special biochemical reaction of the membranes due to increased current density during spike propagation in these axons[11], thus corroborating the interpretation of the physiological role of the nodes given above. Further electrophysiological and electron microscopical work is in progress to elucidate these points[13].

Zusammenfassung. Es werden neuartige, segmental angeordnete Öffnungen in der Myelinscheide der medianen Riesenfaser des Regenwurms *Lumbricus terrestris* beschrieben, durch welche die Axonmembran direkt an die extrazelluläre Kollagenhülle des Bauchmarkperineuriums heranreicht. Dieser Befund wird in Hinblick auf den Mechanismus der Erregungsleitung myelinisierter Nervenfasern von Wirbellosen diskutiert.

J. GÜNTHER

I. Zoologisches Institut der Universität, Abteilung für Zellbiologie, Berliner Strasse 28, D–34 Göttingen (Germany), 2 May 1973.

Identification of Hemocyanin in the Cyanocytes of *Carcinus maenas*

Cyanoblast and cyanocyte are the names given by FAHRENBACH[1] to the cells which synthesize hemocyanin (Hcy) in *Limulus polyphemus*. These cells have been observed, together with granulated hemocytes, in the circulatory sinusoids of the compound eye and, according to this author, they probably originate in the digestive gland. Mature cyanocytes show crystalline bodies within the cytoplasm which have been identified as Hcy on the basis of the dimensional congruence of the crystalline lattice with the size of the Hcy molecule of the same animal.

Hcy is the respiratory pigment of 2 phyla of invertebrates: Mollusca and Arthropoda, and the molecular weight of the main component present in the blood is typical not only of each phylum but also of the classes, orders and suborders of both phyla[2,3]. The Hcy of *Limulus* differs from that of other arthropod species in its high sedimentation constant: 60 S against 24 S and 16 S in Crustacea and 34 S in other Arachnomorpha[4]. Except for the observations by DILLY and MESSENGER[5] on *Octopus vulgaris* and by FAHRENBACH on *Limulus polyphemus*, both based on morphological evidence only, the problem of the biosynthesis of Hcy in Mollusca and Arthropoda is still a matter of speculation.

With the purpose of demonstrating unequivocally the endocellular presence of Hcy, the hemopoietic cells of *Carcinus maenas* have been identified and characterized by electron microscopy and the protein which is synthesized in the cyanoblast has been identified as Hcy by immunofluorescence.

Material and methods. The animals were obtained from the Hydrobiology Station of Chioggia (Venice) and kept in the aquarium until used. The dorsal carapace was removed and the tissues were hardened in situ by short prefixation (30–40 min).

For the optical microscopy, Bouin, formaldehyde or glutaraldehyde at different concentrations were employed. The sections were coloured with hematoxylin-eosin or PAS and with rubeanic acid for the detection of copper. For electron microscopy, the best fixation was obtained by using 3 or 4% gluteraldehyde in sea water and 0.05 *M* cacodylate buffer pH 7.0 (1:1 vol.) plus 0.001 *M* $CaCl_2$ and 0.025 *M* sucrose. The tissues, previously treated in situ with this solution, were removed and kept for 1 h in fresh fixative, then washed 3 times (10 min each) with the cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.1 *M* cacodylate buffer pH 7.2 for 60 min. The material was dehydrated and included in DER (Dow Epoxy Resin, Carlson Co.)[6]. Fine (500 Å ca.) and 1 μm thick sections were prepared with the LKB Ultratome III. The thick sections were coloured with toluidine blue and the thin ones with uranyl acetate (saturated solution in 50° ethanol) for 25–30 min and lead citrate for 3–4 min[7]. The observations were made using a Phylips Electron Microscope Mod. 300.

Hcy was purified from the hemolymph of *Carcinus* by dialysis and ultracentrifugation according to GHIRETTI-MAGALDI et al.[8]. The rabbit Hcy antibody was prepared by injecting 5 ml saline containing 10 mg of protein once

[1] W. H. FAHRENBACH, J. Cell Biol. *44*, 445 (1970).
[2] I. B. ERIKSSON-QUENSEL and T. SVEDBERG, Biol. Bull. *71*, 498 (1936).
[3] F. GHIRETTI, A. GHIRETTI-MAGALDI and B. SALVATO, Proc. int. Congr. comp. Physiol., in press.
[4] K. E. VAN HOLDE and E. F. J. VAN BRUGGEN, in *Subunits in Biological Systems* (Eds. N. S. TIMASHEFF and C. D. FASMAN; Decker, New York 1971), vol. 5, p. 1.
[5] P. N. DILLY and J. B. MESSENGER, Z. Zellforsch. *132*, 193 (1972).
[6] W. R. LOCKWOOD, Anat. Rec. *150*, 129 (1964).
[7] E. S. REYNOLDS, J. Cell Biol. *17*, 208 (1963).
[8] A. GHIRETTI-MAGALDI, C. NUZZOLO and F. GHIRETTI, Biochemistry *5*, 1943 (1966).

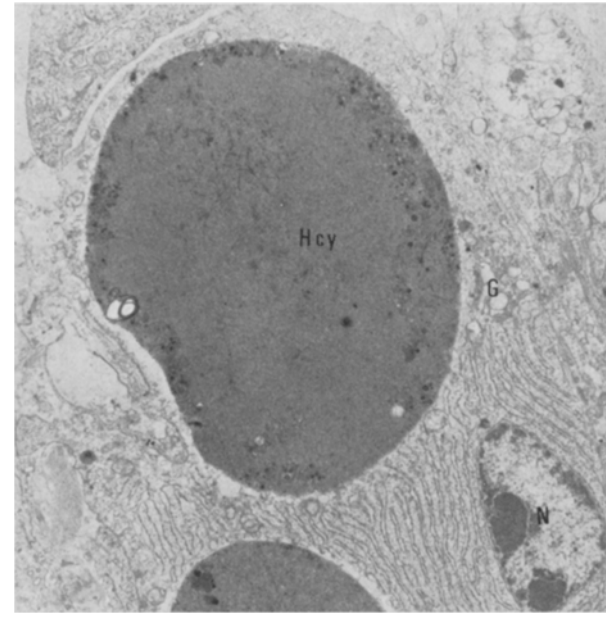

Fig. 1. Electron micrograph of a cyanocyte in the reticular connective tissue of the hepatopancreas of *Carcinus maenas* showing a large inclusion of proteic material (Hcy), the Golgi system (G) and a peripheric nucleus (N). 3% glutaraldehyde. ×9,800.

a week for 5 weeks. The immunological activity of the serum was tested by the precipitin reaction using different amounts of antigen (from 2 to 0.02 mg) dissolved in buffered saline (0.05 *M* phosphate, pH 8.6).

The anti-Hcy serum was purified by ammonium sulfate precipitation and by DEAE cellulose chromatography[9]. Fluorescein or rhodamine isothiocyanate were used as fluorochromes[10] and the conjugates were separated by DEAE cellulose chromatography[9]. The immunological activity was checked at each step of purification by the precipitin reaction; the presence of unreacted fluorescent material was controlled spectrophotometrically. The ratio fluorochrom to protein of 2:1 was considered optimal.

For the immunofluorescent reaction the tissues were prepared according to WESTIN and PERLMAN[11] and the reaction was carried out with the technique recommended by NAIRN[12] using the blocking test for control. Observations were made with a Leitz orthoplan microscope provided with a KBO-200 mercury lamp and BG-12 or UG-5 filters. Photographs were taken with a 'daylight, 23 DIN Kodachrom film and the time of exposure varied from 20 to 65 sec.

Results and discussion. In Crustacea Decapoda the gizzard, the ophthalmic artery and the hepatopancreas have been identified as the sites of hemocytogenic activity[13–15]. Part of the connective tissue of the gizzard walls contains nodules of undifferentiated cells, many of

[9] S. LOWEY and L. A. STEINER, J. molec. Biol. *65*, 111 (1972).
[10] B. T. WOOD, S. H. THOMPSON and G. GOLDSTEIN, J. Immun. *95*, 225 (1965).
[11] M. WESTIN and P. PERLMAN, Expl Cell Res. *72*, 232 (1972).
[12] R. C. NAIRN, *Fluorescent Protein Tracing* (E. and S. LIVINGSTONE, Edinburg 1969).
[13] L. CUENOT, Archs Zool. exp. gén. *3*, sér. 4, 1 (1904).
[14] L. BRUNTZ, Archs Zool. exp. gén. *7*, sér. 4, 1 (1907).
[15] M. KOLLMANN, Annls Sci. nat. *8*, 1 (1908).

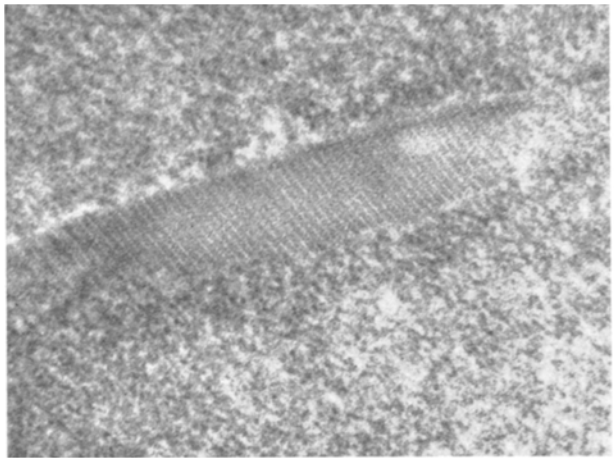

Fig. 2. Crystalline hemocyanin in a cyanocyte showing a parallel succession of dark and clear bands. ×114,00.

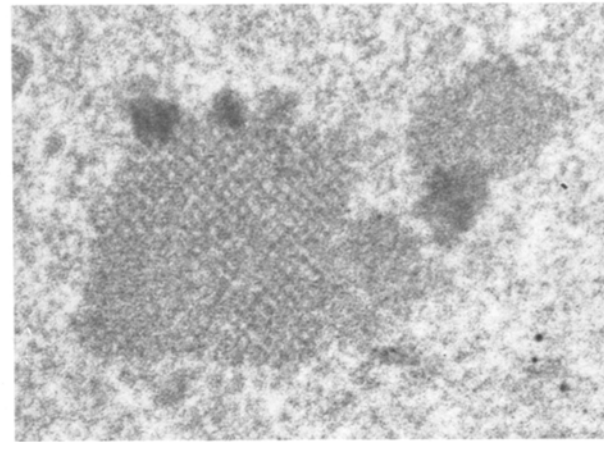

Fig. 3. Another crystalline hemocyanin body showing regularly aligned granules in the dark repeating bands. ×143,000.

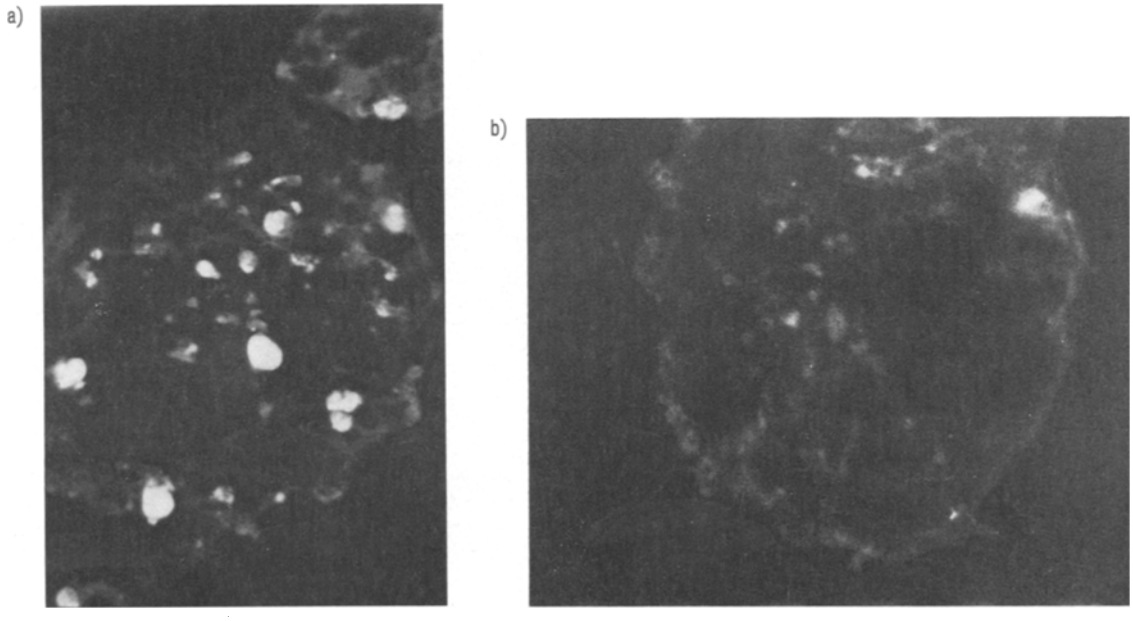

Fig. 4. Immunofluorescence reaction of the cyanocytes from the ophthalmic artery. Rhodamine isothiocyanate as fluorochrom. Blue light, 45 sec exposure. ×90. (a) reaction; (b) control.

which are undergoing mitosis. According to MARREC[16] and DEMAL[17], these are 'stem cells' of the hemopoietic lines; the ophthalmic artery and the hepatopancreas are the places where some lines migrate and differentiate.

In the reticular connective tissue, around the ophthalmic artery and in the spaces between the caeca of the hepatopancreas, several very large eosinophilic, PAS positive cells are present together with granulated hemocytes and fagocytes.

By light and electron microscopy several stages of differentiation of the eosinophilic cells have been identified as cells characterized by the presence of one or few compact inclusions which fill most of the cellular body. The inclusions are not contained in vacuoles, although sometimes membrane profiles are seen around them (Figure 1); the material has a granular structure and in several cases shows a number of crystalline bodies formed by a succession of parallel dark and clear bands repeating every 130 Å (Figures 2 and 3). The dark bands are apparently composed of a regular alignement of dense granules with a periodicity of 120 Å. These dimensions correspond to the 16 S component of the crustacean Hcy[3]. In the last stages of maturation, the cells show a small peripheric nucleus and no longer contain cytoplasmic organelles except for a few reticulum profiles. Supposedly they disrupt and discharge their content in the nearby circulatory sinuses.

On the basis of the dimensions of the granular and the crystalline material, these cells are homologous to the cyanoblasts and the cyanocytes described in *Limulus*. The coloured reaction with rubeanic acid is very faint on account of the low copper concentration of crustacean Hcy (0,17%) whereas the PAS positive reaction is probably due to the polysaccharide fraction of the Hcy molecule[3,17]. As demonstrated by the fluorescent reaction, only the cells which have been identified by light and electron microscopy as cyanoblasts and cyanocytes in the hemopoietic tissue of *Carcinus maenas* react with the specific antibody for *Carcinus* Hcy (Figure 4)[18].

The ferritin conjugated specific antibody will be employed for the identification of the crystalline material with Hcy. This technique will also be used with the aim of establishing the cell lineage. The analysis by optical diffraction of the crystalline bodies is under study.

Riassunto. Nel tessuto emopoietico di *Carcinus maenas* sono state identificate le cellule che sintetizzano l'emocianina. Queste cellule contengono materiale che al microscopio elettronico presenta un aspetto granulare con particelle di dimensioni costanti simili a quelle dell'emocianina circolante e che in molti punti sono organizzate in una struttura cristallina. L'identità di questo materiale con l'emocianina è stata dimostrata col metodo dell'immunofluorescenza.

ANNA GHIRETTI-MAGALDI, CARLA MILANESI and B. SALVATO[19]

Centro per lo studio della Fisiologia e Biochimica delle Emocianine del Consiglio Nazionale delle Ricerche, Istituto di Biologia Animale dell'Università di Padova. Via Loredan 10, I-35100 Padova (Italy), 5 April 1973.

[16] M. MARREC, Bull. Inst. Océanogr. Monaco *867*, 1 (1944).
[17] J. DEMAL, Céllule *56*, 87 (1953).
[18] V. ALBERGONI, A. CASSINI and B. SALVATO, Comp. Biochem Physiol. *41 B*, 445 (1972).
[19] Thanks are due to Mr. G. TOGNON for technical assistance; to Prof. P. OMODEO for advice and criticism and to Prof. V. ALBERGONI and Dr. P. BURIGHEL for help and discussion.

The Occurrence of a Band of Nuclei in Primary Neural Induction in the Chick Embryo

Primary neural induction has been previously deduced by HARA[1] to occur in the very early stage 5 chick embryo[2]. At this point in development the presumptive head mesenchyme cells are present as a mass anterior to Hensen's node but as yet have not formed any notochord. The ectoderm overlying these mesenchyme cells has thickened. Neural folds are not present.

When the very early stage 5 embryo is fixed in Bouin's or Carnoy's fluid and sectioned transversely at 10 µm a band of nuclei can be seen in the ectoderm overlying the presumptive notochord cells (Figure 1). This appearance lasts for approximately one half hour. The band of nuclei appears at the time at which HARA found induction to occur. It is thus a natural marker indicating that induction is occurring.

This band of nuclei is also visible when induction is brought about experimentally. A 'pocket' was formed in a stage 3.3/4 host embryo by inserting a scalpel between the ectoderm and endoderm near the area opaca border on a level with Hensen's node. A stage 4 Hensen's node (0.3 mm × 0.4 mm) was excised from a donor chick embryo and then transferred by pipette to the host embryo. The graft was then inserted into the 'pocket' so that the graft endoderm was adjacent to the host ectoderm. The host embryo was incubated at 38 °C. 72 embryos were prepared in this manner. 6 were fixed at 0 min, and further groups of 6 at 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 16–24 h. Both the ectoderm of the graft and host were examined to determine: 1. the length of time between implanting the graft of Hensen's node into a host and the corresponding neural induction in the host ectoderm and 2. the time between the node graft forming a band of nuclei and the host ectoderm forming a band of nuclei.

A band of nuclei appeared in the graft ectoderm by 3 h (Figure 2). The host ectoderm responded similarly by

[1] K. HARA, Ph. D. Thesis, University of Utrecht (1961).
[2] V. HAMBURGER and H. L. HAMILTON, J. Morph. *88*, 49 (1951).

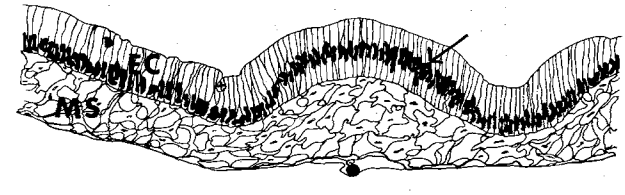

Fig. 1. Transverse section anterior to Hensen's node of the normal early stage 5 chick embryo. Note the band of nuclei (arrow) in the ectoderm. EC, ectoderm; MS, mesoderm.