STUDIES OF INSULIN CRYSTALS AT LOW TEMPERATURES: EFFECTS ON MOSAIC CHARACTER AND RADIATION SENSITIVITY*

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Communicated by David Shemin, October 12, 1966

The low-temperature X-ray diffraction studies of orthorhombic insulin citrate crystals reported here were initially undertaken because of the rapid deterioration at room temperature of certain insulin crystals containing heavy-metal cations. The effects of cooling on mosaic character and radiation sensitivity were studied. Two temperature ranges were employed: (a) below \(-150^\circ C\) and (b) \(0^\circ C\) to \(-13^\circ C\).

In normal laboratory practice orthorhombic insulin citrate crystals are grown and stored at \(1 \pm 1^\circ C\). Immersion studies in heavy-atom reagents are also made at this temperature. All X-ray diffraction work is, however, carried out at room temperature (\(~21^\circ C\)).

We have found that orthorhombic insulin citrate crystals may be cooled rapidly to below \(-150^\circ C\) without impairing the X-ray diffraction pattern. Rapid cooling does, however, enhance the mosaic character of these crystals. X-ray diffraction studies at \(0^\circ C\) and \(-13^\circ C\) have provided evidence for marked reduction in radiation sensitivity in certain heavy-atom-containing crystals.

General Background.—Although metal-free insulin crystals are somewhat more sensitive to radiation damage (nickel-filtered CuK\(\alpha\)) at room temperature than at \(0^\circ C\), the radiation sensitivity at room temperature is not marked enough to prevent the collection of intensity data by counter techniques.\(^1\) Furthermore, these crystals do not deteriorate on standing at room temperature for several weeks without irradiation.

However, after immersion (\(1 \pm 1^\circ C\)) in buffered solutions containing certain salts with heavy-metal cations (including uranyl) insulin crystals deteriorate rapidly at room temperature (sometimes within 1 day or less of exposure) after only the minimal radiation exposure (minutes) necessary to monitor the state of the crystal. The deterioration has been observed both in the presence and in the absence of significant changes in X-ray diffraction intensity distribution after immersion. It is not markedly accelerated by continuous X-ray irradiation. This deterioration was originally attributed to greatly enhanced radiation damage, as laboratory practice avoids keeping the crystals at room temperature prior to X-ray photography. Some apparent enhancement in radiation damage is also observed with a second group of metal-containing crystals which do not show the striking and rapid room-temperature deterioration reported above.

Before the deterioration in the first class of crystals was recognized as largely of thermal origin, studies were made of both classes of metal-containing crystals into which radiation protectors had been introduced. These provided marginal evidence of protection. There was no evidence of protection when the crystals were studied under oxygen-free conditions.

In order to collect X-ray data from the crystals which deteriorate rapidly at room temperature, it would have been necessary to build an apparatus to maintain...
them at approximately 1°C. The use of data collected at different temperatures in isomorphous replacement techniques would present serious problems. Cooling may affect both lattice and thermal parameters and cause structural changes. Therefore, we decided to investigate the general advantages and disadvantages of low-temperature X-ray diffraction studies with insulin crystals.

*Studies at Temperatures below −150°C.*—The effects of cooling crystals to temperatures below −150°C were first investigated. Such intensive cooling should provide maximum radiation protection effects for all types of crystals. The crystals used in this study were metal-free crystals or crystals grown from silver nitrate-containing solution which showed no changes in intensity distribution. The crystals, which contain no organic solvent, were wiped free of adhering mother liquor and mounted in a special cell with mylar windows (thickness 0.15 mil). The cell contained a droplet of mother liquor some distance from the crystal. Still photographs (1-min exposure) were taken of the crystal on a precession camera using Polaroid ASA 3000 film with an intensifying screen, as described by Smith, in a modified film holder. After photography of the crystal at room temperature, the cell was dipped into liquid nitrogen and photographed while a stream of liquid nitrogen flowed over it. The temperature within 2 mm of the crystal position was measured with a thermocouple. It was lower than −150°C, and on cooling reached this temperature in less than 1 min. As shown in Figure 1b, there was an increase in mosaicity of the crystal on cooling which was enhanced after rapid rewarming to room temperature, Figure 1c, and further enhanced on rapid recooling, Figure 1d.

Some ice diffraction pattern was always observed which we attribute to condensation on the mylar film. When it was very heavy, suggesting an accidental wet mounting, the diffraction pattern after warming up was slightly less intense than before cooling. It may be noted in the figure that the crystal orientation changed slightly in the cold, but returned to its original position on warming. This is probably caused by tension in the mylar film.

The mosaic character of these protein crystals after cooling precludes their use for accurate intensity data collection. To us, it appears probable that the enhancement of mosaic character is largely the result of thermal strain on rapid cooling, rather than primarily the disruptive effect of ice crystallite formation between protein crystalline domains. Such enhancement is the well-known consequence of dipping crystals into liquid air.

We do not have sufficient evidence to distinguish between these two possible causes. It should be noted that the original crystal gave a sharp still. On cooling it showed evidence of a mosaic spread of ~0.5–1° and on rewarming a mosaic spread of about 1.5°, presumably here enhanced by the ice crystallites which must form on rewarming. After two cooling-warming cycles, the diffraction pattern from these crystals faded appreciably. In another experiment where a protein crystal enclosed with a film of aqueous solution in a sealed capillary was cooled slowly until the surrounding droplet froze (−20°C), both ice diffraction pattern and a much weakened protein diffraction pattern were present in the cold; the protein diffraction pattern weakened further when the crystal was warmed up. Thus, in the certain presence of external ice crystals, disorder effects in the protein lattice do occur.

The rapid cooling procedure employed in these experiments would reduce the size of ice crystals formed on cooling, the temperature at which the crystals were
maintained (below $-129^\circ$) would totally inhibit spontaneous recrystallization and the presence of the protein might retard crystallization velocities.

Our hypothesis that thermal strain was the prime cause of the enhanced mosaic character suggested the need for slower cooling rates, but these would increase the size of any ice crystals formed. Before accepting this conflict as irresolvable, we attempted first to introduce into the insulin crystals alcohols and glycols which retard crystallization velocities and might permit slower controlled cooling, at least in certain temperature ranges; and second, to prepare a cross-linked insulin crystal using the glutaraldehyde cross-linking reagent developed by Quiocio and
Richards. The first experiment failed because these solvents themselves increase the mosaic character of insulin crystals. The second failed because the glutaraldehyde disordered the structure within the crystalline domains, although it did not increase the mosaicity and did harden the crystal. With other cross-linked protein crystals, slow cooling within certain ranges might well permit studies to be made at these low temperatures.

Efforts to reach very low temperatures as a means of affording maximum protection from both thermal and radiation damage were therefore abandoned and studies were then made of the effectiveness of relatively minor temperature reductions.

*Studies at Room Temperature, 0°C, and −13°C.*—The crystals used in this study were heavy-atom-containing crystals which deteriorate rapidly at room temperature. All crystals were mounted in thin-walled glass capillaries by the normal procedure in the cold room, except that, with adequate insulation for the crystal, one end of the capillary was sealed in a cold flame rather than with wax to avoid turbulence in the cooling gas stream from the protruding drop of wax.

The photographs were taken on a Nonius Weissenberg camera with a cooling attachment, using copper radiation (40 kv 25 ma) and Ilford film. The temperature near the crystal (2 cm) was measured by means of a copper constantan thermocouple, the wires of which are led through the Dewar tube. A cylindrical piece of mylar was used as an extension of the Dewar cylinder to guide the cooling nitrogen stream over the capillary. With this arrangement, condensation was avoided on both the capillary and mylar tube. The crystals were cooled and irradiated continuously and standard-exposure oscillation photographs were taken at periodic intervals. In order to maintain flow conditions when photographs were not being recorded, the layer-line screen was placed close to the crystal and the cassette replaced by a lead shield.

For studies at room temperature, the crystals were allowed 15 min to establish thermal equilibrium after they were brought out of the cold room. They were then mounted on the goniometer head and preliminary alignments made with the polarizing microscope. For studies at low temperature, the crystals were mounted on the goniometer head and aligned optically inside the cold room. They were then rapidly transferred (<10 sec) to the precooled camera.

The onset of deterioration was defined as evident reduction (visually determined), however slight, in the over-all intensity of the diffraction pattern. Complete deterioration was defined by absence of the diffraction pattern.

Although the lowest temperature investigated (∼−13°C) is below the melting point of the immersion medium (∼−3°C) and therefore presumably below the melting point of the liquid of crystallization, it is above the critical nucleation temperature (∼−25°C) of the immersion medium as experimentally determined.

All the crystals studied at 0°C which had not deteriorated completely at the end of the experiment did so in a few hours when warmed to room temperature without further X-ray irradiation. The crystal which showed no deterioration after 47 hr continuous X-ray exposure at −13°C gave no diffraction pattern after 2 additional hr at room temperature without irradiation. This crystal, however, showed some evidence of a normal thermal parameter. It was accidentally permitted to warm up to −10°C between 40 and 43½ hr. The pattern which diminished slightly at −10°C was completely restored on recooling to −13°C.
TABLE 1

CHANGES IN DIFFRACTION PATTERN OBSERVED FOR CRYSTALS CONTINUOUSLY IRRADIATED AT DIFFERENT TEMPERATURES

<table>
<thead>
<tr>
<th>Crystal*</th>
<th>Temperature (°C) †</th>
<th>Exposure Time (hr)</th>
<th>Unchanged</th>
<th>Diminished</th>
<th>Disappeared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg</td>
<td>21</td>
<td></td>
<td>8</td>
<td>...</td>
<td>21</td>
</tr>
<tr>
<td>Hg</td>
<td>21</td>
<td></td>
<td>2.5</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Hg</td>
<td>0</td>
<td></td>
<td>35</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Hg</td>
<td>0</td>
<td></td>
<td>25</td>
<td>42</td>
<td>&gt;72</td>
</tr>
<tr>
<td>UO₂a</td>
<td>21</td>
<td></td>
<td>...</td>
<td>6.5</td>
<td>12</td>
</tr>
<tr>
<td>UO₂b</td>
<td>21</td>
<td></td>
<td>...</td>
<td>...</td>
<td>16</td>
</tr>
<tr>
<td>UO₂c</td>
<td>21</td>
<td></td>
<td>...</td>
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<td>...</td>
</tr>
<tr>
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<td></td>
<td>29</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>UO₂e</td>
<td>0</td>
<td></td>
<td>27</td>
<td>38½</td>
<td>...</td>
</tr>
<tr>
<td>UO₂f</td>
<td>0</td>
<td>8½</td>
<td>21½</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>UO₂g</td>
<td>21</td>
<td></td>
<td>...</td>
<td>...</td>
<td>18</td>
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<td>...</td>
<td>18-20</td>
<td>...</td>
</tr>
<tr>
<td>UO₂i</td>
<td>-13</td>
<td>47</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

* The crystals are here identified in terms of the metal cations which were in the diffusion medium. UO₂ with superscripts a, b, and c, represents three different preparations.
† As the thermocouple was 2 cm from the crystal in the capillary, the temperatures cited are not those at the crystal. The absolute error will therefore be greater at the lower temperature. The fluctuations in the temperature were rarely greater than ±3°C.

The results of these studies (Table 1) show that the crystals deteriorate less rapidly under constant irradiation at 0° than at room temperature. These metal-containing crystals deteriorate at room temperature without irradiation virtually as rapidly as under constant irradiation. This contrasts with the normal metal-free crystals, which deteriorate rapidly at room temperature only as the result of radiation damage. Cooling of the metal-containing crystals to 0° (compare storage temperature 1 ± 1°C) must eliminate thermal damage. Moreover, cooling these crystals to 0° has effectively protected most of them from radiation damage for periods longer than those observed for metal-free crystals at room temperature.

At −13°C, the period before onset of radiation damage is considerably longer than that for normal metal-free crystals at room temperature. These qualitative studies provide clear evidence of enhanced radiation protection which may be achieved by cooling, even though the absolute temperature reduction is very small. Quantitative studies have been made of the effects on the diffraction patterns of crystals kept at temperatures in the range of 21°C to −13°C. These will be reported in detail elsewhere.  

* This investigation was supported in part by U.S. Public Health Service research grant ROI-AM-01320 from the National Institute of Arthritis and Metabolic Diseases; in part (B. W. L.) by a U.S. Public Health Service Research Career Award, 5-K3-GM-15,246; in part by a U.S. Public Health Service fellowship (J. E. B.) 1-F3-GM-19,813; and in part by a U.S. Public Health Service fellowship (J. F. P.) 4-FL-GM-13,858.
3 Low, B. W., unpublished studies.
5 Quiñcho, F. A., and F. M. Richards, these PROCEEDINGS, 52, 833 (1964).