THE CYANOBLAST: HEMOCYANIN FORMATION IN *LIMULUS POLYPHEMUS*

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**INTRODUCTION**

Hemocyanin, the copper-containing respiratory pigment of many molluscs and arthropods, including the horseshoe crab, *Limulus polyphemus*, has been the subject of numerous and diverse studies, many undoubtedly stimulated by the exotic blue color of this blood protein. *Limulus* hemocyanin received early attention when its molecular weight was determined by the newly developed technique of ultracentrifugation (7, 23). Some of the first efforts of biological electron microscopists were likewise directed toward *Limulus* hemocyanin (4, 22) in an effort to discern the shape of individual molecules. Several reviews (5, 13, 14, 21) detail the broad spectrum of biochemical, physiological, and immunochemical studies on the hemocyanins. The macromolecular structure of *Limulus* hemocyanin has been described by the biochemical work of Printz (19, 20) and Bancroft
et al. (1), and by the ultrastructural studies of Levin (17), Fernández-Morán et al. (11), and van Bruggen and associates (2, 3).

Although hemocyanins, with a maximal recorded molecular weight of 10,000,000, constitute the largest known protein molecules (excluding the coat of some viruses), no information on their origin is available. A series of incidental observations, made over a period of several years in connection with studies of the visual system (8, 10), have revealed a new blood cell type, the “cyanoblast” (9). This paper describes its role in the formation and release of hemocyanin in Limulus polyphemus.

MATERIALS AND METHODS

The compound eyes of animals of 5, 10, and 20 cm prosomal width were fixed in several fixatives, the best consisting of 1% glutaraldehyde, 3.5% formalin, 3% NaCl, and 3.5% sucrose in 0.1 M phosphate buffer. The tissue was briefly washed in buffer with 8% sucrose, osmicated, and embedded in Araldite. Standard staining techniques were applied to thick and thin sections.

Hemocyanin was purified by the method of Malley et al. (18). A suitable dilution of this preparation was negatively stained with 1% phosphotungstic acid. Electron microscopic magnifications were calibrated with crystals with a 25 A periodicity prepared by the method of Labaw (15).

RESULTS

The hemocoelic fluid of Limulus contains freely dispersed hemocyanin and many granular hemocytes; the function of these cells in the clotting process of the blood has been documented in detail (6, 16). In addition, a second circulating cell type has been found and named the cyanoblast, in analogy to the vertebrate erythroblast. Cyanoblasts are very rarely found in the general circulation. However, the sinusoidal spaces pervading the neural plexus of the compound eye seem to trap these cells, particularly those of an advanced stage and large size. The proportion of cyanoblasts in this region varies from a norm of less than 1% to a singular instance of 8% of the circulating blood cells. Cyanoblasts have always been found to lie within the hemocoelic space and not underneath the adjacent thick basal lamina. Hence, these cells do not originate in situ despite their occasional equivocal location with respect to the adjacent vascular tissue (Figs. 2, 3).

The youngest recognizable cyanoblast (Figs. 1, 4, and 5) is about 8 ″ in diameter, with basophilic cytoplasm that when viewed in the electron microscope contains abundant free ribosomes; a few cisternae of the endoplasmic reticulum in continuity with the circumnuclear cisterna, both often engorged with a dense granular matrix; a small Golgi system; and a number of mitochondria. The nucleus, with a diameter of about 5 ″, fills most of the cell. An occasional hemocyanin crystal is an unmistakable indication of this cell type.

With the presumptive start of synthetic activity at a high rate, the cell grows to about 30 × 15 ″ and develops a correspondingly large nucleus (10 ″). At this stage, hemocyanin molecules begin to accumulate singly and in numerous crystalline arrays. Helical polyribosomes (16–22 n), though rare, are found attached with some regularity to the faces of growing crystals (Fig. 7).

The hemocyanin crystals are composed of hexagonally packed, hollow-appearing cylinders with a diameter of about 190 A and a center-to-center spacing of 260 A (Fig. 6). Longitudinal stacking of the hemocyanin molecules comprising the cylinders is evident in a 100 A transverse periodicity (Fig. 7). Thin interconnecting bridges between adjacent cylinders are visible in cross-sectioned crystals. Complex moiré patterns appear in crystals cut at an acute angle to the primary crystalline planes. This phenomenon probably means that a greater geometrical complexity exists in the relative orientation of hemocyanin molecules and subunits in the crystals than is apparent in ordinary electron micrographs. The lattice usually contains many lacunar defects (Fig. 6). Immature cyanoblasts commonly contain numerous small crystals, which subsequently grow and fuse into a few larger bodies. This coalescence is evidenced as nonconformities (Fig. 6) in the symmetry of packing in larger crystals, one of which will occasionally come to fill the entire cell. Continued growth of the cyanoblast and accretion of the crystals lead ultimately to a very large cell, which could now be called a cyanocyte; it is totally filled with hemocyanin except for narrow interstices, a few peripheral mitochondria, and the nucleus (Figs. 3, 7). Its nucleus maintains a normal, nonpycnotic appearance until the moment of cell disruption, but in rare instances it builds up sizable (5 ″ long) intranuclear crystals of hemocyanin. Cyanocytes approach 100 ″ in maximal extent, and single crystals 40 × 15 ″ in section have been observed. The terminal cyanocyte is the most frequently encountered stage, since its size
causes it to become trapped in the sinusoidal spaces of the plexus. At this point, the cyanocyte ruptures and liberates its cytoplasm and hemocyanin crystals into the hemocoel. Single or small groups of free-floating crystals are regularly encountered in the vascular spaces of the compound eye (Fig. 8). The crystals are degraded by the dispersal of hemocyanin molecules at the ends of crystal columns into the hemolymph.

Several molecular forms of hemocyanin are present in the hemolymph (Fig. 9). In a negatively stained preparation, the largest of these (56S) presents two views, presumably the projections of a hollow-appearing cylinder 190A in average side diameter with a wall thickness of 75 A. In side view, the molecule is composed of two 80 A tiers with a slight separation, resulting in an over-all height of about 180 A and a width of 190–220 A, the greater width possibly due to deformation during drying in the staining material. Three molecular subunits, formed by dissociation of the
FIGURE 4 Very young cyanoblast. Cisternae of the endoplasmic reticulum and nuclear envelope are filled with an opaque substance. A small hemocyanin crystal is present. Granules in the extracellular space in this and the following pictures are individual hemocyanin molecules. Scale, 1 μ. X 16,000.

FIGURE 5 Cytoplasm of a slightly more advanced cyanoblast than shown in Fig. 4. Granular material is still contained in the lumen of the endoplasmic reticulum, but the granules are much smaller than the hemocyanin molecules in the adjacent crystals. Scale, 1,000 A. X 44,000.
Cross-section of hemocyanin crystals in an advanced cyanoblast. Numerous crystalline lattice defects in the form of lacunae and nonconforming planes of fusion are present. Scale, 1,000 Å X 43,000.
FIGURE 7 A mature cyanocyte packed solidly with hemocyanin crystals (compare with Fig. 3). The upper inset shows a polysome attached to the growing end of a crystal. The lower inset demonstrates the appearance of columns of stacked molecules in the crystal. Scale, 1,000 Å, × 45,000; upper inset, × 80,000; inset × 120,000.
Figure 8  Free-floating hemocyanin crystals in the hemocoel. Peripheral molecules or column of molecules are in the process of dispersing into the hemolymph. Scale, 1,000 A. × 80,000.

Figure 9  Negatively stained preparation of highly associated Limulus hemocyanin at pH 6.8. The 56S component appears as a 190 A ring (a) in surface view, or as a double-tiered structure (b) in side view. The smallest subunit (16S) is seen as a single small square (c), in its dimeric (32S) form (d), or as a tetramer (3S; e). The 3S subunit more commonly has the configuration of two squares attached to an indistinct bar (f). Scale, 1,000 A. × 210,000.
largest molecules, are visible. The smallest of these (16S) presents a square profile 75 A on an edge and often appears in a dimeric form (24S) of two squares joined on edge. The largest subunit configuration (35S) is visible either as a tetradic structure 190 A in diameter, composed of four joined squares, or more commonly as the dimeric unit joined to an indistinct, broad bar.

**DISCUSSION**

The identity of the described protein crystals in the cyanoblast as hemocyanin is assured by the dimensional congruence of the crystalline substructure with the dimensions of isolated hemocyanin purified to meet immunological requirements. A second protein, namely a hemagglutinin present in *Limulus* blood (12), has a cylindrical shape similar to that of the 56S form of hemocyanin, but its diameter (100 A) is about half that of hemocyanin.

The morphology of the cyanoblast is typical of an active protein-synthesizing and storing cell, with the possible exception of the transitory appearance of some metabolic products in the lumen of the endoplasmic reticulum and circumnuclear cisterna at the onset of synthetic activity. Abundant free ribosomes and inconspicuous membrane systems of the cell give the cell the appearance of an erythroblast. The apparent adhesion of occasional polyribosomes to the presumptive growing face of a crystal suggests a tendency of the hemocyanin to aggregate even before the complete polymeric molecule has assembled. Hemocyanin is dispersed in the cell between ribosomes before the formation of crystals, but the increase in size of the cell and crystals requires continuing synthesis rather than only aggregation. The appearance of intranuclear crystals can most reasonably be interpreted as indicating intranuclear synthesis rather than massive diffusion of the very large hemocyanin molecules through nuclear pores.

The site of origin of the cyanoblast is unknown, although several lines of evidence point to the hepatopancreas as the principal organ involved in hemocyanin metabolism. In crustaceans, in which ordinarily 90% of the copper is contained in circulating hemocyanin, this amount decreases to near zero during molting. About half of the withdrawn copper is stored in the hepatopancreas, the remainder being secreted (24). In *Octopus*, more than 75% of the total copper is stored in the hepatopancreas. Hence, it is quite possible that the cyanoblasts in *Limulus* originate in the hepatopancreas and that their varying abundance is related to the molting cycle. The greatest incidence of cyanoblasts was recorded in July, coinciding with the molting time of *Limulus*.

The correlation between the four largest molecular components and their sedimentation values has been made by Levin (17). In untreated hemolymph the 35S unit, with a molecular weight of about 2,000,000 (1), predominates. The association is concentration-dependent and shifts toward the 56S form (Fig. 9), presumably a dimer of the 35S unit, when a high concentration of hemocyanin is stored for some time at physiological pH. The smallest visible subunit (16S), having a molecular weight of about 500,000, is possibly composed of eight 6S components, which represent the smallest hemocyanin subunits functional in oxygen transport, with a molecular weight between 60,000 and 100,000 (1, 19). Inasmuch as two copper atoms are required to bind one molecule of oxygen, the minimum weight of *Limulus* hemocyanin would be about 35,000, a value that is average for arthropod hemocyanins. It is presumably this smallest molecular unit that is synthesized in the cyanoblast, its subsequent aggregation into 56S macromolecules and crystals being a spontaneous and probably concentration-dependent process (1).

Despite the fact that hemocyanins are easily crystallized (13), *Limulus* hemocyanin has been resistant to such efforts. Crystalization in the cyanoblast is no doubt governed by physicochemical characteristics, such as protein concentration and the presence of calcium and magnesium ions (13), but additionally the cell exerts some direct morphogenetic effect on the alignment of separate crystals. This influence is evidenced in the frequent occurrence of rigorously parallel though separate crystals (Fig. 6) that subsequently accrete to larger crystals with but minor flaws. Microtubules on occasion parallel crystals being formed, a juxtaposition that is in keeping with the frequent association of microtubules with morphogenetic events. Inasmuch as the hemocyanin is contained in the cytoplasm, normal channels of merocrine protein secretion in the cell are excluded. The large molecular size of even the smallest subunits precludes other modes of egress, leaving only the rather unusual holocrine method of protein secretion. Liberated crystals evidently dissolve rather slowly, since single crystals or small swarms of them are frequently seen in the hemocoelic spaces.
SUMMARY
A new and rare blood cell type has been found in the circulation of the horseshoe crab, Limulus polyphemus. The cell, which has been named the nephroblast, is replete with free ribosomes and synthesizes the blood pigment hemocyanin. The hemocyanin accumulates in the cytoplasm and aggregates into crystals. The fully mature cell, up to 100 \( \mu \) in maximal extent, is almost completely filled with large hemocyanin crystals, which by disruption of the cell are liberated into the hemocoel.

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This study constitutes publication No. 414 from the Oregon Regional Primate Research Center, and was supported by Grants FR 00163 and NB 02717 from the National Institutes of Health and by a Bob Hope Grant-in-Aid from Fight-For-Sight, Inc. Part of this work was presented in brief form at the Eighth Annual Meeting of the American Society for Cell Biology.

I wish to thank Mrs. Audrey J. Griffin for patient and excellent technical assistance, and Dr. A. Malley for providing the purified hemocyanin.

Received for publication 9 July 1969, and in revised form 22 September 1969.