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THE IONIC ENVIRONMENT OF HEMOCYANIN IN *LIMULUS POLYPHEMUS*

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The extracellular respiratory pigments found in invertebrates are, in general, highly sensitive to their ionic environment in the blood. The ion dependence of molecular structure and *in vitro* oxygen binding of the hemocyanins has been investigated intensively. Small changes in H^+ or inorganic salt concentrations, for example, bring about large changes in the equilibrium between native molluscan hemocyanin and its constituent subunits (DePhillips, Nickerson and Van Holde, 1970). These structural changes are accompanied by shifts in oxygen affinity and the cooperativity of oxygen binding.

The effective ions that have received the most attention are H^+ and the divalent cations Ca^{+2} and Mg^{+2}. Interestingly, H^+ and the ions of inorganic salts have opposite effects on *in vitro* oxygenation. If an increase in H^+ concentration reduces oxygen affinity (*e.g.*, the extracellular heme proteins and crustacean hemocyanins), an increase in salts raises oxygen affinity (Antonini, Rossi-Fanelli and Caputo, 1962; Chantler, Harris and Bannister, 1973; Everaarts and Weber, 1974). Conversely, if an increase in H^+ raises oxygen affinity (*e.g.*, gastropod and xiphosuran hemocyanins), then an increase in inorganic salts lowers oxygen affinity (DePhillips *et al.*, 1970; Sullivan, Bonaventura and Bonaventura, 1974). Although the divalent cations in blood change very little with acclimation salinity, the physiological changes appear to be great enough to induce detectable shifts of the *in vitro* oxygen affinity of hemocyanin from the portunid crab *Carcinus maenas* (Truchot, 1973, 1975). In addition, more variable ions such as Na^+ and Cl^- modify the oxygen affinity of other extracellular respiratory pigments, such as annelid hemoglobins and xiphosuran hemocyanins (Everaarts and Weber, 1974; Sullivan *et al.*, 1974).

The salt dependence of oxygen carrying pigments suggests that the oxygen transport system must be influenced by environmental salinity in estuarine species that are not perfect regulators of the ion concentrations in their body fluids. In a study of oxygen transport in the portunid crab *Callinectes sapidus*, it was shown that *in vivo* oxygenation of hemocyanin remains essentially unchanged even when blood salts are diluted, because the salt effect is opposed by a concomitant elevation of blood pH (Mangum and Weiland, 1975; Weiland and Mangum, 1975). The pH change at low salinity is believed to result from the increased production of ammonia, which binds H^+, raises pH and opposes the effect of salt depletion on hemocyanin oxygenation (Weiland and Mangum, 1975). The increased ammonia, which may be produced in the catabolism of free amino acids as intracellular fluids re-equilibrate to the dilute blood (Gerard and Gilles, 1972), also plays a role in salt absorption at the gill. In microsomal preparations, NH_4^+ serves equally well as K^+ as the counterion for activity of the transport enzyme that moves Na^+ across the gill membrane (Towle and Harris, 1976). When *C. sapidus* osmotically con-

forms to a salinity reduction, no change in NH_4^+ output occurs, but there is a large increase when the medium is diluted further to a salinity at which the crab actively maintains a hyperosmotic blood (Mangum, Silverthorn, Harris, Towle and Krall, 1976).

Thus the oxygen transport system in *C. sapidus* is highly adapted to life in an unstable ionic environment. The mechanism may be categorized as *enantiostatic,* in the sense that the standing condition, a highly integrated response of the respiratory, osmoregulatory and excretory systems, results from opposite and counterbalancing ionic effects on the respiratory pigment (Mangum, 1976). There are ample reasons to expect that these findings are not general among estuarine animals and that the enantiostatic balance observed in portunid crabs is but one of many possible adaptations. Therefore we have investigated the influence of acclimation salinity on oxygen transport in the xiphosuran *Limulus polyphemus* (Linnaeus), which differs in several important respects from decapod crustaceans. First, its hemocyanin has different ionic sensitivities; the Bohr shift is negative and the addition of salt lowers oxygen affinity. The influence of salts on oxygen affinity is due in large part to Cl^- and not only to divalent cations (Sullivan *et al.,* 1974). Stripping the blood of all salts increases oxygen affinity from 6.8 to 1.6 torr at 20° C and pH 7.5, and the addition of NaCl to the stripped preparation induces a logarithmic decrease to about 3.8 torr at 4 M NaCl. The chloride effect on whole blood has been quantitatively explained by the sensitivity of two of the five hemocyanin components separated by ion exchange chromatography. Second, salinity-induced changes in blood NaCl and Ca^{+2} concentrations in *Limulus* are more than twice as great (Robertson, 1970). Thirdly, less than 10% of the change in intracellular osmolality in *Limulus* is due to free amino acids (Bricteux-Grégoire, Dûchateau-Bosson, Jeuniaux and Florkin, 1966; Robertson, 1970), while the comparable figure for *C. sapidus* is 70% (Gerard and Gilles, 1972). Last, horseshoe crabs enter the estuary only in the late spring and summer, and they do not penetrate very far into the deeper and more dilute regions.

Materials and Methods

Animals were obtained from fishermen or by personal collection in high salinity waters (31–33‰) near Wachapreague on the Eastern Shore of Virginia, where the work was conducted. They were maintained in running sea water until a few days prior to experimentation. Sampling was completed within one week of collection, so that no animal was in an extreme state of starvation and hemocyanin depletion.

Animals were exposed to low salinity in aerated containers for two days prior to sampling. A second set of samples was taken after five or seven days at 15.4–16.5‰ salinity, but no significant differences were noted. Dilutions were prepared with distilled water, and the salinities reported were calculated from measurements of the chloride ion (Buchler-Cotlove chloridometer). Acclimation to 8.8–9.4‰ was carried out in two steps, with 24 hr at an intermediate salinity.

Measurements on whole animals

The rate of oxygen consumption was calculated from a recorder trace of oxygen depletion in a closed container. Oxygen concentration was monitored continuously

with a Yellow Springs Instrument Co. Model 5420 polarographic electrode and 180 recorder. Because of the size of the respirometer (12 l, including a tubular extension for the telson), additional stirring was provided at each of two sides, with large magnetic stirrers.

Total outputs of ammonia and acids were calculated from measurements of the two variables in 45 l sea water, made before and after confining an animal for 70–120 min. Net acid output was estimated from titration curves describing the change in pH after the addition of 0.1 N HCl to water at each salinity; the water was aerated for two hr prior to each measurement to equilibrate it to atmospheric CO_2.

The heartbeat of intact, unrestrained animals was recorded with an impedance pneumograph (E and M Instr. Co.). Electrodes were inserted on either side of the heart through small (1 mm diameter) holes drilled in the dorsal prosoma, and the holes sealed with dental wax (Surgident). Animals survived the operation for as long as five months.

Blood and urine sampling

Large samples (2–3 ml) of blood were taken from the pericardial sinus (postbranchial) or the junction of the mesosoma and telson (prebranchial) into iced syringes, within 15 sec of removing an animal from water. One aliquot was immediately injected into the chamber of a Radiometer Corp. BMS1 blood gas analyzer for PO_2 measurement. The remainder was centrifuged at high speed (Sorvall SS-1) to express serum from the clot, and further subdivided for the other measurements.

Urine was allowed to drip into small snap cap vials through a length of polyethylene (No. 10) tubing inserted into the opening of the coxal gland. Most of the measurements on urine were made immediately after sampling. However, the measurements of total solids, Na^+ and a second measurement of Cl^- were made on samples that had been stored for one to two weeks. Evaporation must have occurred in some of the vials, as indicated by comparing the results for total solids with those for osmolality, and by comparing the two sets of Cl^- data. These samples were used only for the calculations of the ion ratios, and the Na^+ concentrations were predicted from the first set of Cl^- values.

Because of an interest in the respiratory role of the excretory organ, it was important to obtain urine samples from animals that were not in a state of respiratory acidosis. And yet catheters inserted into the opening of the coxal glands did not remain in place when the animals were allowed to swim freely in water. Therefore the measurements were made on urine samples obtained within five to ten min of removing the animal from water. Blood samples taken after much longer air exposure indicate that significant acidosis occurs in 60 min ($\Delta pH = -0.19$ to -0.24), but that change would explain only a small fraction of the results reported below. In addition, several samples were obtained from animals submerged in small containers where they could not crawl or swim; the results for these samples do not show perceptible trends and they are statistically homogeneous ($P > .05$) with the others. There is no significant change in the ammonia concentration of urine after one hr air exposure ($\Delta NH_4^+ = -0.20$ to 0.012 mM/l).

Measurements on blood and urine samples

pH was measured with a liquid junction capillary electrode (Radiometer Corp.). Ammonia concentration was determined by the phenol hypochlorite method (Solórzano, 1969), using 0.5 ml samples. At high concentrations, we found it necessary to dilute the samples with distilled water and to allow two hours for completion of the reaction. In these cases, the background concentration of the ammonia in H_2O was subtracted from the results. Otherwise, the procedure was unchanged.

Sodium concentration was measured with an EEL flame photometer (Oglesby, 1970), and chloride with a Buchler-Cotlove chloridometer. Osmolality was determined with an Osmette freezing point osmometer, and total solids with an Atago refractometer.

The absorbance of blood was measured at 340 nm (Beckman DK-2 spectrophotometer) after dilution of the sample with distilled water (1 : 39). The concentration of hemocyanin was computed from the molecular extinction coefficient given by Nickerson and van Holde (1971). Oxygen carrying capacity at 159 torr was calculated from these results on the assumption that 0.17% of the total weight of the hemocyanin molecule is copper, and that Cu combines with molecular oxygen in the ratio 2 : 1 (van Holde and van Bruggen, 1971). A test of this procedure, made by comparing the results with those of more time-consuming but direct measurements (Mangum, Freadman and Johansen, 1975), indicates that it is equally accurate.

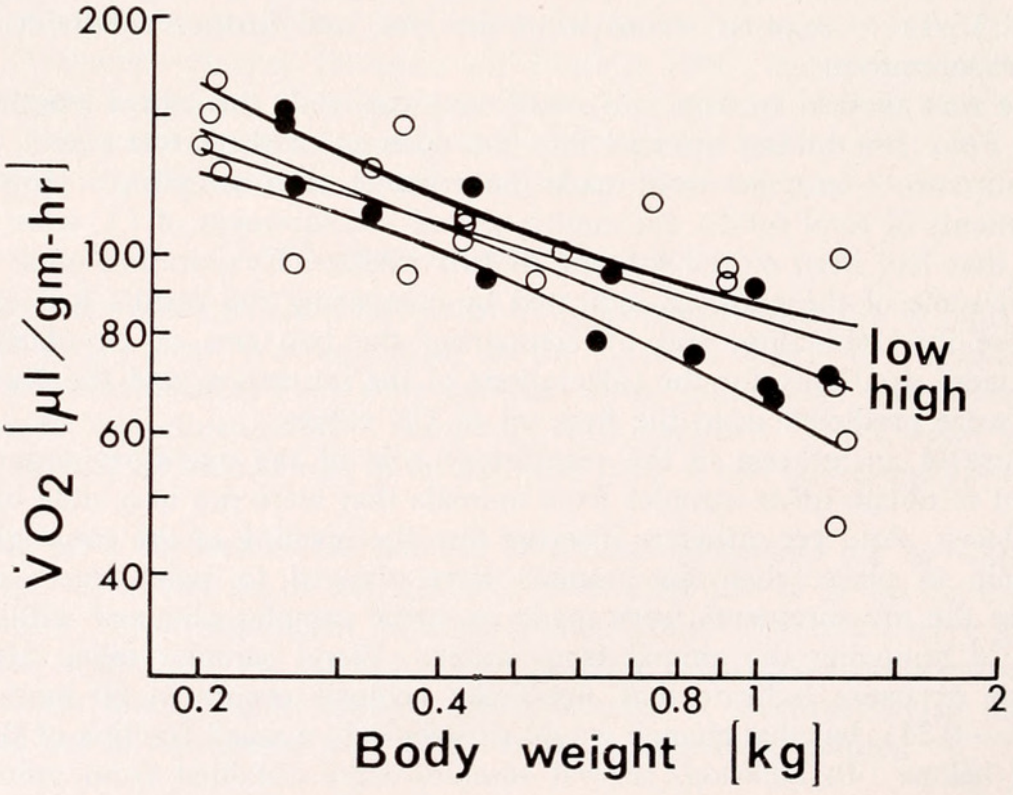

FIGURE 1. The rate of oxygen consumption ($\dot{V}O_2$) in *Limulus polyphemus* acclimated to 32 (closed circles) and 8 (open circles) ‰ salinity and 22° C. The 95% confidence interval encloses the regression line describing the combined data. The slope of that line is −0.39 (± 0.066) and the correlation coefficient (r) is 0.935.

Estimates of hemocyanin oxygenation

The *in vivo* oxygenation of hemocyanin was estimated by extrapolation to *in vitro* oxygen equilibrium curves. These curves were calculated for a temperature of 24° C and each relevant combination of H^+ and NaCl, from data shown by Sullivan *et al.* (1974) and Bonaventura, Sullivan, Bonaventura and Bourne (1974), further details of which were kindly communicated by the authors. The difference between stripped and unstripped blood was assumed to be due to logarithmic increases in NaCl, as shown by Sullivan *et al.* (1974), and also to other inorganic salts, as present in the ratios reported by Robertson (1970). The temperature dependence of oxygen binding was assumed to be conventional ($\Delta H = -13$ kcal/mole) as suggested by the results of Redfield and Ingalls (1933).

The quantitative effects of salts on the cooperativity of oxygen binding (the Hill constant n) are not known, but they are believed to be due to changes in divalent cations as well as NaCl. The physiological changes in Ca^{+2} reported by Robertson (1970) would cause a large change in the cooperativity of crustacean hemocyanins (Larimer and Riggs, 1964). For the present purposes, a linear reduction in the values of n reported by Redfield and Ingalls (1933) and by Sullivan *et al.* (1974) was assumed to occur with changes in blood Ca^{+2} and Mg^{+2} at low salinity.

Results

Body size and salinity tolerance

Despite the precaution of a stepwise dilution, the larger animals in the sample died at 8.8–9.4‰ salinity. After two such losses of the material available, the larger animals were saved for experimentation at a less extreme dilution (15.4–16.6‰). The result is an unfortunate discrepancy in body size: the control group is representative, the group at 15.4–16.6‰ consists of atypically large animals (> 4 kg), and that at 8.8–9.4‰ of small animals (0.3–1.3 kg). Many of the measurements were performed as paired observations on the same individuals, and therefore the results are unaffected. However, the bias does affect several of the respiratory calculations, as discussed below.

This observation suggests that the surprisingly wide range of salinity tolerance reported by Robertson (1970), who studied only animals weighing less than 0.7 kg and who noted a size trend even within that sample, is misleading. In the Chesapeake Bay region horseshoe crabs are rarely found in waters of less than 18‰ (Wass, 1972 and personal communication). On one occasion we have personally observed animals in more dilute waters, 13–16‰ in Long Island Sound, but they were very small. Although Robertson (1970) cites several reports that would suggest otherwise, the observations made by the present authors and those of more experienced observers indicate that the species is largely confined to the polyhaline regions of estuaries, and that only the very young penetrate into mesohaline waters.

Oxygen consumption

Regression lines describing the data (Fig. 1) for oxygen consumption as a function of body weight at high (N = 13) and low (N = 21) salinities are not

TABLE I

Measured respiratory, osmotic and ionic parameters in blood and urine of Limulus polyphemus *held in waters of different salinity. Mean* (± *s.e.*). *Number of animals given in parentheses; number of observations given in brackets. High* O_2 = ≥ *140 torr; reduced* O_2 = *98–105 torr; 22–24° C.*

	Salinity (‰)		
	32–33	15.4–16.5	8.8–9.4
1. Prosomal width (cm)	23.3 (±1.5) (10)	>30 (6)	18.4 (±1.1) (8)
2. Wet weight (kg)	1.882 (±0.371) (10)	>4.0 (6)	0.824 (±0.142) (8)
3. Sex			
Male	(3)	(3)	(3)
Female	(7)	(3)	(5)
4. Postbranchial blood PO_2 (torr) high O_2	75 (±3) (6)	62 (±8) (5)	66 (±3) (4)
reduced O_2	62 (4)	50 (5)	50 (4)
5. Prebranchial blood PO_2 (torr), high O_2	14.0 (±0.5) (9)	8.0 (±0.7) (6)	11.8 (±1.1) (6)
reduced O_2	8.0 (±1.3) (45)		5.8 (±0.7) (6)
6. Blood hemocyanin concentration (gm/100 ml)	9.08 (±1.08) (9) [16]	12.14 (±1.97) (5) [10]	9.87 (±1.58) (6) [10]
7. Prebranchial blood pH, high O_2	7.495 (±0.007) (9) [46]	7.522 (±0.010) (6) [17]	7.537 (±0.010) (6) [35]
Blood H^+ concentration/water H^+ concentration	1.94 (±0.06)	1.52 (±0.05)	1.12 (±0.08)
8. Urine pH	6.849 (±0.049) (6) [18]	7.216 (±0.026) (3) [6]	7.772 (±0.036) (5) [10]
Urine H^+ concentration/blood H^+ concentration	3.59 (±0.34)	0.98 (±0.25)	0.46 (±0.06)
9. Blood NH_4^+ concentration (mM/l)	0.092 (±0.003) (9) [42]	0.051 (±0.003) (6) [7]	0.048 (±0.006) (6) [21]
Blood NH_4^+ concentration/water NH_4^+ concentration	8.4 (±2.1)	11.3 (±1.4)	9.6 (±1.6)
10. Urine NH_4^+ concentration (mM/l)	0.650 (±0.085) (6) [8]	0.139 (±0.041) (3) [7]	0.052 (±0.009) (4) [5]
Urine NH_4^+ concentration/blood NH_4^+ concentration	8.35 (±1.12)	1.87 (±0.43)	1.57 (±0.31)

TABLE I—*Continued*

	Salinity (‰)		
	32–33	15.4–16.5	8.8–9.4
11. Blood osmolality (mOsmol/l)	935 (±5) (10) [19]	540 (±15) (6) [13]	491 (±10) (8) [12]
Osmotic difference across gill membrane	6 (±2)	70 (±18)	209 (±13)
12. Urine osmolality (mOsmol/l)	948 (±5) (6)	562 (1)	410 (±12) (4)
Osmotic difference across coxal gland membrane	1 (±9)	25	68 (±20)
13. Total solids in urine (gm/100 ml)	3.0 (±0.1) (4) [7]	2.0 (±0.2) (4) [4]	1.4 (±0.1) (2) [4]
14. Blood Na^+ concentration (meq/l)	459 (±9) (10) [15]	272 (±12) (6) [11]	266 (±11) (7) [10]
Blood Na^+ concentration/water Na^+ concentration	0.95 (±0.01)	1.09 (±0.04)	2.17 (±0.09)
15. Urine Na^+ concentration (meq/l)	504 (±16)	282*	211*
Urine Na^+ concentration/blood Na^+ concentration	1.07 (±0.02)	0.97*	0.79*
16. Blood Cl^- concentration (meq/l)	488 (±11) (10) [23]	277 (±6) (6) [22]	226 (±13) (8) [22]
Blood Cl^- concentration/water Cl^- concentration	0.92 (±0.02)	1.10 (±0.04)	1.53 (±0.08)
17. Urine Cl^- concentration (meq/l)	517 (±16) (9) [21]	297 (±12) (3) [6]	229 (±7) (5) [7]
Urine Cl^- concentration/blood Cl^- concentration	1.06 (±0.02)	0.99 (±0.02)	0.88 (±0.04)

* See text.

significantly different from one another ($P = 0.35$, F test). This conclusion is supported by two sets of paired observations of oxygen consumption in the same individuals at high and low salinity, which are also homogeneous. If the salt effect on the oxygenation of hemocyanin is great enough to perceptibly influence its role in oxygen transport, then other respiratory compensations must occur.

Oxygen consumption does not decrease significantly ($P > 0.05$ according to

Student's *t* test) from the values shown in Figure 1 when the water PO_2 is lowered from 159 to about 80 torr. This finding agrees with the previous report of Johansen and Petersen (1975).

Effect of acclimation salinity on osmotic and ionic constituents of blood and urine

The results (Table I) on blood NaCl and osmotic concentrations are essentially the same as those reported earlier by Robertson (1970), indicating that body size does not influence the osmotic and ionic steady state, even though it is related to salinity tolerance. No sexual differences were noted in either his experiments or ours. At high salinity the blood of horseshoe crabs is slightly hypoionic and hyperosmotic to the medium. The hypoionic condition of blood appears to be maintained, at least in part, by the excretory organ, as in crustaceans. There is no evidence, however, that blood hyperosmoticity results from the reabsorption of osmotically active substances in primary urine.

At 8.8–9.4‰ the reduction of osmotically active substances in blood is opposed by the output of a dilute urine, and the difference between blood and urine is largely explained by the reabsorption of NaCl from the urine. Despite the regulatory response, the reduction of blood NaCl at low salinity is quite great. The decrease in Cl^-, the ion responsible for much of the change in hemocyanin oxygen affinity when salts are removed from blood (Sullivan *et al.*, 1974), is greatest when the acclimation salinity is lowered to 15.4–16.5‰. Further change at 8.8–9.4‰ is disproportionately small.

The ammonia concentration in the blood of horseshoe crabs at 32–33‰ is high relative to that in decapod crustaceans (Gerard and Gilles, 1972; Siebers, Lucu, Sperling and Eberlein, 1972). Unlike the portunid crabs, the level of ammonia in blood decreases at low salinity, a finding which is consistent with the evidence that reduction of the free amino acid pool is not a major mechanism of intracellular osmotic adjustment (Bricteux-Grégoire *et al.*, 1966; Robertson, 1970). The concentration of ammonia in urine decreases at low salinity to a level that, at 8.8–9.4‰, is no longer significantly higher than blood. At the same time, the total output of ammonia and net output of acids also decrease. Ammonia output in six animals decreased from 0.33 (± 0.10 s.e.) mM/kg-hr at 32.1‰ to 0.18 (± 0.03) mM/kg-hr at 15.4–16.5‰; net acid output decreased from 0.78 (± 0.08) mM/kg-hr at 32.1 to 0.20 (± 0.02) mM/kg-hr at 15.4–16.5‰. These results suggest that NH_4^+ in xiphosurans does not play an important role in salt absorption, in contrast with its importance in the blue crab *Callinectes sapidus,* and they do not suggest H^+ secretion. Due to the methodological difficulties of distinguishing between the movement of H^+ *per se* and changes in the output of a base or some other acid, this result is not a conclusive finding of lower H^+ output at low salinity, however. (The properties of the transport enzyme in the *Limulus* gill are presently under investigation by D. W. Towle.)

Thus the reduction of blood NaCl is not accompanied by the large increase in pH that occurs in portunid crabs, which would oppose the salt effect on the oxygen transport system and, possibly, maintain a respiratory balance. Unless other respiratory or environmental parameters change, the role of hemocyanin in aerobic respiration must be smaller at lower salinity.

TABLE II

Respiratory parameters of Limulus polyphemus *in waters of different salinity, calculated from data in Figure 1, Table I and Sullivan* et al. *(1974).*

	Salinity (‰)		
	32–33	15.4–16.5	8.8–9.5
1. Postbranchial oxyhemocyanin (%)			
high O_2	100	99	99
2. Postbranchial oxygen concentration (ml/100 ml)			
		3.49	
high O_2	2.95	3.83	3.15
reduced O_2	2.90		3.13
3. Prebranchial oxyhemocyanin (%)		69	
high O_2	72	58	81
reduced O_2	50		55
4. Prebranchial oxygen concentration (ml/100 ml)		2.30	
high O_2	2.00	2.13	2.42
reduced O_2	1.38		1.64
5. Postbranchial—prebranchial oxygen concentration (m/100 ml)		1.19	
high O_2	0.95	1.70	0.73
reduced O_2	1.52		1.49
6. Oxygen uptake (ml/kg-min)		0.991	
high O_2	1.00	0.767	1.215
7. Cardiac output (ml/kg-min)		83	
high O_2	105	45	166
reduced O_2	66		82
8. % oxygen uptake by hemocyanin		80	
high O_2	72	89	74
reduced O_2	89		89

Effect of acclimation salinity on oxygen transport

The data in Table I do not unequivocally demonstrate a change in hemocyanin concentration with acclimation salinity, although the interpretation is complicated by the body size bias of the samples. Animals weighing 1 kg or less have significantly lower amounts of hemocyanin in their blood ($P < 0.05$; Student's t test), regardless of acclimation salinity. Comparison of the data for animals of the same size acclimated to different salinities suggest an increase in hemocyanin concentration in dilute waters, but the data are too few for quantitative analysis. In the discussion below it is assumed that an increase in hemocyanin concentration, if it occurs, involves a proportional increase in chloride sensitive as well as chloride insensitive components (Sullivan *et al.*, 1974).

The values for hemocyanin concentration predict an oxygen carrying capacity [2.96 ($\pm$ 0.28) ml/100 ml] which is higher than several of the values in the

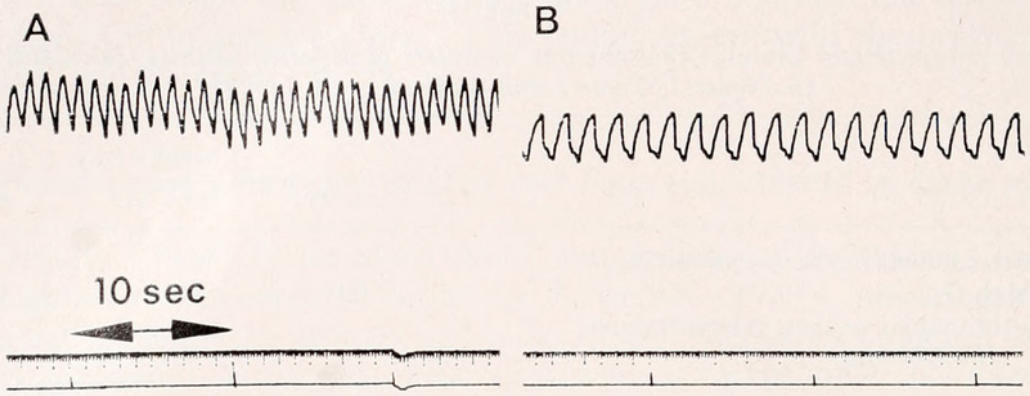

FIGURE 2. The effect of a reduction in salinity on heartbeat of *Limulus polyphemus*. (A) 32 (B) 22‰, four days later; 20–22° C; $PO_2 > 140$ torr.

literature (Redfield, Coolidge and Hurd, 1926; Mangum, Freadman and Johansen, 1975), but they are not significantly different from that mentioned by Sullivan *et al.* (1974). The animals studied by Mangum, Freadman and Johansen (1975) had been kept in the laboratory for at least three months, and it is very likely that the hemocyanin concentration was unnaturally low, due to starvation. Although the values in Table I are higher than those usually found in aquatic brachyurans, they are well within the range found in freshly caught or well fed shrimp (Djangmah, 1970) and in land crabs (Cameron and Mecklenberg, 1973).

Preliminary measurements of postbranchial blood PO_2 indicated that acclimation salinity does not appreciably affect the process of hemocyanin oxygenation at the gill, as long as ambient PO_2 is high (Table I). Subsequent observations were restricted to the parameters in prebranchial blood, which are more sensitive indices of changes in the per cent oxyhemocyanin.

The data for prebranchial blood PO_2 also reflect the body size bias of the samples (Table I). Larger animals clearly have lower prebranchial blood PO_2's, regardless of acclimation salinity ($P < .05$; Student's t test). Despite this confounding factor, the decrease in blood PO_2 with acclimation salinity is apparent; however, it is exaggerated in the data for 15.4–16.6 and minimized at 8.8–9.4‰. The response differs from that in blue crabs, which show a very small increase in blood PO_2 at low salinity (Weiland and Mangum, 1975).

The calculated parameters in Table II support the previous conclusion that, in well oxygenated waters ($PO_2 > 140$ torr), hemocyanin is fully oxygenated at the gill and it delivers only a small fraction of its oxygen to the tissues (Johansen and Petersen, 1975), despite the higher temperature and use of freshly collected animals in the present experiment. At an ambient PO_2 (98–105 torr) which is more typical of the bottom layer of water in the stream channels where *Limulus* enters the estuary (Deaton and Mangum, 1976), the role of hemocyanin becomes greater regardless of salinity, due to the changes in both pH and PO_2 (Johansen and Petersen, 1975; Table II).

The size bias of the samples becomes more important in interpreting the respiratory consequences of low salinity adaptation (Table II). If the results for 15.4–16.5 and 8.8–9.4‰ are combined, the mean body weight is about 2.2 kg, which is not significantly different from that of the high salinity sample, and the

effects of low salinity on oxygen uptake and transport are not obscured. The lower blood PO_2 in dilute media induces greater deoxygenation of hemocyanin at the tissues (Table II). The predicted effect of low blood salt is in fact more than compensated, and the net result is an increase rather than a decrease in the volume of oxygen carried by hemocyanin. These calculations also suggest a decrease in blood flow at low salinity, which is consistent with recordings of heartbeat (Fig. 2). The measured decrease of heartbeat in different animals ranged from 13–33% ($P < .05$).

The respiratory consequences of the data in Tables I and II are summarized in Figure 3. Figure 3A shows the combined data for animals at low salinity, so that water PO_2 and body size are not variables. Figure 3B shows the data for animals of average size at $PO_2 > 140$ torr and 32‰ in comparison with those for small animals at $PO_2 = 98$–105 torr at 8.8–9.4‰, which more closely simulates the events occurring in natural estuarine migrations. Oxygen uptake is maintained at a nearly constant level, regardless of salinity and water PO_2, by decreases in blood flow and blood PO_2. Thus the mechanisms of respiratory adaptation at low salinity are in part passive, the increase in efficiency of oxygen transport by hemocyanin, and in part active, the bradycardia.

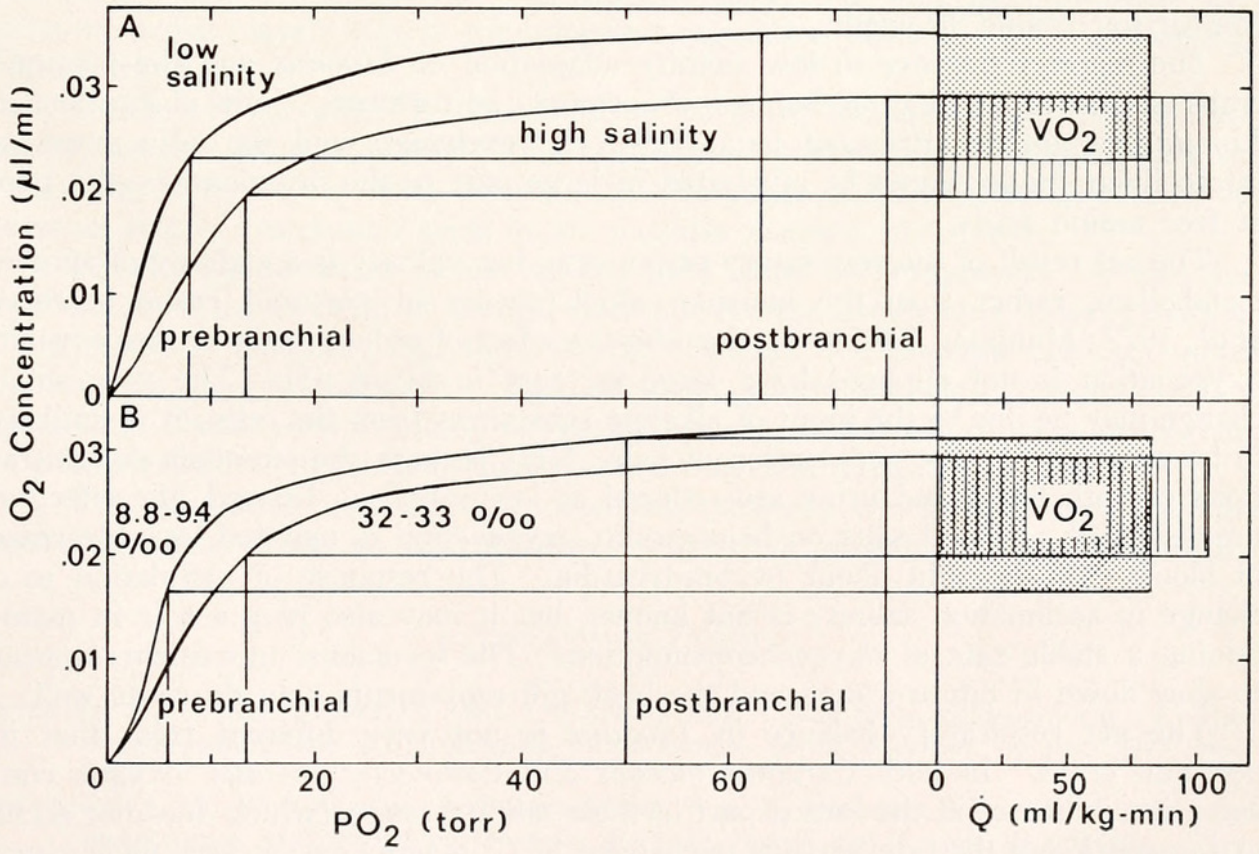

FIGURE 3. The changes in oxygen transport by the blood of *Limulus polyphemus* induced by adaptation to low salinity. The volume of oxygen delivered to tissue is represented by the height of the stippled or hatched boxes, and cardiac output ($\dot{Q}$) by the width. Thus, according to the Fick principle the area of the boxes represents oxygen consumption (µl/ kg-min). Curves are calculated from oxygen equilibrium data for *Limulus* hemocyanin (Redfield and Ingalls, 1933; Sullivan *et al.*, 1974), and from data in Tables I and II.

As indicated above, these conclusions rest upon the assumption that an increase in hemocyanin concentration at low salinity entails a proportional increase in each of the five electrophoretic components. If this assumption is incorrect, the error could have either of two effects. On the one hand, selective increase in the two chloride sensitive components would result in a greater difference between each member of the pairs of curves in Figure 3, and the increase in hemocyanin function might be mitigated or reversed. On the other hand, selective increase in the three chloride insensitive components, the more adaptive response, would result in less difference between the two curves and the response might be enhanced.

Discussion

The osmotic and ionic responses of the blood of *Limulus polyphemus* differ from those in decapod crustaceans only in quantitative terms. The animal osmotically conforms to a dilution of the medium at high salinities, and then begins to maintain a hyperosmotic blood at some point in the range 22–24‰ (Robertson, 1970). Unlike many crustaceans, however, the excretory organ contributes to the hyperosmotic condition of blood at very low salinity by forming a urine which is more dilute than blood. The coxal glands also contribute to acid-base regulation by forming a urine which is acidic to blood at 32–33 and 15.4–16.5‰, and alkaline at 8.8–9.4‰. Since the acid-base status of crustacean urine is not known, this comparison cannot be made.

The major difference in low salinity adaptation of *Limulus* and the portunid crabs seems to be the mechanism of osmotic adjustment. Salt absorption in *Limulus* cannot be attributed to a Na^+–NH_4^+ exchange, and the adjustment of intracellular fluids cannot be attributed in large part to the deamination of a pool of free amino acids.

The net result of the respiratory response to low salinity is a stability of aerobic metabolism, rather than the increase which occurs in portunid crabs (Siebers *et al.*, 1972; Mangum, 1976). In *Limulus* the effect of reduced salts on hemocyanin oxygenation is not opposed by a large increase in blood pH. The very small change may be due to the input of alkaline substances from the cells; it is unlikely to be explained by the protonation of more NH_3 because the ammonia concentrations of both blood and urine are reduced at low salinity. Instead, the effect of the reduction of blood salts on hemocyanin oxygenation is opposed by a decrease in blood PO_2, brought about by bradycardia. The response of ventilation to a change in acclimation salinity is not known, but it may also play a role in maintaining a stable rate of oxygen consumption. The locomotor movements appear to slow down in dilute waters and the book gill movements may do so as well.

The net respiratory balance in *Limulus* is not very different from that in portunid crabs. In both *Carcinus maenas* and *Callinectes sapidus,* oxygen consumption increases in the lowest part of their salinity range (which does not occur in *Limulus*), but the rate remains unchanged in *C. sapidus* over a very wide range of acclimation salinities (Siebers *et al.,* 1972; Laird and Haefner, 1976; Mangum, 1976.)

If blood PO_2 in *Limulus* did not change at low salinity, the function of hemocyanin would be greatly diminished, both in highly oxygenated waters and in the stream channels of estuaries. The salt effect on *Limulus* hemocyanin is so

large that it would not be opposed completely by an elevation of pH, even one as great as that in *C. maenas* (0.3) or *C. sapidus* (0.2). In fact the large changes in salts and PO_2, and the small change in pH result in a greater importance of the oxygen carrying pigment when the animal enters dilute waters.

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Summary

1. Oxygen affinity of *Limulus* hemocyanin is lowered by the addition of Cl^- and raised by the addition of H^+. Therefore the oxygen transport system should be influenced by ionic changes in the blood, such as those which accompany the spring migrations into estuaries.

2. When *Limulus* enters dilute waters, blood NaCl is reduced by almost half, even though active hyperosmotic regulation occurs. At very low salinities, the excretory organ plays a role in the imperfect regulation of blood NaCl by forming a dilute urine.

3. Unlike crustaceans, H^+ concentration of blood changes very little at low salinity, because little additional NH_3 is produced and no appreciable increase in H^+ binding to molecular NH_3 occurs. This response is believed to be related to the small role of free amino acids in intracellular osmotic adjustment.

4. Blood PO_2 decreases in low salinity as a result of bradycardia. While the oxygenation of hemocyanin at the gill does not change appreciably, deoxygenation at the tissues is enhanced and thus, in dilute waters, the pigment becomes more important in oxygen transport. This response, which also differs from that in crustaceans, occurs both in highly oxygenated waters and at the oxygen levels that are characteristic of the stream channels in the natural habitat.

5. The adaptation consists of concomitant changes which have opposite but counterbalancing effects on oxygen consumption in an unstable ionic environment.

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