling the sample down to lower temperatures. Newton's Law of Cooling states that dT/dt is proportional to Delta T. Teng and Moffat (1998), reported that at 20K and a helium flow rate of 40l/min, a cooling rate of >500K/s was achieved between 300-150K and a rate of <100K/s between 120-20K. The lower helium temperature and heat transfer properties of helium must thus be an advantage in providing a more efficient cooling regime. Although this does not directly impact the rate of radiation damage during data collection, it does decrease the amount of cryoprotectant-agent required to obtain a vitreous glass. The relationship between the rate of radiation damage for different cryo-solution concentrations has not yet been investigated, and is thus another uncertainty when considering damage avoidance.

Thus there are too many simultaneous variables to make a prediction of whether using helium as a cryogen will be effective in reducing radiation damage inflicted by

very hot beams. The answer will depend on the balance of advantages/disadvantages outlined above and controlled experiments are required to investigate their relative effects.

This discussion can now be found in full in Section 7 of `Cool Data: Quantity and Quality', E.Garman Acta Cryst D55 (1999) 1641-1653.

In the second session the issue of collecting data at different wavelengths was first addressed:

10) ANA GONZALEZ (EMBL, DESY, Germany)
`Radiation damage and related studies in protein crystals.'

A.G. summarised the results of 3 published experiments she has performed over the last several years which investigated:

1) the limiting total radiation dose a 100K protein crystal can receive before being significantly damaged, and how this limit compares with the theoretical limit predicted by Henderson of 1.3 x 10^17keV/mm^3. A.G. used white radiation with wavelengths between 0.5 and 4A and tested a lysozyme crystal cryoprotected with a layer of oil and cooled to 100K within a hair loop. The team observed damage after a dose of 4 x 10^17 keV/mm^3, in reasonable agreement with prediction [see Gonzalez, Thompson and Nave in Rev.Sci.Instrum, (1991) 63, 1177-1180]. It would be very instructive to repeat this with monochromatic radiation.

2) the relationship between incident wavelength and data quality at room temperture at wavelengths of 0.55A and 0.92A, [see Gonzalez, Denny and Nave in Acta Cryst D (1994) 50, 276-282.] They concluded that there was no improvement in data quality or in signal-to-noise ratios at the shorter wavelength.

3) a) a more quantitative estimate of the radiation damage in terms of the effect on the diffraction pattern as a function of resolution and absorbed radiation dose, b) effect of changing the wavelength (aluminium absorbers were used to shift the white beam spectrum towards high energies), and c) the maximum size of crystal from which it will be possible to collect a data set at cryo-temperatures.

Their results [see Gonzales and Nave, Acta Cryst D (1994) 50, 874-877.] seemed to indicate that a change in wavelength does not lead to a large improvement in the life-time of the crystal at cryo-temperatures. This is consistent with the radiation damage being proportional to the energy deposited rather than the number of photons absorbed (as per Arndt, J.Appl.Cryst (1984) 17, 118-119.)

As in 1) it would be very instructive to repeat this experiment with monochromatic radiation.

A general discussion followed:

11) GERD ROSENBAUM (ANL, U.S.A.)
`Summary of the information discussed in the first part of the workshop; determination of the areas of lacking information; prioritising of experiments and assignment of tasks.'

Before summarising the data presented by the speakers so far, G.R. presented a calculation of the ratio of energy absorbed in a crystal per number of photons into a reflection, which to first order is independent of photon energy and of crystal volume.

G.R. then moderated an open forum which tried to summarise the topics presented or discussed, the areas of lack of knowledge and the priorities for experiments. The Workshop then assigned tasks to particular synchrotron radiation labs. The results of this discussion were as follows:

a) What constitutes or is a measure for "damage"? What needs to be tested?
It was determined that for most studies the overall B-factor should be regarded as a measure of "damage" because of the lesser amount of work as compared with the more specific determination of the loss of occupancy of S-S-bridges. All damage measurements should be accompanied by a careful estimate of the absorbed dose, not only the incident dose. Damage measurements at high temperature as well as cryo-temperatures should be carried out. No specific synchrotron radiation centre was named for these measurements.
b) Dose rate and overall dose effects?
The onset of dose rate effects is not well documented. Need to determine limiting dose above which sample decay is very rapid. The two 3rd generation sources ESRF (McSweeney) and APS (Rosenbaum) are charged with well designed experiments.
c) Time delayed effects? Was assigned low priority.
d) Energy dependence?
No firm data on energy dependence were presented. Rosenbaum and Kuhn reported a trend of their users to higher energy, the users claiming to obtain better data. ESRF (McSweeney) and APS (Rosenbaum) for Ephoton > 18 keV, SSRL (Kuhn) and SRS (Nave) were charged with well calibrated tests to put claims on an objective basis.
e) Crystal size? No tasks assigned.
f) Radioprotectant?
Garman was to characterise scavengers with room temp experiments in-house at U of Oxford and then with McSweeney at ESRF.
g) Crystal heating?
Simulation with finite element analysis should be continued by group that reported it. Experiments to verify results to be devised.
h) 40 K vs. 100 K?
Important to record flow rate as well as temperature. SSRL (Kuhn) and ESRF (McSweeney) were assigned these tests because they have the hardware in their labs.
i) Pulsed vs. CW?
Question was raised. Is connected to question of dose rate effect. No priority set. No lab assigned.

The experiments necessary to answer the questions require careful design, and are not trivial, since there are many convoluted variables. For instance, the same sized crystals should be used within each experiment for proper comparison.

Some useful references addressing questions which came up in the discussion:
1) Henderson, R. `Cryo-protection of protein crystals against radiation damage in electron and X-ray diffraction.' (1990) Proc.R.Soc.Lond. B241, 6-8.
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Peter Lindley brought the Workshop to a conclusion with a plea for more attention to be focused on designing and building faster detectors with lower noise. Since it is likely that radiation damage rates can only be slowed, but not made insignificant, collecting the data in a shorter time would be the best option. Investment of money and resources are crucial for further detector development.

Overall the workshop was felt to have met its objectives: to focus attention on the issue, to encourage scientific discussion of facts as opposed to anecdotes, and to plan a set of realistic experiments which were divided up between laboratories. The participants contributed to the informal discussions in a very positive and open way, and as a result the workshop was scientifically stimulating.

It was agreed to convene again in about a year, possibly at the ESRF, to report on the results and progress of the investigations, and to plan future experiments.

The Chairpersons [Elspeth Garman and Gerd Rosenbaum] would like to express their gratitude to Dr. Peter Lindley and Dr. Yves Petroff at the ESRF for their support and encouragement, both financially and scientifically, which enabled the workshop to take place successfully, and also to all the attendees for their active participation. They would also like to thank Fabienne Mengoni for her organisation and help.

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