AN ABSTRACT OF THE THESIS OF

John Warren Nichols for the degree of Doctor of Philosophy in Toxicology presented on December 10, 1987.

Title: An Assessment of the Cardiac Toxicity of Compounds that Cause Methemoglobinemia Using a Non-Vascularized Fish Heart Model

Abstract approved: Lavern J. Weber

Non-vascularized hearts of buffalo sculpin (Enophrys bison) were used to investigate the cardiac toxicity of compounds that cause methemoglobinemia. The affinity of sculpin cardiac myoglobin for oxygen (P50 = 1.10 torr at 20 C°, pH 7.8) was lower than that of mammals studied (P50 = .44 and .76 torr for sperm whale red skeletal and rat cardiac muscle myoglobin, respectively, at 20 C°, pH 7.8). This difference probably reflects an adaptation to temperature and should not compromise sculpin myoglobin as a model for vertebrate myoglobins generally.

Hemoglobin in the buffalo sculpin was oxidized rapidly and reversibly following intraperitoneal injection with sublethal levels of sodium nitrite ($NaNO_2$) or hydroxylamine. Myoglobin in hearts excised at the time of peak effect on hemoglobin was also oxidized. For $NaNO_2$, the oxidation of myoglobin exceeded that of hemoglobin. The reverse was true of hydroxylamine. In both cases, the effect was dose-dependent. The demonstration of oxidation of cardiac myoglobin in vivo by heme oxidants raises the possibility that

cardiac myoglobin is oxidized in occupational or other exposures to these compounds.

The cardiac toxicity of $NaNO_2$, aniline, and the aniline metabolite, phenylhydroxylamine (PHA), was investigated in isolated perfused sculpin hearts. $NaNO_2$ had little effect on myoglobin oxidation state or cardiac performance, except at high concentrations (> 1.0 x 10-3 M). Aniline did not oxidize myoglobin but was acutely toxic to the heart at concentrations exceeding 1.0 x 10-3 M. The character of the response to aniline (rapid arrest, AV block) suggested an electrical effect, although electrically paced hearts also exhibited dimished levels of contractile performance. Low concentrations (1.0 x 10-5 M) of PHA oxidized 100% of myoglobin in the heart but did not affect cardiac performance at ambient (150 torr) or physiological (32 torr) oxygen tensions. Thus, functional myoglobin did not appear to be necessary to maintain cardiac performance. I conclude that the test conditions of O_2 supply and demand did not provide an adequate test of the importance of functional myoglobin.

I have shown that myoglobin can be oxidized <u>in vivo</u> in sculpin by two compounds ($NaNO_2$, hydroxylamine) that cause methemoglobinemia in humans. I was unable, however, to verify the recently reported role of myoglobin in maintaining cardiac performance and oxygen consumption in the isolated heart.

AN ASSESSMENT OF THE CARDIAC TOXICITY OF COMPOUNDS THAT CAUSE METHEMOGLOBINEMIA USING A NON-VASCULARIZED FISH HEART MODEL

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TABLE OF CONTENTS

Table of Contents, Continued

LIST OF FIGURES

List of Figures, Continued

LIST OF TABLES

AN ASSESSMENT OF THE CARDIAC TOXICITY OF COMPOUNDS THAT CAUSE METHEMOGLOBINEMIA USING A NON-VASCULARIZED FISH HEART MODEL

INTRODUCTION

The iron in hemoglobin can exist in either the ferrous (+2) or ferric (+3) oxidation state. Ferrous hemoglobin binds O_2 reversibly and is the predominant form in healthy humans. Ferric, or methemoglobin, is formed by the oxidation of ferrous hemoglobin and is incapable of binding O_2. Hemoglobin in red blood cells is continually oxidized to methemoglobin at a low rate by molecular oxygen. However, methemoglobin levels are normally low (< 2%; Kiese, 1974) due to the action of NADH-dependent methemoglobin reductase (Scott et al., 1965).

Many compounds are capable of oxidizing hemoglobin in vivo. Some, like sodium nitrite and hydroxylamine, oxidize hemoglobin directly, while others, such as aniline and nitrobenzene, require metabolic activation to the oxidizing form. The condition resulting from oxidation by chemical exposure is called toxic methemoglobinemia to distinguish it from the normal low rate of methemoglobin formation. Toxic methemoglobinemia is recognized clinically by the characteristic brown color of the methemoglobin pigment. Extensive oxidation results in generalized tissue hypoxia and may be fatal. The causes, consequences, and treatment of methemoglobinemia are reviewed by Kiese (1974).

Most reported cases of toxic methemoglobinemia result from poisoning by nitrites or arylamine compounds. Sodium nitrite is

widely used as a meat preservative and has often been mistaken for table salt. Nitrite may also be produced internally by bacterial reduction of ingested nitrate. Nitrate is a common contaminant of well water and accumulates in some vegetables from the use of nitrate fertilizers. Newborn infants are especially susceptible to nitrate-induced methemoglobinemia because they possess underdeveloped methemoglobin reducing systems.

Arylamines are used in the chemical industry as intermediates in a variety of synthetic processes. Aniline is the best known example of this group of compounds and is believed to be the leading cause of methemoglobinemia in adults (Kiese, 1974). Other compounds in this group include nitroaniline, toluidine, and aminophenol. Toxic exposures are usually accidental, often occuring via dermal or inhalation routes. Arylamines are also used as therapeutic agents (eg., sulfanilamide, primaquine) and can cause methemoglobinemia at high dosages or in patients with deficient methemoglobin reducing pathways.

Heme oxidants, as a group, are chemically reactive compounds and can be expected to have acute toxic effects apart from oxidation of hemoglobin. Two observations suggest that additional toxicities do occur. First, dogs (Vandenbelt et al., 1944) and mice (Vacek and Sugahara, 1967) survive methemoglobin levels to 80% when injected intraperitoneally (i.p.) with p-aminopropriophenone, while in rats (Lester et al., 1944) lethal i.p. doses of p-aminophenol or phenylhydroxylamine (PHA) increase methemoglobin content to only 30

and 65%, respectively. Second, aniline is toxic to animals (mice, rabbits) that form little of the oxidizing metabolite, PHA (Kiese, 1974).

The purpose of my research was to use fish hearts to model the cardiac toxicity of compounds that cause methemoglobinemia. Most fish and all mammalian hearts contain the intracellular heme protein myoglobin. Ferrous myoglobin binds oxygen with high affinity and was recently shown to support mammalian cardiac function in vitro (Braunlin et al., 1986; Taylor et al., 1986). But the potential for and hazard posed by its oxidation in vivo have not, to my knowledge, been reported.

It is difficult to assess changes in myoglobin oxidation state when hemoglobin is present because visible spectra for the two proteins overlap. Moreover, biochemical methods for separating myoglobin from hemoglobin in tissue extracts take many hours, and it is not possible to determine if the oxidation state at the time of measurement reflects what it was at the time that the tissue was collected. This is an especially difficult problem in highly vascularized tissues like the mammalian heart. Fish may provide a useful model for studying the cardiac toxicity of heme oxidants because many species do not possess coronary arteries (Santer and Greer Walker, 1980). Hemoglobin-free heart extracts may be obtained in minutes by perfusing freshly excised fish hearts with physiological saline.

I chose for a fish model the buffalo sculpin (Enophrys bison). Buffalo sculpin are available in Yaquina Bay, Newport, Oregon

throughout the year and are easily obtained with an otter trawl. They are available in a range of sizes (50 - 1000 g) and adapt well to captivity. Sculpin hearts do not possess coronary arteries and are well supplied with myoglobin. The sculpin heart can be easily isolated and perfused, and made to work in a physiologically relevant manner (Stuart et al., 1983).

I have organized this dissertation into three chapters. The initial purpose in Chapter I was to characterize the O_2 affinity of sculpin cardiac myoglobin. When it became clear that it bound O_2 with somewhat lower affinity than sperm whale myoglobin, I expanded the study and developed it as a comparison of fish and mammalian myoglobins. Chapter II establishes the potential for myoglobin oxidation in vivo and demonstrates the value of the fish heart model. In addition, methemoglobin time course studies illustrate a strong similarity of response between sculpin and mice (Smith et al., 1967; Smith and Layne, 1969). Chapter III was devoted to the study of isolated perfused sculpin hearts and is comprised of two parts: In the first part, a dose-response approach was developed to distinguish between the effects of myoglobin oxidation and other cardiac toxicities. The second part describes an unsuccessful attempt to demonstrate the importance of functional myoglobin in the fish heart, contradicting two studies (Dreidzic et al., 1982; Bailey and Driedzic, 1986) that provide much of the very limited evidence for a physiological role for myoglobin in cardiac muscle. Appendices I and II support the methods used in Chapter III. The results of the dissertation are integrated and discussed in the

final section entitled "Summary and Conclusions".

CHAPTER I

COMPARATIVE OXYGEN AFFINITY OF FISH AND MAMMALIAN MYOGLOBINS

INTRODUCTION

Myoglobins are monomeric heme proteins, which in the ferrous (+2) form, bind O_2 reversibly. Myoglobin is present in vertebrates in red skeletal and cardiac muscle and has long been thought to contribute to oxygenation of these tissues (Millikan, 1939; Wittenberg, 1970). All mammalian myoglobins tested bind O_2 with high affinity (reviewed by Antonini and Brunori, 1971). We questioned whether fish myoglobins bind O_2 with the same high affinity.

Myoglobin is present in most fishes, but amounts vary widely. Generally, myoglobin content is correlated with the physiological ecology of a species. The highest levels are found in very active fishes such as tuna (scombridae) (Giovane et al., 1980), while benthic ice fish (chaenichthyidae) possess little or no myoglobin (Douglas et al., 1985). The relationship between myoglobin content and activity level is not always clear, however, and two species that occupy a similar ecological niche may possess quite different amounts (Driedzic and Stewart, 1982).

Tuna myoglobins are the best characterized of fish myoglobins. The amino acid sequence of yellowfin tuna (Thunnus albacares) myoglobin differs considerably from that of sperm whale and other mammalian myoglobins, attesting to a lack of molecular conservation

(Watts et al., 1980). Nevertheless, its three-dimensional structure resembles that of mammalian myoglobins (Lattman et al., 1971). Bluefin tuna (Thunnus thynnus) myoglobin is to our knowledge the only fish myoglobin for which O_2 binding curves have been developed. The O_2 affinity of bluefin tuna myoglobin is intermediate to that of myoglobins from the rat and sperm whale (Rossi Fanelli et al., 1960; Antonini and Brunori, 1971). Comparisons between tuna and other fish species cannot be made at this time because stuctural and affinity data are lacking.

Our purpose was to characterize the O_2 binding affinity of myoglobins from several fishes representing different behavioral and physiological strategies. The yellowfin tuna, coho salmon (Oncorhynchus kisutch) and buffalo sculpin (Enophrys bison) were chosen. Yellowfin tuna are extremely active and utilize a vascular heat-exchange network (the retia mirabile) to maintain core temperatures up to 5 C° above that of the environment (Carey et al., 1972). Coho salmon are an active, poikilothemic and cold-adapted species. Buffalo sculpin are sedentary, poikilothemic and cold-adapted. The results of this study reflect a diversity of myoglobin O_2 binding affinity heretofore unrecognized among vertebrates.

MATERIALS AND METHODS

Experimental Animals

Buffalo sculpin, weighing 200 to 400 g, were caught with an otter trawl in Yaquina Bay, Newport, Oregon, and maintained in aerated, flowing seawater, at 12 $\pm$ 2 C°. Fish held for more than one week were fed a gelatin-based synthetic diet (Choromanski, 1985). Adult spawning coho salmon were processed on site at the Fall Creek Hatchery, Alsea River, Oregon. Yellowfin tuna myoglobin was precipitated by ammonium sulfate and shipped on dry ice from the Duke University Marine Laboratory, Beaufort, North Carolina. Six-week old male, Sprague-Dawley rats, were obtained from the Animal Science Department, Oregon State University, Corvallis, Oregon.

Tissue Collection and Storage

Buffalo sculpin and coho salmon were killed by a blow to the head, rats by decapitation. Hearts were rapidly excised and perfused with ice cold physiological saline (350 mOsm, pH 8.2 at 4 C°) containing (in mM): 124 NaCl, 5.1 KCl, 1.6 $CaCl_2.2H_2O$, 11.9 $NaHCO_3$, 0.9 $MgSO_4.7H_2O$, 5.5 glucose, 6.6 Trizma base, and 43.4 Trizma HCl. Hearts, while still beating, were frozen in liquid nitrogen and stored at -70 C°.

Myoglobin Extraction and Purification

Reduced, oxygenated myoglobins from rat, coho salmon and buffalo sculpin hearts, and yellowfin tuna red skeletal muscle, were

partially purified by salt fractionation and chromatography on size exclusion gel (Wittenberg and Wittenberg, 1981). Frozen tissues were ground to a fine powder in a mortar cooled on dry ice and the myoglobin extracted by addition of 4.25 ml/g ice-cold 10 mM Tris-Cl, 1 mM EDTA, pH 8.2. Oxidation was minimized by adding ammonium hydroxide to maintain pH at or above 8.0 (Yamazaki et al., 1964). Homogenates were spun for 10 min at 20,000 g in a refrigerated centrifuge (4 C°) and the supernatants decanted and saved. Salt cuts were determined empirically. Ammonium sulfate was added to the supernatant to 65% (fish) or 85% (rat) saturation to precipitate contaminating protein and the samples centrifuged as before. Myoglobin was precipated by adding ammonium sulfate to 80% (fish) or 100% (rat) saturation, collected by centrifugation and dissolved in a minimum volume of ice-cold 20 mM Tris-Cl, 1 mM EDTA, pH 8.2. Samples of 1.5 to 2 ml were loaded onto a 2.5 x 65 cm column of Sephadex G-100 that was equilibrated with the same buffer and eluted at 0.5 ml/min.

Purified sperm whale myoglobin was obtained from Sigma Chemical Company (St. Louis, MO). The oxidized protein was reduced with sodium dithionite under N_2 atmosphere and filtered on Sephadex G-25 to yield the reduced, oxygenated form.

A modest amount of oxidation inevitably occurs when myoglobin is extracted. In our experience, fish myoglobins oxidized more readily than mammalian myoglobins. Oxidation of the purified protein was greatly reduced by adding an enzymatic reducing system composed of ferredoxin, ferredoxin-NADP reductase, an

NADPH-generating system, and catalase (Hayashi et al., 1973).

Myoglobin Oxygen Binding

The affinity of myoglobin for O_2 was determined using a modification of the tonometric method of Riggs (1951). Samples were transferred directly from the fraction collector to a tonometer vessel fused to a 1 cm pathlength optical cuvette. Spectra from 500 to 700 nm were recorded onto floppy disc with a Perkin-Elmer diode array spectrophotometer (model 3840; Perkin Elmer Ltd., Beaconsfield, U.K.), and could be displayed at any time on a computer terminal. Myoglobin was deoxygenated by alternately applying a vacuum then gassing with N_2. The oxygenated form was regenerated by sequential addition of room air and the spectra were recorded after equilibrating samples for 10 min in a temperature-controlled rotating water bath. The percentage of myoglobin in the oxygenated form was calculated for each air addition at 542, 560, and 580 nm, averaged, and correlated with O_2 tension in the tonometer vessel.

RESULTS

Purification

The elution behavior of fish and mammalian myoglobins from size exclusion gel was characterized by the ratio of elution volume (VE) to void volume (VO)(Figure I.1). As a group, fish myoglobins eluted earlier than mammalian myoglobins. Apparent molecular weights, estimated from the column calibration curve, are given in Table I.1. Coho salmon myoglobin was purified on four separate occasions over a period of several months and eluted each time in approximately the same volume attesting to the stability of the column. Hemoglobin eluted much earlier than any of the myoglobins tested and was absent from sculpin heart extracts.

Oxygen Binding

Oxygen affinities and Hill coefficients for fish and mammalian myoglobins at 20 C°, pH 7.8 are given in Table I.2. Our initial efforts were directed toward validating the tonometric O_2 binding method. The O_2 affinity of commercially prepared sperm whale myoglobin was found to be very close to the value (P50 = 0.51 torr at 20 C°, pH 7.0) reported by Antonini and Brunori (1971). Estimated O_2 affinities for rat and yellowfin tuna myoglobin were also consistent with values reported in the literature (Antonini and Brunori, 1971) and Hill coefficients were all approximately one as expected for a monomeric binding interaction.

Myoglobins from the buffalo sculpin and coho salmon bound

O_2 with lower affinity than rat, sperm whale, or yellowfin tuna myoglobin (Figures I.2 and I.3). The significance of this result was investigated by performing O_2 binding experiments at physiologically relevant temperatures (Figure I.4). All myoglobins tested displayed the previously documented inverse relationship between binding affinity and temperature (Antonini and Brunori, 1971). Corrected for physiological conditions, buffalo sculpin and coho salmon myoglobins bound O_2 with higher affinity than mammalian myoglobins. Physiological conditions for tuna are less well known and may vary considerably within the same individual because tuna warm their red muscle but not their heart (Carey et al., 1972). Yellowfin tuna myoglobin was therefore excluded from this analysis.

DISCUSSION

The elution rate of a molecule from size exclusion gel depends upon several factors including molecular volume and shape. Therefore, molecular weights given in Table I.1 should only be considered apparent. The values reported do, however, point to a general difference between fish and mammalian myoglobins. Fosmire and Brown (1976) attribute different elution rates for yellowfin tuna and sperm whale myoglobin to differences in molecular size and shape, rather than molecular weight. Yellowfin tuna myoglobin has a more open configuration and lower alpha helical content than sperm whale myoglobin (Fosmire and Brown, 1976), and lacks the electrostatic interactions characteristic of mammalian myoglobins (Colonna et al, 1983). The elution data obtained in this study suggest the possibility that gross structural attributes of yellowfin tuna myoglobin are a feature of fish myoglobins generally.

Myoglobins isolated from buffalo sculpin and coho salmon bound O_2 with lower affinity than myoglobins from the rat or sperm whale. Oxygen binding studies at physiological temperatures suggest that this difference is adaptive. Oxygen, once bound, must be released if myoglobin is to contribute to O_2 flux (Wittenberg, 1970). Excessively high O_2 affinity is therefore incompatable with biological function. Were cold-adapted fish to possess a myoglobin having mammalian-like O_2 affinity, significant desaturation would not occur until O_2 partial pressures were reduced to less than 0.5 torr. Instead we suggest that sculpin and salmon have evolved

relatively lower affinity myoglobins that are well suited to the temperatures at which these fish live.

The purification and O_2 binding data obtained in this study describe three types of myoglobin : 1) a fast eluting, low affinity form (buffalo sculpin and coho salmon), 2) a fast eluting, high affinity form (yellowfin tuna), and 3) a slow eluting, high affinity form (rat and sperm whale). In terms of structure and function, vertebrate myoglobins are among the best characterized of all molecules. To date, however, the tendency has been to minimize the physiological significance of minor differences in O_2 binding affinity. Bluefin tuna myoglobin is the only fish myoglobin for which O_2 binding curves have been developed (Rossi Fanelli et al., 1960). This is unfortunate insofar as it may have led to the conclusion that all fish myoglobins bind O_2 with mammalian-like affinity. We suggest that this is not the case and that myoglobins from cold-adapted fish evidence a heretofore unrecognized diversity of myoglobin O_2 affinity among vertebrates.

Table I.1. Apparent molecular weights of partially purified myoglobins from rat, coho salmon, and buffalo sculpin hearts, and yellowfin tuna red skeletal muscle. Molecular weights were estimated from the column calibration curve (Figure I.1).

Species	VE	VE/VO	Apparent molecular weight
Rat	222.0	2.08	18,700
Buffalo sculpin	213.0	2.00	21,500
Yellowfin tuna	210.0	1.97	22,300
Coho salmon	207.8 ± 1.5*	$1.95 \pm .01$*	23,000

* Mean $\pm$ SD, N = 4.

Abbreviations: VE - elution volume, in ml; VO - void volume, in ml, determined with blue dextran (Sigma).

Table I.2. Oxygen affinities and Hill coefficients for sperm whale, rat, yellowfin tuna, coho salmon, and buffalo sculpin myoglobins determined at 20 C°, pH 7.8. Oxygen affinities, expressed as P50, the O_2 partial pressure giving half-maximal saturation, were read directly from O_2 dissociation curves. Hill coefficients (N) were estimated from the slope of the linear relationship between log Y/1-Y and log PO_2, where Y = fractional saturation.

Species	P50 (torr)	N
Sperm whale	0.44	1.02
Yellowfin tuna	0.63	1.00
Rat	0.76	0.88
Buffalo sculpin	1.10	0.97
Coho salmon	1.78	0.96

Figure I.1. Elution of fish and mammalian myoglobins from a calibrated column of Sephadex G-100 size exclusion gel. Molecular weight markers (represented by dots) were obtained from Sigma and made up to 1 mg/ml in 20 mM Tris-Cl, 1 mM EDTA, pH 8.2. Aliquonts of 1 ml were eluted at 0.5 ml/min and the resulting fractions stained with Coomassie brilliant blue G (Eastman Kodak Co., Rochester, N.Y.). Sperm whale myoglobin was assumed to have a molecular weight of 17,000. Void volume (VO) was determined with blue dextran (Sigma) having a molecular weight of approximately 2,000,000. Open circles indicate values for partially purified myoglobins and were plotted directly onto the calibration curve.

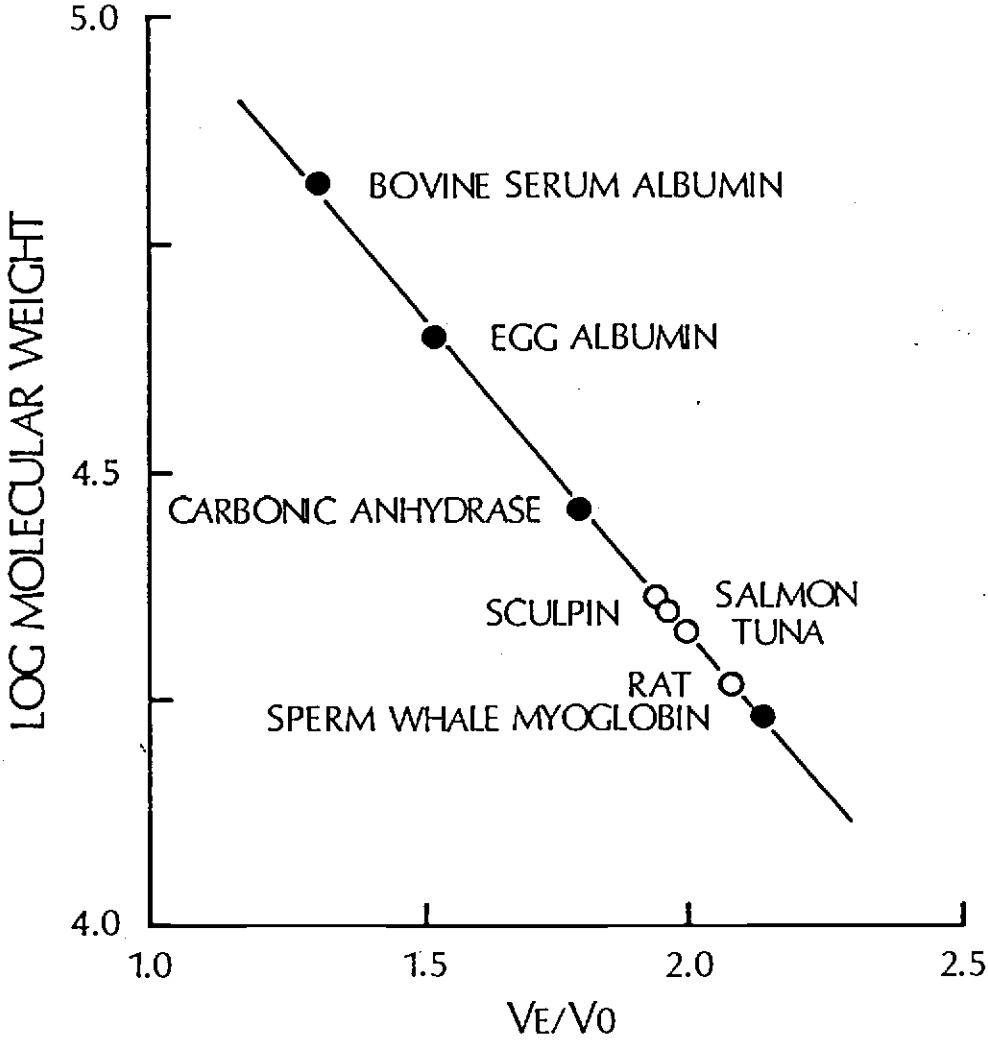
5.0
4.5
4.0
LOG MOLECULAR WEIGHT
BOVINE SERUM ALBUMIN
EGG ALBUMIN
CARBONIC ANHYDRASE
SCULPIN
SALMON
TUNA
RAT
SPERM WHALE MYOGLOBIN
1.0
1.5
2.0
2.5
VE/V0

Figure I.1.

Figure I.2. Oxygen dissociation curves for rat cardiac, and yellowfin tuna and sperm whale red skeletal muscle myoglobin determined at 20 C°, pH 7.8.

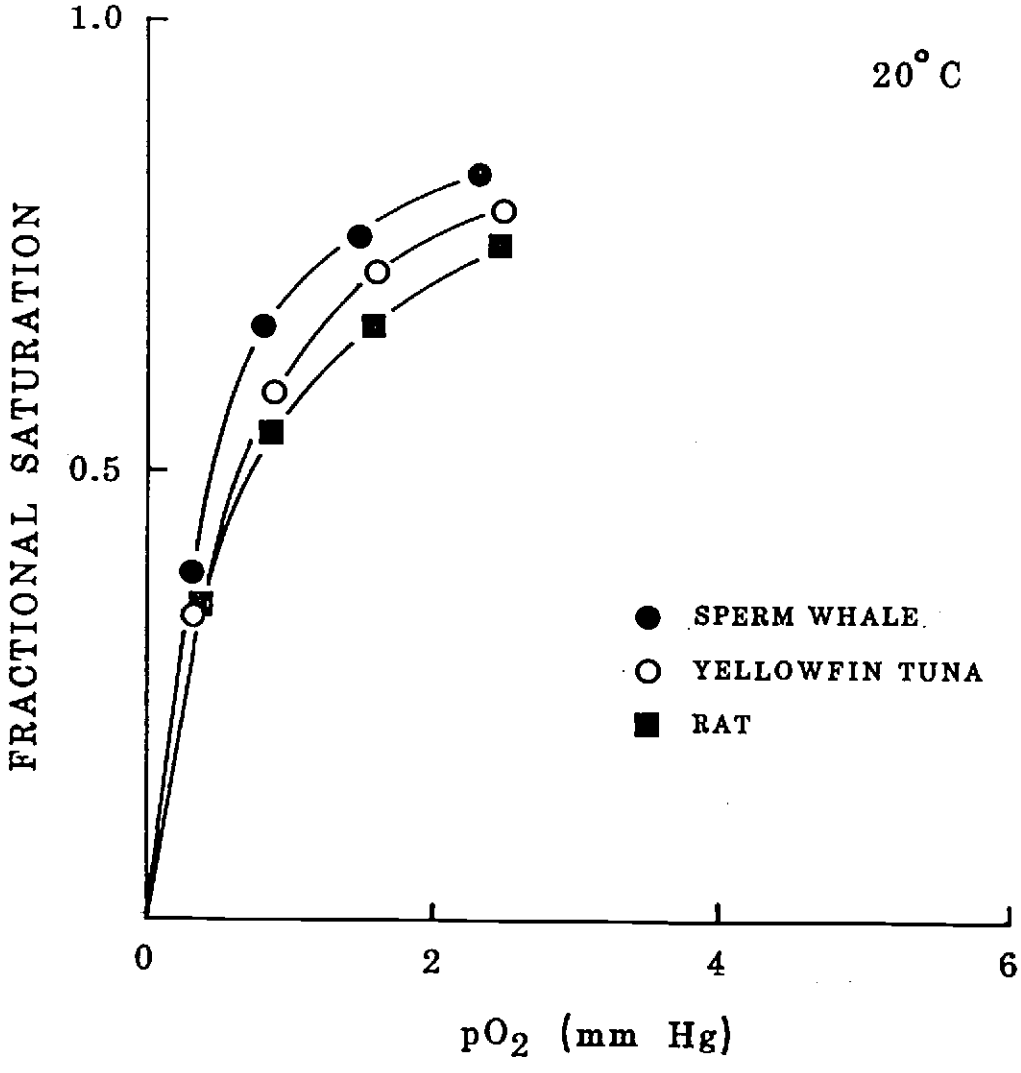
1.0
0.5
FRACTIONAL SATURATION
20° C
SPERM WHALE
YELLOWFIN TUNA
RAT
0
2
4
6
pO2 (mm Hg)

Figure I.2.

Figure I.3. Oxygen dissociation curves for buffalo sculpin and coho salmon cardiac, and sperm whale red skeletal muscle myoglobin determined at 20 C°, pH 7.8.

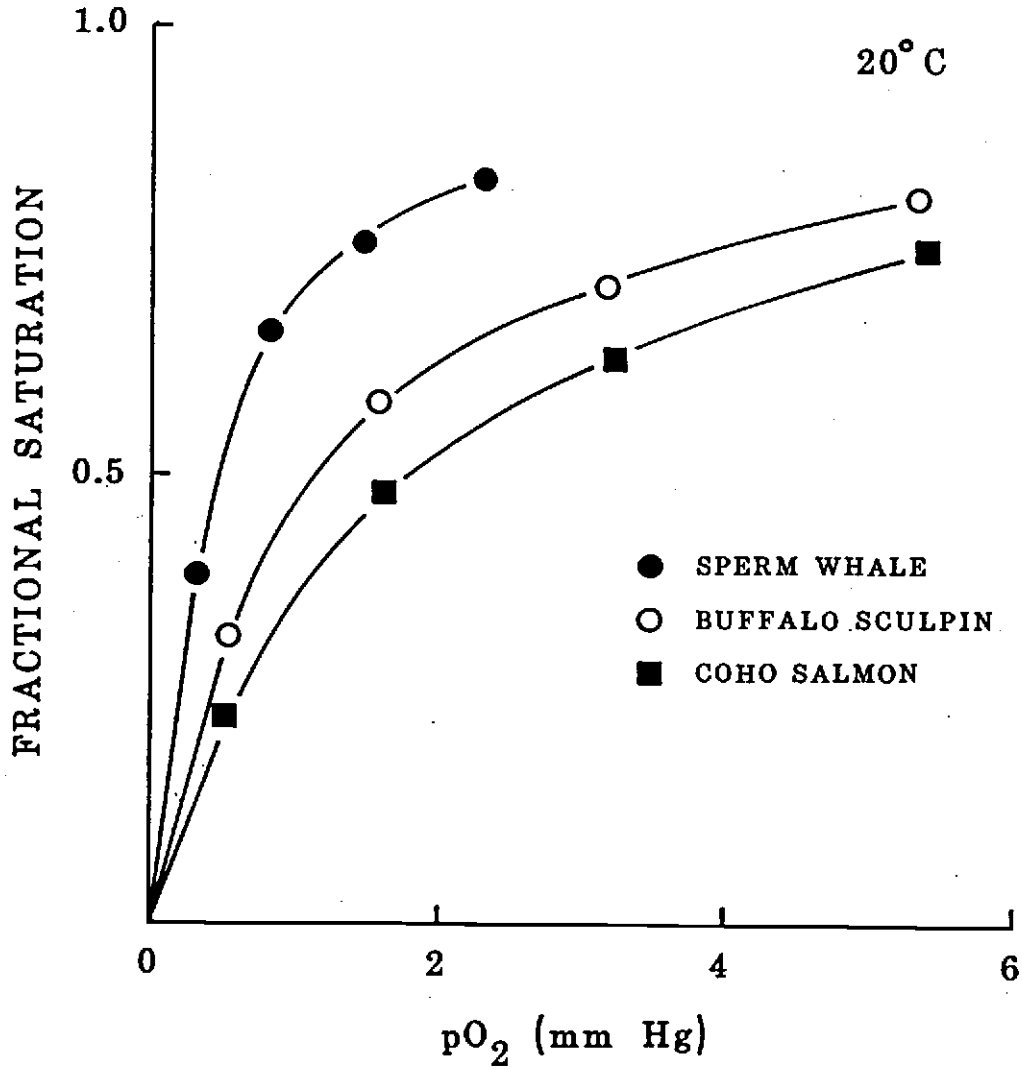
1.0
0.5
FRACTIONAL SATURATION
20° C
SPERM WHALE
BUFFALO SCULPIN
COHO SALMON
0
2
4
6
pO2 (mm Hg)

Figure I.3.

Figure I.4. Changes in oxygen binding affinity of fish and mammalian myoglobins with temperature. Affinity is expressed as P50, the O_2 partial pressure for half-maximal saturation. Arrows indicate the direction of change from 20 C° to physiological temperatures (12 or 37 C°). Samples were adjusted to pH 7.8 at each temperature prior to deoxygenation.

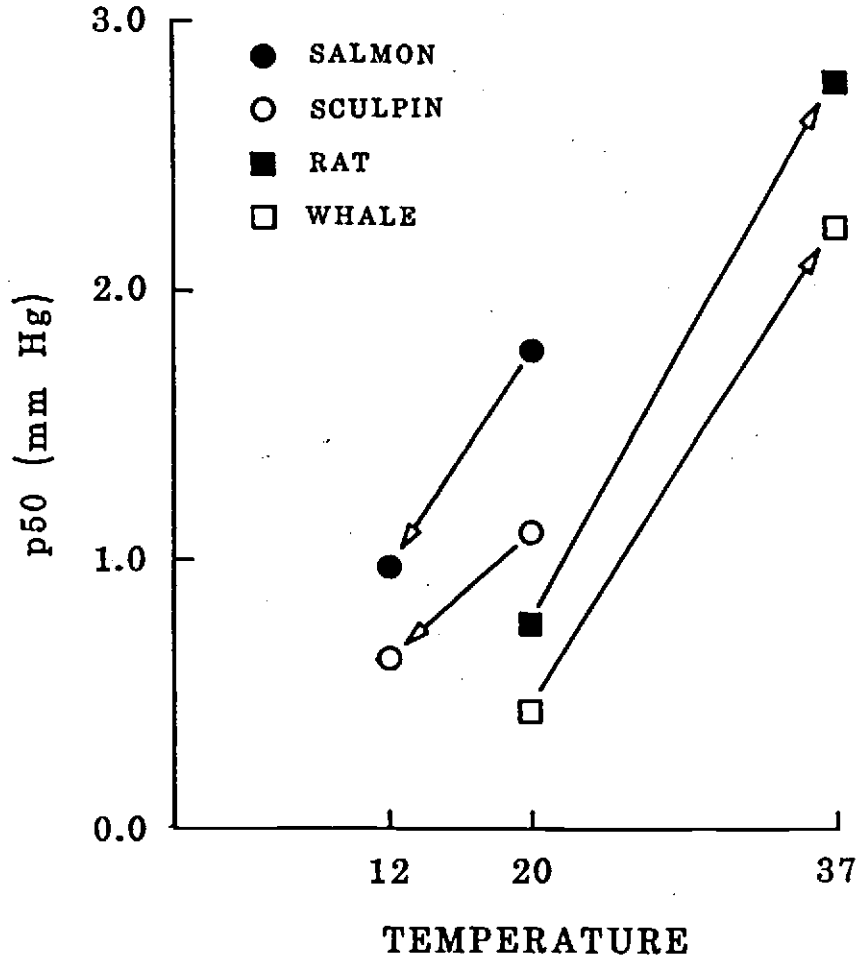
3.0
2.0
1.0
0.0
p50 (mm Hg)
SALMON
SCULPIN
RAT
WHALE
12
20
37
TEMPERATURE

Figure I.4.

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CHAPTER II

OXIDATION OF CARDIAC MYOGLOBIN *IN VIVO* BY SODIUM NITRITE OR HYDROXYLAMINE

INTRODUCTION

A wide variety of compounds oxidize hemoglobin rendering it incapable of binding O_2. The resulting condition, called methemoglobinemia, is potentially fatal. Most exposures are accidental, usually occurring via dermal or inhalation routes. Many of these compounds, including nitrites, hydroxylamines, and various quinones and dyes, oxidize hemoglobin directly. Others, such as aniline and nitrobenzene, are metabolized to the oxidizing form (reviewed by Kiese, 1974).

Our interest in the cardiovascular system led us to ask whether cardiac myoglobin is oxidized as a consequence of exposure to these compounds. Myoglobin is a monomeric heme protein, which in the ferrous (+2) form, binds O_2 with high affinity facilitating its diffusion in model systems (reviewed by Wittenberg, 1970). Myoglobin is present in vertebrates in red skeletal and cardiac muscle and has long been thought to contribute to oxygenation of these tissues (Millikan, 1939). The importance of functional myoglobin in skeletal muscle was demonstrated by selectively oxidizing the protein, resulting in decreased O_2 consumption (Wittenberg et al., 1975) and reduced isometric tension generation (Cole, 1982). The role of myoglobin in cardiac muscle is not as well established, but recent evidence collected *in vitro* suggests

that myoglobin supports cardiac function hypoxia (Braunlin et al., 1986; Taylor et al., 1986). The potential for and hazard posed by the oxidation of cardiac myoglobin *in vivo* has not, to our knowledge, been investigated.

We employed a nonvascularized fish heart model to address this question. Buffalo sculpin (*Enophrys bison*), like many benthic fish, do not possess coronary arteries, but are well supplied with cardiac myoglobin. Hemoglobin-free heart extracts were obtained by perfusing freshly excised hearts with physiological saline. We found that both sodium nitrite and hydroxylamine oxidized cardiac myoglobin to a significant degree when injected intraperitoneally.

MATERIALS AND METHODS

Experimental Animals

Buffalo sculpin were caught with an otter trawl in Yaquina Bay, Newport, Oregon, and maintained in aerated, flowing seawater, at 12 ± 2 C°. Acute toxicity tests were performed with fish weighing 100 to 250 g. All other experiments employed fish weighing 250 to 500 g. Fish held for more than one week were fed a gelatin-based synthetic diet (Choromanski, 1985).

Acute Toxicity Testing

Ninety-six hour LD 50 values, for bolus intraperitoneal (i.p.) injection, were estimated using the multiple sample up-and-down method of Hsi (1969). Compounds were dissolved in 40% propylene glycol (balance 50 mM Tris-Cl) such that 1 to 2 ml/100 g body weight contained the appropriate dose. Each solution was prepared immediately before injection and adjusted to pH 7.8 at 12 C°. A sequence of four trials was carried out using two animals per trial. Starting dosages and dosing levels, in .25 log unit steps, were chosen ahead of time based on preliminary experiments. In the second and subsequent trials, dose was determined by the outcome of the preceeding trial; the dose was increased if neither animal died and decreased if both died. The dose was not changed if only one animal died. Reagent grade sodium nitrite ($NaNO_2$), aniline, and hydroxylamine monohydrochloride were obtained from Sigma Chemical Company (St. Louis, MO).

Branchial Cannulation

Sculpin were cannulated from the afferent branchial artery to permit repeated blood sampling (Choromanski et al., 1987). Sculpin were anesthetized in seawater containing MS 222 (70 mg/L, Sigma Chemical Co., St. Louis, MO), weighed, and placed ventral side up on a fish operating table. The second gill arch on the left side was isolated by dorsal and ventral ligatures, the filaments trimmed away, and a shallow notch cut to expose the afferent branchial artery. A 40 cm length of PE 50 cannula was cut at an angle and filled with heparinized (500 IU/ml) physiological saline (350 mOsm, pH 7.83 at 12 C°) containing (in mM): 124 NaCl, 5.1 KCl, 1.6 $CaCl_2.2H_2O$, 11.9 $NaHCO_3$, 0.9 $MgSO_4.7H_2O$, 5.5 glucose, 6.6 Trizma base and 43.4 Trizma HCl. The ventral ligature was then loosened briefly and the cannula was inserted and advanced a short distance toward the ventral aorta. The cannula was secured by additional sutures around the gill arch and along the back. Blood flow was controlled by fitting each cannula with a 23 G needle and plastic 1 ml tuberculine syringe filled with heparinized saline (500 IU/ml). Gill perfusion with anesthetic-treated water (70 mg MS 222/L) was maintained throughout the operation, which lasted about 30 min. Sculpin were allowed 24 h to recover from anesthesia and surgery.

Methemoglobin Determination

Experiments were performed to characterize the time course of methemoglobin formation by the test compounds. Toxicants were dissolved in 20% propylene glycol (balance 50 mM Tris-Cl) and the

fish injected i.p. Samples (0.1 ml) were withdrawn from branchial cannulae with a Hamiliton gas-tight syringe and lysed in 50 ml ice-cold 50 mM Tris-Cl, 1 mM EDTA, pH 8.2. Methemoglobin content as a percentage of total hemoglobin was determined by the two-wavelength (575 and 525 nm) method of Salvati and Tentori (1981).

Metmyoglobin Determination

The effects of $NaNO_2$ and hydroxylamine on cardiac myoglobin were determined by excising hearts at previously established times of peak effect on hemoglobin (Figure II.1). Aniline, which did not cause methemoglobinemia in sculpin, was not tested. A cannulated fish was injected i.p. and, at the appropriate time, a blood sample was drawn and lysed to determine methemoglobin content. Shortly thereafter, the fish was killed by a blow to the head. The heart, including the bulbous arteriosus and a portion of the sinus venosus, was excised and placed in ice-cold physiological saline. Blood was removed by perfusing the heart with additional ice-cold saline. The atrium and ventricle were dissected away, weighed and homogenized in 29.25 ml/g ice-cold 50 mM Tris-Cl, 1 mM EDTA, pH 8.2. The homogenate was spun for 10 min at 20,000 g in a refrigerated (4 C°) centrifuge and the supernatant decanted and saved. The absence of hemoglobin in heart preparations was confirmed by chromatography on Sephadex G-100 size exclusion gel (Chapter I).

A small amount of oxidation inevitably occurs when myoglobin is extracted. Consequently, samples from treated fish may contain

metmyoglobin from two different sources. The percentage of total myoglobin oxidized by chemical treatment was determined by modifying the two-wavelength method used to measure methemoglobin (Salvati and Tentori, 1981). Myoglobin extracted from the heart of an untreated fish was transferred to a tonometer vessel fused to a 1 cm pathlength optical cuvette and equilibrated for 10 min with humidified pure O_2. The absorption spectrum from 500 to 700 nm was recorded with a Perkin-Elmer model 124 dual beam spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, U.K.) and the molar concentration of heme estimated using a millimolar extinction coefficient of 13.6 at 543 nm (Antonini and Brunori, 1971). Potassium ferricyanide was then added in 2.5 molar excess (approximate because heme concentration was determined from a partly oxidized sample) to obtain the spectra for 100% oxidized myoglobin. Spectra were then overlapped and peak and isobestic wavelengths noted. The first spectrum was adopted as that of 100% reduced, oxygenated myoglobin and a calibration curve was developed relating the ratio of absorbance at 576 and 590 nm to the percent of total myoglobin oxidized by chemical exposure (as by potassium ferricyanide _in vitro_ or sodium nitrite _in vivo_). Interference by cytochrome c or cytochrome oxidase is minimal in this region of the optical spectrum (Wittenberg and Wittenberg, 1985). The method assumes that a constant fraction of the reduced myoglobin remaining after chemical treatment oxidizes during the extraction step.

Approximately 30 min elapsed from the time a fish was killed until its myoglobin spectrum was read. Recognizing that the

oxidation state of myoglobin at any one time reflects a balance of oxidation and reduction, we sought to determine whether the spectrum obtained reflected the oxidation state of myoglobin at the time the fish was killed. Heart homogenates from two fish injected with 100 mg/kg $NaNO_2$ were each divided into three aliquots. The first aliquot was processed as usual and the other two incubated on ice for 20 and 40 additional minutes, respectively. There were no differences between spectra from any one heart over this time period. The eight minutes required to excise, perfuse and weigh a heart remain unaccounted for, but we believe that changes were minimized by maintaining temperatures at or below 4 C° (Wittenberg and Wittenberg, 1981). Metmyoglobin control and treatment data were compared using a one-way analysis of varience and Dunnett's multiple range test.

RESULTS

Acute Toxicity

Ninety-six hour i.p. LD50 values for aniline, $NaNO_2$, and hydroxylamine in buffalo sculpin were estimated to be 890, 440, and 44.0 mg/kg, respectively. Fish that died did so during the first six hours after injection. Death was preceeded in each case by loss of color, rapid respiration and sustained flaring of one or both operculae. Fish treated with aniline or hydroxylamine also evidenced neuromuscular and nervous system involvement including twitching, convulsions, asymmetric color changes, arching of the head and back, and paralysis.

Methemoglobin Formation

Methemoglobin was generated by sublethal levels of $NaNO_2$ or hydroxylamine (Figure II.1). For hydroxylamine, the time to peak effect was less than one hour. For $NaNO_2$ the onset was less rapid and the effect more prolonged. With both compounds recovery took many hours. No overt signs of toxicity were observed at these concentrations. Aniline had no effect at any concentration tested. These data were used to establish times at which oxidation of myoglobin would be most likely to occur.

Metmyoglobin Formation

Cardiac myoglobin was oxidized *in vivo* in a dose-dependent manner by $NaNO_2$ or hydroxylamine (Figures II.2 and II.3). At the two higher doses of $NaNO_2$ (50 and 100 mg/kg), the oxidation of

myoglobin exceeded that of hemoglobin. The reverse was true of hydroxylamine at all concentrations tested. This point is emphasized by comparing data for 100 mg $NaNO_2$/kg and 10 mg hydroxylamine/kg. These treatments produced a similar degree of methemoglobin but quite different amounts of metmyoglobin. As during the time course studies, there were no overt signs of intoxication at any of these concentrations.

In Figures II.1-3 it may be seen that methemoglobin levels in untreated fish ranged from 15 to 25%. Methemoglobin levels can be determined with excellent accuracy because hemoglobin constitutes an overwhelming percentage of the light absorbing species in blood. By comparsion, the method for determination of metmyoglobin is only approximate and assumes that there is no oxidation in untreated fish.

DISCUSSION

Acute toxicity values for fish are usually determined by adding toxicants to water, thus modeling the environmental route of exposure. Our objective, however, was to use sculpin to model the effects of heme oxidants in mammals, including man. Toxicants were injected i.p. to give fish the opportunity to metabolize compounds in the liver before they reached the heart. Moreover, $NaNO_2$ and hydroxylamine react rapidly when added to water causing dosing to become problematic.

Toxicant losses to the water were not assessed but may have been substantial. Plasma NO_2^- levels in rainbow trout (Salmo gairdneri) steadily declined after fish were transferred from dilute seawater (16 ppt) containing NO_2^- (22.5 mM) to NO_2^--free water (Eddy et al., 1983). The authors suggested that this decline was mainly due to a passive efflux of NO_2^- across the gills. Elimination routes for aniline and hydroxylamine have not, to our knowledge, been investigated in fish.

Methemoglobin was generated in sculpin by $NaNO_2$ or hydroxylamine. With either compound, the time course of the effect resembled that observed using the same compounds in mice (Smith and Layne, 1969). Similar results were reported by Huey et al. (1980) and Eddy et al. (1983) using NO_2^--treated channel catfish (Ictalurus punctatus) and rainbow trout, respectively. Hydroxylamine is consumed in its reaction with hemoglobin, rapidly terminating its effect. In contrast, the kinetics of oxidation by $NaNO_2$ are

characterized by a lag phase followed by autocatalysis (Kiese, 1974). The mechanism of this effect is incompletely understood but involves the formation of hydrogen peroxide, itself an oxidizing species (Kosaka and Uozumi, 1986; Spagnuolo et al., 1987). Methemoglobin is reduced *in vivo* in both mammals and fish by NADH-dependent methemoglobin reductase (Freeman et al., 1983; Scott and Harrington, 1985). The presence of a reducing system in sculpin was inferred by the recovery of reduced hemoglobin levels 24 h after treatment.

Aniline did not promote the formation of methemoglobin in sculpin at concentrations to 250 mg/kg. Aniline is metabolized in mammals to the oxidizing form, phenylhydroxylamine, by N-oxidation. This reaction is thought to be mediated by a mixed function oxidase. Phenylhydroxylamine enters an intraerythrocytic cycle of oxidation and reduction such that each mole may produce many equilvalents of methemoglobin. There are, however, notable interspecies differences; methemoglobin is readily formed in dogs following treatment with aniline, but not in mice or rabbits. These differences are due to differences in rate and pattern of metabolism, and rate of methemoglobin reduction (reviewed by Kiese, 1974).

The metabolism of aniline by fish is not well known. Aniline hydroxylase activity was detected in liver extracts from rainbow trout (Buhler and Rasmusson, 1968; Gerhart and Carlson, 1978) and sunfish (*Lepomis spp.*; Carter et al., 1984). However, Abram and Sims (1982) could not detect any para-aminophenol in the test

environment after exposing rainbow trout to aniline in water. They noted, instead, an increase in ammonia levels and suggested that trout were capable of deaminating aniline.

Relatively high concentrations of methemoglobin were detected in untreated sculpin. Similar observations were made by Cameron (1971) and Margiocco et al. (1983), using rainbow trout, and by Graham and Fletcher (1986) in five species of marine teleosts. In the latter study methemoglobin levels varied seasonally in three of the five species, including two species of sculpin. The significance of these observations is not clear. As already noted, fish appear to be able to efficiently reduce methemoglobin formed by chemical treatment.

Cardiac myoglobin was oxidized in vivo in sculpin as a consequence of i.p. dosing with $NaNO_2$ or hydroxylamine. Sodium nitrite and hydroxylamine, or reactive metabolites thereof, must have been absorbed into and carried by the bloodstream to reach the heart. We would not have been surprised to find that hemoglobin in blood spared cardiac myoglobin by acting as a reactive sink, but this was not the case. Sodium nitrite appeared to oxidize cardiac myoglobin more efficiently than hydroxylamine. However, this conclusion must be viewed cautiously because we sampled at only one time for each toxicant and do not know whether the time course of metmyoglobin formation follows that of methemoglobin.

We do not know how well sculpin model the effects of heme oxidants in mammals. Sculpin were utilized because they do not possess coronary arteries, thus providing a fast and simple way to

obtain essentially hemoglobin-free heart extracts. However, they possess very little red skeletal muscle which, like hemoglobin in blood, might be expected to spare cardiac myoglobin. Nevertheless, the extent and time course of methemoglobinemia in sculpin bore a strong resemblance to that observed in mice (Smith and Layne, 1969).

Sodium nitrite, hydroxylamine, and aniline represent the two major categories of heme oxidants: 1) those which act directly (sodium nitrite and hydroxylamine) and 2) those requiring metabolic metabolic activation (aniline). Each has been used to generate methemoglobinemias in mammals (Kiese, 1974). Sodium nitrite and hydroxylamine have also been used to selectively oxidize myoglobin in vitro (Wittenberg et al., 1975; Cole et al., 1978; Braunlin et al., 1986; Taylor et al., 1986). We are not aware, however, of any previous demonstration of myoglobin oxidation in vivo following exposure to these or any other compounds.

Our study raises the possibility that cardiac myoglobin is oxidized in occupational or other exposures to compounds which cause methemoglobinemia. Oxygen transport would be thus impaired both in the lungs and within the cardiac myocyte. Moreover, the effect on the heart might occur at a particularly inopportune time. Heart rate and cardiac output increase in dogs when methemoglobin content exceeds 40% (Clark et al., 1943). Cardiac demand therefore increases even as the supply of O_2 diminishes. The heart would be poorly equipped to deal with an increase in cardiac demand if myoglobin was oxidized to the same extent as hemoglobin.

Figure II.1. Time course of methemoglobin generation by sodium nitrite, hydroxylamine and aniline. Sculpin were cannulated from the branchial artery to permit repeated blood sampling and the toxicants were injected i.p. Values are means, N = 3 (sodium nitrite) or 2 (hydroxylamine and aniline). The dosage of hydroxylamine is expressed as that of the monohydrochloride salt.

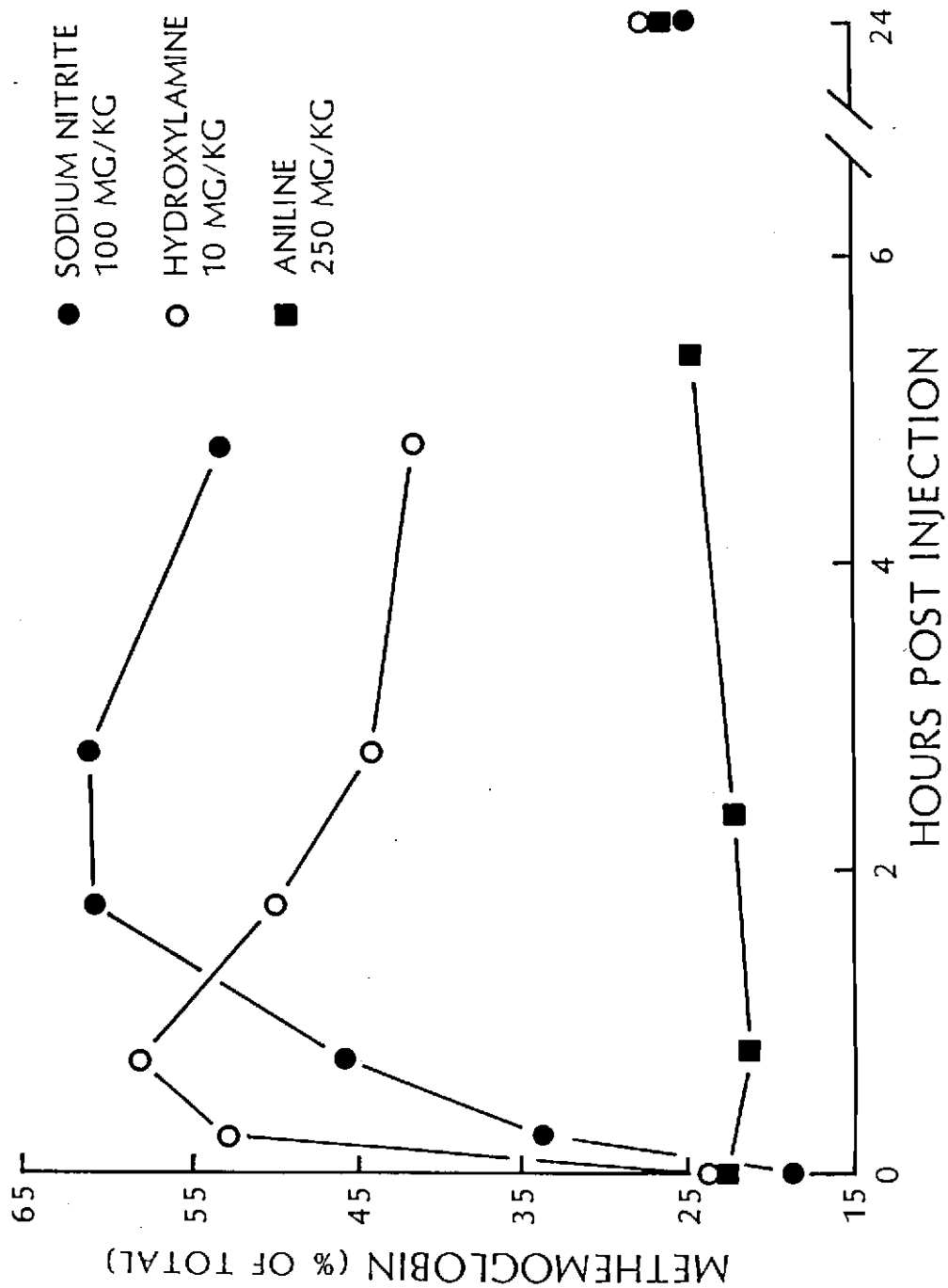
SODIUM NITRITE
100 MG/KG
HYDROXYLAMINE
10 MG/KG
ANILINE
250 MG/KG
METHEMOGLOBIN (% OF TOTAL)
65
55
45
35
25
15
0
2
4
6
24
HOURS POST INJECTION

Figure II.1.

Figure II.2. Formation of methemoglobin and metmyoglobin by sodium nitrite. Hearts were excised 2 h after treatment. Each column represents the mean $\pm$ SD, N = 3. An asterisk (*) denotes a significant difference ($p < 0.05$) from respective control (0.0 mg/kg) means.

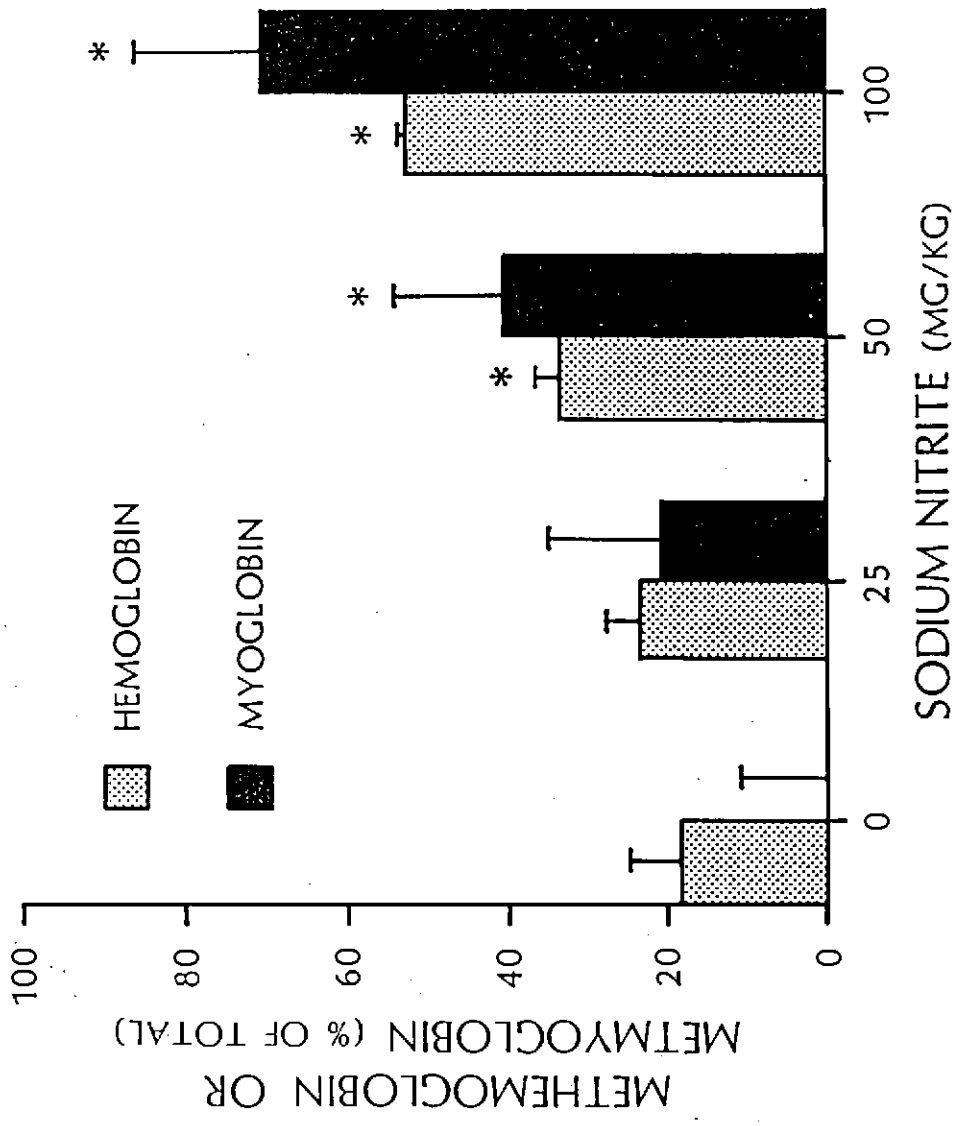
METHEMOGLOBIN OR
METMYOGLOBIN (% OF TOTAL)
100
80
60
40
20
0
HEMOGLOBIN
MYOGLOBIN
0
25
50
100
SODIUM NITRITE (MG/KG)
*
*
*
*

Figure II.2.

Figure II.3. Formation of methemoglobin and metmyoglobin by hydroxylamine. Hearts were excised 1 h after treatment. Each column represents the mean $\pm$ SD, N = 3. An asterisk (*) denotes a significant difference ($p < 0.05$) from respective control (0.0 mg/kg) means. The dosage of hydroxylamine is expressed as that of the monohydrochloride salt.

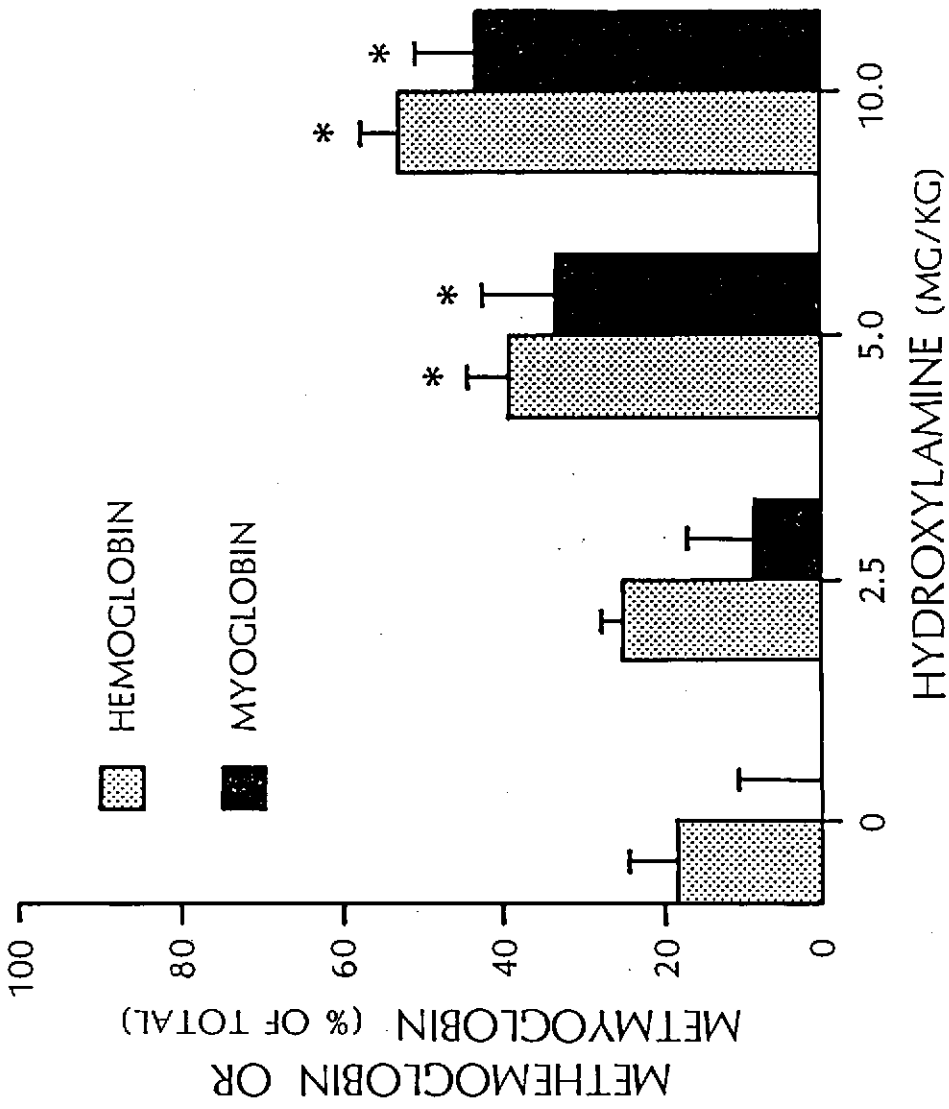
METHEMOGLOBIN OR
METMYOGLOBIN (% OF TOTAL)
100
80
60
40
20
0
HEMOGLOBIN
MYOGLOBIN
0
2.5
5.0
10.0
HYDROXYLAMINE (MG/KG)
*
*
*
*

Figure II.3.

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CHAPTER III

TOXICITY OF HEME OXIDANTS TO THE ISOLATED PERFUSED BUFFALO SCULPIN (*Enophrys bison*) HEART: COMPLETE OXIDATION OF MYOGLOBIN BY PHENYLHYDROXYLAMINE DOES NOT AFFECT CARDIAC PERFORMANCE OR OXYGEN CONSUMPTION

INTRODUCTION

Sodium nitrite ($NaNO_2$) and aniline exhibit as a common mechanism of toxicity the ability to cause oxidation of hemoglobin. Oxidized, or methemoglobin, is incapable of binding O_2, reducing the O_2 carrying capacity of blood in proportion to the amount formed. Extensive oxidation results in generalized tissue hypoxia and may be fatal (Kiese, 1974). $NaNO_2$ is able to oxidize hemoglobin directly. The mechanism of this reaction is incompletely understood but involves the formation of hydrogen peroxide, itself an oxidizing species (Kosaka and Uozumi, 1986; Spagnuolo et al., 1987). Aniline is metabolized to the oxidizing form, phenylhydroxylamine (PHA; Kiese, 1974; Harrison and Jollow, 1987). Other compounds capable of causing methemoglobinemia include hydroxylamine, nitrobenzene, and various quinones and dyes. The causes, consequences, and treatment of methemoglobinemia are reviewed by Kiese (1974).

Heme oxidants, as a group, are chemically reactive compounds and can be expected to have acute toxic effects apart from oxidation of hemoglobin. Two observations suggest that additional toxicities do occur. First, dogs (Vandenbelt et al., 1944) and mice (Vacek and Sugahara, 1967) survive methemoglobin levels to 80% caused by intraperitoneal (i.p.) injection of p-aminopropriophenone, while in

rats (Lester et al., 1944) lethal i.p. doses of p-aminophenol or PHA increase methemoglobin content to only 30 and 65%, respectively. Second, aniline is toxic to animals (mice, rabbits) that form very little of the oxidizing metabolite, PHA (Kiese, 1974).

We found that $NaNO_2$ and hydroxylamine oxidize cardiac myoglobin in buffalo sculpin (Enophrys bison) when injected i.p. (Chapter II). Myoglobin is a monomeric heme protein, which in the ferrous form, binds O_2 with high affinity facilitating its diffusion in model systems (Wittenberg, 1970). Myoglobin is present in the red skeletal and cardiac muscle of vertebrates and has long been thought to contribute to the oxygenation of these tissues (Millikan, 1939). The importance of functional myoglobin in skeletal muscle was demonstrated by selectively oxidizing the protein, resulting in decreased O_2 consumption (Wittenberg et al., 1975) and reduced isometric tension generation (Cole, 1982). The role of myoglobin in mammalian cardiac muscle is not as well established. Taylor et al. (1986) used phosphorous nuclear magnetic resonance spectroscopy to measure high energy phosphate levels in isolated hearts treated with $NaNO_2$. ATP and phosphocreatine levels were unaffected by inactivation of myoglobin under normoxic conditions but declined more rapidly than control levels when myoglobin was oxidized during hypoxia. However, the mechanical performance of fluorocarbon-perfused dog hearts was unaffected by oxidation of myoglobin during hypoxia (Cole et al., 1978).

Some of the best evidence for a physiological role for myoglobin in cardiac muscle has been obtained in studies of isolated

fish hearts. Using fish hearts with and without myoglobin, Driedzic et al. (1982) evaluated cardiac performance at high (150 torr) and low (38 torr) O_2 tensions in the presence and absence of hydroxylamine. The performance of myoglobin-containing sea raven (Hemitripterus americanus) hearts was reduced by treatment with hydroxylamine at low O_2 tensions, while performance of myoglobinless ocean pout (Macrozoarces americanus) hearts did not change. In a later study, O_2 consumption by sea raven hearts was reduced at low O_2 tensions and elevated afterloads by treatment with hydroxylamine, while O_2 consumption by ocean pout hearts was unaffected (Bailey and Driedzic, 1986).

The purpose of our study was to use isolated fish hearts to investigate the cardiac toxicity of $NaNO_2$, aniline, and PHA. Recognizing that few toxicants have only one action, we sought ways to distinguish between the effects of myoglobin oxidation and other cardiac toxicities. Finally, we reexamined the question of myoglobin's role in cardiac muscle by exposing hearts to PHA at a physiological level of O_2 tension (32 torr). Cardiac performance and O_2 consumption were unaffected by treatment with PHA, despite greater than 95% oxidation of myoglobin.

MATERIALS AND METHODS

Experimental Animals

Buffalo sculpin weighing 350 to 900 g were caught with an otter trawl in Yaquina Bay, Newport, Oregon, and maintained in aerated, flowing seawater, at 12 ± 2 C°. Fish held for more than one week were fed a gelatin-based synthetic diet (Choromanski, 1985).

Isolated Heart Preparation

Sculpin hearts were isolated and perfused as described by Stuart et al. (1983), with minor modifications. A sculpin was killed by a blow to the head and its heart, including the bulbous arteriosus and a portion of the sinus venosus, was excised and placed in ice-cold physiological saline. The heart was then flushed briefly with additional ice-cold saline to remove blood from the lumen and mounted on a modified Langendorff perfusion apparatus. Preload and afterload pressures were adopted from Driedzic (1983). Afterload was fixed at 15 cm H_2O by the height of the postperfusion column and was not changed. Preload was initially set at 1.27 cm H_2O and could be adjusted as required by the experimental protocol. Control and treatment perfusates were equilibrated with room air, unless otherwise indicated. The heart and perfusing solutions were maintained at 12 ± 1 C°.

Hearts were allowed to equilibrate for 30 min after mounting and were discarded if they failed to develop an intrinsic rhythm, usually 35-40 beats/min. Electrical pacing, when required, was

begun after the equilibration period with a Grass model SD9 stimulator (Grass Instruments Inc., Quincy, MA). Duration was set to 20 ms and voltage adjusted as necessary to entrain the heart.

Pressure was monitored continuously from the bulbous arteriosus with a Stratham P23 ID pressure transducer (Stratham Instruments Co., Hato Rey, P.R.) connected to a Gould 11-4307-04 transducer amplifier (Gould, Inc., Cleveland, OH). The pressure signal was split and one-half fed to a resistance-capacitance differentiating circuit (modified from Carr, 1978) to give the rate of change of pressure (dP/dt). The maximum rate of change of pressure (peak dP/dt) associated with the rising phase of the pressure pulse was used as an index of myocardial contractility (Mason, 1969). A complete description of the differentiating circuit, including experiments designed to validate its use, is given in Appendix A.I. The pressure signal and its first derivative were recorded with a Clevite Brush Mark 220 chart recorder (Brush Instruments Division of Gould, Inc., Cleveland, OH). Cardiac output was determined by collecting saline exhausted from the postperfusion column.

Cardiac Oxygen Consumption

Cardiac O_2 consumption was estimated from cardiac output and the arterial to venous difference in O_2 content. Perfusate samples (0.5 ml) were obtained anaerobically from ports on both sides of the heart and injected into a Radiometer type D616 thermostatted cell (Radiometer Copenhagen, Copenhagen, Denmark) fitted with a Radiometer O_2 electrode (type E5046). The cell and electrode were

maintained at 12 C° by a circulating water bath. The electrode was zeroed before each experiment with alkaline sulphite solution and the span set using physiological saline equilibrated with air at 12 C°. Subsequent calibration was accomplished with humidified air, after taking into account the air/water correction factor. Calibration was performed before and after each sample determination and the sample data were discarded if bracketing calibration values differed by more than 2 torr. Samples were equilibrated in the cell for 3 min and O_2 tensions (PO_2) read directly from a Radiometer PHM 73 blood gas monitor. O_2 content was determined from PO_2 using the solubility constant, 1.89 x 10-6 M O_2/L/torr at 12 C° (Boutilier et al., 1984).

Venous Blood Gas and Acid-Base Status

Sculpin were cannulated from the sinus venosus to determine resting levels of PO_2, total O_2 content (TO_2), total CO_2 content (TCO_2), and pH (Table III.1). The cannulation method and analytical procedures are described in detail in Appendix A.II. Briefly, a fish was anesthetized and placed ventral side up on a fish operating table and the abdominal cavity was opened to expose the liver and hepatic veins. The largest vein was tied off at the liver, retracted and opened, and a 40 cm length of PE 60 cannulae was inserted and tied in place. The fish was then closed and allowed to recover for 20-24 h before sampling. The mean O_2 tension in venous blood from cannulated sculpin (32.2 torr) was subsequently adopted as the "physiological" value for studies of myoglobin

function in the isolated perfused heart. "Ambient" PO_2 was approximately 155 torr.

Toxicants and Perfusing Solutions

Hearts were perfused with physiological saline (350 mOsm, pH 7.83 at 12 C°) containing (in mM): 124 NaCl, 5.1 KCl, 1.6 $CaCl_2.2H_2O$, 11.9 $NaCO_3$, 0.9 $MgSO_4.7H_2O$, 5.5 glucose, 6.6 Trizma base, and 43.4 Trizma HCl. Toxicants were added to the perfusion media immediately before treatment and the pH adjusted if necessary to 7.83 at 12 C°. PHA was synthesized from nitrobenzene by Dr. Robert Bodysky of the Medical University of South Carolina using the method of Kamm (1951). Purity was confirmed by the appearence of a single peak using high pressure liquid chromatography (HPLC) and electrochemical detection. The product was shipped on dry ice and stored under N_2 atmosphere at 0 C°. Reagent grade aniline and $NaNO_2$ were purchased from Sigma Chemical Company (St. Louis, MO).

Experimental Protocols

Fish were randomly divided into four experimental groups: 1) dose-response, ambient PO_2, 2) sham treatment, ambient PO_2, 3) sham treatment, physiological PO_2, and 4) PHA treatment, physiological PO_2. Group 1 hearts were allowed to equilibrate for 30 min before treating for 20 min with PHA, aniline, or $NaNO_2$. Heart rate, pulse pressure and peak dP/dt were monitored continuously. At the end of each experiment the heart was dismounted, weighed and assayed for metmyoglobin content (Chapter II). Attempts were made to pace a heart only if it arrested or slowed to the point that arrest

appeared imminent. Pacing was then initiated at the pretreatment rate and maintained for the duration of the experiment. On the basis of these experiments, it was determined that 1.0 x 10-5 M PHA oxidized greater than 95% of the myoglobin present but did not affect pulse pressure, peak dP/dt or heart rate.

The testing protocol for groups 2-4 is shown in Figure III.1. Electrical pacing at 42 beats/min was begun at the end of the 30 min equilibration period and maintained for the remainder of the experiment. Each heart was taken through the 40 min test protocol twice, allowing 20 min to elapse between the end of the first testing period and the beginning of the second. Group 3 and 4 control and treatment (or sham treatment) perfusates were gassed with O_2 and N_2 to result in a PO_2 of approximately 32 torr. Group 4 hearts were perfused with 1.0 x 10-5 M PHA from the end of the first testing period to the end of the experiment, at which time they were dismounted, weighed and assayed for metmyoglobin content.

Data Analysis

Power output was calculated using the formula (Bailey and Driedzic, 1986):

$$\text{Power (mWatts/g)} = [Q] \times \frac{[((\text{pulse pressure} \times .33) + \text{afterload}), - \text{preload}] \times [980 \times 10\text{-}4]}{\text{heart weight (g)}}$$

where Q = cardiac output in ml/s. Pre- and posttreatment (or sham treatment) means were compared within groups using a paired sample t test. Differences of means between groups were analyzed using the Student's t test. A P value of < 0.05 was considered significant.

RESULTS

Dose-Response (Group 1)

PHA had dose-dependent effects on both myoglobin oxidation state and cardiac performance (Figure III.2). The EC50 for oxidation of myoglobin was approximately 1.0 x 10-6 M, while that for toxicity, appearing as a steady decline in pulse pressure, was 2.5 x 10-4 M. In Figure III.2 it can be seen that 1.0 x 10-5 M PHA oxidized greater than 95% of the myoglobin present but did not reduce pulse pressure. This observation suggested that functional myoglobin was not necessary to maintain cardiac performance under these conditions. PHA had no effect on heart rate at any concentration tested.

Dose-reponse curves for myoglobin oxidation and toxicity (defined as for PHA as a reduction in pulse pressure) by $NaNO_2$ appeared to overlap (Figure III.3). Moreover, the effect of $NaNO_2$ on pulse pressure differed from that of PHA, appearing at all but the highest concentration (1.0 x 10-1 M) as a transient reduction, followed by partial recovery. The EC50 for oxidation was 6.3 x 10-3 M, while that for toxicity was 2.5 x 10-2 M. Independently, these data suggested a relationship between myoglobin oxidation and toxicity. But in view of the results obtained with PHA, we questioned this association, noting instead that high concentrations of $NaNO_2$ were required to elicit either oxidation or toxicity. We speculated that the reduction in pulse pressure was due to the osmotic and ionic effects of $NaNO_2$ addition and performed two

experiments to test this possibility. In the first experiment, 1.0, 3.16 and 10.0 x 10-2 M $NaNO_2$ were substituted for like amounts of NaCl in the perfusion media. In so doing, however, the concentration of Cl^- ions was reduced. To control for this effect a second experiment was performed in which 1.0 x 10-1 M sodium glutamate was substituted for 1.0 x 10-1 M NaCl in the perfusion media. Assuming glutamate to be nontoxic, we reasoned that the toxicity of NO_2^- could be estimated by subtracting the effect of sodium glutamate from that of $NaNO_2$. The results of these studies are shown in Figure III.4. Substituted for NaCl, $NaNO_2$ oxidized myoglobin approximately as it had when added to the perfusion media, but its effect on pulse pressure was diminished. Moreover, 1.0 x 10-1 M sodium glutamate reduced pulse pressure to nearly the same extent as 1.0 x 10-1 M $NaNO_2$. Subtracting one effect from the other, it is clear that NO_2^-, per se, had little effect on cardiac performance, even at the highest concentration tested.

Aniline did not oxidize myoglobin but was acutely toxic to the isolated heart at high concentrations (Table III.2). Toxicity appeared as a rapid, simultaneous decline in heart rate, pulse pressure and peak dP/dt. Cardiac arrest often occurred and was usually preceeded by atrioventricular (AV) block. Dose-response relationships were difficult to determine but appeared to be steep. Cardiac rhythym was abolished in seconds in 3 of 3 hearts perfused with 5.6 x 10-3 M aniline and could be reestablished by electrical pacing in only one of these. In contrast, 2 of 3 hearts exposed to 1.78 x 10-3 M aniline arrested, but both were easily paced. Electrically paced performance, although depressed relative

to pretreatment values, did not appear to be dose-dependent. Aniline had no apparent effect on cardiac performance at concentrations less than 1.78 x 10-3 M.

Sham Treatment (Groups 2 and 3)

The isolated sculpin heart preparation was stable at both ambient (155 torr) and physiological (32 torr) O_2 tensions for the duration of the study period (100 min). Cardiac output, power output, and peak dP/dt tended to decrease with time, but the changes were not significant (Figures III.5 and 6, and Table III.3). There were no significant differences in normalized O_2 consumption within or between groups 2 and 3 (Table III.3).

PHA Treatment (Group 4)

Treatment with PHA had no apparent effect on the performance of isolated perfused sculpin hearts despite the fact that myoglobin was greater than 95% oxidized (Figure III.7 and Table III.3). Cardiac output, power output, and peak dP/dt tended to decrease in time, but no more so than with either sham treatment group. Weight normalized O_2 consumption was similarly unaffected by exposure to PHA (Table III.3).

DISCUSSION

Isolated Heart Preparation

The sculpin heart model offers several advantages for studies in cardiovascular toxicology. Because it does not possess coronary arteries, it can be easily perfused and made to work in a physiologically relevant manner. The absence of coronaries also eliminates the possibility of vascular effects on cardiac performance. The preparation is stable for a period of two hours or more and remains viable when perfused at physiological O_2 tensions.

Fish hearts, like mammalian hearts, rely almost exclusively on oxidative metabolism to meet their cardiac requirement for ATP (Driedzic, 1983). The sculpin heart ventricle is well supplied with myoglobin (mean $\pm$ SD = 1.45 $\pm$ .17 mg/g wet weight, N = 5, using the method of deDuve, 1948), although the O_2 affinity of sculpin myoglobin (P50 = 1.10 torr at 20 C°, pH 7.8) is somewhat lower than that of mammalian myoglobins (P50 = 0.4 - 0.8 torr at 20 C°; Chapter I). Preload and afterload were modeled after levels used by Driedzic (1983). Increases in preload were accompanied by increases in pulse pressure and cardiac output in accord with Starling's law of the heart.

Dose-Response Studies (Group 1)

Dose-response studies with PHA and sodium $NaNO_2$ were conducted as a means of distinguishing between the acute effects of these

compounds on myoglobin oxidation state and cardiac performance. The contribution of myoglobin to cardiac performance was minimized by maintaining O_2 tension at the ambient level (155 torr). Aniline was not expected to oxidize myoglobin but was included in these studies because it was reported to have cardiac effects in vivo (Clark et al., 1943).

$NaNO_2$, although well known for its ability of oxidize hemoglobin, exhibited little activity toward myoglobin in the isolated heart. To correctly interpret these data it is necessary to consider both the kinetics of oxidation by NO_2^- and the duration of the exposure period. The reaction of NO_2^- with oxyhemoglobin is characterized by a lag phase followed by an autocatalysis (Rodkey, 1976). PHA rapidly oxidizes hemoglobin when injected into mice, the peak effect occuring within 20 min (Smith et al., 1967). $NaNO_2$, by comparison, oxidizes hemoglobin slowly (time to peak effect approximately 1 h), but the effect is more prolonged (Smith and Layne, 1969). Judging from the color change in treated hearts (red turning to pale brown as myoglobin oxidizes), we conclude that these generalizations also apply to the effects of PHA and $NaNO_2$ on myoglobin. In a continuous exposure the percentage of metmyoglobin at any one time is determined by the balance between chemical oxidation and reduction by endogenous metmyoglobin reductase (Hagler et al., 1979). The duration of the exposure period dictates the extent to which the system approaches steady state. The exposure period was limited in this study to 20 min to provide sufficient time for physiological testing before and afterward (Groups 2-4).

Given more time, it is possible that low concentrations of $NaNO_2$ would oxidize a significant amount of the myoglobin present. However, this remains to be verified.

The utility of the dose-response approach becomes apparent when the results obtained for PHA are compared to those for $NaNO_2$. Dose-response curves for myoglobin oxidation and cardiac toxicity by PHA were separated by a wide margin suggesting that functional myoglobin was not required to maintain cardiac performance. We therefore questioned the apparent relationship between oxidation and toxicity found with $NaNO_2$, and later determined that most of the "toxicity" was due to the ionic and osmotic effects of $NaNO_2$ addition. $NaNO_2$ was subsequently dropped from consideration for use in myoglobin inactivation studies (Groups 2-4).

Aniline had marked effects on cardiac performance when present at high concentrations. The frequent appearance of AV block and rapid cardiac arrest were indicative of an electrically-based effect, although paced hearts also exhibited diminished levels of contractile performance. Clark et al. (1943) found that aniline caused significant electrocardiographic changes when infused intravenously into dogs, the most consistent of which was a reduction in the height of the R wave. In a more recent and possibly related study, aniline increased the amplitude of the endplate potential at the frog neuromuscular junction (Enomoto and Maeno, 1985). These authors suggested that the site of action of aniline was the same as that of 4-aminopyridine, namely the voltage dependent potassium channel on the presynaptic terminal. It would

be of interest to investigate the cardiac effects of aniline in a system which is more amenable to electrical recording, such as isolated heart cells.

It is interesting to note that the profile of cardiac toxicity for aniline bears no resemblance to that for PHA. Aniline is encountered in large quantities in a variety of occupational settings. Many mammals, including man, are capable of metabolizing aniline to PHA, which has in turn been implicated as the primary cause of aniline-induced hemolytic anemia (Harrison and Jollow, 1986) and methemoglobinemia (Kiese, 1974; Harrison and Jollow, 1987). The results of our study suggest that in cases of aniline intoxication there is a potential for multiple cardiovascular effects involving independent actions of aniline and PHA on the heart and blood.

Myoglobin Inactivation (Group 2-4)

Treatment with PHA had no effect on cardiac performance or O_2 consumption, despite oxidizing more than 95% of the myoglobin present. We therefore conclude that functional myoglobin was not required to maintain cardiac function under the conditions of this study. At first glance, this outcome appears similar to that observed for 1.0 x 10-5 M PHA in dose-reponse studies (Group 1). However, the conditions employed in myoglobin inactiviation studies (including lower O_2 tensions, longer exposure periods and changes in preload), constituted a much stronger test of the importance of functional myoglobin.

Cardiac function depends upon a balance of O_2 supply and demand. Assuming that the supply of O_2 to cardiac muscle is limited to diffusive and myoglobin-facilitated flux, the results of this study suggest that diffusive flux alone was sufficient to satisfy cardiac O_2 demand. The theory of myoglobin-facilitated oxygen transport predicts that as extracellular PO_2 decreases the relative contribution of myoglobin-facilitated flux to total O_2 flux increases (Wittenberg, 1970). PO_2 is therefore critical to the outcome of any study of myoglobin function. The O_2 tension (32 torr) used in this study was modeled after values in venous blood from resting sculpin may be higher than levels commonly occuring in sculpin in natural settings. O_2 tensions in the venous return of exercising fish, particularly those in otherwise sedentary fish during "burst" swimming activity, are poorly known, as are O_2 tensions when ambient O_2 levels are low. It is possible that the O_2 tension employed in myoglobin inactivation studies was not low enough to demonstrate the physiological role of myoglobin in the sculpin heart.

As indicated earlier, some of the strongest evidence for a physiological role for myoglobin in cardiac muscle has been obtained in studies of isolated sea raven and ocean pout hearts (Driedzic et al., 1982; Bailey and Driedzic, 1986). We do not know the origin of the discrepency between these and the present study but suggest that it is not due to differences in O_2 supply or demand. Sculpin hearts

(preload = 1.27 cm H2O, afterload = 15 cm H2O)) developed power output levels equal to those of isolated sea raven or ocean pout hearts (0.5-0.7 mW/g at an afterload of 15 cm H2O, preload not given; Bailey and Driedzic, 1986), while operating at lower O_2 tensions (32 as compared to 38 torr). We question, however, Bailey and Driedzic's (1986) use of increased afterload as a means of increasing cardiac O_2 demand. Cardiac output and pulse pressure vary in a working heart as complex functions of preload and afterload. Generally, as preload increases cardiac output and pulse pressure increase also (Starling's law of the heart) resulting in an increase in power output, hence O_2 demand. The effect of increasing afterload is more difficult to predict because increases in pressure tend to be offset by decreases in cardiac output, resulting in little or no change in power output. Power output by the sculpin heart doubled (to 1.0-1.3 mW/g) when preload was increased to from 1.27 to 2.54 cm H_2O. In contrast, power outputs by sea raven and ocean pout hearts were unchanged or declined when afterload was increased from 15 to 25 cm H_2O (Bailey and Driedzic, 1986).

Blood pressure in the ventral aorta of fish in vivo is probably higher than the afterloads employed in this study (Farrell (1984) reports a range of from 30 - 50 cm H2O), but it is important to remember that the heart is supported in vivo by a relatively rigid pericardial cavity and is working against a compliant vascular system. Isolated sculpin hearts become visibly distended at afterloads in excess of 20 cm H_2O, apparently due to failure of the

aortic valve. The result is a marked decrease in cardiac performance (R.E. Stuart, personal communication).

In conclusion, we employed a non-vascularized fish heart model to investigate the cardiac toxicity of $NaNO_2$, aniline, and the aniline metabolite, PHA. A dose-response approach was used to distinguish between effects on myoglobin oxidation state and other cardiac toxicities. This approach was well suited for use with PHA, which oxidized myoglobin rapidly. $NaNO_2$, in contrast, oxidized myoglobin slowly, and high concentrations were required to elicit a measurable effect. Toxicity appearing at these high concentrations was due to the ionic and osmotic effects of $NaNO_2$ addition and not to oxidation of myoglobin or to the toxicity of NO_2^-. Isolated hearts treated with aniline arrested and exhibited other signs of electrical and contractile failure.

The importance of functional myoglobin in cardiac muscle was tested by treating hearts with 1 x 10-5 M PHA. Cardiac performance and O_2 consumption were unaffected by PHA despite greater that 95% oxidation of myoglobin. The toxicological significance of this result is not clear. Studies with sculpin suggest that myoglobin is oxidized <u>in vivo</u> by compounds that cause methemoglobinemia (Chapter II). Although not demonstrated by this study, we believe that myoglobin plays an important role in oxygen transport and that oxidation <u>in vivo</u> would be detrimental. Lower O_2 tensions may be required to illustrate the role of myoglobin in the isolated perfused sculpin heart.

Table III.1. Blood gas and acid-base status of the venous return in unanesthetized, unrestrained buffalo sculpin.

Parameter	Fish sampled	N	Mean ± SD
PO_2 (torr)	3	8	32.2 ± 6.4
TO_2 (ml %)	4	11	2.3 ± 0.6
Hematocrit (%)	4	11	21 ± 4.0
pH	5	17	7.83 ± 0.04
TCO_2 (mM/L)	5	16	4.30 ± 0.8
PCO_2 (torr)*	5	16	1.5 ± 0.3

* Calculated from pH and TCO_2 (see Appendix A.II).

Table III.2. Aniline data summary. Pulse pressure and peak dP/dt are expressed as the mean of the corresponding number of observations.

Treatment (mM/L)	Arrested	Responsive to stimulation	Paced performance (10 min post tx; % of control)		
			N	Pulse pressure	dP/dt
10.0	1/1	0/1			
5.62	3/3	1/3	1	65	70
3.16	2/5	4/5	4	74	76
1.78	2/3	2/2	2	64	76

Table III.3. Changes in peak dP/dt and oxygen consumption during myoglobin inactivation studies. Peak dP/dt was measured just prior to increasing preload from 1.27 to 1.9 cm H_2O. Values are means ± SD, N = 3 (peak dP/dt) or 6 (O_2 consumption).

Treatment group and testing period	Peak dP/dt (cm H2O/s)	Oxygen consumption (M x 10-6/min/g wet wt)
Group 2		
Control	9.2 ± 1.8	.29 ± .04
Sham treatment	8.6 ± 2.0	.30 ± .06
Group 3		
Control	9.0 ± 0.5	.29 ± .09
Sham treatment	7.6 ± 0.8	.34 ± .06
Group 4		
Control	11.0 ± 0.0	.24 ± .03
PHA treatment	9.2 ± 1.4	.24 ± .04

Figure III.1. Testing protocol for groups 2-4. Perfusion pressure (preload) was stepped up and then down. Arrows indicate times at which O_2 consumption was determined. Boxes indicate periods during which cardiac output was measured. Pulse pressure and peak dP/dt were monitored continuously.

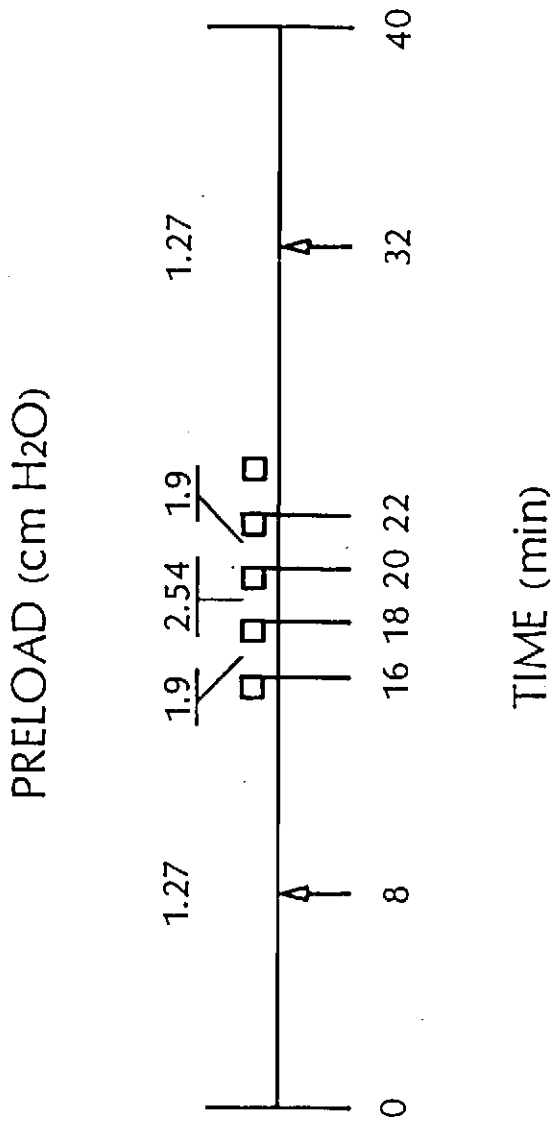

Figure III.1.

Figure III.2. Dose-reponse curves for myoglobin oxidation and pulse pressure reduction by PHA. Individual metmyoglobin and pulse pressure reduction values are represented by dots and open circles, respectively.

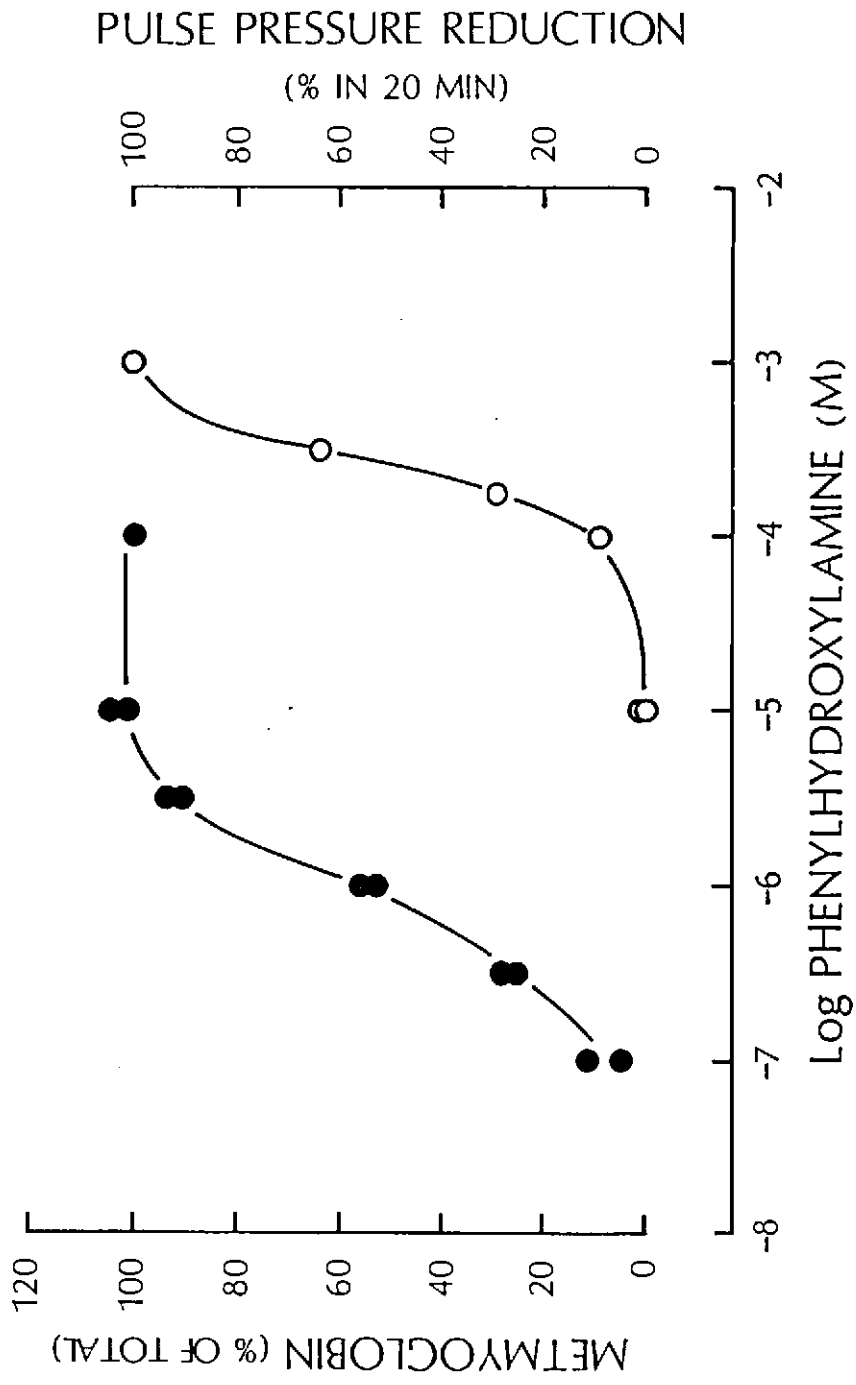
PULSE PRESSURE REDUCTION
(% IN 20 MIN)
100
80
60
40
20
0
-2
-3
-4
-5
-6
-7
-8
Log PHENYLHYDROXYLAMINE (M)
120
100
80
60
40
20
0
METMYOGLOBIN (% OF TOTAL)

Figure III.2.

Figure III.3. Dose-response curves for myoglobin oxidation and pulse pressure reduction by $NaNO_2$, when $NaNO_2$ was added to the perfusion media. Individual metmyoglobin and pulse pressure reduction values are represented by dots and open circles, respectively.

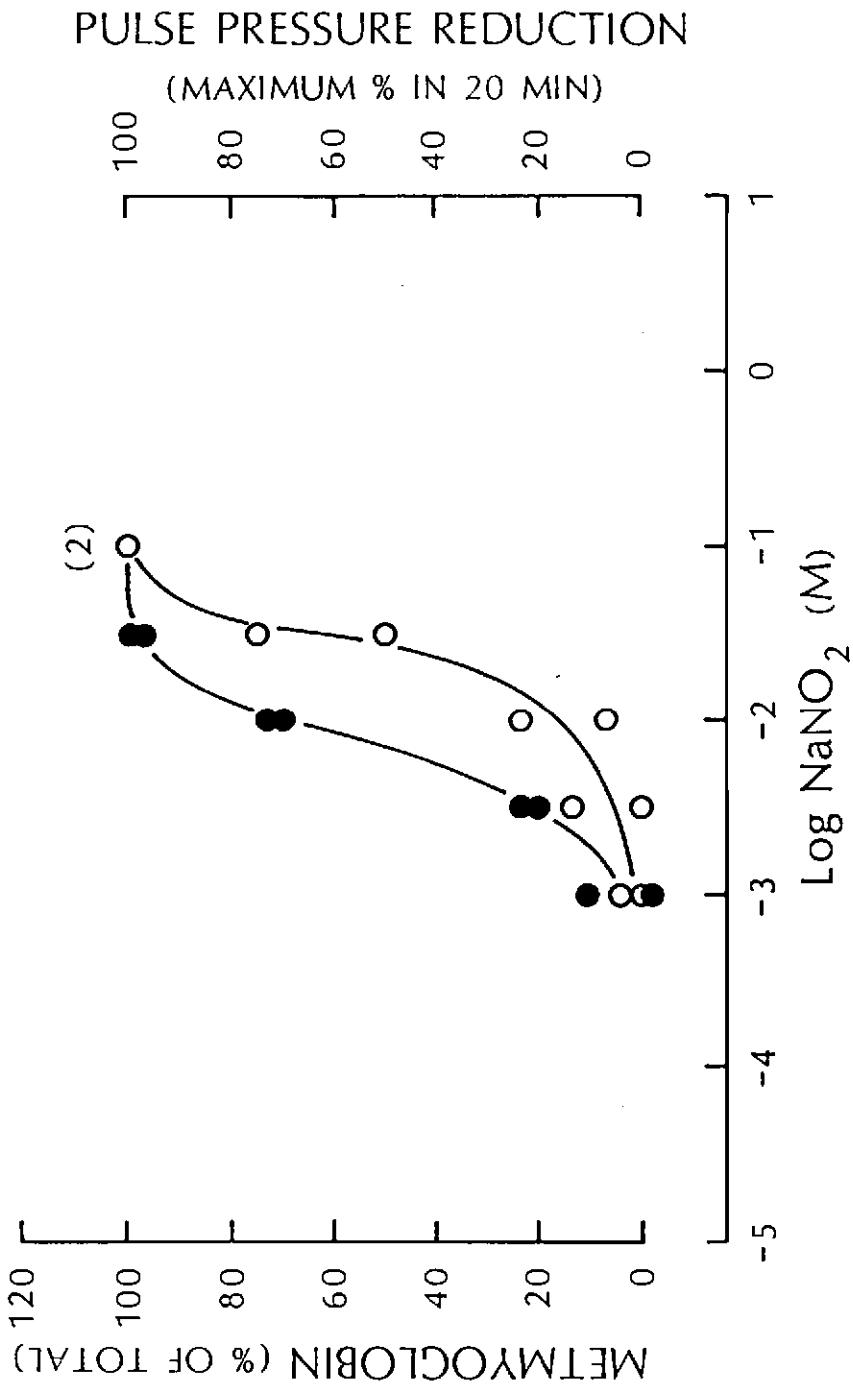
PULSE PRESSURE REDUCTION
(MAXIMUM % IN 20 MIN)
100
80
60
40
20
0
(2)
1
0
-1
-2
-3
-4
-5
Log $NaNO_2$ (M)
METMYOGLOBIN (% OF TOTAL)
120
100
80
60
40
20
0

Figure III.3.

Figure III.4. Myoglobin oxidation and reduction in pulse pressure by $NaNO_2$, when $NaNO_2$ was substituted for NaCl in the perfusion media. Dose-response curves from Figure III.3 (solid lines) have been redrawn for comparison. Individual values for metmyoglobin and pulse pressure reduction are represented by dots and open circles, respectively. Pulse pressure reduction values are joined by a dashed line. The effect of sodium glutamate on pulse pressure is indicated by solid squares.

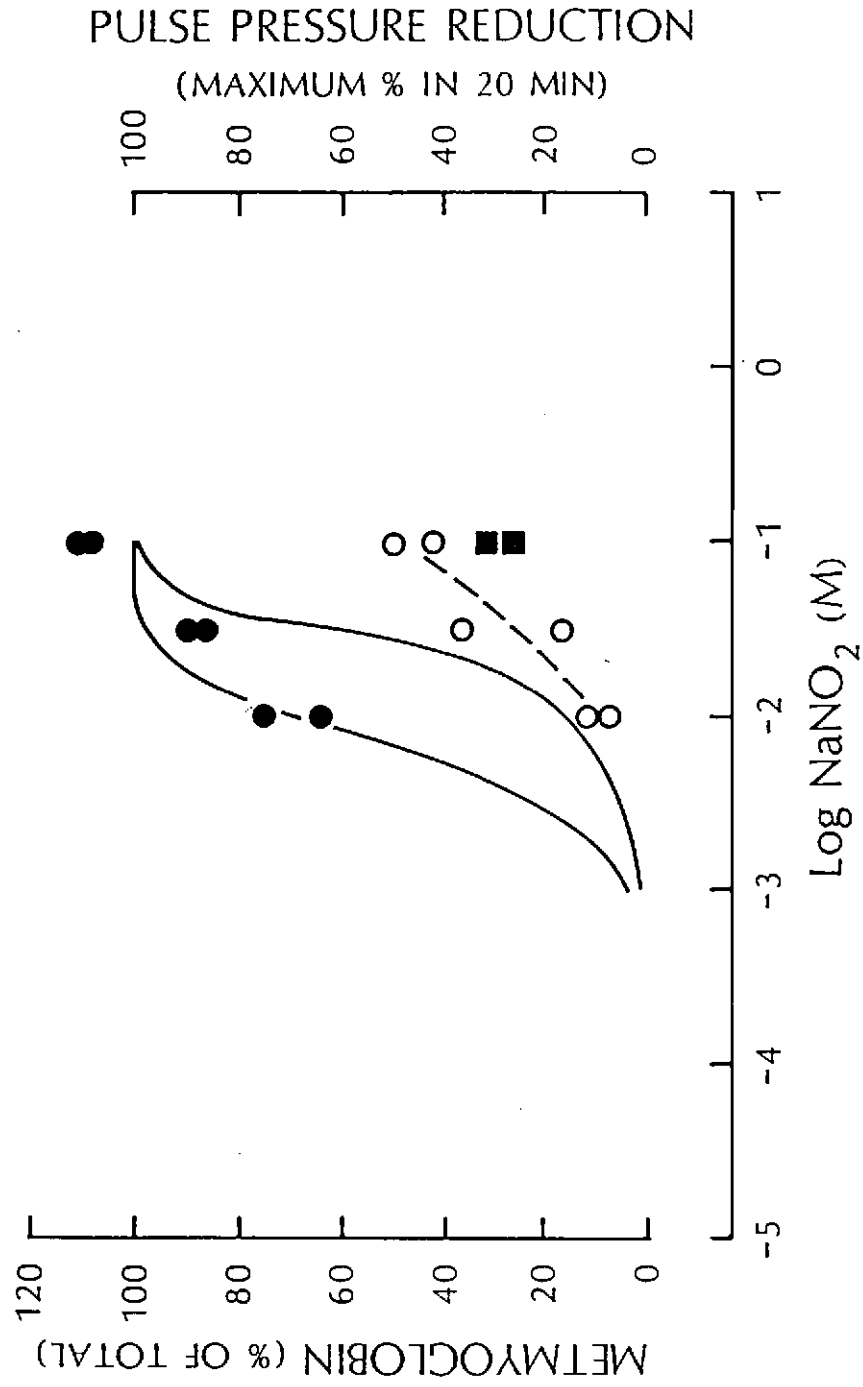
PULSE PRESSURE REDUCTION
(MAXIMUM % IN 20 MIN)
100
80
60
40
20
0
1
0
-1
-2
-3
-4
-5
Log $NaNO_2$ (M)
120
100
80
60
40
20
0
METMYOGLOBIN (% OF TOTAL)

Figure III.4.

Figure III.5. Effect of changes in preload on cardiac output and power output at ambient O_2 tensions (150 torr). Data from the first and second testing periods are represented by dots and open circles, respectively. Values are means $\pm$ SD, N = 3.

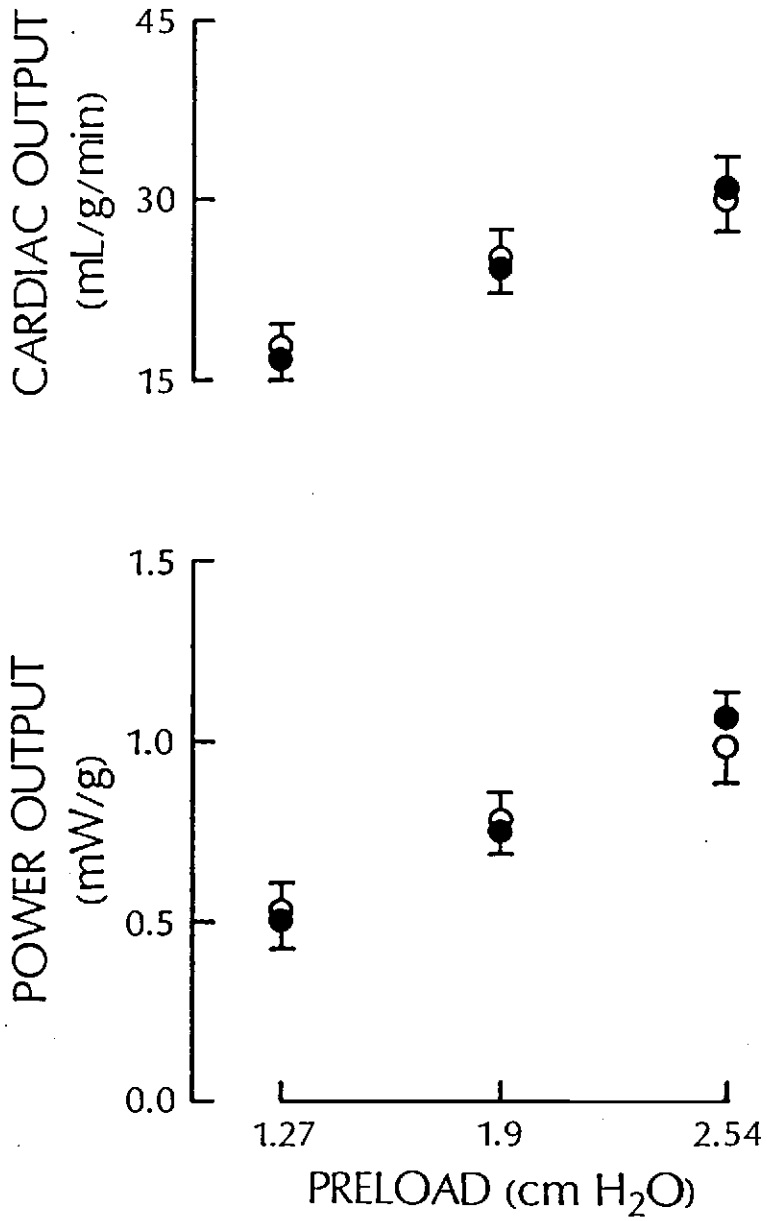
CARDIAC OUTPUT
(mL/g/min)
45
30
15
POWER OUTPUT
(mW/g)
1.5
1.0
0.5
0.0
1.27
1.9
2.54
PRELOAD (cm H_2O)

Figure III.5.

Figure III.6. Effect of changes in preload on cardiac output and power output at physiological O_2 tensions (32 torr). Data from the first and second testing periods are represented by dots and open circles, respectively. Values are means $\pm$ SD, N = 3.

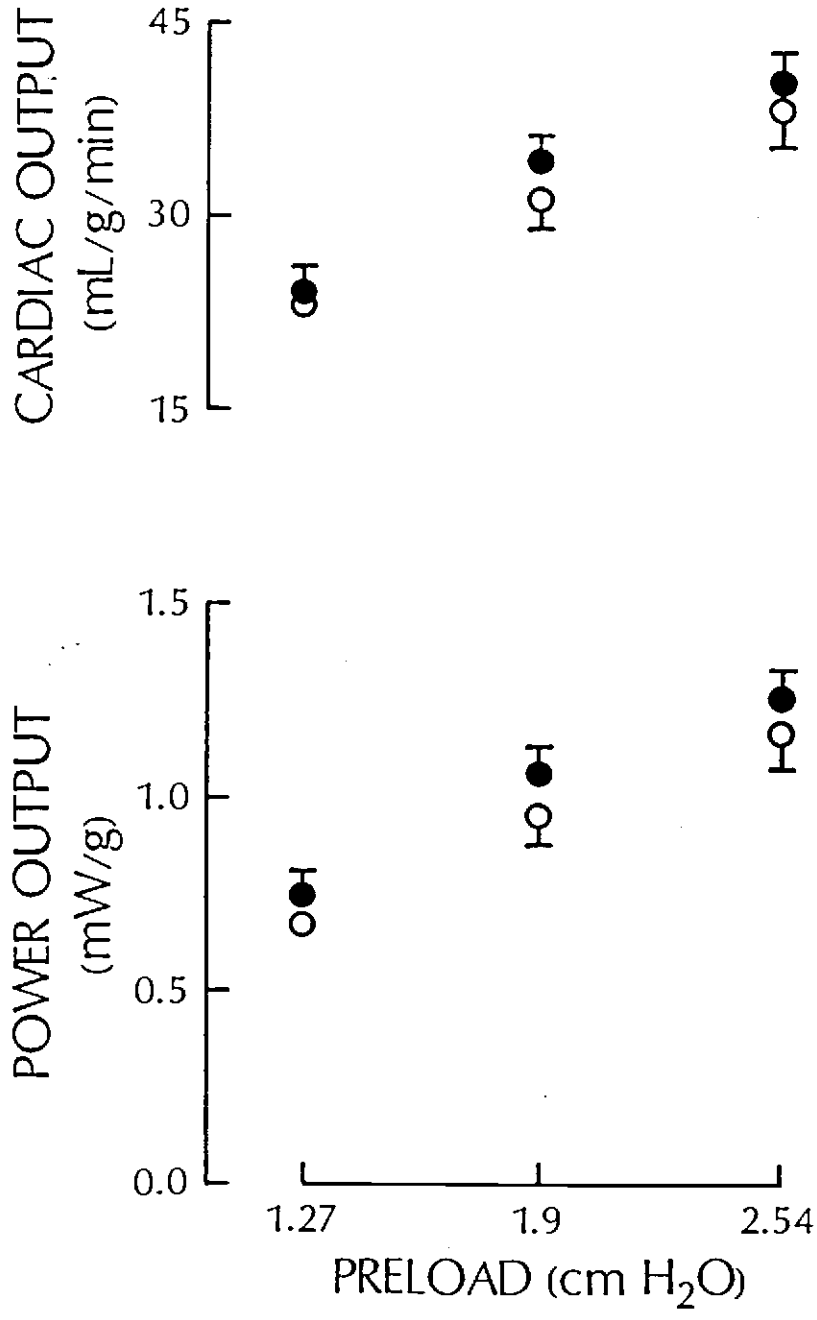
CARDIAC OUTPUT
(mL/g/min)
45
30
15
POWER OUTPUT
(mW/g)
1.5
1.0
0.5
0.0
1.27
1.9
2.54
PRELOAD (cm H_2O)

Figure III.6.

Figure III.7. Effect of changes in preload on cardiac output and power output at physiological O_2 tensions (32 torr) before and after the addition of PHA. Hearts were perfused with 1.0 x 10-5 M PHA from the end of the first test period to the end of the experiment. Data from the first and second testing periods are represented by dots and open circles, respectively. Values are means $\pm$ SD, N = 3.

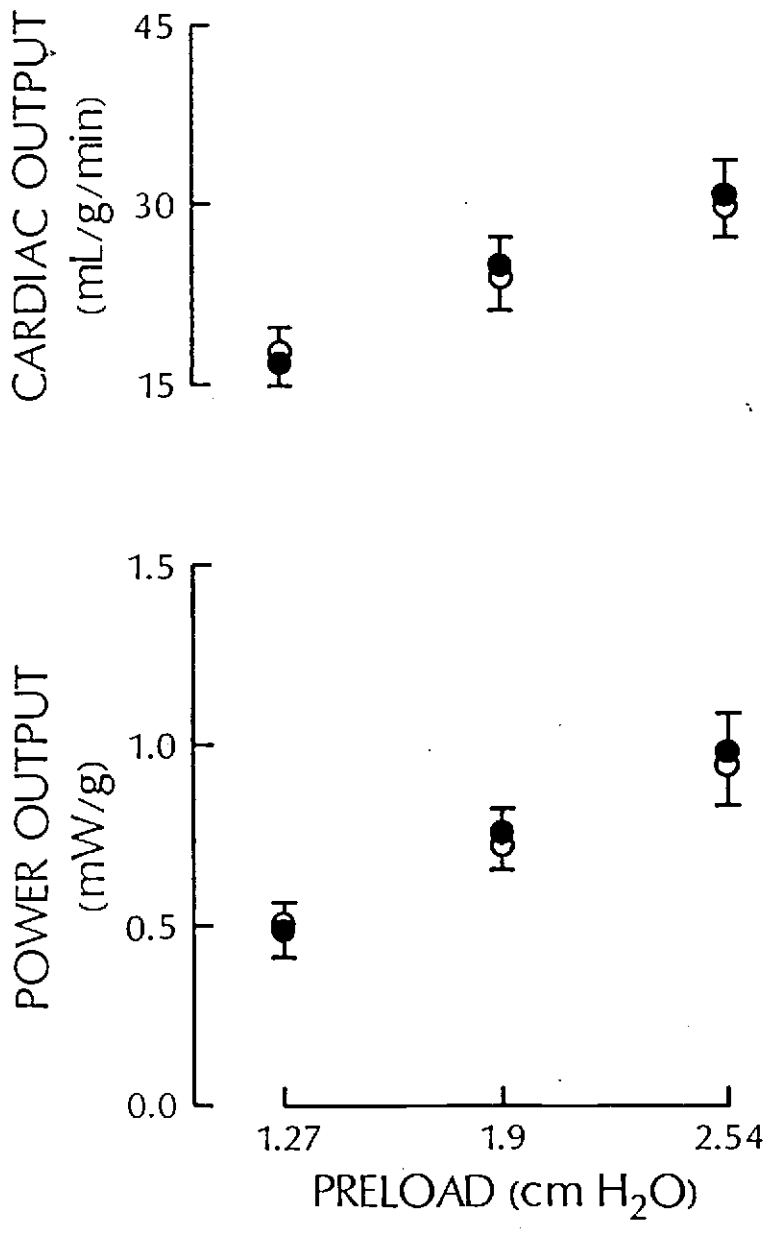
CARDIAC OUTPUT
(mL/g/min)
45
30
15
POWER OUTPUT
(mW/g)
1.5
1.0
0.5
0.0
1.27
1.9
2.54
PRELOAD (cm H_2O)

Figure III.7.

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SUMMARY AND CONCLUSIONS

The objective of my research was to investigate the cardiac toxicity of compounds that cause methemoglobinemia by using a non-vascularized sculpin heart model Anticipating that these compounds would oxidize cardiac myoglobin, I first characterized the O_2 binding affinity of sculpin myoglobin and compared it to that of mammalian myoglobins. The affinity of sculpin myoglobin for O_2 at 20 C° was lower than that of sperm whale or rat myoglobin. This difference probably reflects an adaptation to temperature, permitting sculpin myoglobin to function as an O_2 transport molecule at physiological temperatures (12 C°).

Sculpin myoglobin bound O_2 with higher affinity than mammalian myoglobins when O_2 binding experiments were performed at physiological temperatures. Assuming that the function of myoglobin in muscle is similar to that of the isolated protein (Tamura et al., 1978; Wittenberg and Wittenberg, 1985), one may expect that this difference also exists *in vivo*. Thus, the question arises, can sculpin myoglobin be utilized to model the function of vertebrate myoglobins generally? I believe that it can. Wittenberg (1970) showed that myoglobin can facilitate O_2 diffusion in model systems *in vitro* and defined three criteria for the existence of this phenomena *in vivo*. Briefly, they are: 1) diffusion of myoglobin within the cytoplasm, 2) an O_2 gradient from the blood to the mitochondria, and 3) a gradient of myoglobin fractional saturation from the sarcolemma to the mitochondria. Myoglobin fractional

saturation depends in turn upon O_2 affinity and partial pressure. Myoglobin O_2 affinity and physiological O_2 tension vary interspecifically but I am not aware of any evidence suggesting that the nature of the interaction between these factors changes.

Cardiac myoglobin was oxidized *in vivo* following i.p. injection with sublethal levels of $NaNO_2$ and hydroxylamine. This result demonstrates the potential for oxidation of cardiac myoglobin in toxic exposures to these and related compounds. The time course for this effect was not established and the possiblity remains that oxidation was maximal at times other than those sampled.

The percentage of myoglobin in the oxidized form at any time is determined by the prevailing balance of oxidation and reduction. Myoglobin, like hemoglobin, is slowly oxidized by molecular oxygen. It is reduced in mammals by NADH-dependent metmyoglobin reductase (Hagler et al., 1979). Myoglobin purified from untreated yellowfin tuna, coho salmon, and buffalo sculpin occured as the reduced oxygenated form suggesting that a reducing system is also present in fish.

Although not demonstrated by this study, I believe that myoglobin plays an important role in O_2 transport in cardiac muscle and that oxidation *in vivo* would be deterimental. Moreover, oxidation of myoglobin may occur as only one of several effects on the heart. Dose-response studies with isolated perfused hearts indicated that both aniline and PHA have toxic effects apart from oxidation of myoglobin. Dose, route and duration of exposure are important because the concentrations required to elicit various

effects differ.

In vivo as in vitro, the physiological significance of myoglobin oxidation depends upon conditions of O_2 supply and demand. O_2 supply to the heart is reduced during toxic exposure to heme oxidants due to oxidation of hemoglobin. The resulting hypoxia initiates in turn a reflexive stimulation of the heart, causing it to work harder (Clark et al., 1943). The heart would be poorly equipped to respond to this increase in cardiac demand if myoglobin was significantly oxidized or if cardiac performance was compromised by some other toxic effect. Cardiac insufficiency might thus contribute to the described syndrome by inhibiting the circulation, hence oxygenation, of blood.

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APPENDICES

APPENDIX I

THE USE OF dP/dt, RECORDED FROM THE BULBOUS ARTERIOSUS, AS AN INDEX OF MYOCARDIAL CONTRACTILITY IN THE ISOLATED PERFUSED SCULPIN (*Enophrys bison*) HEART

INTRODUCTION

Contractility is an intrinsic property of muscle cells that determines their ability to perform work under imposed loading conditions. This property is relatively constant in skeletal muscle cells and a graded response of the whole muscle derives from differential recruitment of motor units. In contrast, cardiac muscle acts as an electrical syncytium, and the intensity of the response of an individual cell is highly variable. The concept of contractility is often developed in terms of the force-velocity relationship of isolated muscle strips. A change in contractility is indicated if the force-velocity curve changes at a fixed muscle length. The change may be due to changes in the maximal velocity of shortening, maximal isometric tension generation, or both. The biochemical basis for these changes is incompletely understood but probably involves both calcium delivery and myosin ATPase activity (reviewed by Katz, 1977).

A change in contractility in the intact heart is defined as a change in stroke work that does not result from a change in initial fiber length. These changes can be illustrated using ventricular function curves, which relate preload (venous pressure or ventricular end-diastolic volume) to stroke work. An increase in

contractility, as by norepinephrine, shifts the curve to the left. A decrease, as by ischemia or acetylcholine, shifts the curve to the right. Alternatively, changes in myocardial contractility may be monitored using the maximal rate of change of pressure (peak dP/dt) in the contracting left ventricle (Mason, 1969). In a healthy mammalian heart, peak dP/dt occurs at approximately the same time that the aortic valve opens and is primarily dependent upon the contractile state of the muscle and the loading conditions under which it is operating. Peak dP/dt is influenced by heart rate, venous return (preload) and arterial diastolic pressure (afterload). Controlling for these variables, changes in contractility may be assessed directly. Unlike the use of ventricular function curves, the measurement of peak dP/dt enables the investigator to monitor changes in contractility on a beat-to-beat basis.

Peak dP/dt is usually recorded by introducing a micromanometer into the ventricle, or by direct needle puncture. Unfortunately, the small and relatively thin walled sculpin heart ventricle does not lend itself to these manipulations. Therefore, we turned our attention to the rising phase of the pressure trace recorded from the bulbous arteriosus. In this study, we used epinephrine and acetylcholine to demonstrate that the rate of change of pressure recorded from the bulbous arteriosus can be used as an index of contractility in the sculpin heart.

MATERIALS AND METHODS

The interpretation of pressure data from an extraventricular site such as the bulbous arteriosus is complicated by a variety of factors including vascular resistance and compliance, the hydrodynamics of ejection, and the nonlinear relationship between fiber length and tension development. For this reason, we restricted our observations to the initial, rapidly rising phase of the pressure trace. We assumed that these data best represent the contractile status of the ventricle and are relatively unaffected by elastic elements in the bulbous.

Pressure was monitored continuously with a Stratham P23 ID pressure transducer (Stratham Instruments Co., Hato Rey, P.R.) connected to a Gould 11-4307-04 transducer amplifier (Gould, Inc., Cleveland, OH). The first derivative of this signal was generated by a resistance-capacitance circuit (modified from Carr, 1978) (Figure A.1), and filtered at 12 Hz (-3 db point) with an 8-pole low-pass Bessel filter (Frequency Devices, model 902 LPF, Haverhill, MA). Both the pressure signal and its derivative were recorded with a Brush 220 chart recorder (Brush Instruments Division of Gould Inc., Cleveland, OH). The system was calibrated with a triangle wave generator and oscilloscope so that the rate of change of pressure, in cm H_2O/sec, could be read as mm of pen deflection x 10.

Sculpin were killed by a blow to the head and their hearts were excised and perfused as previously described (Chapter III). Preload and afterload were fixed at 1.27 and 15 cm H_2O, respectively. Stock

solutions (1 mM in .01 M HCl) of (-)-epinephrine bitartrate and acetylcholine chloride (Sigma Chemical Co., St. Louis, MO) were made up daily and an appropriate volume added to the treatment perfusate reservoir immediately before each experiment. Control and treatment perfusates were equilibrated with room air. The heart and perfusing solutions were maintained at 12 $\pm$ 1 C°.

RESULTS AND DISCUSSION

Representative recordings of the pressure transducer signal and its electronically generated derivative (dP/dt) are shown in Figure A.2. Both traces are similar to those recorded intraventricularly in mammals (eg. Mason, 1969). Pressure in the bulbous arteriosus increased most rapidly (peak dP/dt) during the earliest phase of the pressure pulse, rising less rapidly thereafter until it reached a maximum level and began to decline. The inflection (indicated by the letter B in Figure A.2) described by this pattern was of unknown origin but was a common feature of these recordings. The lag time between signals (about 0.04 seconds) was due to the electronic circuitry and did not affect signal amplitude. Oscillations at the end of each pulse were due to a brief period of backflow before actuation of a one-way valve.

In the first of two preliminary experiments, an unpaced heart was treated for 2 min with 0.5 x 10-6 M epinephrine, perfused for 30 min with drug-free saline, then treated for 2 min with 1.0 x 10-6 M acetylcholine. A second heart was treated with the same concentrations of epinephrine and acetylcholine but the treatment order was reversed. Heart rate, pulse pressure and peak dP/dt were increased by epinephrine and decreased by acetylcholine (Table A.1). Both hearts developed arrythmia when treated with acetylcholine, the second arresting until perfused with drug-free saline. The maximal response to either compound occurred during the 2 min treatment period or very soon thereafter. In both experiments, a steady level

of performance was observed 30 min after the first treatement.

As noted previously, peak dP/dt has been positively correlated with heart rate in mammalian preparations. Thus, inotropic changes observed in preliminary experiments may have been due entirely or in part to chronotropic effects. A second experimental design was therefore developed that incorporated electrical pacing to eliminate changes in heart rate. Two hearts were isolated and allowed to stabilize unpaced for 30 min. The intrinsic heart rate was then determined and electrical pacing begun at the same rate with a Grass SD9 stimulator (Grass Instruments Inc., Quincy, MA) connected to stainless steel cannulae. Pulse duration was set to 20 ms and the voltage adjusted as necessary to entrain the heart. Both hearts were treated for 1 min with 0.5 x 10-6 M epinephrine, perfused for 30 min with drug-free saline, then treated for 1 min with 0.5 x 10-6 M acetylcholine.

The effects of epinephrine and acetylcholine on electrically paced sculpin hearts are presented in Table A.2. Pulse pressure and peak dP/dt were increased significantly by epinephrine even though there was no change in heart rate. Both hearts arrested briefly when treated with acetylcholine necessitating an increase in stimulating voltage. Paced several seconds later, both hearts responded with modest reductions in pulse pressure and peak dP/dt. These observations are in agreement with numerous studies in mammalian systems which suggest that epinephrine has profound inotopic activity while acetylcholine acts primarily on heart rate. The drug dosages used in this study were selected on the basis of

preliminary experiments and were intended to produce measurable effects in the isolated heart system. The dose-response curve for acetylcholine was particularly steep; dosages less than 0.1 x 10-6 M had little or no effect on heart rate, pulse pressure or peak dP/dt. We do not know what these dosages mean in physiological terms but believe that with caution they may be used to evaluate the utility of the derivative circuit. In conclusion, the measurement of peak dP/dt from the bulbous arteriosus appears to offer a viable alternative to cardiac function curves for the assessment of inotropic effects in the isolated, perfused and electrically paced sculpin heart.

Table A.I.1. Effects of epinephrine and acetylcholine on the performance of isolated, unpaced sculpin hearts. Epinephrine (0.5 x 0-6 M) and acetylcholine (1.0 x 10-6 M) were administered for 2 min in physiological saline. Changes in heart rate (b/m), pulse pressure (cm H_2O), and peak dP/dt (cm H_2O/s) are maximal values, averaged over one minute, and are expressed as a percentage of pre-treatment values.

Treatment	Heart Rate	Pulse Pressure	Peak dP/dt
Experiment No. 1			
Epinephrine	+70	+16	+77
Acetylcholine	-53	-20	-19
Experiment No. 2			
Epinephrine	+25	+60	+104
Acetylcholine	- Arrested -	--	--

Table A.I.2. Effects of epinephrine and acetylcholine on the performance of isolated, electrically paced sculpin hearts. Epinephrine (0.5 x 10-6 M) and acetylcholine (0.5 x 10-6 M) were administered for 1 min in physiological saline. Changes in heart rate (b/m), pulse pressure (cm H_2O) and peak dP/dt (cm H_2O/s) are maximal values, averaged over one minute, and are expressed as a percentage of pre-treatment values.

Treatment	Pulse Pressure	Peak dP/dt
Experiment No. 1		
Epinephrine	+27	+47
Acetylcholine	-18	-12
Experiment No. 2		
Epinephrine	+44	+99
Acetylcholine	-18	-18

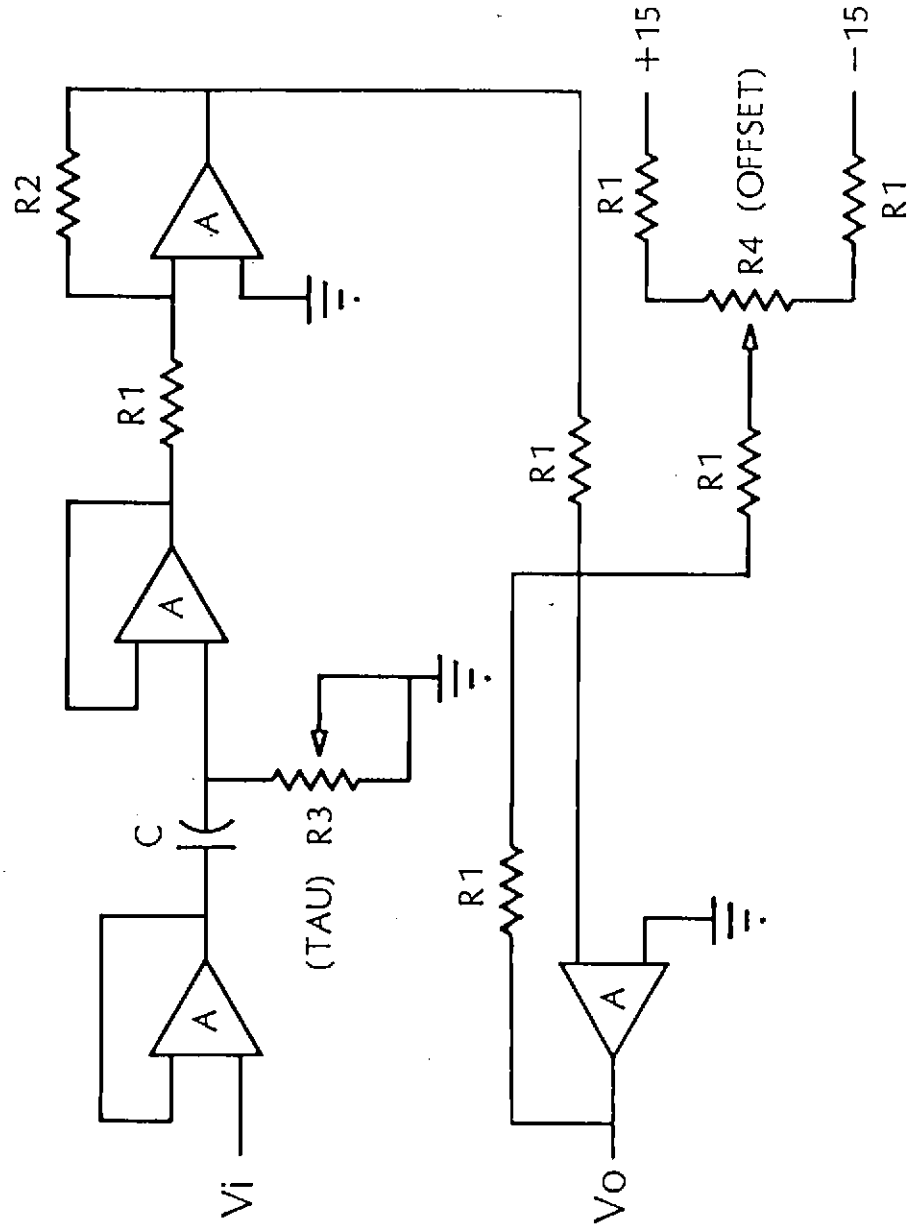

Figure A.I.1.

Figure A.I.2. Representative recording of the pressure transducer signal and its first derivative, dP/dt. Abbreviations: A - peak dP/dt; B - inflection between the initial, rapidly rising phase of the pressure pulse and the remaining pressure increase. Chart speed was 25 mm/s. The transducer signal was calibrated so that 50 cm H_2O gave full scale (50 mm) deflection.

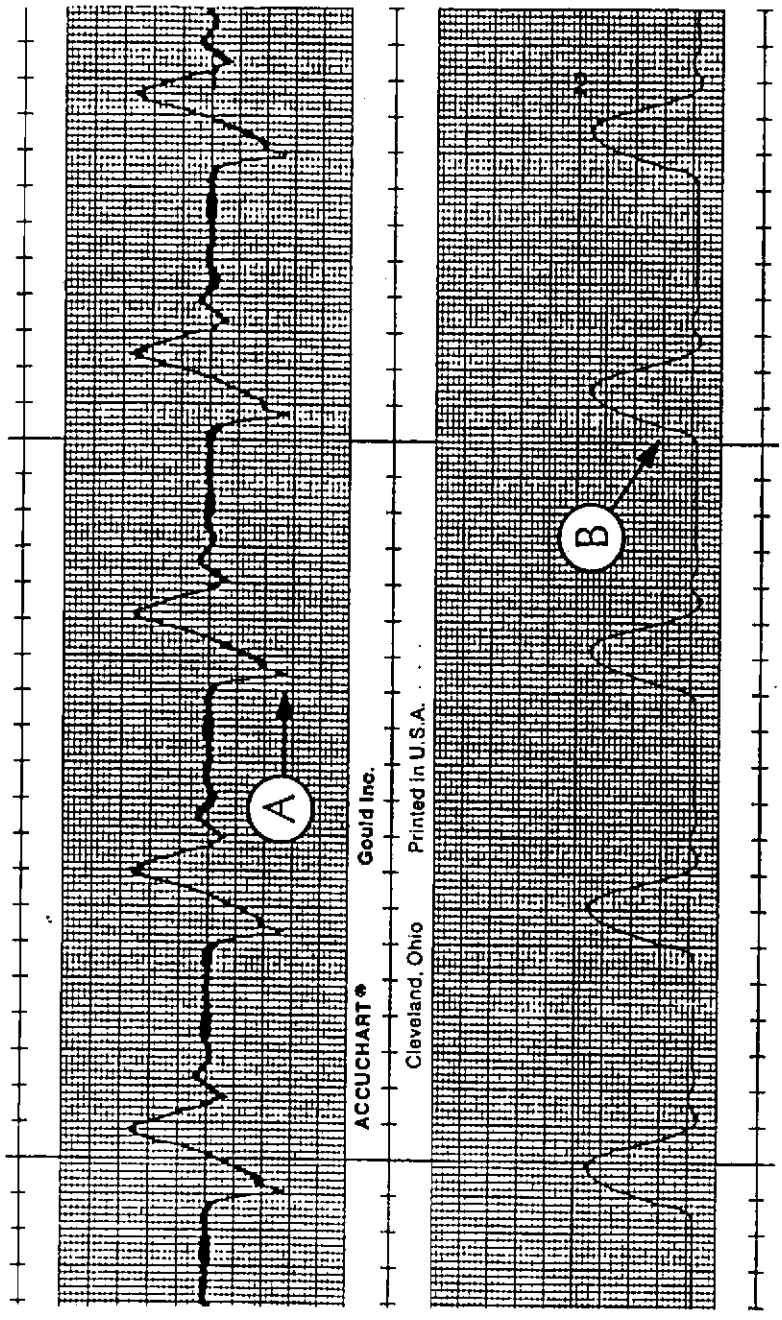

Figure A.I.2.

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APPENDIX II

BLOOD GAS AND ACID-BASE STATUS OF THE VENOUS RETURN IN UNANESTHETIZED, UNRESTRAINED BUFFALO SCULPIN (*Enophrys bison*)

INTRODUCTION

The purpose of this investigation was to characterize the blood gas and acid-base status of the venous return in unanesthetized, unrestrained buffalo sculpin. In doing so we hoped to characterize the physiological "problem" facing a heart that must obtain its O_2 from the venous return. Our intention was to use this information to design physiologically relevant experiments involving the isolated perfused sculpin heart (Chapter III).

MATERIALS AND METHODS

Experimental Animals

Buffalo sculpin, weighing 400 to 600 g, were caught with an otter trawl in Yaquina Bay, Newport, Oregon, and maintained in aerated, flowing seawater, at 12 ± 1 C°. Fish held for more than one week were fed a gelatin-based synthetic diet (Choromanski, 1985).

Sinus Cannulation

The sinus venosus was cannulated via the largest of the hepatic veins to permit repeated sampling of venous blood. A sculpin was anesthetized in seawater containing MS 222 (70 mg/L, Sigma Chemical Co., St. Louis, MO), weighed and placed ventral side up on a fish operating table. The body cavity was opened from the anterior end, well to the right of the pectoral girdle, to the midline, halfway between the posterior margin of the pectoral girdle and the vent. The liver, so exposed, was pushed aside to reveal one large and one or two smaller veins. The largest vein was tied off at the liver, gently retracted and opened with a very fine pair of scissors. A 40 cm length of PE 60 cannula, flared at the end and filled with heparinized saline (500 IU/ml), was then inserted and tied in place. Blood flow usually began immediately, but if not could be encouraged by holding the end of the cannula below the level of the table. Blood flow was stopped by fitting the cannula with a 21 G needle and plastic 1 ml tuberculine syringe filled with heparinized saline (500 IU/ml). The cannula was sutured to the body wall and the incision

closed by sewing muscle and skin layers seperately. The muscle was joined with inverting Lembert sutures (Markowitz et al., 1964) and the skin with simple interrupted sutures. Lembert sutures were originally developed to join intestinal segments and provide protection against leakage. This was considered essential to prevent dehydration. Gill perfusion with anesthetic-treated water (70 mg/L) was maintained throughout the operation, which lasted about one hour.

Sculpin were allowed 20-24 h to recover from anesthesia and surgery. A longer recovery time, while desireable, was precluded by clotting problems caused by very low pressures in the sinus. Despite the invasiveness of the surgery, sculpin respired and behaved normally, their habit of lying motionless for long periods being of great value to this investigation. Sculpin were reopened following the first few experiments to confirm that cannulae had remained in place.

Blood Gas and Acid-Base Measurements

Sculpin were divided into three groups for determination of O_2 partial pressure (PO_2), O_2 content (TO_2) and hematocrit (Hct), and plasma CO_2 content (TCO_2) and pH. Samples were obtained by unplugging cannulae to allow a free flow of blood. Blood pressure, estimated by raising cannulae above the surface of the water, remained positive (approximately 1-3 cm H_2O) throughout the cardiac cycle. Blood was withdrawn from a cannula with a Hamilton gas-tight syringe and kept on ice awaiting analysis (1-2 min maximum).

PO_2 was measured at 12 C° with a Radiometer O_2 electrode (type E5046) and thermostatted cell (type D616). The electrode was zeroed before each experiment with alkaline sulphite solution (Radiometer, S4150) and the span was set using physiological saline equilibrated with air at 12 C°. Subsequent calibrations were accomplished with humidified air, after taking into account the air/water correction factor. Calibration was performed before and after each sample determination and the sample data were discarded if bracketing calibration values differed by more than 2 torr. Physiological saline was degassed before each experiment to a PO_2 level approximating that of venous blood (20-40 torr) and injected ahead of each sample to equilibrate the cuvette (.040 ml). Blood was then transferred from the syringe to a heparinized capillary tube (.100 ml) and injected using a Radiometer micro sample injector (type D654). In all, .150 ml of blood was drawn for each PO_2 determination. Contamination by simple gaseous diffusion was minimized by expelling blood at the blood/air interface when transferring or injecting a sample. Samples were equilibrated for three minutes and PO_2 values read directly from a Radiometer PHM 73 blood gas monitor.

The TO_2 of whole blood was determined as described by Tucker (1967) except that the cuvette was thermostatted to 37 C° to improve the response time of the electrode (Radiometer E5047). Plasma TCO2 was determined with a Radiometer carbon dioxide electrode (type E5037) using the method of Cameron (1971). The pH of whole blood was measured with a Radiometer glass capillary electrode (type

C299A) and BMS 3 MK 2 blood micro system, thermostatted to 12 C°.

Calculation of Plasma Carbon Dioxide Partial Pressure

Plasma PCO_2 was calculated from pH and plasma TCO_2 using a form of the Henderson-Hasselbach equation:

$$pH = pK\ App + \log [TCO_2/(alpha\ CO_2)(PCO_2) - 1],$$

where alpha CO_2 is the solubility of CO_2 in plasma and pK App is the apparent pK of carbonic acid (Albers, 1970; Boutilier et al., 1984). Trout plasma values for alpha CO_2 (.059 mM/L/torr at 12 C°) and pK App (6.12 at 12 C°, pH 7.83) were adopted from Boutilier et al., (1984).

RESULTS AND DISCUSSION

The blood gas and acid-base status of venous sculpin blood is compared in Table A.II.1 to that of venous blood from resting starry (Platichthys stellatus) (Wood et al., 1979) and winter flounder (Pseudopleuronectes americanus)(Cech et al., 1977). Like sculpin, flounder are benthic fish and do not possess coronary arteries. The agreement between data sets was good and we felt confident in applying the values determined for sculpin to studies of the isolated perfused sculpin heart (Chapter III).

Fish that do not possess coronary arteries must depend upon the venous return to satisfy their cardiac requirement for O_2. Farrell (1984) concluded from his review of cardiac performance in fish that venous blood contains more O_2 than is required by the heart. The results of the present investigation tend to confirm this assertion. However, caution is advised, because most of the limited in vivo data have been obtained from resting fish residing in aerated water. O_2 tensions in the venous return of exercising fish, particularly those in otherwise sedentary fish during "burst" swimming activity, are poorly known, as are O_2 tensions when ambient O_2 levels are low. Blood O_2 tensions determined in this study should be considered "best case" values and may be greater than levels commonly occuring in sculpin in natural settings.

Table A.II.1. Blood gas and acid-base status of the venous return in unanesthetized, unrestrained buffalo sculpin: a comparison with other teleosts.

Parameter	Buffalo sculpin			Starry flounder (Wood et al. 1979)	Winter flounder (Cech et al. 1977)
	Fish sampled	N	Mean ± SD		
PO_2 (torr)	3	8	32.2 ± 6.4	13.4	31.0
TO_2 (ml %)	4	11	2.3 ± 0.6	3.34	3.10
Hct (%)	4	11	21 ± 4.0	14.5	----
pH	5	17	7.83 ± 0.04	7.87	7.89
TCO_2 (mM/L)	5	16	4.30 ± 0.8	7.15	----
PCO_2 (torr)*	5	16	1.5 ± 0.3	3.02	----

* Calculated from pH and TCO_2 (see methods).

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