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X-ray studies on lysozyme crystals at − 50°C.

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Cross-linked orthorhombic lysozyme crystals were transferred to 50% glycerol, and X-ray photographs taken with the crystals cooled to −50°C. 9° precession photographs taken with the crystal at room temperature and −50°C showed only small intensity differences.

The cross-linking of lysozyme crystals (Haas, 1968) has permitted numerous X-ray experiments to be performed which otherwise were impossible (Quiocho & Richards, 1966). The series of low temperature experiments presented here was possible only through the use of this technique. Studies similar to those of Low, Chen, Berger, Singman & Pletcher (1966) were performed in order to determine the effect of below-zero temperatures on cross-linked lysozyme crystals.

Large orthorhombic lysozyme crystals (needles with ½ mm diameter), grown in 2% sodium chloride at *p*H 9·5 (Steinrauf, 1959), were cross-linked by the following two alternative procedures. Fully cross-linked crystals were obtained by slowly adding glutaraldehyde to washed crystals (4% sodium chloride) until a concentration of 12% was obtained. The rate of addition of glutaraldehyde was about 2% a day with the *p*H maintained around 8 by a sodium acetate buffer. Partially cross-linked crystals were prepared by placing native crystals in 1% glutaraldehyde for 6 hours. Crystals prepared in this way were insoluble in water, but when cut open their interiors were found to be quite soluble. Presumably the glutaraldehyde requires much more than 6 hours to diffuse into the crystal interior, indicating that the crystals were cross-linked primarily near the surface.

Reaction of the lysines with glutaraldehyde during cross-linking produces a limited amount of disorder in the crystals. Fully cross-linked crystals show a greatly increased fall-off of X-ray diffraction intensities with $\sin\theta$ such that the diffraction pattern disappears at about 3 Å resolution. Even though some individual intensities show considerable changes, the overall diffraction pattern remains unchanged except for the increased fall-off. However, surface cross-linked crystals give a diffraction pattern identical with that of native crystals.

Ice formation is the main problem in cooling protein crystals much below 0°C, both in the mother liquor and within the crystals. This problem was overcome by using water–glycerol mixtures which formed a glass upon freezing. This mixture gives little or no diffraction pattern in the solid state and caused no apparent movement of the room-temperature-aligned crystals. While native lysozyme crystals dissolve when as little as 10% glycerol is added to the mother liquor, both types of cross-linked crystals remain unchanged in salt-free 50% glycerol. The fully cross-linked crystals showed no intensity changes due to the substitution.

The protein crystals were mounted near the sealed end of 1 mm diameter glass capillaries. Liquid was left at the other end, and the capillary was sealed with wax. Cold gas, obtained by boiling liquid nitrogen, was delivered through a 3 mm glass tube coaxial with the capillary. Surrounding this tube was a second tube delivering dry air to prevent ice formation. A measure of the temperature was obtained by placing a thermocouple at the position of the crystal. The working temperature was −50°C, well below the freezing point of the water–glycerol mixture. A comparison of precession photographs taken before cooling, at −50°C, and after returning to room temperature showed only small differences. The observations were made both on 'still' setting photographs and 9° precession photographs (Fig. 1).

Analogous experiments were performed with the surface cross-linked crystals. Even though these crystals are not disordered by the cross-linking, no change or improvement of the diffraction pattern was observed at low temperatures.

The lack of improvement of the diffraction pattern at low temperatures suggests that the high rate of fall-off in protein crystals is due to disorder in the packing of the molecules (or in the molecules themselves) and not thermal motion. However, disorder produced by the freezing solvent cannot be ruled out as a cause for the lack of improvement.

This freezing technique for protein crystals may be of some practical value in order to hold unstable intermediates in enzyme crystals as well as to prolong the useful X-ray life of frozen surface cross-linked protein crystals (Low *et al.*, 1966; Haas & Rossmann, 1968).

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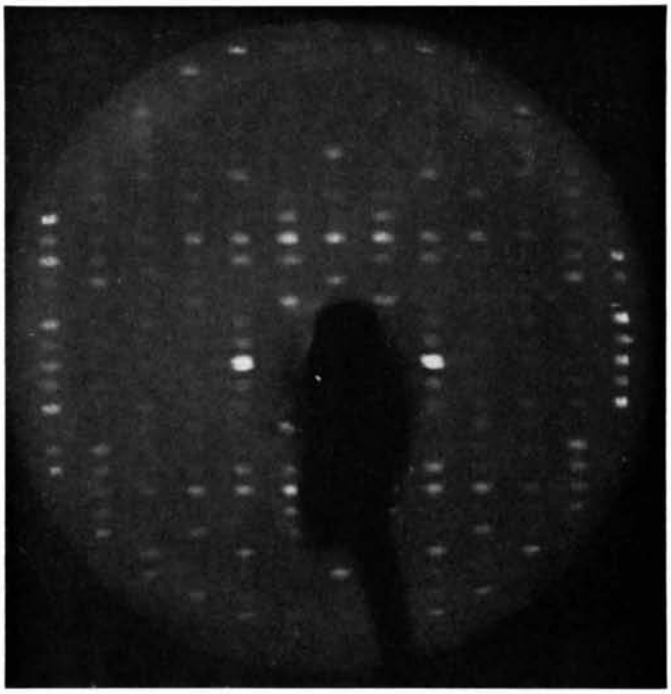
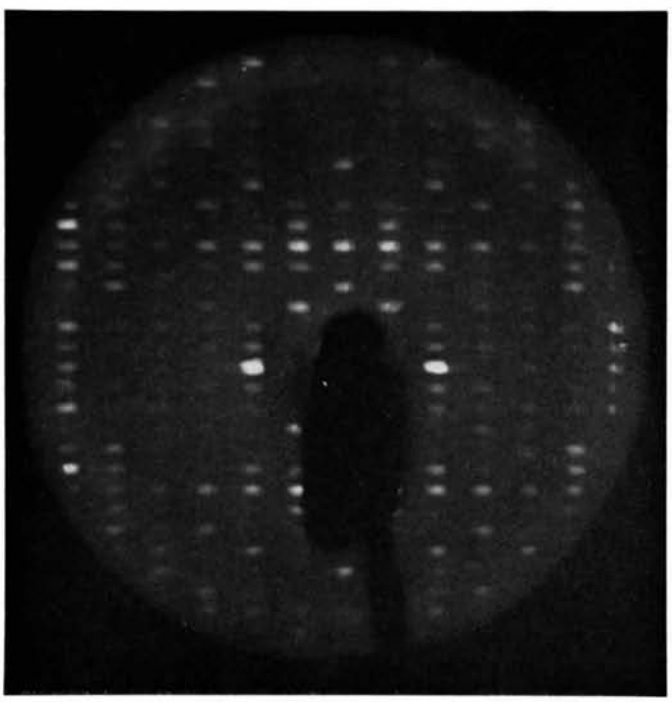


(*a*)



(*b*)

Fig. 1. (*a*) 9° precession photograph of fully cross-linked orthorhombic lysozyme at −50 °C. (*b*) Same as (*a*) after warming to room temperature.

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