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LETHAL AND SUBLETHAL EFFECTS OF HEMOXIDANTS, PARTICULARLY NITRITE, ON SELECTED AQUATIC ANIMALS

DISSERTATION

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By

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Selected aquatic animals were exposed to water-borne nitrite under various environmental conditions. Larvae of the salamander, *Ambystoma texanum* were the most sensitive species tested with a 96-h LC_{50} of 1.09 mg/L. Resistance times to 50% death in crayfish, *Procambarus simulans*, were highly significantly correlated (r = 0.95) to nitrite concentrations: log resistance time (h) = 2.4-0.80 log nitrite concentration. In bluegill, *Lepomis macrochirus*, NO_2^- toxicity increased at low pH regardless of chloride concentrations. Chloride concentrations at molar ratios of 17:1 ($Cl^-:NO_2^-$) or greater, ameliorated nitrite toxicity at neutral pH in aquatic salamanders and crayfish. Nitrite toxicity increased and chloride "protection" was less effective at low pH in all species tested.

Nitrite oxidizes hemoglobin to methemoglobin, a form incapable of binding oxygen. *Rana catesbiana* larvae exposed to nitrite developed methemoglobin within 24-h. Channel catfish (*Ictalurus punctatus*) were exposed for 24-h to nitrite, tricaine methanesulphonate (TMS-222), nitrite

and TMS-222, and a control in which neither chemical was added. Mean methemoglobin in each of the three treatment groups was significantly greater than the control and differed significantly among each other. Nitrite induced methemoglobinemia was significantly reduced in the prescence of TMS-222; however, catfish exposed to TMS-222 had methemoglobin concentrations six times greater than those of controls.

Methemoglobin levels in channel catfish were significantly correlated to time during formation caused by exposure to TMS-222 and during recovery when placed in TMS-222 free water. Channel catfish acclimated to 30°C had significantly greater rates of methemoglobin formation and loss than fish acclimated to either 20°C or 10°C. A modified spot screening test for methemoglobin reductase indicated that this enzyme occurs in channel catfish.

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CHAPTER I

INTRODUCTION

Nitrite (NO_2^-) is an intermediate compound formed during nitrification of ammonia, the major nitrogenous waste product of aquatic animals. In high density fish culture, self-poisoning by ammonia is possible; however, in water, ammonia is biologically converted by bacteria as follows:

Ammonia--(*Nitrosomonas*)→Nitrite--(*Nitrobacter*)→Nitrate

Ammonia can be a serious toxicant to fishes at low concentrations, with lethal concentrations as low as 0.5 mg/L (NH_3) reported for cutthroat trout, *Salmo clarki* (Thurston et al., 1978). Colt and Tchobanoglous (1976) reported lethal concentrations of NH_3 to channel catfish, *Ictalurus punctatus*, of less than 1.0 mg/L. Nitrite is quite toxic, with lethality reported for several fish species at less than 10 mg/L NO_2^- in 96-h exposures (eg. Smith and Williams, 1974; Westin, 1974; Russo, 1974; Brown and McLeay, 1975). Nitrate (NO_3) is much less toxic, with 96-h lethal concentrations of 6,000 mg/L reported for

rainbow trout, *Salmo gairdneri* (Westin, 1974). Nitrate does not cause hemoxidation, thus it is much less toxic than nitrite.

In high density culture ponds, biofiltered recirculating culture systems, laboratory holding facilities, sewage plant receiving waters (Russo et al., 1974), and in nature, where animal biomass is high, nitrite can reach lethal concentrations. Accumulation of nitrite, which is usually an intermediate product of nitrification, can result from imbalances in relative abundances of *Nitrosomonas* and *Nitrobacter* (Haug and McCarty, 1972), inhibition of nitrification by un-ionized ammonia (Anthonisen et al., 1976), and denitrification in anaerobic sediments (Boyd and Hollerman, 1980). Antibiotics, parasiticides, and algalcides commonly employed in fish culture can suppress nitrification, causing toxic amounts of nitrite to accumulate. In most cases, elevated nitrite concentration is a short-term phenomenon; however, due to its quite toxic nature to aquatic organisms, even a short-term elevation in nitrite concentration may cause problems. Understanding nitrite toxicity is economically important due to the production of channel catfish (35,000 metric tons 1978) and rainbow trout (13,600 metric tons 1978) in commercial fish farms (Lovell, 1979). In intensively fed channel catfish production ponds, high

nitrite concentrations may result in the loss of a majority of the pond stock (Lovell, 1979).

Acute nitrite toxicity data have been reported by several investigators (eg. Gillette et al., 1952; Wallen et al., 1957; Klinger, 1957; Westin, 1974; Smith and Williams, 1974; Russo et al., 1974; Konikoff, 1975; Colt and Tchobanoglous, 1976; Crawford and Allen, 1977; Perrone and Meade, 1977; Thurston and Russo, 1978; Tomasso et al., 1979; Russo et al., 1981). Reported 96-h LC_{50}[1] values range from 0.7 mg/L for rainbow trout (Russo et al., 1974) to 43 mg/L for channel catfish (Colt, 1974). In aquatic vertebrates which utilize hemoglobin as a respiratory pigment, nitrite exerts toxic action by converting normal hemoglobin (Hb) to methemoglobin (MHb) (Smith and Williams, 1974; Russo et al., 1979; Huey et al., 1980). Methemoglobin is an oxidative derivative of hemoglobin and is incapable of binding oxygen (Bodansky, 1951). Iron in the normal heme moiety is in the ferrous (Fe^{++}) state, and methemoglobin is the oxidative derivative having iron in the ferric (Fe^{+++}) form. Methemoglobin imparts a brownish color to the blood and can be spectrophotometrically assayed at 635 nm (Evelyn and Malloy, 1938). Small amounts

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[1] LC_{50} is the median lethal concentration, or that concentration which kills 50% of the test organisms in a specified time.

of methemoglobin are found in normal vertebrates (Peters and Van Slyke, 1931). Concentrations of methemoglobin in mammalian red blood cells rarely exceed 1.0% (Beutler, 1968); however, Cameron (1971) found methemoglobin concentrations in rainbow trout (Salmo gairdneri) to range from 2.9 to 17% of total hemoglobin.

Hemoglobin may be oxidized in several ways, including (1) auto-oxidation, (2) the direct action of oxidants, (3) the action of hydrogen donors (eg., aniline dyes), and (4) complexes which render hemoglobin more sensitive to oxidation (Beutler, 1968). An example of the latter is nitrite, which complexes with hemoglobin making it more oxidizable by oxygen (Van Assendelft and Zijlstra, 1965). Each mole of oxyhemoglobin iron converted to methemoglobin causes the oxidation of 1.5 moles of nitrite to nitrate with the release of no oxygen (Rodkey, 1976). Enhanced generation of hydrogen peroxide may cause methemoglobinemia in animals exposed to aniline, sulfonamides, lidocaine, and many other aniline derivatives (Bodansky, 1951). Nitrite induced methemoglobinemia has been reported in several salmonids (Cameron, 1971; Brown and McLeay, 1975; Smith and Russo, 1975; Meade and Perrone, 1980), and in channel catfish, Ictalurus punctatus (Huey et al., 1977; Tomasso et al., 1979; Huey et al., 1980).

The literature contains little information concerning nitrite toxicity to aquatic animals other than fishes. Hemocyanin, the copper-based respiratory pigment found in crayfish, has an oxidized methemocyanin form. *In vitro* oxidation of hemocyanin to methemocyanin was accomplished by Felsenfeld and Printz (1959) with hydrogen peroxide.

In evaluating the toxicity of nitrite, pH and ionic composition of the water source must be considered because of the potential for large differences in nitrite uptake rate across the gills (Crawford and Allen, 1977; Huey et al., 1977; Perrone and Meade, 1977; Wedemeyer and Yasutake, 1978; Tomasso et al., 1979; Huey et al., 1980; Russo et al., 1981). Presence of monovalent anions such as chloride (Cl^-) and bicarbonate (HCO_3^-) can inhibit nitrite toxicity (Huey et al., 1980). A molecular ratio of approximately 16 chloride ions to one nitrite ion completely inhibited increased methemoglobin concentrations in nitrite exposed channel catfish (Tomasso et al., 1979). The toxicity of nitrite is augmented in low pH water (Russo et al., 1981). Colt (1974) suggested that the toxicity of nitrite is due to the nitrous acid form (HNO_2) which would increase with decreasing pH, while Russo et al. (1981) states that both ionic forms of nitrite, HNO_2 and NO_2^- are toxic. Coho salmon (*Oncorhynchus kisutch*) exposed to nitrite at low pH had increased plasma nitrite concentrations when compared

to groups exposed at higher pH (Meade and Perrone, 1980). Huey et al. (1980) reported increased methemoglobin formation in channel catfish exposed to nitrite at low pH.

No published study has examined the effect of acclimation temperature on nitrite-induced methemoglobinemia. Temperature toxicity interactions are often complex; however, in a literature survey Cairns et al. (1975) noted elevated temperatures increase the acute toxicity of most pollutants in short-term (48-h) exposures. Temperature will affect processes concerned with toxicity such as: (1) uptake, (2) metabolism, and (3) elimination rate which will influence survival rates in lethal tests or rate of methemoglobin formation and reduction in sublethal tests.

In mammalian red blood corpuscles, a complex enzymatic system exists which reduces MHb to Hb (Jaffe, 1964). The nicotinamide adenine dinucleotide, reduced form, (NADH) methemoglobin reductase system is the major method by which methemoglobin is reduced in mammalian systems (Harris and Kellermeyre, 1970). This system has not been described in fish; however, from thermodynamic considerations, one must exist (Cameron, 1971), otherwise all hemoglobin would eventually accumulate as methemoglobin. Channel catfish (*Ictalurus punctatus*) with profound nitrite-induced methemoglobinemia recover in 24-h when placed in

nitrite-free water, which gives indirect evidence that a MHb-reductase system is present (Huey et al., 1980). No direct evidence exists to show that an operating MHb-reductase system is present in fishes.

Research Program

A research program was developed to investigate basic and applied aspects of toxicity, both lethal and sublethal, of hemoxidants, particularly nitrite, on fish, non-fish aquatic vertebrates, and crayfish. The major objectives of this research were to determine

A. Acute and sublethal toxicity of nitrite to selected aquatic organisms:

1. aquatic salamander larvae, *Ambystoma texanum*,
2. swamp crayfish, *Procambarus simulans*,
3. bluegill, *Lepomis macrochirus*,
4. bullfrog, tadpoles, *Rana catesbiana*,
5. channel catfish, *Ictalurus punctatus*.

B. The influence of environmental chloride on acute and sublethal exposures to hemoxidants

1. on acute nitrite toxicity to salamander larvae, crayfish, and bluegill,
2. on nitrite-induced methemoglobinemia in bullfrog tadpoles, *Rana catesbiana*.

C. The effect of environmental hydrogen ion concentrations (pH) on acute nitrite toxicity

1. to the crayfish, *Procambarus simulans*,

2. to the bluegill, *Lepomis macrochirus*.

D. The effect of temperature in sublethal exposures to nitrite

1. methemoglobin formation in channel catfish exposed at different acclimation temperatures,

2. recovery from methemoglobinemia at different acclimation temperatures.

E. The effect of the fish anesthetic TMS-222 on nitrite-induced methemoglobinemia in channel catfish

1. supression of nitrite-induced methemoglobinemia,

2. dose-response curve for TMS-222 induced methemoglobinemia.

F. If a methemoglobin reductase system is present in channel catfish.

CHAPTER II

TOXICITY OF NITRITE TO LARVAE OF THE SALAMANDER AMBYSTOMA TEXANUM

Introduction

Nitrite toxicity in non-fish vertebrates has received little research attention. For comparative purposes, research was designed to determine the 96-h LC_{50} for aquatic larvae of the salamander, *Ambystoma texanum*. Environmental chloride levels were increased in one trial to determine if this monovalent anion functions to suppress nitrite lethality as reported for fishes.

Materials and Methods

Larvae of the salamander, *Ambystoma texanum*, (0.45± 0.08 g, $\bar{x} \pm s$)[2] obtained by seining a pond in Denton County, Texas were placed in 190-L filtered aquaria and held postabsorptive for four days prior to testing. Standard methodology for static bioassay (E.P.A., 1975) was employed. Trials were conducted in 25°C, O_2 saturated, medium hardness (140 mg/L, total hardness), low chloride (5.0 mg/L) water at pH 7.0, except for one trial at 300

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[2] Throughout this dissertation all statistics reported in xx ± yy format represent the mean ± 1 standard deviation.

mg/L chlorides. Reagent grade chemicals were added to 30-L test tanks and initially mixed with a mechanical stirrer. Appropriate quantities of nitrite, as sodium nitrite, were added to yield 11 test concentrations ranging from 0.08 to 103.4 mg/L NO_2^-. A concentration of $\geq$0.01 mg/L NO_2^- served as a control. All nitrite concentrations were quantified using an azo-dye technique (APHA, 1971; see pages 85-90 of the Appendix). Temperature, dissolved O_2, pH, nitrite, and ammonia were monitored at 12-h intervals of the 96-h trials. Total hardness and chlorides were measured at the beginning and termination of each trial. Ten larvae were exposed to each concentration. Loss of equilibrium, i.e. ecological death, was the chosen endpoint for lethality. Lethal concentration, as 96-h LC_{50}, was calculated by a computer program developed by Dr. C. E. Stephan, E.P.A. laboratory, Duluth, MN.

Results and Discussion

The 96-h LC_{50} for Ambystoma texanum in low-chloride (5.0 mg/L) water equalled 1.09 mg/L NO_2^- with conservative 95% confidence limits extending from 0.48 to 2.5 mg/L (Table I). Nearly 100% mortality was observed in trials with NO_2^- as low as 2.5 mg/L. At exposures $\leq$10 mg/L NO_2^-, larvae exhibited stress symptoms such as excessive mucus production and air gulping. Differences in chemical composition of test water make relative nitrite toxicity

comparisons tenuous; however, values for *A. texanum* indicate that this species is highly susceptible to NO_2^-. The 96-h LC_{50} approaches the lowest concentration causing lethality, 0.7 mg/L, reported for vertebrates, the rainbow trout (Russo et al., 1974).

None of the 10 larva exposed to 300 mg/L chlorides at 10 mg/L nitrite suffered mortality in 96 h. This increase in nitrite tolerance in the presence of chlorides was expected and corroborates the work of Crawford and Allen (1977), who demonstrated reduced nitrite lethality for chinook salmon (*Oncorhynchus tshawytscha*) in seawater relative to freshwater. Results of this experiment support a monovalent ion-competitive uptake model hypothesized by Perrone and Meade (1977) to occur in fish. In this model, monovalent anions compete with nitrites for ionic uptake sites on the respiratory surfaces. I suggest that decreased nitrite mortality in the presence of chlorides is not an outcome of increased physiological tolerance, but rather relates to lower NO_2^- uptake rates.

Larvae of this salamander genus often inhabit warm, stagnant, temporary ponds with low dissolved oxygen, "gulp" atmospheric O_2, and are highly sensitive to nitrites. Results suggest that their tissue tolerance to reduced oxygen produced by nitrite oxidized hemoglobin is low. Tissue anoxia caused by methemoglobinemia may trigger air

gulping in these animals; however, hemoglobin bound oxygen cannot be increased owing to the inability of methemoglobin to bind oxygen. Sensitivity to nitrites is probably a result of high ionic uptake of NO_2^- and a poorly developed or nonexistent enzyme system to convert methemoglobin to hemoglobin.

TABLE I

LETHALITY OF NITRITE TO LARVAL SALAMANDERS (*AMBYSTOMA TEXANUM*) ALL TESTS INCLUDE TEN ANIMALS EXPOSED FOR 96-H AT PH 7.0.

Nitrite mg/L as NO_2^-	Percentage Dead
103.40	100
51.80	100
10.10	90
6.60	100
2.50	90
0.98	10
0.90	70
0.48	10
0.09	0
0.08	0
0.01	0

CHAPTER III

ACUTE TOXICITY OF NITRITE TO CRAYFISH *PROCAMBARUS SIMULANS* IN VARIED ENVIRONMENTAL CONDITIONS

Introduction

Although considerable research has documented the toxic effects of nitrite to fishes, I could find no published studies concerning nitrite toxicity to aquatic invertebrates. Similar to fish, crayfish excrete ammonia. Also some species are cultured for food in ponds and rice fields (Chien and Avault, 1980), where exposure to nitrite is possible. Nitrite is known to be a potent oxidizer of hemoglobin and is very toxic to vertebrates. The crayfish was selected for testing nitrite toxicity in an aquatic species which uses an oxygen carrying molecule other than hemoglobin. Unlike fishes, crayfish possess the copper-containing hemocyanin as their blood oxygen-carrying pigment. Research was designed to measure the resistance of the swamp crayfish, *Procambarus simulans*, to nitrite and, secondarily, to determine the effects of chlorides and pH on nitrite toxicity in this species.

Materials and Methods

Crayfish, *Procambarus simulans*, (4.7 ± 1.2 g) were seined from a pond in Denton County, Texas, and placed in a "Living Stream" (Frigid Units, Inc.) containing plastic pipe refugia. Crayfish were fed daily during the 10- to 14-day holding period. Static toxicity tests were performed according to the methodology of the U.S.E.P.A. (1975) for 96-h exposure with 24-h water and toxicant replacement. All tests were conducted in 30-L aquaria at 25°C and O_2 saturated conditions. During trials nitrite, temperature, dissolved O_2, and pH were measured at 6-h intervals, while total hardness and alkalinity were measured at 24-h intervals (Table II). Nitrite (NO_2^-) concentrations were confirmed by the azo-dye technique (APHA, 1971).

Loss of equilibrium was the chosen endpoint criterion. Crayfish exhibiting symptoms of nitrite toxicity could neither orient their movements nor maintain balance. Often they were found inverted in the test chamber. Although their legs and swimmerets occasionally moved slowly, they did not respond to prodding with a glass rod. If such crayfish were not removed, they were cannibalized; however, when removed and placed into nitrite-free water, they did not recover and died within less than 24 h.

A total of 20 separate 96-h trials was conducted; ten crayfish were used in each trial. Eleven trials were

conducted with nominal nitrite concentrations ranging from 1.0 to 100 mg/L. Four additional trials with nitrite concentrations of $\leq$1.0 mg/L served as controls. In these 15 trials, medium hard (140 mg/L, total hardness), low chloride ($\leq$5.0 mg/L), and pH 7.0 (maintained by 0.02 M phosphate buffer) water was used. Five additional trials examined the effects of increased chloride concentrations and decreased pH on nitrite toxicity. In the former, groups of 10 crayfish were exposed to three nominal nitrite concentrations ranging from 1.0 to 100 mg/L at pH 7.0 and 300 mg/L chloride. In the remaining two trials, crayfish were exposed to nitrite concentrations of 100 mg/L, chloride concentrations of 5.0 and 300 mg/L and a pH of 5.6. These trials were designed to examine possible mechanisms of nitrite uptake and toxicity to crayfish.

Results and Discussion

In eight of the original 15 trials (including the four controls), only 11 of 80 test crayfish died during 96-h exposures (Table III). Mortalities did not exceed 30% in any of these trials. In the other six trials, at least 50% of the crayfish died. Nitrite concentrations effective to 50% of these samples (EC_{50})[3] were highly significantly

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[3] EC_{50} is the median effective concentration, or that concentration which has a defined effect on 50% of the test organisms.

correlated to exposure time in a double logarithmic fashion ($r = -0.953$; $p = 0.003$). The best fit, least squares regression model equalled log time (h) = 2.64-0.84 log nitrite concentration (mg/L). Standard error for the regression slope equalled 0.134. This model predicts EC_{50} values for 24, 48, 72, and 96-h of 31.5, 13.8, 8.6, and 6.1 mg/L, respectively (Fig. 1).

These represent the only published nitrite EC_{50} values for a nonvertebrate species, to my knowledge. Comparison of these EC_{50} values for *P. simulans* with other aquatic species is tenuous. The literature reporting nitrite toxicity in fishes contains not only large interspecific, but also large intraspecific variation in reported LC_{50} values. Much of this variation is related to the differences in water chemistry which drastically influence the toxicity of nitrite. The 96-h EC_{50} of 6.1 mg/L NO_2^- for *P. simulans* is intermediate to the LC_{50} values of 27.0 and 0.7 mg/L NO_2^- reported for channel catfish (Konikoff, 1975) and rainbow trout, *Salmo gairdneri*, (Russo et al., 1974) tested under similar water quality conditions. These two represent the range in nitrite tolerance for piscine species thus far tested. These results indicate that nitrite is toxic to a hemocyanin utilizing species. Although fish and crayfish possess different oxygen-carrying blood pigments, Felsenfeld and Printz (1959)

produced methemocyanin *in vitro* with hydrogen peroxide. Nitrite oxidized hemocyanin likely prevented efficient oxygen transfer to the tissues in these crayfish, resulting in death.

Ninety-six-hour exposures to 50.8 and 101.2 mg/L NO_2^- in water containing 300 mg/L chlorides resulted in no deaths in two trials of 10 crayfish (Table III). These nitrite concentrations are approximately 8.3 and 16.6 times greater than the 96-h EC_{50} for crayfish tested in 5 mg/L chlorides. Trials with similar nitrite concentrations at 5 mg/L chlorides resulted in 100% mortality in 96-h, and resistance times equalled 15 and 9-h respectively (Fig. 1). The observed increase in nitrite tolerance in the presence of chlorides was expected and compares favorably with results reported by Crawford and Allen (1977) for chinook salmon, *Oncorhynchus tshawytscha*, which experienced a 10% mortality in seawater containing 815 mg/L NO_2^- relative to 70% mortality in freshwater, with only 27 mg/L NO_2^- during 48-h exposures. These results and those of Meade and Perrone (1980) suggest that the increased nitrite tolerance observed in crayfish at higher chloride concentrations is not an outcome of increased physiological tolerance but, rather, of lower NO_2^- uptake rates. This corroborates

results seen in salamander larvae (Chapter II), which also have increased nitrite tolerance in the presence of chlorides.

Nitrite dissociates in water to nitrous acid (HNO_2) and nitrite ions (NO_2^-). Colt and Tchobanoglous (1976) suggested that the uncharged HNO_2 diffuses across gill membranes, and hence may enter the body differently from the anionic form, which utilizes a transport mechanism or anion gate (Huey et al., 1980). Comparisons of the effects of variation in external chloride concentration and pH on resistance times of crayfish (Table IV) confirm an hypothesis that chlorides ameliorate nitrite toxicity. At both pH 5.6 and 7.0, resistance times of crayfish were considerably shorter at chloride concentrations of 5 mg relative to 300 mg/L. In addition, comparison of resistance times at constant chloride concentration (either 5 or 300 mg/L) suggests that both an increase in nitrite toxicity and a decreased efficacy of chloride antagonism may have occurred at the lower pH. Resistance times of crayfish to 100 mg/L NO_2^-, although not substantially different at pH's of 7.0 and 5.6 when chlorides equalled 5.0 mg/L, were considerably shorter at pH 5.6 relative to pH 7.0 when chlorides were held at 300 mg/L.

These experiments indicate that the hemocyanin-carrying invertebrate _P. simulans_ is susceptible to nitrite toxicity and possesses an intermediate level of tolerance to nitrites relative to fishes. Uptake of nitrites by crayfish appears to be similar to that of fishes with anions such as chlorides acting as antagonists to NO_2^- uptake. Molar ratios of 3.8:1 - $Cl^-:NO_2^-$ provide a substantial amelioration of nitrite lethality. Finally, a reduction in pH simultaneously appears to enhance the toxicity of nitrites and reduce the protective efficacy of chlorides.

Fig. 1--Relationship between resistance times to 50% mortality and mean nitrite concentrations (mg/L as NO_2^-) for crayfish, *Procambarus simulans*.

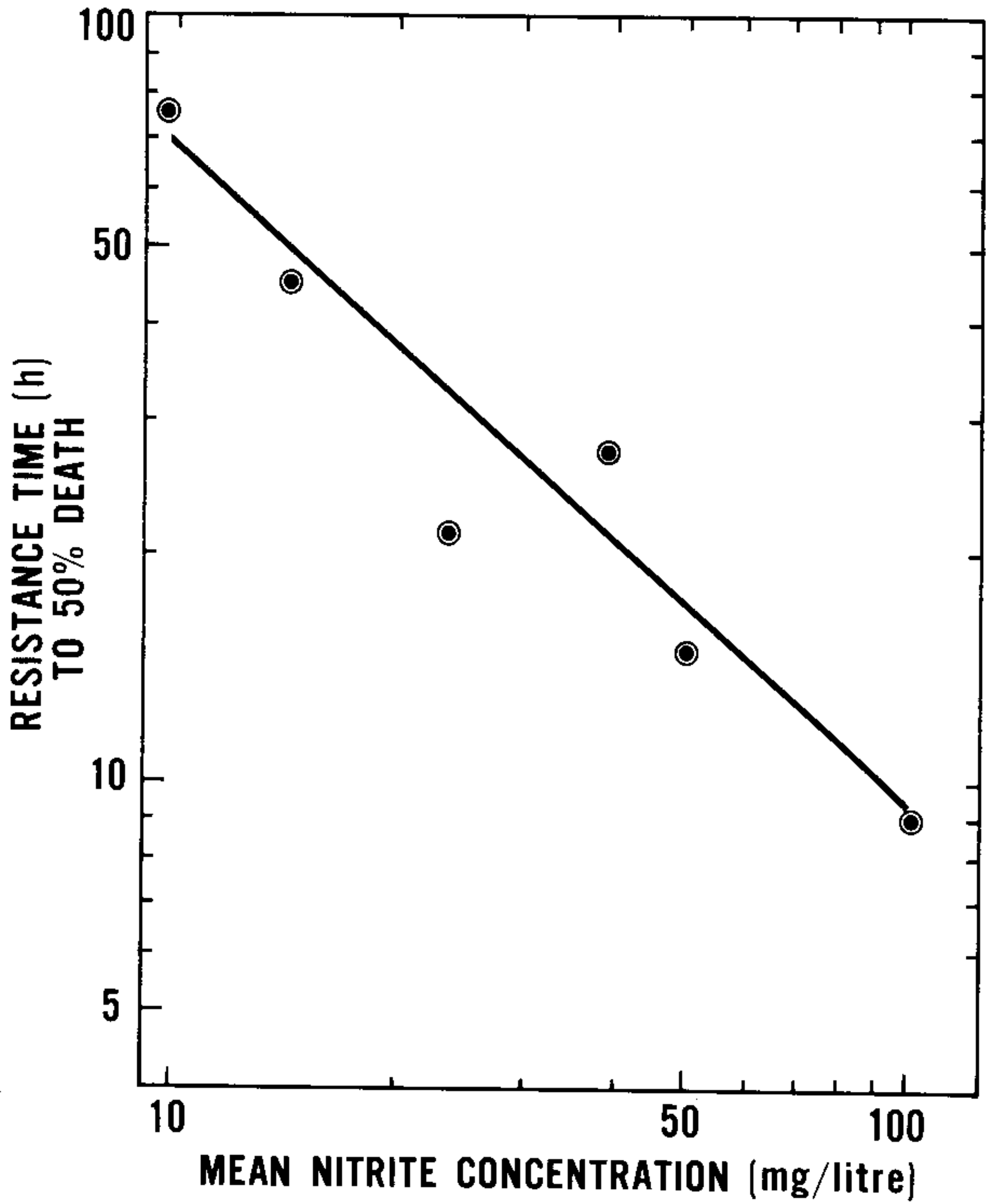

100
50
10
5
RESISTANCE TIME (h)
TO 50% DEATH
10
50
100
MEAN NITRITE CONCENTRATION (mg/litre)

TABLE II

WATER QUALITY VARIABLES DURING CRAYFISH TRIALS. TEMPERATURE, DISSOLVED OXYGEN, AND PH WERE MEASURED AT 6-H INTERVALS, WHILE TOTAL HARDNESS AND ALKALINITY WERE DETERMINED AT 24-H INTERVALS FOR EACH TRIAL. MEAN ($\bar{x}$) $\pm$ ONE STANDARD DEVIATION(S) ARE GIVEN FOR EACH VARIABLE.

Variable	Units	Trials	N	$\bar{x}$	s
Temperature	^{o}C	18	288	25.1	0.7
Oxygen	mg/L	18	288	8.1	0.2
Hydrogen ion	pH	15	255	7.1	0.7
Hydrogen ion	pH	3	51	5.6	0.2
Hardness	mg/L	18	90	158	27.3
Alkalinity	mg/L	18	90	106	11.1

TABLE III

NOMINAL AND MEASURED CONCENTRATIONS OF NITRITE ($\bar{x} \pm s$, AS NO_2^-), CHLORIDE CONCENTRATIONS AND PERCENTAGE OF DEAD CRAYFISH DURING EACH OF 18 96-H TRIALS. NITRITES WERE QUANTIFIED BY THE AZO-DYE METHOD AND MEASURED AT 6-H INTERVALS THROUGHOUT EACH TRIAL.

Nominal	Measured ($\bar{x} \pm s$)	Chlorides (mg/L)	% Dead
Controls:			
≤ 1.0	0.03 $\pm$ 0.04	≤ 5	0
≤ 1.0	0.43 $\pm$ 0.25	≤ 5	0
≤ 1.0	0.50 $\pm$ 0.04	≤ 5	10
≤ 1.0	0.83 $\pm$ 0.13	≤ 5	10
Experimental:	Low Chloride Concentrations		
1.0	1.0 $\pm$ 0.09	≤ 5	10
5.0	5.1 $\pm$ 0.62	≤ 5	20
5.0	4.8 $\pm$ 0.26	≤ 5	10
7.0	7.1 $\pm$ 0.42	≤ 5	30
10.0	9.9 $\pm$ 0.46	≤ 5	70
10.0	10.4 $\pm$ 1.01	≤ 5	10
15.0	14.4 $\pm$ 0.76	≤ 5	90
25.0	23.6 $\pm$ 0.90	≤ 5	100
40.0	39.0 $\pm$ 0.93	≤ 5	100
50.0	50.2 $\pm$ 4.08	≤ 5	100
100.0	104.2 $\pm$ 3.25	≤ 5	100
Experimental:	High Chloride Concentrations		
≤ 1.0	0.39 $\pm$ 0.08	305 $\pm$ 3.9	0
50.0	50.8 $\pm$ 3.51	308 $\pm$ 5.5	0
100.00	101.2 $\pm$ 3.21	311 $\pm$ 6.4	0

TABLE IV

EFFECTS OF PH ON NITRITE TOXICITY IN CRAYFISH. ALL TESTS INCLUDED 10 CRAYFISH (*PROCAMBARUS SIMULANS*) EXPOSED TO 100 MG/L NITRITE (NO_2^-) FOR 96-H.

pH	Cl^- (mg/L)	Percent Dead				
		6-h	12-h	24-h	48-h	96-h
7.0	5	10	80	90	90	100
7.0	300	0	0	0	0	0
5.6	5	40	80	90	100	100
5.6	300	0	0	0	50	50

CHAPTER IV

EFFECT OF PH AND CHLORIDE ON NITRITE-INDUCED LETHALITY IN BLUEGILL (*LEPOMIS MACROCHIRUS*)

Introduction

Toxicity of nitrite both among and within species varies and is highly dependent upon water quality. Reported lethal concentration as 96-h LC_{50} s range from 27 mg/L (Konikoff, 1975) to 43 mg/L (Colt, 1974) for channel catfish, *Ictalurus punctatus*. Rainbow trout, *Salmo gairdneri*, are more sensitive to nitrite with 96-h LC_{50} values of 0.7 mg/L reported by Russo et al. (1974). Aquatic salamanders (Chapter II) have a 96-h LC_{50} of 1.09 mg/L, and crayfish (Chapter III) are less sensitive, with 6.1 mg/L as a 96-h EC_{50}. Various anions influence the toxicity of nitrite (Perrone and Meade, 1977; Wedemeyer and Yasutake, 1978; Huey et al., 1980; Russo, et al., 1981). Tomasso et al. (1979) reported that a molar ratio of 18:1 $Cl^-:NO_2^-$ eliminated NO_2^- lethality in channel catfish. In contrast, enhanced toxicity of nitrite has been observed in channel catfish (Huey et al., 1980), rainbow trout (Russo et al., 1981), and crayfish (*Procambarus simulans*) at increased hydrogen ion concentrations.

Experiments were conducted to evaluate the combined effects of hydrogen ion and chloride concentrations on nitrite toxicity in bluegill (Lepomis macrochirus). My working hypotheses proposed (1) no chloride amelioration of nitrite toxicity at low pH, and (2) significant nitrite toxicity reduction would occur at high pH.

Materials and Methods

Bluegill (7.4-17.3 g) obtained from a pond in Denton County, Texas were held for at least 5 days in 200-L tanks containing dechlorinated, continuously filtered tapwater at 29-30°C. Holding water was analyzed daily for ammonia and nitrite concentrations using an Orion specific ion probe and azo-dye method (Standard Methods, 1975), respectively. Standard methodology for static toxicity testing (E.P.A., 1975) was employed, and all fish were post-absorptive 4 days prior to testing. Trials were conducted in 30°C, O_2 saturated, soft (48 mg/L, total hardness) water. Toxicity tests were conducted under four experimental conditions with pH and chloride (Cl^-) as variables. Composition of test waters varied as follows: (1) pH 4.0 and 18:1 Cl^-:NO_2^- molar ratio, (2) pH 4.0 and 5.0 mg/L Cl^-, (3) pH 7.2 and 18:1 Cl^-:NO_2^- molar ratio, (4) pH 7.2 and 5.0 mg/L Cl^-. A 0.02 M phosphate buffer was used to maintain pH at 7.2, and a potassium hydrogen phthalate buffer maintained

pH at 4.0. Chloride was added as sodium chloride, nitrite as sodium nitrite, and all chemicals were mixed with a mechanical stirrer. Chloride ion concentrations were quantified using a mercuric nitrate titration (APHA, 1975). During the 32 trials, nitrites and hydrogen ion concentrations were monitored at 6-h intervals and adjusted as necessary. Chlorides were determined at the beginning of the experiment. Seven to 10 trials with various constant nitrite concentrations were conducted at each of the four test conditions. Eleven bluegill were exposed to each test concentration, and four groups (N = 11) were used for controls. Each experimental condition had one control group in which nitrite concentrations did not exceed 0.01 mg/L. Lethal concentration, as 48-h LC_{50}, was calculated using the Statistical Analysis System probit package (Finney, 1971) for each of the four test conditions.

Results and Discussion

The 48-h LC_{50} for bluegill, *Lepomis macrochirus*, in pH 4.0 water was 4.4 mg/L NO_2^- in high chloride water and 4.6 mg/L NO_2^- in low chloride water. At pH 7.2, LC_{50} equalled 211.3 mg/L NO_2^- in high chloride water and 281.9 mg/L in low chloride water (Table V).

Fish placed into low pH water exhibited immediate stress at concentrations of 6.9 mg/L NO_2^- and higher.

These fish gulped at the surface and produced excess slime. None of the 33 bluegill survived the 48-h test period. At pH 7.2, all fish survived 48-h exposures at nitrite concentrations 13-fold higher than those that killed all fish at pH 4.0. No mortalities were observed in the control groups. Although pH had a dramatic effect on nitrite toxicity, chloride concentration had little or no effect.

Enhanced nitrite toxcity at high hydrogen ion concentrations was expected and corroborates results in Chapter III and results obtained by Huey et al. (1980), Meade and Perrone (1980), and Russo et al. (1981). This toxicity increase is due to the permeability of the uncharged nitrous acid form of nitrite predominant at low pH (Huey et al., 1980). I suggest that the HNO_2 molecule diffuses rapidly across the gills,causing profound methemoglobinemia and death by anoxia. Klinger (1959) describes nitrite as a slow-acting fish poison, which appears to be correct for pH levels of 7.0 and above, where NO_2^- is the predominant form of nitrite. Both forms of nitrite (HNO_2) and (NO_2^-) are known to be toxic (Russo et al., 1981); however, I suggest that the HNO_2 uptake is much more rapid and the sudden nitrite load converts a majority of the fish's hemoglobin to methemoglobin resulting in death.

At pH levels where NO_2^- is predominant, uptake occurs by means of gill anion gates and is much slower (Huey et al., 1980). A low rate of toxicant uptake allows a methemoglobin reductase system, hypothesized to exist in fishes, time to function before lethal methemoglobin concentrations occur. Although this enzyme system cannot supply enough reducing power in prolonged high nitrite exposure, it would significantly increase short term resistance to nitrite toxicity. In contrast, HNO_2 floods across the gill membranes causing a lethal concentration of methemoglobin, and the methemoglobin reductase system functions too slowly to prevent rapid lethality.

At low pH no decrease in nitrite toxicity was observed in high chloride exposures. This was expected, because chloride competitively interferes with NO_2^- at ionic uptake sites on the gills; however, chloride cannot interfere with uptake of HNO_2, the predominant nitrite form at pH 4.0. At pH 7.2, results were expected to be similar to those obtained by Crawford and Allen (1977), Perrone and Meade (1977), Tomasso et al. (1979), and Huey et al. (1980); however, 48-h LC_{50} values were similar in high and low chloride tests. It is possible that interaction between chloride, nitrite, phosphate buffers, and high temperature

($30^{o}C$) caused the chloride dosed groups to suffer lethality that could not be explained by nitrite toxicity alone. These data support both hypotheses that nitrite is more toxic at low pH and that chloride protection is lost at low pH.

TABLE V

LETHAL CONCENTRATION 50%, LC50 (48-H) FOR BLUEGILL, *LEPOMIS MACROCHIRUS*, EXPOSED TO NITRITE UNDER FOUR ENVIRONMENTAL CONDITIONS. ELEVEN FISH WERE USED IN EACH TRIAL.

Group	Number of Trials	pH	Chlorides	48-h LC_{50} mg/L NO_2^-	95% Confidence Limits mg/L NO_2^-	
I	7	4.0	a	4.4	3.0	12.9
II	7	4.0	b	4.6	3.9	5.8
III	8	7.2	a	211.3	210.5	212.3
IV	10	7.2	b	281.9	251.0	345.7

Chlorides (a) indicates an 18:1 Cl^-:NO_2^- molar ratio was used in each dose. (varied - high).
Chlorides (b) indicates 5.0 mg/L Cl^- was used for each dose. (fixed - low).

CHAPTER V

HEMATOLOGICAL RESPONSES OF LARVAL *RANA CATESBIANA* TO SUBLETHAL NITRITE EXPOSURES

Introduction

Nitrite induced methemoglobin formation has been reported for various salmonid fishes (Brown and McLeay, 1975; Smith and Russo, 1975),including chinook salmon, *Oncorhynchus tschawytscha*, in freshwater and seawater (Crawford and Allen, 1977), and channel catfish, *Ictalurus punctatus* (Huey et al., 1977; Tomasso et al., 1979; Huey et al., 1980). Methemoglobin is oxidized hemoglobin, a derivative incapable of binding oxygen. The iron moiety in methemoglobin is ferric (Fe^{+++}) in contrast with the ferrous (Fe^{++}) of normal hemoglobin. Methemoglobin imparts an easily visible brownish color to blood. Methemoglobinemia is reduced in the presence of monovalent anions (Perrone and Mead, 1977; Russo and Thurston, 1977; Wedemeyer and Yasutake, 1978; Tomasso et al., 1979).

With the exception of a single short paper (Sullivan and Riggs, 1964) reporting methemoglobin in the redear turtle (*Chrysemys scripta*), an extensive literature search revealed no other reports of methemoglobin in nonfish aquatic vertebrates. For comparative purposes, research

was designed to determine if bullfrog tadpoles (*Rana catesbiana*) would develop methemoglobin when exposed to nitrite and, if so, whether increases in ambient chloride concentrations would offer protection against methemoglobinemia.

Materials and Methods

Bullfrog tadpoles (15-26 g) obtained during spring from a local supplier were held in 200-L tanks containing dechlorinated, continuously filtered tapwater at 23-25°C. Prior to and following a 5-day holding period, holding water was analyzed for ammonia and nitrite with calibrated Orion specific ion probes.

Tadpoles were transferred to 25°C, medium hardness (137 total hardness, mg/L), low chloride (5.0 mg/L), pH 7.3 water for static dose-response tests. Chemicals were added to 30-L aerated test tanks and mixed by a mechanical stirrer. After two hours 6 tadpoles were distributed into each test tank. Nitrite levels were monitored at 0, 12, and 24-h intervals, while other water quality variables (ammonia, total hardness, chloride, pH, temperature) were measured at 0 and 24-h intervals. Chemicals were reagent grade, and sodium nitrite was used as the source of nitrite.

Tadpoles were exposed to nitrite, concentrations (mg/L) of 50, 10, 5, 3, 1, and control (< 0.01) in the dose response experiment. Chloride inhibition tests employed 50 and 10 mg/l NO_2^- exposures with molar $Cl^-:NO_2^-$ ratios of 13:1 and 5.2:1, respectively.

Following 24-h exposure, blood was collected from individual tadpoles in heparinized capillary tubes. Total hemoglobin was quantified by the cyanomethemoglobin method, (Hainline, 1958) and blood methemoglobin was determined by a method modified from that of Evelyn and Malloy (1938).

Results and Discussion

Dose response experiments indicated that, although total hemoglobin levels were not altered, a significant, positive relationship was found between methemoglobinemia and nitrite concentration over the test range of 1.0 to 50 mg/L (Table VI). Percentage methemoglobin was highly significantly correlated ($p < 0.001$) to nitrite concentration in a double logarithmic fashion. The best fit regression model was log MHb (per cent total Hb) = 1.328 + 0.271 log NO_2^- (mg/L). Standard errors for the intercept and slope equalled 0.0209 and 0.0178, respectively; and the coefficient of determination (i.e. R^2 was 0.98.

The percentage MHb of all exposure groups shown in Table VI is significantly different from the mean of 5.7 percent MHb (ANOVA $p < 0.001$) for the control group. These results indicate that tadpoles are more resistant to nitrite induced methemoglobinemia than channel catfish under similar conditions (Huey et al., 1980). Channel catfish exposed to 5.0 mg/L nitrite developed methemoglobin levels of 90%; which are approximately three times those observed in tadpoles (Table VI). The reduced methemoglobin response of tadpoles probably is related to reduced nitrite uptake or to the presence of an efficient methemoglobin reductase system, or to both.

Tadpoles exposed to 50 mg/L nitrite at molar ratios of 13:1 ($Cl^-:NO_2^-$) and 5.0 mg/L at 5.2:1 ($Cl^-:NO_2^-$) had methemoglobin concentrations that were not elevated above the controls (Table VI). This suggests that chloride provides protection against methemoglobinemia at much lower concentrations in tadpoles than channel catfish. The latter species require a 17:1 ($Cl^-:NO_2^-$) ratio for full protection (Tomasso et al., 1979).

Resistance to nitrite induced methemoglobinemia in tadpoles is most likely an uptake phenomenon. Bullfrog tadpoles probably show gill ion absorption similar to fishes. Tadpoles at this stage (late larval) probably

have less gill surface area compared to fish, and, hence, a lower dose response would be expected. This could also account for increased efficacy of chlorides as nitrite toxicity protectors.

TABLE VI

HEMATOLOGICAL RESPONSES OF BULLFROG TADPOLES FOLLOWING 24-H EXPOSURES TO NITRITE FOR EACH EXPOSURE, SAMPLE SIZE EQUALLED 6 AND MEAN ± 1 STANDARD DEVIATION ARE GIVEN.

Exposure	Hemoglobin g/100 ml	Methemoglobin g/100 ml	Methemoglobin %
Controls	5.4 ± 0.9	0.3 ± 0.1	5.7 ± 5.7
1.0	5.1 ± 1.1	1.1 ± 5.1	21.4 ± 12.9
3.0	6.4 ± 0.7	1.5 ± 0.6	32.1 ± 11.5
5.0	6.4 ± 1.3	2.1 ± 0.4	34.3 ± 9.8
10.1	6.1 ± 0.7	2.6 ± 0.3	43.7 ± 8.6
50.0	5.7 ± 0.6	2.9 ± 0.7	51.0 ± 11.1
10.0a	6.1 ± 0.9	0.3 ± 0.1	5.6 ± 0.9
50.0b	4.9 ± 1.0	0.1 ± 0.1	2.0 ± 0.7

a and b included addition of chloride at molar $Cl^-:NO_2^-$ ratios of 13:1 and 5.2:1, respectively

CHAPTER VI

METHEMOGLOBIN LEVELS IN CHANNEL CATFISH *ICTALURUS PUNCTATUS*, EXPOSED TO NITRITE AND TRICAINE METHANESULPHONATE

Introduction

The most important hematological change in fishes and larval amphibians caused by exposure to nitrite is oxidation of hemoglobin to methemoglobin, a derivative incapable of binding oxygen (Smith and Williams, 1974; Russo et al., 1974; Huey et al., 1980). Channel catfish, *Ictalurus punctatus*, with about 90% of hemoglobin as methemoglobin can survive when quiescent, but die within minutes when disturbed, exhibiting symptoms (gulping of air, erratic swimming) that suggest anoxia as the cause of death (Huey et al., 1980). Fish with severe methemoglobinemia may survive by reducing locomotor activity.

Research was conducted to determine if nitrite induced methemoglobinemia in channel catfish would be ameliorated by concurrent exposure to tricaine methanesulphonate: TMS-222, MS-222. Tricaine methanesulphonate is a primary aromatic amine used for sedation or anesthetization (Dick, 1975) in fish research. My major hypothesis was that

TMS-222 would decrease methemoglobinemia by reducing locomotor activity, ventilation rates and, hence, nitrite uptake in exposed fish.

Materials and Methods

Channel catfish (11.3 ± 3.4 g) were maintained in a recirculating 500-L stream containing dechlorinated, continuously filtered tapwater at 24-25°C and fed trout pellets equal to ca. 1% wet body weight per day. Holding water was monitored daily for ammonia and nitrite with Orion specific ion probes. Twenty-four-hour static bioassay procedures were conducted in 30-L aerated glass tanks containing medium hardness (170 mg/L total hardness), low chloride (5.0 mg/L), pH 7.0 water at 25°C. Chemicals were added to each tank and mixed by a mechanical stirrer. TMS-222 was obtained from Ayerst Laboratories, Inc. Nitrite was added as reagent grade sodium nitrite, and a 0.02 M phosphate buffer was used to maintain pH at 7.0. Nitrite (NO_2^-), ammonia, alkalinity, chlorides, pH, dissolved O_2, and total hardness (Table VII) were measured at the beginning and conclusion of each trial. Catfish postabsorptive for 48-h were randomly allocated to one of the following three experimental treatments: 40 mg/L TMS-222, 40 mg/L TMS-222 and 4.0 mg/L nitrite, 4.0 mg/L nitrite, and a control in which neither chemical was added.

Following 24-h exposures, blood from the hemal arch of each of the 62 test fish was collected in heparinized capillary tubes. Total hemoglobin was determined from 20-µL samples by the cyanomethemoglobin method (Hanline, 1958), and blood methemoglobin was measured from 50-µL samples by the method of Evelyn and Malloy (1938). Sulfhemoglobin was quantified by a method described by Fairbanks (1976).

Results and Discussion

Hemoglobin for the 62 test fish averaged 7.6 g/100 ml with a standard deviation of 2.3 g/100 ml. Not only did methemoglobin levels differ significantly among the four groups (1-way ANOVA, $P < 0.0001$), multiple range testing indicated that each group was significantly different from each other (Student-Newman-Keuls, SNK, $\alpha = 0.05$). Similar results occurred when only the 3-treatment groups (without controls) were compared (1-way ANOVA, $P < 0.0001$), and again SNK indicated that each treatment group was drawn from populations with statistically different ($\alpha = 0.05$) methemoglobin levels. Mean methemoglobin concentrations occurred in the following sequence: controls < TMS-222 < TMS-222 and nitrite < nitrite (Fig. 2). TMS-222 produced a significant, but rather mild, ameliorating effect (ca. 17%

decrease) on nitrite induced methemoglobin formation. The combination exposure exceeded methemoglobin concentrations in controls by approximately 25 fold. Unexpectedly, TMS-222 alone resulted in a 6-fold increase in methemoglobin levels relative to controls. An additional exposure of 10 fish to TMS-222 confirmed that methemoglobin and not sulfhemoglobin was present. These fish had less than 0.1% sulfhemoglobin.

Nitrite at 4.0 mg/L produced the highest concentrations ($\bar{x}$ = 65.9%) of methemoglobin. This finding was expected due to the strong oxidizing potential of this chemical and corroborates results of Huey et al. (1980) obtained from experiments designed to define the nitrite-methemoglobin dose-response curve for channel catfish.

Significant amounts of methemoglobin appeared in catfish exposed to TMS-222 alone. Extensive work has documented many physiological and behavioral effects of TMS-222, including effects on fish blood components (eg. Phillips et al., 1957; Wedemeyer, 1970; Houston et al., 1971; Soivio et al., 1974; Hattingh, 1977; Smit et al., 1979a, b, c). Nevertheless, the finding of high amounts methemoglobin following TMS-222 exposure apparently constitutes the first mention of this phenomenon. TMS-222 is similar in chemical structure to aniline. Sulfonamides,

lidocaine, and many other aniline derivatives are known to enhance production of hydrogen peroxide and, hence, can cause the formation of methemoglobin by this mechanism (Bodansky, 1951; Kiese and Waller, 1950). When methemoglobin is present, oxidation of the two sulfhydral groups of hemoglobin can occur in the presence of some hemoxidants resulting in sulfhemoglobin formation (Allen and Jandl, 1961). However, negligible amounts of sulfhemoglobin ($\leq 0.1\%$) were found relative to methemoglobin.

Neither additive nor synergistic effects were noted in catfish exposed to nitrite and TMS-222 in combination. In fact, the presence of TMS-222 significantly reduced methemoglobin levels relative to those of fish receiving nitrite alone. I suggest that fish exposed to TMS-222 had reduced nitrite uptake owing to reduced ventilation rates. Reduction in ventilatory rate is a result of respiratory center depression, decreased locomotor activity, or both. Nitrite competes with chloride and bicarbonate ions for entrance sites on the gill surfaces (Tomasso et al., 1979; Huey et al., 1980) and some uptake competition with TMS-222 is possible.

Tricaine methanesulphonate-induced methemoglobin should be considered by researchers who use large doses for fish immobilization and surgery, because treated fish may

have methemoglobin levels which could lead to decreased oxygen carrying capacity and partial anoxia. Potentially false or misleading results from TMS-222-induced methemoglobinemia can be reduced or eliminated if fish are allowed sufficient time to recover from anesthesia.

Fig. 2--Mean ($\bar{x} \pm s$) methemoglobin levels as percentage of total hemoglobin in channel catfish (*Ictalurus punctatus*) following 24-h exposures to test conditions. Numbers in parentheses indicate number of fish sampled.

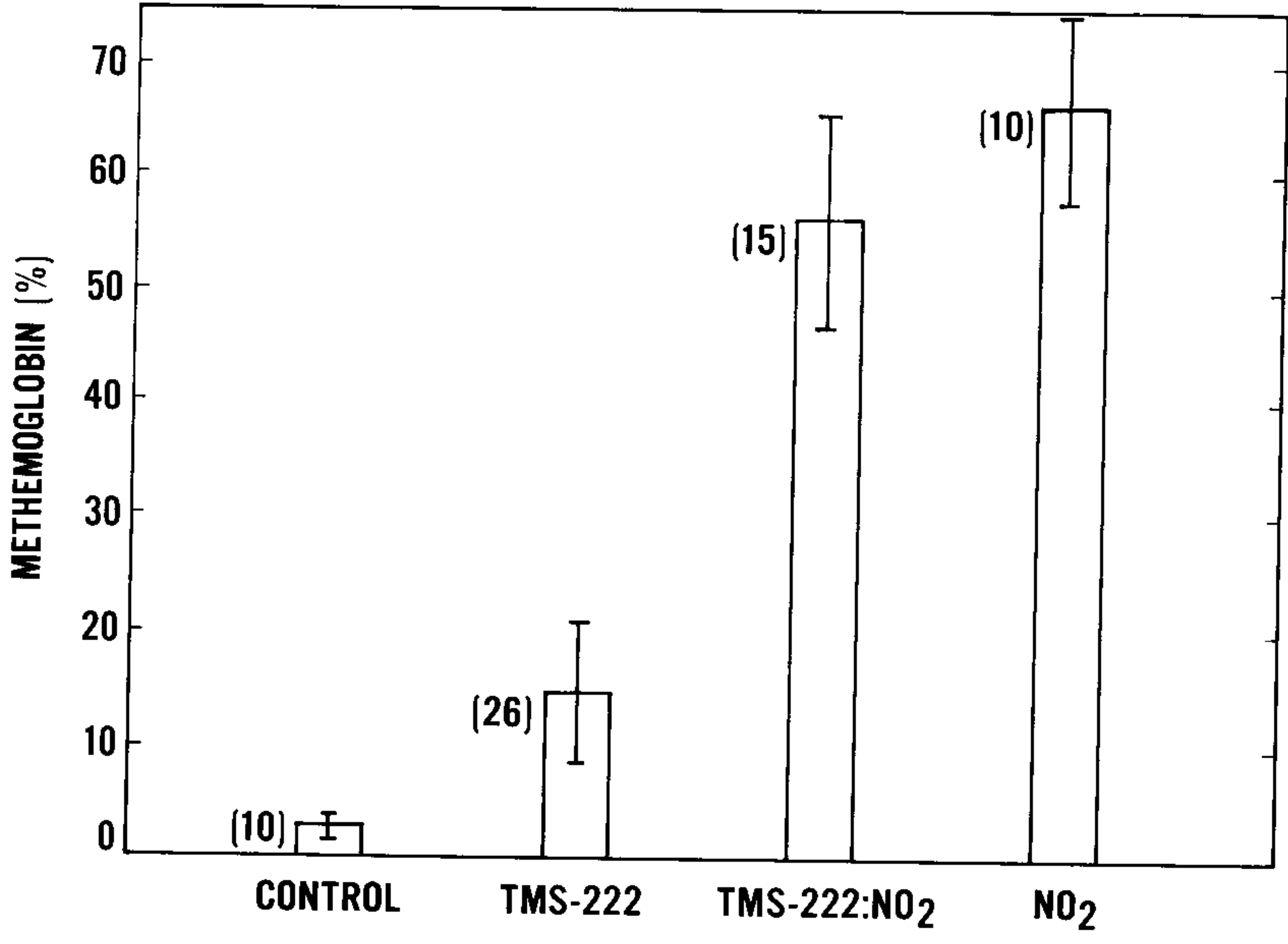

METHEMOGLOBIN (%)
70
60
50
40
30
20
10
0
(10)
(26)
(15)
(10)
CONTROL
TMS-222
TMS-222:NO_2
NO_2

TABLE VII

WATER QUALITY VARIABLES MEASURED AT THE BEGINNING AND END OF EACH 24-H EXPERIMENT.

Group	Concentration mg/L Nitrite		Ammonia		Alkalinity		Total Hardness		Chlorides		pH	
	0	24	0	24	0	24	0	24	0	24	0	24
Control	0.1	0.1	0.1	0.1	112	-	171	171	4.9	4.9	7.0	7.0
TMS-222	0.1	0.1	0.1	0.1	117	117	171	171	5.0	5.0	7.0	6.9
Nitrite + TMS-222	4.0	3.8	0.1	0.1	120	120	120	171	5.0	4.9	7.0	6.9
Nitrite	4.0	3.7	0.1	0.1	120	115	171	171	5.0	4.9	7.0	6.9

CHAPTER VII

TMS-222 INDUCED METHEMOGLOBINEMIA IN CHANNEL CATFISH

Introduction

Recent research conducted in the laboratory indicated that exposure to 40 mg/L TMS-222 for 24-h produced 13% methemoglobin in channel catfish. Experiments were designed to describe the TMS-222 methemoglobin dose/time response curve and to quantify the rate of recovery of channel catfish from methemoglobinemia induced by a commonly-applied dose of TMS-222.

Methods and Materials

Channel catfish (8.3 ± 1.7 g) obtained from a local fish hatchery were maintained in a 500-L biofiltered water-recirculating system containing dechlorinated tapwater at 25°C. Fish were fed Purina Catfish Chow at a maintenance diet of ca. 1.0% body weight per day, but were held postabsorptive 48-h prior to experimentation. Holding water was monitored daily for ammonia (Orion specific ion probe) and nitrite (APHA, 1975), neither of which exceeded a concentration of 0.1 mg/L during the 10-day holding period prior to testing.

Two separate series of trials investigated the rates of development and loss (i.e. recovery) of MHb in channel catfish. In the former, four groups of fish (N = 16 to 21) were exposed to 70 mg/L TMS-222 for intervals of 10, 20, 30, and 40 minutes. A group of 10 fish in TMS-222-free water served as a control. In the recovery phase of this research fish were exposed to 70 mg/L TMS-222 for 30 min., then placed in TMS-222-free water. At intervals of 30, 60, 90, and 120 min., a group of 5-6 fish were removed, and methemoglobin levels were measured. All trials were conducted in 25°C, O_2-saturated hard (total hardness 168 mg/L), low-chloride (5.0 mg/L) water. Water pH was maintained at pH 7.0 ± 0.1 with a 0.02 M phosphate buffer. TMS-222 was obtained from Ayerst Laboratories Inc., and all chemicals were mixed with a mechanical stirrer. TMS-222 concentrations were quantified using a method modified from Walker and Schoettger (1967). The maximum deviation from nominal TMS-222 concentrations was less than 10.0%.

Blood was collected from the hemal arch of each fish in heparinized capillary tubes. Methemoglobin was measured from 50-µL samples by the method of Evelyn and Malloy (1938).

Results and Discussion

Mean methemoglobin concentrations for the 10 control fish equalled 0.2 g% (Table VIII). Subsequent to a 40-min. exposure to 70 mg/L TMS-222, MHb concentrations increased to 0.6 ± 0.27 g%. Lethality did not occur in any group; however, exposed fish were immobilized except for opercular movements. This condition is described as stage one anesthesia by Huish (1972). Time and g% MHb were significantly correlated ($r = 0.63$, $p < 0.0001$) during exposures. The best-fit least squares regression equation equalled MHb (g%) = 0.186 + 0.012 time (min). Standard errors for the slope and intercept were ± 0.002 and 0.043, respectively (Fig. 3).

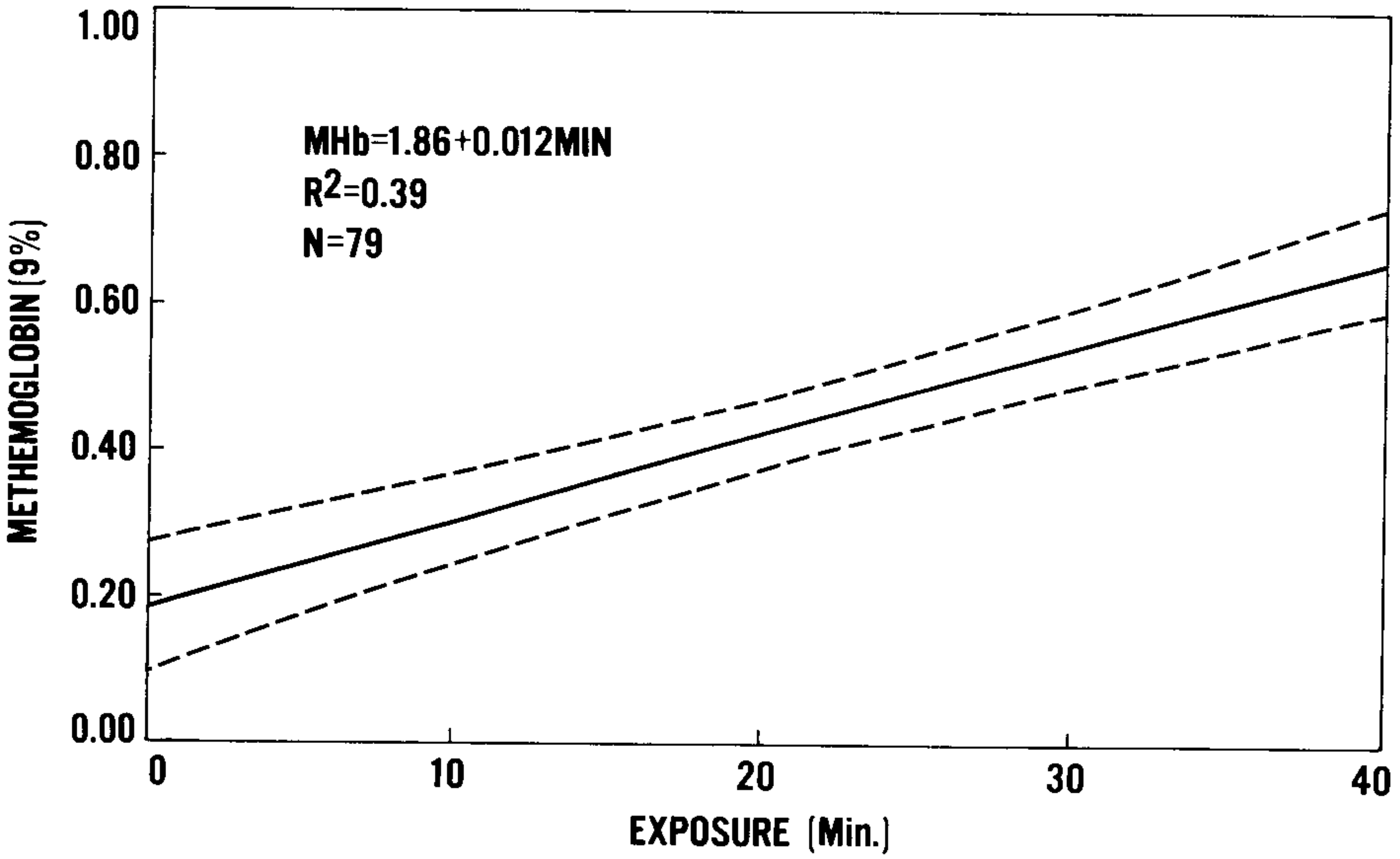
In the recovery experiment, all fish were in stage one anesthesia when transferred; however, after 30 min. they were capable of swimming, a condition Huish (1972) termed stage two anesthesia. Recovered fish appeared normal and reactive to stimuli after 120 min. (Huish, stage 3). During recovery, time and MHb concentrations were significantly negatively correlated ($r = 0.53$, $p = 0.004$). The recovery regression equation was MHb (g%) = 0.363 - 0.002 time (min). Standard errors for the slope and intercept were ± 0.0006 and 0.0434, respectively (Fig. 4).

Methemoglobin formation during TMS-222 exposure is rapid, although total methemoglobin concentration did not

approach lethal concentrations of near 90% described by Huey et al. (1980) in nitrite exposed fish. Recover from TMS-222-induced methemoglobin occurs rapidly when fish are placed in anesthetic free water. After 90 min. catfish had MHb concentrations similar to controls (Fig. 4).

Methemoglobin formation should be considered by researchers who use tricaine methanesulphonate for fish immobilization and surgery, because treated fish have increased methemoglobin concentrations which could lead to decreased oxygen carrying capacity. Similarly, commercial fish farmers should be careful when using TMS-222 to sedate fish for transport. Fish researchers should avoid collecting physiological or behavioral data on fish exposed to TMS-222 until sufficient recovery time has elapsed and MHb has returned to normal concentrations. Following exposure to 70 mg/L of TMS-222 for 30 min. at 25°C, recovery from methemoglobin in channel catfish of this size range requires 90 to 120 minutes.

Fig. 3--Relation between MHb concentrations and time in channel catfish (*Ictalurus punctatus*) exposed to 70 mg/L TMS-222. The solid line is the regression line and dashed lines indicate the 95% confidence belts about the regression line.



MHb=1.86+0.012MIN
R2=0.39
N=79
METHEMOGLOBIN (9%)
1.00
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
EXPOSURE (Min.)

Fig. 4--Relation between MHb concentrations and time in channel catfish (*Ictalurus punctatus*) recovering from a 30 minute exposure to 70 mg/L TMS-222. The solid line is the regression line and the dashed lines represent the 95% confidence belts about the regression line.

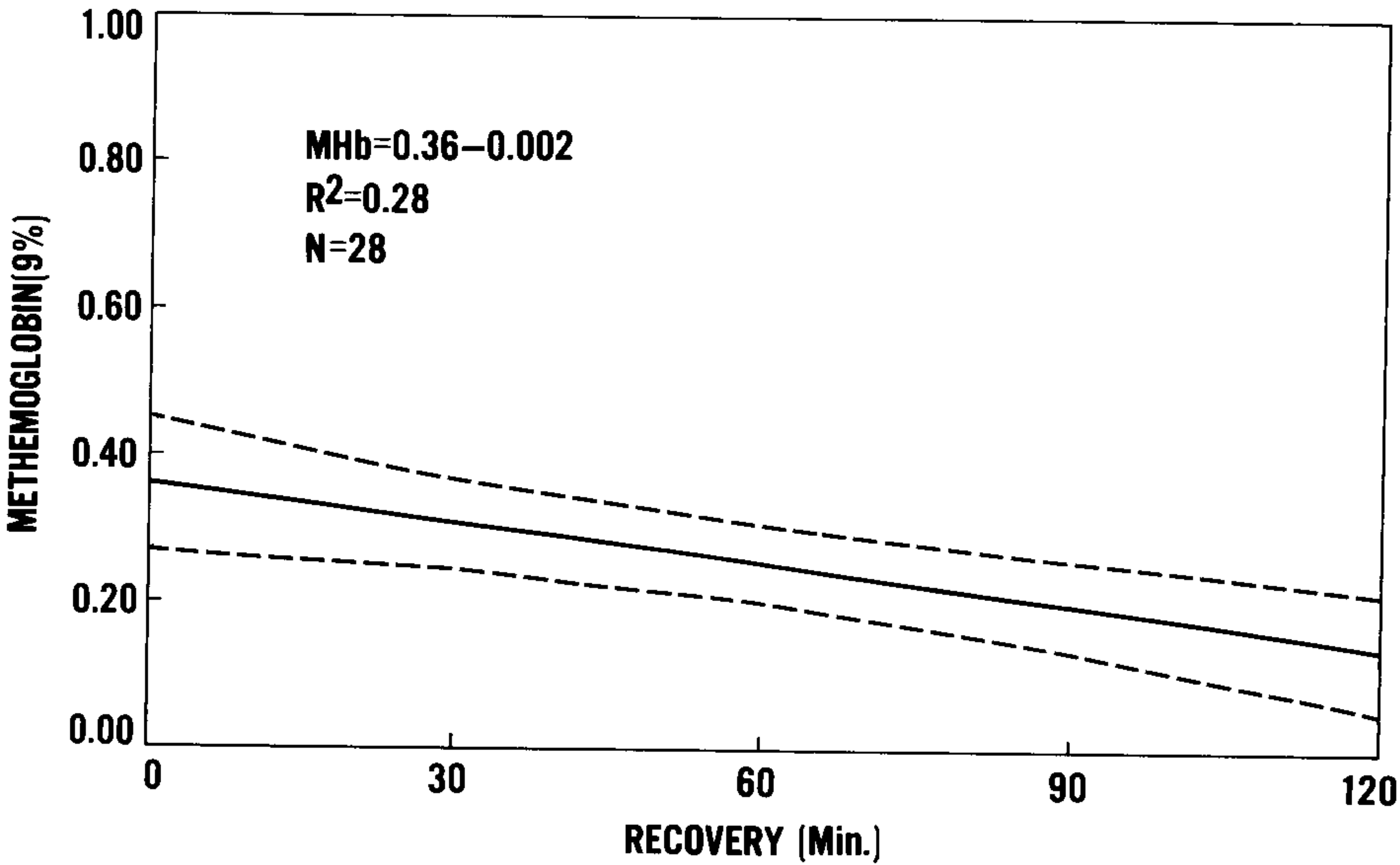

MHb=0.36–0.002
R2=0.28
N=28
METHEMOGLOBIN(9%)
1.00
0.80
0.60
0.40
0.20
0.00
0
30
60
90
120
RECOVERY (Min.)

TABLE VIII

METHEMOGLOBIN CONCENTRATIONS OF CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, EXPOSED TO 70 MG/L TMS-222. RECOVERY GROUP HAS INITAL 30 MINUTE EXPOSURE, THEN VARIOUS RECOVERY TIMES IN TMS-222 FREE WATER.

Group	Time (minutes) for exposure and recovery	N	Methomoglobin Concentration (g%) x ± s
Exposure	0 (control)	10	0.20 ± 0.00
	10	16	0.20 ± 0.12
	20	16	0.57 ± 0.20
	30	21	0.52 ± 0.16
	40	16	0.62 ± 0.28
Recovery	0 (initial)	5	0.32 ± 0.16
	30	6	0.33 ± 0.16
	60	6	0.30 ± 0.13
	90	5	0.16 ± 0.09
	120	6	0.13 ± 0.10

CHAPTER VIII

THE EFFECTS OF ACCLIMATION TEMPERATURE ON NITRITE-INDUCED METHEMOGLOBIN FORMATION AND RECOVERY IN CHANNEL CATFISH *ICTALURUS PUNCTATUS*

Introduction

Temperature-toxicity interactions are known to be complex; however, in a literature survey, Cairns et al. (1975) concluded that elevated temperatures increase the acute toxicity of most pollutants in short (48-h) exposures. Research was designed to determine the effects of acclimation temperatures on methemoglobin formation and recovery in catfish exposed to sublethal concentrations of nitrite.

Materials and Methods

Channel catfish (12.5 ± 3.2 g) obtained from a local fish hatchery were held in the laboratory at 24°C for 5 days, after which fish were randomly separated into 3 groups and acclimated to 10°, 20°, or 30°C at a rate not exceeding 1°C per day. Acclimation temperatures were controlled by Haake circulating thermoregulators. Once at the desired acclimation temperature, fish were held for 10

days prior to testing. During acclimation, fish were fed at 1% body weight per day (Purina Catfish Chow), except the $10^{o}C$ group which were fed ca. 0.5% body weight per day. Fish were held postabsorptive 4 days prior to testing. External biofiltration systems were employed, and both ammonia and nitrite concentrations were monitored daily with an Orion specific ion probe and azo-dye methods (APHA, 1975). Neither ammonia nor nitrite concentrations exceeded 0.2 mg/L.

Six control, 10 exposure, and 10 recovery fish were used for each of the three acclimation temperatures. Twenty fish were exposed to 3.0 mg/L nitrite (NO_2^-) for 24h, and blood was collected from 10 of these fish by removing their tails at the caudal peduncle. The MHb of each fish was determined from a 50-µL sample (Evelyn and Malloy, 1938). MHb was determined in the other 10 fish after they were in nitrite free water for 12-h. Controls were handled in the same manner, with MHb determined on three fish bled after 24-h in nitrite-free water and three other fish after sham handling and 12-h "recovery".

Tests were conducted in 30-L glass aquaria containing hard (160 mg/L total hardness), low chloride (5.0 mg/L), O_2-saturated water, which was prepared by reconstituting distilled water (U.S.E.P.A., 1975). Water pH was maintained at 7.0 ± 0.1 using a 0.02 M phosphate buffer,

and nitrite was added as reagent grade sodium nitrite. A mechanical stirrer was employed to mix chemicals added to each tank. Nitrite (NO_2^-) was measured initially and at 12-h intervals. When necessary, sodium nitrite was added to return the test tanks to their nominal concentrations (Table IX).

A two-way ANOVA was used to test the data for significant 2-way (MHb x Temp) interactions. Since significant interactions ($p < 0.05$) were noted, a quasi-F ratio test was employed to subdivide variation within the data matrix. All possible column and row combinations were tested using an aposterori, Duncan's multiple range test with $\alpha = 0.05$.

Results and Discussion

Control groups consisting of six fish per acclimation temperature had methemoglobin concentrations of 0.15 ± 0.5 (g%) and were not significantly different from each other (Fig. 5). At 10°C, methemoglobin formation occurred (2.18 ± 0.79 g); however, the MHb concentration of the exposure and recovery group acclimated to 20°C had mean MHb concentrations (g%) of 2.18 ± 0.79 as compared to 1.80 ± 0.48 for the recovery group; again these differences were not significant. No significant differences in MHb

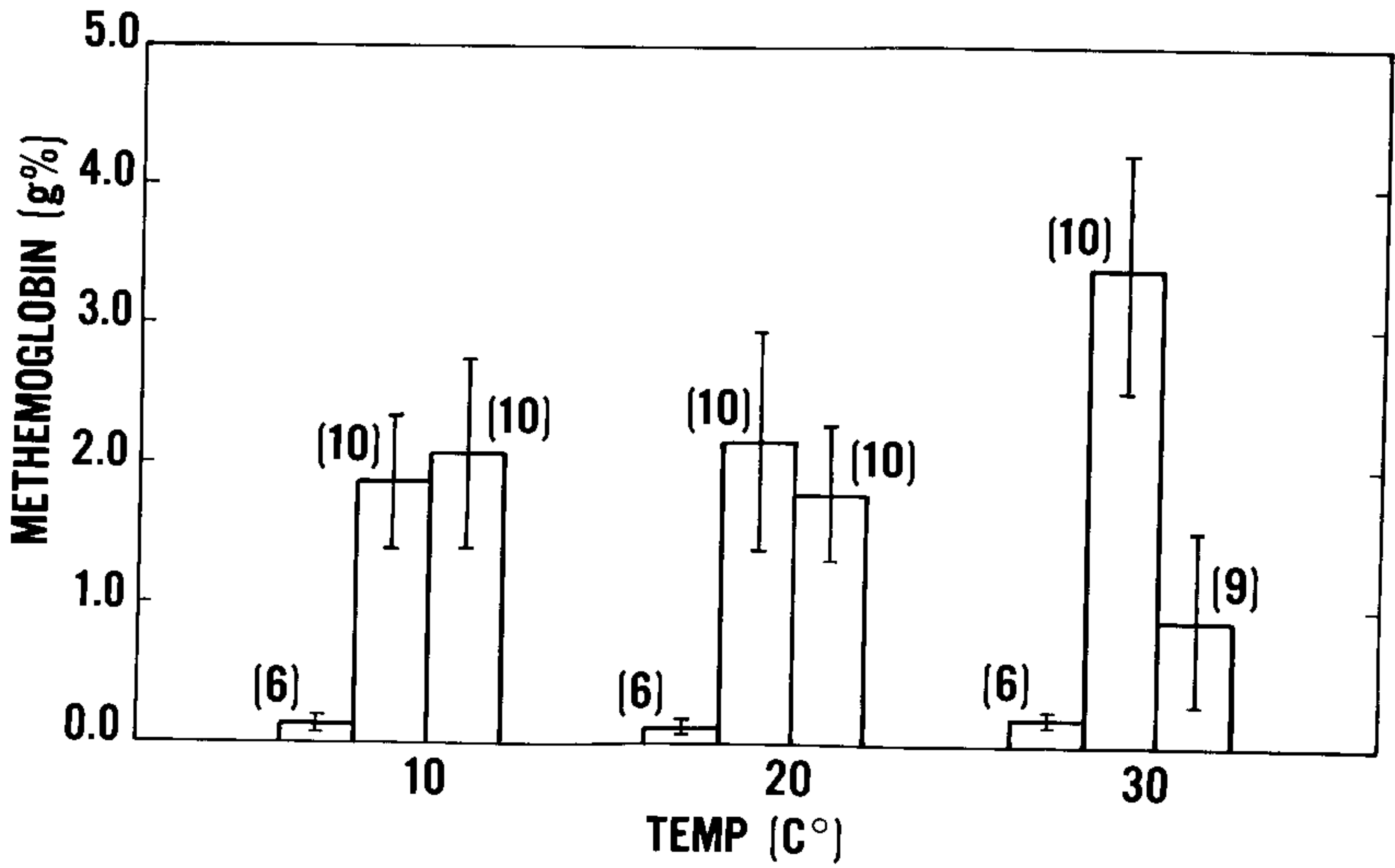
concentration were noted among the four exposure and recovery groups at $10^{o}C$ and $20^{o}C$ (Fig. 5). However, in the $30^{o}C$ group, MHb concentration was significantly greater than the two groups acclimated to lower temperatures. MHb concentration (g%) at $30^{o}C$ was 3.42 ± 0.85 after 24-h of exposure (Fig. 5). This group had a MHb formation rate of 0.13 g/h over the 24-h period, compared to 0.09 g/h at $20^{o}C$ and 0.07 g/h at $10^{o}C$ (Table IX). Significant recovery from methemoglobinemia occurred at $30^{o}C$ in 12-h (Table IX). Loss of MHb amounted to 0.21 g/h for the 12-h recovery period compared to 0.03 g/h at $20^{o}C$ and no loss of MHb at $10^{o}C$. Total decrease in MHb (g%) of 73% in 12-h to 0.92 ± 0.61 was observed in the $30^{o}C$-acclimated fish. One fish in the $30^{o}C$ recovery group was moribund and was not sampled.

Nitrite-induced methemoglobin formation increased with each $10^{o}C$-increase in temperature. Toxicant temperature interactions in the exposure groups indicate increased MHb concentrations occur at higher temperatures, most notably the significant increase in MHb formation at $30^{o}C$ (Fig. 5). These data do not necessarily indicate that nitrite would be more lethal at $30^{o}C$, since the rate of recovery from nitrite-induced methemoglobinemia also is increased at $30^{o}C$ (Fig. 5). Only a slight change is noted in recovery rates between 10° and $20^{o}C$, but the rate increases seven-fold between $20^{o}C$ and $30^{o}C$.

Increased ventilation rates at elevated temperature may result in increased nitrite uptake through the gill surface promoting elevated blood nitrite concentrations and higher concentrations of nitrite oxidized hemoglobin (MHb). Also recovery may be facilitated by increased loss of unbound nitrite through the gills at higher temperatures. The seven-fold increase in recovery rate noted between 20°C and 30°C is of a greater magnitude than the expected two-fold increase in metabolic rate for a 10°C increase in temperature following the Van't Hoff rule. Cairns et al. (1978) noted that, in some cases, animals exposed to low concentrations of toxicants had increased survival at high temperatures, and my results seem to corroborate these data. As temperature and metabolic rate increase, a fish's toxicant uptake will increase (Cairns et al., 1978), but rates of mechanisms which act to metabolize toxicants directly or to counteract toxicity-induced conditions (such as MHb) also increase. I speculate that the activity of the methemoglobin-reductase system, proposed by Cameron (1971) and shown to be present in channel catfish (see Chapter IX), increases at higher acclimation temperatures. This could be a factor in the rapid recovery rate observed at 30°C.

Smith and Heath (1979) exposed several species of fishes to toxicants at different acclimation temperatures and noted varied responses between species. Some of this variation might be explained by differing temperature responses of various enzymatic systems involved in counteracting toxicity in different fish species. In short-term exposures of low doses of nitrite, elevated temperature seems to accelerate the recovery process; however, the interactions between toxicity, temperature, and physiological mechanisms for resisting toxicity are complex and further investigations are indicated.

Fig. 5--The effect of acclimation temperature on methemoglobin formation and recovery in nitrite exposed channel catfish, Ictalurus punctatus. The three vertical bars at each acclimation temperature represents mean MHb concentrations for controls, 24-h exposure, and 12-h recovery groups, respectively. The vertical lines indicate a ± 1 standard deviation from the mean; and the number in parenthesis gives the sample size.



METHEMOGLOBIN (g%)
5.0
4.0
3.0
2.0
1.0
0.0
(6)
(10)
(10)
(6)
(10)
(10)
(6)
(10)
(9)
10
20
30
TEMP (C°)

TABLE IX

NITRITE (NO_2^-) CONCENTRATIONS (MG/L), PH, AND TEMPERATURE FOR 24-H ACCLIMATION TEMPERATURE EXPOSURES.

Group		30°C			20°C			10°C		
		NO_2^-*	pH	°C	NO_2^-*	pH	°C	NO_2^-*	pH	°C
Exposure	0-h	2.92	7.15	30.4	3.10	6.94	20.2	3.01	7.12	10.2
	12-h	2.74	7.38	30.0	2.60	7.20	20.3	2.79	7.30	10.2
	24-h	2.60	7.37	30.0	3.00	7.25	20.0	2.71	7.41	10.4
Recovery	0-h	0.01	7.40	30.5	0.00	6.98	20.1	0.00	7.30	10.2
	12-h	0.01	7.41	30.0	0.05	7.37	20.0	0.01	7.40	10.3
Control	0-h	0.00	7.30	30.5	0.00	7.01	20.2	0.00	7.15	10.2
	12-h	0.00	7.43	30.0	0.00	7.47	20.3	0.00	7.19	10.2
	24-h	0.01	7.70	30.0	0.03	7.45	20.0	0.00	7.20	10.3
	36-h	0.03	7.71	30.0	0.05	7.43	20.0	0.01	7.26	10.4

*Nitrite was added in exposure groups to replace decay; NH_3 was below 0.1 mg/L in all trials.

CHAPTER IX

A METHEMOGLOBIN REDUCTASE SYSTEM IN CHANNEL CATFISH *ICTALURUS PUNCTATUS*

Introduction

Channel catfish, *Ictalurus punctatus*, exposed to water-borne nitrite (5.0 mg/L) rapidly develop methemoglobin. Concentrations approaching 90% MHb have been observed (Huey et al., 1980). Rapid recovery occurs when previously dosed fish are placed in nitrite-free water. Within 24 hours, methemoglobin concentrations have been observed to decrease to about 13% (Huey et al., 1980). The mechanism by which recovery from methemoglobin occurs is the subject of speculation. Mammals possess a methemoglobin reductase system capable of converting methemoglobin to hemoglobin (Jaffe, 1964). Cameron (1971) hypothesized that a methemoglobin reductase system could be functioning in fishes. Indirect evidence supporting this hypothesis for channel catfish is provided by Huey et al. (1980). Lovell (1979) stated that recovery from methemoglobinemia results from the release of erythrocytes from the spleen and suggests that methemoglobinemia is not a reversible condition in channel catfish. A screening

method (Kaplin et al., 1970) used to detect NADH-methemoglobin reductase activity in humans was modified to test for the presence of this enzyme system in channel catfish.

Materials and Methods

Channel catfish, Ictalurus punctatus, ($\bar{x}$ = 28 g) obtained from a local fish hatchery, were maintained in a biofiltered water recirculating system containing dechlorinated tapwater, and were fed Purina Catfish Chow at a maintenance diet of ca. 1% body weight per day. Holding water was monitored daily for ammonia (Orion specific ion probe) and nitrite (APHA, 1976), neither of which exceeded 1.0 mg/L during the 20-day holding period.

Ten channel catfish were randomly selected, their tails were severed at the caudal peduncle, and four capillary tubes of blood were collected from each fish. The presence of NADH-MHb reductase activity was examined by the spot screening method of Kaplan et al. (1970). In this screening test the dye dichlorophenolindophenol (DCIP) is reduced by nicotinamide adenine dinucleotide (NADH). During this reaction NADH, which fluoresces in the presence of long wave UV light, is oxidized to NAD which is not fluorescent. Blood is treated with sodium nitrite to oxidize the hemoglobin. The method was modified to compensate for the relatively small hematocrits (30.0 ±

3.0 of these catfish, which would prolong the time required for disappearance of fluorescense. The collected blood was sealed in heparinized capillary tubes and centrifuged in a clinical centrifuge for 3 minutes. Packed red blood cells were removed and adjusted with 0.75% saline to a hematocrit of approximately 45. An undiluted 20-µL sample of this blood was then used in the screening test. Two MHb-reductase "deficient" controls with fish and human blood (described by Kaplin et al., 1970), a reagent blank containing no blood, and an active human blood standard were also prepared. Each 20-µL blood sample was placed into a test tube, 5 µL of freshly prepared (1.24%) $NaNO_2$ was added, and the mixture was allowed to stand for 30 min. at room temperature. Then 40 µL of 1% saponin and 200 µL of a mixture containing 0.7 mM NADH, 0.19 mM DCIP, and 0.27 mM EDTA in a 0.06 M Tris-HCL buffer (pH 7.6) were added to the nitrited blood. Ten fish samples, both "deficient" controls, the human active standard, and the reagent blank were then incubated at 45°C in a water bath. All samples, controls, the standard, and the reagent blank were spotted (10 µL) on Whatman filter paper at 0, 10, and 30 minutes. The spots were dried under a hot air blower and examined under long wave UV light for fluorescence.

Results and Discussion

All fish tested showed MHb-reductase activity. All "0 minute" spots fluoresced in the presence of NADH, but after 30 min. no fluorescence was noted in any of the fish samples or the human active standard. The two "deficient" controls and the reagent blank exhibited fluorescence at 0, 30, and 60 minutes (Table X).

These results comprise the first report that a MHb-reductase system is present in a fish species. Normal mammalian blood defluoresces in 30 min. as did our channel catfish samples. With our modifications, this method can be used to test fish blood for MHb-reductase activity.

These findings suggest that this active reductase system contributes to the rapid recovery from methemoglobinemia in channel catfish placed in nitrite free water (Huey et al., 1980). This also supports Cameron's (1971) hypothesis that a methemoglobin reductase system is present in fish and refutes Lovell's claim of non-reversible methemoglobinemia in channel catfish. Work is now in progress to quantify MHb-reductase activity in channel catfish.

TABLE X

METHEMOGLOBIN REDUCTASE ACTIVITY IN CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) WITH + INDICATING FLUORESCENCE, - INDICATING LOSS OF FLUORESCENCE AND MHB-REDUCTASE ACTIVITY.

Group	0 min.	30 min.	60 min.
10 channel catfish	+	-	-
Active human standard	+	-	-
Heat inactivated human control	+	+	+
Heat inactivated channel catfish control	+	+	+
Reagent blank	+	+	+

CHAPTER X

INTEGRATION AND CONCLUSIONS

Comparative Toxicology

Nitrite in appropriate amounts was toxic to all species of animals tested in this study. Aquatic salamander larvae (*Ambystoma texanum*) were selected for study because they often inhabit stagnant, warm, temporary ponds where oxygen tensions are low. It is probable that elevated nitrite concentrations may exist in such pools, if high living biomass is present and if anerobic denitrification in the sediments occurs (Boyd and Hollerman, 1980). Low nitrite concentrations were found (<1.0 mg/L) in the shallow pond where salamander larvae were collected, and these organisms were the most nitrite sensitive species tested in this study. A 96-h LC_{50} of 1.09 mg/L was calculated, which makes the salamanders six times more sensitive than crayfish (*Procambarus simulans*). Bluegill (*Lepomis macrochirus*) seemed much less sensitive with an LC_{50} greater than 200 mg/L; however, this comparison is tenuous because the bluegill toxicity test was only a 48-h exposure. Larvae of another amphibian species (*Rana catesbiana*) tested as larvae had 100% survival after a 24-h

exposure to 50 mg/L nitrite. Crayfish resistance to nitrite lethality is partially explained by their blood oxygen carrying pigment, since the copper moiety in hemocyanin is more difficult to oxidize than the iron in hemoglobin (Felsenfeld and Printz, 1959). Acute and sublethal studies show a wide interspecific and some intraspecific variation response to nitrite exposure. This information is summarized in Table XI.

Nitrite-induced lethality in vertebrates is caused by tissue anoxia from methemoglobinemia. Resistance to nitrite toxicity in aquatic animals is probably a function of the uptake rate of the NO_2^- ion through the gills, the ability of the respiratory pigment to resist oxidation, and the conversion rate of an oxidized pigment back to the reduced state. Intraspecific differences in toxicity are due to differences in these phenomena. Recovery from methemoglobinemia most likely depends on how efficiently the NADH-methemoglobin reductase system functions. Gill permeability and the efficiency of monovalent ion uptake sites are two variables that could affect NO_2^- uptake in aquatic species. Epidermal absorption of nitrite may occur in amphibians in addition to gill absorption, which may increase nitrite uptake rates. All intraspecific toxicity comparisons are somewhat tenuous due to differences in biochemical responses to a given toxicant and in water

quality. Since the mode of action of nitrite is generally known to be hemoxidation, interspecific comparisons probably have some validity in animals with the same respiratory pigment.

Methemoglobin

Methemoglobin formation was used an an indicator of nitrite toxicity during this study. Crawford and Allen (1977) suggest that nitrite may oxidize compounds other than hemoglobin, leading to toxic reactions and death; however, no data have been produced to indicate what these compounds or toxic reactions might be. It is suspected that, in short term exposures, methemoglobinemia is the mechanism causing lethality. Channel catfish and bullfrog tadpoles had increased quantified concentrations of methemoglobin after exposure to nitrite (Fig. 2, Table VI), and qualitative examination of the blood from nitrite-exposed salamander larvae and bluegill revealed the brown-colored blood characteristic of methemoglobinemia. Channel catfish were more susceptible to nitrite-induced methemoglobin than were the tadpoles. Most likely this result was influenced by the developmental stage of the tadpoles. Test tadpoles had already absorbed most of their external gill structures and thus had limited gill area for nitrite absorption. No visual difference was noted in the

"color" of the hemolymph of nitrite exposed and control crayfish. All species of aquatic vertebrates tested were susceptible to nitrite-induced methemoglobinemia when ionic conditions of the test water permitted trans-epithelial (gill) transport of nitrite. Alimentary uptake of nitrite is possible in rats (Newberne, 1979), but this type of uptake has not been reported in aquatic organisms.

Oxidation of Hemoglobin by TMS-222

Nitrite was known to be a strong hemoxidant; however, the hemoxidation potential of TMS-222 had not been previously investigated. In an experiment designed to test the potential of TMS-222 as an agent to treat nitrite-induced methemoglobinemia, the anesthetic caused hemoxidation (13% MHb) in channel catfish. This value represents a six-fold increase over the controls and followed a normal sedation dose of 40 mg/L for 24-h. The oxidation potential of TMS-222 was significant, but much less potent than nitrite. In combination with nitrite, TMS-222 caused a significant decrease in the percentage methemoglobin formed in a 24-h exposure relative to fish exposed to nitrite alone.

Chloride Protection

The toxicity of nitrite is dependent on the composition of test water. Chlorides, bicarbonate, and

other monovalent anions can alter the uptake of nitrite. In all species examined in sublethal or lethal tests, with the exception of bluegill, chloride ions had a significant ameliorating effect upon nitrite toxicity. Salamander larvae in water containing 300 mg/L chloride (Cl^-) survived 96-h of nitrite exposure at a dose 10 times greater than their low chloride (5.0 mg/L) LC_{50}. Crayfish treated in a similar manner survived 16 times their low chloride exposure EC_{50}. Tomasso et al. (1979) found that a 17:1 $Cl^-:NO_2^-$ ratio gave full protection against nitrite toxicity in channel catfish over a wide range of doses. Complete protection against nitrite-induced methemoglobinemia was noted at a ratio of 5.2:1 in bullfrog tadpoles. These results are similar to data obtained by other researchers for salmonid species (Perrone and Meade, 1977; Wedemeyer and Yasutake, 1978; Russo et al., 1981). In the experiment with bluegill, no decrease in toxicity was found in fish exposed to nitrite in high chloride (18:1) $Cl^-:NO_2^-$ water, conversely the toxicity was somewhat greater than in the low chloride group. Since tests were of shorter duration, concentrations exceeded those used in other trials indicating that apparently chlorides have a maximum protection limit. Nevertheless, these findings warrant further study.

Huey et al. (1980) and Russo et al. (1981) found enhanced nitrite toxicity at reduced pH, and Meade and Perrone (1980) found increased plasma nitrite concentrations at reduced pH. Investigators have hypothesized that nitrite toxicity results in part from HNO_2 (Wedemeyer and Yasutake, 1978; Huey et al., 1980). Nitrite dissociates in water to HNO_2 and NO_2^-, with the former being the predominant ion at pH's below 4.5. These results indicate that toxicity is enhanced at low pH for the species tested (Chapters III and IV).

The overall relationship between nitrite toxicity, chloride and hydrogen ion concentration can be diagramed as follows:

Ionic Form	NO_2^-	HNO_2
pH	(8.0) High	Low (4.5)
Main Transport Mechanism	Anion Transport	Diffusion
Chloride "Protection"	Yes	No

At a pH of 7.0 and higher, most nitrite is present as NO_2^- and is transported across the gills by means of anion gates (Huey et al., 1980). Chloride offers competitive inhibition to this type of transport and, thus, provides amelioration of nitrite uptake; however, at low pH (5.0-4.0), sufficient amounts of HNO_2 appear and cross the

gill epithelium by simple diffusion processes which external chloride concentrations do not influence. Enhanced toxicity at low pH is the result of cross-gill diffusion which apparently causes death before homeostatic mechanisms such as excretion and the MHb-reductase system can function. Apparently, the anionic transport is slow and gives time for the protective mechanisms to intercede and extend resistance time. This extension of resistance time may be the result of an activated methemoglobin reductase system. Both forms of nitrite are toxic, but increased transport rate of the free acid form (HNO_2) causes increased toxicity at low pH.

Recovery and Methemoglobin Reductase

Channel catfish recover from severe nitrite-induced methemoglobinemia (80%) in 24-h when placed in nitrite-free water (Huey et al., 1980). Recovery mechanisms are the subject of some speculation. Lovell (1979) stated that methemoglobinemia is not a reversible process in fishes, and that recovery is due to the synthesis of new red blood cells. Data collected during this research indicate that recovery from nitrite or TMS-222 induced methemoglobinemia occurs too rapidly to support Lovell's contentions. Cameron (1971) found no red blood cell synthesis in rainbow trout (Salmo gairdneri) held in hypoxic conditions for a

period of 10 days. Cellular oxygen starvation caused by methemoglobinemia should activate homeostatic mechanisms similar to those caused by hypoxia. If this contention is correct, no additional erythrocyte synthesis should result from short-term exposure to nitrite or other hemoxidants. Experiments conducted with TMS-222 and nitrite show no significant differences in total hemoglobin between experimental groups (Chapter VI), indicating no change in red blood cell count during these short-term exposure periods.

Results reported in Chapter IX indicate that a NADH-methemolobin reductase system is present in channel catfish. An active MHb-reductase system would explain, at least in part, the rapid recovery from methemoglobinemia seen in channel catfish. This system may be activated when hemoxidation is occurring.

Temperature Effects

Temperatures can effect the rate of methemoglobin formation in nitrite-exposed channel catfish (Chapter VIII), and increased MHb formation at higher temperatures is probably a function of increased ventilation and NO_2^- uptake rates. In these 10°, 20°, and $30^{o}C$-groups, the only significant loss of methemoglobin occurred in the $30^{o}C$ group. One likely explanation for this thermal effect on recovery is that the MHb-reductase system does not operate

efficiently at temperatures of 20°C and lower but is active at 30°C. Other mechanisms which function to actively eliminate NO_2^- from the plasma may also be thermally sensitive. Interactions between all these "recovery influencing" factors is complex and difficult to explain from the data present.

Treatment and Prophylaxis

From data collected in this research and that of other investigators, a number of recommendations can be made concerning treatment and prophylaxis for nitrite exposed fish. These treatment methods can be useful to fish researchers, fish farmers, and aquarium hobbyists, since all these people may have problems on occasion with nitrite toxicity. Increasing environmental chloride concentration is the best method of preventing nitrite problems. A 17:1 $Cl^-:NO_2^-$ ratio will eliminate nitrite toxicity under most conditions found in ponds, recirculating systems, or aquaria (see Appendix for dosage-treatment information). Chloride concentration can be increased by adding NaCl to the water. Chloride should not be limiting in most species of fishes, unless it exceeds ca. 5.0 g/L. Low pH can compound nitrite toxicity problems, since it decreases the efficacy of the chloride treatment scheme. Maintaining pH between 6.5 and 9.0 will decrease the rate of nitrite

uptake and insure effective chloride treatment. Since several monovalent anions interfere with nitrite uptake, other chemicals such as sodium bicarbonate will decrease nitrite toxicity also, but due to cost, this recommendation only applies to small systems and not ponds or large raceways.

Once "brown blood disease" has appeared in a culture pond, immediate steps should be taken to minimize losses. The following scheme is recommended:

1) Replace contaminated water if feasible.
2) Stop feeding temporarily.
3) Add sodium chloride.
4) Adjust the pH if necessary.
5) Use supplemental aeration.

These procedures should be followed with _minimum_ disturbance to the fish, or increased mortality will result (Huey et al., 1980). Possible reoccurrence of this problem can be prevented by reducing the biomass in the pond, using some flow-through water (if economically feasible), and reducing feeding rates. This treatment scheme can be used in small scale systems also. The treatment method is most effective for freshwater systems and not for marine fish culture systems, since chloride protection does not occur in marine fish species.

Recommendations for Further Research

1. Methemoblobin reductase activity in channel catfish should be quantified. Experiments should be designed to test the effect of varied acclimation temperature and prior nitrite exposure on this enzyme system.

2. Qualitative determination of methemoglobin reductase activity in primitive and advanced forms of fishes should be determined.

3. Isolated gill studies could prove useful in defining nitrite uptake/loss dynamics through the gills. The role of kidneys in nitrite removal could be determined through methods using bladder cannulization and urine collection.

4. Acclimation to nitrite toxicity may be possible, and resistance to nitrite induced lethality may increase in animals exposed to sub-lethal nitrite concentrations for extended periods of time. Experiments should be designed to test these hypothesis.

5. The specific effects of nitrite on the blood oxygen-disassociation curve of fishes should be determined.

TABLE XI

SUMMARY OF DATA ON NITRITE TOXICITY TO AQUATIC ANIMALS.

Species	Temp. (°C)	pH	Alkalinity mg/L	Chloride mg/L	Results Reported mg/L NO_2^-	Ref.
minnows 5-8 cm *Phoxinus laevis*	19	NR*	NR	NR	33.3 fatal in 14 days 6767.0 fatal in 1.5 h	Klinger (1957)
logperch *Percina caprodes*	NR	NR	NR	NR	16.6 mortality in 3-h	McCoy (1972)
Brook stickleback *Culaea inconstans*	NR	NR	NR	NR	16.6 mortality in 3-5 h	
black bullhead *Ictalurus melas*	NR	NR	NR	NR	133.3 no mortality in 48-h	
carp *Cyprinus carpio*	NR	NR	NR	NR	133.3 no mortality in 48-h	
common white sucker *Catostomus cormersoni*	NR	NR	NR	NR	133.3 mortality in 24-h	
quillback *Carpiodes cyprinus*	NR	NR	NR	NR	133.3 survived for 36-h	
rainbow trout *Salmo gairdneri*	10	7.9	NR	NR	1.8 55% mortality in 24-h	Smith and Williams (1974)

* = not measured

TABLE XI CONTINUED

SUMMARY OF DATA ON NITRITE TOXICITY TO AQUATIC ANIMALS.

Species	Temp. (°C)	pH	Alkalinity mg/L	Chloride mg/L	Results Reported mg/L NO_2^-	Ref.
chinook salmon *Oncorhynchus tschawytscha*	16	7.0	NR	NR	2.9 96-h LC_{50}	Westin (1974)
channel catfish *Ictalurus punctatus*	30	8.7	220	NR	43.0 96-h LC_{50}	Colt (1974)
rainbow trout	11	7.9	199	NR	0.7 96-h LC_{50}	Russo et al (1974)
rainbow trout	12	6.5	28	NR	0.8 96-h LC_{50} methemoglobin	Brown and McLeay (1975)
channel catfish	NR	7.6	70	NR	25.0 96-h LC_{50}	Konikoff (1975)
chinook salmon	NR	7.6	NR	38,000	815.0 10% mortality in 48-h	Crawford and Allen (1977)
coho salmon *Oncorhynchus kisutch*	NR	6.5	12.5	261.3	99.0 no mortality in 48-h	Perrone & Meade (1977)

TABLE XI CONTINUED

SUMMARY OF DATA ON NITRITE TOXICITY TO AQUATIC ANIMALS.

Species	Temp (°C)	pH	Alkalinity mg/L	Chloride mg/L	Results Reported mg/L NO_2^-	Ref.
channel catfish	23	7.0	NR	5.0	methemoglobin	Huey et al. (1977)
channel catfish	24	7.0	47.0	5.0	methemoglobin	Tomasso et al (1979)
channel catfish	24	7.0	40.0	V**	methemoglobin	Huey et al. (1980)
bullfrog tadpoles *Rana catesbiana*	25	7.3	NR	V	methemoglobin	Huey and Beitinger (1980)
salamander larvae *Ambystoma texanum*	25	7.0	NR	V	1.1 96-H LC_{50}	Huey and Beitinger (1980)
crayfish *Procambarus simulans*	25	7.0	NR	V	6.1 96-h EC_{50}	Beitinger and Huey (1981)
bluegill *Lepomis macrochirus*	30	V	NR	V	V 48-h LC_{50}	Huey et al. (in press)

** = Varied

APPENDIX

APPENDIX

Treatment of Nitrite Toxicity

Prophylactic measures

Protection against nitrite toxicity up to (NO_2^-) concentrations of 10 mg/L can be obtained by using a 17:1 molar ratio of $Cl^-:NO_2^-$. This equals 0.23 g sodium chloride per L of water in small fish culture systems and aquaria. In ponds this dose rate equals 0.62 pounds per acre foot.

Treatment

Use a standard method for determining water nitrite concentration. The azo-dye method or a specific ion probe are best. Add chloride at a molar ratio of 17:1 $Cl^-:NO_2^-$. Molecular wt. of nitrite is 46 and chloride is 35.5.

Example for 25 mg/L nitrite:

NO_2^- M = 5.43×10^{-4} =

Cl^- M = $9.78 \times 10^{-3} \times 35.5$ =

0.35 g Cl^-/L x 1.65 (sodium chloride conversion)

0.57 g NaCl per L or 1.5 lbs. per acre foot.

Mercuric Nitrate Method for Chlorides

From: Standard Methods, 1975.

1. General Discussion

a. <u>Principle</u>: Chloride can be titrated with mercuric nitrate because of the formation of soluble, slightly dissociated mercuric chloride. In the pH range 2.3 to 2.8, diphenylcarbazone indicates the end point of this titration by formation of a purple complex with the excess mercuric ions. The error in titration is about 1% of the volume of titrant used per change of 0.1 pH unit in the pH range 2.1 to 2.8. Because exact pH adjustment is not feasible except by use of a pH meter, it is felt that keeping within a range of ± 0.1 pH unit is sufficient for most water analyses. Therefore, in this method, a specific mixture of nitric acid and diphenylcarbazone is added to a water sample, adjusting the pH of most potable water to pH 2.5 ± 0.1. A third substance in this alcoholic mixture, xylene cyanol FF, is used as a pH indicator and as a background color to facilitate end-point detection. The introduction of 10 mg sodium bicarbonate to both the blank and the standard titration provides a pH of 2.5 ± 0.1 when 1.0 ml indicator-acidifier reagent is added. Increasing the strength of the titrant and modifying the indicator mixture

enable determination of the higher chloride concentrations common in wastewater.

b. Interference: Bromide and iodide are titrated with mercuric nitrate in the same manner as chloride. Chromate, ferric, and sulfite ions interfere when present in excess of 10 mg/1.

2. Reagents

a. Standard sodium chloride, 0.0141 N.

b. Nitric acid, HNO_3, 0.1 N.

c. Sodium hydroxide, NaOH, 0.1 N.

d. Reagents for low-chloride titrations:

1) Indicator-acidifier reagent: The nitric acid concentration of this reagent is an important factor in the success of the determination and can be varied as indicated in a) or b) to suit the alkalinity range of the sample being titrated. Reagent a) contains sufficient nitric acid to neutralize a total alkalinity of 150 mg/l as $CaCO_3$ to the proper pH in a 100-ml sample.

a) Dissolve, in the order named, 250 mg s-diphenylcarbazone, 4.0 ml conc HNO_3, and 30 mg xylene cyanol FF in 100 ml of 95% ethyl alcohol or isopropyl alcohol. Store in a dark bottle in a refrigerator. This reagent is not stable indefinitely. Deterioration causes a slow end point and high results.

b) Because pH control is critical in this method, adjust the pH of highly alkaline or acid samples to 2.5 ± 0.1 with 0.1 N HNO_3 or NaOH, not with Na_2CO_3. Use a pH meter with a nonchloride type of reference electrode for the pH adjustment. If only the usual chloride-type reference electrode is available for pH adjustment, determine the amount of acid or alkali required to achieve a pH of 2.5 ± 0.1 and discard this sample portion. Treat a separate sample portion with the determined amount of acid or alkali and continue the analysis to its prescribed end. Under these circumstances, omit the nitric acid from the indicator reagent to maintain the proper sample pH. Alternatively, vary the nitric acid concentration of the indicator-acidifier reagent to accommodate conditions wherein water samples of very high or very low alkalinity are being analyzed.

2) Standard mercuric nitrate titrant, 0.014 N: Dissolve 2.3 g $Hg(NO_3)_2$ or 2.5 g $Hg(NO_3)_2 . H_2O$ in 100 ml distilled water containing 0.25 ml conc HNO_3. Dilute to just under 1 L. Use replicates containing 5.00 ml standard NaCl solution and 10 mg $NaHCO_3$ diluted to 100 ml with distilled water. Adjust the mercuric nitrate titrant to exactly 0.0141 N and make a final standardization. Store away from the light in a dark bottle. Standard mercuric nitrate titrant, exactly 0.0141 N, is equivalent to 500 ug Cl/1.00 ml.

e. Reagents for high-chloride titrations:

1) Mixed indicator reagent: Dissolve 5 g diphenylcarbazone powder and 0.5 g bromphenol blue powder in 750 ml 95% ethyl or isopropyl alcohol and dilute to 1 L with ethyl or isopropyl alcohol.

2) Strong standard mercuric nitrate titrant, 0.141 N: Dissolve 25 g $Hg(NO_3)_2.H_2O$ in 900 ml distilled water containing 5.0 ml conc HNO_3. Dilute to just under 1 L, and perform a preliminary standardization. Use replicates containing 25.00 ml distilled water. Adjust the titrant to 0.141 N and make a final standardization. The chloride equivalence of the titrant is 5.00 mg/1.00 ml.

3. Procedure

a. Titration of low chloride concentrations: Use a 100-ml sample or smaller portion so that the chloride content is less than 10 mg.

Add 1.0 ml of indicator-acidifier reagent to the sample. (The color of the solution should be green-blue at this point. A light green indicates a pH of less than 2.0; a pure blue indicates a pH of more than 3.8. For most potable waters, the pH after this addition will be 2.5 ± 0.1. For highly alkaline or acid waters, adjust pH to about 8 before adding the indicator-acidifier reagent.)

Titrate the treated sample with 0.0141 N mercuric nitrate titrant to a definite purple end point. The solution will turn from green-blue to blue a few drops from the end point.

Determine the blank by titrating 100 ml distilled water containing 10 mg $NaHCO_3$.

b. Titration of high chloride concentrations: Place 50 ml sample in a 150 ml beaker (5.00 ml sample may be used when more than 5 ml titrant are needed). Add approximately 0.5 ml mixed indicator reagent and mix well. The color should be purple. Add 0.1 N HNO_3 dropwise until the color just turns yellow. Titrate with 0.141 mercuric nitrate titrant to the first permanent dark purple. Titrate a distilled water blank using the same procedure.

4. Calculation

$$\text{mg/l Cl} = \frac{(A - B \times N \times 35{,}450}{\text{ml sample}}$$

where A = ml titration for sample, B = ml titration for blank, and N = normality of $Hg(NO_3)_2$.

mg/l NaCl = mg/l Cl x 1.65

Nitrite Determination

From: Standard Methods, 1975.

1. General Discussion

a. Principle: The nitrite concentration is determined through the formation of a reddish-purple azo-dye produced at pH 2.0 to 2.5 by the coupling of diazotized sulfanilic acid with N-(1-naphthyl)-ethylenediamine dihydrochloride. The diazotization method is suitable for the determination of nitrite nitrogen in the range 1 to 25 ug/L N. Photometric measurements can be made in the ranges 5 to 50 ug/L if a 5-cm light path and a green color filter are available. The color system obeys Beer's law up to 180 ug/l N with a 1-cm light path at 543 nm.

b. Interference: Chemical incompatibility makes it unlikely that nitrite, free available chlorine, and nitrogen trichloride will coexist in a sample. Nitrogen trichloride imparts a false red color when the normal order of reagent addition is followed. Although this effect may be minimized somewhat by adding the N-(1-naphthyl)-ethylenediamine dihydrochloride reagent first and then the sulfanilic acid reagent, an orange color still may result when a substantial nitrogen trichloride concentration is present. A check for a free available chlorine and nitrogen trichloride residual is advisable under such circumstances. The following ions interfere because of

precipitation under the conditions of the test and therefore should be absent: antimonous, auric, bismuth, ferric, lead mercurous, silver, chloroplatinate, and metavanadate. Cupric ion may cause low results by catalyzing the decomposition of the diazonium salt. Colored ions that alter the color system also should be absent.

Remove suspended solids by filtration through a 0.45 - um membrane filter before color development.

c. Storage of sample: Make the determination promptly on fresh samples to prevent bacterial conversion of the nitrite to nitrate or ammonia.

Never use acid preservation for samples to be analyzed for nitrite. For short-term preservation for 1 to 2 days deepfreeze at -20°C or add 40 mg $HgCl_2$ to sample and store at 4°C.

2. Apparatus

Colorimetric equipment: One of the following is required:

a. Spectrophotometer, for use at 543 nm, providing a light path of 1 cm or longer.

b. Filter photometer, providing a light path of 1 cm or longer and equipped with a green color filter having maximum transmittance near 540 nm.

c. Nessler tubes, matched, 50-ml tall form.

3. Reagents

Prepare all reagents from chemicals that are white in color.

a. Nitrite-free water: If it is not known that the distilled or demineralized water is free from nitrite, use either of the following procedures to prepare nitrite-free water:

1) Add to 1 L distilled water one small crystal each of potassium permanganate and barium or calcium hydroxide. Redistill in an all-pyrex apparatus and discard the initial 50 ml of distillate. Collect the distillate fraction that is free of permanganate.

2) Add 1 ml conc H_2SO_4 and 0.2 ml managanous sulfate solution (36.4 g $MnSO_4 . H_2O$/100 ml aqueous solution) to each 1 L distilled water, and make pink with 1 to 3 ml potassium permanganate solution (400 mg $KMnO_4$/L aqueous solution).

b. Sulfanilamide reagent: Dissolve 5 g sulfanilamide in a mixture of 50 ml conc HCl and about 300 ml distilled water. Dilute to 500 ml with distilled water. The solution is stable for many months.

c. N-(1-naphthyl)-ethylenediamine dihydrochloride solution: Dissolve 500 mg dihydrochloride in 500 ml distilled water. Store in a dark bottle. Renew the solution monthly or immediately when it develops a strong brown coloration.

d. <u>Hydrochloric acid, HCl, 1+3</u>.

e. <u>Stock nitrite solution</u>: Commercial reagent-grade $NaNO_2$ assays at less than 99%. Because nitrite is readily oxidized in the presence of moisture, use a fresh bottle of reagent for preparing the stock solution. Determine $NaNO_2$ content immediately before preparing the stock solution and keep bottles tightly stoppered against the free access of air when not in use. To determine the sodium nitrite content, add an excess of standard potassium permanganate solution, discharge the permanganate color with a standard reductant such as sodium oxalate or ferrous ammonium sulfate solution, and finally back-titrate with standard permanganate solution.

1) Preparation of stock solution- Dissolve 1.232 g $NaNO_2$ in nitrite-free water and dilute to 1,000 ml; 1.00 ml = 250 ug N. Preserve with 1 ml chloroform.

2) Standardization of stock solution- Pipet, in order, 50 ml standard 0.05N $KMnO_4$, 5 ml conc H_2SO_4, and 50 ml stock nitrite solution into a glass-stoppered flask or bottle. Submerge the tip of the nitrite pipet well below the surface of the permanganate acid solution. Shake gently and warm to 70° to 80° C on a hot plate. Discharge the permanganate color by adding sufficient standard 0.05N sodium oxalate (3.350 g $Na_2C_2O_4$, primary standard grade, per 1,000 ml solution) in 10,000 ml portions. Titrate the

excess sodium oxalate with standard 0.05N $KMnO_4$ to the faint pink end point. Carry a nitrite-free water blank through the entire procedure and make the necessary corrections in the final calculation.

If standard 0.05N ferrous ammonium sulfate solution is substituted for sodium oxalate, omit heating to 70° to 80°C and extend the reaction period between the permanganate and ferrous ions to 5 min before making the final $KMnO_4$ titration. This standard 0.05N ferrous solution contains 19.607 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 20 ml conc H_2SO_4/L solution.

Calculate the nitrite nitrogen content of the stock solution by the following equation:

$$A = \frac{(B \times C)\ (D \times E) \times 7}{F}$$

where:

A = mg/ml nitrite nitrogen in stock nitrite solution.

B = total ml standard $KMnO_4$ used.

C = normality of standard $KMnO_4$.

D = total ml standard reductant added.

E = normality of standard reductant.

F = ml stock $NaNO_2$ solution taken for titration.

Each 1.00 ml 0.05N $KMnO_4$ consumed by the nitrite corresponds to 1,725 µg $NaNO_2$, or 350 µg N.

f. Intermediate nitrite solution: Calculate the volume, G, of stock nitrite solution required for the intermediate nitrite solution by means of the following equation: $G=12.5/A$. Dilute to 250 ml the calculated volume, G (approximately 50 ml), of the stock nitrite solution with nitrite-free water; 1.00 ml = 50.0 µg N. Prepare daily.

g. Standard nitrite solution: Dilute 10.00 ml intermediate nitrite solution to 1,000 ml with nitrite-free water; 1.00 ml = 0.500 µg N. Prepare daily.

4. Procedure

a. Removal of turbidity: If the sample contains suspended solids filter through a 0.45 µm membrane filter.

b. Color development: To 50.0 ml clear sample neutralized to pH 7, or to a portion diluted to 50.0 ml, add 1 ml sulfanilamide solution from an automatic pipet. Allow the reagent to react for a period of more than 2 min. but not longer than 8 min. Add 1.0 ml 1-naphthyl-ethylenediamine solution and mix immediately.

c. Photometric measurement: Between 10 min. and 2 hr afterwards measure the absorbance of the solution at 543 nm. As a guide use the following light paths for the indicated NO_2^-N concentrations.

Light Path Length cm	NO_2^-N µg/L
1	2-20
5	2-6
10	2

Run parallel checks frequently against known nitrite standards, preferably in the nitrogen range of the samples. Redetermine complete calibration curves after preparing new reagents.

5. Calculation

$$\text{mg/l nitrite N} = \frac{\mu\text{g nitrite N}}{\text{ml sample}}$$

Recommended Reconstituted Fresh Waters

Quantities of reagent-grade chemicals required to prepare recommended reconstituted fresh waters and the resulting water qualities.

Name	Salts Required (mg/l)				pH	Hardness	Alkalinity
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl			
Very Soft	12	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Hard	192	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very Hard	384	240.0	240.0	16.0	8.0-8.4	280-320	225-245

From: U. S. E.P.A. Publication 660/3-75-09.

Determination of TMS-222 in Water

From: Walker and Schoettger, 1967.

Reagent and Concentration	Blank (ml)	Sample (ml)
HCl 1:10	5.0	5.0
$NaNO_2$ 0.2%	0.5	0.5
Ammonium Sulfamate 0.5%	0.5	0.5
Naphthylethylenediamine 2 HCl 0.5%	0.5	0.5
Distilled H_2O to 100 ml in volumetric flask	x	x

Read at 543 nm on a suitable spectrophotometer.

Determination of Methemoglobin in Blood

From: Evelyn and Malloy, 1938.

Reagents

1. Phosphate buffer, M/15, pH 6.6. Dissolve 1.89 g of Na_2HPO_4 (anhyd.) and 2.85 g of KH_2PO4 (anhyd.) in water to make 500 ml of solution. Dilute this 1:4 with water to form fresh M/60 buffer. Store the reagent refrigerated and discard when microbial growth is evident.
2. Neutral sodium cyanide. This is prepared fresh as needed in 10 ml lots from 10% (w/v) NaCN and 12% (v/v) acetic acid. Working in a well ventilated hood, add 5 ml of the NaCN to a 10 ml glass-stoppered cylinder, followed by 5 ml of acetic acid. Stopper the cylinder and mix the contents by inversion. Use appropriate care in pipetting the poisonous NaCN. Carefully pour the unused reagent into the hood drain and thoroughly flush the drain.
3. Triton X-100.
4. Potassium Ferricyanide, 5% (w/v), prepared fresh.
5. Two or three specimens of fresh, nonclotted blood which are to be used as normal controls.
6. Blood hemoglobin by the cyanomethemoglobin method.

Specimen

Use whole blood, collected preferably in heparin or citrate, although oxalated blood is satisfactory. The specimen should be fresh, and should be analyzed within 2 hours.

Procedure

1. Pipet 10.0 ml of M/60 buffer into 15 x 120 mm tubes and add 100 µl each of the well mixed test and control specimens.
2. Add 1 drop of Triton X-100 to each tube, mix the tubes by gentle inversion six to eight times, and then allow to set for 5 minutes. (The Triton promotes lysis of the cells and minimizes turbidity.)
3. After 5 minutes, pour the solutions into cuvets and read the absorbances at 633 nm in any convenient spectrophotometer. Record this reading at A_1.
4. Add 1 drop of neutral cyanide to the solutions in the cuvets, mix the cuvet contents, and re-read the absorbances after 5 minutes at 633 nm. Record these readings as A_2.
5. Then determine the total hemoglobin content of the blood specimens (tests and controls) using the cyanomethemoglobin procedure described in a previous section.

Calibration

Lyse 100 µl of each of two normal blood specimens in 10 ml buffer (step 1). Then add 0.10 ml of 5% ferricyanide, mix the tubes, and allow to stand for 2 minutes. (All hemoglobin is converted to MetHb.) Read the absorbance (= A_3) at 633 nm. Add 1 drop of neutral cyanide (MetHb ⇌ MetHbCN), and read the absorbance (= A_4) after 2 minutes. Calculate the difference (A_3 - A_4), which represents the absorbance of all the hemoglobin, expressed as methemoglobin. The total Hb being known (step 5), calculate the calibration factor Fm for MetHb from:

$$F_m = \frac{\text{g Hb/100 ml}}{(A_3 - A_4)}$$

The F_m values of the two normal control bloods will usually check within 2 or 3%. The average of the two is used in calculating the MetHb value of the unknown.

Calculations

$$\text{g MetHb/100 ml} = (A_1 - A_2) \times F_m$$

$$\text{MetHb, as \% of total Hb} = \frac{\text{g MetHb}}{\text{g total Hb}} \times 100$$

Interpretation

The quantity of MetHb found in presumably normal blood specimens is from 0.00 to 0.25 g/100 ml of blood, averaging about 0.10 g/100 ml. This is equivalent to about 0.4 to 1.5% of the total Hb.

Determination of Blood Hemoglobin by the Cyanomethemoglobin Procedure

From: Hainline, A., 1958.

Reagents

1. Hemoglobin Standard. This is now available commercially in the form of 5 ml bottles containing 80 mg/100 ml of hemoglobin, the concentration of which is certified to be correct within ± 2%. The standard is reasonably stable if kept refrigerated. The hemoglobin is in the cyanomethemoglobin form.

2. Cyanomethemoglobin reagent. This contains 1.0 g of $NaHCO_3$, 50 mg of KCN, and 200 mg of potassium ferricyanide ($K_3Fe(CN)_6$ in 1 L of solution. The solution is stable, but should never be frozen, since recent observations have shown that such treatment results in false low values. The reagent is also available commercially.

Procedure

1. For instruments requiring cuvet volumes of 5.0 ml or less, add 0.02 ml (20 µl) of blood to 5.0 ml of reagent contained in the appropriate cuvet. If 19 mm Coleman cuvets are used, add the 20 µl of specimen to 6.0 ml of reagent. The dilution of blood is 1:251 or 1:301 when diluent volumes of 5 and 6 ml, respectively, are used.

2. Mix the contents of the cuvet and allow to set for 20 minutes before reading.

3. Read the unknowns and standards against the blank (reagent), using a wavelength of 540 nm (or equivalent filter). The hemoglobin of the blood specimen can be obtained from the calibration curve or by use of a calibration factor.

Calibration

Dilute 1.0, 2.0, 3.0, 4.0, and 5.0 ml of the 80 mg/100 ml standard with cyanomethemoglobin reagent to 5.0 ml or 6.0 ml, respectively. The five standards represent 4, 8, 12, 16, and 20 g of hemoglobin/100 ml of blood respectively. Read the standards against the blank as outlined in step 3. Construct a standard curve by plotting per cent transmittance or absorbance against the hemoglobin concentration.

Although the standard curve is reproducible with any given lot of reagents, it is good practice to run one working standard (highest) daily to verify the validity of the calibration.

Quantitative Methemoglobin Reductase Assay

From: Hegesh et al., 1968.

Reagents

A. EDTA, 10 mM.: Dissolve 0.186 g of EDTA, disodium salt. $2H_2O$ in water to and make to 50 ml.

B. Citrate buffer, pH 4.7, 50 mM.

C. Potassium ferricyanide, 0.5 mM.

D. NADH, 10 mM.: dissolve 0.019 g of NADH in 2 ml of water.

E. Cyanide-ferricyanide reagent: dissolve 0.05 g of KCN, 0.2 g of potassium ferricyanide, and 1 g of sodium bicarbonate in water and make to 1,000 ml.

F. Hemoglobin freed of ferrihemoglobin reductase, 0.18 mM.: centrifuge heparinized or citrated blood at 2,000 r.p.m. and discard the plasma. Wash erythrocytes twice with 10 times their volume of 0.9% NaCl solution and pack by centrifugation at 3,000 r.p.m. To each 10 ml of washed, packed cells add 60 ml of water and 1.6 g of dry diethylaminoethyl-cellulose (DEAE) (Whatman DE 11). Let stand for 10 minutes, mixing the suspension occasionally. Centrifuge and decant the supernatant fluid into another beaker. Repeat the adsorption procedure by adding a new portion of 1.6 g of DEAE-cellulose. Dilute the clear supernatant fluid with water to a final hemoglobin concentration of 0.18 mM. determined by the hemoglobin

assay described below. Ferrihemoglobin reductase remains adsorbed on the DEAE-cellulose. Check if the solution is free of ferrihemoglobin reducing activity. Use the enzyme assay (see Methods) but employ 1.5 ml of the enzyme-free solution and omit the addition of sample. The purified hemoglobin preparation is stable for at least 2 weeks if kept refrigerated.

Methods

Sample. Withdraw from the fingertip two samples of blood, each one of 40 µl. Use one sample for the hemoglobin assay and the second for the enzyme assay. Heparinized or citrated blood can also be employed.

The assay can also be performed with red blood cells packed by centrifugation at 3,000 r.p.m. for 15 minutes, but 20 µl of sample should be used in this case for each determination.

The hemoglobin content of Reagent F is determined with 250 µl sample.

Hemoglobin assay. Transfer to 10 ml volumetric flask about 5 ml of cyanide-ferricyanide solution. Add the sample and make to volume with the same reagent. Let stand for 10 minutes. Centrifuge. Measure the optical density of the clear solution at 540 nm. in a 1 cm cuvette versus water.

Enzyme assay. Pipette into a graduated 10 ml cylinder 0.5 ml of EDTA, 1.0 ml of citrate buffer, 1.8 ml of potassium ferricyanide of 0.5 mM., 1.0 ml of hemoglobin, the sample, and water to bring the total volume to 9.9 ml. Mix and let stand for 5 minutes. Centrifuge at 3,000 r.p.m. for 5 minutes to eliminate the cell membranes. Transfer 2.97 ml of the clear centrifugate into each of two 1 cm spectrophotometric cuvettes. Add 0.03 ml of water to the first cuvette (blank). Mix and set the spectrophotomter to zero. To the second cuvette add 0.03 ml of NADH at zero time. Measure the change in optical density at 575 nm. for 3 minutes at 30 second intervals. The change in optical density from the first to the second minute after the addition of NADH is a measure of the enzymatic activity.

Qualitative Methemoglobin Reductase Test

From: Kaplin et al., 1970.

Reagents

2,6-Dichlorophenolindophenol (DCIP), 19 mM. Dissolve 0.0625 g of the sodium salt in water and bring to 100 ml in a volumetric flask. Dispense 5 ml aliquots into individual plastic vials and store frozen.

Tris Buffer, pH 7.6, 60 mM. Dissolve 0.7267 g Tris-tris (hydroxymethyl) aminomethane-in 85 ml water; adjust pH to 7.5 with conc HCl before diluting to 100 ml.

Ethylenediamine Tetraacetic Acid (EDTA), 0.27 mM. Dissolve 0.0112 g of the tetrasodium dihydrate salt in 60 mM Tris buffer and bring to 100 ml with the buffer. Dispense 5 ml aliquots into individual vials and store frozen.

NADH, Disodium Salt. Place 1 mg aliquots of NADH into vials. (Preweighed vials containing 1 mg NADH per vial are commercially available from P-L Biochemicals.) Refrigerate for prolonged storage. Refrigeration is not as important as keeping the vial perfectly dry and protected from light.

Sodium Nitrite, 1.24%. Add 0.62 g $NaNO_2$ to 50 ml of water. Prepare fresh daily.

Saponin, 1%. Dissolve 1 g saponin in 100 ml water.

Reaction Mixture. Add 1.33 ml EDTA solution and 60 ul 19 mM DCIP to the vial containing 1.0 mg NADH. Mix. The reaction mixture is stable at room temperature for 7 hours. The above volume is adequate for four samples.

Deficient Control. If a known or pretested deficient blood sample is not available, place a normal blood sample collected in glucose-EDTA in a 56° waterbath for 6 hours. Store at 4°C in the refrigerator. This is usable for 1 month.

Procedure

1. For each unknown, the deficient control, and the reaction mixture (to be used as a reagent blank), draw three squares (2 x 2 cm) on Whatman 1 filter paper for spotting assay mixture at 0, 30, and 60 min.
2. To 10 x 75 mm test tubes labeled "unknown" and "control" add 2 µl freshly prepared 1.24% $NaNO_2$.
3. Add 20 µl whole blood sample (Alsever's solution/blood, 1:1) to the "unknown." (Use 10 µl of blood if dried Alsever's mixture was used). Add 20 µl of deficient control blood to the "control." Mix and allow to stand at room temperature for 30 min.
4. Add 0.04 ml 1% saponin to each tube. Mix.
5. Add 0.2 ml reaction mixture. Mix.

6. Using the same pipets as in step 3, immediately spot 10 µl unknown and deficient control on the appropriate filter paper squares marked 0 min. Also spot 10 µl reaction mixture as a reagent control.

7. Incubate the samples at 45°C for 30 min and again spot 10 µl from each tube on the designated square. Return the tube to the heating block or water bath for continued incubation.

8. Dry the spots under a hot air blower.

9. Examine under long wave UV light. Continue incubation of all fluorescing samples for another 30 min. at 45°C.

10. At the end of the 60 min. incubation, spot 10 µl on the designated squares.

11. Dry spots under a hot air blower and examine under long wave UV light.

Interpretation of Results

a. All "0 min." spots should fluoresce from the presence of NADH.

b. At 30 min., no fluorescence of specimen spot indicates normal enzyme activity. Analysis is completed for that sample.

c. Fluorescence at 30 min. does not necessarily indicate enzyme deficiency. If fluorescence disappears by 60 min., the sample is normal.

d. Persistence of fluorescence at 60 min indicates enzyme deficiency.

e. For the test to be valid, fluorescence must persist at 60 min in both the reagent control and the deficient cell control.

LITERATURE CITED

Allen, D. W. and J. H. Jandl. 1961. Oxidative hemolysis and precipitation of hemoglobin II. Role of thiols in oxidant drug action. J. Clin. Invest. 40: 454-474.

American Public Health Association. 1971. Standard methods for the examination of water and wastewater. 13th ed. Washington: APHA. Pp. 874.

American Public Health Association. 1975. Standard methods for the examination of water and wastewater. 14th ed. Washington: APHA. Pp. 874.

Anthonisen, A. C., R. C. Loehr, T. B. S. Prakasam, and F. G. Srinath. 1976. Inhibition of nitrification by ammonia and nitrous acid. J. Water Poll. Cont. Fed. 48: 835-852.

Beitinger, T. L. and D. W. Huey. 1981. Acute toxicity of nitrite to crayfish Procambarus simulans in varied environmental conditions. Environ. Poll. 26: 305-311.

Bell, G. R. 1964. A guide to the properties, characteristics, and uses of some general anesthetics for fish. Fish. Res. Board of Can., Bulletin 148, Pp. 4.

Beutler, E. 1968. Hereditary disorders of erythrocyte metabolism. New York: Grune and Stratton. Pp. 236.

Bodansky, O. 1951. Methemoglobinemia and methemoglobin producing compounds. Pharmacol. Rev. 3: 144-196.

Boyd, C. E. and B. Hollerman. 1980. Sources of nitrite in channel catfish ponds. (Abst.) Aquaculture-80: (Catfish farmers of America water quality section).

Brown, D. A. and D. J. McLeay. 1975. Effect of nitrite on methemoglobin and total hemoblobin of juvenile rainbow trout. Prog. Fish-Cult. 37: 36-38.

Cairns, J., Jr., H. G. Heath, and B. C. Parker. 1975. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia 47: 135-171.

Cairns, J., Jr., A. L. Buikema Jr., and A. G. Heath. 1978. Effects of temperature on aquatic organism sensitivity of selected chemicals. Bull. of Virginia Water Resources Research Center. VPI-VWRRC-Bull 106. Pp. 88.

Cameron, J. N. 1971. Methemoglobin in erythrocytes in rainbow trout. Comp. Biochem. Physiol. 40: 743-749.

Chien, Y. and J. W. Avault. 1980. Production of crayfish in rice fields. Prog. Fish-Cult. 42: 68-70.

Colt, J. E. 1974. Evaluation of the short-term toxicity of nitrogenous compounds to channel catfish. M.S. Thesis, Univ. of California, Davis. Pp. 94.

Colt, J. and G. Tchobanoglous. 1976. Evaluation of the short-term toxicity of nitrogenous compounds to channel catfish, *Ictalurus punctatus*. Aquaculture 8: 209-224.

Crawford, R. E. and G. H. Allen. 1977. Seawater inhibition of nitrite toxicity to chinook salmon. Trans. Am. Fish. Soc. 106: 106-109.

Dick, G. L. 1975. Some observations on the use of the MS-222 Sandoz with grey mullet (*Mugil chelo*). J. Fish Biol. 7: 263-268.

Evelyn, H. A. and H. T. Malloy. 1938. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem. 126: 655-662.

Fairbanks, V. F. 1976. Hemoglobin, hemoglobin derivatives, and myoglobin. Pages 401-454 *in* N. W. Tietz (ed.) Fundamentals of Clinical Chemistry. Philadelphia: W. B. Saunders.

Felsenfeld, G. and M. Printz. 1959. Specific reactions to hydrogen peroxide with active site of hemocyanin. J. Amer. Chem. Soc. 81: 6259-6261.

Finney, D. J. 1971. Statistical method in biological assay. 3rd ed. New York: MacMillan. Pp. 508.

Gillette, L.A. D. L. Miller, and H. E. Redman. 1952. Appraisal of a chemical waste problem by fish toxicity tests. Sewage Ind. Wastes. 24: 1397-1401.

Hainline, A. 1958. Hemoglobin. Pages 268-269 in D. Seligsoln (ed.) Standard methods of clinical chemistry. Philadelphia: W. B. Saunders.

Harris, J. W. and R. W. Kellermeyre. 1970. The red cell production, metabolish, destruction: normal and abnormal. Cambridge: Harvard University Press. Pp. 795.

Hattingh, J. 1977. The effect of tricaine methanesulphonate (MS-222) on the microhaematocrit of fish blood. J. Fish. Biol. 10: 453-455.

Haug, R. T. and P. L. McCarty. 1972. Nitrification with submerged filters. J. of the Water Poll. Cont. Fed. 44: 2068-2102.

Hegesh, E., N. Calmanovici, and M. Avron. 1968. New method for determining ferrihemoglobin reductase (NADH-methemoglobin reductase) in erythrocytes. J. Lab. and Clin. Med. 72: 339-344.

Houston, A. H., M. A. DeWilde, and J. A. Madden. 1971. Some physiological effects of handling and tricaine methanesulphonate anaesthetization upon the brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 28: 635-642.

Huey, D. W., D. Criswell, B. A. Simco, and K. B. Davis. 1977. Nitrite toxicity and methemoglobin formation in channel catfish. Assoc. Southeastern Biol. Bull. 24: 60 (Abst.).

Huey, D. W. and T. L. Beitinger. 1980a. Hematological responses of larval Rana catesbiana to sublethal nitrite exposures. Bull. Environm. Contam. Toxicol. 25: 574-577.

Huey, D. W. and T. L. Beitinger. 1980b. Toxicity of nitrite to larvae of the salamander (Ambystoma texanum). Bull. Environm. Contam. Toxicol. 25: 909-912.

Huey, D. W., B. A. Simco, and D. W. Criswell, 1980. Nitrite-induced methemoglobin formation in channel catfish. Trans. Amer. Fish. Soc. 109: 558-562.

Huey, D. W. and T. L. Beitinger. 1982. Effect of pH and chloride on nitrite-induced lethality in bluegill (Lepomis macrochirus). Bull. Environm. Contam. Toxicol. 28: 3-6.

Huey, D. W. and T. L. Beitinger. A methemoglobin reductase system in channel catfish, Ictalurus punctatus. Can. J. of Zoology. (In press).

Huey, D. W. and T. L. Beitinger. Methemoglobin levels in channel catfish, Ictalurus punctatus, exposed to nitrte and tricaine methanesulphonate. Can J. Fish. and Aquatic Sco. (in press).

Huish, M. T. 1972. Some responses of the brown bullhead to MS-222. Prog. Fish-Cult. 34: 27-32.

Jaffe, E. R. 1964. Metabolic processes involved in the formation and reduction of methemoglobin in human erythrocytes. Pages 397-422 in C. Bishop and D. Surgener (eds.) The red blood cell. New York: Academic Press.

Kaplin, J. C., A.M. Nicolas, A. H. Leroux, and E. Beutler. 1970. A simple spot screening test for fast detection of red cell NaDH diaphorase deficiency. Blood 36: 330-333.

Kiese, M. and H. D. Waller. 1950. Kinestic der hamoglobibildung: Die stoffwechselvorgange in roten zellen bei der hamoglobinbildung durch den kreisprogress phenyllhydroxylaminnitrosobensol. Arch. Exp. Path. u. Phar. 211: 345.

Klinger, K. 1959. Sodium nitrite, a slow acting fish poison. Water Poll. 32: 142 (Abst.).

Konikoff, M. 1975. Toxicity of nitrite to channel catfish. Prog. Fish-Cult. 37: 96:98.

Lovell, R. T. 1979. Brown blood disease in pond-raised catfish. Comm. Fish Farmer. 5: 33.

Lovell, R. T. 1979. Fish culture in the United States. Science. 206: 1368-1372.

McCoy, E. F. 1972 Role of bacteria in the nitrogen cycle in lakes. Water Poll. Cont. Res. Ser. 16010 EHR 03/72. Office of Research and Monitoring, E.P.A. Washington, D. C.

Meade, T. L. and S. J. Perrone. 1980. Effect of chloride ion concentration and pH on the transport of nitrite across the gill epithelia of coho salmon. Prog. Fish-Cult. 42: 71-72.

Newberne, P. M. 1979. Nitrite promotes lymphoma incidence in rats. Science 204: 1079-1081.

Perrone, S. J. and T. L. Meade. 1977. Protective effect of chloride on nitrite toxicity to coho salmon (Oncorhynchus kisutch). J. Fish Res. Board Can. 34: 486-492.

Peters, J. P. and D. D. VanSlyke. 1931. Quantitative clinical chemistry; interpretations. London: Bailliere. Pp. 631.

Phillips, A. M., H.A. Podoliak, D. R. Brockway, and R. R. Vaughn. 1957. The nutrition of trout. New York Conservation Dept. Fish. Research Bull. 21, Cortland Hatchery Report. 26: 93.

Rodkey, F. L. 1976. A mechanism for the conversion of oxyhemoglobin to methemoglobin by nitrite. Clin. Chem. 22: 1986-1990.

Russo, R. C. and R. V. Thurston. 1977. The acute toxicity of nitrite to fishes. Pages 118-131 in R. A. Tubb (ed.) Recent advances in toxicology. U.S. E.P.A., National Research Center. Ecological Research Series EPA-60013-77-085.

Russo, R. C., R.V. Thurston, and K. Emerson. 1981. Acute toxicity of nitrite to rainbow trout (Salmo gairdneri) effects of pH, nitrite species, and anion species. Can. J. Fish. Aquat. Sci. 38: 387-393.

Smit, G. L., J. Hattingh, and A. P. Burger. 1979a. Haematological assessment of the effects of the anaesthetic MS-222 in natural and neutralized form in three freshwater fish species: interspecies differences J. Fish Biol. 15: 633-643.

Smit, G. L., J. Hattingh, and A. P. Burger. 1979b. Haematological assessment of the effects of the anaesthetic MS-222 in natural and neutralized form in three freshwater fish species: intraspecies differences. J. Fish Biol. 15: 645-653.

Smit, G. L., J. Hattingh, and A. P. Burger. 1979c. Haematological assessment of the effects of the anaesthetic MS-222 in natural and neutralized form in three freshwater fish species: haemoglobin electrophoresis, ATP levels, and corpuscular fragility curves. J. Fish Biol. 15: 655-663.

Smith, C. E. and W. G. Williams. 1974. Experimental nitrite toxicity in trout and chinook salmon. Trans. Amer. Fish. Soc. 103: 389-390.

Smith, C. E. and R. C. Russo. 1975. Nitrite induced methemoglobinemia in rainbow trout. Prog. Fish-Cult. 37: 150-152.

Smith, M. J. and A. G. Heath. 1979. Acute toxicity of copper, chromate, zinc, and cyanide to fresh water fish: effect of different temperatures. Bull. Environm. Contam. Toxicol. 22: 113-119.

Soivio, A., K. Westman, and K. Nyholm. 1974. Changes in hematocrit values in blood samples treated with and without oxygen: A comparative study of four salmonid species. J. Fish Biol. 6: 763-769.

Sullivan, B. and A. Riggs. 1964. Haemoglobin: reversal of oxidation and polymerization in turtle red cells. Nature 204: 1098-1099.

Thurston, R. H. and R. C. Russo. 1978. Acute toxicity of ammonia and nitrite to cutthroat trout fry. Trans. Am. Fish. Soc. 10: 361-368.

Tomasso, J. R., B. A. Simco, and K. B. Davis. 1979. Chloride inhibition of nitrite induced methemoglobinemia in channel catfish. J. Fish Res. Board Can. 36: 1141-1144.

United States Environmental Protection Agency. 1975. Methods for acute toxicity tests with fish, macroinvertebrates and amphibians. E.P.A. 660/3-75-009. Corvallis, Ore.

Van Assendelft, O. W. and W. G. Zijlstra. 1965. The formation of hemoglobin using nitrites. Clin. Chim. Acta. 11: 571-577.

Walker, C. R. and R. A. Schoettger. 1967. Method for determining MS-222 residues in fish. U. S. Bureau of Sport Fisheries and Wildlife, Investigations in Fish Control. 14: 10-15.

Wallen, I. E., W. C. Greer, and R. Lasater. 1957. Toxicity to Gambusia affinis of certain pure chemicals in turbid waters. Sewage Ind. Wastes. 29: 695-711.

Wedemeyer, G. A. 1970. Stress of anaesthesia with MS-222 and benzocaine in rainbow trout (Salmo gairdneri). J. Fish Res. Board Can. 27: 909-914.

Wedemeyer, G. A. and W. T. Yasutake. 1978. Prevention and treatment of nitrite toxicity in juvenile steelhead trout (Salmo gairdneri). J. Fish. Res. Board Can. 35: 822-827.

Westin, D. T. 1974. Nitrate and nitrite toxicity to salmonid fishes. Pro. Fish-Cult. 36: 86-89.