The pre-history of hemocyanin. The discovery of copper in the blood of molluscs

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Abstract. The presence of copper in molluscs was discovered in 1833 by Bartolomeo Bizio, a Venetian chemist, who found copper in marine gastropods of the family Muricidae during his studies on the purple dye he had isolated from these animals. Bizio was so surprised by this finding that he extended his analysis to several other invertebrate species in which he identified the metal.
Key words. Hemocyanin; copper.

The story of hemocyanin begins in 1878 when Léon Fredericq, while studying the physiology of *Octopus vulgaris* at the Zoological Station of Roscoff, isolated a blue pigment from the blood of the animal and recognized it as a protein which carries oxygen to the tissues. Fredericq wrote: "*Je propose de l'appeler Hémocyanine (de haima, sang, et cyanos, bleu), terme rappelant la parenté étroite avec l'hémoglobine du sang des Vertébrés. La combinaison avec l'oxygène serait naturellement l'oxyhémocyanine*"[6].

A scientific discovery is, like a river, the confluence of several tributary streams apparently less important because of their minor size. Upstream, there is the discovery that hemocyanin is the blood oxygen carrier of some invertebrate species, there is the change from blue to colorless of the blood when oxygen is removed, observed by former investigators, and there is the finding of copper instead of iron in the blood and tissues of those animals which have the blue pigment.

It was Paul Bert, the pupil of Claude Bernard, who observed in 1867 that the blood of cuttlefish turned from colorless to blue when exposed to air. "*L'action de l'air*", he says, "*se manifeste même à travers les parois des vaisseaux sanguins ... A l'état normal même dans l'eau, on aperçoit une légère différence de teinte entre le liquide des veines efférentes branchiales et celui des artères afférentes*"[1].

As for the presence of copper rather than iron in the blood, Bert was aware of the paper published by Emil Harless in 1847: "*Das blaue Blut einiger wirbellosen Tiere und dessen Kupfergehalt*"[7]. "*Je ne manquerai pas*", says Bert in his paper on the physiology of the cuttlefish, "*de rechercher dans le sang la présence du cuivre qui a été signalée par Harless et Bibra, et qui, s'il faut en croire les analyses de plusieurs chimistes, remplacerait le fer dans le sang de beaucoup d'invertébrés*"[1].

"*S'il faut en croire ...*". The scepticism of Bert was shared by many scientists and persisted for years. In 1919 Philippi was still looking for the presence of iron (and of porphyrin) in the hemocyanin molecule[11].

Actually, Harless was interested in the influence of gases on the color change of the blood of invertebrates, and erroneously believed that carbonic acid turned the blood of Cephalopods from colorless to blue. As mentioned before, this was refuted by Bert who unequivocally demonstrated the oxygenation reaction of the blood.

Harless collected the biological material in Trieste from the Adriatic sea. He used the blood and the organs of *Octopus, Sepia, Sepiola, Cancer*, etc. and the chemical analyses were carried out by his friend Bibra. The dried blood and organs were incinerated and the ashes dissolved in nitric acid. Copper was determined electrochemically, as well as copper oxide. It was found that in both the blood and the liver no iron was present, this metal being entirely replaced by copper.

Harless' paper is well known in the scientific literature. Does, therefore, the story of hemocyanin go back only to 1847?

Fourteen years before that, Bartolomeo Bizio described the presence of copper in marine gastropod species of the family Muricidae. He announced this finding in a short note entitled "*Scoperta recentissima del rame nei murici porporiferi*". This note, which was followed by several lengthy papers on the same subject, appeared in 1833 as an appendix to a study on the purple "tyrian" dye he had discovered in the same gastropod species[2].

Bizio, who was born near Vicenza in 1791, became a chemist and apothecary in Venice[9]. He was the author of more than one hundred papers on topics of physical and analytical chemistry which were published, mostly in Italian, in the annual Proceedings of the local scientific institutions and academies. The most relevant are the investigations on the nature of red spots which appeared on some foods and the discovery of the purple dye in marine animals.

Bartolomeo Bizio demonstrated that the contaminant growing as red spots on homemade maize flour puddings and upon other foods, were of biological origin and due to "a new microscopic organism" which he called *Serratia marcescens*, today classified in the family of Enterobacteriaceae. (In bacteriological taxonomy this genus is indicated with his name: *Serratia*, Bizio, 1823)[8,10].

The observation that sunlight caused a color change in the secretion from the hypobranchial gland of two marine gastropod species, *Murex brandaris* and *Murex trunculus*, and the discovery of the tyrian dye, was the starting point of a long series of studies which gave Bizio a high reputation in his country and abroad. It was during these studies that he found considerable amounts of copper in the body and the organs of these animals.

Bizio incinerated the dried biological material and dissolved the ashes in hydrochloric acid, filtered the precipitate and added ammonia. The presence of copper was indicated by the appearance of a blue color. From this solution, after acidification with acetic acid, the metal was precipitated as red copper cyanide by addition of few drops of potassium-ferrocyanide.

Copper was known to be a toxic element and it was quite unexpected in living animals. Bizio wondered whether the intoxications which frequently occurred to *Murex* eaters were due to copper, "*il pernicioso metallo*" as he says. He repeated the analyses over and over again and extended them to a number of other molluscan species[3,4]. He also investigated the source of the metal: did it come from the water or from the soil? Or was it present in the food? Did copper in the animals originate from the hulls of the ships which crowded the Adriatic Sea? It is interesting to remember that Harless had considered exactly the same points. He too investigated the environment where the animals lived and checked whether the sea was polluted with some copper material. Both authors excluded any such copper contamination originating from the sea. Bizio analyzed very carefully the stomach and its contents and also excluded any alimentary origin of copper in *Murex*. He concluded that copper is a normal component of the body of the animal as are other elements like sodium, potassium, calcium and all the inorganic substances known in the elementary composition of living organisms.

The presence of copper in marine animals, however, was received skeptically by the scientific community. Aiming to present incontrovertible proof, Bizio finally succeeded in obtaining metallic copper by electrochemical precipitation onto the surface of an iron thread immersed in the solution. Touch it, if you don't believe![5].

Bizio never analyzed the blood of the animals as Harless did. He used the whole body of the *Murex* and of other animals species; in many cases he carefully dissected out the muscle foot and the spiral body ("*la spira*"), the stomach, and analyzed them separately. The animals used by Bizio are not suitable for collecting blood, certainly less so than the Cephalopods used by Harless. It is possible, however, that Bizio never tried to bleed his animals because blood was simply not in the field of his interest. Bizio also found a faintly positive reaction with iron cyanide when testing marine species (such as molluscan Bivalves) which are lacking in the copper blood respiratory pigment. However, he says: "Unequivocally, among all molluscan species investigated, none contained so much copper as *Murex* species". And *Murex* species have hemocyanin.

Did Harless know the work of Bizio? Harless never mentions this author in his paper. Yet his work closely parallels the investigations of Bizio. Apart from the biological material used (Cephalopod species by Harless, Gastropod species by Bizio), the problems they had in mind are much the same. It cannot be excluded that rumors of the surprising discovery of copper made by Bizio, crossed the border between Venice and Trieste. Trieste was a well-known cultural center of 'Mitteleuropa'. In this city German scientists lived and worked long before the foundation of the Marine Station in 1875. The local fauna and flora were described in 1845 by J. J. C. Gravenhoret in a publication called 'Tergestina' (quoted by Specchi[12]). In his paper Harless tells of his visit to the Zoological Museum of Trieste (at that time a section of the Union of Natural History) where he met the Director, Enrico Koch, a Swiss malacologist, and asked for information about the ecology of Cephalopods in the Adriatic sea.

Copper is, after iron, the most abundant trace element in animals. For a long time, however, the only copper proteins with a known function have been hemocyanin and tyrosinase. In the thirties, other copper proteins were isolated from mammalian blood and liver, and were named generically hemocuprein and hepatocuprein. The function of these and other tissue copper proteins were unknown until 1971 when they were identified as superoxide dismutase. Today more than 20 copper proteins are known, each having a specific function and a copper-containing active site. Hemocyanin, therefore, is a member of a large family of copper proteins, where it stands out not only because of its molecular size, but also because of its historical rôle.

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