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X-stream™ Cryocrystallography

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Introduction

The technique of flash-cooling [1-3] crystals of biological macromolecules represents an important advance in the field of macromolecular crystallography. Flash-cooling crystals results in improved data quality since the entire data set can usually be collected from a single crystal because radiation damage is minimized. Additionally, flash-cooling using glass or fiber loops [4] simplifies crystal mounting, produces less mechanical stress on the crystal during mounting, eliminates effects of capillary absorption and allows for the storage of crystals for future use after exposure.

In the flash-cooling experiment, the crystal is quickly lifted out of its mother liquor using a glass spatula or fiber loop (usually attached to a metal pin) and centered in a nitrogen gas cold-stream (T = 100K) which has been temporarily obstructed. After the crystal is in place, the object used to obstruct the cold stream is quickly removed and the crystal flash-cools to cryogenic temperature. Several commercial cryogenic crystal cooling devices employing a nitrogen gas cold stream are currently available. Most of these commercial devices require liquid nitrogen (LN2) either to generate (boil off) or cool the nitrogen gas cold stream. However, the X-stream™ cryogenic crystal cooler produced by Molecular Structure Corporation (MSC), a Rigaku company, uses an innovative gas separator to produce nitrogen gas which is then cooled to cryogenic temperatures by means of a two-stage helium compressor*. This completely eliminates the need for LN2. The advantages of the X-stream™ are evident. Since LN2 is not required, the cost and problems associated with the ordering, delivery and storage of LN2 are eliminated. The footprint of the X-stream™ heat-exchanger is small compared to that of the other commercial systems which require some sort of LN2 storage Dewar in close proximity to the heat-exchanger, The small X-stream™ footprint allows easy access to components of the X-ray generator which are normally blocked by the LN2 storage Dewar of the other systems. In addition, unlike most cryogenic systems using LN2 boil-off, the X–stream™ is capable of operation at any temperature between -180° and 4°C.

*Rigaku Corporation has developed a similar system independently of MSC. This system is commercially available in Asia.

The X-stream™ Theory and Design

The X-stream™ consists of six major components (Figure 1); an air compressor (1), a refrigerated air dryer (2), a filter bank and gas separator (3), a two-stage helium compressor (4), a control unit (5), and a heat-exchanger and coldstream delivery nozzle (6). An optional computer (7) is available to control the X–stream™ and monitor temperatures at various points in the system. The air compressor (1) provides a source of high pressure (150 PSI) compressed air. The compressed air is then fed into a refrigerated air dryer (2) where most of the moisture is removed. The dry compressed air is then fed into the filter bank and gas separator (3) where any remaining dispersed oil and water are filtered out by coalescing filters and any volatile organic compounds are removed by an activated charcoal filter. Finally, a particulate filter protects the gas separator membrane from any remaining solid particles. The membrane gas separator is used to remove contaminant gases such as argon, carbon dioxide, oxygen and water, resulting in a nearly pure nitrogen gas supply. This gas is then fed into the heat exchanger-nozzle assembly (6). In the heater-exchanger, the nitrogen gas is passed through the cold side of a two-stage helium compressor (4) where it is cooled to cryogenic temperatures. The cooled gas then passes through a flexible, vacuum jacketed, stainless steel transfer tube to the nozzle assembly. In the nozzle assembly, the gas stream is heated to the desired temperature by two microprocessor-controlled, in-stream, heater-thermocouple loops (HTL). The first HTL, called the vapor loop, is designed to remove any liquefied nitrogen from the gas stream and to heat the gas stream to a temperature that can be regulated by the second HTL, called the nozzle loop. The nozzle loop is designed to perform fine regulation of the gas stream temperature using feedback from a

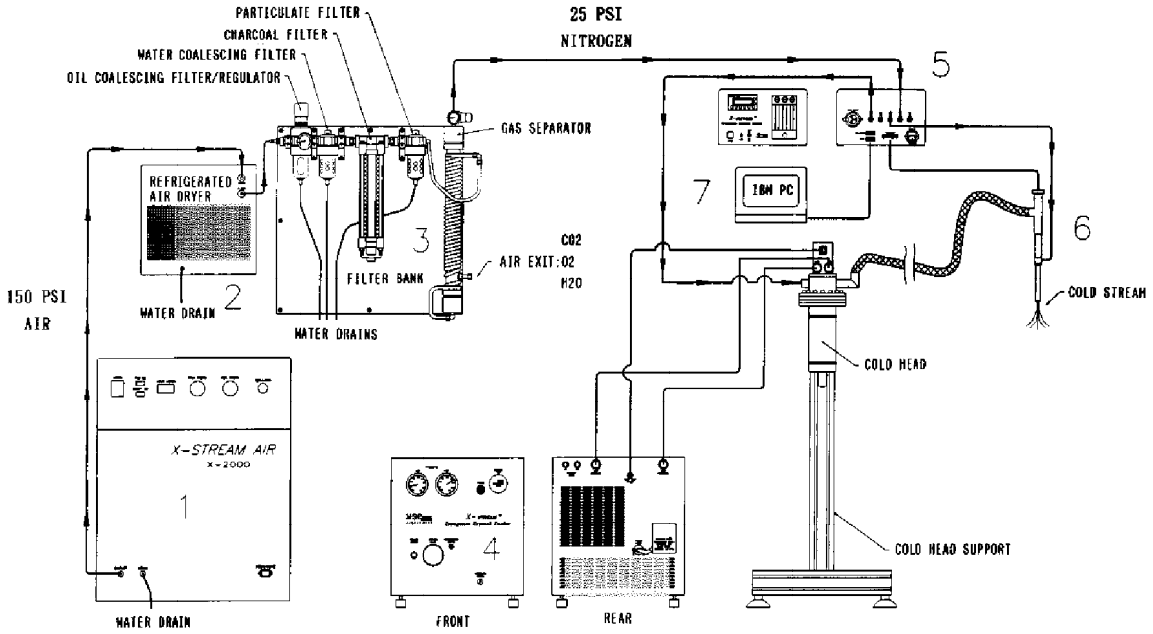

Fig. 1. A schematic of the X-stream™ system. (1) air compressor, (2) refrigerated air dryer, (3) filter bank, (4) helium compressor, (5) control unit, (6) heat exchanger and nozzle assembly and (7) optional computer.

thermocouple placed near the nozzle tip. The nozzle also provides a coaxial warm, dry nitrogen stream around the cold stream to prevent ice formation on the sample.

The control unit (5) houses the flow gauges for both the cold and warm gas streams, as well as the temperature controller. The control unit also provides a serial interface to the optional computer (7) and inputs for an external thermocouple, auxiliary gas supply and optional goniometer head heat shield.. The optional computer can be used to set, display, monitor and plot the temperatures of the vapor, nozzle, goniometer head heat shield and external thermocouple loops. The optional goniometer head heat shield can be placed over the goniometer head to prevent ice buildup when the X-stream™ nozzle is positioned collinear with the phi axis.

The X-stream™ in the Laboratory

On January 5, 1996, the first X-stream™ System was installed in the University of Georgia's (UGA) BioCrystallography Laboratory. As with any new technology, there were some initial concerns about the X-stream™ and its installation. Will the quality of our in-house compressed air supply be good enough? How well will the filter bank and membrane gas separator perform in the laboratory environment? How reliable and stable is the helium compressor and heat exchanger? Given the humid Georgia summers, will improvements in the design of the nozzle assembly eliminate the crystal icing problems sometimes observed with the old style nozzle?

At UGA, the X-stream™ was connected to a dedicated air compressor (Quincy QT-5) rated at 15-18 CFM (160-180 PSI cycle), refrigerated air dryer (Motivan AC-0300) and a water and oil trap located in a basement machine room. A water filter (Dominic Hunter, OH-2858-5), an oil filter (Dominic Hunter, OH-127-5), an activated alumina regenerating air dryer (Hankinson, DHW-15) and a I liter, self-draining, water trap were located in a utility closet next to the X-ray bay (Figure 2). The air compressor, air dryers and the X-stream™ were connected to emergency power to insure that crystals remain at cryogenic temperature during power failures.

The questions about the in-house air quality and how well the MSC-supplied filter bank and gas separator would work in the laboratory environment were soon

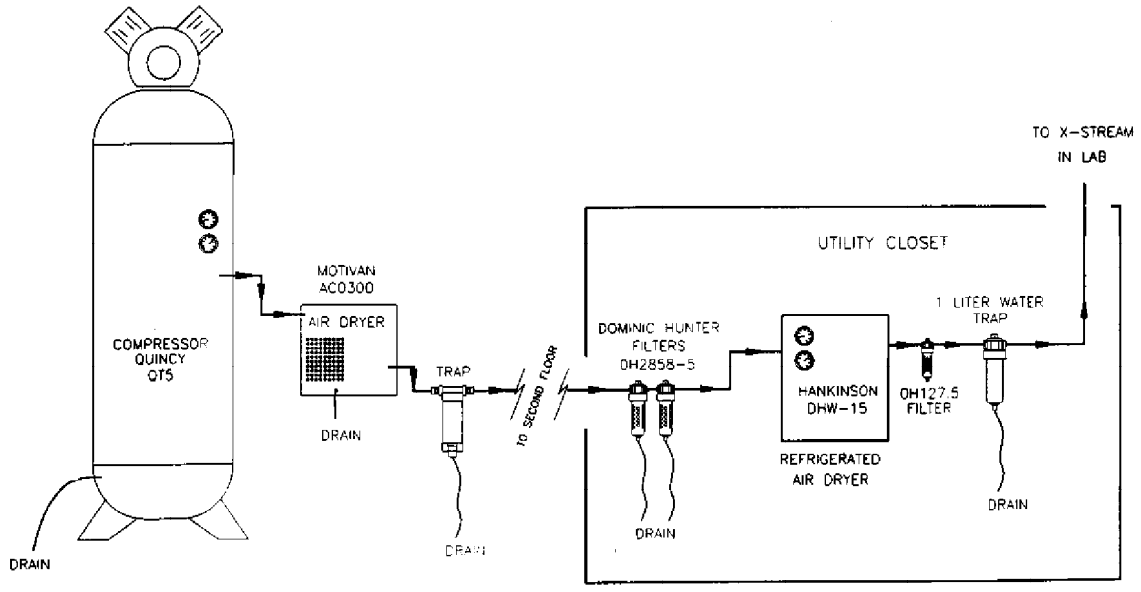

Fig 2. A schematic of the UGA compressed air system. The air supply system shown here is capable of supporting two X-stream™ systems

answered. Liters of water and oil were dumped into the compressed air supply by a malfunction of the refrigerated air dryer and filter traps located in the basement. The X-stream™ continued to function normally (T=-180°C) with no indication of internal ice buildup even as water and oil were dripping from the Hankinson air dryer located only a few feet away. Although the water trap located in the utility closet removed most of the oil and water, the water coalescing filter (the first filter in the filter bank) did show a small amount of discharge. The X–stream™ continued to operate normally under these conditions for a period of 2-3 weeks, while parts for the malfunctioning or damaged components of the air supply were replaced.

The X-stream™ has now been in continuous operation for 15 months. Both the helium compressor and heat exchanger have proven to be reliable and stable, and have required only routine maintenance during this period. The charcoal filter has been replaced every 6 months. The cap seal of the heat exchanger was replaced and the unit recharged with helium at 13 months.

The flexible stainless steel transfer tube of the X–stream™ allows one to position the nozzle assembly in a variety of orientations. In the UGA installation, the nozzle was positioned as illustrated in Figure 3. In this arrangement the angle between the X-ray collimator and the nozzle is approximately 80° and the angle between the nozzle and the phi axis of the goniometer is approximately 150°. This configuration allows one to center the tip of the nozzle assembly to within 1 cm of both the optical center of the goniometer and the end of the X-ray collimator. Positioning the nozzle assembly in this manner avoids the problems of goniometer head icing and condensation on the phi axis components. This arrangement also allows good access to the goniometer for crystal mounting and does not interfere with the optional helium beam path. Using the nozzle configuration illustrated in Figure 3, no ice formation at the crystal or on the pin was observed during data collection runs as long as 3 weeks, even during the humid Georgia summers.

Care and Feeding of the X–stream™

The X-stream™ is quite simple to operate: just feed compressed air at 150 PSI into the filter bank, turn on the control unit and set the gas flow rates and desired running temperature (Table 1) and turn on the helium

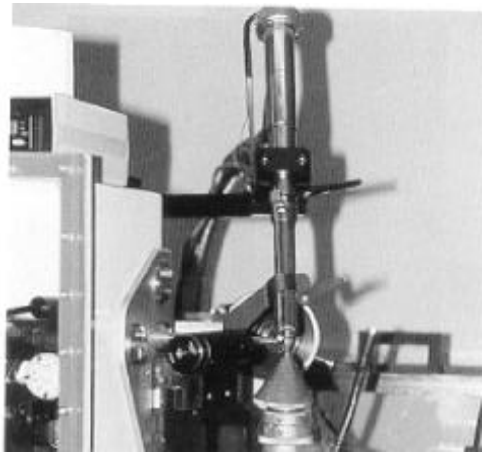

Fig 3. The position of the X-stream™ nozzle with respect to the goniometer as installed at UGA. The angle between the X-ray collimator and the nozzle is approximately 80° while the angle between the nozzle and the phi axis of the goniometer is approximately 150°. With this arrangement, ice formation has not been observed even during the humid Georgia summers.

compressor. The X-stream™ normally takes an hour to reach equilibrium at -180°C.

It is possible to generate LN2 with the X-stream™ since the heat exchanger is cooled to approximately –243°C, well below the boiling point of nitrogen. Generation of LN2 results in large fluctuations in the nozzle loop temperature and can result in ice formation on the crystal since the cold-stream flow at the crystal is erratic. LN2 formation is usually due either to setting the cold-stream flow rate too low or to problems with the compressed air supply system. Routine maintenance calls for replacing the charcoal filter every 6 months, replacing the cap seal of the heat exchanger every 12 months and adding helium as needed to maintain the operating pressures of the helium compressor at 50 (low pressure side) and 250 PSI (high pressure side).

Low Temperature Techniques

This section presents a brief overview of low temperature data collection as used with the X–stream™ at UGA. It represents a compilation of techniques described in the literature and at various meetings. Several excellent cryocrystallography reviews are available [5-8]. Also recommended is an excellent video tutorial (Cryocrystallography by Walter, Ealick, and Slaybaugh) which illustrates one of the more common low temperature crystal mounting techniques. The video may be obtain free of charge from Cornell University (contact Judy Caveny, JAC41@cornell.edu).

Table 1. Flow rates (FR) and HTL settings (°C)

Running temperature	-180	4
$FR_{cold\text{-}stream}$	40	40
$FR_{warm\text{-}stream}$	15	15
HTL_{vapor}	172	-30
HTL_{nozzle}	-180	4

Using these settings the nozzle loop heater output should remain below 20% at -180°C

The flash-cooling process can be divided into several steps: crystal preparation, crystal mounting, flash-cooling and crystal removal and storage.

a. Crystal Preparation

The first step in the flash-cooling process is determining whether the mother liquor will freeze as an amorphous glass. This can be done by freezing a small amount of the mother liquor suspended in a loop [4] and observing whether the mother liquor remains clear (amorphous glass) or turns opaque. If the mother liquor turns opaque upon freezing, cryoprotectant solutions [1, 2, 9, 10] (Table 2) may be added to the mother liquor to aid in amorphous glass formation.

In assessing cryoprotectants, it is often useful to try a range of concentrations. Experience shows that if the crystals are grown from high salt, the upper end of the cryoprotectant concentration range may work better. Once a cryoprotectant/mother liquor solution is found, its effect on the crystal should be determined. This is done by introducing the crystal to the cryoprotectant solution either by (1) growing the crystal in presence of the cryoprotectant, (2) rinsing or soaking the crystal in the cryoprotectant solution or (3) dialysis against the cryoprotectant solution. For some crystals it may be necessary to increase the

Table 2. Some common cryoprotectants [8].

Cryoprotectant	Concentration
(2R-3R)-butane-2,3-diol	8% (v/v)
Erythritol	11% (w/v)
Ethylene glycol	11-30% (v/v)
Glucose	to 25% (v/v)
Glycerol	13-30% (v/v)
Jeffamine	5-20% (w/v)
2-Methyl-2,4-pentanediol (MPD)	20-30% (v/v)
PEG 400	20-35% (v/v)
Sucrose	25% (w/v)
Xylitol	22% (w/v)

cryoprotectant concentration in steps by briefly soaking the crystals in solutions with increasing cryoprotectant concentrations.

b. Crystal Mounting

Several methods for mounting crystals for data collection at cryogenic temperatures have been described: (1) freezing the mounted crystal at the end of a glass fiber mounted on a metal pin [1-3], (2) freezing the crystal on . a small glass spatula mounted on a metal pin [11] and (3) freezing the crystal in a small fiber or wire loop mounted on a metal pin [4], Mounting crystals in loops offers several advantages over the other mounting methods. The loops are easy to make, the size can be tailored to the crystal and can be deformed to aid in orienting the crystal within the loop. Loop mounting also results in less stress on the crystal during mounting, provides a large surface area which improves the cooling rate and minimizes background and absorption effects. The loops can be made from a variety of materials including wire, hair, glass wool, rayon and nylon. However, in order to minimize diffraction from the loop, care should be used in choosing the loop material and its diameter. A loop making device is available from Charles Supper Co. (15 Tech Circle, Natick, MA 01760, (617) 237-2995). Preformed cryoloops are also available from Hampton Research (25431 Cabot Road, Suite 205, Laguna Hills, CA 92653, (909) 699-1040).

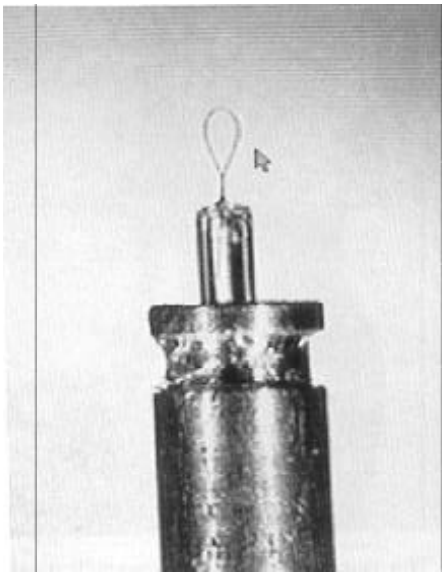

Fig 4. A Lawrence Berkeley style (LBL) loop and pin mount. The pin is secured in a metal magnetic mount. (See Fig. 5.)

The loop is usually glued to the end of a metal mounting pin (Figure 4). A variety of mounting pins have been designed (Figure 5). Several designs use a magnetic base to aid in mounting the crystal onto the goniometer. Using a pin with magnetic base has several advantages: (1) mounting the crystal on the goniometer is easier; (2) crystals can be mounted flash-cooled and stored in LN2 prior to data collection and (3) crystals can be easily removed and stored after data collection for future use. Magnetic pin mounts are ideally suited for synchrotron data collection since crystals may be screened at home for diffraction or derivative quality and stored in LN2 for shipment to the synchrotron. Mounting pins and magnetic mounts can be obtained from the Yale University machine shop, 260 Whitney Ave., New Haven, CT 06520, (203) 432-6300) (contact Ann Pardee) and from Hampton Research (25431 Cabot Road, Suite 205, Laguna Hills, CA 92653,(909) 699-1040).

c. Flash-cooling

Before mounting any crystal in the cold stream one should insure that there is a sufficient supply of LN2, for LN2-based systems, to complete the data collection. We ran into this problem with our LN2-

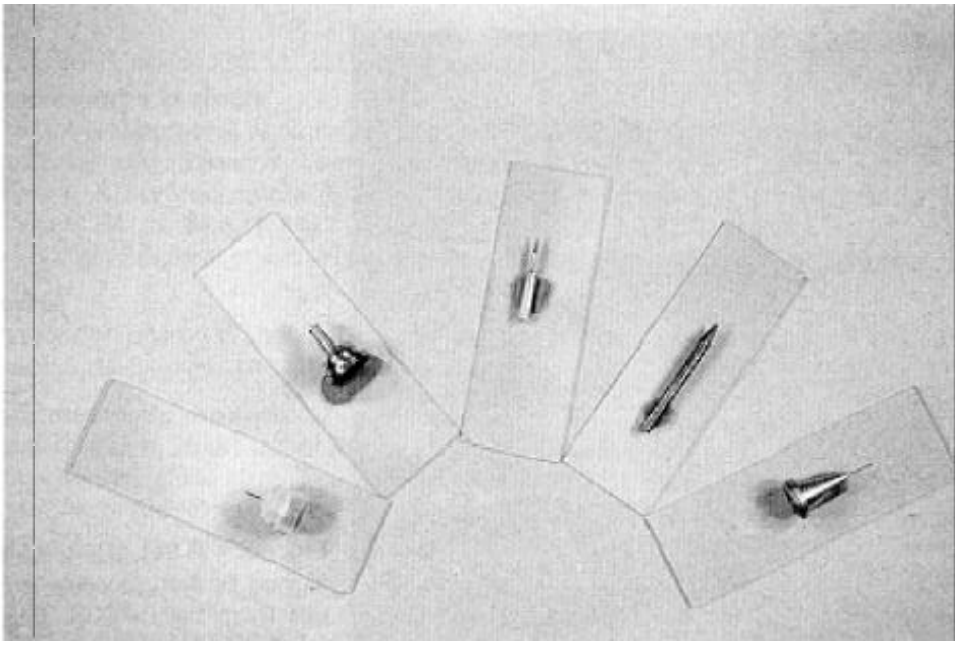

Fig. 5. A selection of mounting pins. From left to right, Hampton Research CrystalCap™, (LBL) style, Huber goniometer pin, Hope style and MSC style. We have found that the metal alignment pin which comes with the Huber goniometer head makes a very good pin for flash-cooling. The MSC pin is a prototype designed by Keith Crane.

based system since there is no LN2 delivery at UGA during Christmas break. Also, since cryogenic data sets can easily run into days or weeks, it is a good idea to check the number of hours the filament of. the X-ray generator has accumulated. This will avoid having the data set interrupted by filament failure and the necessity of removing and storing the crystal in LN2. Finally, if you are using a new or different pin-loop combination, it is a good idea to check for ice formation on the pin or loop before mounting the crystal. This can be done by freezing a loop of the mother liquor in the cold stream and doing a mock data collection overnight. Ice formation on the loop or pin may be caused by several factors, including: (1) misalignment of the cold-stream with respect to the loop, (2) inadequate cold or warm stream flow rates, (3) poor heat transfer along the pin or (4) poor heat transfer between the pin and the goniometer head. These factors should be checked if ice formation is observed. A video microscope is useful in monitoring the crystal for ice formation during data collection

At UGA all crystals are flash-cooled using the X–stream™ cold stream [3]. Another technique, in which the crystal is flash-cooled using a cryogenic liquid (LN2, propane, ethane) [1, 11], is described in more detail in the review by Rodgers [8]. For flash-cooling it is preferable to have the crystals, microscope and related equipment as close as possible to the goniometer since the crystal must be rapidly transferred from the mother liquor into the cold-stream. First the loop is optically aligned, making sure the tip of the metal pin supporting the loop is in the cold stream to prevent ice formation. Next, the loop is removed from the goniometer and is used to gently scoop the crystal out of the mother liquor. The crystal is held within the loop by surface tension. Excess mother liquor can be removed by blotting with filter paper. The cold stream is then momentarily obstructed by a thin plastic wand or microscope slide while the loop is placed back onto the goniometer. The wand or slide is quickly removed and the crystal is flash-cooled.

The transfer of the crystal to the cold stream should be done as quickly as possible to prevent evaporation of the mother liquor within the loop. Evaporation can damage the crystal leading to non-isomorphism, or result in salt crystal formation which can interfere with data collection. If evaporation is a problem,

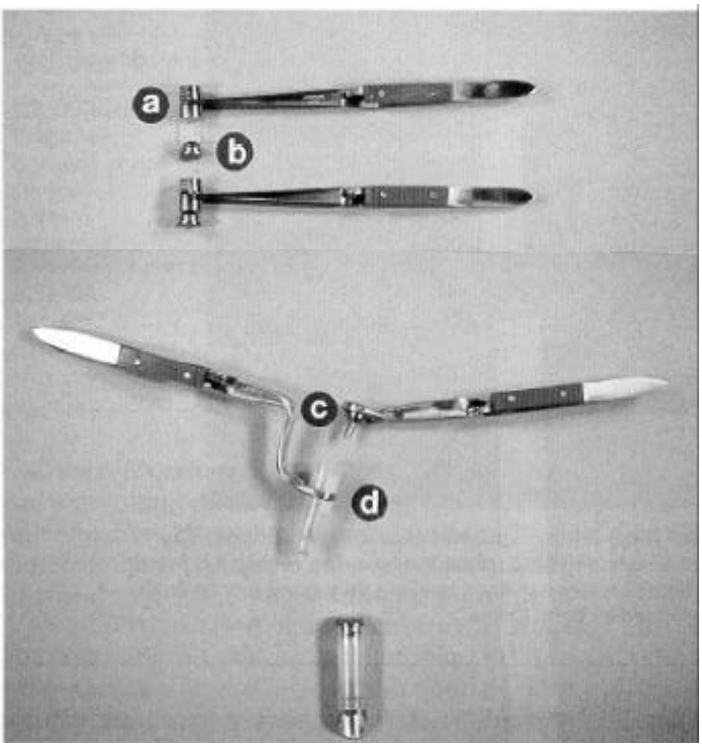

Fig 6. A set of cryotongs (LBL style) designed by Ken Goodwill for recovering crystals from the R-AXIS. The cryotongs (a) are cooled in LN2 and used to grip the pin (b). The pin is then removed from the goniometer and immersed under LN2 where a second set of tongs (c) is used to grip the base of the pin assembly. The cryotongs are removed and the pin is placed in a cryovial held by a third set of tongs (d). The cryovial containing the crystal and LN2 can now be safely transferred to the storage Dewar.

coating the crystal with oil as described by Hope [3] may help. Another problem associated with the flash-cooling technique is the increase of mosaicity which usually accompanies the freezing process. If the mosaicity of the crystal increases as a result of flash-cooling, changing the cryoprotectant [1, 2, 9] (Table 2) or its concentration may limit the increase in mosaicity. If the increase in mosaicity is due to uneven cooling, flash-cooling in a cryogenic liquid, which should provide more even cooling, may help.

d. Crystal Storage

An advantage of the flash-cooling technique is that the crystal may be recovered and stored in LN2 for future use. Crystal recovery is most easily accomplished using a magnetic mount. To recover the crystal, the crystal and pin are immersed in LN2 contained in a cryovial and removed from the goniometer. The base of the magnetic mount is usually designed to serve as a cap. The cryovial is then attached to a cane and the cane is stored in a Dewar containing LN2. For goniometers having a horizontal phi axis, a special goniometer head with an extended arc (Charles Supper Inc., 15 Tech Circle, Natick, MA 01760, (617) 237-2995) has been designed to aid in crystal recovery. For goniometers

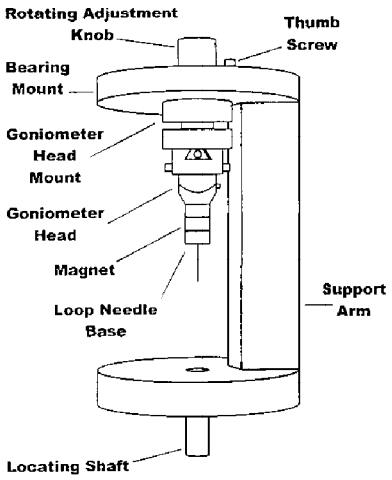

Fig. 7. A diagram of the inverted phi axis designed by Ken Goodwill [12] for recovering crystals from the R-AXIS.

having a vertical phi axis, such as the R-AXIS, recovering the crystal is not as simple and devices have been designed to aid in the recovery. One of the more common techniques for recovering crystals from the R-AXIS involves the use of special tongs

(Figure 6) having a large heat sink at the end which has been milled to grip the pin without disturbing the loop or crystal, The tongs, first described by Hope, are cooled in LN2 and used to remove the crystal from the goniometer, The tongs and crystal are then immersed in LN2 where the crystal is transferred to a.cryovial using a second set of tongs. Cryotongs may be obtained from the University of California at Berkeley, Chemistry Department machine shop (contact Erik Granlund, (510) 642-4486) and Hampton Research (25431 Cabot Road, Suite 205, Laguna Hills, CA 92653, (909) 699-1040).

In another method [12] the phi axis of the R-AXIS is replaced with an inverted phi axis (Figure 7) to aid in crystal retrieval. The crystal and pin are immersed in LN2 contained in a cryovial and removed from the goniometer for storage, The inverted phi axis may be obtained from the University of California at Berkeley, Chemistry Department machine shop (contact Erik Granlund, (510) 642-4486).

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Table 3. Suppliers.

Cryocrystallography reviews

Hope, H., Crystallography of biological macromolecules at ultra low temperature, *Ann. Rev, Biophys Biophys. Chem.*, **19**,107-126 (1990).
Watenpaugh, K. D., Macromolecular crystallography at cryogenic temperatures, *Curr. Opin. Struct. Biol.*, **1**, 1012-1015 (1991).
Rodgers, D. W., Practical cryocrystallography, *Methods Enzymol.* **276**, 183-203 (1997).

Video tutorial

Cryocrystallography by Walter, Ealick, and Slaybaugh, Cornell Unlversity (contact Judy Caveny, JAC41@cornell.edu).

Loops, pins, magnetic mounts

Hampton Research, 25431 Cabot Road, Suite 205, Laguna Hills, CA 92653, (909) 699-1040.
Yale University machine shop, 260 Whitney Ave. New Haven, CT 06520, (203) 432-6300 (contact Ann Pardee)

Loop making device

Charles Supper Inc., 15 Tech Circle, Natick, MA 01760, (617) 237-2995.

Extended arc goniometer heads

Charles Supper Inc., 15 Tech Circle, Natick, MA 01760, (617) 237-2995.

Inverted phi axis for R-AXIS goniometer

University of California at Berkeley, Chemistry Department machine shop, Berkeley, CA 94720, (510) 642-4486 (contact Erik Graniund).

Video microscope

Molecular Structure Corporation, 3200 Research Forest Dr., The Woodlands, TX 77381, (281)363-1033.

Cryovials and cryocane holders

Fisher Scientific, PO Box 360153, Pittsburgh, PA 15250, (800) 776-7000.

Cryotongs

Hampton Research, 25431 Cabot Road, Suite 205, Laguna Hills, CA 92653, (909) 699-1040.
University of California at Berkeley, Chemistry Department machine shop, Berkeley, CA 94720, (510) 642-4486 (contact Erik Graniund).

Storage Dewars

Fisher Scientific, PO Box 360153, Pittsburgh, PA 15250, 800-766-7000.
Traxair, 1545 E. Edinger Ave., Santa Anna, CA 92705, 800 225-8247.

Taylor-Wharton cryogenic shipper (model CP65S)

Traxair, 1545 E. Edinger Ave., Santa Anna, CA 92705, 800-225-8247.