

# The CO<sub>2</sub>-Specific Sensitivity of Hemocyanin Oxygen Affinity in the Decapod Crustaceans

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**ABSTRACT** The CO<sub>2</sub>-specific sensitivity of hemocyanin oxygen affinity and oxygen binding site cooperativity were investigated in five species of crabs. The hypothesis that CO<sub>2</sub>-specific effects on hemocyanin are related to the molecular CO<sub>2</sub> concentrations that occur naturally in crab hemolymph was tested in crabs that experience a variety of hemolymph CO<sub>2</sub> pressures. Crab species were chosen from subtidal, intertidal, and semiterrestrial habitats. Hemolymph samples from each species were added to physiological salines buffered with 50 mM HEPES. O<sub>2</sub> equilibrium curves were determined spectrophotometrically by gassing the hemolymph-saline mixtures with different PO<sub>2</sub>S at constant PCO<sub>2</sub> and temperature. We found no CO<sub>2</sub>-specific effects on oxygen equilibrium properties of hemocyanin in any species under the conditions investigated. It is concluded that molecular CO<sub>2</sub> and bicarbonate ions are not important in directly modulating the oxygen equilibrium properties of crustacean hemocyanins.

Early reports on the respiratory function of hemocyanins described the oxygen affinity of these pigments in a variety of different organisms and the dependence of oxygen affinity on the partial pressures of carbon dioxide (Redfield et al., '26; Redfield and Goodkind, '29). At about the same time, the changes in oxygen affinity in hemoglobin first attributed to CO<sub>2</sub> were assumed to be due totally to the generation of hydrogen ions by the hydration reactions of CO<sub>2</sub> (Barcroft, '28). Subsequent studies on hemocyanins concerned primarily pH effects on oxygen affinity and rarely mentioned CO<sub>2</sub> as a specific modulator. Redfield's classic review of hemocyanins in 1934 fails to discuss the influence of CO<sub>2</sub> on oxygen affinity. From that point in time to the present, most investigators have explored the sensitivity of hemocyanin oxygen affinity to hydrogen ions and have ignored the specific effects of CO<sub>2</sub>.

The specific action of CO<sub>2</sub> on hemoglobin was explored in the early part of this century beginning with evidence presented by Henriques ('28) that CO<sub>2</sub> combines directly with hemoglobin. A specific effect of CO<sub>2</sub> on hemoglobin oxygen affinity was demonstrated clearly by Margaria and Green in 1933. It is now thought that carbamate CO<sub>2</sub> is responsible for the oxygen affinity changes brought

about by molecular CO<sub>2</sub> (see Kilmartin and Rossi-Bernardi, '73). These effects on oxygen affinity occur independently of bicarbonate ions, since specific effects of bicarbonate have been shown to be small (Kernohan et al., '66).

Much less is known of the CO<sub>2</sub>-combining properties of the hemocyanins. Recent investigations demonstrate CO<sub>2</sub> effects on hemocyanin oxygen affinity independent of the changes in pH that occur due to CO<sub>2</sub>. Truchot ('73) found a rather large increase in hemocyanin oxygen affinity in the crab *Carcinus maenas* within the physiological pH range. CO<sub>2</sub> effects on hemocyanin oxygen affinity were also found in another crustacean (Weber and Hagerman, '81) and in some molluscs (Mangum and Lykkeboe, '79; Torensma and Brix, '81). On the other hand, CO<sub>2</sub>-specific effects are absent in different representatives of these groups (Jokumsen et al., '81; Jokumsen and Weber, '82; Wells and Weber, '82).

The significance of CO<sub>2</sub>-specific effects on hemocyanins and the patterns of their occurrence among different animals remains unclear. We wished to test the hypothesis that CO<sub>2</sub>-specific effects are related to the molecu-

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lar CO<sub>2</sub> concentrations that naturally occur in crab hemolymph. We investigated the CO<sub>2</sub>-specific effects on hemocyanin oxygen affinity and oxygen binding site cooperativity in a variety of decapod crustaceans likely to experience different ranges of hemolymph CO<sub>2</sub> pressures according to their habitat. Thus, the animals chosen represent aerial, aquatic, and intermediate modes of breathing.

## MATERIALS AND METHODS

### *Collection of animals*

Five different species of decapod crustaceans were used in this study. Species were chosen based on their habitat and mode of breathing (ranging from completely subtidal to semiterrestrial). *Cancer anthonyi* Rathbun, a completely subtidal crab, was purchased locally and held in running sea water at the Hubbs/Sea World Research Institute. *Pachygrapsus crassipes* Randall, an active intertidal crab, was collected along the rock jetties in Mission Bay, San Diego, and held in aquariums where they had access to both air and water. *Callinectes bellicosus* Stimpson, an active swimming crab, occurs in the Gulf of California, where we have observed it taking short (less than one minute) excursions onto land in pursuit of small fiddler crabs. *Uca princeps* Smith is found in the Gulf of California, where it is active on mudflats during periods of low tide. *Ocyropsis occidentalis* Stimpson, the most terrestrial of the crabs studied, also occurs along the shores of the Gulf of California, inhabiting the sandy burrows of the upper beach. *C. bellicosus*, *U. princeps*, and *O. occidentalis* were collected in the northern Gulf of California at Laguna Percebu, 10 miles south of San Felipe, Mexico, and transported to San Diego.

The experimental temperatures were chosen to approximate *in vivo* conditions. Since *C. bellicosus* and *U. princeps* are exposed to extremely high temperatures during the summer months (36°C in July, 1983, at Laguna Percebu), we investigated the effects of low (23°C) and high (36°C) temperature on hemocyanin oxygen affinity. CO<sub>2</sub> pressures for oxygen equilibrium curve determinations represent the low and high extremes of pressures known to occur in these crabs or assumed to occur based on data available for similar species. In some cases the experimental ranges of PCO<sub>2</sub> exceed the values that occur *in vivo*. Since no hemolymph PCO<sub>2</sub> data are available for *C. anthonyi*, we assumed values to be similar to those of a related

species *Cancer magister*. During strenuous activity in *C. magister*, hemolymph PCO<sub>2</sub> rises from 1.5 to 4.1 torr (McDonald et al., '79). We have measured hemolymph PO<sub>2</sub> and acid-base status in *C. bellicosus*, which were quiescent (P<sub>a</sub>O<sub>2</sub> = 58.5 torr, N = 7; P<sub>v</sub>O<sub>2</sub> = 8.0 torr, N = 7; pH<sub>a</sub> = 7.729, N = 11; pH<sub>v</sub> = 7.620, N = 11; P<sub>a</sub>CO<sub>2</sub> = 3.1 torr, N = 5; P<sub>v</sub>CO<sub>2</sub> = 3.6 torr, N = 6). Similar values were obtained in *C. sapidus* and in addition PCO<sub>2</sub> was found to rise to 5.0 torr as a result of swimming activity. Hemolymph PCO<sub>2</sub> in *P. crassipes* immersed in seawater was found to average 2.3 torr and rose to 8 torr when crabs were air exposed (Burnett and McMahon, unpublished results). Similar CO<sub>2</sub> pressures are presumed to exist in *U. princeps*. We also measured hemolymph variables in three individual *O. occidentalis* and found pH<sub>v</sub> to be 7.917 and P<sub>v</sub>CO<sub>2</sub> 3.9 torr, N = 3. We assumed that during activity such as running on the beach *O. occidentalis* might have a hemolymph PCO<sub>2</sub> about 12 torr higher than that during rest, as found for another land crab (Smatresk et al., '79).

### *Oxygen equilibrium determinations*

Oxygen equilibrium curves were determined tonometrically using a method described by Burnett ('79). Hemolymph samples were drawn from crabs and allowed to clot. The clotted hemolymph was homogenized in a 2-ml glass tissue homogenizer to break up the clot. The homogenate was centrifuged at 13,000g and the supernatant was used in oxygen equilibrium determinations. Supernatants were diluted with buffered saline (Table 1) to varying degrees depending upon the concentration of hemocyanin in the supernatant. Approximately 100–300 μl of supernatant were added to 4 ml of buffered saline. The pH of the buffered saline was adjusted to the desired value by adding either HCl or NaOH and then incubated at the appropriate CO<sub>2</sub> pressure prior to adding hemolymph. Oxygen equilibrium curves were determined by gassing glass tonometers with four to seven different partial pressures of oxygen at constant PCO<sub>2</sub> prepared by Wösthoff gas-mixing pumps and measuring light absorbance spectrophotometrically at 345 nm on a Bausch and Lomb Spectronic 21 spectrophotometer. Gas equilibrations were carried out in tonometers incubated in a water bath thermostatted to the appropriate experimental temperature (±0.1°C). The pH and total CO<sub>2</sub> content of the hemolymph-saline mix-

TABLE 1. Buffered salines used in the determination of oxygen equilibrium properties of hemocyanins for five different species<sup>1</sup>

	<i>C. anthonyi</i>	<i>P. crassipes</i>	<i>C. bellicosus</i>	<i>U. princeps</i> and <i>O. occidentalis</i>
Na <sup>+</sup>	526	465	450	500
K <sup>+</sup>	12	12.1	9.2	6
Ca <sup>++</sup>	24	11.4	13.7	15
Mg <sup>++</sup>	19	29.2	37.5	40
Cl <sup>-</sup>	520	450	500	480
SO <sub>4</sub> <sup>--</sup>	50	10	7.3	30
NH <sub>4</sub> <sup>+</sup>	1	1	1	1
HEPES	50	50	50	50

<sup>1</sup>Recipes were constructed using data from Prosser et al. ('55), Gifford ('62), Hunter and Rudy ('75), Burnett et al. ('81), and Burnett and Woodson (unpublished). All concentrations are expressed as mM.

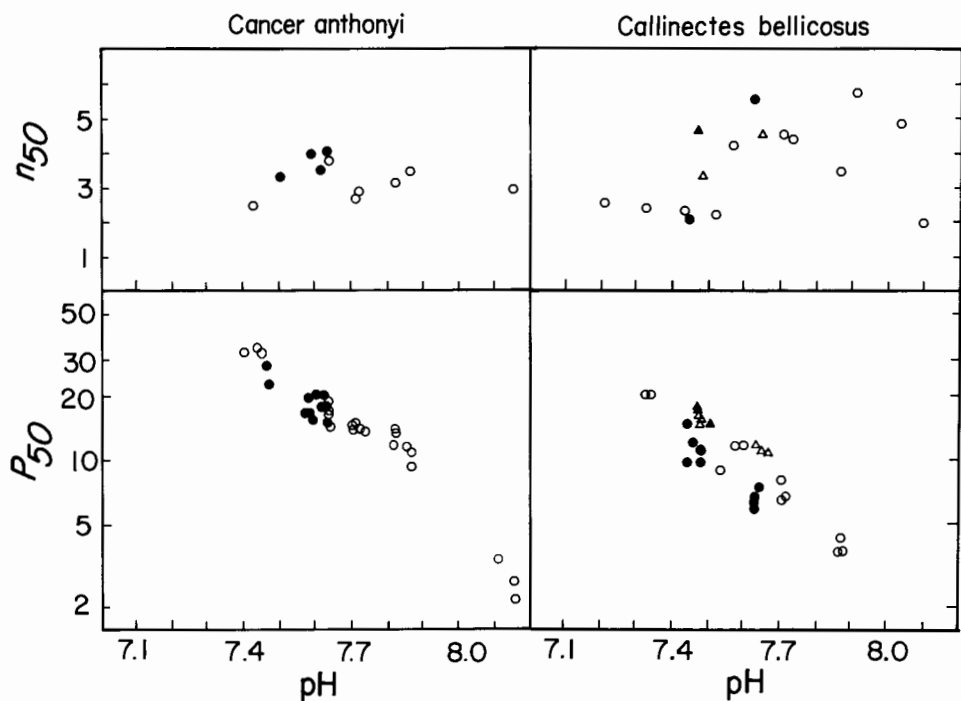


Fig. 1. Hemocyanin oxygen affinity ( $P_{50}$ ) and cooperativity ( $n_{50}$ ) as a function of pH in two subtidal crabs, *Cancer anthonyi* and *Callinectes bellicosus*. Open symbols represent values determined at  $PCO_2 = 1.5$  torr,

closed symbols  $PCO_2 = 7.4$  torr. Temperature = 15°C for *C. anthonyi*. Temperature = 23°C (circles) and 36°C (triangles) for *Callinectes bellicosus*.

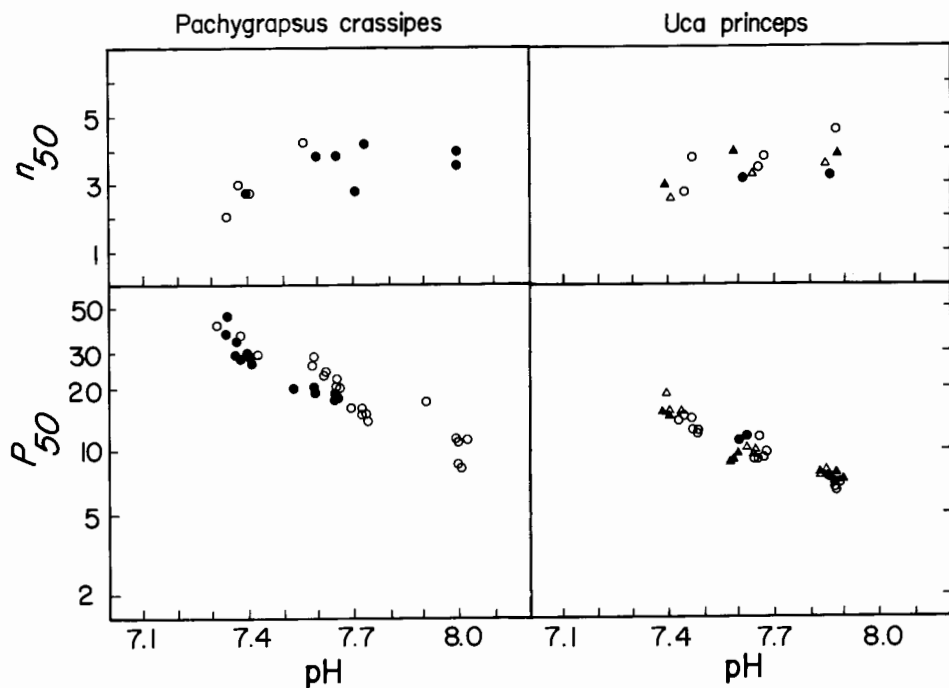


Fig. 2. Hemocyanin oxygen affinity ( $P_{50}$ ) and cooperativity ( $n_{50}$ ) as a function of pH in two intertidal crabs, *Pachygrapsus crassipes* and *Uca princeps*. Open symbols

represent values determined at  $PCO_2 = 1.5$  torr, closed symbols  $PCO_2 = 7.4$  torr. Temperature = 23°C (circles) and 36°C (triangles).

ture were determined after a complete oxygen equilibrium curve was generated. It was found that the oxygenation state of the mixture affected neither pH nor total  $CO_2$  content. pH was determined using a micro-capillary electrode (Radiometer E5021a) thermostatted to the temperature at which oxygen equilibrium curves were determined. Total  $CO_2$  content was determined using the method of Cameron ('71).

#### RESULTS

In each of the five species examined we found no evidence that  $CO_2$  has a specific effect on either oxygen affinity or oxygen binding site cooperativity within the range of physiological pH and  $PCO_2$  (Figs. 1-3). We report the constants that describe the oxygen equilibrium properties of hemocyanin for each of the species (Table 2). In all cases, the constants were determined from data collected at the lowest experimental  $PCO_2$  for that species. Values for  $n_{50}$  are reported but

the slopes of the Hill plots were linear between 10% and 90% oxygen saturation.

The oxygen equilibrium properties of *C. anthonyi* hemocyanin are similar to those found in *C. magister* where the Bohr coefficient is  $-1.2$  and  $n_{50}$  is 4.0 (Graham et al., '83). The values for *C. bellicosus* are higher than those reported for *Callinectes sapidus* ( $\Delta \log P_{50}/\Delta pH = -1.14$  [Booth et al., '82];  $n_{50} = 2.4$  [Bonaventura et al., '74]). The properties of *P. crassipes* and *U. princeps* are similar to those of another intertidal crab *Carcinus maenas* (Truchot, '71; Taylor and Butler, '78). *O. occidentalis* has a Bohr coefficient slightly lower than that found in *Ocyropsis quadrata* ( $-0.95$ ) while  $n_{50}$  is much lower (4.5) (compare values with Table 2 [Burnett, '79]).

Increasing the temperature from 23° C to 36° C has no effect on oxygen affinity in *U. princeps* (Fig. 2) and causes a very small decrease in oxygen affinity in *C. bellicosus* (Fig. 1). Temperature insensitivity of oxygen affin-

ity no doubt reflects an adaptation to a habitat where we have measured temperature fluctuations from 25°C to 36°C over a tidal cycle. CO<sub>2</sub> had no specific effect on oxygen affinity in either species.

It is important to note that at the experimental pHs used in this study, bicarbonate

ions make up most of the total CO<sub>2</sub> in the buffered salines. Bicarbonate concentrations range from 1 mM to 5 mM at low experimental PCO<sub>2</sub> and from 5 mM to 22 mM at high experimental PCO<sub>2</sub>. Thus, we can conclude that there are no effects of either molecular CO<sub>2</sub> or bicarbonate ions on hemocyanin oxygen affinity or cooperativity.

#### DISCUSSION

The sensitivity of hemocyanin oxygen affinity to CO<sub>2</sub> appears to be quite variable both between and within phyla. Truchot ('73) demonstrated a significant increase in oxygen affinity in the hemocyanin of *Carcinus maenas* when PCO<sub>2</sub> was increased from 0.75 to 14.9 torr over the pH range 7.2–7.8. The decrease in P<sub>50</sub> was smaller at high pH in contrast to the results of Weber and Hagerman ('81) who showed a small CO<sub>2</sub>-specific effect in the hemocyanin of the shrimp *Palaeomon adspersus* but only at pH greater than 8.0. Arp and Childress ('81) also claim to have demonstrated a CO<sub>2</sub>-specific sensitivity of hemocyanin O<sub>2</sub> affinity in a brachyuran crab inhabiting deep-sea hydrothermal vents. The basis of their claim is a difference observed in the Bohr coefficient ( $\Delta \log P_{50}/\Delta \text{pH}$ ) between hemolymph samples where pH was varied by incubation at different partial pressures of CO<sub>2</sub> and hemolymph samples where pH was varied in the absence of CO<sub>2</sub> by adding HCl. The comparison is complicated by two factors. First, a Bohr coefficient of -0.81 was calculated for CO<sub>2</sub>-incubated samples at temperatures between 2°C and 15°C, although the authors maintain that the O<sub>2</sub> affinity is insensitive to temperature. Second, the Bohr coefficient of -0.31 was measured on samples kept at -85°C for 9 months

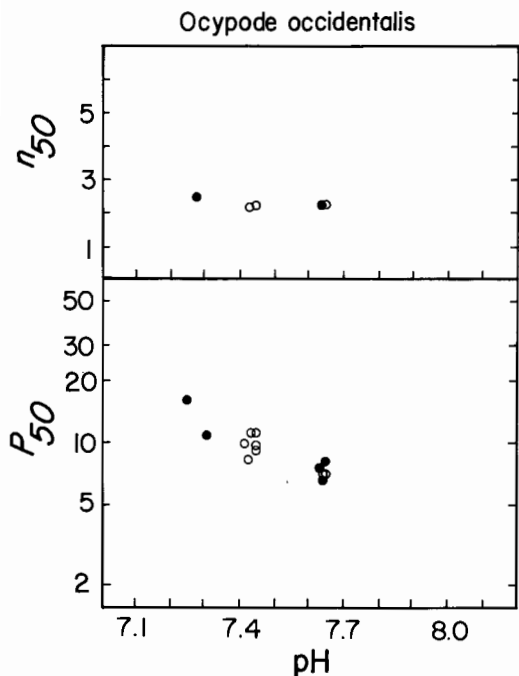


Fig. 3. Hemocyanin oxygen affinity (P<sub>50</sub>) and cooperativity (n<sub>50</sub>) as a function of pH in the semiterrestrial crab *Ocypode occidentalis*. Open circles represent values determined at PCO<sub>2</sub> = 3.7 torr, closed circles PCO<sub>2</sub> = 14.9 torr. Temperature = 20°C.

TABLE 2. Oxygen equilibrium properties of hemocyanins

Species	Temp. (°C)	P <sub>50</sub> (torr)	pH	Pco <sub>2</sub>	$\frac{\Delta \log P_{50}}{\Delta \text{pH}}$	n <sub>50</sub>	SEM	(N)
<i>Cancer anthonyi</i>	15	11.2 <sup>1</sup>	7.8	1.5	-1.44	3.01	0.14	(20)
<i>Callinectes bellicosus</i>	23	6.8	7.67	1.5	-1.32	3.79	0.27	(11)
<i>Pachygrapsus crassipes</i>	23	20.4	7.62	1.5	-0.87	3.49	0.14	(23)
<i>Uca princeps</i>	23	5.9	7.92	1.5	-0.71	3.55	0.24	(14)
<i>Ocypode occidentalis</i>	20	4.2	7.92	3.7	-0.69	2.23	0.23	(8)

<sup>1</sup>Values for P<sub>50</sub> are reported for the physiological pH indicated.

and diluted with an isosmotic solution containing HCl. These authors suggest that the difference in the Bohr coefficients reflects a CO<sub>2</sub>-specific effect on hemocyanin oxygen affinity. However, the effect of the different treatments on the samples is unclear. Our own analysis of their data for freshly drawn hemolymph incubated at different PCO<sub>2</sub>s (and assuming temperature insensitivity) shows no clear CO<sub>2</sub>-specific effect.

We are aware of only two other studies that deal with CO<sub>2</sub>-specific effects on hemocyanins in the crustaceans. One report states that a CO<sub>2</sub>-specific effect is absent in the hemocyanin of the hermit crab *Pagurus bernhardus* but presents no evidence (Jokumsen and Weber, '82). The other study presents evidence for the lack of a CO<sub>2</sub> effect in the hemocyanin of the isopod *Glyptonotus antarcticus* over the pH range 7.0–8.2 (Jokumsen et al., '81). Thus, out of ten different species where CO<sub>2</sub> effects have been investigated, two show an increase in O<sub>2</sub> affinity, six show no change in O<sub>2</sub> affinity, and for two species the data cannot be evaluated.

CO<sub>2</sub> effects on molluscan hemocyanins have been investigated in only three species to our knowledge and the picture presented is as diverse as that found in the crustaceans. Mangum and Lykkeboe ('79) found that the effect of CO<sub>2</sub> on oxygen affinity in the hemocyanin of the gastropod mollusc *Busycon canaliculatum* was variable and depended on the salinity of pigment incubation. Wells and Weber ('82), on the other hand, found no CO<sub>2</sub>-specific effects in the hemocyanin of the terrestrial slug *Arion ater* over the pH range 7.2–7.9. In the only other paper to our knowledge that treats the subject, Torensma and Brix ('81) used very small PCO<sub>2</sub> differences in treatment groups (0 and 0.7 torr) in investigating the hemocyanin O<sub>2</sub> equilibrium properties of the whelk *Neptunea antiqua*. Their results indicate a variable effect of CO<sub>2</sub> on O<sub>2</sub> affinity depending upon pH.

The effects of CO<sub>2</sub> on hemocyanin oxygen binding, therefore, appear to be variable within the crustaceans and the molluscs. Our results indicate that changes in molecular CO<sub>2</sub> and bicarbonate ion concentrations have no effect on hemocyanin oxygen affinity or cooperativity. However, in situations where effects have been observed, the changes in oxygen affinity have been small and cannot have much functional importance within the naturally occurring range of hemolymph PCO<sub>2</sub> and bicarbonate ion concentrations.

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