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Immunocytochemical reaction of a haemocyanin antibody in the midgut gland of *Nautilus* (Cephalopoda, Tetrabranchiata)

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Abstract. The branchial gland of the dibranchiate cephalopods is described as the site of haemocyanin synthesis. Because there is no equivalent to this organ in tetrabranchiate cephalopods the localization of haemocyanin synthesis remained unknown for a long time. In this study we could confirm the conclusions from preliminary investigations concerning the copper content of the midgut gland of *Nautilus*, which gave the first indications for a possible localization of haemocyanin synthesis in this organ. We developed a polyclonal antibody against *Nautilus* haemocyanin, tested its specificity, and used it on ultra-thin sections of the tissue of the midgut gland. It could be shown that there is a clear immunogold precipitation only on the triangular basal cells in the terminal alveoli. All the other types of cell in this organ were free of any immunoreactivity. It can be supposed that the triangular basal cells in the terminal alveoli of the midgut gland are the sites of haemocyanin synthesis in *Nautilus*.
Key words. Haemocyanin synthesis; Cephalopoda; *Nautilus*; midgut gland; immunocytochemistry.

There is little information regarding the site of haemocyanin-synthesis in either arthropods or molluscs. In arthropods, Fahrenbach described 'cyanocytes'[1] within the circulatory system of *Limulus*, which contain large crystals of haemocyanin. 'Cyanocyte-like' cells could be identified immunocytochemically in the heart muscle of the spider *Eurypelma californicum*[2]. In crustaceans, it is known that the midgut gland is the site of haemocyanin synthesis[3–5]. Cyanocytes could be found in the reticular connective tissue surrounding the midgut gland of *Carcinus maenas*. These cyanocytes synthesize and accumulate haemocyanin, and secrete it into the haemolymph by means of a holocrine secretion process[6].

In the gastropods *Lymnea* and *Helix*, special pore-cells located in the connective tissue surrounding the respiratory cavity[7] are responsible for the synthesis of haemocyanin. Numerous rod-like elements with diameters identical to that of haemocyanin could be identified in the cisternae of the rough endoplasmatic reticulum (rER) of these cells[8].

In cephalopods the locus of haemocyanin synthesis is known in some dibranchiate species. Dilly and Messenger identified the branchial gland of *Octopus vulgaris* as the haemocyanin producing organ[9]. Schipp et al. also described this specialized gland in *Sepia officinalis* as the site of haemocyanin synthesis[10]. In both species this gland contains triangular cells with a large amount of rER. Using transmission electron microscopy, it was shown that haemocyanin molecules are released from the cisternae of the rER of those cells into the haemolymph by means of an apocrine secretion process[10].

Tetrabranchiate molluscs, however, do not possess branchial glands[11,12], so the question of the localization of haemocyanin synthesis in the only recent genus of tetrabranchiate cephalopods (*Nautilus*) remains unanswered. In a previous investigation, using analytical scanning transmission electron microscopy, we showed that remarkably high amounts of copper occur in specialized cells called triangular basal cells in the midgut gland of *Nautilus*[13].

The general organization of the midgut gland of *Nautilus* is shown in figure 1. The gland consists of numerous blind-ended tubules, which ramify extensively. Each acinus is connected to the ductus hepatopancreaticus by a so-called ductulus. The ductus hepatopancreaticus connects via the caecum to the midgut. The present study deals with the enlarged terminal parts of the tubules, the terminal alveoli. Triangular basal cells bordering the haemolymphatic are only found within this part of the midgut gland.

Because copper is the oxygen-binding element of haemocyanin, it is possible that haemocyanin synthesis in *Nautilus* is located in these triangular basal cells.

In order to test this hypothesis, we developed a polyclonal antiserum against the haemocyanin of *Nautilus* and used it in immunocytochemical experiments on the midgut gland of the same species. The present paper reports the results of these experiments.

Materials and methods

The investigations were carried out on three adult *Nautilus pompilius*[14] from Pago Pago Bay in American Samoa and two adult *Nautilus pompilius*[14] from the coastal waters of Port Moresby in New Guinea.

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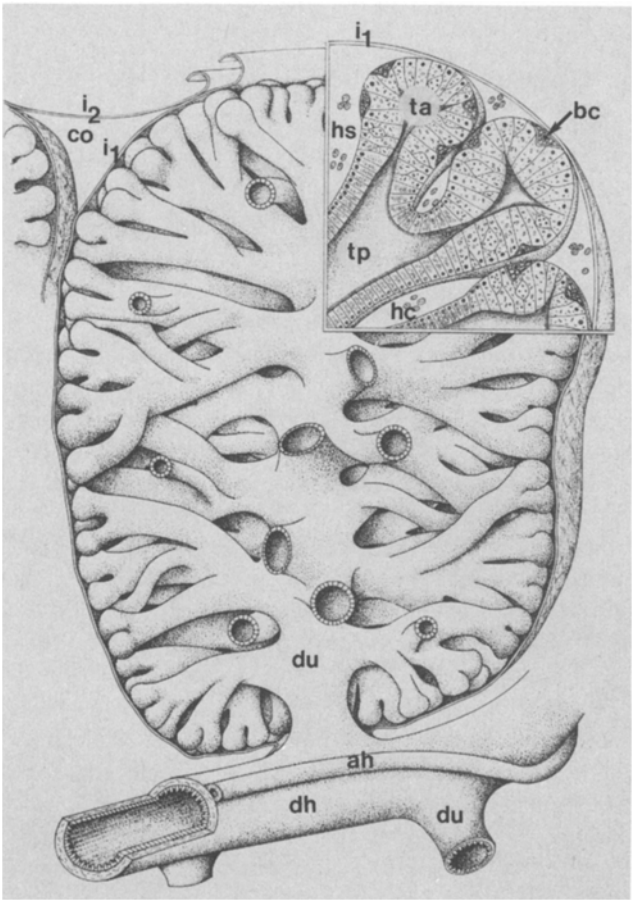

Figure 1. Schematic organization of an acinus of the midgut gland of *Nautilus*. Each acinus is supplied from the ductus hepatopancreaticus (dh) via a ductulus (du). The ductulus is part of the extensively ramified tubules which ends in alveolary enlarged terminal parts, the terminal alveoli (ta). Between the ductuli and the terminal alveoli there are tubulary transitional parts (tp). Each acinus is covered by thin integuments which separate the haemolymphatic space (hs) from the coelom (co), i_1, and the coelom from the entrails, i_2. The triangular basal cells (bc) can only be found in the terminal alveoli (ta) near the haemolymphatic space (hs). Haemocytes (hc). Arteria hepatopancreatica (ah).

Production and specification of the antibody. After the animals had been anaesthetized in a 1%–2% ethanol-seawater solution, the shell was removed and the arteria siphonalis was ligated immediately to prevent bleeding. The haemolymph was taken out of the arteria branchialis and the cellular components were removed by centrifugation at 2500 g at 4° C for 15 min. In order to extract the haemocyanin from the haemolymph, the cell-free suspension was diluted 1:50 with a 0.1 M Tris/HCl-buffer containing 10 mM $CaCl_2$ and 50 mM $MgCl_2$ and ultracentrifuged in a DuPont ultracentrifuge (rotor AH-627) at 150 000 g at 4 °C for 16 h. The resulting dark blue pellet was resuspended in the Tris buffer described above, and the centrifugation was repeated three times. After the last centrifugation the pellet, containing a high concentration of haemocyanin, was resuspended in a small amount of Tris buffer and the concentration of protein was determined according to the method described by Bradford[15]. A stock solution with a protein concentration of 1 mg protein in 1 ml Tris buffer was made and deep frozen for further investigations.

The immunization was carried out in rabbits following standard methods, using complete adjuvant[16]. A preimmune serum was taken before the start of the immunization of the rabbits. After six weeks of immunization the serum was collected and the immunoglobulins were purified according to the method of McKinney and Parkinson[17]. The specificity of the antibody was tested by immunodiffusion[18] and Western blots.

SDS-polyacrylamide-gel electrophoresis. The SDS-PAGE was carried out according to Laemmli[19] with a mini-gel cell Type G42 (Biometra). For this investigation the protein samples of the haemolymph and the extracted haemocyanin were used. The gels were stained with Coomassie brilliant blue R-250 and Coomassie brilliant blue G-250 (1:1). As a molecular weight standard the high molecular weight standard mixture Sigma H6 was used.

Western blot. The proteins were blotted with a mini-trans blot electrophoretic transfer cell (Bio-rad) overnight at 4 °C on a 0.45 micron nitrocellulose membrane (Bio-rad). The first antibody was tested at different dilutions (1:500; 1:1000; 1:2000; 1:4000; 1:8000). The antigen-antibody reaction was detected with alkaline phosphatase (Protoblot-system, Promega) using nitro-blue-tetrazolium-chloride and 5-bromo-4-chloro-3-indolylphosphate as substrate. For the control, the primary antibody was omitted from the procedure.

Transmission electron microscopy. The animals were anaesthetized as described above. The tissue of the midgut gland was removed immediately and prefixed in 2% glutardialdehyde in phosphate buffer (pH 7.3). The temperature of the fixation solution was gradually increased from 0 °C to 25 °C over a period of 1.5 h. The postfixation treatment was carried out with 1% OsO_4 in phosphate buffer (pH 7.3) at a temperature of 25 °C for 1 h. After dehydration in a graded series of acetones the tissue was embedded in Durcupan (Fluka). Semi-thick sections (1 µm) were cut and stained with methylene blue azure II and basic fuchsin according to Humphrey and Pittman[20] and studied with a Zeiss Photomikroskop II. The ultra-thin sections (70 nm) were contrasted according to Reynolds[21] and viewed with a Zeiss EM 9A transmission electron microscope.

Immunocytochemistry. Anaesthesia and preparation of samples were performed as described above. The tissue was prefixed in 4% paraformaldehyde also containing 0.4% glutaraldehyde and 0.2% picric acid in phosphate buffered saline (PBS). The postfixation was carried out with 1% OsO_4 in 0.2 M cacodylate buffer.

After the dehydration in a graded series of ethanols the tissue was embedded in LR-white (medium) and cut into 70 nm thick sections on a Reichert and Jung OM/U2 ultramicrotome. For immunocytochemistry the sections were processed according to a slightly modified

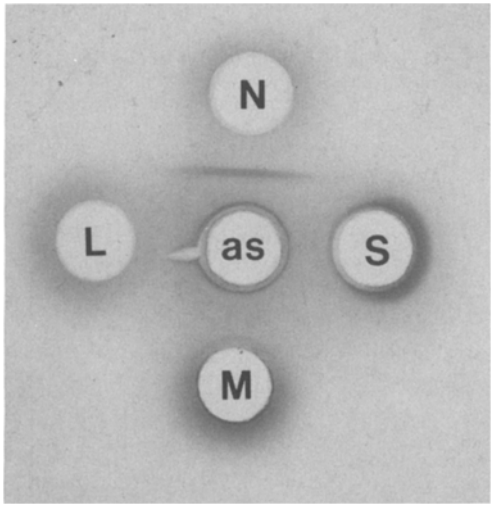

Figure 2. Ponceau-2R staining of a two-dimensional immunodiffusion according to the method of Ouchterlony[18]. Around the antiserum (as) with a high level of polyclonal antibodies against *Nautilus* haemocyanin are the haemocyanins of *Nautilus* (N), *Sepia* (S), *Limulus* (L) and *Megathura* (M).

version of the protocol of Vardell et al.[22]. All the sections were incubated in PBS (phosphate buffered saline) containing 5% preimmune serum to block non-specific binding. Our polyclonal antiserum against *Nautilus* haemocyanin was diluted 1:5000 in PBS/BSA (1%) and the sections were incubated in this solution for 16 h at 4 °C. The protein-A-gold solution (diameter of the gold particles: 15 nm) was diluted 1:1000. It contained 1% glycerol. The ultra-thin sections were incubated for 1 h at room temperature. Afterwards the sections were rinsed in Tris/BSA (1%), Tris and distilled water each for 10 min. All the solutions were filtered through a 0.2 µm pore filter. In the control, the primary antibody was omitted from the procedure. After drying for 1 h at 50 °C the sections were contrasted according to Reynolds[21] and studied with a Zeiss EM 9 A transmission electron microscope.

Results

Specification of the antiserum. After the purification of the immunglobulins from the whole serum, the antiserum was tested by two-dimensional immunodiffusion against different antigens. To study possible cross-reactions between our polyclonal antiserum and other haemocyanins, the haemocyanins of *Nautilus*, *Sepia*, *Megathura* and *Limulus* were used as antigens. As shown in figure 2 there is a distinct precipitation line between the antiserum against *Nautilus* haemocyanin and the purified *Nautilus* haemocyanin. There is no reaction of the antiserum to the haemocyanins of *Sepia*, *Megathura* and *Limulus*.

A more sensitive method to document the specificity of an antiserum is the western blot technique. The full haemolymph of *Nautilus*, and the purified haemocyanin which was used for the immunization of the rabbits, were

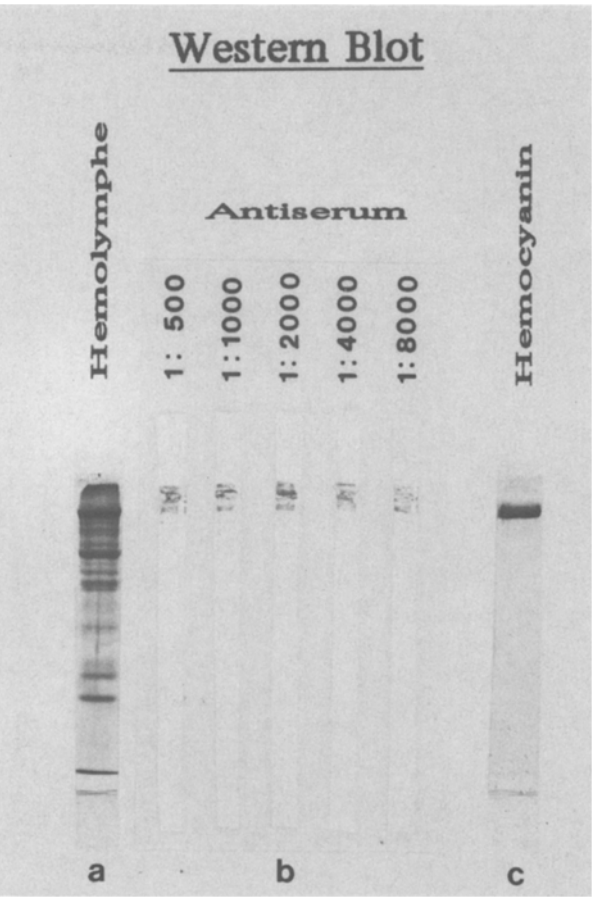

Figure 3. Western blot. *a*) Coomassie stained SDS gel of the *Nautilus* haemolymph, which was blotted onto nitrocellulose membrane. *b*) Blots of the *Nautilus* haemolymph after incubation with the polyclonal antibody against *Nautilus* haemocyanin in different dilutions. *c*) SDS gel of the purified *Nautilus* haemocyanin.

loaded on a SDS polyacrylamide gel, separated, blotted on a nitrocellulose membrane and incubated with different dilutions of the antiserum. Coomassie blue stained SDS gels of the full haemolymph (left) and of the purified haemocyanin (right) are shown in figure 3. The blot membranes of the full haemolymph, treated with different dilutions of the haemocyanin-antiserum, are shown between the gels. The reaction of the separated proteins of the haemolymph and the antiserum is exactly in the molecular weight range of the purified haemocyanin (fig. 3, right). Based on these results, the polyclonal antiserum was used on the ultra thin sections.

Immunocytochemical findings. In a semi-thick section of a terminal alveolus three different types of cells can be distinguished: principal cells, vacuolized cells, and triangular basal cells (fig. 4a). The basal cells are characterized by sharply outlined nucleoli and a more or less round nucleus (fig. 4b). Under the transmission electron microscope it can be seen that the rough endoplasmatic reticulum (rER) is abundant and fills up almost the whole volume of the triangular basal cell (fig. 4d). Under higher magnification a large number of ribo-

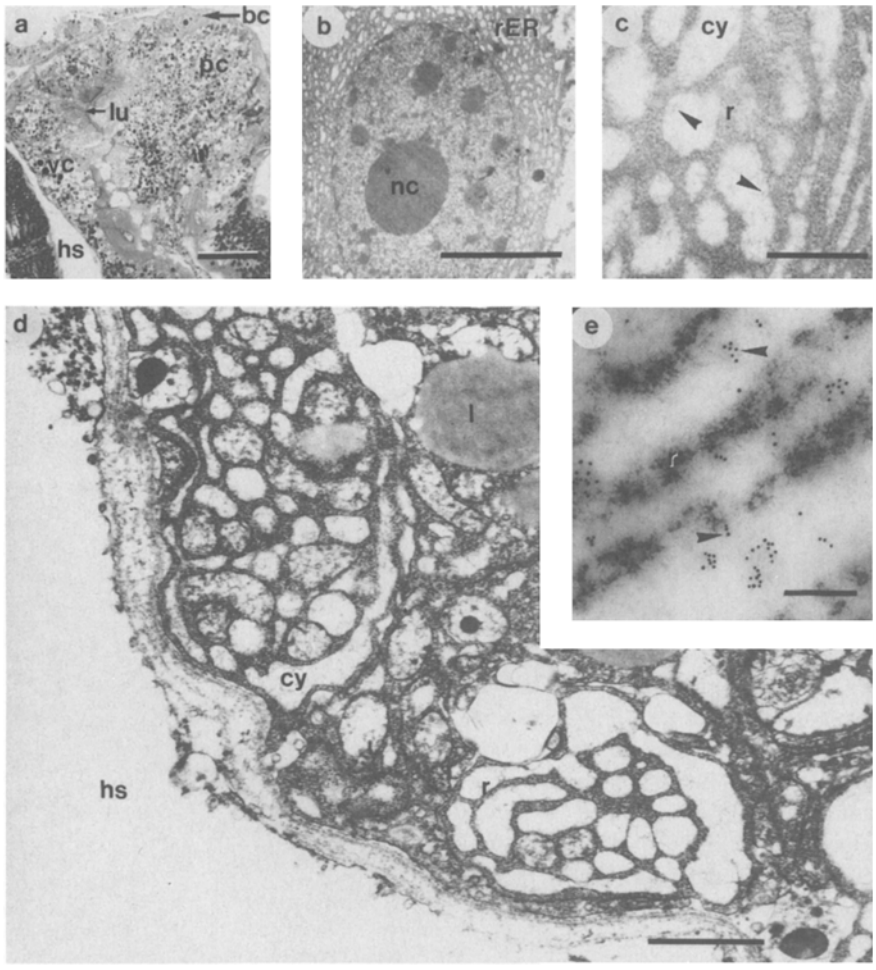

Figure 4. The semi-thin section shows a terminal alveolus of the midgut gland of *Nautilus*. Haemolymphatic space (hs), lumen (lu), principal cell (pc), triangular basal cell (bc), vacuolized cell (vc), bar: 50 µm. *b*) The typical nucleus of a triangular basal cell with a sharply outlined nucleolus (nc), rough endoplasmatic reticulum (rER), bar: 5 µm. *c*) Higher magnification of the rER of the triangular basal cell. Ribosomes (r), products of synthesis of the rER of this cell (arrowheads) in the cisternae (cy), bar: 0.5 µm. *d*) Transmission electron micrograph of a triangular basal cell. Cisternae of the rER (cy), haemolymphatic space (hs), lipid (l), ribosomes (r), bar: 1 µm. *e*) Incubation of the polyclonal antibody against *Nautilus* haemocyanin visualized with a protein-A gold complex, diameter of the gold particles 15 nm. Ribosomes (r), immunoprecipitation of the products of synthesis of the triangular basal cell (arrowheads), bar: 0.3 µm.

somes and the products of synthesis in the cisternae of the rER become visible (fig. 4c). In ultra-thin sections which were processed immunocytochemically it was evident that immuno-gold precipitation occurs only in the triangular basal cells. The gold particles (diameter: 15 nm) are located especially on the products of synthesis of the rER of the basal cells (fig. 4e). All the other types of cells were free of any precipitation, except for some reactions in the haemolymphatic space and a very few gold particles dispersed diffusely over the whole tissue. On the control sections there was no distinct immuno-gold reaction.

Discussion

The present data suggest that the triangular basal cells in the midgut gland are the site of haemocyanin synthesis in *Nautilus*. This finding is in accordance with previous studies on the haemocyanin synthesising system of tetra- and dibranchiate cephalopods. Transmission electron microscopical studies have shown that the cisternae of the rER of the triangular basal cells of *Nautilus* contain rod-like elements[13] with diameters identical to haemocyanin molecules[23].

Since copper is the oxygen binding element in haemocyanin the concentration of this element was studied in the midgut gland and the branchial gland of *Sepia* and in the midgut gland of *Nautilus* using atomabsorption spectroscopy. The highest value could be found in the midgut gland of *Nautilus* ($473 + 79$ µg/g wet weight)[13]. The level of copper in the midgut gland of *Sepia* was distinctly lower ($100 + 49$ µg/g wet weight)[24]. In the braqnchial gland of *Sepia*, which is supposed to be the site of haemocyanin synthesis in this species, the copper

concentration was 22 + 7 µg/g wet weight[24]. Because of this relatively low value, it has been supposed that the copper is incorporated extracellularly in *Sepia*[24]. By using analytical scanning transmission electron microscopy it was possible to detect the element copper in cells and cell compartments in the midgut gland of *Nautilus*. Only the triangular basal cells in the terminal alveoli of the midgut gland showed a clear copper peak, and the copper was located in the cisternae of the rER, in dense bodies, and in vesicles leaving the basal cells[13]. The antibody we produced against the haemocyanin of *Nautilus* was first used on paraffin sections of the midgut gland of *Nautilus*, and showed a clear immunoreactivity in the triangular basal cells[25]. The present immunocytochemical investigation on the electron microscopical level confirms our earlier studies on the location of haemocyanin synthesis in *Nautilus*[13]; it shows that the immunoprecipitation is located only in the triangular basal cells of the midgut gland of *Nautilus* and especially on the products of synthesis of the rER of these cells.

If one compares the cytomorphology of haemocyanin producing cells in dibranchiate cephalopods and tetrabranchiate cephalopods there is a remarkable conformity. In the branchial gland of *Octopus*, which is the site of haemocyanin synthesis in this specimen[9], there are triangular to polygonal cells with an abundant rER, and an almost round nucleus with a sharply outlined nucleolus. Within the cisternae of these cells haemocyanin molecules could be identified. The same cell type could be identified in the branchial gland of *Sepia*[10]. The midgut gland of *Sepia* also contains this type of cell[26]; however, the number in this gland is rather small as compared to *Nautilus*. In *Nautilus* there is no equivalent of the branchial glands of the dibranchiate cephalopods[12]. However, in the so-called ligamentum branchiale a small number of cells could be identified that show the typical cytomorphology of triangular basal cells[12]. It might thus be concluded that haemocyanin synthesis in cephalopods is associated with an identifiable cell type which is predominantly found in the midgut gland (*Nautilus*) or the branchial gland (*Sepia*, *Octopus*).

These findings raise the question of the phylogenetic polarity of the obvious difference between tetrabranchiates (*Nautilus*) on the one hand and the dibranchiates (*Sepia*, *Octopus*) on the other. Since it cannot be assumed a priori that the condition seen in *Nautilus* is plesiomorphic (even though this species retains many plesiomorphic characters, for example a shell), it is necessary to determine the plesiomorphic character state by means of an out-group analysis[27]. Gastropods are believed to be the sister-group of cephalopods[28] and in this group haemocyanin synthesis occurs in the connective tissue surrounding the respiratory cavity[7], and not in the midgut gland. Thus, one might argue that haemocyanin production was primarily associated with respiratory organs in gastropods, thus the condition seen in dibranchiates should be rated as plesiomorphic. However, it should be emphasized that this phylogenetic analysis is based on only one out-group comparison. Further studies, particularly on bivalve molluscs, are needed in order to determine the phylogenetic polarity with more certainty.

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