Reference: *Biol. Bull.* **171**: 248–263. (August, 1986)

THE CO_2 SENSITIVITY OF THE HEMOCYANINS AND ITS RELATIONSHIP TO Cl^- SENSITIVITY

CHARLOTTE P. MANGUM AND LOUIS E. BURNETT, JR.*

Department of Biology, College of William and Mary, Williamsburg, Virginia 23185

ABSTRACT

The effect of CO_2 on hemocyanin-oxygen binding is not generally related to the effect of Cl^-. Some hemocyanins respond to both and some to either one alone. The direction of the responses of O_2 affinity of the various hemocyanins to CO_2 is poorly correlated with the direction of responses to other effectors. The influence of CO_2 on *Busycon* and *Limulus* hemocyanins reaches its maximum at high pH. Since the effect can be abolished by restoring divalent cation activities to the control levels prior to the addition of CO_2, we suggest that the effect is not specific but rather indirect, by the pairing of the allosteric effectors Ca^{+2} and Mg^{+2} with the CO_2 anions. In contrast the effect of CO_2 on crustacean hemocyanins is greater at low pH and it can be enhanced by maintaining HCO_3^- levels within narrow limits and permitting PCO_2 to vary by a large factor. This finding suggests that the effective species is molecular CO_2. While Cl^- influences only oxygen affinity, CO_2 may influence cooperativity as well. In different species the effects of both Cl^- and CO_2 may or may not be great enough to be physiologically important.

INTRODUCTION

A site on the hemocyanin (Hc) molecule which is linked to the active site competitively binds divalent cations and H^+. Its molecular features and their physiological importance have been explored in detail (Arisaka and Van Holde, 1979; reviewed by Mangum, 1980; Miller and Van Holde, 1981).

The nature and significance of anion binding to the Hcs are less clear. While the effects of the organic anion L-lactate on the crustacean Hcs are beginning to be elucidated by a number of investigators (reviewed by Bridges and Morris, 1986), the influence of Cl^- is not as well understood. Cl^- specifically influences HcO_2 binding in the chelicerate arthropod *Limulus* (Sullivan *et al.*, 1974; Diefenbach and Mangum, 1983) and the gastropod mollusc *Busycon* (Mangum and Lykkeboe, 1979), but its effect on crustacean Hcs ranges from strong (Brouwer *et al.*, 1978) to absent (Truchot, 1975; Mason *et al.*, 1983). Brix and Torensma (1981) and Torensma and Brix (1981) concluded that the effects of Cl^- and CO_2 on gastropod Hcs are both allosteric and linked to one another, suggesting a Cl^--HCO_3^- site analogous to the divalent cation-H^+ site.

In fact, the effects of CO_2 in whatever form are even less clear than those of Cl^-. As pointed out earlier (Burnett and Infantino, 1984), only one of the several investigations on the subject convincingly demonstrates a significant and specific effect of CO_2 on O_2 affinity of the crustacean Hcs (Truchot, 1973). In contrast, a number of negative reports have appeared (reviewed by Burnett and Infantino, 1984; see also

Received 27 February 1986; accepted 25 April 1986.

* Permanent address: Department of Biology, University of San Diego, Alcala Park, San Diego, California 92110.

Morris and Bridges, 1985). After the paper by Burnett and Infantino (1984) was in press a large specific effect of CO_2 on a crab Hc was reported by Greenaway *et al.* (1983) but, curiously, the response is opposite to that found in *Carcinus* (Truchot, 1973).

An effect of very low levels of CO_2 on O_2 affinity of gastropod Hc has been found in three species (Mangum and Lykkeboe, 1979; Torensma and Brix, 1981) but the mechanism remains unclear. Mangum and Lykkeboe (1979) suggested that it may be either direct and specific, *viz.* by means of CO_2 binding to a site linked to the active site, or indirect and non-specific, *viz.* by means of ion-pair formation of HCO_3^- and CO_3^{-2} with the divalent cations, thus inhibiting the action of other allosteric effectors. Brix and Torensma (1981) and Torensma and Brix (1981) described the CO_2 effect as allosteric, but presented as supporting evidence only the fit of their data to the Monod-Wyman-Changeux model. However, the model, which describes a particular form of allosteric behavior, would not distinguish between direct and indirect allosteric actions.

We have investigated the effect of CO_2 and Cl^- on HcO_2 binding in a variety of species chosen because they are either the same as or closely related to those studied earlier and also because they may represent various combinations of CO_2 and Cl^- sensitivity. We have investigated the mechanisms of CO_2 effects on gastropod (and also chelicerate) Hcs, by controlling the levels of divalent cation activity while varying those of CO_2 anions. If the divalent cations are the immediate effectors, we would expect to see no effect of CO_2. We have investigated the mechanism of the effect on crustacean Hcs by controlling (in large part) the levels of the CO_2 anions while allowing CO_2 to vary by a large factor. In this case we would expect the CO_2 effect to vary if molecular CO_2 is the immediate effector.

MATERIALS AND METHODS

Collection and holding of animals

The crustaceans *Callinectes sapidus* Rathbun and *Palaeomonetes pugio* Holtuis, the chelicerate *Limulus polyphemus* (Linnaeus), and the gastropod mollusc *Busycon canaliculatum* Linnaeus were collected locally. The crustacean *Penaeus duorarum* Burkenroad was collected offshore of Beaufort, North Carolina and *Carcinus maenas* (Linnaeus) at Mt. Desert Island, Maine. The polyplacophoran mollusc *Cryptochiton stelleri* Middendorff was purchased from commercial sources. Animals were held at 16–25°C, depending on origin, and salinities (20–35‰) either identical to those at the collection sites or approximating those specified in previous investigations. The experimental temperature was chosen similarly.

Procurement and preparation of samples

If the ionic composition of the blood of a species was unknown it was determined with ion-selective electrodes (Mangum and Lykkeboe, 1979).

Blood samples were obtained by syringe sampling. The samples were centrifuged to remove debris, in the case of the arthropods after first declotting with a tissue grinder. The sera were then either used for O_2 binding measurements without modification or first dialyzed against the desired saline (4°C, 24 h).

O_2 binding

O_2 binding was determined by two methods: (1) for measurements in the presence of different levels of CO_2 a spectrophotometric method was employed (Burnett, 1979;

Burnett and Infantino, 1984). Mixtures of N_2 (99.9995%, scrubbed with Oxisorb), CO_2 (99.5%), and, depending on PO_2, either air (scrubbed with soda lime and Dri-Rite) or O_2 (99.9%) were prepared with Wosthoff pumps, humidified in a gas washing bottle, and passed over samples incubated in a thermostatically controlled shaker bath. The samples had been diluted by factors ranging from 13.5 to 101 with 0.05 *M* Tris Maleate buffered saline. Changes in absorbance (1 cm light path) were determined at 335–345 nm, depending on species, with a Bausch & Lomb Spectronic 20 colorimeter. These data are illustrated with open symbols.

(2) The cell respiration method (Mangum and Lykkeboe, 1979) was used to determine the effects of inorganic ions. Samples were diluted by 10% with buffered saline or buffered test solution (final concentration 0.05 *M* Tris Maleate buffer), and pH (Fisher Accumet with Ross electrode) and PCO_2 (Radiometer electrode, calibrated with 1.05 and 3.03% CO_2) were measured at the end of an experiment. These data are illustrated with closed symbols. In this procedure PO_2 is lowered at a constant rate by respiring yeast cells, which also excrete CO_2. To ascertain that PCO_2 in a buffered preparation does not change during an experiment, a second measurement was performed in parallel, but PCO_2 was measured instead of PO_2. Following equilibration of the electrode with the sample, no change was detected (Fig. 1).

O_2 carrying capacity

The total O_2 capacity of polyplacophoran blood is known from a single observation on a single individual (Redmond, 1962). After first subtracting the absorbance of deoxyHc to eliminate light scatter, an estimate was made from the spectrophotometric data using the extinction coefficient for gastropod Hc (Nickerson and Van Holde, 1970). Total O_2 was also obtained from the records made during the cell respiration procedure as follows (Fig. 2): The area under the (dashed) line representing a constant rate of O_2 depletion describes the volume of free O_2, which can be evaluated knowing

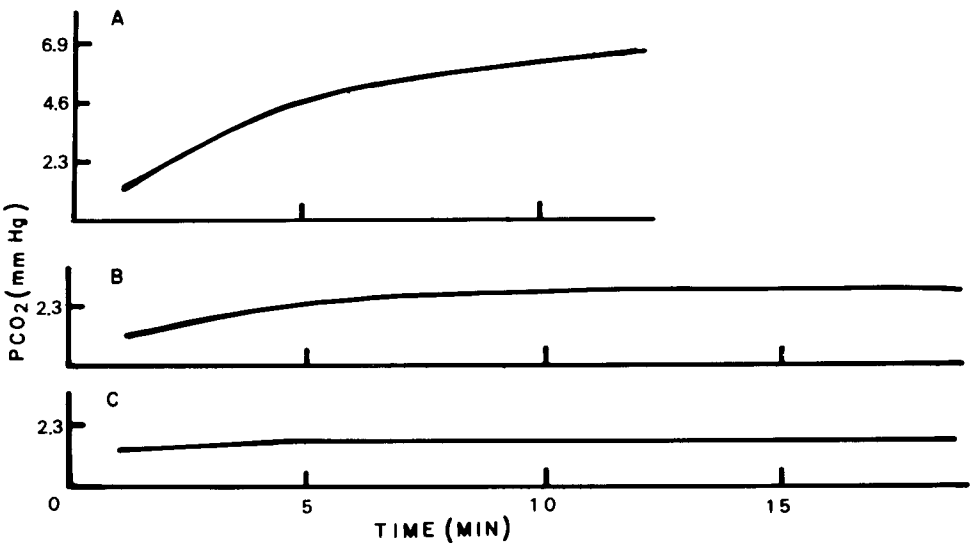


FIGURE 1. Recording of PCO_2 during depletion of O_2 in: A. Saline, with no Hc and no exogenous buffer. B. Saline + *Penaeus duorarum* Hc but no exogenous buffer. C. Saline + *P. duorarum* Hc and 0.05 *M* Tris Maleate buffer (pH 7.6). 20°C.

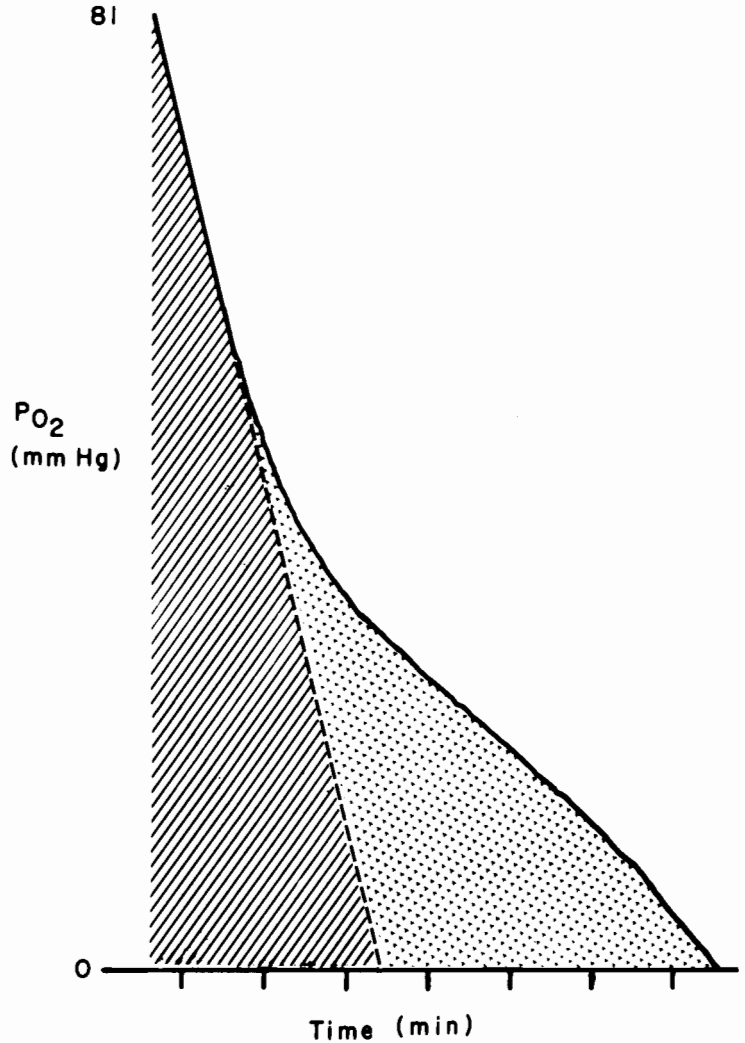


FIGURE 2. O_2 depletion by yeast cells in presence of *Cryptochiton stelleri* Hc. pH 7.43, 15°C. Hatched area shows volume of free O_2; stippled area shows volume of HcO_2.

temperature and solute concentration. The area under the curve representing the deviation from that line describes the volume of Hc-bound O_2, which can be evaluated as a fraction or, more often, a multiple of the free O_2 area.

Data analysis

When pH, CO_2, and Cl^- were either controlled or when all three had no detectable effect, the significance of differences between mean values of P_{50} or n_{50} was estimated by Student's *t*-test; the probability of a significant difference is specified below. When pH and CO_2 were varied simultaneously and Cl^- controlled, the data were described by semilogarithmic (log Y) regression lines and the overlap (if any) between 95% confidence intervals around the lines used as the criterion of significance. When NaCl

was varied while pH and PCO_2 were controlled, the P_{50} data were described by semilogarithmic regression lines and the difference of the slope from zero (at $P = .05$) used as the criterion. Finally, when HCO_3^- and NaCl were controlled while pH and PCO_2 were varied simultaneously, the data were described by semilogarithmic regression lines and the overlap (if any) between 95% confidence intervals around the slopes was used as the criterion. If a substance is said below to have an effect these criteria were exceeded.

RESULTS

Busycon canaliculatum

Mangum and Lykkeboe (1979) showed that in the channeled conch Cl^- has a specific effect on HcO_2 binding and that, at high pH, the addition of molecular CO_2 lowers the oxygen affinity of low salinity samples and raises the oxygen affinity of high salinity samples, but only above pH 7.9 where the reverse Bohr shift is quenched by divalent cations. They suggested that, if the CO_2 effect were indirect (*viz.* by means of ion-pair formation with the divalent cations), the difference between high and low salinity could be explained quantitatively by their findings on the effects of individual inorganic ions: at high salinity the addition of CO_2 immobilizes some of the divalent cations that normally lower oxygen affinity but enough Ca^{+2} and Mg^{+2} remains to quench the reverse Bohr shift; the net result is an increase in oxygen affinity. At low salinity, where Ca^{+2} and Mg^{+2} are already scarce, their immobilization by CO_2 does not leave enough free divalent cations to quench the reverse Bohr shift. Na^+ and Cl^- become the dominant inorganic ions, and they lower oxygen affinity, which is in fact the net result. The present findings confirm the effect of CO_2 at high salinity, only above about pH 7.9 (Fig. 3).

To more directly demonstrate the mechanism the following experiment was performed: first a sample of serum was dialyzed against buffered physiological saline for 24 h and O_2 binding determined. Then 25 m*M* $NaHCO_3^-$ was added to the stirred preparation in which a Ca^{+2} selective electrode was immersed, and the change in Ca activity noted. O_2 binding was determined again. While the sample was stirred with the Ca electrode immersed, $Ca(NO_3)_2$ was then added in sufficient quantity to restore the original level of Ca activity; O_2 binding was measured a third time. This experiment controls for pair-formation of the CO_2 anions with Ca^{+2} but not Mg^{+2}. It could not be performed with a total divalent cation selective electrode for a variety of reasons, the most important of which are the lower sensitivity and resolution of the electrode. Normally these problems are mitigated by chelating Ca with EGTA and then measuring pMg alone; the remedy would not have been possible in the present context without eliminating an important effector of O_2 binding. Since the percent pair formation of Ca and Mg with the CO_2 anions is the same (Kester and Pytkowicz, 1969) and since the effects of Ca^{+2} and Mg^{+2} on O_2 binding are very nearly so (Mangum and Lykkeboe, 1979), the pair formation with Mg was calculated and additional $Ca(NO_3)_2$ was added to simulate the original Mg^{+2} activity using Ca^{+2} instead. Changes in monovalent anions of those small magnitudes have no detectable effect (Mangum and Lykkeboe, 1979). O_2 binding was determined a fourth time.

As reported earlier (Mangum and Lykkeboe, 1979), the addition of $NaHCO_3$ to the blood raises the O_2 affinity of high salinity blood and lowers the O_2 affinity of low salinity blood (Table I). It does not clearly influence cooperativity ($P = .09$–0.70). The third and fourth steps of the experiment were performed on high salinity blood. When the original activity of only Ca was restored, O_2 affinity dropped slightly though sig-

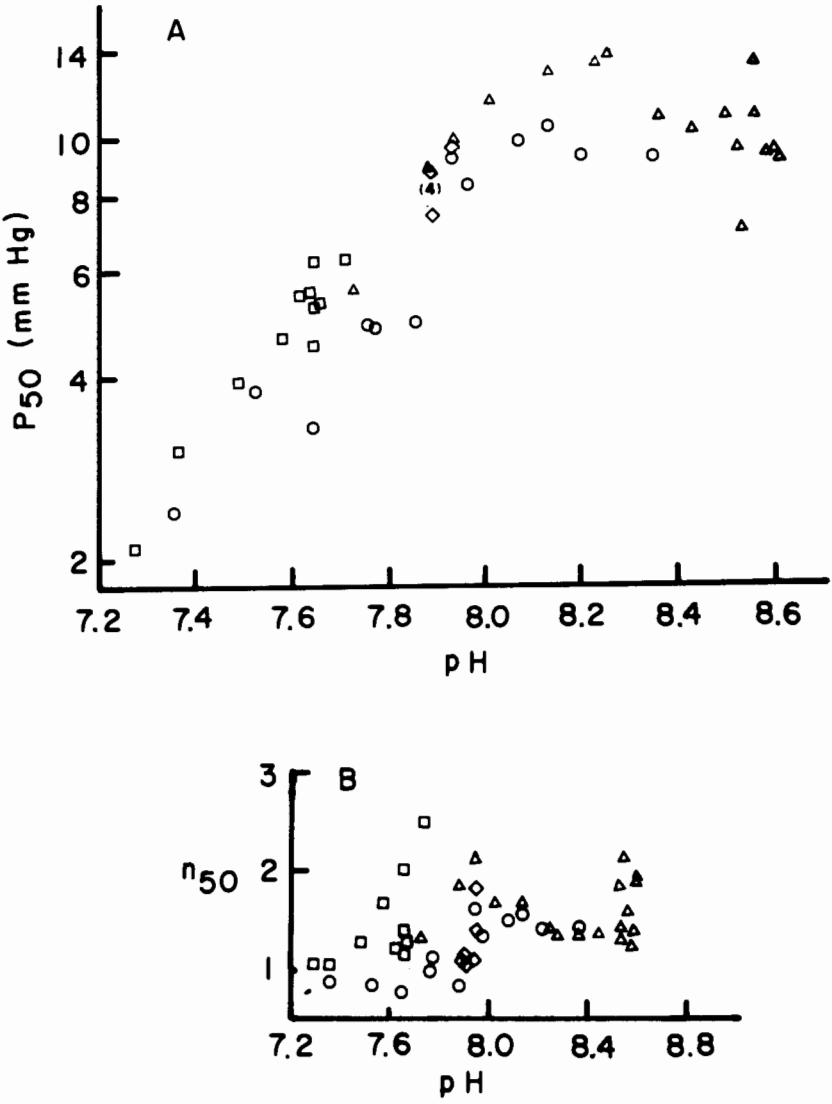


FIGURE 3. Effect of CO_2 on *Busycon canaliculatum* HcO_2 binding. Tonometric method. (○) PCO_2 = 0; (△) PCO_2 = 0.74 mm Hg; (◇) PCO_2 = 7.4 mm Hg; (□) PCO_2 = 14.8 mm Hg. 20°C. Serum was diluted with 0.05 *M* Tris maleate buffered high salinity saline (from Mangum and Lykkeboe, 1979). A. Oxygen affinity. B. Cooperativity.

nificantly, but still differed from the control value. When additional Ca was added to simulate the change in total divalent cations, O_2 affinity decreased further to a value that is indistinguishable from the control.

Cryptochiton stelleri

The Hc of the giant sea cradle is clearly cooperative and it has a small (though significant) normal Bohr shift (Fig. 4). The present results essentially agree with earlier

TABLE I

Effect of $NaHCO_3$ and $Ca(NO_3)_2$ on Busycon canalicalatum HcO_2 binding[1]

	HS		LS	
	P_{50}	n_{50}	P_{50}	n_{50}
Control, PCO_2 1.50–1.59 mm Hg	10.75 ± 0.31 (8)	1.31 ± 0.04 (8)	9.70 ± 0.40 (6)	1.36 ± 0.13 (6)
+ 25 m*M* $NaHCO_3$ PCO_2 5.5–5.7 mm Hg	9.31 ± 0.17 (6)[2]	1.40 ± 0.03 (6)	10.70 ± 0.30 (8)	1.43 ± 0.25 (8)
Same, + Ca^{+2} to Equal pCa in control. PCO_2 5.7 mm Hg	9.95 ± 0.20 (9)[3]	1.33 ± 0.05 (9)	—	—
Same, + Ca^{+2} to Equal pCa + pMg in control	10.72 ± 0.20 (8)[4]	1.33 ± 0.06 (8)	—	—

[1] Cell respiration method:serum was dialyzed against 0.05 *M* Tris maleate buffered high (HS) or low (LS) salinity saline (from Mangum and Lykkeboe, 1979). 19.7–20.1°C, pH 8.18–8.23. Mean ± S.E. (N).

[2] $P = 0.0035$ *vs* control.

[3] $P = 0.025$ *vs* control, 0.0375 *vs* + $NaHCO_3$ alone.

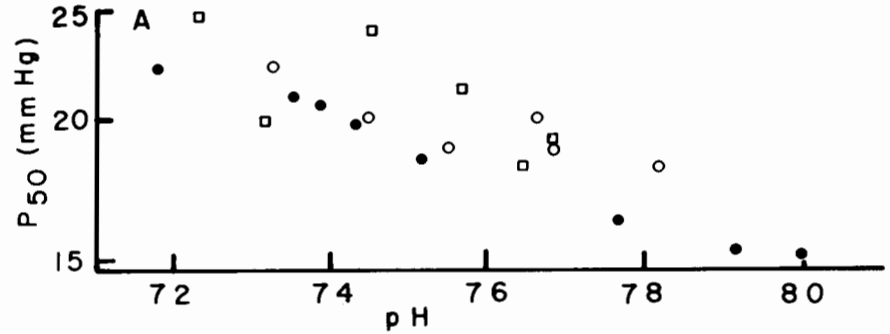
[4] $P = 0.04$ *vs* + Ca^{+2} to equal pCa in control, 0.97 *vs* control.

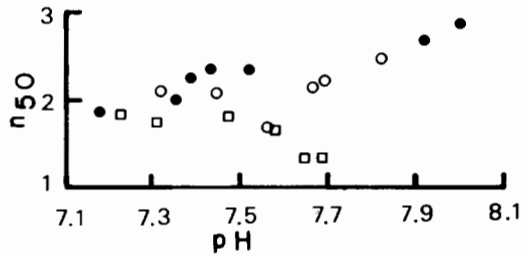
findings (Manwell, 1958), although the comparison is made somewhat difficult by the different pH ranges investigated. The slope of a regression line describing the two sets of control data for P_{50} in Figure 4A is -0.20 ± 0.04 (95% C. I.). Also in the combined control data, there is a small but significant increase in cooperativity with pH.

The O_2 affinity data for 0 and 14.8 mm Hg PCO_2 differ significantly in the pH range 7.2–7.6 but not in the range 7.7–7.85 (Fig. 4A). At high PCO_2 cooperativity is lower ($P = 0.002$) and there is a small but significant decrease with pH. Neither HcO_2 affinity (Fig. 4B) nor cooperativity (1.87 ± 0.09 S. E., N = 6) respond to NaCl.

The data in Figure 4 were obtained using blood taken from the hemocoel directly into a hypodermic needle passed through two adjacent dorsal shell plates. When an initial attempt to perform the operation was unsuccessful, a blood sample was obtained by slitting the foot and draining the hemocoel. No other organs were damaged although some mucus was produced. The sample formed a blue precipitate and, after centrifugation at low speed, the supernatant fluid did not combine reversibly with oxygen. When the pellet was washed with saline, it did not go into solution but it still combined reversibly with oxygen. Measured with the cell respiration technique, oxygen affinity was higher than that of the syringe sample, but only by about 25%; cooperativity was lower but still easily detected ($n_{50} = 1.72 \pm 0.19$ S. E., N = 12). Apparently the native higher order structure is not critical to cooperativity and a relatively low O_2 affinity.

The difference in absorbance between oxygenated and deoxygenated samples, corrected for the dilution factor indicates that the native Hc concentration in one animal was about 0.74 g/100 ml, yielding an HcO_2 capacity of only about 0.33 (total O_2 capacity of 0.91) ml/100 ml. The integrals obtained from the records made during the cell respiration procedure indicate an HcO_2 carrying capacity in another animal of 0.22 ml/100 ml or a total blood O_2 carrying capacity of 0.80 ml/100 ml. The results support the inference of an extremely low O_2 carrying capacity of the blood in this class (Redmond, 1962).





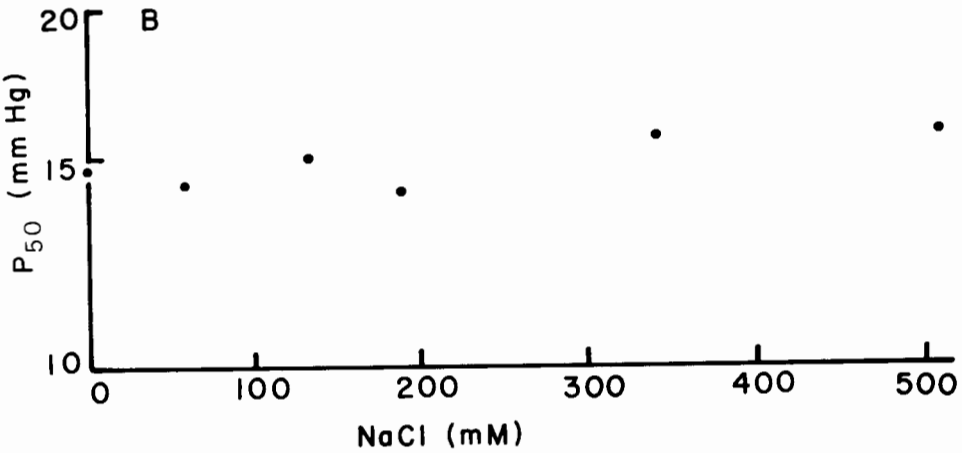


FIGURE 4. A. Effect of CO_2 on *Cryptochiton stelleri* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered, filtered seawater (35‰) in which the animals had been shipped. (○) $PCO_2 = 0$, (□) $PCO_2 = 14.8$ mm Hg. 15°C. Cell respiration method: after dialysis against seawater, Tris maleate buffer (final concentration 0.05 *M*) was added to the serum. (●) PCO_2 1.32–5.41 mm Hg. B. Effect of NaCl on HcO_2 affinity. Cell respiration method: serum was dialyzed against 10 m*M* $Ca(NO_3)_2$ + 0.05 *M* Tris maleate buffer, pH 7.62–7.65. PCO_2 3.0–3.3 mm Hg. 15°C.

Penaeus duorarum

At pH 7.6, which is likely to approximate the physiological value, the Hc of the pink shrimp has a moderate oxygen affinity and pronounced cooperativity. In the pH range studied it also has a large normal Bohr shift (Fig. 5); the slope of a regression line describing the two sets of control data is -1.09 ± 0.19 (95% C. I.). The data are virtually indistinguishable from those reported earlier for *P. setiferus* (Brouwer *et al.*, 1978). As also in *P. setiferus* (Brouwer *et al.*, 1978), *P. duorarum* HcO_2 affinity clearly responds to NaCl (Fig. 5B). However, it responds identically to $NaNO_3$, Na_2SO_4 (plotted as m*M* cation), and choline Cl^-. There is a significant decrease in cooperativity ($P = 0.05$–0.001, Fig. 5B). Neither property responds to CO_2, although the trend in cooperativity is the same as that in the data for CO_2 sensitive Hcs ($P = 0.15$, Fig. 5A).

Palaeomonetes pugio

At putative physiological pH the Hc of the grass shrimp has an extremely low O_2 affinity and fairly little cooperativity (Fig. 6). The slope of a semilogarithmic regression line describing the control data for P_{50} is -1.33 (± 0.56 95% C. I.). HcO_2 affinity clearly responds to CO_2 (Fig. 6A), which does not appear to influence cooperativity ($P = 0.18$). The pH dependence of P_{50} at high PCO_2 appears to be about the same as that at low PCO_2, but the estimate made by regression analysis is much greater (-1.75 ± 0.11). In view of the far greater variability in the control data ($r^2 = 0.756$) the numerical estimate of the Bohr shift at high PCO_2 ($r^2 = 0.998$), which shows extremely great pH dependence, is probably the more accurate of the two. NaCl clearly raises HcO_2 affinity but does not change cooperativity (Fig. 6B). Due to the great difficulty of obtaining ample volumes of blood from these always small animals, which were even smaller than usual at the time of the experiment on NaCl sensitivity because the population had just reproduced, we did not investigate the specificity of this response.

Callinectes sapidus

The Hc of the blue crab does not respond specifically to Cl^- (Mason *et al.*, 1983). Oxygen affinity (but not cooperativity) does respond to CO_2 (Fig. 7). Because it was concluded earlier that the Hc of a species belonging to the same genus (*C. bellicosus*) is not sensitive to CO_2 within the range 1.5–7.4 mm Hg, the response of *C. sapidus* was examined in more detail. While there may appear to be little or no difference in P_{50} at 0 and 1.5 mm Hg (Fig. 7), the two sets of data are in fact significantly different. When the data for *C. bellicosus* are analyzed similarly, however, an effect of CO_2 can be demonstrated only at 23°C and only at pH 7.5 and above (Burnett and Infantino, 1984). Of the other four species examined by Burnett and Infantino (1984) a similar trend may be present in the data for *Pachygrapsus crassipes* (though $P > .05$); however, no sign of an effect can be perceived in the remaining three (Burnett and Infantino, 1984). Thus we suggest that our original conclusion, *viz.* that CO_2 is not an important effector of HCO_2 binding in the five species, was essentially correct (Burnett and Infantino, 1984). Regardless, only at much higher PCO_2 is there an appreciable response of HCO_2 affinity in *C. sapidus;* cooperativity does not respond even at that level (Fig. 7).

In an attempt to identify the CO_2 species responsible for the effect, we dialyzed aliquots of a sample against either (1) saline containing no exogenous CO_2, or (2) saline containing high levels of $NaHCO_3$. When pH was adjusted by adding Tris maleate buffered saline (final concentration 0.05 *M*), PCO_2 varied by more than an order of magnitude while HCO_3^- varied by only about 25% (Fig. 7B). The oxygen affinity of this sample was depressed even more at low pH and high PCO_3 than at high pH and low PCO_2. If this result were due to the greater change in pCa at high

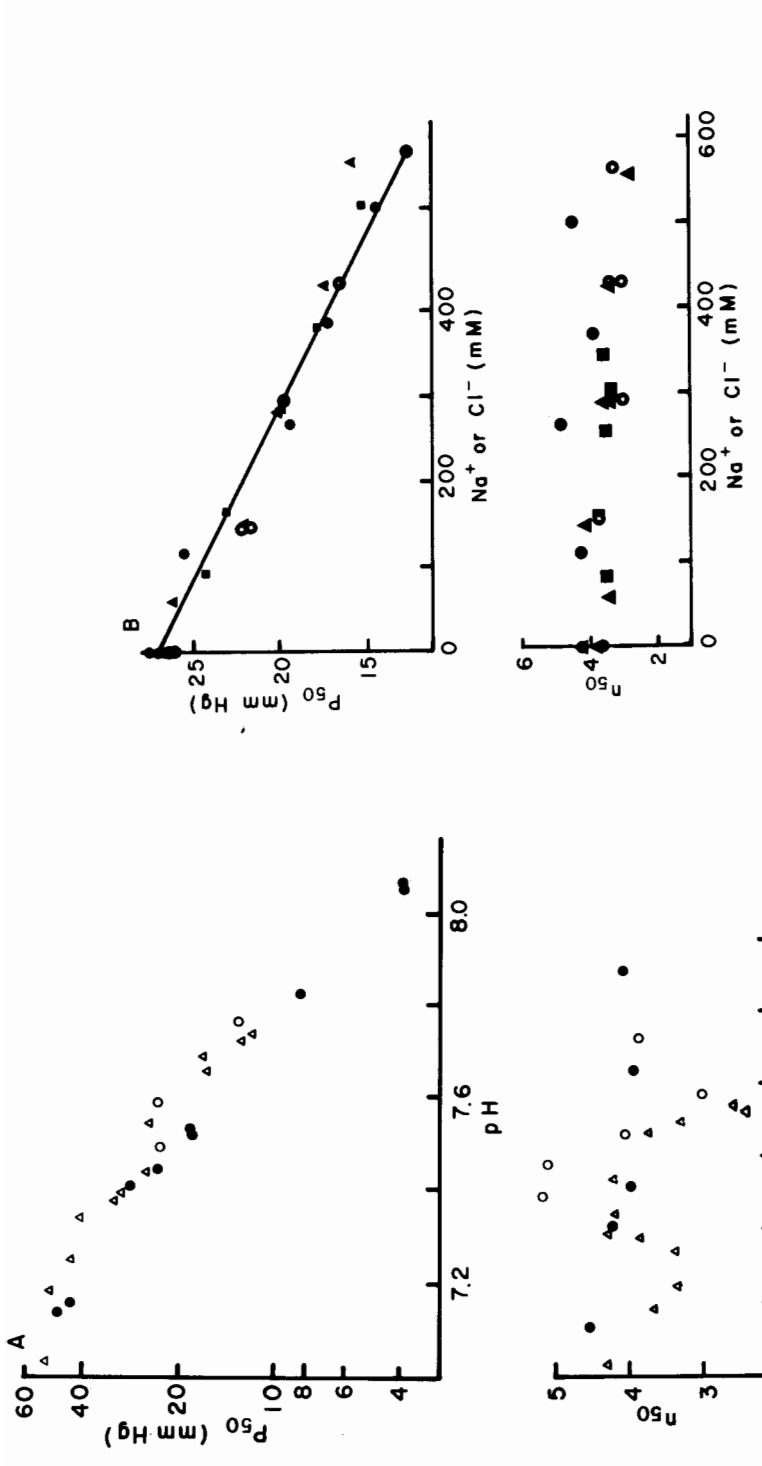


FIGURE 5. A. Effect of CO_2 on *Penaeus duorarum* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered saline containing 11 m*M* KCl, 14 m*M* $CaCl_2$, 39 m*M* $MgCl_2$, 433 m*M* NaCl, and 32 m*M* Na_2SO_4. (○) PCO_2 = 0, (△) PCO_2 = 14.8 mm Hg. Cell respiration method: serum was dialyzed against buffered saline described above, PCO_2 = 1.30–6.60 mm Hg. B. Effect of (●) NaCl, (■) $NaNO_3$, (▲) Na_2SO_4, and (○) choline Cl^-. Cell respiration method: serum was dialyzed against 0.05 *M* Tris maleate buffered $Ca(NO_3)_2$ (15 m*M*, pH 7.58–7.69, PCO_2 = 2.0–3.3 mm Hg). 20°C.

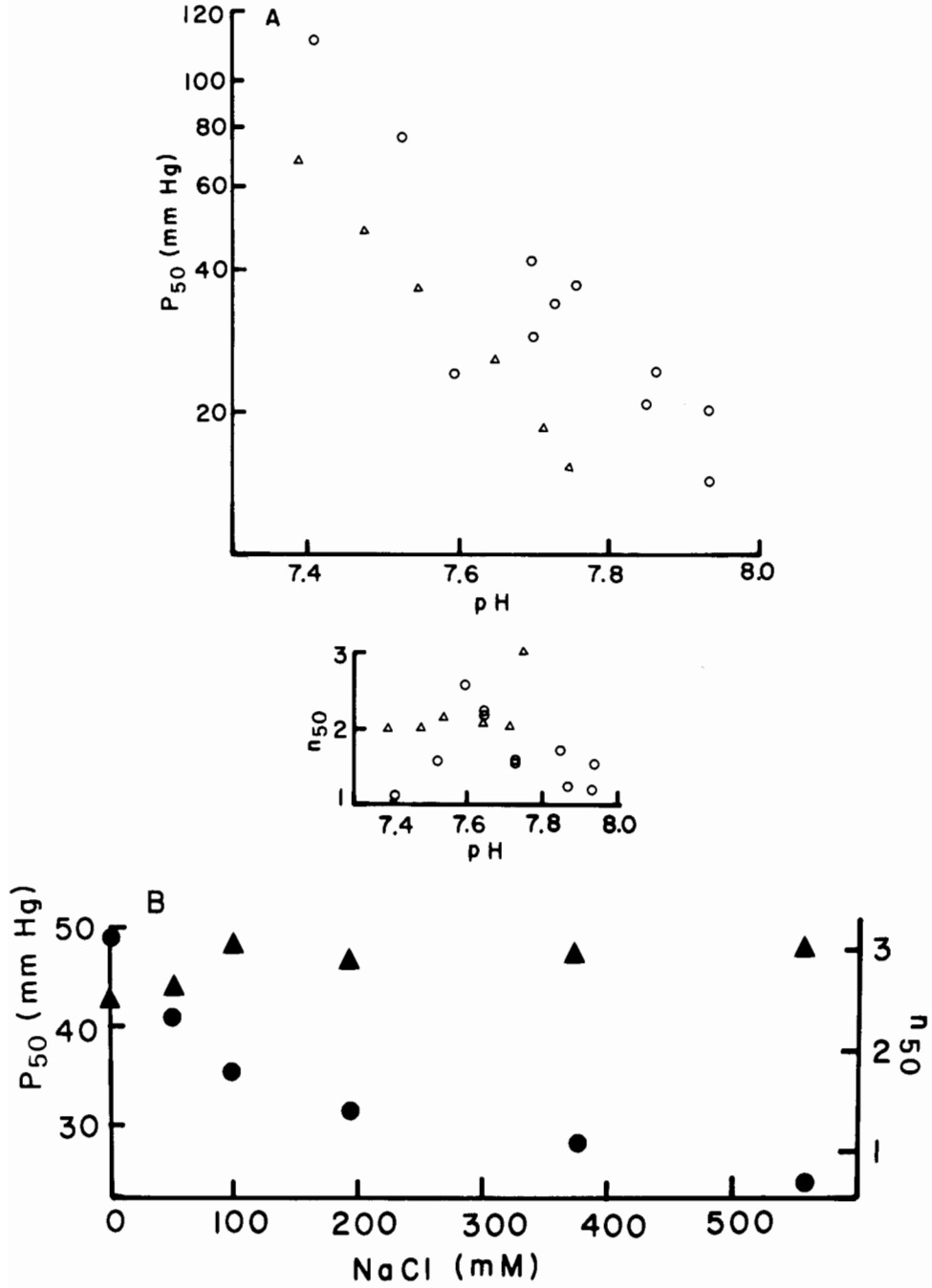


FIGURE 6. A. Effect of CO_2 on *Palaemonetes pugio* HcO_2 binding. Tonometric method: serum was dialyzed against saline containing 261 m*M* NaCl, 8.1 m*M* KCl, 6.3 m*M* $CaCl_2$, 4.4 m*M* $MgCl_2$, and 29.5 m*M* Na_2SO_4 (from Mantel and Farmer, 1983), and diluted with 0.05 *M* Tris maleate buffered saline. (○) PCO_2 = 0, (△) PCO_2 = 14.8 mm Hg. 17°C. B. Effect of NaCl on HcO_2 binding. Cell respiration method: serum was dialyzed against 10 m*M* $Ca(NO_3)_2$ + 0.05 m*M* Tris maleate buffer, pH 7.65–7.68, PCO_2 1.3–1.5 mm Hg. 25°C. (●) P_{50}, (▲) n_{50}.

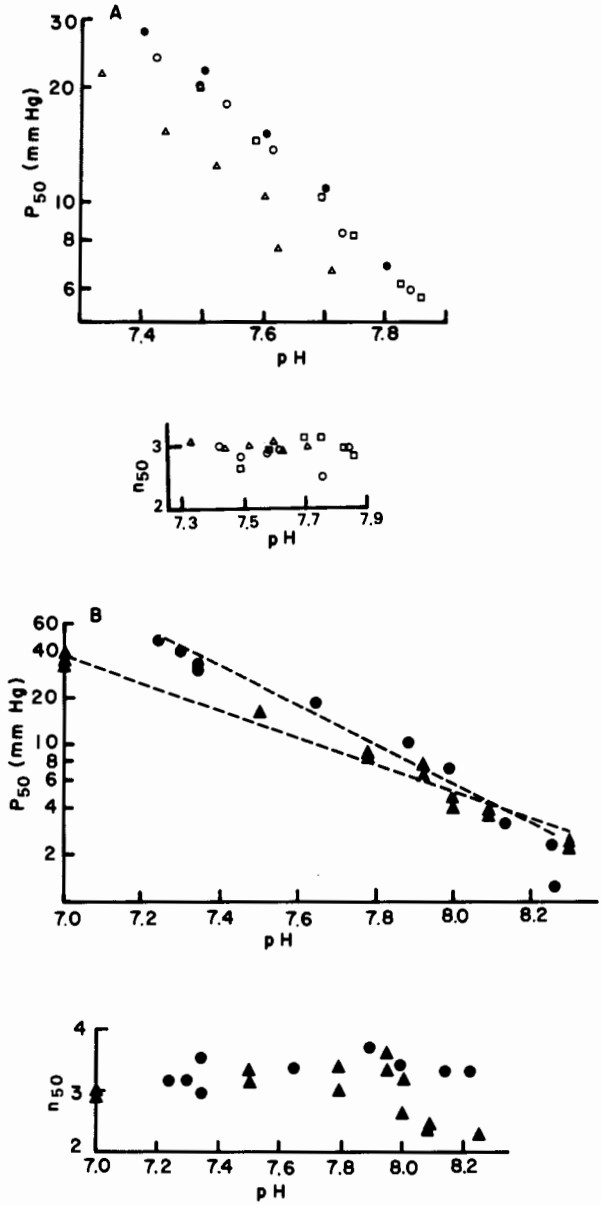


FIGURE 7. A. Effect of CO_2 on *Callinectes sapidus* HcO_2 binding. Tonometric method: serum was diluted with 0.5 *M* Tris maleate buffered high salinity saline (from Mason *et al.*, 1983). 25°C. (○) PCO_2 = 0, (□) PCO_2 = 1.5 mm Hg; (△) PCO_2 = 14.8 mm Hg. (●) For comparison, unpublished data collected earlier by cell respiration method: serum was dialyzed against same Tris maleate buffered saline. 25°C, PCO_2 unknown. B. Effect of high $NaHCO_3$. Cell respiration method: in controls (●), PCO_2 and $[NaHCO_3]$ vary from 0.32 mm Hg and 4.09 m*M* at pH 8.25 to 6.98 mm Hg and 1.69 m*M* at pH 7.24. In experiments (▲), PCO_2 and $[NaHCO_3]$ vary from 1.2 mm Hg and 13.7 m*M* at pH 8.25 to 1.4 mm Hg and 10.61 m*M* at pH 7.00. 25°C. Dashed lines, which differ significantly, were fitted by regression analysis.

than at low pH, O_2 affinity should have been lowered at high pH and unchanged at low pH rather than the other way around.

Carcinus maenas

Truchot (1973, 1975) showed earlier that, in European members of this species, Cl^- has no effect whereas CO_2 clearly raises O_2 affinity. Our data confirm the CO_2 effect, which occurs in about the same magnitude in the North American population (Fig. 8). They also show that CO_2 significantly lowers cooperativity and its pH dependence.

Limulus polyphemus

Cl^- has a specific effect on HcO_2 affinity (but not cooperativity) in the horseshoe crab (Sullivan *et al.*, 1974; Diefenbach and Mangum, 1983). CO_2 has a significant effect on O_2 affinity as well; an apparent decrease in cooperativity is not quite significant ($P = 0.11$; Fig. 9). The experiment designed to identify the mechanism of the CO_2 effect on *Busycon* Hc was also performed on *Limulus* Hc, with the exception that the step simulating the effect of changing only pCa was omitted. The addition of $NaHCO_3$ significantly raises O_2 affinity (Table II) but not cooperativity ($P = 0.19$) and the addition of $Ca(NO_3)_2$ to restore the original levels of free Ca^{+2} and Mg^{+2} lowers O_2 affinity back again to a value that does not differ from the control.

DISCUSSION

Various combinations of Cl^- and CO_2 sensitivity of the Hcs can be found. In any particular species HcO_2 affinity may be sensitive to both (*e.g., Busycon, Limulus,* and *Palaeomonetes*), to Cl^- but not CO_2 (*Penaeus*) or to CO_2 but not Cl^- (*Cryptochiton,*

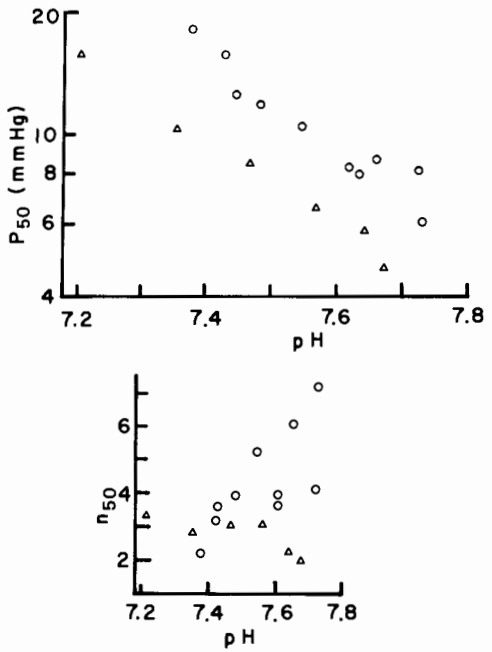


FIGURE 8. Effect of CO_2 on *Carcinus maenas* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered saline (from Robertson, 1960). 16°C, (○) $PCO_2 = 0$, (△) $PCO_2 = 14.8$ mm Hg.

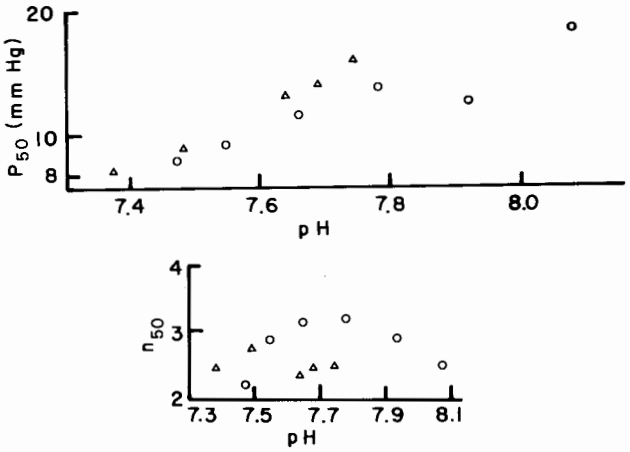


FIGURE 9. Effect of CO_2 on *Limulus polyphemus* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered high salinity saline (from Towle and Mangum, 1982). (O) PCO_2 = 0, (Δ) PCO_2 = 14.8 mm Hg. 25°C.

Carcinus, and *Callinectes*). In contrast the effect of CO_2 (if it occurs) is poorly correlated with sensitivity to other ionic effectors. While CO_2 consistently raises the O_2 affinity of Hcs (*Busycon, Limulus*) with reverse Bohr shifts (and, more importantly, positive divalent cation responses), it may either raise (*Callinectes, Carcinus,* and *Palaeomonetes*), lower (*Cryptochiton*) or have no effect on (*Penaeus*) the O_2 affinity of Hcs with normal Bohr shifts. Different combinations of CO_2 sensitivity of HcO_2 affinity and cooperativity can also be found. CO_2 may affect O_2 affinity but not cooperativity (*e.g., Busycon, Limulus,* and *Callinectes*), both (*Carcinus, Cryptochiton,* and perhaps *Palaeomonetes*) or perhaps neither (*Penaeus*). If CO_2 does have an effect, it generally lowers cooperativity and reduces its pH dependence.

Our results suggest that the chemical species responsible for the effect of CO_2 on portunid crab HcO_2 affinity is molecular carbon dioxide. The effect is greater at low than at high pH and the effector changes O_2 affinity in the same direction as the divalent cations. We can think of no indirect mechanism that would produce such a response. Moreover, the experiment in which HCO_3^- was held within a relatively narrow range while PCO_2 was allowed to change by a large factor produced a much greater effect of CO_2 at low pH, also implicating the molecular species.

Our results also support the hypothesis that the mechanism responsible for the effect on *Busycon* and *Limulus* Hcs is the action of the CO_2 anions on the allosteric effectors free Ca^{+2} and Mg^{+2} rather than a direct effect. It occurs only at high pH, it

TABLE II

Effect of $NaHCO_3$ and $Ca(NO_3)_2$ on Limulus polyphemus *HcO_2 binding*[1]

	P_{50}	n_{50}
Controls PCO_2 1.06 mm Hg	17.1 ± 0.2 (5)	1.89 ± 0.03 (5)
+ 25 m*M* $NaHCO_3$ PCO_2 5.05 mm Hg	15.4 ± 0.3 (5)[2]	1.82 ± 0.05 (5)
+ $Ca(NO_3)_2$ to equal pCa + pMg in control	16.9 ± 0.5 (6)[3]	1.38 ± 0.05 (6)

[1] Cell respiration method:serum was dialyzed against 0.05 *M* Tris maleate buffered high salinity saline (from Towle and Mangum, 1982). 19.7°C. pH 8.21–8.23. Mean ± S.E. (N).

[2] $P < 0.001$ *vs* control.

[3] $P = 0.05$ *vs* + $NaHCO_3$, 0.75 *vs* control.

requires more CO_2 at high than at low salinity and it can be quantitatively explained by the restoration of total divalent cation activity to levels that existed prior to the addition of CO_2. There is no reason to postulate a binding site linked to the active site for which anions compete. The similar responses in these two species also support the conclusion drawn earlier that the fundamentally different quaternary structures of the molluscan and arthropod Hcs do not mandate different O_2 binding properties (Mangum *et al.*, 1985).

The surprising effect of CO_2 on the Hc of the freshwater crab *Holthuisana* (Greenaway, Bonaventura, and Taylor, 1983; Greenaway, Taylor, and Bonaventura, 1983) may be due to the same phenomenon; this Hc may not be directly sensitive to carbon dioxide. Unlike the response of other crustacean Hcs, the O_2 affinity of this molecule decreases with the addition of molecular CO_2, (9.9 mm Hg), and the effect is slightly greater at pH 7.6 than at 7.22–7.30. Moreover, the levels of divalent cations in the blood of this freshwater crab are presumably relatively low (Greenaway and MacMillen, 1978), even at low levels of CO_2. On the other hand, relatively few of the CO_2 anions would be formed by the addition of 9.9 mm Hg PCO_2 in that pH range (Greenaway, Bonaventura, and Taylor, 1983). Its Hc would have to be especially sensitive to divalent cations.

Typically the slopes of Hill plots of HcO_2 equilibria increase with oxygenation. Since these data are often collected by a tonometric procedure that involves the stepwise addition of unscrubbed room air, we suggest that at least a fraction of the slope change may result from an increase in O_2 affinity with CO_2 rather than oxygenation, especially in species in which cooperativity is not very sensitive to CO_2.

The Cl^- sensitivity of *Penaeus* Hc may be either (1) specific and mimicked exactly by a specific effect of Na^+, at least within the physiological range, or (2) a general effect of ionic strength. The information presently available does not decide the question.

Since CO_2 sensitivity is not related to the sensitivity of inorganic anions (and since the nature of the binding sites is totally unknown), it is not possible at present to predict the effects of CO_2 (or Cl^-) on the O_2 affinity of an unknown Hc. Regrettably, if the knowledge is needed it must be acquired in each case. Since it may be quite large, the effect of Cl^- may be an important factor in understanding how a HcO_2 transport system works. In *Penaeus,* for example, a change from 330 to 560 m*M* Cl^-, well within the physiological range in many crustaceans (though perhaps not in these offshore species), causes a 50% change in P_{50} (pH 7.6). Though more often small, the CO_2 effect may become similarly large at 25°C and physiological pH and Cl^-, the maximum being 46% in the range 0–14.8 mm Hg (*Palaemonetes*). This is considerably greater than the effect of CO_2 on mammalian hemoglobin at the same temperature and at 100 m*M* Cl^- and pH 7.4 (calculated from data shown by Imai, 1982). The effect of CO_2 on Hc cooperativity is probably even more important. At pH 7.6 the maximum is a change of about 100% (*Carcinus*), which could radically alter oxygenation states at both the gill and the tissues. A more quantitative assessment awaits extensive measurements of physiological extremes of PCO_2 made simultaneously with measurements of HcO_2 binding and its other determinants.

ACKNOWLEDGMENT

Supported by NSF DCB 84-14856 (Regulatory Biology).

LITERATURE CITED

ARISAKA, F., AND K. E. VAN HOLDE. 1979. Allosteric properties and the association equilibria of hemocyanin from *Callianassa californiensis. J. Mol. Biol.* **134:** 41–73.

BRIDGES, C. R., AND S. MORRIS. 1986. Modulation of haemocyanin-oxygen affinity by L-lactate—a role for other cofactors. In *Invertebrate Oxygen Carriers,* B. Linzen, Ed. Springer-Verlag, Heidelberg. (In press.)

BRIX, O., AND R. TORENSMA. 1981. The molecular basis for the reversed Root effect in the blood of the marine prosobranch, *Buccinum undatum. Mol. Physiol.* **1:** 209–212.

BROUWER, M., C. BONAVENTURA, AND J. BONAVENTURA. 1978. Analysis of the effect of three different allosteric ligands on oxygen binding by hemocyanin of the shrimp, *Penaeus setiferus. Biochemistry* **17:** 2148–2154.

BURNETT, L. E. 1979. The effects of environmental oxygen levels on the respiratory functions of hemocyanin in the crabs *Libinia emarginata* and *Ocypode quadrata. J. Exp. Zool.* **200:** 289–300.

BURNETT, L. E., AND R. L. INFANTINO. 1984. The CO_2-specific sensitivity of hemocyanin oxygen affinity in the decapod crustaceans. *J. Exp. Zool.* **232:** 59–65.

DIEFENBACH, C. O. DA C., AND C. P. MANGUM. 1983. The effects of inorganic ions and acclimation salinity on oxygen binding of the hemocyanin of the horseshoe crab, *Limulus polyphemus. Mol. Physiol.* **4:** 197–206.

GREENAWAY, P., AND R. E. MACMILLEN. 1978. Salt and water balance in the terrestrial phase of the inland crab *Holthuisiana (Austrothelphusa) transversa* Martens (Parathelphusoidea: Sundathelphusidae). *Physiol. Zool.* **51:** 217–229.

GREENAWAY, P., J. BONAVENTURA, AND H. H. TAYLOR. 1983. Aquatic gas exchange in the freshwater/land crab, *Holthuisiana transversa. J. Exp. Biol.* **103:** 225–236.

GREENAWAY, P., H. H. TAYLOR, AND J. BONAVENTURA. 1983. Aerial gas exchange in Australian freshwater/land crabs of the genus *Holthuisiana. J. Exp. Biol.* **103:** 237–251.

IMAI, K. 1982. *Allosteric Effects on Haemoglobin.* Cambridge University Press, Cambridge. 275 pp.

KESTER, D. R., AND R. M. PYTCOWICZ. 1969. Sodium, magnesium and calcium sulfate-ion pairs in seawater at 25°C. *Limnol. Oceanogr.* **14:** 686–692.

MANGUM, C. P. 1980. Respiratory function of the hemocyanins. *Am. Zool.* **20:** 19–38.

MANGUM, C. P., AND G. LYKKEBOE. 1979. The influence of inorganic ions and pH on oxygenation properties of the blood in the gastropod mollusc *Busycon canaliculatum. J. Exp. Zool.* **207:** 417–430.

MANGUM, C. P., J. L. SCOTT, R. E. L. BLACK, K. I. MILLER, AND K. E. VAN HOLDE. 1985. Centipedal hemocyanin: its structure and its implications for arthropod phylogeny. *Proc. Natl. Acad. Sci. USA* **82:** 3721–3725.

MANTEL, L. H., AND L. L. FARMER. 1983. Osmotic and ionic regulation. Pp. 54–161 in *The Biology of Crustacea,* L. H. Mantel, ed. Academic Press, New York.

MANWELL, C. 1958. The oxygen-respiratory pigment equilibrium of the hemocyanin and myoblogin of the amphineuran mollusc *Cryptochiton stelleri. J. Cell. Comp. Physiol.* **52:** 341–358.

MASON, R. P., C. P. MANGUM, AND G. GODETTE. 1983. The influence of inorganic ions and acclimation salinity on hemocyanin-oxygen binding in the blue crab *Callinectes sapidus. Biol. Bull.* **164:** 104–123.

MILLER, K. I., AND K. E. VAN HOLDE. 1981. The effect of environmental variables on the structure and function of hemocyanin from *Callianassa californiensis.* I. Oxygen binding. *J. Comp. Physiol.* **143:** 253–260.

MORRIS, S., AND C. R. BRIDGES. 1985. An investigation of haemocyanin oxygen affinity in the semi-terrestrial crab *Ocypode saratan* Forsk. *J. Exp. Biol.* **117:** 119–132.

NICKERSON, K. W., AND K. E. VAN HOLDE. 1971. A comparison of molluscan and arthropod hemocyanin. I. Circular dichroism and absorption spectra. *Comp. Biochem. Physiol.* **39B:** 855–872.

REDMOND, J. R. 1962. The respiratory characteristics of chiton hemocyanins. *Physiol. Zool.* **35:** 304–313.

ROBERTSON, J. D. 1960. Ionic regulation in the crab *Carcinus maenas* (L.) in relation to the moulting cycle. *Comp. Biochem. Physiol.* **1:** 183–212.

SULLIVAN, B., J. BONAVENTURA, AND C. BONAVENTURA. 1974. Functional differences in the multiple hemocyanins of the horseshoe crab, *Limulus polyphemus* L. *Proc. Nat. Acad. Sci.* **71:** 2558–2562.

TORENSMA, R., AND O. BRIX. 1981. Oxygen binding of *Neptunea antiqua* hemocyanin. The significance of allosteric ligands. *Mol. Physiol.* **1:** 213–221.

TRUCHOT, J-P. 1973. Action spècifique de carbone sûr l'affinité pour l'oxygène de l'hémocyanin de *Carcinus maenas* (L.) (Crustace Décapode Brachyoure). *Comptes Rendus Acad. Sci. Paris* **276:** 2965–2968.

TRUCHOT, J-P. 1975. Factors controlling the *in vivo* and *in vitro* oxygen affinity of the hemocyanin in the crab *Carcinus maenas* (L.). *Respir. Physiol.* **24:** 173–179.