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The influence of inorganic ions on the oxygen binding of hemocyanin in the blue crab, Callinectes sapidus Rathbun

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THE INFLUENCE OF INORGANIC IONS ON THE OXYGEN BINDING OF HEMOCYANIN IN THE BLUE CRAB, *CALLINECTES SAPIDUS* RATHBUN

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Master of Arts

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Richard Patrick Mason

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of

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TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The effects of the inorganic ions in the blood on the oxygen binding properties of hemocyanin have been examined in the blue crab Callinectes sapidus Rathbun. Ca^{+2}, Mg^{+2} and Na^{+}, but not K^{+} or Cl^{-}, measurably increase oxygen affinity. However, the presence of Ca^{+2}, alone and in physiological concentrations, suffices to restore oxygen affinity to the range observed in the presence of a complete physiological saline. The presence of Ca^{+2} alone also restores the Bohr shift to the physiological level. The cooperativity of oxygen binding, however, is independent of physiological variation in inorganic ions or pH.

Physiological variation in Ca^{+2} explains only a minor part of the actual change in hemocyanin-oxygen binding that accompanies acclimation to a new salinity. The acclimation is reversible within 8 da., and it requires the presence of a non-dialyzable factor in the blood. An obvious hypothesis that a new hemocyanin molecule is synthesized must be considered tentative on the basis of present information.

THE INFLUENCE OF INORGANIC IONS ON THE OXYGEN BINDING OF HEMOCYANIN IN THE BLUE CRAB, *CALLINECTES SAPIDUS* RATHBUN

INTRODUCTION

The effect of the ionic environment on the oxygenation properties of the hemocyanins (Hcs) has been known for many years. Among the crustacean Hcs, O_2 affinity increases with the addition of inorganic ions and decreases with the addition of H^+ (Redfield, 1933; Larimer and Riggs, 1964; Chantler et al., 1973; Truchot, 1975; Mangum and Towle, 1977; Brouwer et al., 1978; Miller and Van Holde, 1981). The opposite responses to pH and inorganic ions are especially important in many estuarine species because they actually occur in vivo, with the net effect of counterbalancing the performance of the HcO_2 transport system (Truchot, 1975; Weiland and Mangum, 1976). A detailed theoretical analysis of the opposite effects of Mg^{+2} and H^+ suggests that the mechanism is competitive binding at the same ion sites, a few of which are linked to O_2 binding sites (Arisaka and Van Holde, 1979). The effect of physiological changes in the ionic environment on the cooperativity of HcO_2 binding has not been extensively investigated in the crustaceans, and the available information does not permit a very knowledgeable assessment. Recent data for the major component prepared from the thalassinid shrimp Callianassa californiensis suggests that the effect is either small or absent (Miller and Van Holde, 1981).

The influences of the different inorganic ions found in the blood on HcO_2 affinity are not equal. The available information agrees that, on a molar basis, the divalent cations have a greater effect than any of

the monovalent ions. On a molar basis, however, the physiological changes in inorganic ions are unequal. Divalent cations are both less abundant and, in most species, regulated more strongly than Na^+ and Cl^- (Mangum, 1981).

The literature on the effect of Cl^- appears to be contradictory. A specific effect of Cl^- on $Hc O_2$ affinity in the chelicerate arthropod Limulus was first demonstrated by Sullivan et al. (1974) and later elucidated by Brouwer et al. (1977). Perhaps more relevant here, a very large and allosteric effect of Cl^- is found in the dendrobranchiate crustacean Panaeus setiferus (Brouwer et al., 1978). The addition of NaCl and KCl to the experimental medium also raises $Hc O_2$ affinity in Callianassa which, in this case, may be the general effect of ionic strength (Miller and Van Holde, 1981). In contrast, Truchot (1975) concluded that, in the brachyuran crustacean Carcinus maenas, the effects of both total ionic strength and of Cl^- on $Hc O_2$ affinity are very small and that the response to a change in total salinity can be explained virtually in full by changes in the level of divalent cations. In view of the nearly homeostatic regulation of the divalent cations in the brachyuran crab Callinectes sapidus Rathbun (Colvocorresses et al., 1974), a member of the same family as C. maenas, these conclusions seemed difficult to reconcile with a clear change in $Hc O_2$ affinity (Mangum and Towle, 1977).

With the exception of the investigation of Callianassa Hc (Miller and Van Holde, 1981 and pers. comm.), the experimental protocols have involved first removing inorganic ions by dialysis, in some instances at high pH and with the addition of EDTA, followed by the addition of salts in the desired ratios. Even at physiological pH and without EDTA,

this procedure causes at least some dissociation of the native Hc polymers to their monomeric subunits although, in C. sapidus, the amount of dissociation is small (Herskovits et al., 1981). Following more complete dissociation, the monomers reassemble again in a physiological saline, but perhaps only to polymers smaller than the native molecule and with an "incorrect" subunit composition that alters O_2 affinity and its own ion sensitivity (e.g. Jeffrey and Treacy, 1980).

We have investigated in detail the basis of the salinity effect on HcO_2 binding in Callinectes sapidus Rathbun. We have examined the specificity of the various inorganic ions, and have ascertained the presence of an alternative factor.

MATERIALS AND METHODS

Collection and maintenance of animals

The effects of inorganic ions on HcO_2 binding were first examined using blood taken from crabs purchased commercially during March, April and May from suppliers in the lower Chesapeake Bay. Therefore, the precise environmental history of the animals is not known. In most experiments, the blood from 27 to 36 individuals, held in recirculating water at 18 o/oo salinity and 21 to 23° C for less than 24 hr, was pooled and the pool used for a series of experiments; an exception is noted below. All animals were adult males in intermolt stage C, ranging in carapace length from 6.0 to 7.3 cm.

Preparation of hemocyanin

Blood was extracted from the infrabranchial sinuses of each walking leg, allowed to clot in a tissue homogenizer, and then the clot broken and separated from the serum by centrifugation. Ten ml aliquots of the pooled sera were dialyzed at 5° C against 1 l of the test solution (see below), buffered with 0.05 M Tris maleate. The dialysis medium was changed after 24 hr and the dialysis continued for an additional 24 hr. The preparation was then centrifuged prior to the O_2 binding measurements.

Preparation of test solutions

Since our purposes were to examine the effects of maximum physiological changes in inorganic ions and to clearly distinguish the effective from the ineffective factors in the blood, the entire range of values reported in the literature was used (Mangum and Amende, 1972; Colvocoresses et al., 1974; Lynch et al., 1973). We should emphasize that the values are derived from acute measurements made on freshly collected animals, and therefore the average physiological variation is highly exaggerated. This point is considered further in the Discussion.

The test solutions were prepared so that the activity of each ion in the single salt solutions and in the mixtures of two or three salts closely approximated the value in a physiological saline containing all of the major inorganic ions found in vivo. The activity coefficients were taken from Robinson and Stokes (1970) and from Pytkowicz et al. (1975). The composition of the series of physiological salines is given in Table I.

Oxygen binding measurements

Oxygen binding measurements were initiated within 30 min after completing the dialysis. In order to use physiological concentrations of Hc, the undiluted protein (1 ml) was placed into the tonometer/cuvette illustrated in Figure 1. The sample was allowed to equilibrate at atmospheric pressure for 10 min with 4 to 7 humidified mixtures of N_2 (ultra high purity, scrubbed further in a 120 x 3 cm column of Ridox) and either O_2 (99.5 % pure) or air (scrubbed of CO_2 with KOH). The

Table I

Ionic compositions of the test solutions in which C. sapidus blood was dialyzed. The free concentration of the ions in the single and multiple salt solutions approximated the free concentration of the ions in the complete salines. Solutions were buffered with 0.05 M Tris maleate.

Ions	Concentration (meq/l)							
	Solution 1		Solution 2		Solution 3		Solution 4	
	Total	Free	Total	Free	Total	Free	Total	Free
Na^{+}	138	90	278	181	410	270	546	359
K^{+}	4	2.5	8	5.0	12	7.5	16	10.0
Ca^{+2}	15	2.6	25	4.8	35	7.0	45	9.2
Mg^{+2}	15	1.1	25	1.8	35	2.5	45	3.3
Cl^{-}	150	94	300	190	450	283	600	377
HCO_3^{-}	2	1.2	2	1.2	2	1.2	2	1.2
SO_4^{-2}	20	1.0	30	1.4	40	2.8	50	3.3
Total salinity	342	194	664	386	984	574	1304	764

mixtures were prepared with a Wosthoff gas mixing pump. During the equilibration period, the vessels were immersed in a water bath (25° C), and the samples mixed by manually shaking the tonometer/cuvette. At each PO_2, changes in absorbance at 335 nm were noted in a Bausch and Lomb Spectronic colorimeter.

Measurements of pH were made at $PO_2 = 0$ and, depending on HcO_2 affinity, at either 159 or 760 mm Hg. A Fisher Accumet 520 pH meter and a Radiometer electrode were used. The pH of the oxygenated and deoxygenated Hc preparations never differed by more than 0.02 pH units.

Two to four replicate measurements were performed on each preparation, and the data were treated as a homogeneous sample. P_{50} (PO_2 at which 50 % of HcO_2 sites are bound to O_2) and n_{50} (value of the slope (b) at P_{50}) values were determined from logarithmic regression lines fit to Hill plots (Hill, 1910) of the pooled data in the range 15 to 85 % HcO_2. The significance of differences between the values for n_{50} were estimated from the 95 % confidence intervals around the slope and, for P_{50}, from 95 % confidence belts constructed around the regression lines.

Experimental design

In the single and multiple salt experiments, the response to total salinity is considered the control, and the response to the test ions corresponding to a particular salinity is considered the experiment. For example, the point at 194 meq/l salinity is considered the control for 1.1 meq/l $MgCl_2$, 2.6 meq/l $CaCl_2$, 90 meq/l NaCl and 2.5 meq/l KCl in Figure 2. A single pool of blood was used for each of the four sets of concentrations shown in Figures 2 and 3. Therefore, each point obtained

for the different concentrations of a particular salt or salinity represents a different pool of blood. This may be responsible for some of the scatter around the curves shown in Figures 2 and 3.

In the Bohr shift experiments, the data obtained at a particular concentration represent a single pool of blood. Hence the closer fit of the points to the curves in Figures 4 through 7.

Acclimation to high and low salinity

In June, adult, intermolt males were purchased from a commercial supplier who had caught them in the upper York River estuary (0-3 o/oo salinity) within the previous 6 hr. Adult, intermolt males were also captured in pots in inlets of the Atlantic Ocean near Wachapreague, Virginia (34 o/oo). While we cannot exclude the possibility of exchange between the two populations, the distance clearly would require a travel period of weeks. Each group was held for less than 12 hr in natural water (21° C to 23° C), the low salinity group at 5-8 o/oo (York River estuary water) and the high salinity group at 35 o/oo (Wachapreague Inlet water). Two ml of blood was taken from each crab, the crab transferred to the alternative salinity, held there for 8 days, and the sampling repeated.

Half of the blood from each crab taken prior to the transfer was dialyzed against a complete saline representing high salinity, and the other half was dialyzed against a complete saline representing low salinity. Eight days after the transfer, the sampling and dialysis were repeated. Oxygen binding measurements were made and the Hc concentration estimated, from the absorbance of $\left[HcO_2 - Hc\right]$ at 335 nm (Nickerson and

Van Holde, 1971), under each of the four conditions. The data were analyzed as paired observations according to Student's t-test, using the 0.05 level as the criterion for significance.

RESULTS

The effect of total salinity

When the preparation is dialyzed against distilled water, HcO_2 affinity becomes very low (Figures 2 and 3), but cooperativity remains unchanged (Table II). When the preparation is dialyzed against a physiological saline containing all of the major ions found in the blood, HcO_2 affinity increases with an increase in total salt concentration (Figures 2 and 3). Cooperativity, however, is independent of total salt concentration (Table II).

The effects of single salts

At similar activities as in the complete salines, no single salt clearly raises the HcO_2 affinity in full to the level observed in the complete salines (Figure 2). In the presence of small amounts of KCl, the response does not differ significantly from that observed after the Hc has been stripped by dialysis against distilled water. The presence of either NaCl or $MgCl_2$ increases HcO_2 affinity, but P_{50} is independent of NaCl concentration above 90 meq/l free Na^+. By far the most important single salt is $CaCl_2$, which very nearly restores HcO_2 affinity to the control level. Indeed the difference between the data for $CaCl_2$ and for the complete saline, although significant, is very small. Ca^{+2} has a

specific effect, an inference supported by the value at 9.2 meq/l $MgCl_2$; at this level, the free Mg^{+2} concentration approximates the highest free Ca^{+2} concentration used. At similar activities, Mg^{+2} has a much smaller effect than Ca^{+2}, which therefore rules out a general divalent cation effect (Figure 2). The effect of Ca^{+2} is also independent of the anion, as indicated by the identity of the values for $CaCl_2$ and $Ca(NO_3)_2$ at virtually equal activities (Figure 2).

No significant difference between the values for cooperativity is found in the presence of any single salt (Table III).

The effects of salt mixtures

In the presence of two or three salts, the responses of P_{50} are not always simple sums of the single salt values (Figures 2 and 3). In the presence of NaCl and $MgCl_2$, HcO_2 affinity behaves very much as it does in the presence of NaCl alone (Figure 3). The mixture of NaCl and $CaCl_2$ appears to mitigate the effect of $CaCl_2$ alone. The addition of $MgCl_2$ to either $CaCl_2$ or the mixture of NaCl and $CaCl_2$ produces a result virtually indistinguishable from that of the complete saline. Again, the replacement of Cl^- with NO_3^- in the mixture of Na^+, Mg^{+2} and Ca^{+2} has no significant effect on P_{50} (Figure 3), and n_{50} does not change regardless of the mixture (Table II).

The effects of inorganic ions on the Bohr shift

When the preparation is dialyzed against distilled water, the Bohr shift is virtually eliminated ($\Delta \log P_{50}/\Delta$ pH = -0.16 in the pH range

6.97-8.07). However, in the most dilute complete saline, it is restored in full ($\Delta \log P_{50}/\Delta pH = -0.99$ in the pH range 6.95-8.03) and it does not increase further in the most concentrated complete salines (Figure 4). While the Bohr factor increases with $MgCl_2$ concentration (Figure 5), it remains the same in the presence of various levels of $CaCl_2$ alone, or in a mixture of the two (Figures 6 and 7).

In addition, only in the presence of pure $MgCl_2$ does pH influence cooperativity (Table III); n_{50} is independent of pH in the presence of $CaCl_2$, in the mixture of $MgCl_2$ and $CaCl_2$, and in the complete saline (Table III).

In contrast to some of the data in Figures 2 and 3, the more extensive observations in Figures 4 through 7 suggest that the presence of $CaCl_2$ alone does restore HcO_2 affinity to the control level, at least in the range 7.0 to 9.2 meq/l free Ca^{+2}, and that the presence of Mg^{+2} is not necessary.

The effect of salinity acclimation

Animals collected at high and low salinities have significantly different HcO_2 affinities (Figure 8). The high salinity population has a higher HcO_2 affinity than the low salinity population. The difference disappears completely 8 days after transfer of the low salinity crabs to high salinity, and it disappears in a large part 8 days after transfer of the high salinity crabs to low salinity. In the latter case, the trend suggests incomplete acclimation, even though only one point is significantly different. No change in cooperativity can be detected (Table IV).

Although the data in Table V suggest a small increase in Hc concentration at low salinity and a small increase at high salinity, the differences are not significant.

Figure 1. Diagram of the tonometer/cuvette apparatus. The sample is introduced (A) and equilibrates with a known PO_2 in compartment D, a clear plastic tonometer. The gases enter at B and exit at C. After equilibration, the sample is agitated into compartment E, a 1 mm quartz cuvette. The tonometer and cuvette are held together with two brass screws and silicone grease is applied at the junction (F) to insure an airtight seal.

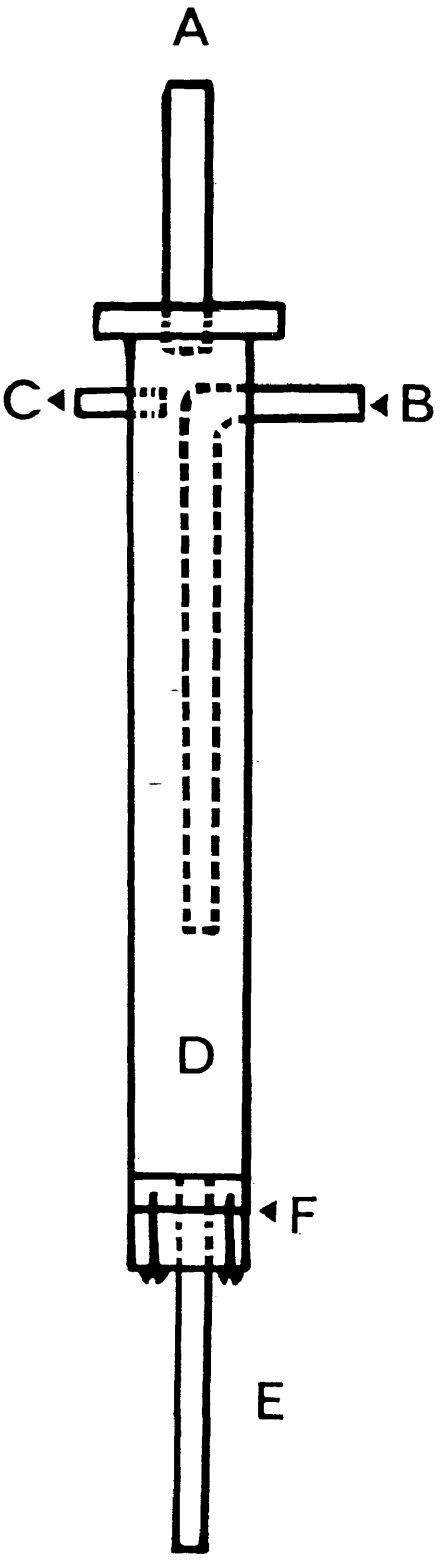
A
C
B
D
F
E

Figure 2. Effects of a complete saline and of single inorganic salts on O_2 affinity (P_{50}) of C. sapidus Hc. 0.05 M Tris maleate, pH 7.50 ± 0.02, 25° C. Free ion concentration is given. (◇) 7.0 meq/l $Ca(NO_3)_2$.

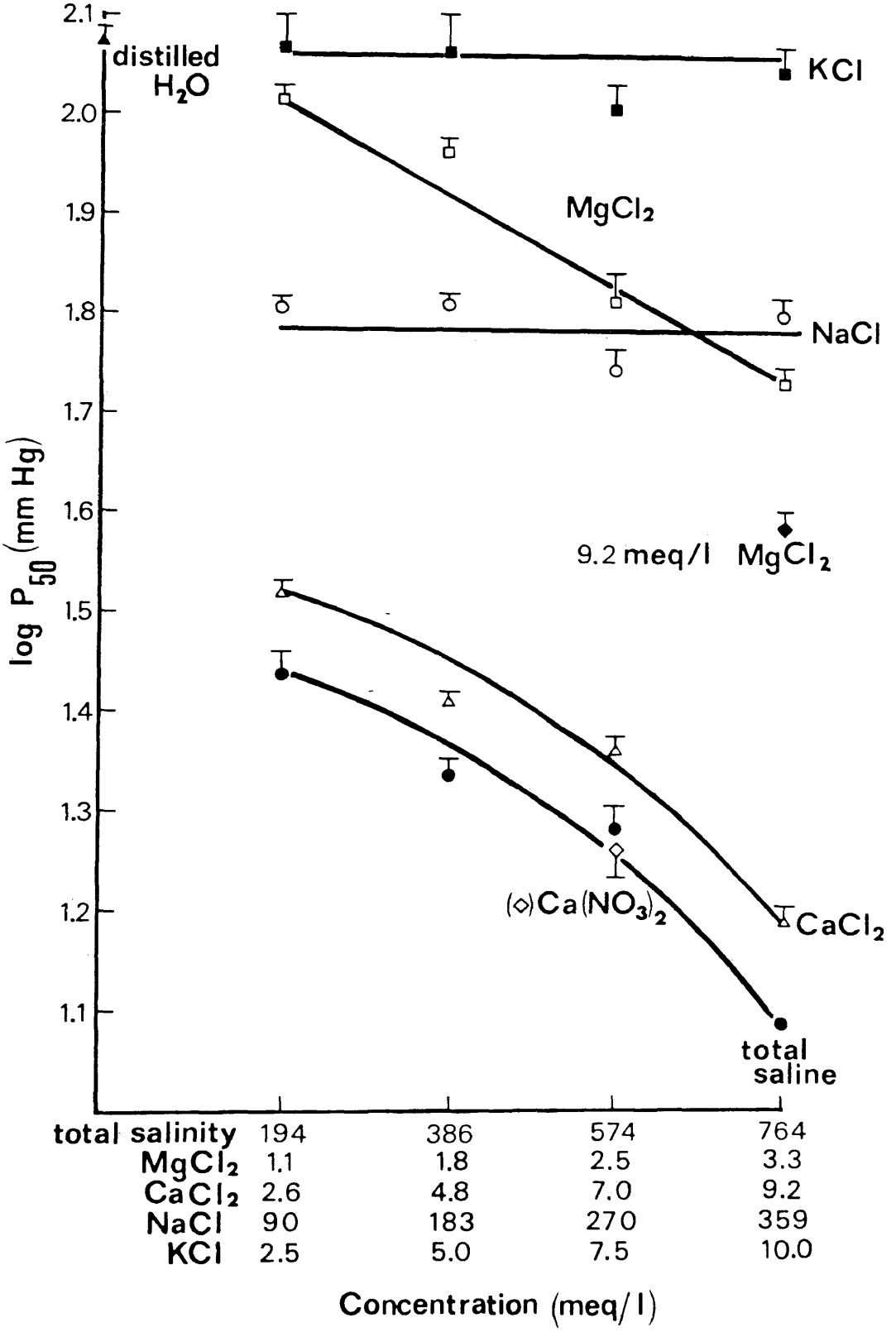
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
log P_{50} (mm Hg)
distilled H_2O
KCl
$MgCl_2$
NaCl
9.2 meq/l $MgCl_2$
$CaCl_2$
(◇) $Ca(NO_3)_2$
total saline
total salinity 194 386 574 764
$MgCl_2$ 1.1 1.8 2.5 3.3
$CaCl_2$ 2.6 4.8 7.0 9.2
NaCl 90 183 270 359
KCl 2.5 5.0 7.5 10.0
Concentration (meq/l)

Figure 3. Effects of mixtures of inorganic salts on O_2 affinity (P_{50}) of C. sapidus Hc. 0.05 M Tris maleate, pH 7.50 ± 0.02, 25° C. Free ion concentration is given. Curve for the complete saline (●) is from Figure 2. (◇) 270 meq/l $NaNO_3$/ 7.0 meq/l $Ca(NO_3)_2$/ 2.5 meq/l $Mg(NO_3)_2$.

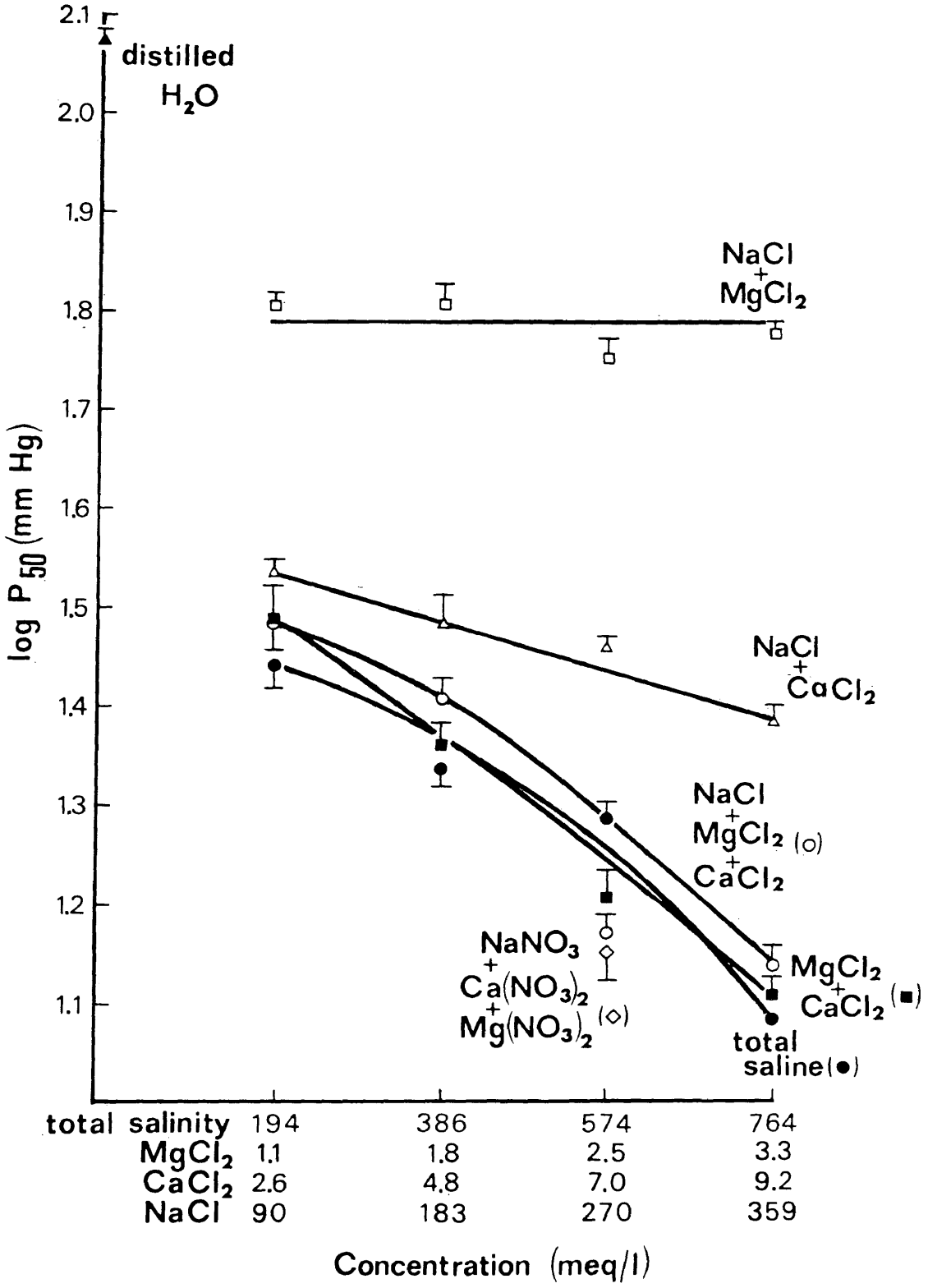
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
log P_{50} (mm Hg)
distilled H_2O
NaCl + $MgCl_2$
NaCl + $CaCl_2$
NaCl + $MgCl_2$ (○) + $CaCl_2$
$NaNO_3$ + $Ca(NO_3)_2$ + $Mg(NO_3)_2$ (◇)
$MgCl_2$ + $CaCl_2$ (■)
total saline (●)
total salinity 194 386 574 764
$MgCl_2$ 1.1 1.8 2.5 3.3
$CaCl_2$ 2.6 4.8 7.0 9.2
NaCl 90 183 270 359
Concentration (meq/l)

Figure 4. Effect of total salinity on the Bohr shift of C. sapidus Hc. 0.05 M Tris maleate, 25° C. Curves are designated by free ion concentration.

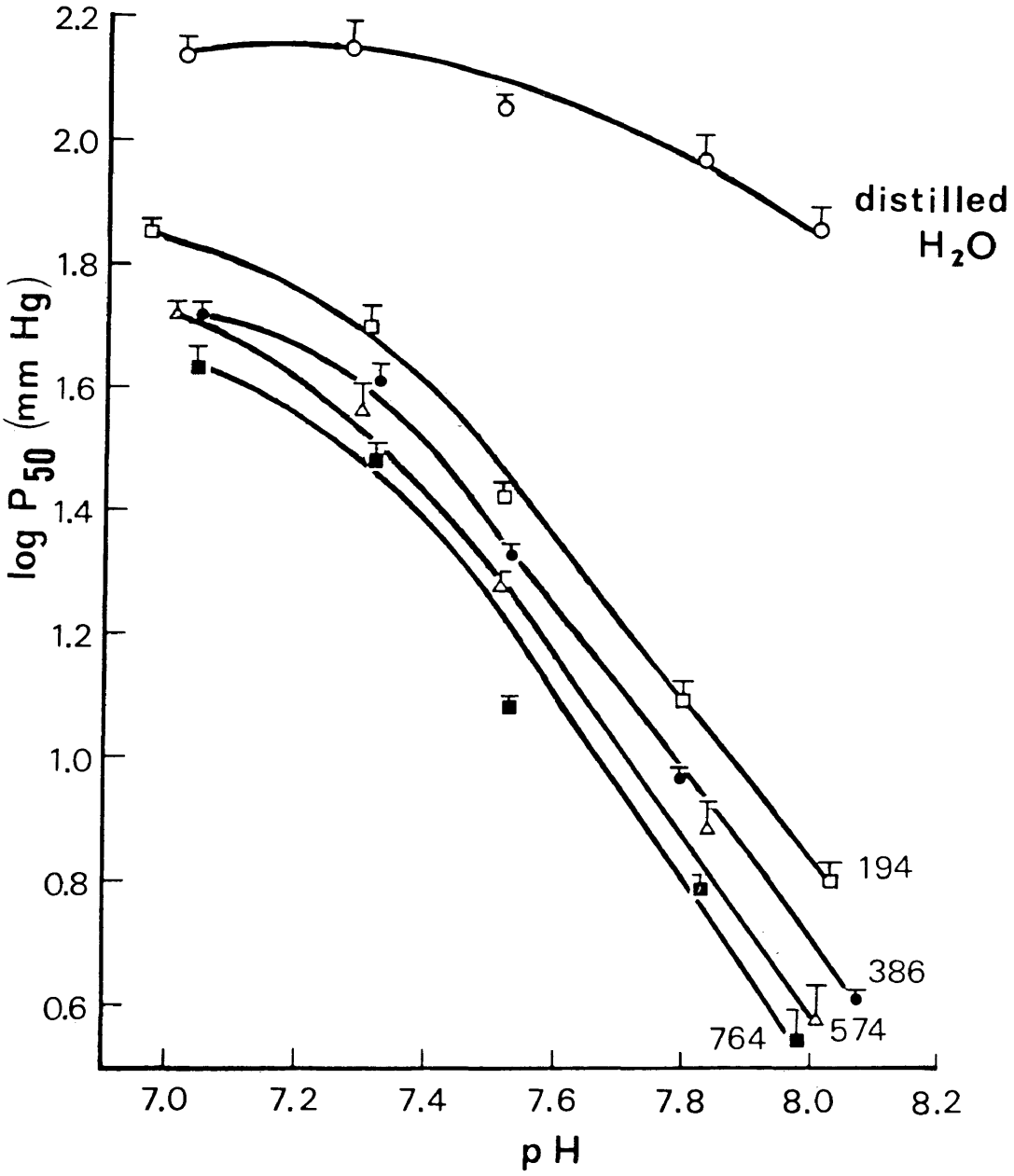
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
log P50 (mm Hg)
distilled H2O
194
386
574
764
7.0
7.2
7.4
7.6
7.8
8.0
8.2
pH

Figure 5. Effect of $MgCl_2$ on the Bohr shift of C. sapidus Hc. 0.05 M Tris maleate, 25° C. Curves are designated by free ion concentration.

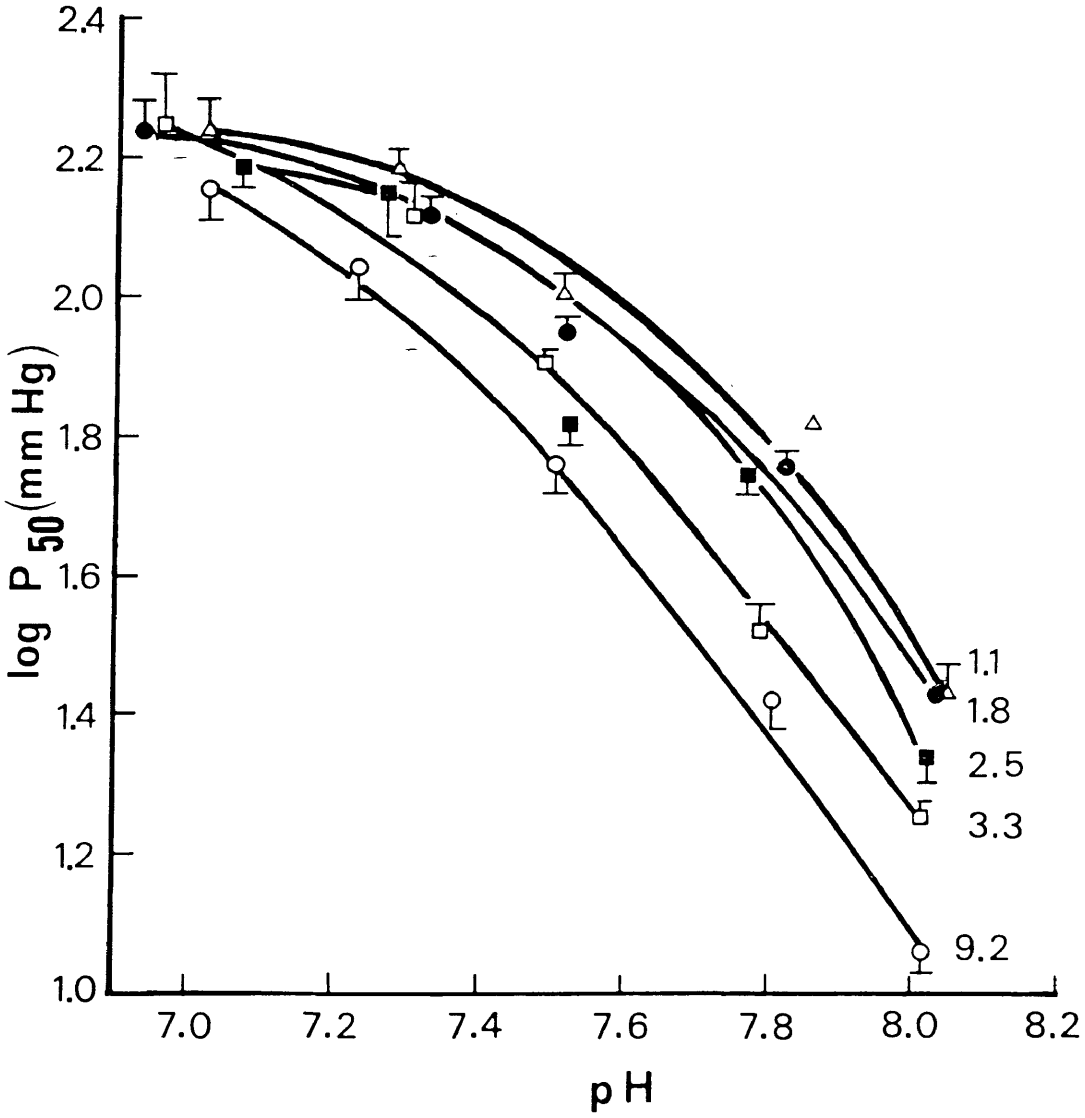
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
log P 50 (mm Hg)
7.0
7.2
7.4
7.6
7.8
8.0
8.2
pH
1.1
1.8
2.5
3.3
9.2

Figure 6. Effect of $CaCl_2$ on the Bohr shift of C. sapidus Hc. 0.05 M Tris maleate, 25 ° C. Curves are designated by free ion concentration.

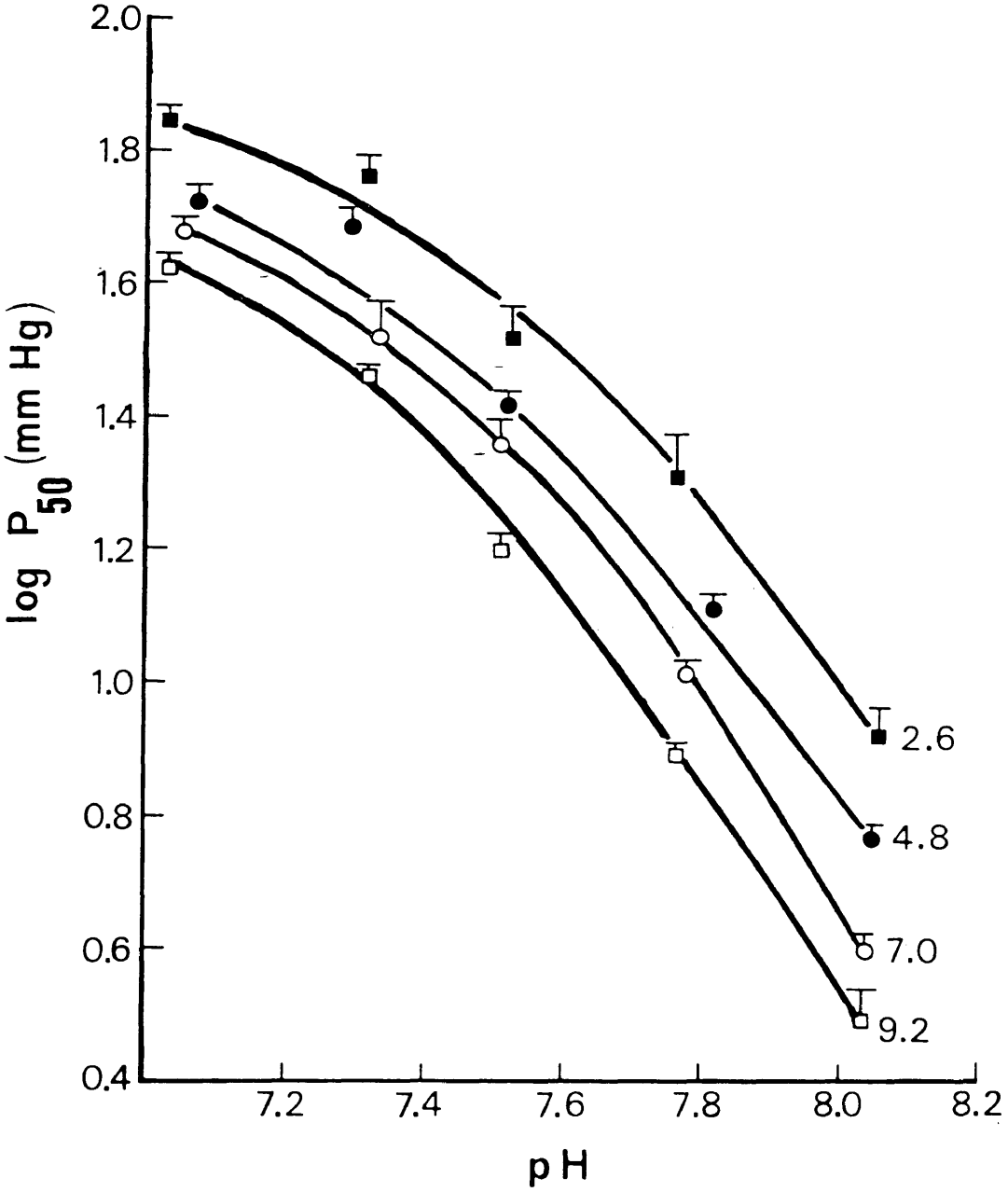
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
log P50 (mm Hg)
7.2
7.4
7.6
7.8
8.0
8.2
pH
2.6
4.8
7.0
9.2

Figure 7. Effect of a mixture of $MgCl_2$ and $CaCl_2$ on the Bohr shift of C. sapidus Hc. 0.05 M Tris maleate, 25° C. Curves are designated by free ion concentration. $MgCl_2$ value is given first.

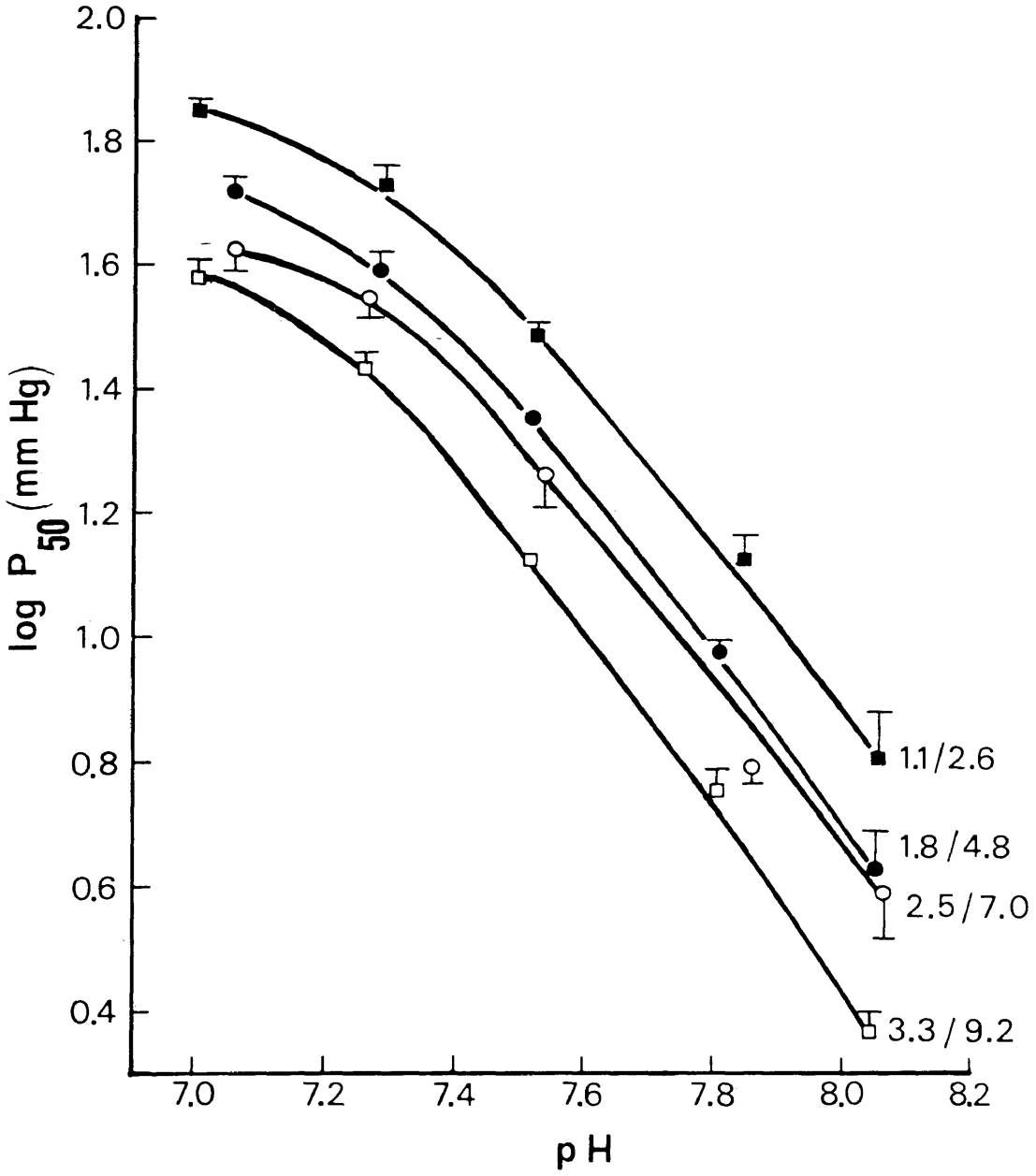
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
log P50 (mm Hg)
7.0
7.2
7.4
7.6
7.8
8.0
8.2
pH
1.1/2.6
1.8/4.8
2.5/7.0
3.3/9.2

Figure 8. The effect of acclimation salinity on O_2 affinity of C. sapidus Hc. Low salinity (0-3 o/oo) population from the upper York River (l.s. at l.s.), low salinity population after 8 days at 35 o/oo (l.s. at h.s.), high salinity population from the Wachapreague Inlet (34 o/oo) (h.s. at h.s.), and high salinity population after 8 days at 5-8 o/oo (h.s. at l.s.) blood dialyzed against 194 and 764 meq/l complete saline solutions, 0.05 M Tris maleate, 25° C, pH 7.53 ± 0.02. The data are paired observations, N = 6 for the low salinity population, N = 7 for the high salinity population.

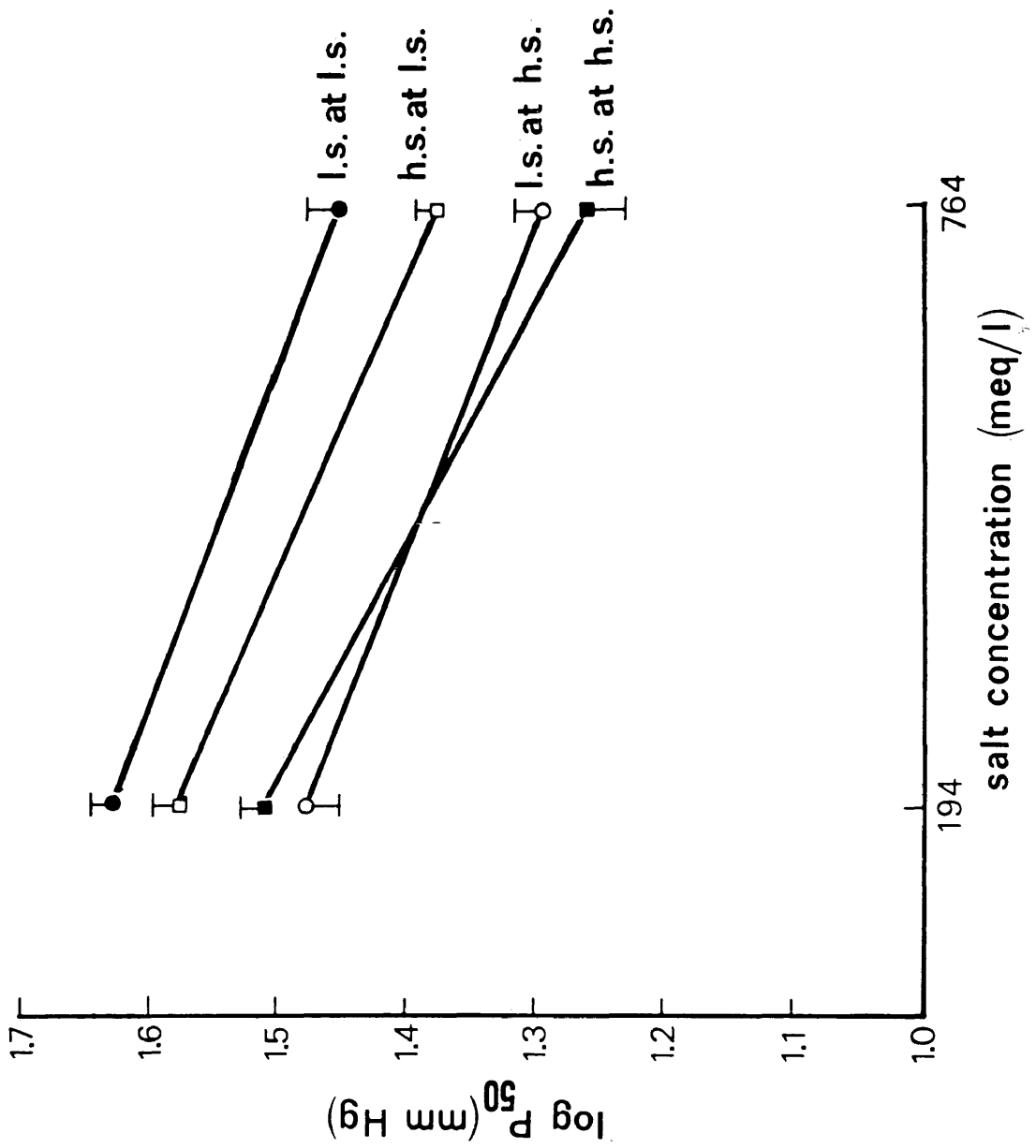
log P_{50}(mm Hg)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
194
764
salt concentration (meq/l)
l.s. at l.s.
h.s. at l.s.
l.s. at h.s.
h.s. at h.s.

Table II

The effect of inorganic ions on cooperativity (n_{50}) of HcO_2 binding in C. sapidus Hc. 0.05 M Tris maleate, 25° C, pH 7.48-7.52. N = 2-4.

Solution	Free ion concentration (meq/l)	n_{50} (mean ± S.E.)
Distilled water	0	2.9 ± 0.2
Complete salines	194	3.0 ± 0.3
	386	3.2 ± 0.4
	574	3.4 ± 0.4
	764	2.9 ± 0.2
Single salts:		
NaCl	90	3.2 ± 0.2
	183	2.8 ± 0.2
	270	2.7 ± 0.3
	359	3.0 ± 0.3
KCl	2.5	2.6 ± 0.8
	5.0	1.7 ± 0.9
	7.5	2.5 ± 0.2
	10.0	2.6 ± 0.3
$CaCl_2$	2.6	2.5 ± 0.5
	4.8	3.6 ± 0.4
	7.0	3.1 ± 0.4
	9.2	2.8 ± 0.3
$MgCl_2$	1.1	2.9 ± 0.2
	1.8	3.3 ± 0.2
	2.5	3.2 ± 0.6
	3.3	3.8 ± 0.6
	9.2	3.4 ± 0.4
$Ca(NO_3)_2$	7.0	2.8 ± 0.4
Salt mixtures:		
$Nacl/MgCl_2$	90/1.1	3.3 ± 0.2
	183/1.8	3.8 ± 0.3
	270/2.5	3.1 ± 0.2
	359/3.3	3.6 ± 0.2
$NaCl/CaCl_2$	90/2.6	3.3 ± 0.2
	183/4.8	3.8 ± 0.4
	270/7.0	3.1 ± 0.3
	359/9.2	3.7 ± 0.2

Table II (con't)

Solution	Free ion concentration (meq/l)	n_{50} (mean ± S.E.)
$CaCl_2/MgCl_2$	2.6/1.1	3.3 ± 0.2
	4.8/1.8	3.7 ± 0.4
	7.0/2.5	3.1 ± 0.6
	9.2/3.3	2.8 ± 0.3
$NaCl/MgCl_2/CaCl_2$	90/1.1/2.6	3.6 ± 1.8
	183/1.8/4.8	3.6 ± 0.5
	270/2.5/7.0	3.1 ± 0.3
	359/3.3/9.2	2.9 ± 0.6
$NaNO_3/Mg(NO_3)_2/Ca(NO_3)_2$	270/2.5/7.0	2.8 ± 0.2

Table III

The effect of inorganic ions and pH on cooperativity (n_{50}) of HcO_2 binding in C. sapidus. 0.05 M Tris maleate, 25° C. N = 2-4.

Sample	Free ion concentration (meq/l)	pH (± 0.02)	n_{50} (mean ± S.E.)
Distilled water	0	7.01	2.2 ± 0.3
		7.27	2.3 ± 1.0
		7.51	3.0 ± 0.2
		7.82	2.6 ± 0.3
		8.00	3.3 ± 0.8
Complete salines	194	6.97	2.6 ± 0.3
		7.30	2.8 ± 0.5
		7.49	3.0 ± 0.3
		7.79	3.9 ± 0.9
		8.03	3.1 ± 0.3
	386	7.04	2.6 ± 0.6
		7.30	2.8 ± 0.8
		7.49	3.2 ± 0.4
		7.79	3.6 ± 0.5
		8.06	2.3 ± 0.8
	574	7.00	2.7 ± 0.3
		7.29	2.8 ± 0.7
		7.50	3.4 ± 0.2
		7.83	3.5 ± 0.6
		8.01	2.8 ± 0.8
	764	7.03	3.0 ± 0.7
		7.32	3.1 ± 0.5
		7.52	3.0 ± 0.2
		7.82	3.8 ± 0.6
		7.98	2.7 ± 0.8
$CaCl_2$	2.6	7.02	2.9 ± 0.2
		7.31	2.7 ± 0.4
		7.52	2.5 ± 0.6
		7.76	2.8 ± 0.5
		8.05	2.7 ± 0.9
	4.8	7.06	2.6 ± 0.9
		7.29	3.2 ± 0.6
		7.49	3.6 ± 0.4
		7.81	3.4 ± 0.3
		8.04	3.8 ± 0.6

Table III (con't)

Sample	Free ion concentration (meq/l)	pH (± 0.02)	n_{50} (mean ± S.E.)
$CaCl_2$	7.0	7.05	2.8 ± 0.3
		7.33	2.2 ± 0.8
		7.49	3.1 ± 0.4
		7.78	3.1 ± 0.4
		8.04	2.6 ± 0.6
	9.2	7.03	2.5 ± 0.2
		7.31	3.1 ± 0.3
		7.50	2.8 ± 0.3
		7.76	3.2 ± 0.2
		8.03	2.9 ± 0.4
$MgCl_2$	1.1	7.02	1.8 ± 0.3
		7.28	2.2 ± 0.2
		7.50	2.9 ± 0.2
		7.85	3.9 ± 0.3
		8.05	3.4 ± 0.8
	1.8	6.95	1.9 ± 0.4
		7.32	2.5 ± 0.3
		7.48	3.3 ± 0.2
		7.82	3.9 ± 0.3
		8.03	3.8 ± 0.4
	2.5	7.06	2.2 ± 0.2
		7.27	2.4 ± 0.6
		7.52	3.2 ± 0.4
		7.77	4.0 ± 0.4
		8.02	4.1 ± 0.5
	3.3	6.96	2.0 ± 0.6
		7.31	2.7 ± 0.6
		7.50	3.5 ± 0.4
		7.79	4.0 ± 0.3
		8.01	4.5 ± 0.6
	9.2	7.02	2.3 ± 0.5
		7.24	3.2 ± 0.4
		7.50	3.8 ± 0.7
		7.80	4.2 ± 0.4
		8.01	4.8 ± 0.5
$MgCl_2/CaCl_2$	1.1/2.6	7.00	3.0 ± 0.2
		7.28	2.8 ± 0.3
		7.49	3.3 ± 0.2
		7.84	3.5 ± 1.1
		8.05	2.6 ± 0.7

Table III (con't)

Sample	Free ion concentration (meq/l)	pH (± 0.02)	n50 (mean ± S.E.)
$MgCl_2/CaCl_2$	1.8/4.8	7.05	3.2 ± 0.2
		7.28	2.8 ± 0.6
		7.50	3.7 ± 0.4
		7.80	3.3 ± 0.4
		8.04	2.4 ± 0.8
	2.5/7.0	7.05	2.5 ± 0.3
		7.25	2.6 ± 0.3
		7.49	3.1 ± 0.6
		7.85	3.9 ± 0.8
		8.06	2.5 ± 1.0
	3.3/9.2	6.99	3.2 ± 0.4
		7.26	3.1 ± 0.5
		7.51	2.8 ± 0.3
		7.81	3.0 ± 0.9
		8.04	2.5 ± 0.9

Table IV

Cooperativity (n_{50}) of HcO_2 binding in native and acclimated C. sapidus populations obtained by dialysis against 194 meq/l and 764 meq/l complete saline solutions. 0.05 M Tris maleate, 25° C, pH 7.51-7.55.

Sample	Free ion concentration (meq/l)	n_{50} (mean ± S.E.)
Low salinity population from upper York River (0-3 o/oo) (N = 6)	194 764	3.0 ± 0.2 3.5 ± 0.3
Low salinity population after 8 days at 35 o/oo (N = 6)	194 764	3.4 ± 0.5 3.2 ± 0.9
High salinity population from Wachapreague Inlet (34 o/oo) (N = 7)	194 764	3.3 ± 0.5 3.4 ± 0.3
High salinity population after 8 days at 5-8 o/oo (N = 7)	194 764	3.8 ± 0.7 3.3 ± 0.4

Table V

The effect of acclimation salinity on Hc concentration of C. sapidus Values are from paired observations on the same individuals before and after transfer to the alternative salinity.

Sample	Protein concentration (mean ± S.D.)
Low salinity population from upper York River (0-3 o/oo) (N = 6)	59.4 ± 17.6 mg/ml
Change in low salinity population after 8 days at 35 o/oo (N = 6)	-3.2 ± 2.3 mg/ml
High salinity population from Wachapreague Inlet (34 o/oo) (N = 7)	61.2 ± 12.6 mg/ml
Change in high salinity population after 8 days at 5-8 o/oo (N = 7)	+2.2 ± 0.8 mg/ml

DISCUSSION

The O_2 affinity of *C. sapidus* Hc increases measurably when total salinity increases within the physiological range. The levels of K^+ in the blood are apparently too small to have a measurable effect, and the effects of Na^+ are also relatively small, reaching their maximum below 90 meq/l free Na^+. Ca^{+2} and Mg^{+2}, on the other hand, continue to increase HcO_2 affinity within the physiological range. Of the two, Ca^{+2} is clearly the more important, by a factor approaching six. This conclusion agrees in general with earlier results (Larimer and Riggs, 1964; Pickett *et al.*, 1966; Chantler *et al.*, 1973; Truchot, 1975). Indeed, the data suggest that no other inorganic ion must be present to restore HcO_2 affinity to the level found in a complete physiological saline, as long as free Ca^{+2} exceeds 7 meq/l.

The effect is highly specific for Ca^{+2}, and not at all for Cl^-. The small change in Hc molecular weight following the removal of Ca^{+2} at pH 7.8 (Herskovits *et al.*, 1981) suggests that little, if any, of the effect involves dissociation of the native dodecamer molecule. The absence of a Ca^{+2} effect on cooperativity also supports this conclusion. It is likely that there are O_2 binding sites specifically influenced by Ca^{+2}.

Using chelating agents to remove divalent cations, other investigators have reported a Ca^{+2} effect on the cooperativity of portunid crab HcO2 binding in the pH range 6.9 to 8.1 (Chantler *et al.*, 1973).

Similarly, the removal of Mg^{+2} at pH 10 causes complete dissociation to monomers (Hamlin and Fish, 1977). However, the present data findings clearly indicate that orders of magnitude in excess of the physiological range are necessary to influence cooperativity.

The present results also indicate that very little Ca^{+2} is required to maintain the Bohr shift at the physiological level, even less than that needed to restore HcO_2 affinity.

Although highly variable, the acute measurements made on freshly collected crabs by Colvocoressess et al. (1974) suggest that the average physiological variation in the environmental salinity range 0 to 35 o/oo is about 27 to 34 meq Ca^{+2}/l blood. The average changes in Ca^{+2} would be expected to alter HcO_2 by 3 to 4 mm Hg (Figure 2); the actual differences observed between populations acclimated to salinities slightly less different than that above is about 14 mm Hg (Figure 8). Thus the effect of low salinity acclimation on the intrinsic HcO_2 affinity is considerably larger than that estimated by Mangum and Towle (1977) on the basis of the salt effect alone. The increment is due to changes in a non-dialyzable factor which is induced, in large part or even in full, within 8 days.

The most probable hypothesis might seem to be a change in the protein per se, resulting in Hcs with different intrinsic O_2 affinities in the high and low acclimation states. Using unpaired observations on different individuals, a number of investigators have examined the relationship between environmental salinity and either total protein (Horn and Kerr, 1963; Lynch and Webb, 1973; Péqueux et al., 1979) or Hc in the blood (Boone and Schoffeniels, 1979). Possibly due to the enormous variation in the data, the conclusions are various; we believe

that it is correct to conclude that the question of a net increase in Hc synthesis at low salinity has not been decided.

Our own data, based on paired observations following transfers in both directions and thus eliminating individual variation as well as the effects of nutritional state, indicate that a net increase in synthesis, if it occurs at all, is considerably smaller than reported by Boone and Schoffeniels (1979) and Péqueux et al. (1979). Although a net Hc synthesis might be masked by an increase in blood volume at low salinity, this effect would also be expected to be small in a strong osmoregulator such as C. sapidus. Finally, it is essential to keep the physical properties of the blood, and thus the Hc concentration, from exceeding a level which can be handled efficiently by the cardiovascular system (Snyder and Mangum, 1982). Therefore, it is possible that a rather precise balance between synthesis and degradation is maintained. Thus the available information argues neither for nor against the replacement of one Hc molecule by another as the mechanism of acclimation.

APPENDIX

Activity coefficients for single salt solutions from Robinson and Stokes (1970).

Salt	Concentration (M)	Activity coefficient
NaCl	0.1	0.778
NaCl	0.2	0.735
NaCl	0.3	0.710
NaCl	0.4	0.693
NaCl	0.5	0.681
NaCl	0.6	0.673
$NaNO_3$	0.1	0.762
$CaCl_2$	0.1	0.518
$Ca(NO_3)_2$	0.1	0.486
$MgCl_2$	0.1	0.528
$Mg(NO_3)_2$	0.1	0.522
KCl	0.1	0.770

Activity coefficients for salts in a multiple salt solution from Pytkowicz *et al.* (1975).

Salt	Activity coefficient
NaCl	0.659
KCl	0.621
$MgCl_2$	0.463
$CaCl_2$	0.445
$MgSO_4$	0.146
$CaSO_4$	0.137
$NaHCO_3$	0.590

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