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Anion and Urea Sensitivity of Three Elasmobranch Hemoglobins

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ANION AND UREA SENSITIVITY OF THREE

ELASMOBRANCH HEMOGLOBINS

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A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Masters of Arts

———————

by

David Allen Scholnick

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of

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ii

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The sensitivity of elasmobranch hemoglobins to effectors that vary with salinity were determined on the euryhaline cownose ray <u>Rhinoptera</u> <u>bonasus</u> and the stenohaline butterfly ray <u>Gymnura</u> <u>altavela</u>. High concentrations of urea, TMAO, NaCl, and ATP have no significant effect on oxygen binding (P_{50}) or cooperativity (n_{50}) of RBCs or stripped hemoglobins from <u>R. bonasus</u>. RBCs from late gestation fetal <u>R. bonasus</u> have a slightly higher oxygen affinity and a greater pH dependence than those of the adult. Measurements of plasma concentrations of urea, TMAO and inorganic ions from high and low salinity adults show that these animals are either extremely impermeable to ions or that they are osmoregulating at low salinity. Neither urea, NaCl nor ATP have a significant effect on oxygen binding of stripped Hbs from <u>G. altavela</u>. Further studies on Hbs from the spiny dogfish <u>Squalus</u> <u>acanthias</u> show that urea and NaCl have little or no effect on Hb oxygen binding, which conflicts with some earlier studies.

ANION, UREA AND ATP SENSITIVITY OF THREE

.ELASMOBRANCH HEMOGLOBINS

INTRODUCTION

Like many other proteins, the hemoglobins (Hbs) of higher
vertebrates denature in the presence of urea (Tanford, 1968).
In contrast most elasmobranchs studied have Hbs that are
insensitive to urea. The insensitivity of elasmobranch Hbs
to urea was first reported in 1963 by Manwell on the spiny
dogfish Squalus acanthias (= suckleyi). In 1974 Bonaventura,
Bonaventura and Sullivan demonstrated urea insensitivity
using three representative elasmobranchs, a shark, a skate
and a ray. Since that time two additional studies (Mumm et
al., 1978; Martin et al., 1979), one of which included a
freshwater Amazon ray, also found elasmobranch Hbs to be
insensitive to urea, at concentrations up to 4 M. From these
studies it was proposed that elasmobranch Hbs have evolved an
increased structural stability to high concentrations of
urea, which is normally a protein denaturant (Tanford, 1968).

In 1983, Weber et al. reported that Hb from the spiny
dogfish Squalus acanthias is sensitive to urea. In the
original work the Hb may have been influenced by cyanates
that form in aged urea solutions (Marier et al., 1964; Stark
et al., 1960). However, Weber's (1983) second study in which
deionized solutions of urea were used, reported sensitivity
to concentrations of urea as low as 0.45 mM. Thus, it

appears that most of the few elasmobranch Hbs studied are insensitive to urea, compared to the urea sensitive Hbs of higher vertebrates, with the one exception being S. acanthias.

The sensitivity of elasmobranch Hbs to chloride seems to be much more variable. Of the four species for which data have been reported, high concentrations of NaCl dramatically increased Hb oxygen affinities in the electric ray Torpedo nobiliana (Bonaventura et al., 1974) and in the clearnose skate Raja eglanteria (Bonaventura et al., 1974b), while in the common stringray Dasyatis sabina (Mumm et al., 1978) and a freshwater stingray Potamotrygon (Martin et al., 1979) high concentrations of NaCl have virtually no effect on Hb oxygen binding. Moreover, unpublished data for the smooth dogfish Mustelis canis show that NaCl can lower oxygen affinity of an elasmobranch Hb (C. P. Mangum, J. Bonaventura, pers. comm.). Evolutionarily, this is of interest because Cl^- consistently lowers Hb oxygen affinity in most vertebrates, including at least one marine teleost (Bonaventura et al., 1976). In addition, Mangum (1989) recently found that stripped coelacanth Hbs are insensitive to both urea and Cl^-. It appears that primitive Hbs (i.e. uncooperative or poorly cooperative monomers or oligomers) are generally insensitive to Cl^-, but exceptions can occur in the elasmobranchs. From

3

an evolutionary point of view, environmental changes in chloride may be important in determining Hb sensitivity, but a highly euryhaline and an obligate stenohaline species have yet to be compared.

In elasmobranchs and coelacanths adapted to high salinities, high concentrations of urea and methylamines, particularly trimethylamine oxide (TMAO), are used to raise internal osmotic pressure to conform to that of seawater. These compounds, which are readily reabsorbed by the kidney (Goldstein et al., 1968), account for about half the osmotic pressure in elasmobranch blood, which is usually maintained in stenohaline species at about 900-1100 milliosmolal (Holmes and Donaldson, 1969). In the moderately euryhaline skate Raja eglanteria, urea and Cl$^-$ change with salinity by about the same factor (Price and Creaser, 1967). The presence of TMAO is of physiological importance because methylamines can neutralize the denaturing effects of urea on elasmobranch enzymes and have a maximum effect when present in a 1:2 ratio with urea (Yancey and Somero, 1979, 1980). It thus seems possible that the TMAO may protect otherwise urea sensitive Hbs, much like other elasmobranch proteins (Yancey and Somero, 1980). Thus far, the only study (Weber, 1983b) has shown that TMAO does not affect Hb oxygen affinity of S.

acanthias, in either the absence or presence of urea and an organic phosphate (ATP).

Organic phosphates are well known modulators of Hb function in most higher vertebrates (Benesch and Benesch, 1974). Organic phosphates lower Hb oxygen affinity in higher vertebrates by preferentially binding to a common anion binding site in the deoxy-conformation. Interestingly, organic phosphate sensitivity also appears to be variable among the elasmobranchs, while showing the expected effects in other marine teleosts (Bonaventura et al., 1975) and the coelacanths (Wood et al., 1972). Thus, the elasmobranchs seem to be a transitional group between the more primitive insensitive Hbs of the lower vertebrates and invertebrates (Mangum, 1985), and the sensitive Hbs of the higher vertebrates.

The responses of elamobranch Hbs to effectors that vary with salinity appear to differ among the few species studied. In this paper we report the sensitivity of Hbs from the cownose ray Rhinoptera bonasus, a rare example of a highly euryhaline elasmobranch, to urea, Cl^-, TMAO and ATP. As a control species, we chose the stenohaline butterfly ray Gymnura altavela. Due to its apparently exceptional status, further studies were also conducted on the Hb of the spiny dogfish Squalus acanthias using concentrations of NaCl well

5

above physiological levels and freshly prepared solutions of urea.

The opportunity also arose to further study elasmobranch fetal and adult Hbs at late stages of development. It is well established that blood from many fetal vertebrates have higher oxygen affinities than adult. While distinct fetal Hbs have been found in several elasmobranchs, McCutcheon (1947) found no difference in adult and late gestation fetal oxygen affinities in three species of rays. In addition, Manwell (1958) found only slight differences in oxygen affinities between the early embryo and adult and no difference between the late fetus and adult in the clearnose skate. Thus, the general relationship between the ontogenetic Hbs in elasmobranchs is unclear.

MATERIALS AND METHODS

Eleven specimens of the euryhaline cownose ray _Rhinoptera_
bonasus (Michill), together with water samples from the site
of collection, were collected during the summer months of
1988. Specimens were obtained from pound nets operated by
commercial fishermen at Gloucester Point, Virginia and in the
upper Chesapeake near the mouth of the Potomac. One high
salinity _R. bonasus_ blood sample was provided by Dr. B.
Kirby-Smith from Duke University Marine Laboratory, North
Carolina. Five specimens of the stenohaline butterfly ray,
Gymnura altavela (Linnaeus) were collected by trawl off the
coast of Virginia Beach in mid. June and Drs. M. Freadman and
C. P. Mangum kindly provided the _Squalus acanthias_ (Linnaeus)
blood samples from Woods Hole Marine Laboratory,
Massachusetts.

Within a few hrs of removing the fish from water blood
was collected by cardiac or caudal puncture into cold
syringes coated with less than 0.5 ml heparinized isotonic
saline. Large samples (>4 ml) were taken to minimize the
effects of the saline. RBCs were diluted and stored on ice
in isotonic saline with 5mM ATP and 5mM lactate to maintain
integrity. Samples were kept on ice and immediately

transported to the laboratory. Phosphate sensitivity and RBC
oxygen equilibrium experiments were carried out within 2-24
hrs of sampling in all cases.

Red cells were separated from plasma by centrifuge,
washed 4 or 5 times in isotonic saline and suspended in the
experimental medium for oxygen binding measurements.
Remaining erythrocytes were lysed with cold distilled water,
centrifuged and the supernatant dialyzed overnight against
0.05 M Tris Maleate buffer for NaCl sensitivity experiments,
buffered saline without urea for urea sensitivity experiments
or buffered saline with urea for all other experiments, and
then frozen for oxygen binding experiments.

Oxygen binding

Oxygen equilibrium curves were determined by the non-
optical cell respiration method (Mangum and Lykkeboe, 1979)
and also by the tonometric method (Burnett, 1979), using
micro- tonometers with 1 mm light path cuvettes. All
experiments were carried out at $20^{\circ}C$, except for $\underline{S}$. $\underline{acanthias}$
where oxygen binding curves were also determined at $35^{\circ}C$
because of unusually high oxygen affinities. Samples used in
optical measurments were checked spectrophotometrically for
presence of methemoglobin and reduced with sodium borohydride
when necessary. The half saturation point (P_{50}) and

8

cooperativity (n_{50}) were determined from Hill plots. Hb sensitivity to effectors was statistically analyzed using confidence intervals around regression lines describing log P_{50} vs pH.

Elasmobranch saline (with urea) was modified from Rabinowits and Gunther (1973) and stock solutions of NaCl, TMAO, ATP and calcium acetate were buffered with 0.05 M Tris Maleate. Urea solutions were prepared fresh immediately before each experiment and kept on ice to prevent cyanate formation, as recommended by Strunk et al. (1960) and Marier et al. (1964).

Chemical analysis

Serum urea concentrations were determined as enzymatically produced ammonia (Sigma #640). Frozen plasma samples were diluted 1:10 and urea was determined at 570nm using Sigma urea standard (#535-150).

Serum TMAO concentrations were determined by Conway microcell diffusion (Forster et al., 1985). TMAO was reduced to trimethylamine using metallic zinc and allowed to diffuse into the inner chamber of the cell in the presence of KOH and formaldehyde. Boric acid with an indicator was used to combine with the amine in the inner chamber and

concentrations were determined by back titration using 0.005 N HCl (Appendix A).

Serum and environmental water sample Na^+, Cl^-, Ca^{+2} and Mg^{+2} ion activity were determined with ion selective electrodes (Radiometer, Orion and A. H. Thomas Cos.) using stirred, buffered samples and a double junction reference electrode, and IAPSO sea water for ion activity standards as described by Mangum et al. (1978).

Measurements of serum and environmental water osmotic pressures were made using Precision Systems freezing point or Wescor vapor pressure osmometers.

RESULTS

Disc widths (distance between the tips of the pectoral fins in cm), salinity (‰) and location of collection are given in Table 1, for adult and two fetal rays, which were delivered upon capture.

Blood osmotic variables

Chemical analysis data for $\underline{R}$. $\underline{bonasus}$ and $\underline{G}$. $\underline{altavella}$ plasma and sea water samples are given in Table 2. $\underline{Rhinopera}$ $\underline{bonasus}$ plasma urea concentrations were 38% lower and Cl concentrations were 20% lower in animals collected from low salinity than those collected from high salinity. Plasma Na concentrations for high salinity $\underline{R}$. $\underline{bonasus}$ (362 mmol/l) are higher than other elasmobranchs studied, which normally range from 230 to 290 mmol/l (Holmes and Donaldson, 1969). Our results for both urea and Cl are similar to those for the lemon shark $\underline{Negaprion}$ $\underline{brevirostris}$ (Goldstein $\underline{et}$ $\underline{al}$., 1968) after acclimation to half sea water. Changes in serum Cl^-, Na concentrations and osmotic concentration were surprisingly low considering that these rays are usually thought to be osmoconformers at high salinity. Plasma osmotic concentration only changed from 1108 to 813, a 27%

decrease, while sea water changed from 1034 to 368, a 64%
decrease. Thus, these rays appear to be osmoregulating at
low salinity. In addition, TMAO concentrations remained
relatively low for both high and low salinty $\underline{R}$. $\underline{bonasus}$,
compared to $\underline{G}$. $\underline{altavella}$ (Table 2.).

Oxygen binding of Rhinoptera RBCs

The Bohr plots for $\underline{R}$. $\underline{bonasus}$ RBC oxygen binding are
illustrated in Fig. 1. Values for log P_{50} are about 1.0 at
pH 7.5, which is similar to those reported by McCutcheon
(1947) on $\underline{Rhinoptera}$ $\underline{quadriloba}$ (= $\underline{bonasus}$) at 25°C.
Concentrations well above the physiological range of urea,
NaCl and ATP have no significant effect on oxygen binding
(P>.05 based on overlapping confidence intervals around
regression lines). The Bohr coefficient (log P_{50} / pH) is
-0.77 and cooperativity ranges from about 1.2 to 1.8 for all
treatments.

The oxygen affinity and cooperativity of adult and fetal
R. $\underline{bonasus}$ RBCs are shown in Fig. 2. Fetal RBCs have a
slightly higher oxygen affinity and significantly greater pH
depencence (P>.05) than adult RBCs. P_{50} values are
significantly different above pH 7.2 (P<.05) and the largest
differences occurr at very high pH (e.g. change of 0.4 at pH

of 8.1). Cooperativity appears to be the same (P=0.5
according to Student's t test).

Oxygen binding of Rhinoptera Hbs

The oxygen affinity of stripped R. bonassus hemolysates
examined in the presence of urea, NaCl, TMAO and ATP (ratio
of 1.0 ATP:0.8 heme and 5 ATP:0.8 heme) are presented in
Figs. 3-6. No effector has any significant effect on oxygen
binding (P>.05) and cooperativity varies little more than
0.4, except for treatments with NaCl where n_{50} varies little
more than 1. The effects of substituting calcium acetate for
NaCl were also measured, to eliminate any possible effect of
NaCl while still maintaining ionic strength (Fig. 7);
stripped Hbs are insensitive.

Oxygen binding of Gymnura Hbs

The oxygen affinity of stripped Gymnura altavela
hemolysates was examined at $20^{\circ}C$ and pH of 7.6 ($\pm$ 0.2) as a
function of urea and NaCl concentrations (Fig. 11). Neither
urea or NaCl has a significant effect on oxygen binding
(slope does not differ from zero at P=.05) up to 5 M and log
P_{50} varies little more than 0.18 over the entire range.

Oxygen binding of Squalus Rbcs and Hbs

The response of <u>Squalus</u> <u>acanthias</u> RBCs to urea at 35°C

are shown in Fig 8. Urea concentrations up to 2.5 M had no

significant effect on oxygen binding (P>.05). There was

considerably more variability (the correlation coefficiant r

for a regression line describing the Bohr plot for the 2.5 M

urea data in Fig. 8 is not significant at P=.05) than found

for the other Hbs studied. Bohr plots for stripped <u>Squalus</u>

Hbs in the presence of urea at 20°C are shown in Fig. 9. We

chose to repeat these experiments at a higher temperature

because the unusually high oxygen affinities made the

resolution of possible differences very difficult. Bohr

plots for stripped <u>Squalus</u> Hbs in the presence of high

concentrations of urea and NaCl are shown in Fig. 10,

considerable scatter was also observed. High concentrations

of urea significantly increase Hb oxygen affinity above pH

7.7 (.01<P<.05), while 2 M NaCl significantly decreases

oxygen binding below pH 7.5 (.01<P<.05). The relationship

between urea concentration and oxygen affinity of dialyzed <u>S</u>.

<u>acanthias</u> Hb at pH 7.6 is shown in Fig 11. There is a slight

decrease in oxygen affinity but log P_{50} varies little more

than 0.15 over the entire range. However, the effect of urea

is very small. Moreover, our data do not agree numerically

with those of Weber <u>et</u> <u>al</u>. (1983), who showed much lower

oxygen affinities (log P_{50} =0.6 at 15 C, pH=7.5) and a much larger urea sensitivity (log P_{50} =0.3).

DISCUSSION

The Hbs of R. bonasus, G. altavela and S. acanthias are
either insensitive or virtually insensitive to urea. These
results are similar to those for Hbs of most other
elasmobranchs previously examined (Bonaventura et al., 1974;
Mumm et al., 1978; Martin et al., 1979). It seems probable
that this insensitivity protects these Hbs from the
denaturing effects of urea. In the presence of urea most
higher vertebrate Hbs, including human, dissociate into
dimers, associated with a large increase in oxygen affinity
(Toulmond, 1985). Knowing that urea tends to dissociate
hydrogen bonds, Mumm et al. (1978) suggested that
elasmobranch Hbs may have different amino acid residues at
crucial points of contact between subunit chains than are
found in higher vertebrates. At least on the alpha chains
(Aschauer et al., 1985) these residues appear to be
histidine. They are important in preventing the dissociation
of the chains and probably maintain the integrity of the
molecule in the presence of urea.

The urea insensitivity of all but one elasmobranch Hb
studied lends support to the notion that urea sensitivity is
an important physiological adaptation to the presence of high

levels of urea $\underline{in}$ $\underline{vivo}$. These results do not eliminate the possibility that urea insensitivity is a primitive, conservative feature that was inherited by the elasmobranchs. If these molecules do represent a primitive insensitive state, one would expect to find many similarities between elasmobranch Hbs and tissue heme proteins, which are widely believed to represent the primitive condition. Bonaventura $\underline{et}$ $\underline{al.}$ (1974) suggested that myoglobins which, like elasmobranch Hbs, are also unresponsive to all effectors may be a precursor-like molecule to the elasmobranch Hbs; this hypothesis, however, assumes that Hbs arose $\underline{de}$ $\underline{novo}$ in the vertebrates, which is by no means clear (Mangum $\underline{et}$ $\underline{al.}$, 1986). Moreover, the response of primitive Hbs to urea is completely unknown.

The insensitivity of $\underline{R.}$ $\underline{bonasus}$ and $\underline{G.}$ $\underline{altavela}$ Hbs to organic phosphate appears to be consistent with some other studies, but this insensitivity is not uniform in the elasmobranchs. While the primary effectors differ in each group of vertebrates, all preferentially bind to the deoxyconformer lowering oxygen affinity, and enhancing the Bohr effect (Coates and Riggs, 1980). In addition, the difference between phosphate sensitivity and insensitivity entails only one or two amino acid substitutions (Coates and Riggs, 1980; Perutz $\underline{et}$ $\underline{al.}$, 1980). Since these responses are

not found in invertebrates and agnathans, are variable within the elasmobranchs, and are found in all classes of higher vertebrates studied, phosphate sensitivity would appear to be closely related to Hb evolution. Thus, elasmobranchs would be considered a transitional Hb group and may represent a very primitive phosphate response.

While R. bonasus was collected from a broad range of salinities in this study, from 12.5 ‰ to 34 ‰, they have been reported in areas as low as 7 ‰ (Smith and Marriner, 1986). Moreover, these animals migrate into low salinities in enormous numbers. These large changes in salinity, which significantly alter the concentration of effectors in elasmobranch blood plasma (Table 2), do not influence the respiratory function of the oxygen transport system. The insensitivity of R. bonasus Hbs to Cl$^-$ is surprising considering the Cl$^-$ sensitiive Hbs from the moderately euryhaline clearnose skate (Bonaventura et al., 1974). However, the present results do indicate insensitivity of R. boanasus Hb to organic phosphate, which is believed to bind to the same site as Cl$^-$. The variability of elasmobranch Hbs to Cl$^-$ and phosphate is of evolutionary interest considering the vast majority of tetrameric vertebrate Hbs studied have shown large responses to these effectors. Recently Mangum et al. (1988) reported the insensitivity of RBC's from the blood

worm Glycera to Cl⁻, but at this time the responses of other primitive Hbs to Cl⁻ is not known.

The insensitivity of R. bonasus Hb to TMAO is consistent with Weber's (1983) results on S. acanthias Hb. Considering the insensitivity of R. bonasus Hb to urea and ATP, these results are not surprising. It appears that TMAO does not protect R. bonasus Hb from urea. If methylamines are neutralizing the denaturing effects of urea on elasmobranch proteins, this mechanism is not uniform. Interestingly, plasma TMAO concentrations were found to be relatively low in R. bonasus compared to G. altavela (Table 2) and S. acanthias (Goldstein et al., 1967). Also, Goldstein et al. (1968), showed a 60% decrease in plasma TMAO concentration in the lemon shark Negaprion brevirostris after acclimation to dilute sea water, which we did not find. While these results differ, a euryhaline ray had never been examined. It seems possible that in R. bonasus urea may vary with salinity (Table 2) and TMAO may be maintained at relatively constant low levels.

There are at least two possible explanations for the osmotic profile in R. bonasus collected from low salinity: 1) It is possible that this ray is osmoregulating at lower salinities. While urea concentrations greatly decreased in the low salinity animals, sodium and chloride changes were

19

much smaller and TMAO did not contribute to this osmoregulatory process. Also, Price et al. (1967) found low Cl⁻ levels in clearnose skate blood plasma, before dilutions with sea water, which supports the possibility that R. bonasus might not be a strict osmoconformer. 2) It is also possible that these large and fast-swimming animals undergo such rapid diurnal feeding migrations (which usually occur in groups or schools) that they do not have enough time to acclimate osmotically. Price and Creaser (1967) showed that the clearnose skate Raja eglanteria requires at least 48 hrs to acclimate to a salinity change of only 2.4 ‰ (at about 24°C). If both integument and gill epithelia are extremely impermeable, R. bonasus may not reach osmotic equilibrium during the time they invade very low salinities.

The higher oxygen affinity of R. bonasus fetal Hb observed in this study agrees with similar findings in other vertebrates but has not been well established in the elasmobranchs (McCutcheon, 1947; Manwell, 1963; Penelly et al., 1975). While it is known that very extensive uterine villi (trophonemata) invade the spiracles and gill slits of embryos from some rays, it is not known how important these structures are late in the development of the fetus (J. Musick, pers. comm.). Studies on the skate Raja binoculata (Manwell, 1958) separated early embryonic Hb from

20

adult but showed no difference in oxygen binding properties between the late embryo or fetus and the adult. Electrophoresis and fingerprinting of adult and fetal Hb of the spiny dogfish, showed distinct Hb proteins and oxygen affinities for adult and fetus (Manwell, 1963). In addition, Ingermann and Terwilliger's (1984) data on fetal and adult seaperch, which have a pseudoplacental connection, suggest that there are structural and functional differences between Hbs from early and late stages of development. It would be interesting to examine the difference between embryonic and fetal Hbs in the rays, to determine whether the villi are of importance and if there is an in-utero shift from fetal to adult hemoglobin.

Our results indicate that S. acanthias Hbs do not show considerable sensitivity to urea or NaCl. These findings do not agree with previous studies by Weber et al. (1983) and Weber (1983). The discrepancy may prove to be related to the fact that S. acanthias Hb has been shown to be highly polymorphic when it is in the oxyconformation (Fyhn and Sullivan, 1965). In addition, our results show considerable scatter in oxygen affinity compared to the other elasmobranch Hbs studied, which could conceivably involve variability of the molecule. Considering the numerous Hb phenotypes observed by Fyhn and Sullivan (1965), it seems at least

21

possible that the Eastern Atlantic Coast species Hb may be phenotypically different than those collected from the North Sea. At this time S. acanthias Hb should be regarded as at least various.

The overall insensitivity of the elasmobranch Hbs studied seems to indicate a molecular stability in the presence of urea not found in the higher vertebrates. Environmental changes in chloride do not appear to be an important factor in determining Hb sensitivity in the euryhaline species studied but the variability in Cl^- and phosphate sensitivity found in the elasmobranchs may indicate a primitive response to these effectors compared to the highly sensitve Hbs of most higher vertebrate. It will be interesting to learn the responses of a large number of primitive Hbs, especially teleosts and the yet studied monomeric Hbs of the lamprey, considering these molecules may represent primitive states before the separation and differentiation of higher vertebrate Hbs.

APPENDIX A

Procedure for determination of TMAO using Conway
microcell diffusion.

Materials:

 Metallic Zinc - zinc mesh
 Boric acid solution with indicator -
     Use a mixed indicator of 25 ml 0.033% bromocreosol green
         and 100 ml of 0.066% methyl red in alcohol.
     Add 10 grams boric acid to 200 ml alcohol, add 700 ml
         distilled water and about 40 ml indicator solution.
     Adjust pH until soution is yellowish green, with 1N NaOH
         and bring up to 1 liter with distilled water.
     40% KOH
 Formaldehyde solution - about 37% W/W

     If plastic Bel-Art Microdiffusion chambers are used,
replace lids with glass plates and seal using excess vacuum
grease and use the following volumes.

In this order add:
     Outer Chamber
         excess zinc (>0.5 grams)
         0.5-1 ml plasma
         1ml formaldehyde
     Inner Chamber
         0.5 ml Boric acid solution with indicator

     Let stand for 5 min., and then add 1ml of 40% KOH to
outer chamber, cover immediately and let stand 6-10 hrs.
     Back titrate using 0.005N HCl into boric acid solution
wich should go from green to bright red. Titration volumes
are usually no larger than 1-2 ml so titration must be
carried out with microliter pipetts. Standard curve is
sigmoidal possibly due to second order chemical reactions.

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Table 1. The number of species collected (N), disc widths (cm), salinity (parts per thousand) and the location of collection.

Species	N	Disc Width	Salinity	Location
R. bonasus				
(adults)	6	89-100	19	Gloucester Pt., VA
(fetuses)	2	13	--	
R. bonasus	4	50-97	12.5	Potomac River, VA
R. bonasus	1	------	34.5	Beaufort, NC
G. altavela	5	40-185	30	Virgina Beach, VA
S. acanthias	1	------	31-32	Woods Hole, MA

Table 2. The concentrations of urea (mmol/l), TMAO (umol/l),
free ions (millimolar), and the osmotic concentration.

	Urea	TMAO	Na$^+$	Ca^{+2}	Mg^{+2}	Cl$^-$	MOSM
LOW SALINITY							
R. bonasus	256 $\pm$6.0	15.9	221 $\pm$14	3.24 $\pm$0.3	6.9 $\pm$1.3	317 $\pm$15	813 $\pm$4.3
Sea water	---	---	166 $\pm$7.4	3.65 $\pm$0.1	12.3 $\pm$0.5	228 $\pm$13	368 $\pm$1.0
HIGH SALINITY							
R. bonasus	411 $\pm$9.2	13.8	362 $\pm$12	4.47 $\pm$0.1	9.14 $\pm$1.8	395 $\pm$24	1108 $\pm$1.0
Sea water	---	---	400 $\pm$23	8.67 $\pm$0.6	12.5 $\pm$2.3	531 $\pm$16	1034 $\pm$2.1
G. altavela	252	64.1 $\pm$13.8	137 $\pm$5.7	1.65 $\pm$.02	3.78 $\pm$0.2	211 $\pm$14	792 $\pm$13
Sea water	---	---	340 $\pm$10	7.24 $\pm$.05	10.3 $\pm$0.9	475 $\pm$9.3	920 $\pm$1.5

 values given are means $\pm$ standard deviation for replicate
measurements on pooled samples, where N$\geq$3 in all cases except
for TMAO determination where N=1

Figure 1. The effect of urea, Cl^- and ATP on whole cell oxygen affinity (Log P_{50}) and cooperativity (n_{50}) as a function of pH in the cownose ray <u>Rhinoptera</u> <u>bonasus</u> at 20°C. Log P_{50} and n_{50} were determined at 1M urea (+), 0.5M NaCl (✗), normal saline (■), and 1mm (△) and 5mm (□) ATP.

Figure 1
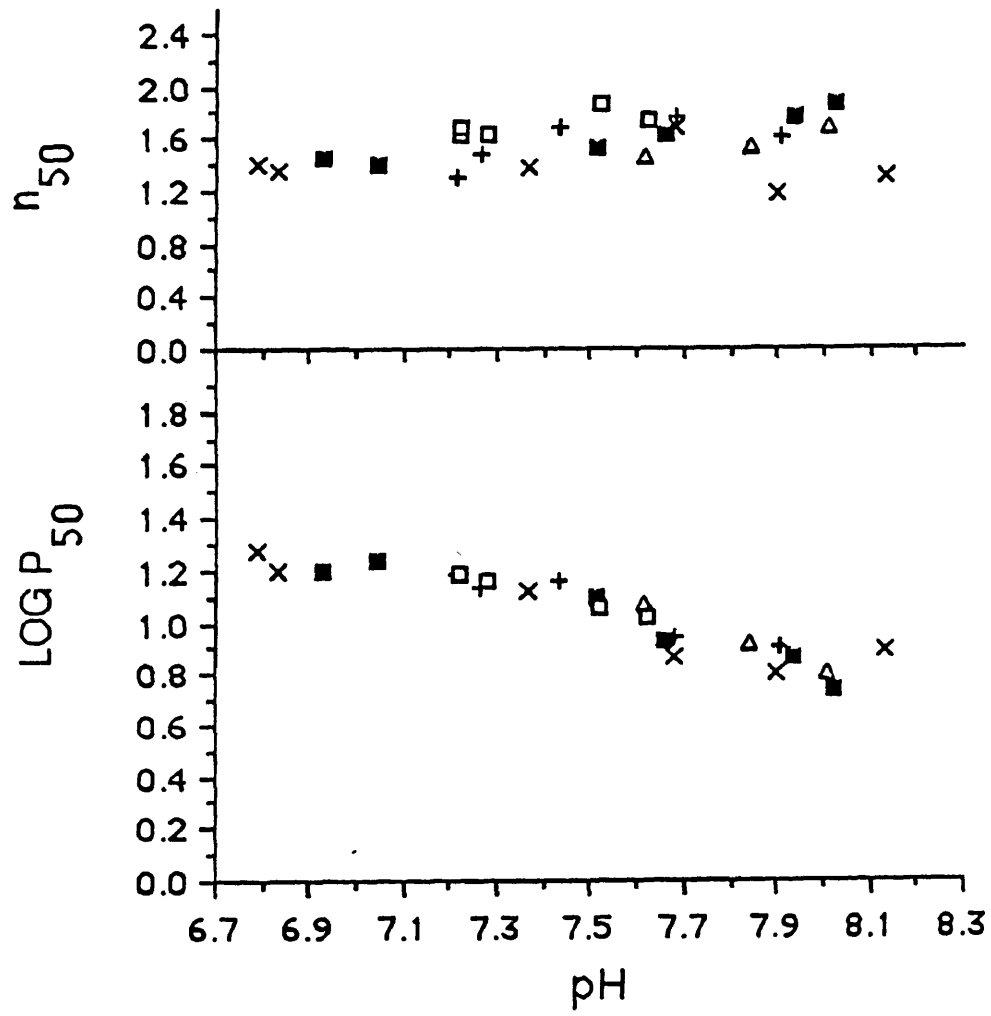
Figure 2. The difference between adult (■) and fetal (□) <u>Rhinoptera</u> <u>bonasus</u> whole cell oxygen affinity on Log P_{50} and n_{50} as a function of pH at 20°C.
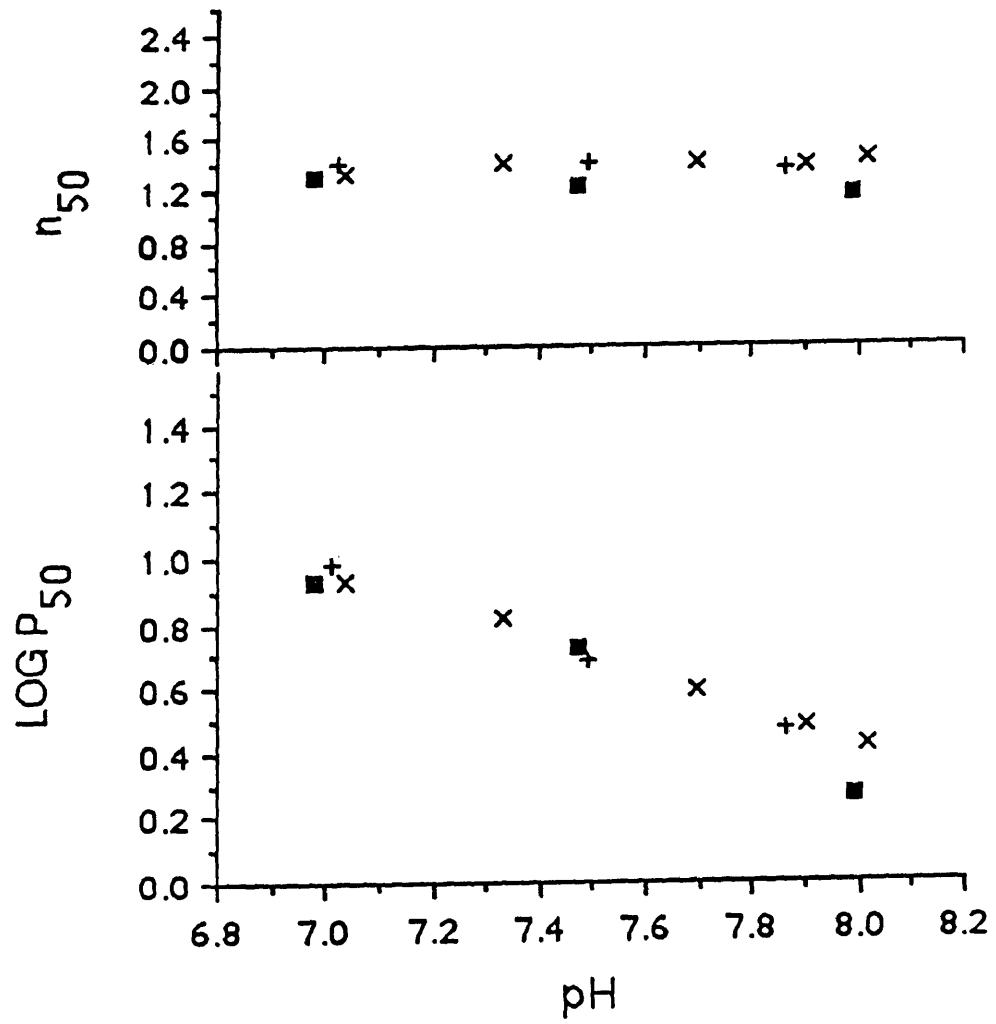
Figure 2
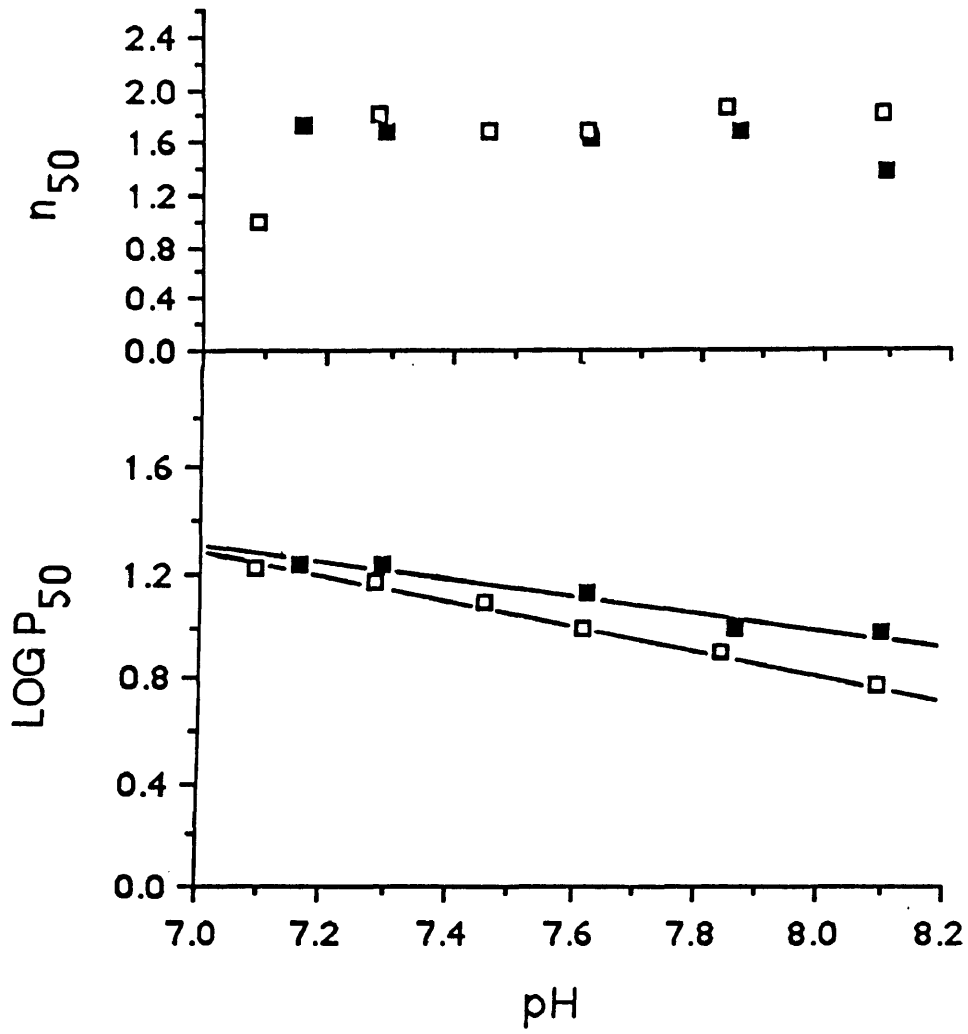
Figure 3. The effect of urea on oxygen affinity (P_{50}) and cooperativity (n_{50}) of stripped <u>Rhinoptera</u> <u>bonasus</u> Hbs as a function of pH at 20°C. Log P and n were determined at 0.3M urea (■) and 1M urea (□).
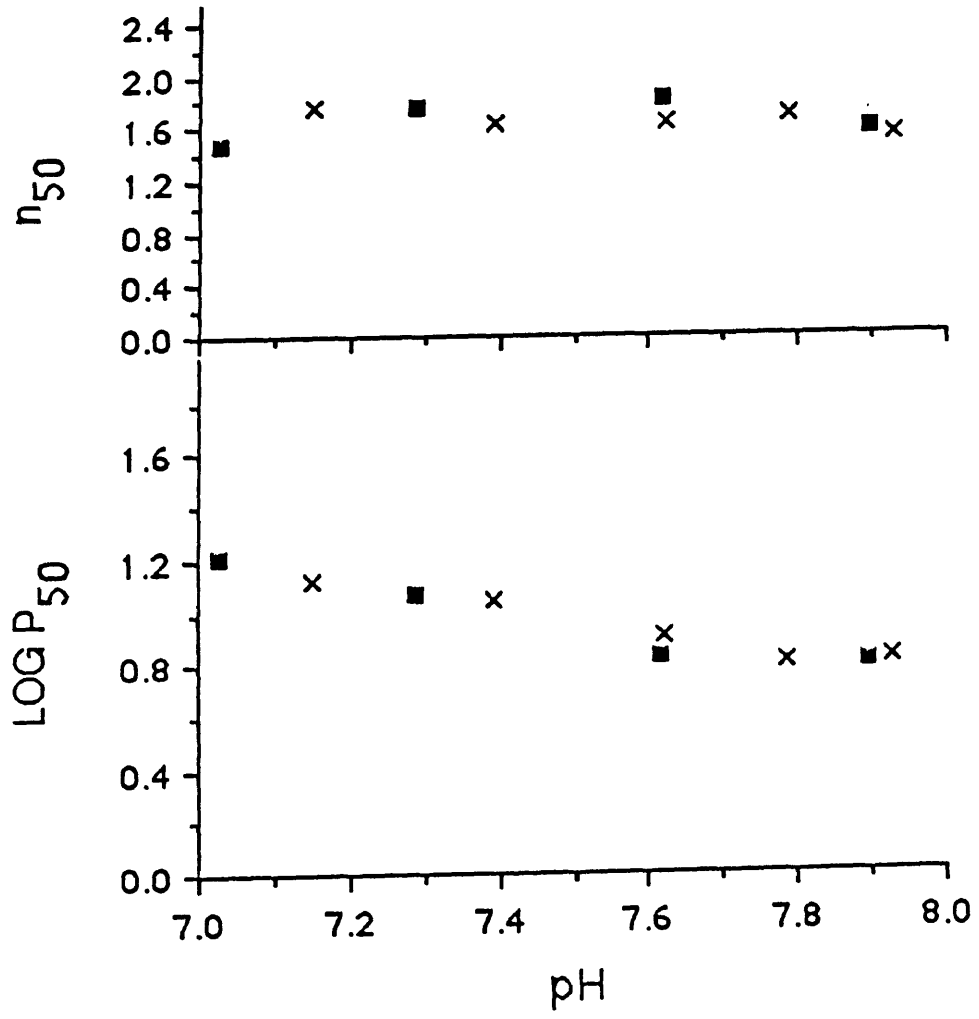
Figure 3
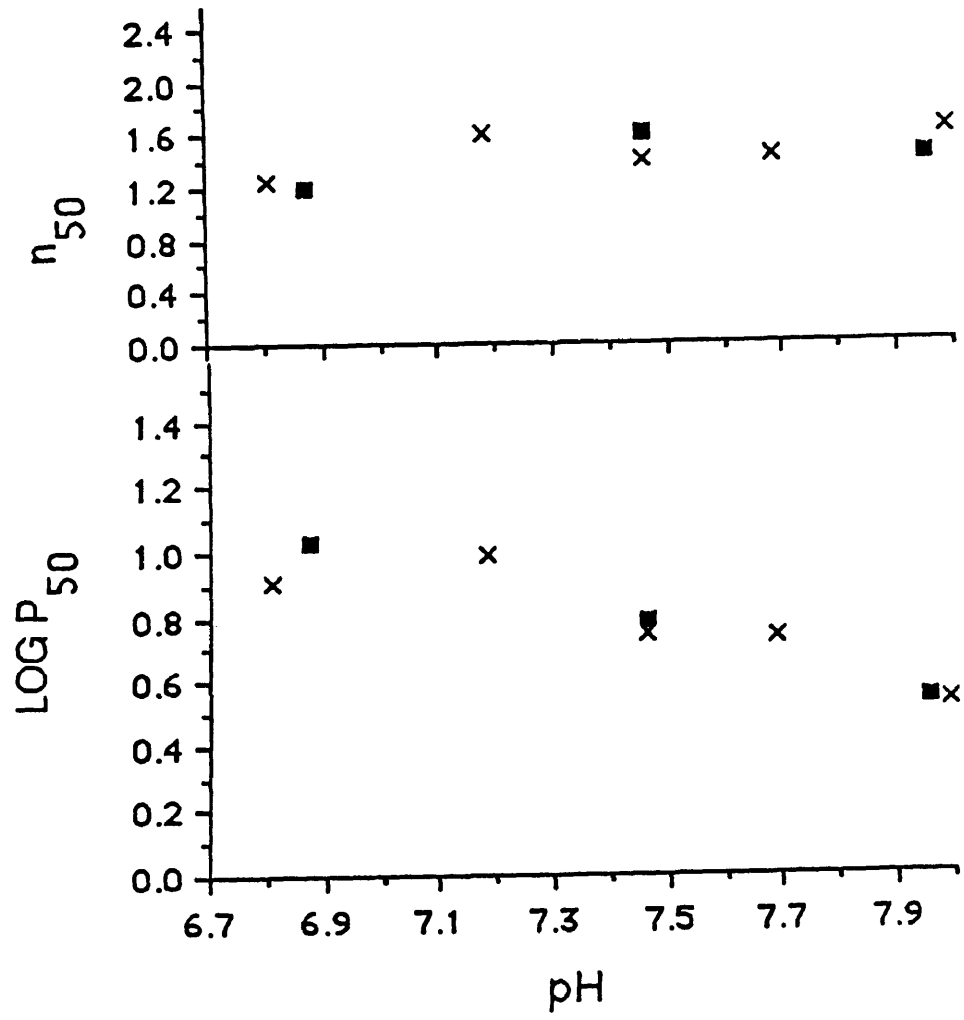
Figure 4. The effect of NaCl on oxygen affinity (P_{50}) and cooperativity (n_{50}) of stripped <u>Rhinoptera bonasus</u> Hbs as a function of pH at 20°C, using the optical analysis method. Log P_{50} and n_{50} were determined at 0.2M (■), 2.5M (+) and 5M NaCl (✗).
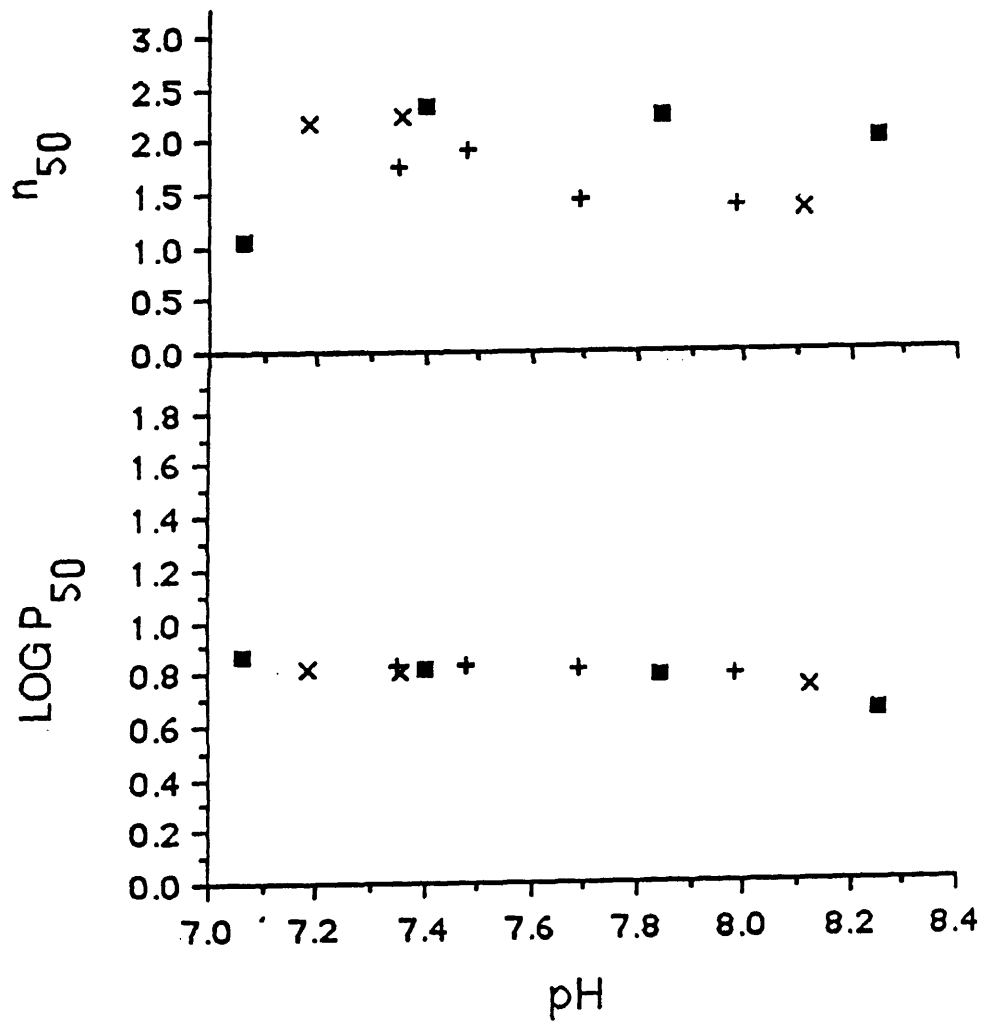
Figure 4

Figure 5. The effect of ATP on oxygen affinity (P_{50}) and cooperativity (n_{50}) of stripped <u>Rhinoptera bonasus</u> Hbs as a function of pH at $20°C$. Log P_{50} and n_{50} were determined at 0 (■), 1mM (✗), and 5mM ATP (✦).

Figure 5

Figure 6. The effect of TMAO on oxygen affinity (P_{50}) and cooperativity (n_{50}) on stripped <u>Rhinoptera</u> <u>bonasus</u> Hbs as a function of pH at 20°C. Log P_{50} and n_{50} were determined at 0 (■) and 2M TMAO (x)

Figure 6

Figure 7. The effect of substituting calcium acetate for NaCl on oxygen affinity (P_{50}) and cooperativity (n_{50}) on stripped <u>Rhinoptera</u> <u>bonasus</u> Hbs as a function of pH at 20°C. Log P_{50} and n_{50} were determined at normal saline (■) and and 300 mM calcium acetate (✗).

Figure 7
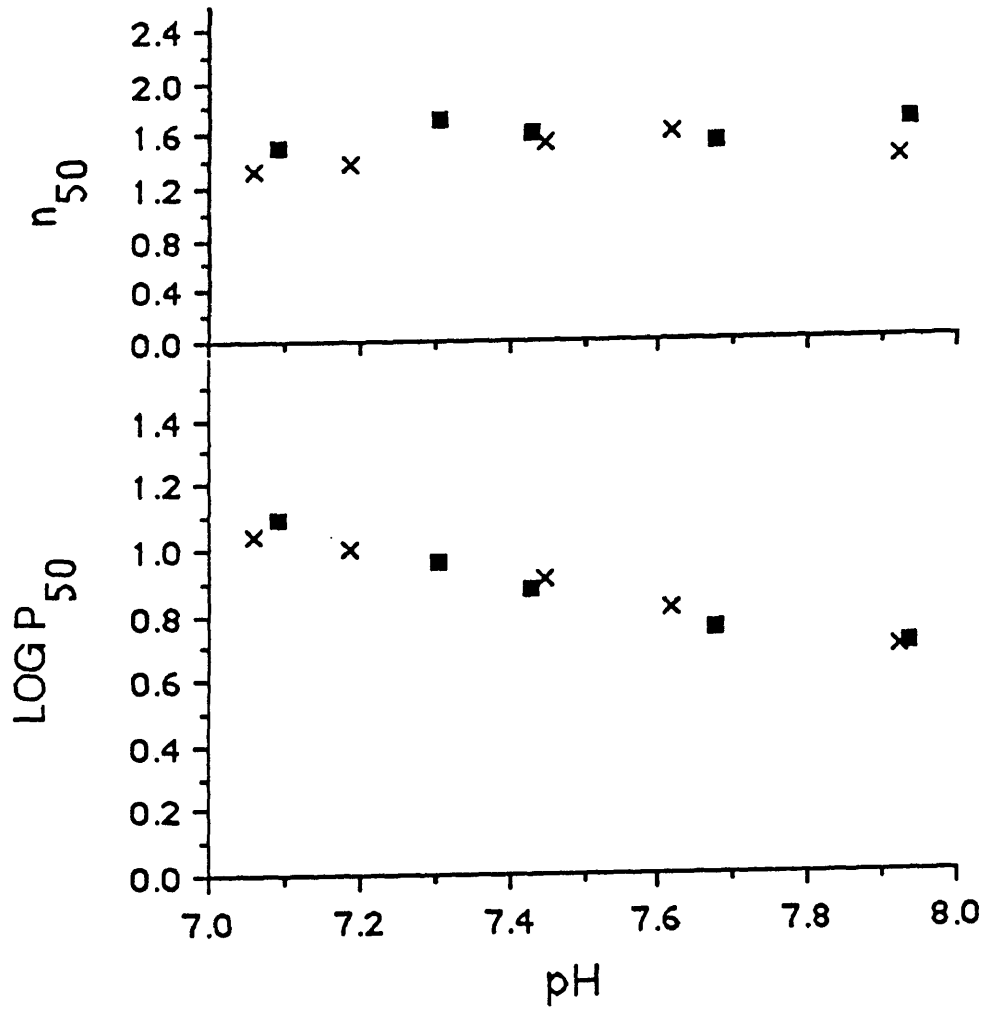
Figure 8. The effect of urea on whole cell oxygen affinity (Log P_{50}) and cooperativity (n_{50}) as a function of pH in the shark <u>Squalus</u> <u>acanthias</u>. Log P_{50} and n_{50} were determined at 0.3M (■), 1M (✗) and 2.5M urea at 35°C.
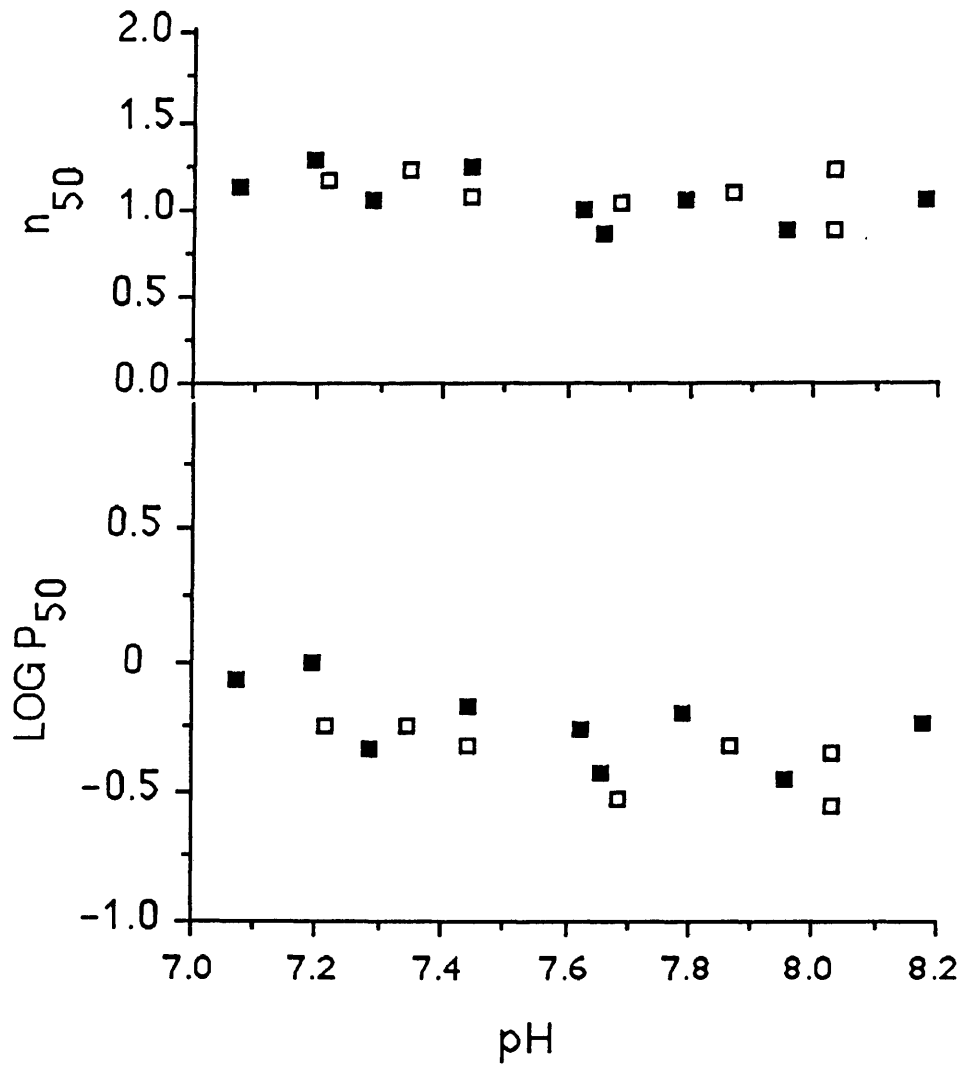
Figure 8
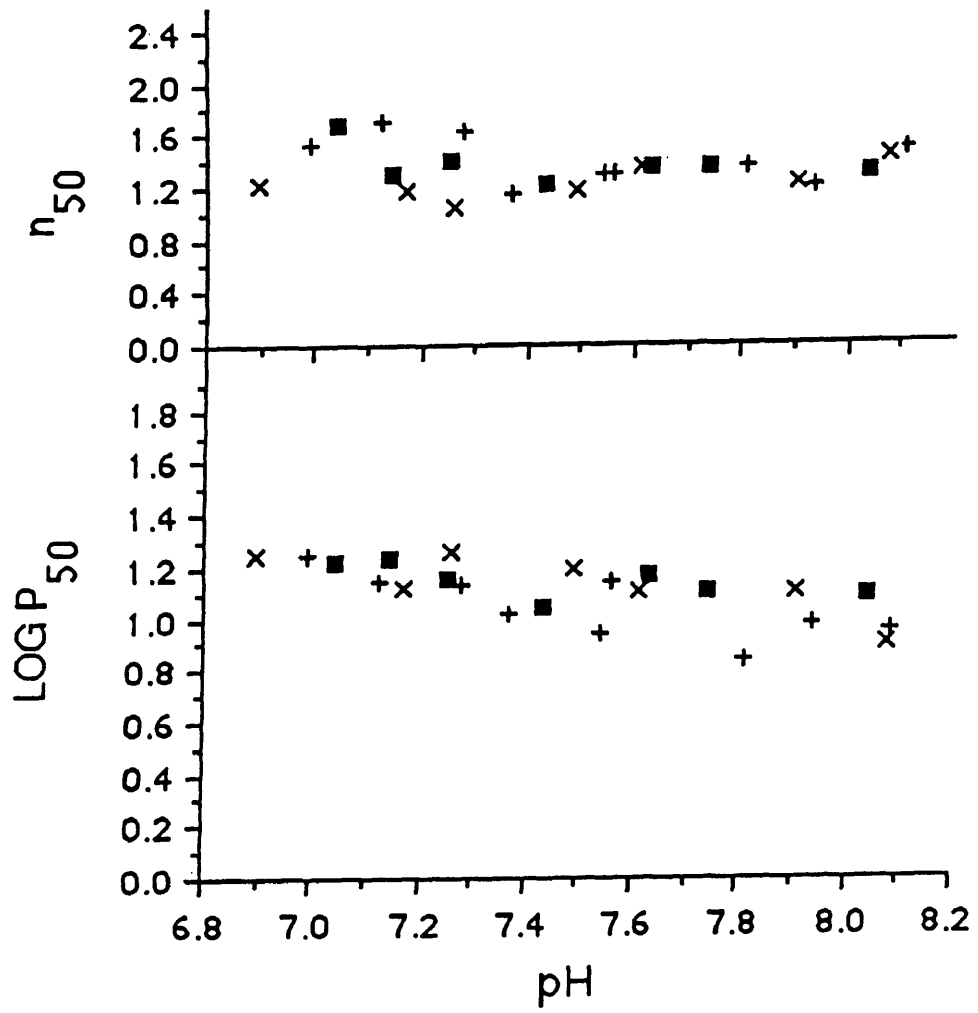
Figure 9. The effect of urea on oxygen affinity (P_{50}) and cooperativity (n_{50}) on stripped <u>Squalus</u> <u>acanthias</u> Hbs as a function of pH at 20°C. Log P_{50} and n_{50} were determined at normal saline (■) and 2.5M urea (□).

Figure 9

Figure 10. The effect of urea and NaCl on oxygen affinity (P_{50}) and cooperativity (n_{50}) on stripped <u>Squalus</u> <u>acanthias</u> Hbs as a function of pH at 35°C. Log P_{50} and n_{50} were determined at normal saline (■), 2M urea (+) and 2M NaCl(×).
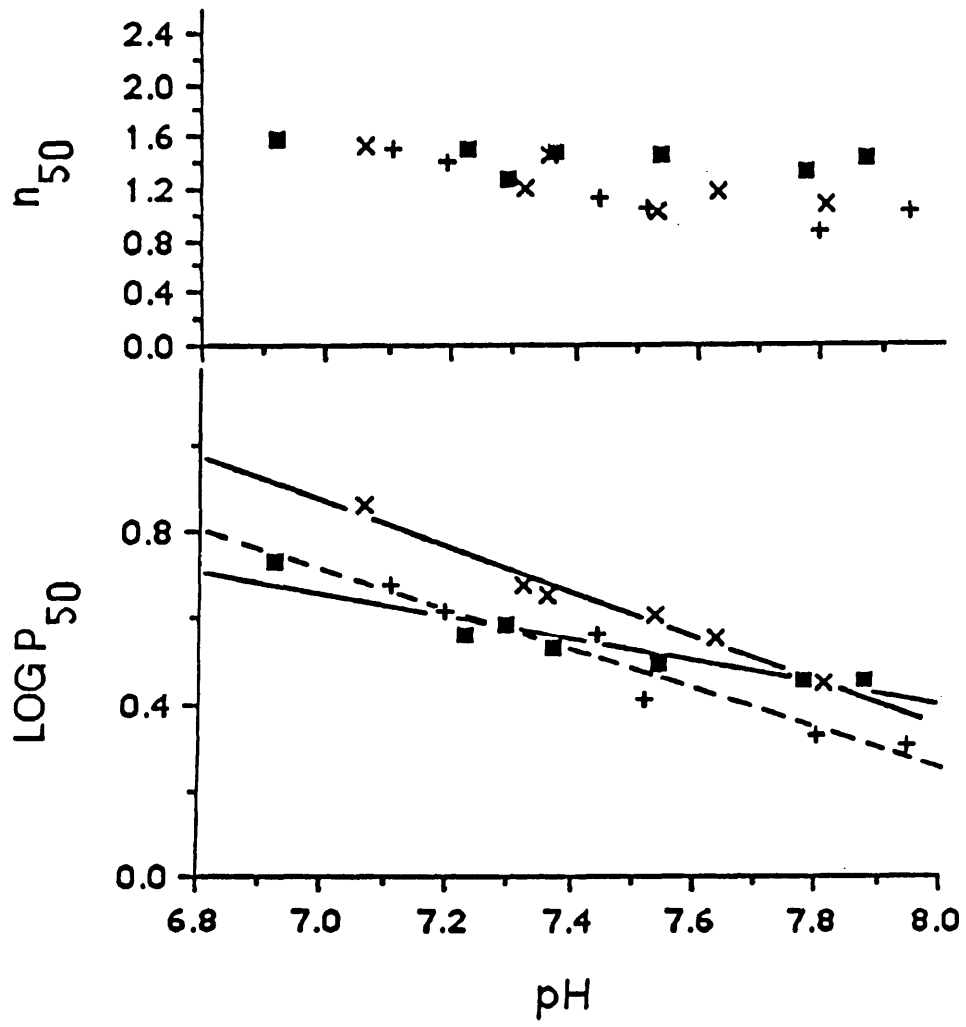
Figure 10

Figure 11.   The effect of urea on _Squalus_ _acanthias_
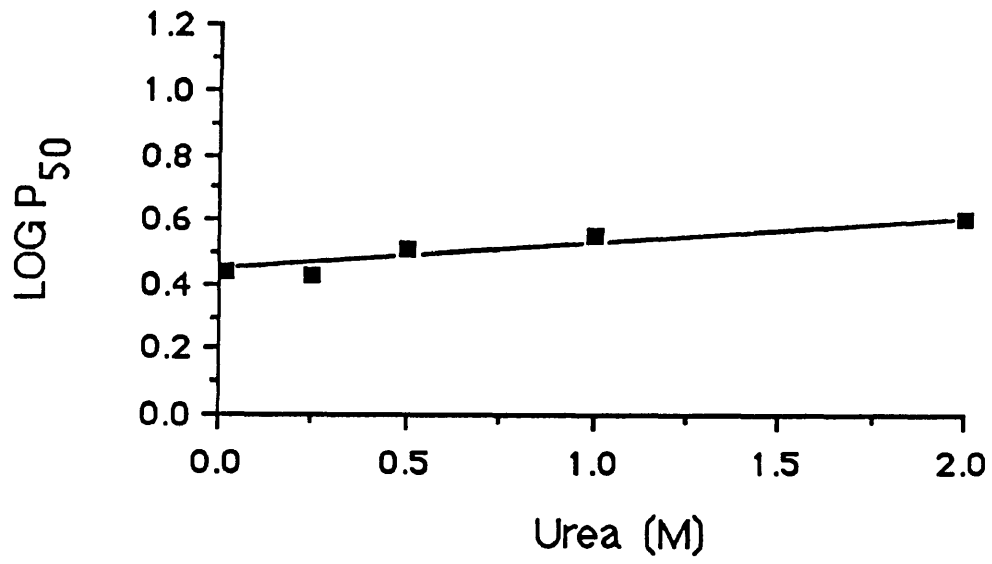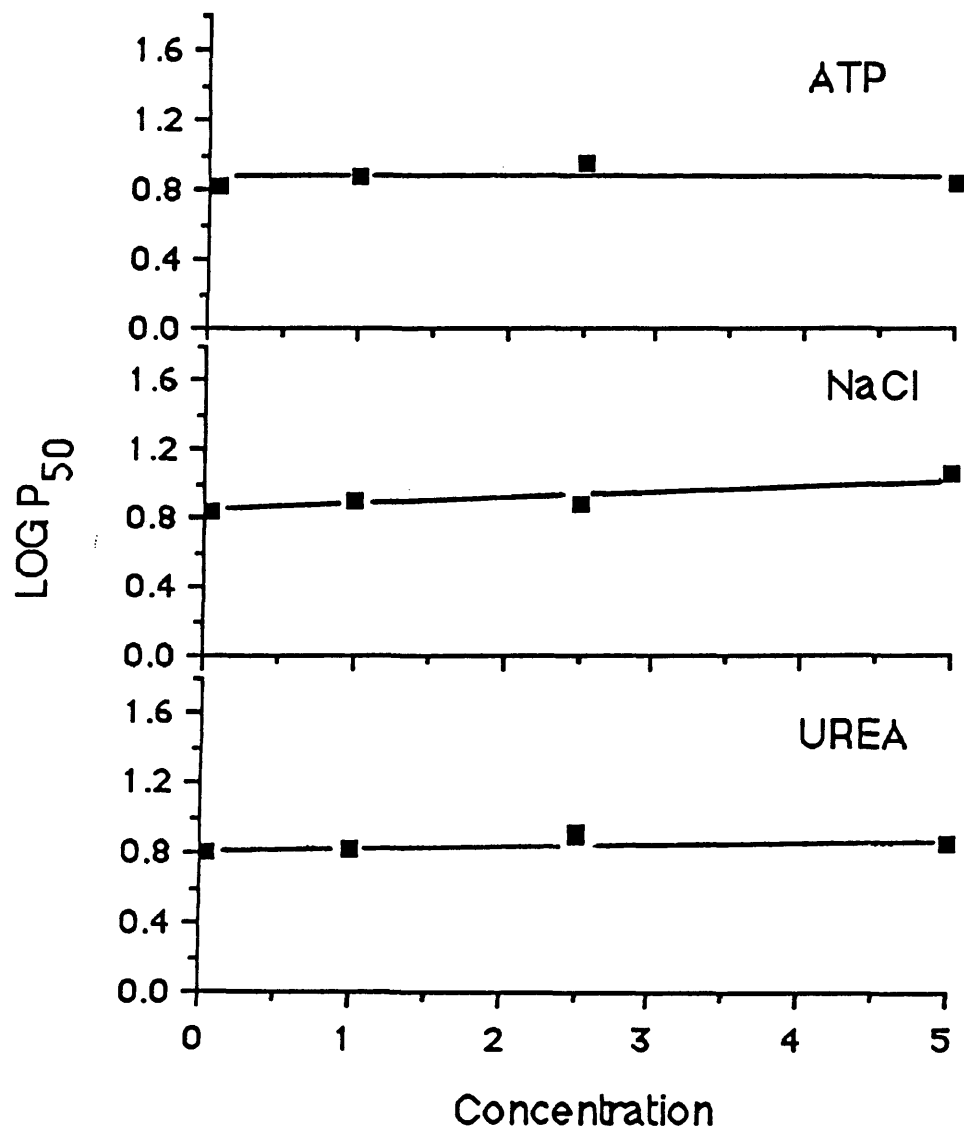stripped Hb at 35°C and pH of 7.6.

Figure 11

Figure 12. The effect of ATP, NaCl and urea on oxygen affinity (P_{50}) of stripped <u>Gymnura</u> <u>altavela</u> Hbs at 20°C. Concentration is given in mM for ATP and M for NaCl and Urea.

Figure 12

VITA

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