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THE INFLUENCE OF SERUM LIPIDS ON OXYGEN BINDING OF *CALLINECTES SAPIDUS* HEMOCYANIN

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Abstract—1. Addition of the detergent Triton X100, which removes lipid moieties from lipoproteins, to the serum of *Callinectes sapidus* did not change hemocyanin–oxygen affinity.

2. Addition of the soluble fraction and/or vesicles of phosphatidyl choline, the major lipid in crustacean blood, to Triton X-treated hemocyanin did not alter oxygen affinity. Addition of lipids prepared from native serum did not influence oxygen affinity.

3. Despite the close correlation between the two in nature, the seasonal changes in serum phospholipids cannot explain the seasonal changes in hemocyanin–oxygen affinity in this species.

INTRODUCTION

Recently Zatta (1981) reported that the hemocyanin (Hc) of the green crab *Carcinus maenas* has a phospholipid content of about 1.5% by weight. Dialysis against Triton X100 to remove the lipid moiety altered the kinetics of the reaction of the molecule with KCN and also the circular dichroism spectrum. Since the former alteration was abolished in part by simply adding extracted lipid (Zatta, 1981), the association appeared to be quite loose. Although not reported in full, Zatta mentioned data collected by L. Tallandini showing that delipidated Hc has a 30% greater O_2 affinity, an alteration that was also abolished in part by simply adding lipid to a solution of Hc. Tallandini and Zatta kindly communicated to us an example of these data in which the Triton exposed Hc retains its cooperativity in full but has a higher O_2 affinity. Zatta (1981) concluded that the liquid moiety serves to maintain the active site in a more stable, closed configuration.

Earlier, Kerr (1969) had shown that a serum phospholipid found in the blue crab *Callinectes sapidus* Rathbun varies seasonally: its levels increase in the fall and decrease in the spring (see also Lee, 1985). If the phospholipid formed a loose association with the Hc in this species, one would expect Hc–O_2 affinity to be higher after its levels increase and lower after they decrease, with no change in cooperativity. Since this is exactly the seasonal trend in O_2 binding observed in animals collected at the two different times of the year, Mauro and Mangum (1982) suggested that the phospholipid might be the effector.

We have examined the effects of Triton and the subsequent addition of lipid on O_2 binding of *C. sapidus* Hc. It was pointed out to us by W. Schartau that an appreciable lipid content would retard sedimentation in aqueous media and thus bias estimates of mol. wt obtained from ultracentrifugation, whereas in fact the estimates agree with data obtained by alternative methods. In our experience, however, serum lipids often undergo a phase inversion and thus are removed from aqueous solution during the purification procedures that precede determination of molecular weight. Therefore we have examined whole serum as well as purified Hc.

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MATERIALS AND METHODS

Animals were collected by trawling in the waters off Skidaway Island, Georgia and maintained in running seawater (20–23°C, 28–30‰) for periods ranging from 1 to 10 days. Blood was taken from the infrabranchial sinuses into a hypodermic syringe, allowed to clot in a tissue grinder and the clot removed by homogenization followed by centrifugation.

The serum was added to a solution of Triton X100 (final concentration 0.02 g detergent/g Hc or protein) and stirred for 1 hr at room temperature. Protein and Hc concentrations were measured by adding 0.1 ml serum to 3.9 ml dissociating buffer (0.05 M Tris + 0.05 mM EDTA, pH 8.9) and measuring absorbance at 280 and 338 nm (extinction coefficients from Nickerson and Van Holde, 1971). To remove the emulsified lipid–detergent complex, Bio-Beads (SM-220-50 mesh; BioRad Co.) were then added in a concentration of 1 g/g Hc (or protein) and the solution stirred for another hour. The Bio-Beads were removed by filtration through cotton.

Since more than 90% of the lipids in crustacean blood are phospholipids (Lee and Puppione, 1978), phosphatidyl choline was chosen for addition to the Triton treated Hc. It was added to either whole serum or purified (centrifuged at speeds up to 100,000 *g* for several hours) Hc in either or both of two forms: (1) soluble, by dissolution of the phospholipid in the appropriate saline, and (2) phospholipid vesicles. Phosphatidyl choline vesicles were prepared by adding 10 mg to 1 ml methanol: chloroform (1:1). After mixing, the organic solvents were volatilized with N_2 and 0.1 M $MgCl_2$ and 0.05 M Tris HCl buffer (pH 7.5) were added to the residue. The preparation was then sonicated on ice for 30 min at 30% of maximum setting and added to

serum at a ratio of 1 mg Hc:1 mg phosphatidylcholine vesicles. This mixture was stirred gently for at least 30 min at 30°C.

In a final experiment the lipids prepared from native blood were added to Triton treated Hc. The blood was extracted with methanol and chloroform (Bligh and Dyer, 1959), allowed to stand overnight and the organic phase evaporated under N_2. Serum was then added to the residue.

Because they are important effectors of O_2 binding, the levels of Ca^{2+} and L-lactate were also determined. Ca^{2+} activity was measured with an ion selective electrode (Mangum and Lykkeboe, 1979) and lactate was determined by enzymatic conversion to pyruvate (Graham *et al.*, 1983).

O_2 binding was determined by the cell respiration method (Mangum and Lykkoboe, 1979).

RESULTS

Using either male or mature female crabs, we found no effect of the delipidation procedure on HcO_2 affinity (Fig. 1). Regression lines fitted to the data are not significantly different ($P > 0.05$) throughout the pH range investigated. The Triton treated Hc may appear to have either a lower (Fig. 1A) or a higher (Fig. 1B) O_2 affinity, or the regression lines may cross in the middle of the pH range investigated (Fig. 1C). The addition of either dissolved phosphatidyl choline + phosphatidyl choline vesicles (Fig. 1A), or the soluble fraction alone (Fig. 1B), also failed to influence O_2 affinity either significantly or in a consistent direction. Finally the addition of the native lipid fraction to Triton treated Hc fails to influence O_2 affinity (Fig. 1C). To ensure that our negative results were not due to low levels of serum phospholipid, we deliberately selected the sera of mature females which were extremely orange, indicating a very high serum lipid level (Lee, 1985). Moreover, we conducted these experiments in May, a time when Georgia crabs are experiencing a temperature regime similar to that at which the seasonal increase in HcO_2 affinity was originally observed (Mauro and Mangum, 1982).

DISCUSSION

We have not shown that *C. sapidus* Hc does, in fact, have a lipid moiety. However, our results clearly show that removing the lipids from whole serum and/or subjecting purified Hc to a delipidation procedure has no detectable effect on HcO_2 affinity in this species, even when high levels of the orange lipoprotein are present. It seems highly unlikely that a lipid moiety of the Hc molecule is responsible for the seasonal adaptation of the O_2 transport system

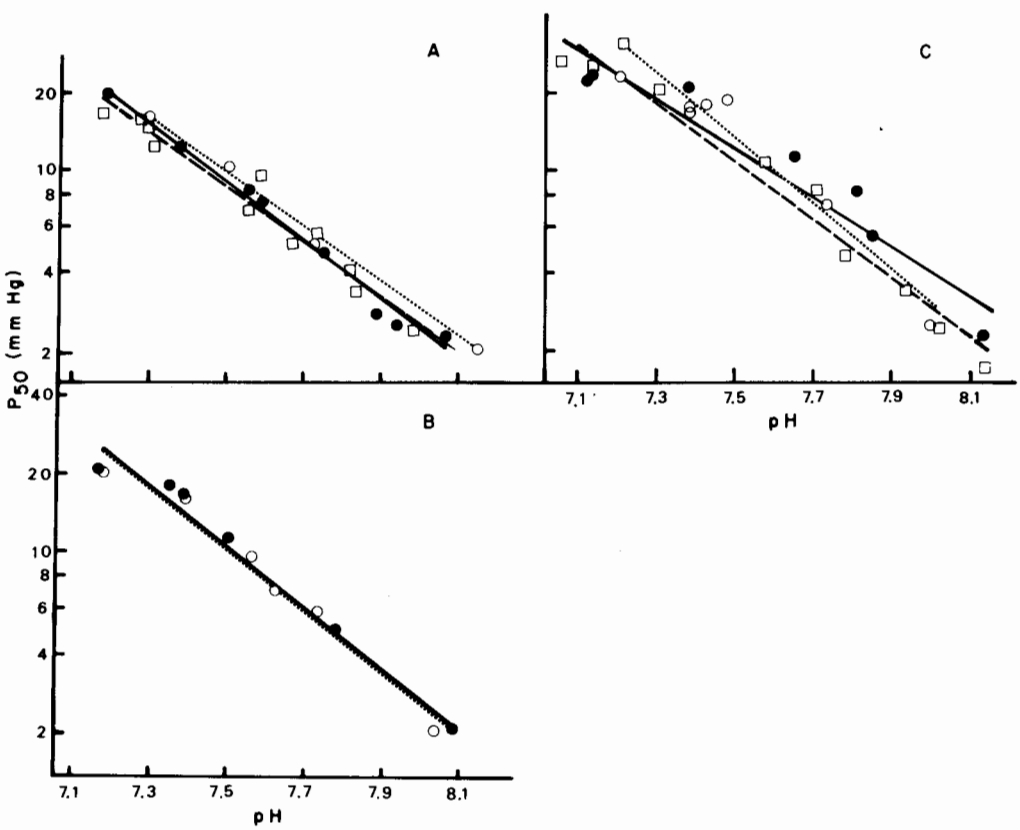

Fig. 1. Effects of Triton treatment and the subsequent addition of lipid to the hemocyanin of the blue crab *Callinectes sapidus*. 20°C, 8.40–9.22 mM Ca^{2+}, ≤0.61 mM lactate, Hc concentration 2.6–6.6 g/100 ml. Lines fitted by semilogarithmic regression analysis. (A) Serum from seven males, 11.5 ± 0.4 (SE) cm carapace width. (●) Controls, r^2 0.989; (○) + Triton × 100, r^2 0.998; (□) + vesiculated and soluble phosphatidyl choline (33 mg/ml), r^2 0.956. (B) Serum from four mature females, 13.2 ± 0.6 cm. (●) Controls, r^2 0.976; (○) + Triton × 100, r^2 0.969. (C) Hc from seven males, 11.2 ± 0.4 cm. (●) Controls, r^2 0.914; (○) + Triton 100X, r^2 0.939; (□) + soluble lipids extracted from native serum, r^2 0.950.

reported by Mauro and Mangum (1982), even though the correlative relationships between lipid concentrations and HcO_2 binding, and also the seasonal changes in both, support the hypothesis.

What is responsible for the seasonal adaptability of HcO_2 transport systems? The known, naturally occurring effectors of HcO_2 binding in crustaceans include H^+, divalent cations, heavy metals, Cl^-, L-lactic acid and uric acid (reviewed by Mangum, 1980, 1983a; also Brouwer *et al.*, 1982 and Morris *et al.*, 1985). There are also believed to be yet unidentified effectors that raise O_2 affinity (reviewed by Bridges and Morris, 1986). Of these, only L-lactic acid and Ca^{2+} are known to vary *in vivo* in a sufficiently adaptive fashion to be designated modulators (Truchot, 1980; Booth *et al.*, 1983; Graham *et al.*, 1983; Mangum, 1985). However, none of the above are likely to be responsible for thermally or seasonally induced adaptations, which have been detected in Hc preparations diluted by a large factor for the purpose of making optical measurements of O_2 binding using a 1 cm light path (Rutledge and Pritchard, 1981; Mauro and Mangum, 1982). It seems unlikely that ion or lactate levels in these preparations differed enough to cause detectable changes in O_2 affinity. Although all properties, including concentration, of the unknown effectors are by definition unknown, these effectors are also unlikely to be responsible because they do not occur in summer blue crabs (Mangum, 1983b), which have the high affinity Hc.

Following thermal acclimation of crayfish Hc Rutledge and Pritchard (1981) observed no difference in the composition of subunits denatured with urea and electrophoresed on polyacrylamide gels. In many species, however, additional subunits are revealed by more sensitive procedures (e.g. Brenowitz *et al.*, 1981; Larson *et al.*, 1981; Markl and Kempter, 1981). A preliminary examination of the subunit composition of winter and summer blue crabs revealed no differences in number (C. P. Mangum and G. Godette, unpublished data). However, we have since found that considerable quantitative variation occurs (Mason *et al.*, 1983; Rainer *et al.*, 1985), suggesting that subunit composition should be analyzed more carefully in relation to season. Finally, the role (if any) of the carbohydrate moiety of the Hcs is unknown (Van Holde and Miller, 1982).

REFERENCES

Bligh E. G. and Dyer W. J. (1959) A rapid method of total lipid extraction and purification. *J. Biochem. Physiol.* **37,** 911–917.

Booth C. E., McMahon B. R. and Pinder A. W. (1983) Oxygen uptake and the potentiating effects of increased hemolymph lactate on oxygen transport during exercise in the blue crab, *Callinectes sapidus*. *J. comp. Physiol.* **148,** 111–121.

Brenowitz M., Bonaventura C., Bonaventura J. and Gianazza E. (1981) Subunit composition of a high molecular weight oligomer: *Limulus polyphemus* hemocyanin. *Archs Biochem. Biophys.* **210,** 748–761.

Bridges C. R. and Morris S. (1986) Modulation of haemocyanin-oxygen affinity by L-lactate—a role for other cofactors. In *Invertebrate Oxygen Carriers* (Edited by Linzen B.) Springer-Verlag, Heidelberg.

Brouwer M., Bonaventura C. and Bonaventura J. (1982) Heavy metal interactions with *Callinectes sapidus* hemocyanin: structural and functional changes induced by a variety of heavy metal ions. *Biochem.* **21,** 2559–2538.

Graham R. A., Mangum C. P., Terwilliger R. C. and Terwilliger N. B. (1983) The effect of organic acids on oxygen binding of hemocyanin from the crab *Cancer magister*. *Comp. Biochem. Physiol.* **74A,** 45–50.

Kerr M. S. (1969) The hemolymph proteins of the blue crab, *Callinectes sapidus*. II. A lipoprotein serologically identical to oocyte lipovitellin. *Devl Biol.* **20,** 1–17.

Larson B. A., Terwilliger N. B. and Terwilliger R. C. (1981) Subunit heterogeneity of *Cancer magister* hemocyanin. *Biochim. biophys. Acta* **667,** 294–302.

Lee R. F. (1985) Female-specific lipoprotein in the hemolymph of the blue crab, *Callinectes sapidus*. *Am. Zool.* **25,** 34A.

Lee R. F. and Puppione D. L. (1978) Serum lipoproteins in the spiny lobster *Panulirus interruptus*. *Comp. Biochem. Physiol.* **59B,** 239–243.

Mangum C. P. (1980) Respiratory function of the hemocyanins. *Am. Zool.* **20,** 19–38.

Mangum C. P. (1983a) Oxygen transport in the blood. In *Biology of the Crustacea* (Edited by Mantel L. H.), Vol. 5, pp. 373–429. Academic Press, New York.

Mangum C. P. (1983b) On the distribution of lactate sensitivity among the hemocyanins. *Mar. Biol. Lett.* **4,** 139–149.

Mangum C. P. (1985) Oxygen transport in the invertebrates. *Am. J. Physiol.* **248,** R505–R514.

Magnum C. P. and Lykkeboe G. (1979) The influence of inorganic ions and pH on the oxygenation properties of the blood in the gastropod mollusc *Busycon canaliculatum*. *J. exp. Zool.* **207,** 417–430.

Markl J. and Kempter B. (1981) Subunit heterogeneity in crustacean hemocyanins as deduced by two-dimensional immunoelectrophoresis. *J. comp. Physiol.* **141,** 495–502.

Mason R. P., Mangum C. P. and Godette G. (1983) The influence of inorganic ions and acclimation salinity on hemocyanin–oxygen binding in the blue crab *Callinectes sapidus*. *Biol. Bull.* **164,** 104–123.

Mauro N. A. and Mangum C. P. (1982) The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. I. Intraspecific responses to seasonal differences in temperature. *J. exp. Zool.* **219,** 179–188.

Morris S., Bridges C. R. and Grieshaber M. K. (1985) A new role for uric acid: modulator of haemocyanin oxygen affinity in crustaceans. *J. exp. Zool.* **235,** 135–139.

Nickerson K. W. and Van Holde K. E. (1971) A comparison of molluscan and arthropod hemocyanin. I. Circular dichroism and absorption spectra. *Comp. Biochem. Physiol.* **39B,** 855–872.

Rainer J., Mangum C. P. and Godette G. (1985) Subunit heterogeneity of the blue crab (*Callinectes sapidus*) hemocyanin along a salinity gradient. *Am. Zool.* **25,** 47A.

Rutledge P. S. and Pritchard A. W. (1981) Effects of temperature acclimation on crayfish hemocyanin oxygen binding. *Am. J. Physiol.* **240,** R93–R98.

Truchot J.-P. (1980) Lactate increases the oxygen affinity of crab hemocyanin. *J. exp. Zool.* **214,** 205–208.

Van Holde K. E. and Miller K. I. (1982) Haemocyanins. *Q. Rev. Biophys.* **15,** 1–129.

Zatta P. (1981) Protein–lipid interactions in *Carcinus maenas* (Crustacea) hemocyanin. *Comp. Biochem. Physiol.* **69B,** 731–735.