Comp. Biochem. Physiol., 1970, *Vol.* 34, *pp.* 203 *to* 211. *Pergamon Press. Printed in Great Britain*

A STUDY OF THE BLOOD PROTEINS IN *SEPIA OFFICINALIS* L. WITH SPECIAL REFERENCE TO EMBRYONIC HEMOCYANIN

W. DECLEIR and A. RICHARD

Department of Physiological Chemistry, Casinoplein, Ghent, Belgium, and
Institut de Biologie Maritime et Régionale, Wimereux, France

(*Received* 4 *September* 1969)

Abstract—1. Blood proteins in *Sepia officinalis* have been identified by an ultramicroelectrophoretic technique.

2. The hemocyanin of adult animals shows a constant electrophoretic pattern, characteristic for cephalopods.

3. The hemocyanin of young animals shows electrophoretic patterns that differ significantly from those obtained with adult hemocyanin, with gradual change into the adult pattern during the first weeks after eclosion.

4. First-day animal's blood shows a characteristic hemocyanin pattern that disappears after a few days. This hemocyanin is considered to be embryonic.

5. While the embryonic hemocyanin bands disappear, other bands arise. These are considered to be young hemocyanin which are gradually converted into the adult protein.

INTRODUCTION

HEMOCYANINS are copper proteins found in the blood serum of five classes of invertebrates. These are the Gastropoda and the Cephalopoda among the Mollusca and the Xiphosura, Arachnida and Crustacea among the Arthropoda. Detailed investigations have mainly been carried out with the larger species.

The molecular weights of hemocyanins range from about 300,000 to 900,000 in Arthropods and from a few hundred thousand to several million in Molluscs. Amino acid composition of several hemocyanins has been determined by Ghiretti-Magaldi *et al.* (1966) and by Witters & Lontie (1968), but the mode of binding of copper in the protein is still unknown.

As shown for the first time by Erikson-Quensel & Svedberg (1936) hemocyanin molecules dissociate reversibly into sub-units. Whole molecules exist in a pH range including the isoelectric point. This pH stability region is influenced by electrolytes such as Ca^{2+} and Mg^{2+}, dilution and other factors. In very acid or alkaline solutions this dissociation becomes irreversible. Recently this dissociation into sub-multiples has been confirmed by electron microscopic studies (Fernandez Moran *et al.*, 1966; Eskeland, 1967; Van Bruggen, 1968).

Hemocyanin is mostly considered as the only protein present in blood serum (Goodwin, 1960). Besse & Mocquard (1968) and Lagarrigue & Trilles (1969)

found in the blood of some Isopods, besides hemocyanin and fibrinogen, a protein fraction restricted to female animals. In *Cancer pagurus* a slowly migrating copper containing protein fraction with hemocyanin-like properties has been shown to occur only in female animals with ripening eggs and a protein fraction which does not contain any copper appears in the blood prior to moulting (Decleir, 1966; 1968). Although hemocyanin is found in high concentrations and very often forms the only protein present in the blood, nothing is known concerning either the site or the biosynthesis of this molecule.

The present study was undertaken to determine when hemocyanin is first synthetized during the life of the cuttlefish *Sepia officinalis*. We have, therefore, made a study of the blood proteins from eclosion to adulthood. The results of this study are presented in this paper.

MATERIALS AND METHODS

All the animals used were collected during Summer 1968 in the region of Wimereux. Emphasis was placed on *Sepia officinalis* for two reasons. First, these animals can be caught in great numbers during May–June in the coastal waters, and second, we possessed a good rearing method for newly hatched animals (Richard, 1966). The newly hatched specimens are isolated daily and kept in separate aquaria at constant temperature (18°C), so we had at our disposal a whole range of animals whose ages were accurately known.

The blood was taken directly from the heart with a Pasteur pipette and in the case of young animals the total amount of blood (1–3 μl) was introduced directly into the sample slot in the gel for electrophoretic analysis.

Agar gel electrophoresis

We used the agar gel ultramicroelectrophoretic technique of R. Wieme (1959). The buffer was either a phosphate buffer pH 6·5 ($\mu = 0{\cdot}08$) or a boric acid buffer pH 9·0 ($\mu = 0{\cdot}05$). The concentration of the gel (Difco Bacto Agar) was 0·9%. The tension between the electrodes was kept at 150 V and electrophoresis time was 45 min.

The identification of hemocyanin

Hemocyanin is a copper protein with pseudoperoxidase (Ghiretti, 1956) and pseudophenoloxidase (Bhagvat & Richter, 1938; Decleir & Vercauteren, 1965) activity. These properties can be used for the identification of the pigment. The following tests were performed on all specimens:

1. Copper was demonstrated with rubeanic acid as described earlier (Decleir, 1961).
2. Peroxidase activity was identified according to the method of Manwell & Baker (1963).
3. Phenoloxidase activity was demonstrated by incubating the electropherograms in a mixture of: Pyrocatechol 0·15 M—20 ml; Sodium oleate 0·01 M—20 ml; Phosphate buffer 0·1 M pH 7·3—20 ml; Ethanol 20 ml. After 60 min a red-brown colour indicates phenol oxidase activity (Decleir, 1966).
4. When several bands with hemocyanin characteristics were found on the electropherograms, the association–dissociation properties were checked by using a buffer with and without Ca^{2+} 0·01 M.

Electron microscopy

Gills were fixed by glutaraldehyde, followed by a post-fixation with osmium tetroxide. After inclusion with Epon, the tissue was cut with an ultratome LKB and the slices were examined with a Siemens Elmiskop I.

Spectroscopy

Spectra were taken with an Optica Milano automatic spectrophotometer.

RESULTS

1. *A comparative study of adult blood proteins*

Freshly drawn blood of *Sepia officinalis* is mostly uncoloured or a very weak blue. After exposure to the air it turns dark blue in a few seconds. This colour is due to oxygenated hemocyanin. In cephalopods we found no pigments in the blood other than hemocyanin. In crustaceans the weak blue colour of the oxygenated freshly drawn blood is mostly obscured by yellow, brown or red pigments which sedimentate easily after dialysis of the blood with running tap water or a phosphate buffer pH 7·0. In *Cancer pagurus* we showed these to be protein-bound astaxanthine. The absorption spectrum of oxygenated *Sepia* hemocyanin shows peaks at 575, 345 and 277 mμ. These peaks are shifted to the longer wavelengths as compared to crustacean hemocyanins. In *Cancer pagurus* hemocyanin, for instance, we found the corresponding peaks at 560, 335 and 276 mμ (Decleir, 1968).

Electrophoresis of adult blood from *Sepia officinalis* reveals at pH 9·0 two equally important protein fractions that can be shown to contain copper and that give a positive reaction for peroxidase and phenoloxidase. Moreover in the presence of Ca^{2+} these two bands fuse to a single band, Hence they are considered to be hemocyanin. The two hemocyanin fractions are always preceded by a smaller fraction, which does not show any of the tests mentioned for hemocyanin. Furthermore it gives a negative lipoprotein and a positive glycoprotein reaction.

Figure 1 compares the electrophoretic mobilities of hemocyanin fractions from different adult molluscs and Crustacea at pH 9·0. We can see that the electrophoretic pattern of *Sepia officinalis* is characteristic for all the cephalopods tested and that the migration velocity of the two bands is very slow as compared to crustacean hemocyanin fractions.

At pH 6·5 we find only one hemocyanin fraction, as is the case for most other hemocyanins, the family of the Helicidae excepted. This fraction is preceded by the already mentioned glycoprotein fraction. Figure 2 shows the electrophoretic patterns of different hemocyanins at pH 6·5. We see that the electrophoretic mobility of *Sepia officinalis* hemocyanin at this pH, like other cephalopods, is higher than those of many other molluscs tested and is similar to those of the slowest crustacean hemocyanins.

2. *Study of the blood proteins in newly hatched animals*

While the protein fractions from electropherograms of adult animals remain unchanged, the blood of newly hatched specimens shows a quickly changing protein composition. Figures 3 and 4 represent the evolution of the blood proteins on electropherograms from young animals during the first weeks after eclosion at pH 6·5 (Fig. 3) and at pH 9·0 (Fig. 4). All the protein bands found in the blood of young animals show a positive reaction to the tests for hemocyanin. The non-hemocyanin glycoprotein fraction found in adults is formed only from the

third month after eclosion. When Ca^{2+} is added to the buffer we always find only one slowly migrating fraction, the mobility of which increases slightly from the first to the sixtieth day.

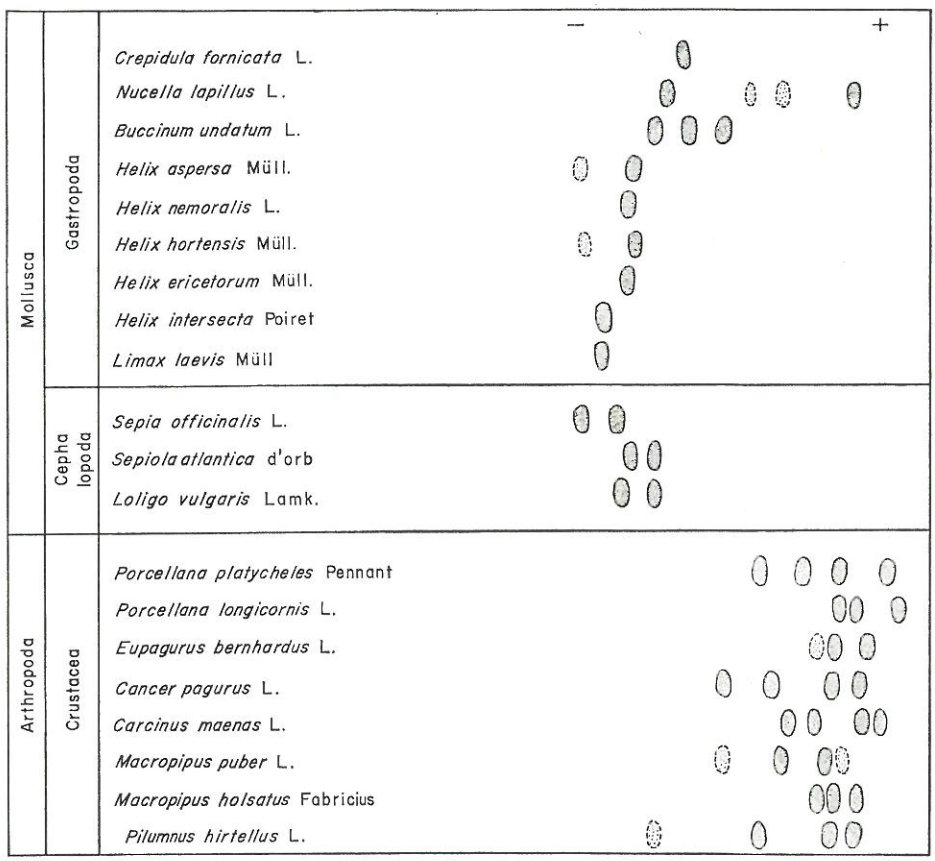

FIG. 1. Electrophoretic patterns of the hemocyanin from the blood of some arthropods and molluscs at pH 9·0.

In the electrophoretic patterns of 1-day-old animals we find three fractions, IV, V and VI (Fig. 3) at pH 6·5 which disappear after a few days, while new bands are formed (III C, III B, III A and II B, II A) with higher and higher mobility until, after 50 days, the relatively fast migrating single adult band is obtained.

At pH 9·0 we also find three fractions (A 1, A 2 and A 3) in 1-day-old animals, which disappear after a few days. These fractions migrate in front of a diffuse protein zone which could not be separated into different fractions. From the ninth day on, sharp separations can easily be obtained in this zone and from the thirty-fifth day on the two adult bands can be found (Fig. 4).

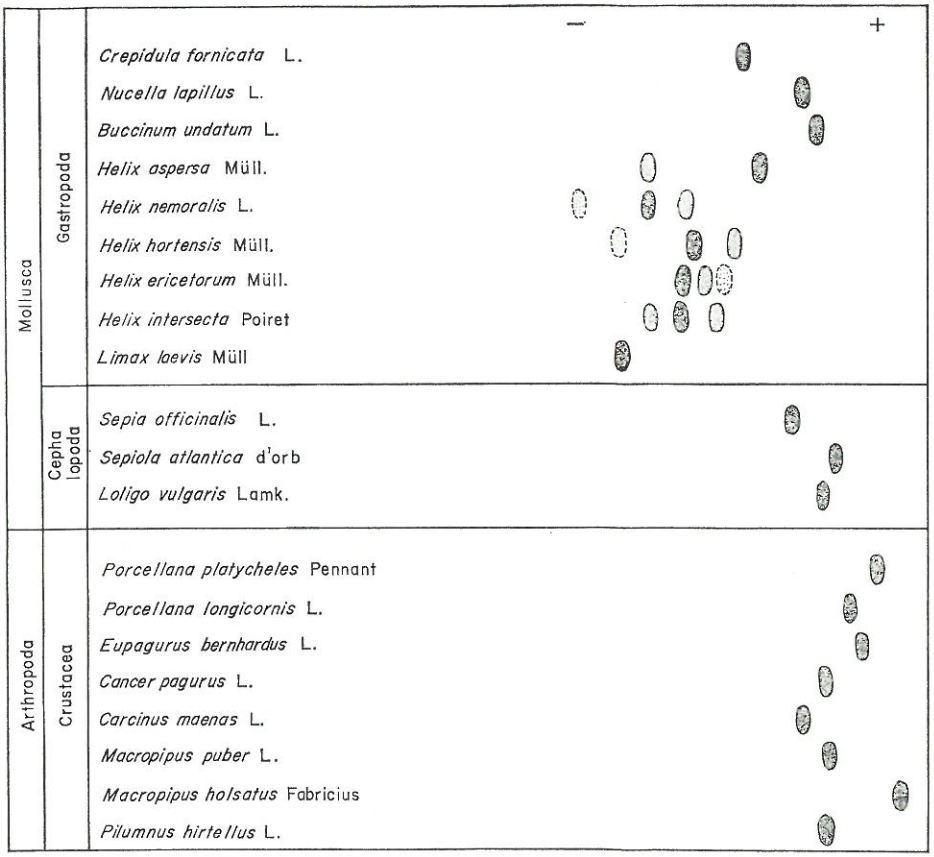

FIG. 2. Electrophoretic patterns of the hemocyanin from the blood of some arthropods and molluscs at pH 6·5.

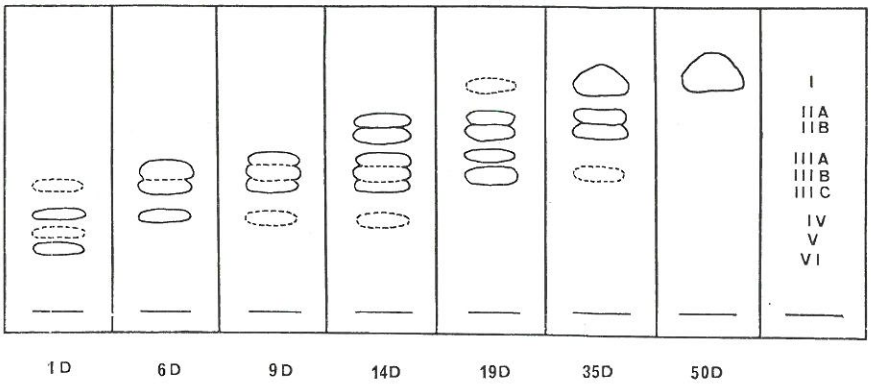

FIG. 3. Electrophoretic patterns of the blood from young *Sepia officinalis* specimens at pH 6·5 during the first days after eclosion.

These results were obtained with several hundred animals showing that the described changes in the electrophoretic pattern of newly eclosed *Sepia* specimens are quite reproducible. When the protein pattern was found to be delayed as compared to the age, then without exception, other parameters such as weight, length, quantity of yolk, state of liver development etc. were also retarded. So the change in hemocyanin composition during the first weeks of life of *Sepia officinalis* seems to be in strict correlation with the development of embryo to adult. This allowed us to divide the post-embryonic life of *Sepia* into different stages as published earlier (Richard & Decleir, 1969).

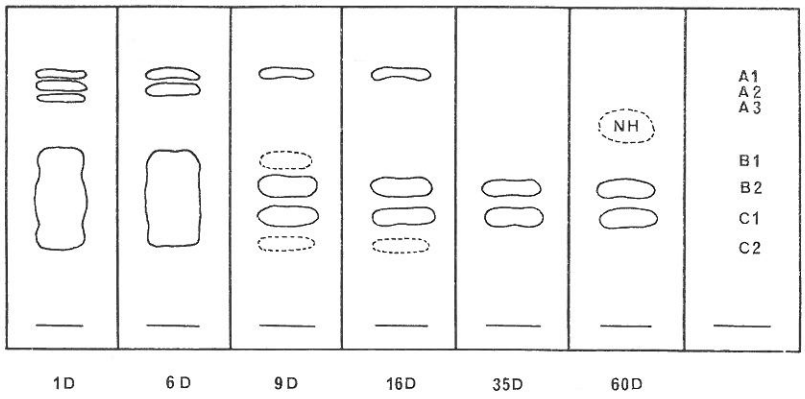

FIG. 4. Electrophoretic patterns of the blood from young *Sepia officinalis* specimens at pH 9·0 during the first days after eclosion. The only protein fraction with no hemocyanin properties is indicated with the letters NH.

Electron micrographs of gills of young and adult *Sepia officinalis* seem to support the stated difference between hemocyanin from young and adult specimens. In the blood vessels of adult gills (Fig. 5A) the big hemocyanin molecules aggregate and form linearly orientated rows. This phenomenon has already been described by Barber & Graziadei (1965), Eskeland (1967). Blood from gills of young animals (Fig. 5B) shows hemocyanin molecules of different sizes which do not tend to form chains like the adult molecules. These observations on slices of blood vessels in gills are now being compared with other electron microscopic techniques which are more appropriate to molecular studies of hemocyanins. The results of this comparison will be published later.

DISCUSSION AND CONCLUSION

The hemocyanin of *Sepia officinalis* was studied by an ultramicroelectrophoretic method using agar gel as supporting medium. This was done at pH 6·5 and pH 9·0. Intermediate values of pH gave large diffuse protein zones probably due to continuous association–dissociation reactions of the large hemocyanin polymers.

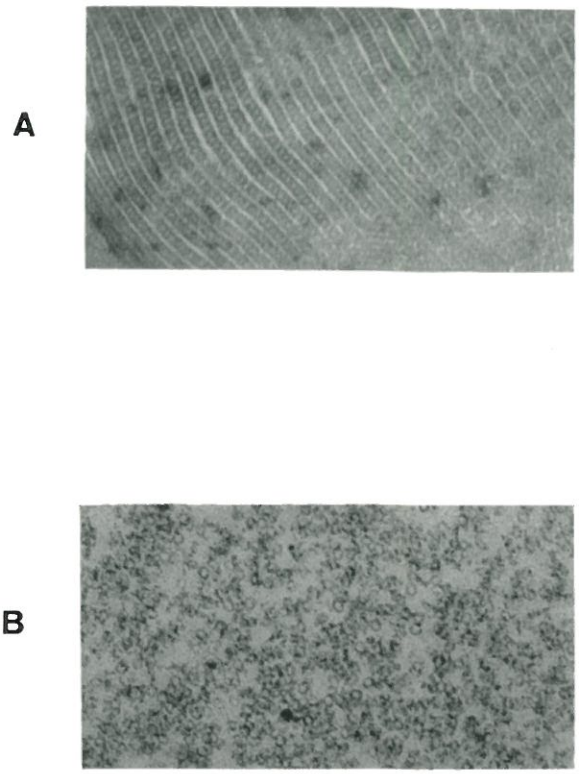

FIG. 5. Blood from adult animals reveals in the electron microscope linearly polymerized hemocyanin molecules (A). In young animals (B) we find a heterogeneous population of molecules which do not tend to aggregate. × 60,000.

At the two selected pH values we obtain electrophoretic patterns that are constant for animals older than 2 months, but that change considerably during the first weeks after eclosion. This has already been the subject of a short preliminary publication (Decleir & Richard, 1969).

Adult animals have in their blood a glycoprotein and hemocyanin. The latter forms the bulk of the blood proteins. It migrates in the electric field as a single fraction at pH 6·5 and splits into two equally important fractions at pH 9·0. This electrophoretic behaviour is shown by all the cephalopods tested, but not by animals belonging to other classes.

Electrophoretic patterns of young animals show at pH 6·5 and at pH 9·0 many protein fractions that all have hemocyanin properties. They contain copper, they give peroxidase and phenoloxidase reactions, and in the presence of Ca^{2+} they fuse to one single protein fraction. The non-hemocyanin glycoprotein fraction of adults emerges only during the third month after eclosion.

Immediately after eclosion three hemocyanin bands can be found at the two pH values. They disappear by the time the animal is 6 days old. This favours the idea of the existence of an embryonic hemocyanin. Together with the disappearance of these embryonic bands, other bands arise until, after about 50 days, the adult blood composition is obtained. It seems that the embryonic hemocyanin is replaced after eclosion by a young hemocyanin, which is gradually converted into the adult molecule.

We do not yet know the exact relationship between the proteins found at pH 6·5 and those found at pH 9·0. To resolve this problem we intend to continue our investigations by searching for the right circumstances to obtain corresponding separations at different intermediate pH values.

The results described in this paper point to a change in hemocyanin composition from the embryo to a 3-month-old specimen. This change is in correlation with other factors such as weight, length, quantity of yolk, colour of the liver etc. This change probably corresponds also with a change in size and properties of the molecule as revealed by electron microscopy.

Since the embryonic hemocyanin fractions fuse together in a Ca^{2+} containing buffer, the difference between embryonic and adult hemocyanin must be due to differences in the constituent monomers. If this is correct the difference between embryonic and adult hemocyanin can be explained as follows:

1. The hemocyanin monomers are synthetized in an organ that already exists in the embryo. In this case the difference between embryonic and adult monomers might be explained by, for instance, the subsequent incorporation of different quantities of copper.

2. The monomers are synthetized in different organs in the embryo and in the adult. The gradual formation of the adult organ may then be responsible for the gradual conversion of embryonic hemocyanin into the adult form. These organs may very well be the yolk sac in the embryo and the liver in the adult.

3. Both phenomena may occur together.

Which of these three hypothetical pathways for hemocyanin formation is

most likely to be correct cannot yet be proved. We must first learn more about the site and the mode of synthesis of this important invertebrate blood pigment.

Acknowledgements—We wish to thank Professor Vercauteren (University of Ghent) and Professor Defretin (University of Lille) for their constant interest in this work. We also thank Professor Wieme for his help and advice concerning the electrophoretic technique. The electron micrographs were obtained thanks to the collaboration of Mrs. Bonet from the Centre de Recherche sur la Cellule (Lille). We are indebted to Miss Coulombez for technical assistance. We are also indebted to the Centre National de la Recherche Scientifique for a grant to one of us (W. D.).

REFERENCES

BARBER V. C. & GRAZIADEI P. (1965) The fine structure of Cephalopod blood vessels. *Z. Zellforsch.* **66**, 765–781.

BESSE J. & MOCQUARD J. P. (1968) Étude par électrophorèse des quantités relatives des protéines de l'hémolymphe d'individus normaux et de femelles castrées chez deux crustacés Oniscoides. *C. r. hebd. Séanc. Acad. Sci., Paris* **267**, 2017–2019.

BHAGVAT K. & RICHTER D. (1938) Animal phenolases and adrenaline. *Biochem. J.* **32**, 1397–1406.

DECLEIR W. (1961) The localisation of copper in agar gel electrophoretic patterns of crustacean blood. *Naturwissenschaften* **48**, 102–103.

DECLEIR W. (1966) Phenoloxidase in Crustacea. Doctor's Thesis, University of Ghent, Belgium.

DECLEIR W. (1968) Study of the blood proteins in *Cancer pagurus*. *Archs int. Physiol. Biochim.* **76** (2), 368–370.

DECLEIR W. & VERCAUTEREN R. (1965) L'activité phénoloxydasique dans les leucocytes de crabe au cours du cycle d'intermue. *Cah. Biol. mar.* **6**, 163–172.

DECLEIR W. & RICHARD A. (1969) *Blood proteins in* Sepia officinalis. F.E.B.S. Publications, Madrid. Abstract 1020. Academic Press, London and New York.

ERIKSSON-QUENSEL I. & SVEDBERG T. (1936) The molecular weights and pH stability regions of the hemocyanins. *Biol. Bull.* **71**, 498–574.

ESKELAND T. (1967) Ultrastructure of hemocyanin components from the gastropod Buccinum undatum. *J. Ultrastruct. Res.* **17**, 544–564.

FERNANDEZ MORAN H., VAN BRUGGEN E. F. J. & OHTSUKI M. (1966) Macromolecular organization of hemocyanins and apohemocyanins as revealed by electron microscopy. *J. molec. Biol.* **16**, 191–207.

GHIRETTI F. (1956) The decomposition of hydrogen peroxide by hemocyanin and its dissociation products. *Archs Biochem. Biophys.* **63**, 165–176.

GHIRETTI-MAGALDI A., NUZZOLO C. & GHIRETTI F. (1966) Chemical studies on hemocyanin—I. Amino acid composition. *Biochemistry* **5**, 1943–1951.

GOODWIN T. W. (1960) Biochemistry of Pigments. In *The Physiology of Crustacea* p. 112. (Edited by WATERMAN T. H.). Academic Press, London & New York.

LAGARRIGUE J. G. & TRILLES J. P. (1969) Recherches écophysiologiques chez les Isopodes. Études électrophorétiques des protéines de l'hémolymphe de certains Isopodes marins, halophiles et terrestres. *C. r. hebd. Séanc. Acad. Sci., Paris* **268**, 838–841.

MANWELL C. & BAKER C. M. A. (1963) Starch gel electrophoresis of sera from some marine arthropods. *Comp. Biochem. Physiol.* **8**, 193–208.

RICHARD A. (1966) La température, facteur externe essentiel de croissance pour le Céphalopode *Sepia officinalis* L. *C. r. hebd. Séanc. Acad. Sci., Paris* **263**, 1138–1141.

RICHARD A. & DECLEIR W. (1969) Mise en évidence d'une variation des protéines sanguines au cours des premières semaines de vie post-embryonnaire chez *Sepia officinalis* L. *C. r. hebd. Séanc. Acad. Sci., Paris* **268**, 107–110.

VAN BRUGGEN E. F. J. (1968) Electron Microscopy of Hemocyanins. In *Physiology and Biochemistry of the Hemocyanins* pp. 37–48. (Edited by GHIRETTI F.). Academic Press, London & New York.

WIEME R. (1959) An improved technique of agar gel electrophoresis on microscope slides. *Clinica chim. acta* **4**, 317–321.

WITTERS R. & LONTIE R. (1968) Stability Regions and Amino Acid Composition of Gastropod Hemocyanins. In *Physiology and Biochemistry of the Hemocyanins* pp. 67–73. (Edited by GHIRETTI F.). Academic Press, London & New York.

Key Word Index—Hemocyanin; embryonic hemocyanin; copper proteins; blood proteins; differentiation of invertebrate blood proteins; invertebrate physiology; *Sepia officinalis*; Cephalopoda; Mollusca; proteins; protein subunits.