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FUNCTIONAL PROPERTIES OF THE HEMOGLOBINS OF *ARTEMIA SALINA* L.

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Abstract—1. In hypoxic conditions *Artemia salina* L. forms 3 hemoglobins, which until now had only been characterized by their electrophoretic mobility.

2. We have analysed the oxygen-binding properties of these 3 hemoglobins.

3. HbI shows the lowest oxygen affinity, has an almost negligible Bohr effect and it has no physiological adaptation to temperature changes.

4. HbII shows an intermediate oxygen affinity and a pronounced positive Bohr effect.

5. HbIII has a very high affinity for oxygen, an almost negligible Bohr effect and a good physiological adaptation to temperature changes.

6. By combining the 3 hemoglobins in different proportions *Artemia salina* L. must be able to withstand diverging environmental conditions.

INTRODUCTION

The brine shrimp, *Artemia salina* L., inhabits an ecological niche which shows a great diversity in some environmental factors like salinity, temperature, partial oxygen pressure, etc. to which this animal must show a variety of adaptations. One of these is the development of high concentrations of respiratory pigment in hypoxic conditions. Moreover, Bowen *et al.* (1969) have shown the existence in *Artemia salina* of 3 different hemoglobins which have been called HbI, II and III according to their electrophoretic mobility on cellulose acetate gel strips in 25 mM sodium 5:5-diethylbarbiturate-HCl at pH 8.6.

This paper is a first effort towards the functional characterization of these 3 hemoglobins with regard to the influence of some environmental factors on oxygen affinity.

MATERIAL AND METHODS

Starting with a total homogenate (S105) from deep frozen *Artemia salina* L. (San Francisco) the hemoglobins were isolated by ammonium sulphate precipitation and further purified by stepwise and gradient elutions with NaCl in 50 mM Tris-HCl, pH 7.5 on DEAE-Sephadex A50 (Waring *et al.*, 1970; Moens & Kondo, to be published). The separated HbI, II and III were concentrated by ultrafiltration (Amicon Diaflo system with filter UM-10) and desalted on Sephadex G-25 in 5 mM Tris-glycine, pH 8.3. Finally the purity was controlled by cellulose acetate electrophoresis (Bowen *et al.*, 1969). The 3 separated hemoglobins were dialysed (Visking 18/32-20 hr at 3°C) against buffers with different pH and different ionic strength and subsequently frozen in small fractions and stored at −70°C until use.

Oxyhemoglobin dissociation curves were determined with the diffusion chamber method of Sick & Gersonde (1969). The diffusion chamber was constructed by Eschweiler & Co., Kiel.

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RESULTS

1. *Comparison of the oxygen dissociation curves of* 3 *purified hemoglobins from* Artemia salina *L*

HbI, II and III, dissolved in 0.1 M boric acid buffer, pH 8.5, with 0.6 M NaCl and 0.02 M KCl at 25°C, show P_{50} values of 6.49, 3.99 and 1.83 mm Hg respectively. They all have a typical sigmoid dissociation curve (Fig. 1) with a Hill coefficient, *n*, of 1.81 (HbI), 2.00 (HbII) and 2.05 (HbIII). The hemoglobin concentration of the preparations used for the measurements, estimated from the extinction value at 412 nm, has no influence on the P_{50} value.

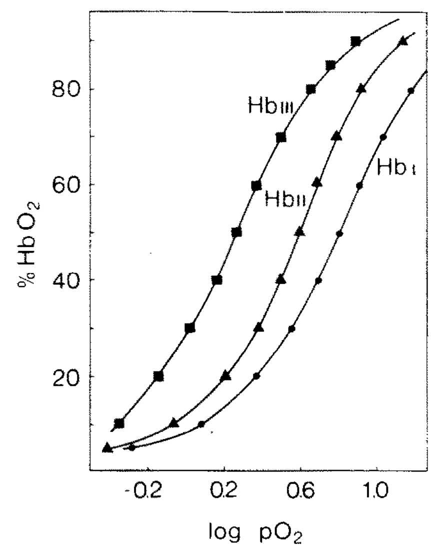

Fig. 1. Oxygen dissociation curves of HbI, II and III from *Artemia salina* L. in 0.1 M boric acid buffer, pH 8.5, with 0.6 M NaCl and 0.02 M KCl at 25°C.

2. *Effect of ionic strength*

P_{50} values were determined in 50 mM phosphate buffers, pH 7.5, with increasing NaCl concentrations from 0.2 up to 1 M and a constant concentration of 0.02 M KCl. No significant difference in P_{50} value could be found.

3. *pH and temperature effect*

The 3 isolated and purified hemoglobins were dialysed against 50 mM phosphate buffers or 0.1 M boric acid buffers with different pH values. The buffer solutions always contain 0.6 M NaCl and 0.02 M KCl.

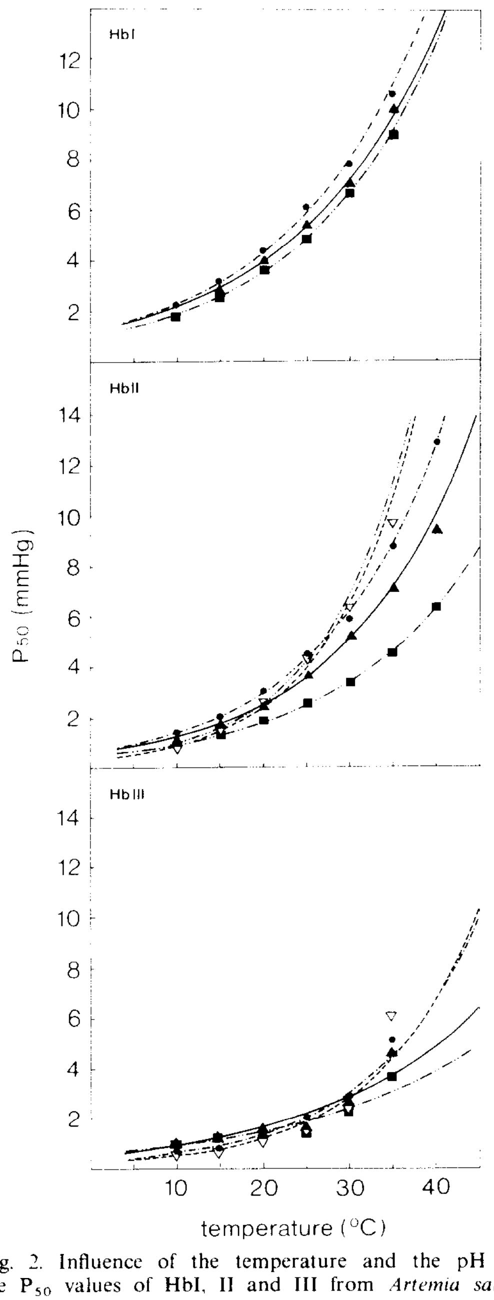

Fig. 2. Influence of the temperature and the pH on the P_{50} values of HbI, II and III from *Artemia salina* L. Symbols: ··········· average P_{50} value at pH 7.0; — — ▽— — — average P_{50} value at pH 7.5; —·—● —·— average P_{50} value at pH 8.0; —— ▲—— average P_{50} value at pH 8.5; —··—■—··— average P_{50} value at pH 9.0.

The oxygen binding properties of the 3 hemoglobins were examined in a temperature range from 10 to 35°C (intervals of 5°C) and at pH values ranging from 7 to 9 (intervals of 0.5 pH units).

In this way the measurements are approximately restricted to physiological conditions: we determined the pH of the hemolymph to be 8.5: the natural habitat shows annual temperature fluctuations from 8 to 33°C (Baker, 1966).

The influence of pH and temperature on the P_{50} values of the 3 hemoglobins is represented in Fig. 2. The P_{50} values plotted are calculated from 5 measurements each.

DISCUSSION

The oxygen binding properties of the 3 hemoglobins in *Artemia salina* L. were analysed by means of the diffusion-chamber technique.

All 3 hemoglobins show a typical sigmoid dissociation curve (Fig. 1). Their oxygen affinities are moderately high and they differ as follows: HbIII > HbII > HbI. This means that the 3 hemoglobins are adapted to cope with different environmental P_{O_2} values, HbI being more efficient in oxygen richer water than the other hemoglobins and HbIII being most efficient in very poorly oxygenated water.

The P_{50} values were not influenced by the ionicity nor by the concentration of the pigments. It is known that *Artemia* can adapt to media varying from one tenth of seawater, wherein it is a hypertonic regulator, to crystallizing brine, wherein the animal is a very effective hyporegulator. Nevertheless between these limits the osmotic pressure of the body fluids and hemolymph may change considerably (Croghan, 1958) and this does not seem to alter the properties of the respiratory pigments.

The effect of temperature on the P_{50} values is in general a decrease in hemoglobin O_2-affinity (increase in P_{50} value) for an increase in hemolymph temperature. In ectothermic animals adaptations to alter the temperature dependence of the oxyhemoglobin dissociation curve are very important. In *Artemia* HbI shows no adaptation at all. HbII only a very slight one, while HbIII is less temperature sensitive than the others and seems to be well adapted for adequate oxygen transport at high temperatures (Fig. 2).

The pH effect on the P_{50} values (or Bohr effect) seems to be temperature-dependent above pH 8.5, and each hemoglobin shows a positive Bohr effect between 15 and 35°C. This positive Bohr effect is most pronounced for HbII which consequently seems to be less suited for oxygen loading in the gills at high environmental P_{CO_2} pressures than the other hemoglobins (Fig. 2).

Other important physiological properties of respiratory pigments such as the Root effect and the capacity cannot be studied with our diffusion chamber technique which only measures relative differences in extinction.

A great deal of critical discussion on the role of the respiratory pigments centers around the relevance of *in vitro* experiments. In the case of *Artemia* we are dealing with a small animal and for the moment it seems impossible to do analogous *in vivo* deter-

minations of oxyhemoglobin dissocation curves. We know almost nothing about the temperature and the pH variations in the hemolymph of these animals. Consequently, at this time, we can only deduce the function of the hemoglobins of *Artemia* from their oxygenation properties *in vitro*. Moreover the hemoglobins from *Artemia* are extracellular pigments and hence they are probably less influenced by specific local factors that may alter the properties of intracellular pigments.

The results of our study show that the 3 hemoglobins in *Artemia salina* L. are differently adapted to different environmental factors. By the formation of three hemoglobins in varying proportions this animal must be able to cope with extremely different environmental conditions.

The results of our oxygen binding studies which can be related to structural aspects of the respiratory pigment will be published later (Kondo *et al.*).

SUMMARY

Artemia salina L. forms 3 hemoglobins in hypoxic conditions. We have isolated these 3 hemoglobins and we have determined their oxygen-binding properties. The ecophysiological role of these pigments is discussed.

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