Insecticyanin, a blue biliprotein from the tobacco hornworm Manduca sexta, has been crystallized in a form suitable for a high resolution x-ray analysis. The crystals grow by vapor diffusion against solutions of polyethylene glycol 8000 at pH 5.5. They belong to the space group P4₁2₁2 or P4₂2₂ with unit cell dimensions of \( a = b = 115.0 \, \text{Å} \); \( c = 71.1 \, \text{Å} \). Insecticyanin is believed to be a tetramer in solution; there are two subunits per asymmetric unit. The crystals diffract to at least 2.2 Å resolution and appear reasonably resistant to radiation damage.

Insecticyanin is a blue biliprotein found in the hemolymph and integument of the tobacco hornworm Manduca sexta. In conjunction with yellow pigmentation provided by carotenoids, it is responsible for giving these insect larvae their green color (Kawooya et al., 1985) and as such is an important component of their camouflage system. Insecticyanin is produced only during the larval life stage of the insect (Riddiford, 1982). However, it persists throughout the pupal stage and into the female hemolymph from which it is ultimately sequestered into the egg (Cherbas, 1973). The protein was first isolated and characterized by Cherbas (1973). Results from his work led to the proposal that biliverdin IX₇, the chromophore responsible for giving insecticyanin its intense blue coloration. The chromophore is tightly bound to the protein and can only be removed under denaturing conditions such as treatment with formamide (Cherbas, 1973) or guanidine hydrochloride (Riley et al., 1984). It is not known whether the strong interaction between the chromophore and protein is due to a labile covalent bond or to noncovalent forces (Schoenleber et al., 1983).

Since the original work of Cherbas, an improved purification scheme has been developed and the amino acid sequence has been determined (Riley et al., 1984). From these studies, which included chemical cross-linking experiments, gel permeation chromatography, and sedimentation studies, it has been shown that insecticyanin is a tetramer of four identical subunits. Each "apo-" subunit contains 189 amino acid residues of total \( M_r \) = 21,378 and two disulfide bridges.

In light of the central role that insecticyanin plays in the camouflage system of the tobacco hornworm larvae and its remarkable stability during insect metamorphosis, we have initiated a structural study of this protein. In this paper we report the crystallization of insecticyanin in a form suitable for a high resolution crystallographic structure determination.

**EXPERIMENTAL PROCEDURES**

Insecticyanin was isolated from the fifth instar M. sexta using the procedure of Riley et al., 1984. The protein was stored in 150 mM NaCl, 50 mM Tris-Cl, pH 7.5 at 4°C until required. Crystals were grown at room temperature by vapor diffusion using either the hanging drop or sitting drop method (for a review of crystallization techniques, see McPherson, 1982). Typically for hanging drop experiments, 10 μl of protein solution were mixed with 10 μl of 20-25% w/v, polyethylene glycol 8000 on silanized glass coverslips. These coverslips were then suspended over wells containing the same polyethylene glycol solution and allowed to equilibrate for a period of several weeks. The protein concentration was at 6 mg/ml in 10 mM potassium succinate, pH 5.5, and the polyethylene glycol was buffered with 50 mM potassium succinate, pH 5.5. Crystals grew as long intensely blue needles with well developed faces and edges and typical dimensions of 0.3 × 0.3 × 1.0 mm (Fig. 1).

To determine the number of molecules of insecticyanin per asymmetric unit, measurements of crystal density were made using a bromobenzene:xylene density gradient column calibrated with droplets of potassium iodide solutions of predetermined density (Low and Richards, 1952). For x-ray diffraction experiments, the crystals were sealed in thin-walled quartz capillary tubes (Charles Supper Co., Natick). X-ray diffraction photographs were recorded using nickel-filtered copper Kα radiation from an Elliot GX20 rotating anode x-ray generator operated at 35 kV and 40 mA with a 200-μm focal cup. The exposure time was typically 20 h for a 10° precession photograph.

**RESULTS AND DISCUSSION**

Crystals of insecticyanin have been known for many years. They were first grown by Cherbas (1973) using 30% ammo-
nium sulfate as the precipitant. We have since grown crystals from either ammonium phosphate or a combination of sodium and potassium phosphate. These crystals, which belong to the orthorhombic space group P2₁2₁2₁, possess a formidable large unit cell with a = 82.3 Å, b = 137.0 Å, and c = 313.0 Å. Moreover, the measured density of these crystals, 1.22 g/cm³, is consistent with 12 subunits in the asymmetric unit for which the calculated density would be 1.216 g/cm³ (Matthews, 1968). Assuming a subunit molecular weight of 21,900 for the holoprotein, the volume/unit molecular weight in the unit cell (Vₚ) is 3.3 Å³/Da. This lies within the normal range (1.68–3.53 Å³/Da) observed for globular proteins (Matthews, 1968). Consequently, the size of the unit cell and the large number of molecules in the asymmetric unit have discouraged structural studies on this protein. Thus, we undertook a search for other crystallization conditions that might yield a crystal form with fewer molecules in the unit cell.

Crystals of insecticyanin have now been grown from polyethylene glycol at pH 5.5. The quality of the crystals is dependent on the pH and ionic strength; above pH 6.5 and at very low ionic strength, the crystals grow as extremely thin needles. The molecules crystallize in the space group P4₁2₁2 or P4₃2₁2 with unit cell dimensions a = b = 115.0 Å; c = 71.1 Å. The crystals show well developed ((100) and (010)) faces with the 4-fold screw axis oriented along their length (Fig. 1).

The observed density of the crystals, 1.17 g/cm³, is consistent with two subunits of insecticyanin in the crystallographic asymmetric unit for which the calculated density would be 1.17 g/cm³. Assuming two subunits/asymmetric unit, Vₚ has a value of 2.68 Å³/Da.

There is some discrepancy in the literature as to the quaternary structure of insecticyanin. One study concludes that the protein is a trimer (Goodman et al., 1985). On the other hand, the studies of Riley et al., 1984, suggest that insecticyanin is biologically active as a tetramer. Our results of two subunits per asymmetric unit is consistent with insecticyanin being a tetramer and positioned in the unit cell with one of its molecular 2-fold axes coincident with the crystallographic dyad. However, if there were three subunits of insecticyanin per asymmetric, then Vₚ would be 1.78 Å³/Da which is still within the range typically observed for globular proteins although at the extreme low end. Furthermore, a value of 1.78 Å³/Da for Vₚ requires an unusually tight packing of the molecules within the crystalline lattice. Also, the calculated density for a trimer in the asymmetric unit would be 1.249 g/cm³ which is not consistent with our observed density measurements. Although the evidence points to insecticyanin being a tetramer, we cannot at this point rule out the possibility that it is a trimer. This question of the quaternary structure of insecticyanin will be resolved once electron density maps have been calculated and studied.

The crystals of insecticyanin are robust and well suited for x-ray data collection. Reflections at 2.2 Å resolution can be clearly seen on still photographs after a 30-min exposure at 35 kV, 40 mA. Fig. 2. A and B shows 10° precession photographs of the hk0 and h01 zones. In addition, the crystals are insensitive to radiation damage. Typically they still diffract to better than 3 Å resolution after 48 h in the x-ray beam at room temperature. Data collection from this crystal form using the oscillation method is in progress together with a search for isomorphous heavy atom derivatives.

One of the interesting aspects of insecticyanin is the strong attachment of the chromophore to the protein and the remarkable stability of the holoprotein during the radical restructuring that accompanies insect metamorphosis. Also, it is an essential ingredient in the cryptic coloration of insects. The three-dimensional structure of insecticyanin will yield insight into the extreme stability of the protein as well as into the strategies and regulation of color mimicry among the insects. Furthermore, in conjunction with biochemical data, the insecticyanin structure may provide a rational basis upon which to design insect control strategies.

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Fig. 2. 10° precession photographs. A, hk0 zone; B, h01 zone. Both photographs were recorded using nickel-filtered copper K, radiation from an Elliot GX20 rotating anode x-ray generator operated at 35 kV, 40 mA with a 200-μm focal cup. Exposure time was 20 h.