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Determination of copper in embryos and very young specimens of *Sepia officinalis*

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Abstract

The total amount of copper in embryos and newly hatched young individuals of *Sepia officinalis* L. has been determined by microtechnique, using bathocuproine-sulfonate as complexing reagent. During embryonic life, the total amount of copper does not change; it remains at a level close to 3.8 µg. The copper is found in the yolk sac of very early embryos; it is subsequently transferred into the embryo proper. After hatching, the copper content diminishes quickly in starved individuals. Fed *S. officinalis* also usually lose copper. The reason for this may be that the inner yolk sac of newly hatched individuals contains a great deal of the total copper, which is excreted with the yolk after the latter has become superfluous. Later on, copper must be taken up from the food. The mobilization of protein and copper from the yolk into the blood may account for the early appearance of embryonic hemocyanin in the blood.

Introduction

Copper is a very important trace element in living matter; it occurs in every cell as part of the cytochromeoxydase-complex. Several tissues contain relatively high concentrations of copper, which may be present in oxydases and blood proteins.

Copper determinations in different animals show that mean values of copper content in invertebrates are much higher than in vertebrates. The following values are given by Clare (1949):

Insects	0.0919 mg copper/g dry weight
Marine invertebrates	0.1736 mg copper/g dry weight
Vertebrates	0.0119 mg copper/g dry weight.

Copper occurs predominantly in the form of phenoloxydase in insects, and in the form of hemocyanin in molluscs and crustaceans. Although there are many reports on the occurrence and localisation of copper in different invertebrate tissues, nothing is known about its uptake and incorporation into proteins.

As a first approach to the problem of "copper metabolism" in the cuttlefish *Sepia officinalis* L., we have measured the total copper content in embryos and newly hatched young individuals. The results of our determinations are presented and discussed in this paper.

Material and methods

All copper determinations presented and discussed here were made using specimens of *Sepia officinalis* L. Adult individuals were caught in the coastal waters of the Wimereux (France) region and allowed to mate in aquaria of the Marine Station. Soon after mating, the female starts oviposition (Richard, 1968). The very large eggs are attached singly to short wooden sticks, resulting in an arrangement comparable to a bunch of black grapes. One such cluster of eggs was reserved for our copper studies. At regular intervals, a few eggs were removed from this cluster and opened. The outside egg membrane was discarded and the periembryonic fluid set aside for ionic content analyses (not discussed in this paper). The embryo and its yolk sac were carefully separated and placed apart, in calibrated micro-Kjeldahl vessels of 1 ml capacity.

Digestion was carried out by adding 0.2 ml perchloric acid to each vessel. The vessels were placed into corresponding holes in an aluminium plate and heated on a warming plate until evaporation was complete. If the resulting ash was still slightly coloured, another 0.2 ml perchloric acid was added and evaporated until the ashes were entirely colourless. Finally, copper was determined by measuring the absorption of a cuprous bathocuproinesulfonate complex. This was obtained by adding the following reagents to the digested material in the Kjeldahl vessels:

— 0.30 ml, containing 0.20 mg 2.9 dimethyl-4.7-diphenyl-1.10 phenantrolinesulfonate (bathocuproine-sulfonate — Fluka) in 20% sodium acetate;
— 0.1 ml saturated ascorbic acid solution;
— 20% sodium acetate to 1 ml.

After thorough mixing, the yellow-orange colour of the copper complex is measured at 484 mµ in 0.4 ml bowls of a Jobin and Yvon spectrophotometer.

The calibration curve was obtained with copper standards made by dissolving pure copper metal in acid bidistilled water.

Results

Eggs of cephalopods are characterized by a very large quantity of yolk. The embryo forms as a small disc on the top of a large yolk sac. As development proceeds, the embryo grows larger while the size of the outer yolk sac diminishes. At the moment of hatching a very small outer yolk sac may remain, but it is lost after a maximum period of 1 day (the period may be

longer in cases of premature hatchings). In our experiments on *Sepia officinalis* embryos we determined the embryonic stage by the length of the dorsal part of the embryo as shown in Fig. 1.

The results of copper determinations before hatching are shown in Fig. 2, and of copper determinations in young individuals immediately after hatching, in Fig. 3.

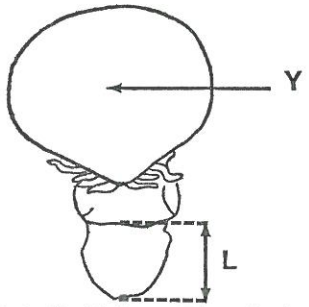

Fig. 1. *Sepia officinalis*. Embryo. *L* length of mantle; *Y* outer yolk sac

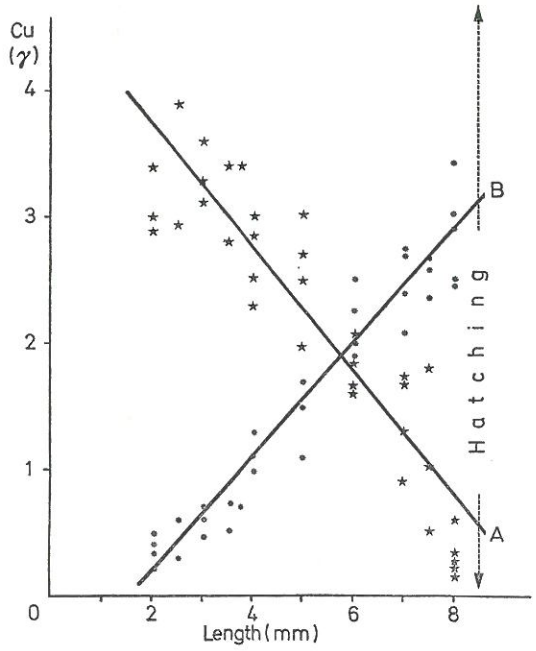

Fig. 2. *Sepia officinalis*. Determinations of copper during embryonic life. *A* Copper content in the outer yolk sac; *B* copper content in the embryo

Before hatching, the copper content in the outer yolk sac decreases constantly. A linear relationship between copper content and embryonic stage exists, which may be described by the following equation:

$$Q_A = -0.49\,L + 4.75 \qquad (1)$$

where Q_A is the copper content in the outer yolk sac in µg and L the length of the embryo in mm.

The copper content in embryos shows a corresponding increase, the equation being:

$$Q_B = 0.47\,L - 0.76 \qquad (2)$$

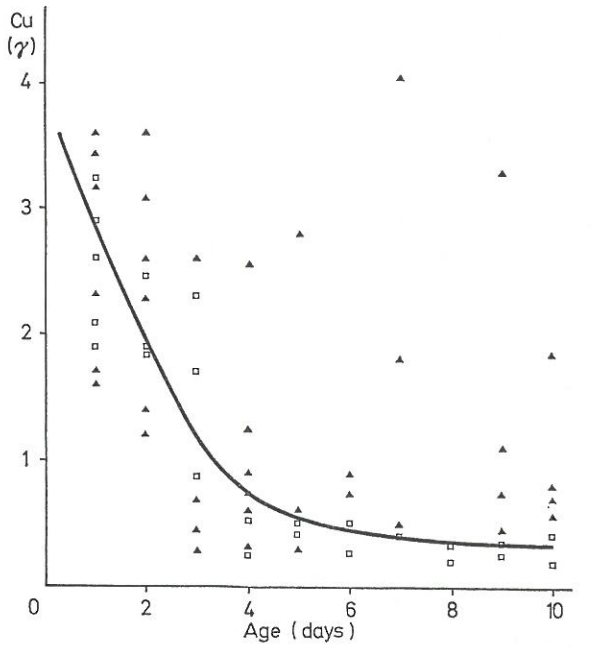

Fig. 3. *Sepia officinalis*. Determinations of copper in young individuals; fed (filled triangles) and starved (open squares and solid line)

where Q_B is the copper content in the embryo in µg and L the length in mm.

The slopes of the 2 curves are opposite to each other, indicating the existence of a reciprocal relationship between the copper content of the outer yolk sac and that of the embryo. We may write:

$$Q_A + Q_B = k. \qquad (3)$$

This constant, under our experimental conditions, has a value of 3.80/4 µg.

After hatching (Fig. 3), the copper is quickly lost by starved individuals. In fed animals the amount of copper varies considerably. Individuals older than 10 days were not analysed since they had become too large to be introduced into our small Kjeldahl vessels. We intend to continue our investigations with other sets of vessels in order to follow the changes in total copper content during the first months after hatching.

Discussion and conclusion

Copper was determined in embryos and young individuals of *Sepia officinalis* employing bathocu-

proinesulfonate. This product immediately forms a very stable complex with monovalent copper, a reaction which is very specific and very sensitive (Zak, 1958).

The values of total copper in embryos and outer yolk sacs show that copper must be incorporated into the yolk before being transferred to the embryo. The total amount of copper in an embryo just before hatching corresponds to the total amount of copper incorporated initially in the yolk sac. These results are not in agreement with those obtained by Ranzi (1938), who found that the copper in embryos of *Sepia officinalis* has its origin in the surrounding seawater, and accumulates as development proceeds.

The divergent results after hatching are difficult to explain. We presume that the following factors play an important part:

(1) At the moment of hatching a very large inner yolk sac exists, which disappears within a few days. The inner yolk sac probably still contains much copper which is possibly excreted together with the yolk.

(2) When food is offered to a group of freshly hatched specimens there is great competition for nourishment. Starting with a homogeneous group we observed, after some time, striking differences in size and activity. The copper content in young animals after hatching may very well be correlated with the quantity of food taken up, giving very different results.

Recently, we were able to prove the existence of an embryonic hemocyanin in *Sepia officinalis* (Decleir and Richard, 1970). This is probably preceded by another embryonic hemocyanin occurring only in early embryos (Decleir and Richard, unpublished). These embryonic hemocyanins probably originate from protein and copper of the yolk sac. Portmann and Bidder (1928) have described the secretory function of the vitelline membrane in *Loligo vulgaris*. They have shown that the membrane of the yolk sac thickens in certain regions, while liquefied yolk particles appear and are transferred from the yolk to the blood.

Our efforts to demonstrate copper histochemically in the yolk sac have been unsuccessful. The embryos were fixed in cold (4 °C) formalin calcium Baker's fluid and imbedded in paraffin. The sections were stained for copper with rubeanic acid according to the method of Okamoto (1938), and Uzman (1956) as cited by Pearse (1960). It has been shown that the minimum amount of copper demonstrable with the rubeanic acid method for electropherograms after agar gel electrophoresis is 0.13 µg put together in a narrow slit (Decleir, 1961). Furthermore, we have found that the minimum amount of copper necessary to obtain a visible green colour in a test tube with the rubeanic acid method which we used for the histochemical localisation, is about 1 µg per ml.

These tests show that the rubeanic acid method to demonstrate copper in thin slices of the yolk sac or embryos is not sufficiently sensitive. They also prove that the copper we demonstrated in the yolk sac is not concentrated in granules, but is probably distributed homogeneously throughout the yolk.

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Summary

1. The total amount of copper is different in embryos and newly hatched individuals of the mollusc *Sepia officinalis* L.

2. In embryos, the amount of total copper remains practically constant. It occurs in the yolk sac of the very early embryo; later, it is transferred into the embryonic tissue.

3. In the freshly hatched young, the amount of copper is quickly reduced. The copper is, possibly, excreted with the remaining yolk. Subsequently, the copper required must be secured from the food consumed.

4. Mobilization of protein and copper from yolk into blood may account for the presence of hemocyanin in the embryonic blood.

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