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Evaluation of the efficacy of the stress protin response as a biomonitoring Method

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The objective of this research, as stated in the proposal, was to evaluate the phenotypic expression of the stress proteins as indicators of biotic stress with potential for adapting the response as a rapid, water quality biomonitoring method. To accomplish this goal, we characterized the induction of the stress proteins in fathead minnows (Pimephales promelas) exposed to a variety of stressors, such as heat, arsenite, chromate, lindane, and diazinon. Stress protein responses were correlated to conventional toxicity endpoints, such as effects on survival. In addition, the stress response was assessed for its environmental relevance via in situ caged fish studies. Through the described research we found that the stress protein response has potential value as a biomonitoring method. Specifically, it is a rapid and sensitive response. Specific translation patterns appear to be chemical class or

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translation patterns, striated muscle, gill, brain, and toxicity

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mode of action specific. Immunological techniques appear to have the greatest sensitivity and relevance as potential monitoring tools. A significant correlation of the induction of the stress proteins with *in situ* species richness verifies the potential of this biochemical parameter as a potential indicator of water qualtiy and/or biotic health.

Evaluation of the Efficacy of the Stress Protein Response as a Biochemical Water Quality Biomonitoring Method

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I. INTRODUCTION

Chemical and physical demands of life in water impose rigorous constraints on aquatic species. Superimposed on such demands may be the additional physiological constraints from anthropogenic contaminants. Stresses of anthropogenic origin are multiple in nature, and aquatic life may be subjected to some or all of the following: exposures to acute and sublethal levels of contaminants; unfavorable fluctuating temperatures, adverse light levels; increased sediment loads, etc. (Wedemeyer et al., 1984). Each of these factors, singly or together, can impose a considerable load, or stress, on physiologic systems.

Early detection of changes in the quality of water resources, especially those impacted by anthropogenic contanimants, is of primary concern to individuals involved with managing these resources. The associated fisheries, both commercial and recreational, represent a valuable resource which can be irreparably impacted, as well. Those charged with managing water resources rely heavily on chemical, physical and biomonitoring techniques. A need exists for a rapid means of assessing the "health" of rivers, reservoirs, and estuaries in the nation. A reliable, biomonitoring method which determines stress levels in fish could provide resource managers with a valuable tool to determine if chemicals from point and non point source pollution are adversely impacting aquatic systems.

Recently, a cellular protective response has been identified which is involved in protecting organisms from such detrimental effects of exposure to a variety of stressors. This response, referred to as the heat shock or stress response, involves the

preferential synthesis of a suite of proteins referred to as heat shock proteins (hsp's) in reaction to exposure to elevated temperatures and other environmental stressors (Atkinson and Walden 1985; Lindquist 1986). Heat shock proteins are ubiquitous and highly conserved. It is becoming clear that stress proteins have important functions in cells under normal conditions and take on dual, protective and repair, roles. Synthesis of hsp's is closely correlated with stress from heat, viral infections, heavy metals, and pesticides, as well as others.

This report describes the results of a research project designed to evaluate the potential of the use of the stress protein response as a biomonitoring method of water quality. The research builds upon an ongoing program of the investigators whose goals have been to develop methods to detect the effects of chemicals on aquatic organisms. The emphasis has expanded to the use of biochemical/physiological biomarkers of exposure and effects as biomonitoring tools.

The objective of this research was to evaluate the efficacy of using the phenotypic expression of stress proteins, a cellular response that increases the organism's capacity to cope with greater stress loads, as a means of determining the degree of stress caused by anthropogenic contaminants in the aquatic environment. A major goal was to adapt the procedure as a rapid, precise water quality biomonitoring method. The following report describes the methods and qualitative and quantitative results of the stress protein response to heat, two metals (arsenic, and chromium) and two pesticides (lindane and diazanon). In addition, preliminary findings from a field validation study are presented. The data accumulated will show

that the stress protein response is rapid, occurring within hours of stressor exposure, the stress protein response is sensitive to sublethal exposures and can be correlated to toxicant type, concentration, and percent mortality, and certain stress proteins are indicative of chemical class or mode of action of specific stressors. While not a part of our original research plan, the usefulness of an antibody test measuring accumulation of certain stress proteins, especially sp70, suggests this method is several-fold more sensitive as an indicator of general stress.

II. HEAT SHOCK EXPERIMENTS

II. A. Introduction

Behavioral and physiological responses which enable organisms to tolerate a wide range of temperatures encountered in their environment have been well characterized (see reviews in Bowler and Fuller, 1987). With respect to temperature tolerance, it has been widely acknowledged that the higher the level of biological organization, the more thermal sensitive are the processes involved. For example, the maximum temperature at which the whole organism can survive is usually lower than the maximum temperature at which many of its constituent tissues and cells can function (Ushakov, 1964; Roberts, 1973; Lagerspetz, 1987).

Explanations of the causative factors responsible for lethality due to acute temperature exposure have involved a search for the "weakest link" or rate limiting physiological process (Roberts, 1973; Lagerspetz, 1987). Studies have shown that a primary source of cellular damage from acute heat exposure involves the malfunction of receptor proteins and other processes associated with intercellular communication (White, 1976, 1983; Calderwood, 1987). Nervous tissue is particularly sensitive to elevated temperatures, and it has been suggested that the release of neurotransmitters from the presynaptic neuron may be extremely thermal sensitive and a limiting step leading to thermal death (Roberts, 1973; Crawshaw, 1979; White, 1983; Lagerspetz, 1987). In fish under extreme thermal stress, regions of the brain appear to be the most sensitive, and lethality may result from failure of the central nervous system to control respiration causing a critical drop in brain pO_2 (Tsukuda, 1961; Roberts, 1973).

Recently a cellular protective response has been identified which might be involved in protecting organisms from such detrimental effects of exposure to elevated temperatures. This response, referred to as the heat shock or stress response, involves the preferential synthesis of a suite of proteins referred to as heat shock proteins (hsp's) in reaction to exposure to elevated temperatures and other environmental stressors (Atkinson and Walden, 1985; Lindquist, 1986). Heat shock proteins are ubiquitous and highly conserved. Synthesis of hsp's is closely correlated with the phenomenon of heat hardening or thermotolerance in which a conditioning heat shock confers the ability to survive a subsequent, more severe heat shock that otherwise would be lethal to the organism (Landry et al., 1982; Gedamu et al., 1983; Mosser et al., 1987; Bosch et al., 1988).

It is now becoming clear that at least two of these hsp's, hsp60 and hsp70, have important functions in cells under normal conditions and take on dual, protective and repair, roles upon exposure to elevated temperatures (Rothman, 1989; Welch, 1990). For example, members of the large hsp70 family are believed to be catalysts of protein folding (Rothman, 1989; Beckman et al., 1990). Under normal conditions hsp70 chaperones proteins in an unfolded configuration from their site of synthesis in the cytoplasm to the appropriate intracellular compartment for transport across the membrane (Chirco et al., 1988; Deshaies et al., 1988; Craig, 1989). Another member of the hsp70 family, which resides in that compartment, then facilitates the appropriate folding of the newly imported protein (Craig, 1989). In addition, hsp60, occurring exclusively in mitochondria, is involved in the folding and

assembly of the numerous large enzyme-protein complexes associated with this organelle's inner membrane (Cheng et al., 1989; Ostermann et al., 1989).

Upon exposure to adverse environmental conditions, such as elevated temperatures, it has been suggested that hsp60 and hsp70 can also perform the related functions of renaturing damaged peptides and resolubilizing protein aggregates (Rothman, 1990; Welch, 1990). Therefore, hsp's might facilitate the repair of proteins and protein complexes associated with critical physiological processes and, thereby, protect cells from heat induced damage. Because of their role in protection from the detrimental effects of elevated temperatures, we hypothesized that if malfunctioning of the nervous system is indeed rate limiting in organismal survival at high temperatures, it may be because this tissue has a limited capacity to synthesize and accumulate hsp's and is more vulnerable to heat induced damage.

We designed a set of experiments with the fathead minnow (*Pimephales promelas*) to address this question. Initially, we exposed 25°-C acclimated fish to a wide range of temperatures to determine the maximum temperature at which they could survive for a 24-h period. We then examined the synthesis of hsp's in three tissues, striated muscle, gill and brain, upon 1-h exposures of the whole organism to a range of temperatures up to the maximum sublethal temperature. The results suggest that, of the three tissues examined, brain synthesized the fewest heat shock proteins and had the least capacity to synthesize hsp70 at 34°C. Further, the extreme sensitivity of brain tissue to elevated temperatures may be the result of the limited capacity of brain tissue to synthesize hsp's, supporting previous observations that

neural tissue might be a rate limiting tissue in organismal survival.

II. B. Materials and Methods

Organisms

Ninety- to 120-day-old fathead minnows (*Pimephales promelas*) were obtained from the University of North Texas, acclimated at 25°C with a 16L:8D photoperiod for at least 7 days, and fed frozen brine shrimp once daily to satiation. In initial experiments, fathead minnows were exposed to a broad range of temperatures to determine the maximum temperature at which they could survive. Fish (5 or 6) acclimated at 25°C were transferred to water at 33, 34, 35, 36, or 37°C for 24 h (three replicates per treatment). Control minnows were maintained at 25°C.

Experimental Design

To examine tissue specific synthesis of hsp's fish were transferred from 25°C to sublethal water temperatures of 28, 31, 32, 33, and 34°C for 1 hr. Control minnows were maintained at 25°C. Brain, gill and striated muscle tissues were dissected immediately and metabolically labeled with ^{35}S-methionine/cysteine. Gill and muscle samples included five replicates of three fish each. Two replicates of brain tissue of five fish each were obtained. After metabolic labeling samples were homogenized, and proteins were separated by SDS-PAGE and fluorographed.

Metabolic Labeling

Gill, muscle (50 ± 5 mg each) and brain tissue (39 ± 15.2 mg) were incubated at 25°C for 2 hr in 125μL of physiological saline (140 mM NaCl, 7.5 mM Na_2SO_4, 1.0 mM $NaHCO_3$, 1.5 mM $CaSO_4$, 1.0 mM $MgCl_2$, 3.0 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM tris) which contained 5.5 mM glucose and 1μCi/μL ^{35}S-methionine/cysteine (Translabel, ICN Biomedicals). Incorporation of radioactive amino acids were halted by freezing the samples at -80°C.

Sample Preparation

Metabolically labeled samples were rapidly thawed and centrifuged at 13,000xg at 4°C for 30 min. The supernatant was discarded, and the tissue was rinsed in saline. The tissue was suspended in 150 μL of tris buffer (150 mM Tris-HCl, pH 7.8, 1 μM phenylmethylsulfonylfluoride) at 4°C, homogenized for 45 sec with an Overhead Stirrer (Wheaton Instruments), and sonicated for 45 sec with a Sonifier Cell Disruptor (Branson). Samples were centrifuged at 13,000 x g at 4°C for 30 min, and the pellet was discarded. Protein concentration was determined by the method of Bradford (1976), and samples were frozen at -80°C.

SDS-PAGE

Samples containing 30 μg of total protein and molecular weight-standards (phosphorylase b, 97.4 kD; bovine serum albumin, 66.2 kD; ovalbumin, 45 kD; carbonic anhydrase 31 Kd; soybean trypsin inhibitor, 21.5 kD; and lysozyme, 14.4 kD)

were boiled in sample buffer and loaded onto 12.5% SDS-polyacrylamide gels using the method described by Laemmli (1970). After electrophoresis, the gels were impregnated with Enhance (New England Nuclear) and exposed to film for 6 days (Kodak XAR-5).

Quantitation

Molecular weights of sample polypeptides for each fluorogram were determined using a standard curve that was generated by plotting the R_f of each standard protein against its molecular weight. Sample band molecular weights were then determined by finding their R_f's on the standard curve and reading the molecular weight from the ordinate.

Relative intensities of bands on the film were quantitated using a laser densitometer (Ultroscan 2202, LKB Instruments; McMullin and Hallberg, 1986). Peaks of identified hsp's from scans were integrated, and intensities were calculated with GELSCAN (LKB) software on an Apple IIe microcomputer interfaced with the densitometer (Fig. 1). The intensities were determined at a scan speed of 100 mm/min, and the values were measured at an absorbance of 2. The actual intensities are arbitrary units such that a full scale peak height of 10 with full width at half maximum of 2.0 has an intensity of 1000 units (Griffen and Spalding, 1988).

Statistics

Kruskal-Wallis nonparametric analysis of variance was used to test for significance between treatment effects (Zar, 1984). Comparisons to controls were determined by one-tailed Dunnett T tests on ranked data. Significance for all statistical analyses was $\alpha = 0.05$.

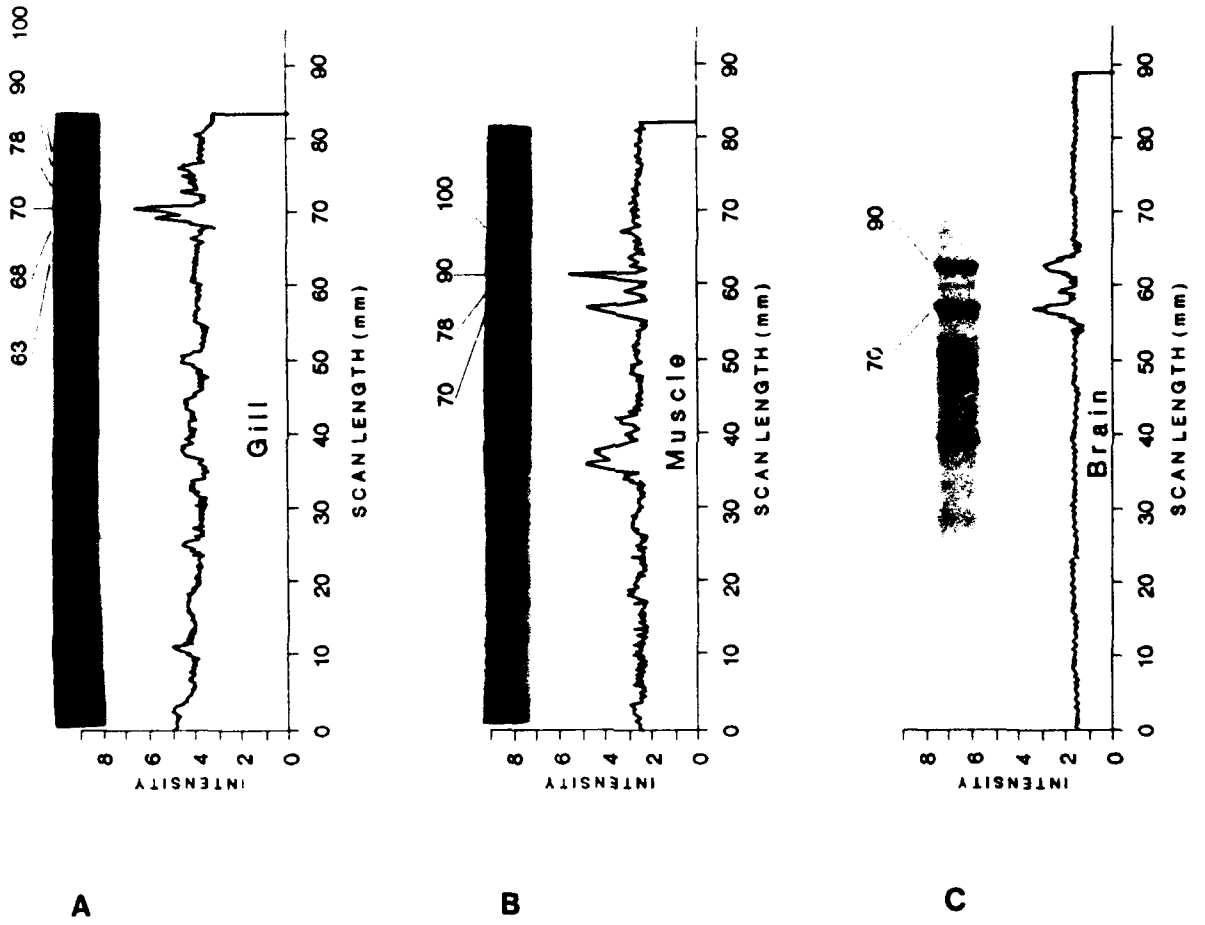

Figure 1. Overlying fluorograms with respective densitometer tracings from tissues of fish exposed to 32°C for 1h. The intensity scale is in arbitrary units. A. Gill- optically dense bands and underlying peaks corresponding to hsp's 60, 68, 70, 78, 90, and 100 are indicated. B. Striated muscle- bands and peaks correspond to hsp's 70, 78, 90, and 100. C. Brain- bands and peaks correspond to hsp's 70 and 90.

II. C. Results

In experiments to determine the maximum sublethal temperature, fish acclimated at 25°C were transferred to water at 33, 34, 35, 36, or 37°C and observed for 24 h. Survival was significantly reduced at 35°C (Dunnett's T test, $p \leq 0.05$), and mortality was 100% at 36 and 37°C. Therefore, the maximum sublethal temperature was defined as 34°C.

Typical results from each of the experiments from which intensities were analyzed statistically are shown in Fig. 1. Each scan corresponds to tissues metabolically labeled with ^{35}S-methionine/cysteine, separated on an SDS-polyacrylamide gels and exposed to X-ray film for 6 days. Molecular weights (kD) of heat shock proteins (hsp's) are indicated above each fluorogram, and optically dense bands correspond to peaks of respective scans.

The heat shock response was tissue specific in fathead minnows exposed in vivo to a broad temperature range. Individual hsp's induced and their biosynthetic rates differed among muscle, gill and brain tissue. Furthermore, temperatures which induced the heat shock response and the maximum temperature at which each tissue synthesized hsp's were also tissue specific. The temperature range of the gill, as well as the number of hsp's elicited, was greatest followed by the muscle and then brain tissue. Synthesis of six hsp's were observed in gill, with apparent molecular weights of 100, 90, 78, 70, 68.3, and 63.4 kD, hereafter referred to as hsp's 100, 90, 78, 70, 68, and 60 (Fig. 2A). Rates of ^{35}S-methionine incorporation into hsp's 100, 90, and 78 increased from 28 to 33°C, while incorporation into hsp's 70 and 68

increased to 34°C. Increased synthetic rates of a 60 kD protein were found from 31 to 34°C. The fluorogram shown indicates a relative decrease in translation rate for hsp's of 100, 90, and 78 kD at 34°C. However, statistical analysis of stress protein band intensities over all replicates showed that five of the six (100, 90, 78, 70, and 68 kD) protein synthetic rates at 34°C were significantly greater than those of controls (Fig. 2B; Dunnett's T test, $p \leq 0.05$). Significant increases above control (25°C) rates of incorporation were observed for hsp's of 78 and 68 kD from 28 to 34°C, whereas the 70-kD protein showed increased incorporation from 31 to 34°C. A significantly increased rate of incorporation for the 100-kD protein was found at 34°C. Rates of incorporation into the 70- and 68-kD hsp's

were greatest, followed by those of hsp's 90, 78, 100, and 60 kD, respectively.

Four hsp's, 100, 90, 78 and 70, were found in striated muscle (Fig. 3A). Incorporation of radiolabel into hsp 70, although apparent at 25 and 28°C, was greater at 31-34°C. Rates of radiolabel incorporation for hsp's 100, 90, and 78 increased at 31 through 33°C and decreased at 34°C. Densitometric scanning of five replicates for each treatment revealed that temperature significantly altered relative rates of synthesis for all four hsp's (Kruskal-Wallis, $p \leq 0.05$). In contrast to the fluorogram, when all replicates were taken into account, significant increases above control (25°C) rates were observed for hsp's of 78 and 90 kD from 31 to 34°C (Fig. 3B; Dunnett's T test, $p \leq 0.05$). Significant increases in accumulation of hsp's relative to control occurred from 32 to 34°C for the 70-kD hsp and only at 32°C for the 100-kD hsp. Lack of radiolabel incorporation into the 100-kD protein in three

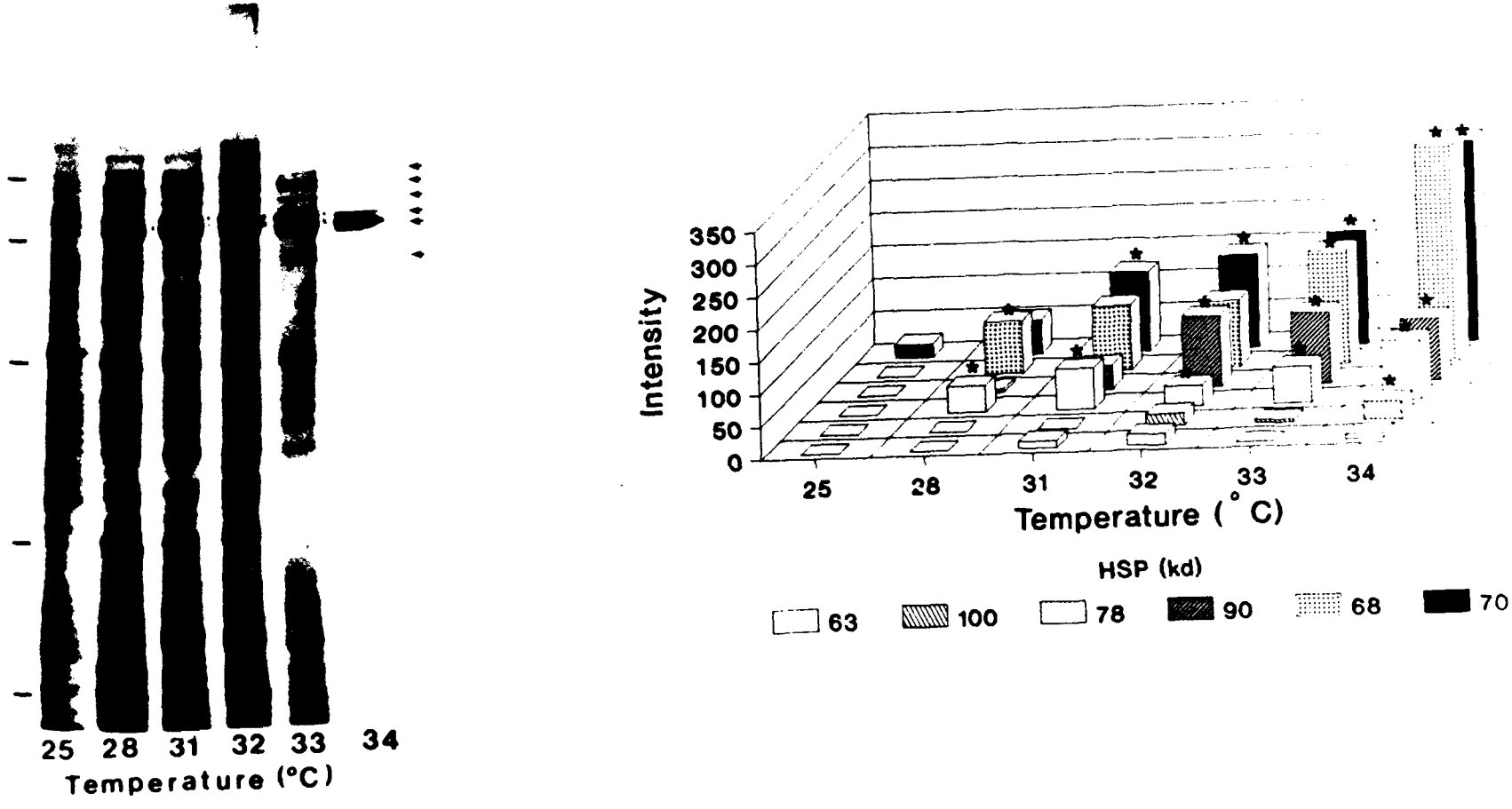

Figure 2. A. A fluorogram of gill tissue proteins from fathead minnows transferred to water at 28 to 34°C for 1 h and control minnows maintained at 25°C. Molecular weight markers of 97, 66, 42, 31, 21, and 14 kD, respectively, are designated on the left. Arrows on the right denote heat shock proteins of 100, 90, 78, 70, 68 and 60 kD respectively. B. Median intensities of 100-, 90-, 78-, 70-, 68- and 60-kD proteins in fluorograms from replicate samples (n = 5) of experiment described in A. Intensities were determined densitometrically and are presented as arbitrary units (see Materials and Methods). Intensities which are statistically significantly higher than controls are indicated by *.

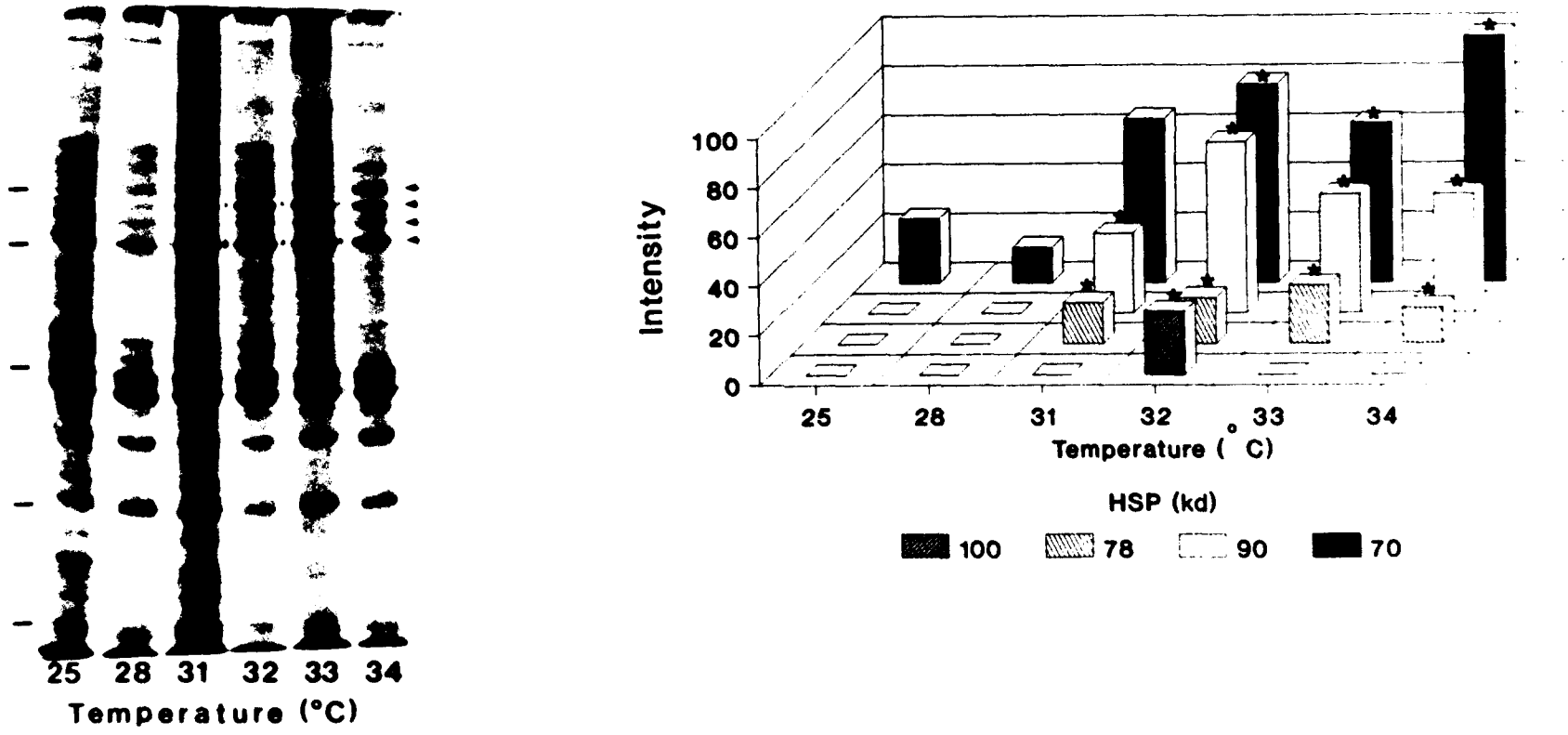

Figure 3. A. A fluorogram of striated muscle proteins from fathead minnows transferred to water at 28 to 34°C for 1 h and control minnows maintained at 25°C. Molecular weight markers of 97, 66, 42, 31, 21, and 14 kD, respectively, are designated on the left. Arrows on the right denote heat shock proteins of 100, 90, 78 and 70 kD respectively. B. Median intensities of 100-, 90-, 78-, and 70-kD proteins in fluorograms from replicate samples (n = 5) of experiment described in A. Intensities were determined densitometrically and are presented as arbitrary units (see Materials and Methods). Intensities which are statistically significantly higher than controls are indicated by *.

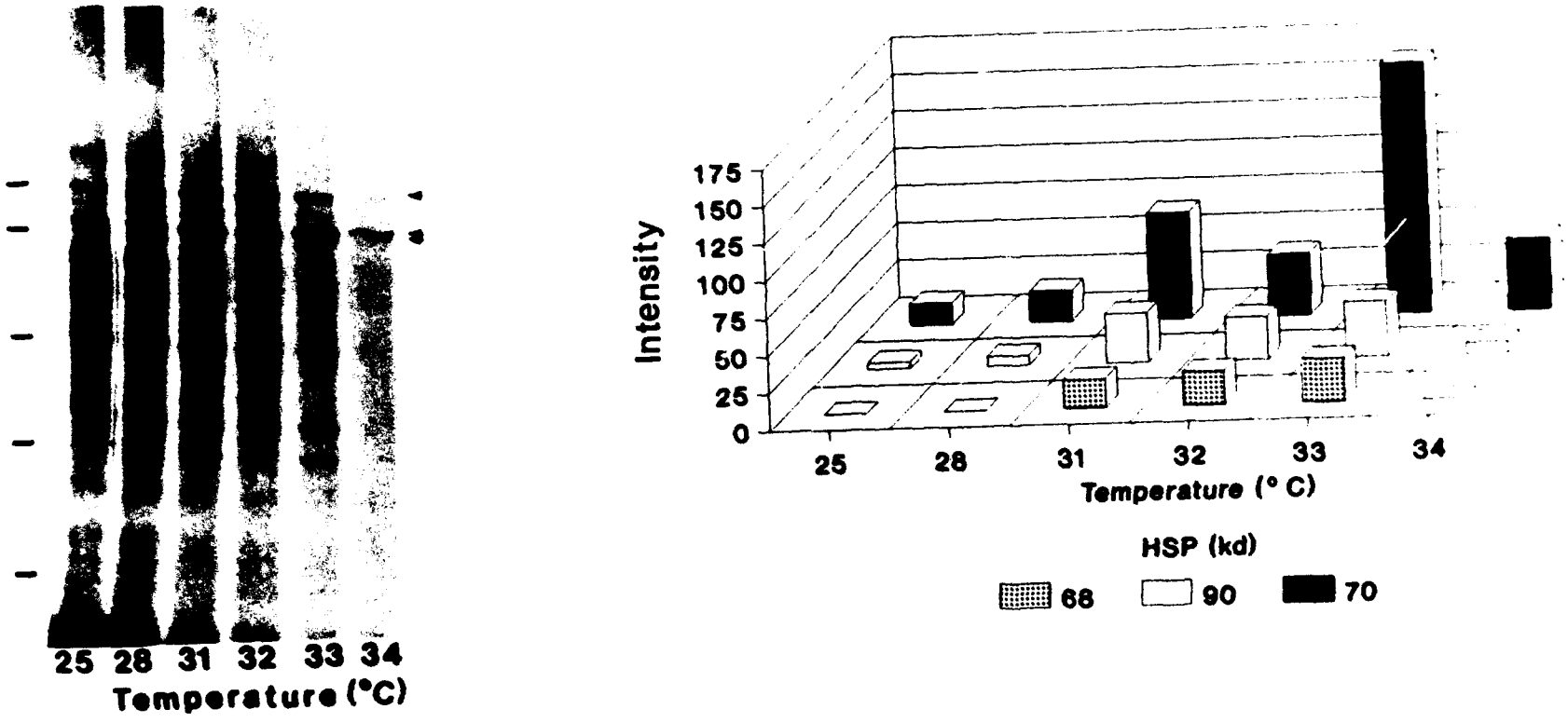

Figure 4. A. A fluorogram of brain tissue proteins from fathead minnows transferred to water at 28 to 34°C for 1 hr and control minnows maintained at 25°C. Molecular weight markers of 97, 66, 42, 31, 21, and 14 kD, respectively, are designated on the left. Arrows on the right denote heat shock proteins of 90, 70, and 67 kD, respectively. B. Mean intensities of 90-, 70-, and 68-kD proteins in fluorograms from replicate samples (n = 2) of experiment described in A. Intensities were determined densitometrically and are presented as arbitrary units (see Materials and Methods).

of the five replicates at 33 and 34°C and the use of the median as our quantile compromised its graphical representation in the place figure 2 here (gill) landscape captionfigure. The heat shock response was dominated by hsp 70 followed by 90, 78, and 100 respectively.

The brain showed low incorporation of radiolabel into hsp's 70 and 90 at 25 and 28°C (Fig. 4A). Induction of the hsp response, as seen by increased band intensities corresponding to hsp's 90, 70, and 68, began at 31°C and continued through 33°C. At 34°C, a sharp decline in hsp synthesis and accumulation was noted, with only hsp 70 being clearly visible. In addition, overall translational activity appeared to be markedly inhibited at 34°C as evidenced by the decrease in overall protein banding or background.

Only three hsp's could be quantitated in brain tissue, and statistical analysis was not possible because of the low number of replicates (n = 2; Fig. 4B). Densitometric analysis indicated that hsp 70 dominated the heat shock protein response, followed by hsp's 90 and 68. Maximum intensities for all heat shock proteins occurred at 33°C. A threefold decrease in radiolabel incorporation into hsp's 70 and 90 was found from 33 to 34°C. In addition, hsp 68 was not quantifiable at 34°C due to low incorporation of label.

II. D. Discussion

Results of this study showed that fathead minnow (*Pimephales promelas*) survival was significantly decreased at 35°C. This corroborates a previous report by

Watenpaugh and Beitinger (1985) in which the critical thermal maxima for this species was found to be 35.12°C. However, Schmidt et al. (1984) found that fathead minnow epithelial cell viability did not decrease significantly until 41°C exposures, well beyond the thermal limits of the whole organism. This supports previous trends noted by Ushakov (1964) and Lagerspetz (1987) in which thermal sensitivity was found to be greatest at the organismal level and lowest at the cellular level. Tissues of fathead minnows exposed *in vivo* to elevated temperatures responded by eliciting a suite of proteins whose synthesis and accumulation followed tissue specific patterns. Gill tissue produced the greatest number of hsp's (six) followed by striated muscle (four) and the brain (three). Molecular weights of hsp's synthesized in the gill were 100, 90, 78, 70, 68, and 60 kD. Heat shock proteins 100, 90, 78, and 70 were observed in the striated muscle and hsp's 90, 70, and 68 were induced in the brain. In comparison, several studies that have involved *in vitro* heat shock exposures with fish cell cultures have yielded similar, but not identical results. Fathead minnow epithelial cells exposed to 32°C elicited hsp 70 plus three low molecular weight hsp's of 39, 36, and 22 kD (Merz and Laudien, 1987). However, our data did not show induction of any low molecular weight hsp's over the sublethal temperature range of this species. Tissue specificity or differences in experimental conditions might account for such differences.

Further, Koban et al. (1987) reported that hsp's 94, 91, 76, 74, and 65 were induced in catfish hepatocytes. Other researchers using *Tilapia sp.* ovary cells (Chen et al., 1988), chinook salmon embryonic cells (Heikkila et al,. 1982; Gedamu et al.,

1983), rainbow trout hepatoma (Misra et al., 1989) and fibroblast cells (Mosser et al., 1986) have not only shown high and medium molecular weight hsp's (100-60 kD) but also low molecular weight hsp's (42-19 kD) upon hyperthermic exposures.

Tissue specific induction patterns of hsp's have been reported in other organisms as well. For example, connective tissue (blood and muscle cells) of the mollusc, *Aplysia californica*, elicited hsp's 110, 90, and 70 upon *in vitro* heat shocks of 34°C, while ganglion cells produced only hsp's 110 and 70 (Greenberg and Lasek, 1985). Similar neuronal heat shock responses have been seen in the laboratory rat and American cockroach (*Periplaneta americana*). Heat shock proteins 110, 74, and 64 were synthesized in optical nerves of the laboratory rat, and hsp's 83, 70, and 68 were synthesized in the nerve cords of the cockroach (Tytell and Barbe, 1987; Ruder et al., 1989). Although few multi-tissue studies have been undertaken with poikilotherms, a study on the lungless salamander, *Desmognathus ochrophaeus*, brain and muscle tissues did not show any differences in the heat shock response (Rutledge et al., 1987).

Two explanations for the more complex heat shock response of the gill as compared to that of brain and striated muscle are plausible. First, only the gill is directly exposed to heated water, whereas the other two tissues experience increased temperatures via diffusion from surrounding tissues. A second explanation is that the evolution of the gill as a highly membranous gas and ion exchange organ required increased protective mechanisms such as the heat shock proteins for membrane structural integrity under constantly changing exposures, i.e. temperatures, gases, ions,

pH, turbidity, etc. In addition, the greater variety of heat shock proteins in the gill may also be indicative of the many cell types and functions performed versus those in brain and muscle. At present, nothing is known about the contribution of cell types to the overall gill hsp response.

In the present study, temperature ranges over which hsp's were synthesized were also highly tissue specific. Gill tissue had the broadest range and showed significantly increased hsp syntheses at 28°C, reaching maximum rates at 34°C, whereas striated muscle showed significantly increased hsp syntheses at 31°C and reached maximum rates at 34°C. In contrast, brain tissue showed increased hsp syntheses at 31°C, reached maximum rates at 33°C, and showed a marked decrease at 34°C. These observed differences in the heat shock protein response suggest that, of the three tissues examined, brain is the most sensitive. The temperature range which elicits the heat shock response in fathead minnow striated muscle was similar to that of connective tissue in a mollusc, *Aplysia* (Greenberg and Lasek, 1985). Both showed significantly increased synthetic rates of hsp 70 at 31°C, and both showed maximum responses at the maximum temperatures tested, 34°C for minnow muscle and 37°C for mollusc connective tissue. In an *in vitro* heat shock exposure, as were the *Aplysia* exposures, we found that striated muscle elicited its maximum heat shock response at 37°C (unpublished results).

Similarly, the minimum temperature required for induction of hsp's in fish brain and mollusc ganglion was 31°C. However, the ganglion showed a heat shock protein response through 37°C, whereas the maximum response was 33°C in the

brain. The heat shock response in brain also may be explained by data suggesting that axons have low synthetic abilities for hsp's and, therefore, require extracellular hsp inputs, specifically from glial cells, to repair damage incurred from hyperthermic exposure (Hightower and Guidon, 1989).

Although our data demonstrated specificity of hsp induction patterns for each tissue, there appears to be a common pattern with all tissues eliciting hsp's 70 and 90 upon *in vivo* hyperthermic exposure. Much is now known about the function of these two hsp's in normal cellular metabolism. Heat shock protein 70 has been shown to have at least three functions, multimeric protein assembly, unfolding for translocation, and disaggregation of protein aggregates (Craig, 1989; Rothman, 1989; Welch; 1990). These functions are also thought to facilitate repair of proteins and protein complexes associated with common and critical physiological processes, and thus protect cells from heat induced damage. Whereas, hsp 90 has been shown to be associated with steroid receptors (Baulieu, 1989), the aryl hydrocarbon hydroxylase receptor (Perdew, 1988), and a translational initiation factor kinase (Rose et al., 1989). Thus, hsp 90 is intimately involved in both transcriptional and translational events.

The fact that the heat shock protein response differs among tissues supports the hypothesis that thermal limits of an organism are governed by certain tissues more than others. The maximal hsp responses of gill and muscle at the maximum sublethal heat shock temperature negate their suspect as "weak link" candidates. However, the coincident decreases in hsp synthesis and accumulation in the brain

with that of the fish's upper thermal limit supports earlier hypotheses that the central nervous system governs the thermal limits of poikilotherms. Sensitivity of brain tissue to elevated temperatures may be the result of the limited capacity of the brain to synthesize hsp's and supporting the suggestion that neural tissue might be a rate limiting tissue in organismal survival. Without an appropriate cellular protective mechanism, such as heat shock proteins, cellular function and integrity may be compromised along with the organism's survival.

III. CHEMICAL EXPOSURES

III. A. Introduction

Biotic responses to environmental perturbations begin at the molecular/cellular level and then extend to tissues and organs, whose responses occur before changes in populations and ecosystems (Bouk, 1984). Assessments of water quality and biotic health have largely centered on whole animal and population responses, such as gross abnormalities, survival, growth, and reproduction (Mayer, 1983). Although these responses have been the best criteria to date for evaluating general stress, they have several limitations: 1) they are not based on the mechanisms which underlie the relationship between general stress physiology and toxicity; 2) they measure stress indirectly; and 3) they function at the organismal level and lack the sensitivity or cellular parameters (Jenkins and Sanders, 1986). Therefore, measurement of biotic health using biochemical/cellular parameters has the potential to provide an early warning of excessive environmental perturbations and would do so comparatively faster and cheaper than monitoring whole animal and population responses.

Over the past decade, investigations into the use of biochemical parameters as indicators of organismal health and water quality have intensified. Unfortunately, much of the body of biochemical indicator (biomarker) research has dealt with the measures of secondary effects (blood and tissue enzyme activity, biochemical composition of blood and tissues; Mazeaud and Mazeaud, 1981; Neff, 1985) rather than parameters intimately involved in protecting and defending the cell from environmental insults. Thus, biomarkers that correspond to events that are coupled

to cellular protection and defense systems could serve as highly sensitive indicators of contaminant exposure and adverse biological effects (Sanders et al., in press).

The stress protein response (SPR), a ubiquitous protective mechanism that enables cells to tolerate environmental perturbations, has potential to serve as an indicator of water quality and organismal health. Induction of the SPR involves the increased transcription of stress protein genes, the products of which function in protein folding, multimeric protein assembly, receptor interactions and heme catabolism, to name a few (Craig, 1989; Keyse and Tyrell, 1989; Rothmann, 1989; Beckmann et al., 1990; Schlesinger, 1990). Synthesis of hsp 70, a major stress protein, has been shown to be induced by a wide variety of stressors (i.e. hyperthermia, sulfhydryl-reactive agents, heavy metals, peroxide, dinitrophenol, steroids, and viral infection; Nover, 1984; Schlesinger, 1990). Increased synthesis of low molecular weight stress proteins, 40 through 20 kD, however, have been shown to be indicative of oxidizing agents, arsenite, and heavy metal exposure (Shibahara et al., 1987; Keyse and Tyrell, 1989). Thus, the SPR has potential to serve as both an indicator of general organismal stress and stressor specific exposure.

To evaluate the preferential translation of the stress proteins as biomarkers of effects and/or exposure we designed an experimental regime in which fathead minnows (*Pimephales promelas*) were exposed to chemicals with different modes of action for various lengths of time and to a wide range of concentrations. The objectives of this research endeavor were fourfold: 1) to determine the time required for the SPR to be induced and time(s) in which maximum stress protein synthetic

rates occurred; 2) to determine the effect of chemical concentration on stress protein synthesis and accumulation; 3) to determine tissue specificity of the SPR; and 4) to correlate the synthesis and accumulation of induced stress proteins to a known effect, namely percent mortality.

Several studies with cell lines have shown that the expression of different arrays of stress proteins is chemical specific (Courgeon et al., 1984; Honda et al., 1986; Shelton et al., 1986). Therefore, we examined the potential chemical specificity of the SPR by exposing fish to toxicants in which mode of action and/or effects were well documented.

Sodium arsenite is the best known chemical inducer of the SPR (Duncan and Hershey, 1987; Kapoor and Lewis, 1987; Hiwasa and Sakiyama, 1986). It binds to thiol groups on proteins thereby inhibiting oxidative pathway, non-lysosomal degradation of proteins, and impairing tissue respiration (Webb, 1966; Goyer, 1986; Munro and Pelham, 1986; Klemperer and Pickart, 1989).

Sodium chromate (CrVI) has been shown to oxidize proteins and DNA (Ono, 1988; Nieboer and Jusys, 1988; Nieboer and Shaw, 1988). When reduced to CrIII, it has high affinity for nucleotides and nuclei acids and can alter their three-dimensional geometry. Thus, it is a potent mutagen and carcinogen (Ono, 1988; Nieboer and Shaw, 1988; Yassi and Nieboer, 1988). In addition, chromate has been shown to cause structural damage to fish gill and concentrate in the nuclear fraction (Van der

Putte, et al., 1981; Van der Putte and Part, 1982).

Lindane is an organochlorine insecticide that has a mode of action similar to toxaphene and cyclodiene insecticides. Toxicity of lindane is a result of its inhibition of the GABA-activated chloride channel in the nerve (Matsumura and Ghiasuddin, 1983; Woolley and Zimmer, 1986; Fishman and Gianutsos, 1987; Joy et al., 1987; Ogata et al., 1988). Thus, it inhibits GABA's ability to suppress excitation in nerves. In aquatic organisms, lindane has been shown to disrupt osmoregulatory mechanisms, alter the membrane lipidic matrix, and inhibit protein synthesis (Demael et al., 1987; Shirley and McKenney, 1987; Al-Chalabi and Al-Khayat, 1989).

Diazinon is an organophosphate insecticide that inhibits cholinesterase activity (Matsumura,1985; Murphy, 1986; Durham, 1987). In fish, this toxicant has been shown to inhibit oxygen comsumption and oxidative enzymes (Rao and Rao, 1979; Vijayalakshmi, 1980; Basha et al., 1984).

III. B. ARSENITE EXPOSURES

III. B. 1. Materials and Methods

Organisms

Ninety-day old fathead minnows (Pimephales promelas) were obtained from a culture located at the University of North Texas. Fish used for the study were acclimated to 25°C for at least 7 days and a 16L:8D photoperiod and were fed frozen brine shrimp twice daily to satiation.

Experimental Design

An initial range-find experiment was carried out to determine a range of sodium arsenite concentrations suitable for a definitive LC50 test and to determine times to first signs of intoxication. Aquaria (19L) were filled to 10L with nominal concentrations of 0, 5, 10, 15, 25, and 35 mg As/L. Each treatment was replicated three times. Fish (3 to 5) were placed into each treatment replicate and observed for onset of intoxication. Fish exposed to 25 mg As/L, showed first signs of intoxication by 8-10 h. Signs of intoxication followed a behavioral progression where fish first displayed lethargy with coughing, followed by erratic swimming at the water/air interface, and finally a loss of equilibrium.

To determine the length of time required for stress protein induction, two replicates of three fish each were exposed to 25 mg As/L and sampled at 0, 2, 4, 6, 8, and 10h. Immediately after sampling, fish were sacrificed and gill tissue (50.6 $\pm$ 3.7 mg) was dissected and pooled within each treatment replicate. Tissues were metabolically labeled for 2h with ^{35}S-methionine/cysteine. Incorporation of label into

proteins was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Resulting fluorograms demonstrated that all stress proteins observed within the 10-h treatment period were maximally induced between 6 and 10h. Thus, 8h was selected as the sampling time to examine the SPR in the subsequent definitive 96-h toxicity test.

To assess the concentration range in which the gill and striated muscle synthesize stress proteins, fish were exposed to five different arsenite concentrations (6.02 $\pm$ 0.24; 9.59 $\pm$ 0.05; 17.72 $\pm$ 1.91; 26.66 $\pm$ 1.91; 35.61 $\pm$ 2.64 mg As/L) , plus control, and sampled at 8h. Each treatment was replicated five times. Thirteen fish were placed into each treatment replicate and three were subsampled at 8h while the rest were exposed for 96h. Tissues of subsampled fish (gill 45.8 $\pm$ 6.6; striated muscle 47.8 $\pm$ 4.9 mg) destined for metabolic labeling, were treated as in the induction study. Time to mortality was recorded every 24h. Metabolic labeling, sample preparation, SDS-PAGE, and quantitation were performed using methods described in the heat shock experiment. X-ray films used for densitometric quantitation were exposed to gels for 3d.

Immunoblotting

Proteins from striated muscle tissue of 96-h toxicity test survivors were separated on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose using a transfer buffer containing 1% SDS, 0.192 M glycine in 0.025 M tris and 20 % methanol (v/v), pH 8.3. The gels were subjected to 30 V for 15 h at 4°C. The

nitrocellulose was then blocked for 30 min in tris buffered saline (TBS) containing 3% bovine serum albumin (BSA), 0.5% Tween (TTBS) and 0.01% thimerosal.

The nitrocellulose was probed with one of two antibodies, a polyclonal antibody (Ab) raised against hsp60 from the moth *Heliothis* (generously provided by S. Miller; Miller, 1987) and a monoclonal Ab raised against hsp72/73 from HeLa cells (generously provided by W. Welch; Riabowol et al., 1988). Blots were incubated at room temperature with hsp60 and hsp 72/73 antibodies for 90 min and rinsed several times with TBS containing 0.5% Tween-20 (TTBS). The hsp60 Ab blots were incubated with a second Ab of goat anti-rabbit alkaline phosphatase (AP) conjugated antibody (BIORAD) for 90 min, whereas hsp 72/73 Ab blots utilized a goat anti-mouse Ab for the second antibody. The blots were washed several times with TTBS and TBS and incubated with the substrates 5-bromo 4-chloro 3-indoyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride (BIORAD) to visualize bound antibody.

Water Chemistry

All exposures used dechlorinated Denton City tap water that had the following mean characteristics: alkalinity 100 mg CaCO3/L; hardness 120 mg CaCO3/L; and conductivity 300 umhos. Dissolved oxygen and pH were monitored for each test. Dissolved oxygen ranged from 4.6 to 9.0 mg O_2/L while pH ranged from 7.2 to 7.5 over all concentrations. Arsenic concentrations were determined using a Perkin Elmer atomic absorption spectrophotometer (EPA method 206.2).

Statistical Analysis

The LC50 was determined by probit analysis. Stress protein intensities were analyzed nonparametrically due to heteroscadacity. Kruskal-Wallis analysis of variance was used to determine the significance of treatment effects (alpha=0.05) on specific stress protein intensities. Comparisons to controls were determined by one-tailed Dunnett T tests on ranked data. Spearman rank correlations were used to compare percent mortality to stress protein intensities.

III. B. 2. Results

Gill tissue of fathead minnows exposed to 25 mg As/L responded by eliciting the stress response. Six stress proteins with apparent molecular weights of 20, 30, 40, 70, 72, and 74 kD were observed (Figure 5). Radiolabel incorporation into stress proteins increased beyond 0-h rates by 2h of exposure. Incorporation remained high throughout the 10h exposure period. Maximum synthetic rates for the 30 kD protein occurred at 4 and 6h. However, densitometric analyses of the other stress proteins (20, 40, 70, 72, and 74) showed that maximum rates occurred at 6, 8, and 10h exposure. An 8h exposure was, therefore, determined to be the appropriate sampling time for the concentration-response study. Our goal was to correlate the synthesis of the stress proteins to an eventual effect, namely percent mortality.

The stress protein response was found to be tissue-specific in fish exposed to a range of arsenite concentrations. The specific stress proteins and their levels of radiolabel incorporation differed between gill and the striated muscle. Moreover, the

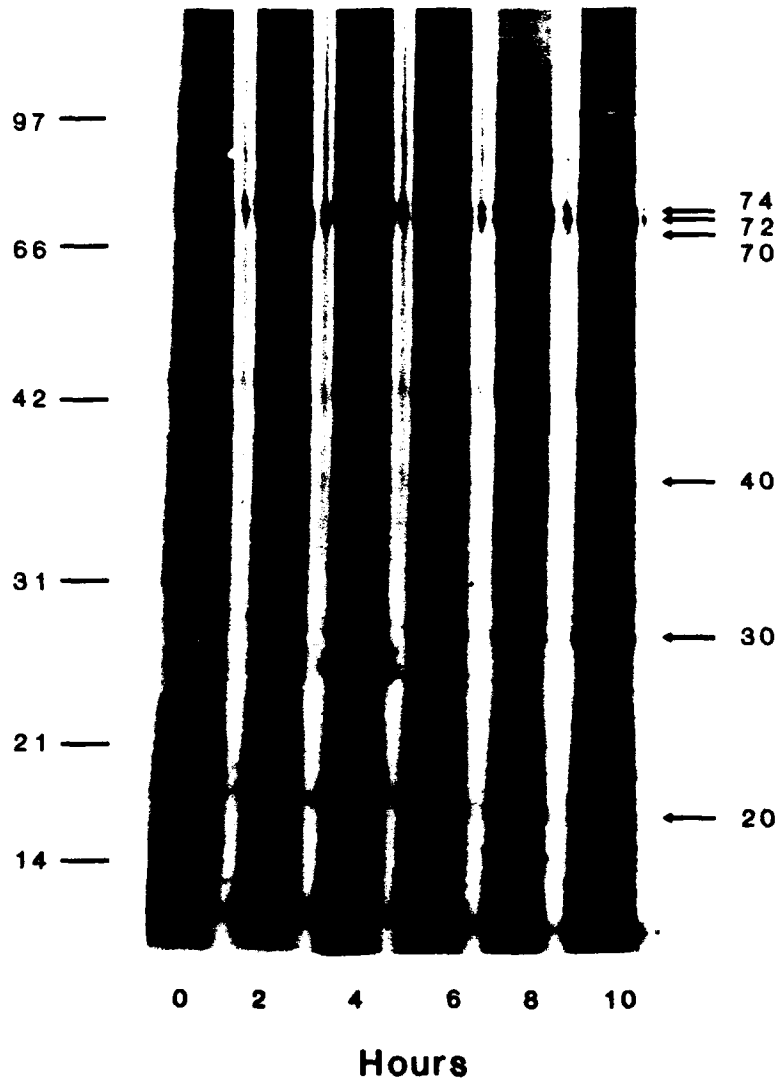

Figure 5. A fluorgram of gill tissue proteins from fish exposed to 25 mg As/L for 0, 2, 4, 6, 8, and 10h. Molecular weight markers are designated on the left. Arrows on the right denote stress proteins of 20, 30, 40, 70, 72, and 74 kD.

range of concentrations required for the increased incorporation of ^{35}S-methionine/cysteine into similar molecular weight proteins was also tissue specific.

Increased radiolabel incorporation into six stress proteins (20, 30, 40, 70, 72, and 74) was observed in the gill (Figure 6A). A qualitative description of one of the replicate fluorograms shows that ^{35}S-methionine/cysteine incorporation into proteins of 20, 40, 72, 74 kD increased above control rates from 17.72 to 35.61 mg As/L, and the 70 kD protein showed an increase at 35.61 mg As/L. Statistical analyses of densitometric scans of all five replicates showed that sodium arsenite significantly altered incorporation into five of the six (20, 40, 70, 72, and 74 kD) stress proteins observed (Figure 6B; Kruskal-Wallis, $p \leq 0.05$). Arsenite treatments did not significantly affect stress protein 30 synthesis and accumulation (Kruskal-Wallis, $p = 0.16$). Dunnett's T test performed on ranked data showed significantly increased ($p \leq 0.05$) radiolabel incorporation above controls for stress proteins 20, 40, and 70 from 9.6 to 35.6 mg As/L. Increased isotope incorporation into stress protein 20 was evident at 6.0 mg As/L but was not significant due to high variance. For stress proteins 72 and 74, significant increases occurred from 17.7 to 35.61 mg As/L. Overall, levels of incorportation were greatest into the 20 kD stress protein, followed by stress proteins 70, 72, 74, 40, and 30, respectively.

Five stress proteins of 20, 30, 68, 70, and 90 kD were found in the striated muscle (Figure 7A). The 70-kD protein was observed at all concentrations and showed increased incorporation rates above controls from 17.7 to 35.61 mg As/L. Increased incorporation of ^{35}S-methionine into stress proteins 20 and 30 occurred

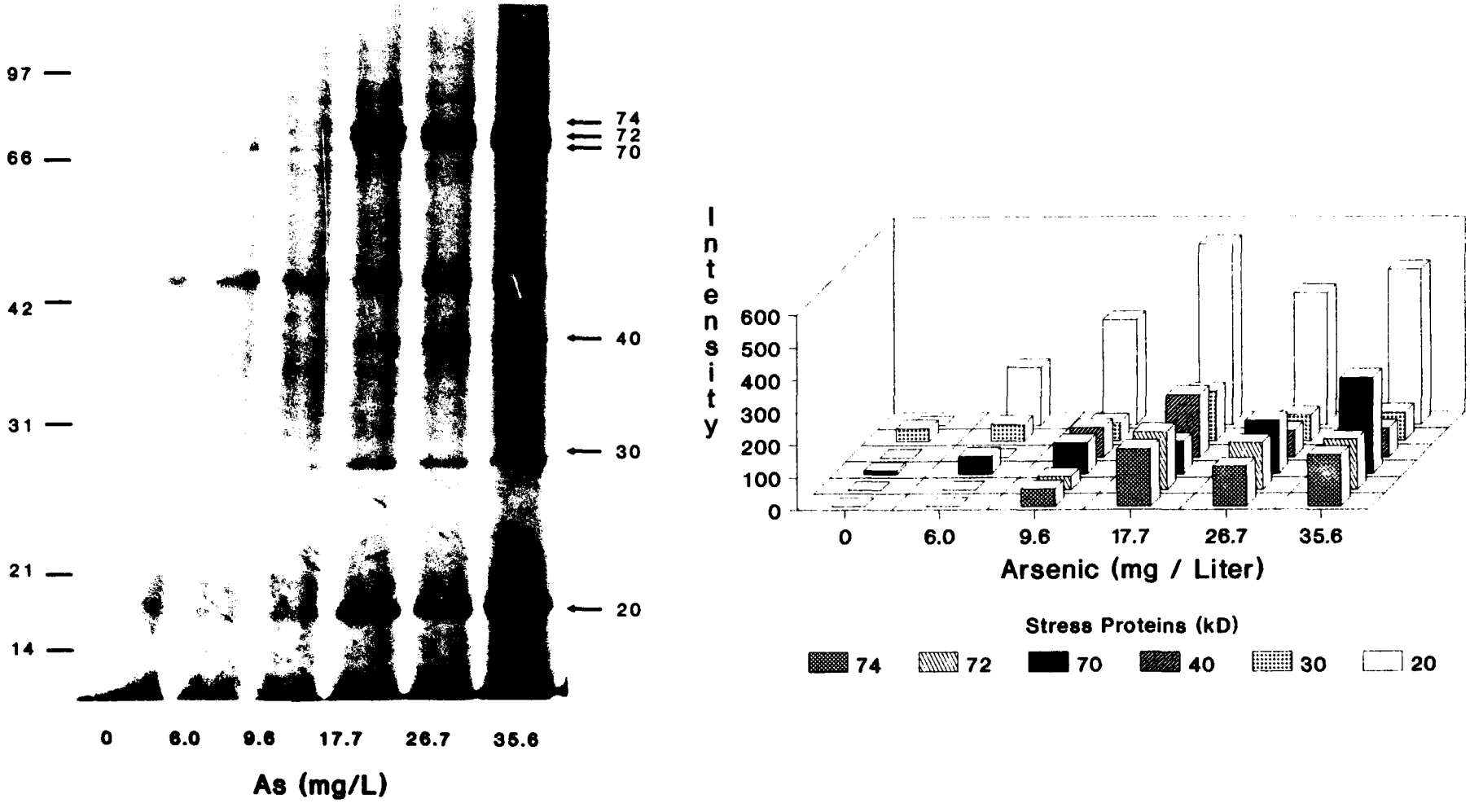

Figure 6. A. A fluorgram of gill tissue proteins from fish exposed to 0, 6.0, 9.6, 17.7, 26.7, and 35.6 mg As/L for 8h. Molecular weight markers are designated on the left. Arrows on the right denote stress proteins of 20, 30, 40, 70, 72, and 74 kD. B. Median intensities of 20-, 30-, 40-, 70-, 72-, 74-kD proteins in fluorograms from replicate samples (n = 5) of experiment described in A.

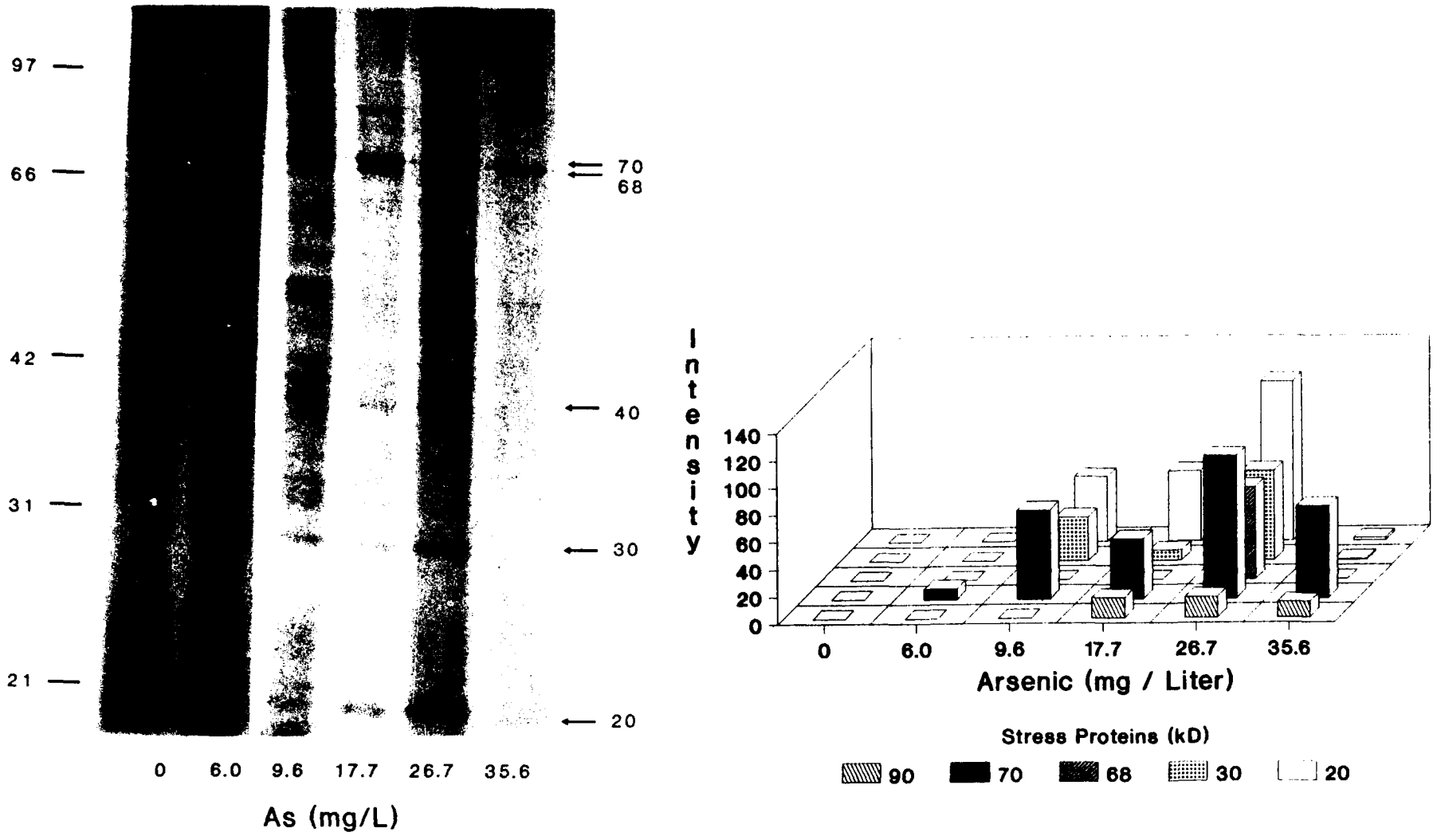

Figure 7. **A.** A fluorgram of striated muscle tissue proteins from fish exposed to 0, 6.0, 9.6, 17.7, 26.7, and 35.6 mg As/L for 8h. Molecular weight markers are designated on the left. Arrows on the right denote stress proteins of 20, 30, 40, 68, and 70 kD. **B.** Median intensities of 20-, 30-, 40-, 68-, 70-kD proteins in fluorograms from replicate samples (n = 5) of experiment described in **A.**

from 17.7 to 26.7 mg As/L. There was no observable incorporation at the highest arsenite concentration for either protein. A 90-kD protein was observed from 17.7 to 35.61 mg As/L and the 68-kD protein was seen from 17.7 to 26.7 mg As/L. Arsenite treatments significantly affected the synthesis and accumulation of all stress proteins quantitated in the striated muscle (Kruskal-Wallis, $p \leq 0.05$). In addition, Dunnett's T tests on ranked data showed significantly increased radiolabel incorporation into the 70-kD protein above control levels from 17.7 to 35.61 mg As/L (Figure 7B). Significantly increased incorporation levels were also observed with the 30-kD protein at 9.6 and 26.7 mg As/L. The remaining of the stress proteins quantitated (20, 68, and 90) showed significant increases above controls at only 26.7 mg As/L.

Intensities from replicate fluorograms for each stress protein from both gill and striated muscle were correlated to 96-h mortalities for each respective treatment replicate (Table 1). In the gill, synthesis and accumulation rates of stress proteins 20, 70, 72, and 74 were significantly correlated with mortality. However, in striated muscle, only stress protein 70 was shown to be significantly correlated with mortality. The lack of radioisotope incorporation at the highest concentration compromised the correlation's significance.

Striated muscle protein from survivors of replicate number one was immunoblotted with antibodies to stress proteins 70 and 60 (Figure 8A and 8B). Increased relative antibody binding for stress protein 70 occurred at all treatment concentrations compared to the control. In comparison, increased hsp 60 Ab binding

occurred at only the highest two arsenite concentrations.

Table 1. Spearman rank correlations of stress protein intensities from gill and striated muscle tissues versus 96-h mortality.

Tissue	Stress Protein	Correlation Coefficient	P > R
Gill	74	0.537	0.0056
	72	0.599	0.0020
	70	0.697	0.0001
	40	0.277	0.1807
	30	0.297	0.1499
	20	0.626	0.0011
Striated Muscle	90	0.240	0.2693
	70	0.417	0.0424
	68	0.201	0.3471
	30	0.064	0.7657
	20	0.192	0.3674

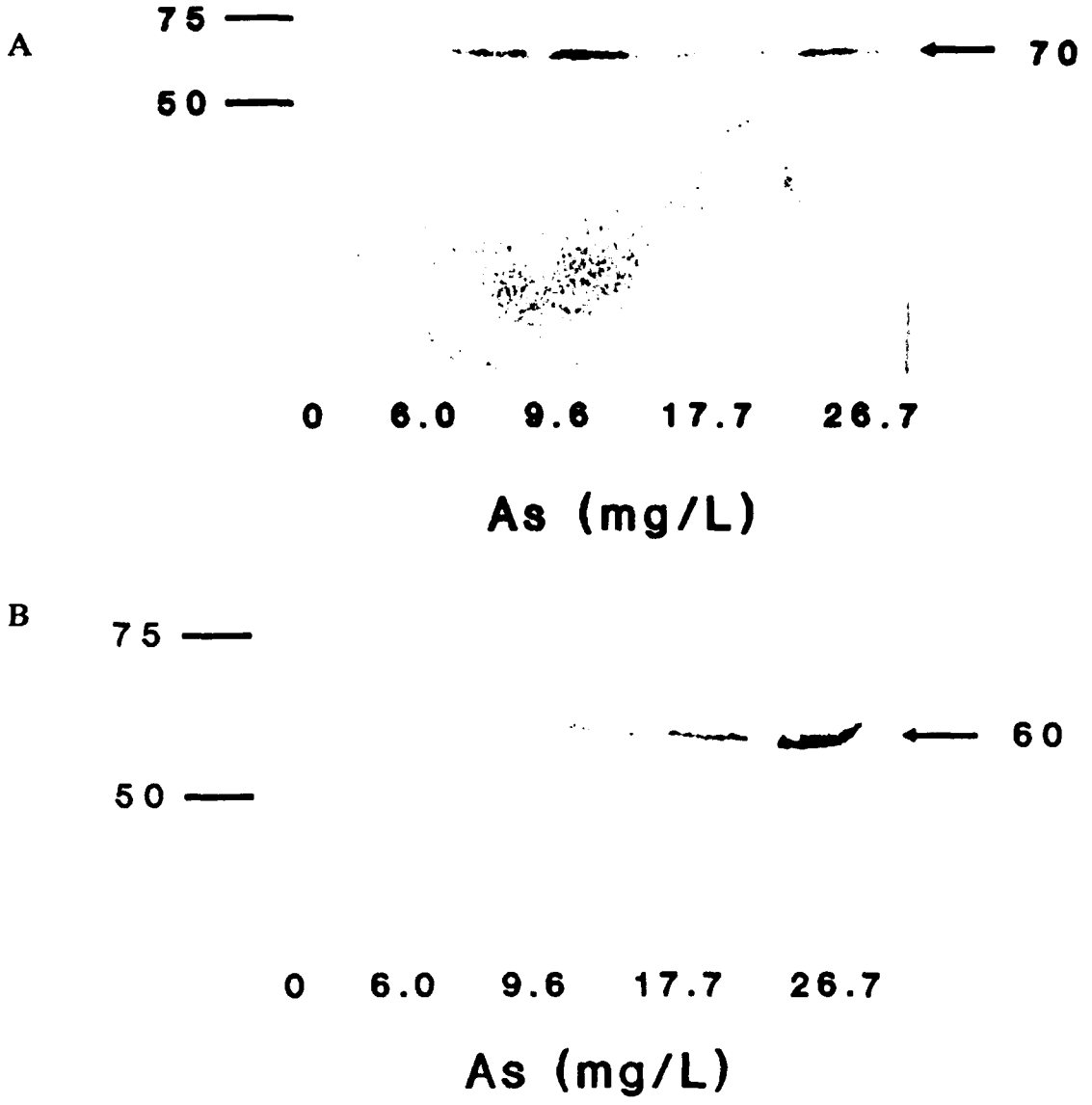

Figure 8. A. Western blot of striated muscle tissue proteins, from fish that survived the 96h toxicity test, against a monoclonal antibody raised against hsp70 (from Dr. W. Welch; Riabowol et al., 1988). Samples of equal protein were separated on a 12.5% polyacrylamide gel before blotting. Prestained molecular weight markers of 75 and 50 kD are designated on the left. The arrow on the right denotes a single band with an apparent molecular weight of 70 kD. B. Western blot of striated muscle tisse proteins, from fish surviving the 96h toxicity test, against a monoclonal antibody raised against hsp60 (from Dr. S. Miller; Miller, 1987). Samples were run as described in A. Prestained molecular weight markers 75 and 50 kD are designated on the left. The arrow on the right denotes a single band with an apparent molecular weight of 60 kD.

III. B. 3. Discussion

The acute exposure of fathead minnows to 25 mg As/L resulted in the rapid and preferential synthesis of the stress proteins in gill tissue. By 2h, ^{35}S-methionine/cysteine incorporation rates into stress proteins with molecular weights of 20, 30, 40, 70, 72, and 74 kD were increased and continued throughout the 10-h exposure period. The rapidity of this response to arsenite exposure has also been reported in HeLa, mouse fibroblast, and *Neurospora crassa* cells which have also been shown to elicite the stress response within 0.5 to 2h (Duncan and Hershey, 1987; Kapoor and Lewis, 1987; Hiwasa and Sakiyama, 1986).

Gill and striated muscle of fish exposed to different arsenite concentrations yielded different translational patterns. Gill synthesized more stress proteins (six) than muscle (five) and required a lower concentration of arsenite to induce the stress response. In addition, all stress proteins induced by the gill showed increased incorporation rates over all concentrations. However, the striated muscle showed little or no translation of stress proteins 20, 30, and 68 at the highest concentration possibly due to the overwhelming of transcriptional/translational processes. Two possible explanations exist that describe the pattern differences between these two tissues. First, is route of exposure. Of the two tissues examined, only the gill is directly exposed to arsenite, whereas the muscle required transport via the circulatory system. A second explanation may be that gill is more sensitive to changes in environmental conditions (i.e. temperature, pH, conductivity, DO, turbidity, etc.)

compared to the muscle and, therefore, requires the inducement of protective mechanisms such as the stress protein response to cope with such changes. In support of this, a previous study in this laboratory showed that the temperature required to elicit the stress response in the gill was 3°C less than that for muscle (Dyer et al., in review).

Over all treatment levels the relative synthetic rates of the stress proteins were significantly correlated to percent mortality. In gill, stress proteins 20, 40, 70, 72, and 74 were significantly correlated, whereas only stress protein 70 was correlated in the striated muscle. Other investigations with *in vitro* arsenite exposures have also yielded similar results. Upon exposures of 1 to 100 uM sodium arsenite, rainbow trout (*Oncorhynchus mykiss*) fibroblasts elicited increased synthetic rates of proteins 30, 32, 42, 62, 70, 87, and 100 with increased concentrations (Kothary and Candido, 1982). Similarly, synthesis of 40, 70, and 80 kD proteins in *Neurospora crassa* cells also were found to be concentration dependent (Kapoor and Lewis, 1986).

Immunoblots with hsp60 and 70 antibodies revealed that relative levels of stress proteins 60 and 70 in striated muscle of fish surviving the 96-h toxicity test were greater in treated fish. Increased hsp 70 Ab binding at 6.0 mg As/L corresponded well with the MATC for fathead minnows, 3.33 mg As/L (Spehar and Fiandt, 1986). Further, Sanders et al., (in press) has recently shown that accumulation of stress protein 60 in mantle tissue of *Mytilus edulis* is an order of magnitude more sensitive than scope for growth, a whole organism index of health. Thus, it appears that usage of immunological techniques to ascertain stress protein levels may serve as a highly

sensitive method to determine chronic cellular and organismal stress.

Historically it has been reported that arsenite exerts its toxicity through interactions with sulfhydryl groups of enzymes. Binding to such groups often causes tertiary structural changes resulting in decreased catalytic activities. Inhibition of metabolic oxidative pathways, such as the TCA cycle, and glucose utilization result in a depletion of high energy substances in the cell (Webb, 1966).

Recent research has shown that structurally altered proteins serve as a signal for the stress response (Munro and Pelham, 1986; Ananthan, et al., 1986; Beckmann, et al., 1990). In addition, non-lysosomal degradation of denatured proteins is also inhibited by arsenite (Munro and Pelham, 1986; Klemperer and Pickart, 1989). The net result is that denatured proteins accumulate rapidly in the cell. The level of transcription or intensity of the response is relative to the level of signal (Mizzen and Welch, 1988).

This study showed that radiolabel incorporation into different stress proteins was tissue specific. However, both tissues elicited stress proteins of 20, 30, and 70 kD. Much is known about the functions of the 70 and 30 kD stress proteins in cellular metabolism but relatively little is known about the 20 kD protein. Stress protein 70 has been shown to have at least three functions, multimeric protein assembly, unfolding for translocation, and disaggregation of protein aggregates (Craig, 1989; Rothman, 1989; Welch, 1990; Schlesinger, 1990). These functions are thought to facilitate repair of proteins and protein complexes associated with common and critical metabolic processes, therefore protecting cells from stressor induced damage.

Recently, a 32 kD stress protein known to be induced by arsenite, heavy metals, and oxidizing agents has been identified to be heme oxygenase (Shibahara et al., 1987; Keyse and Tyrell, 1989). This is an essential enzyme in heme catabolism where it cleaves heme to form biliverdin. Biliverdin is subsequently converted to bilirubin via biliverdin reductase. These heme products have been shown to be efficient peroxyl radical scavengers (Stocker et al., 1987a; Stocker et al., 1987b). Thus, it has been suggested that induction of heme oxygenase may be part of a protective response against oxidative damage (Keyse and Tyrell, 1989).

Little is known about the function of the remaining stress protein, 20. However, several proteins in the 20-27 kD range have been investigated in a variety of cell cultures and all appear to be phosphorylated during cell stress (Shuman et al., 1984; Duncan and Hershey, 1987; Regazzi et al., 1988). These phosphorylated proteins accumulate in the nucleus and other large sedimenting organelles where it is suggested that they perform their protective processes (Shuman et al., 1984; Duband et al., 1986; Arrigo, 1987).

Our data support previous suggestions (Sanders et al., 1990; Sanders, 1990) that the stress protein response should be considered as a potential biomonitoring tool. Increased translation of stress proteins due to arsenite exposure and their significant correlation to percent mortality further suggest that the stress protein response may function as a biochemical indicator of both exposure and effects.

III. C. CHROMATE

III. C. 1. Materials and Methods

Experimental Design

An initial range-find experiment was carried out to determine a range of sodium chromate concentrations suitable for a definitive LC50 test and to determine times to first signs of intoxication. Aquaria (19L) were filled to 10L with nominal concentrations of 0, 10, 50, 100, 150, and 200 mg Cr/L. Each treatment was replicated twice. Fish (7 to 12) were placed into each treatment replicate and observed for onset of intoxication. Fish in 100 mg Cr/L tanks, showed first signs of intoxication by 6-12h, with nearly 30% dead in 24h. Fish in other treatments showed signs of intoxication later than 12h, or the treatments were too toxic, with mortalities occurring within 8h. Thus, 100 mg Cr/L was used as the exposure concentration for the time to stress protein induction study.

In order to determine the length of time required for stress protein induction, two replicates of three fish each were exposed to 100 mg Cr/L and sampled at 0, 2, 4, 6, 8, 10 and 12h. Immediately after sampling, fish were sacrificed and gill tissue (51.1 ± 7.2 mg) was dissected and pooled within each treatment replicate. Tissues were metabolically labeled for 2h with ^{35}S-methionine and cysteine. Incorporation of label into proteins was examined by SDS-PAGE and fluorography. Resulting fluorograms showed that all stress proteins observed within the 12-h treatment period were maximally induced at 12h. Thus, 12h was selected as the appropriate sampling time to examine the stress protein response in the subsequent definitive 96-h toxicity

test.

To assess the concentration range in which gill and striated muscle tissues synthesize stress proteins we exposed fish to five different arsenite concentrations (9.0 $\pm$ 1.3; 49.7 $\pm$ 2.7; 75.4 $\pm$ 2.4; 104.9 $\pm$ 1.6; 159.4 $\pm$ 2.5 mg Cr/L) , plus control, and sampled at 12h. Each treatment was replicated five times. Thirteen fish were placed into each treatment replicate and three were subsampled at 12h while the rest were exposed for 96h. Tissues of subsampled fish (gill 46.7 $\pm$ 7.2; striated muscle 46.7 $\pm$ 6.5 mg) destined for metabolic labeling were treated as in the induction study. Time to mortality was recorded every 24h. Metabolic labeling, sample preparation, SDS-PAGE, and densitometric quantitation of fluorograms was conducted as described in the arsenite experiment's materials and methods section. However, X-ray films used for quantitation were exposed to radiolabeled gels for 5d. Survivors of the 96-h toxicity test were sacrificed and immediately placed into a -80°C freezer and later assayed for stress protein 70 levels using quantitative immunoblots.

Quantitative Immunoblots

Gill and striated muscle proteins from 96-h toxicity test survivors were digested and serially diluted in sample buffer. Dilutions were loaded into seven wells of a 12.5% SDS-polyacrylamide gel and subsequently electrophoresed. Proteins in gels were then transferred to nitrocellulose using a transfer buffer containing 1% SDS, 0.192 M glycine in 0.025 M tris and 20% methanol (v/v), pH 8.3. The gels were subjected to 25 V for 15h at 4 C. The nitrocellulose was then blocked for 1h in tris

buffered saline (TBS) which containing 3% gelatin.

The nitrocellulose was probed using a monoclonal antibody (Ab) raised against hsp72/73 from HeLa cells (generously provided by W. Welch; Riabowol, et al., 1988). Blots were incubated at room temperature with hsp 72/73 Ab for 2h and subsequently rinsed several times with TBS containing 0.5% Tween-20 (TTBS). The blots were then incubated with a second antibody of goat anti-mouse alkaline phosphatase (AP) conjugated antibody (BIORAD) for 2h. The blots were washed several times with TTBS and TBS and incubated with the substrates 5-bromo 4-chloro 3-indoyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride (BIORAD) to visualize bound antibody. The least amount of total protein required to visualize stress protein bands was recorded as the minimum detection level, which was verified using a laser densitometer.

Water Chemistry

All exposures used dechlorinated tap water that had the following characteristics: alkalinity 100 mg $CaCO_3$/L; hardness 120 mg $CaCO_3$/L; and conductivity 300 umhos. Dissolved oxygen and pH were monitored for each test. Mean dissolved oxygen was 6.5 $\pm$ 0.7 mg O_2/L while pH was 7.7 $\pm$ 0.3 over all concentrations. Chromium concentrations were determined using a Perkin Elmer atomic absorption spectrophotometer (EPA Method 218.1).

Statistical Analysis

The LC50 and LC1 was determined by probit analysis. Stress protein intensities were analyzed nonparametrically due to heteroscadacity. Kruskal-Wallis analysis of variance was used to determine the significance of treatment effects (alpha=0.05) on specific stress protein intensities. Comparisons to controls were determined by one-tailed Dunnett T tests on ranked data. Spearman rank correlations were used to compare percent mortality to stress protein intensities. Data from the quantitative immunoblots were analyzed by parametric analysis of variance and one-tailed Dunnett's T test on raw data. Pearson correlations were used to compare stress protein blot results to percent mortality.

III. C. 2. Results

Chromate treatments caused significant changes in mortality (Kruskal-Wallis, $p \leq 0.05$). Percent mortality ranged from 2.0% in controls and 9.9 mg Cr/L to 97.5% and 159.4 mg Cr/L. A Dunnett's T test revealed that treatments of 49.7, 75.4, 104.9, and 159.4 mg Cr/L had significantly higher mortalities versus controls ($p \leq 0.05$). Probit analysis determined the LC50 to be 60.1 mg Cr/L and the LC1 to be 18.1 mg Cr/L.

The stress protein response was elicited in gill tissue of fathead minnows exposed to 75 mg Cr/L. Three different stress proteins with apparent molecular weights of 20, 30, and 70 kD were observed (Figure 9). Incorporation of

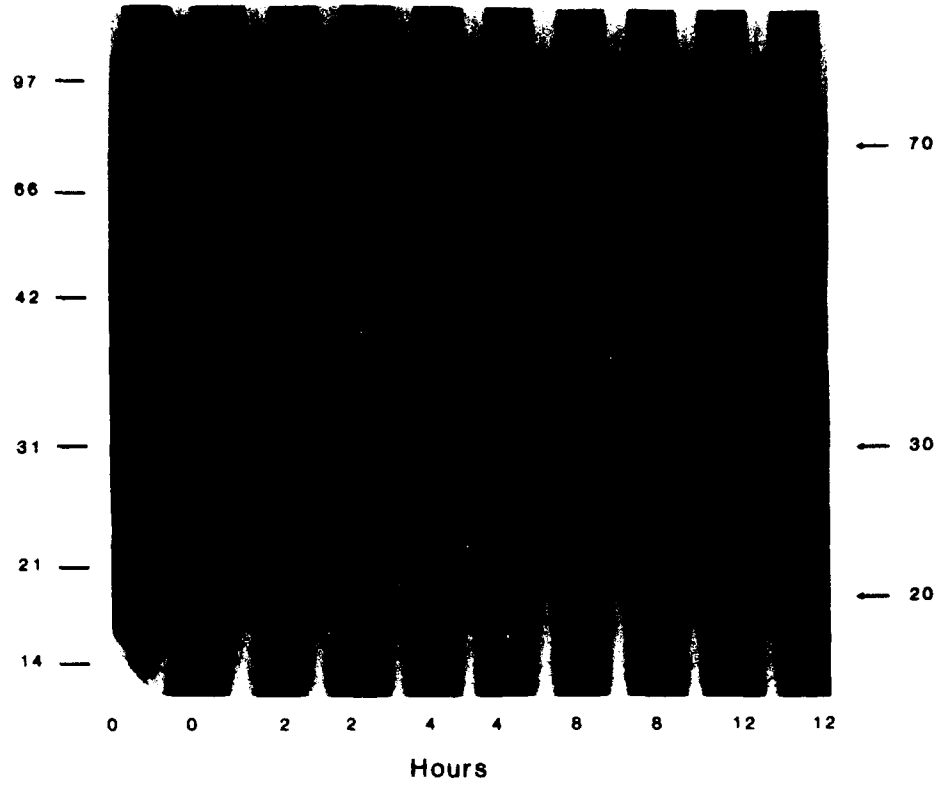

Figure 9. A fluorgram of gill tissue proteins from fish exposed to 100 mg Cr/L for 0, 2, 4, 6, 8, 10, and 12h. Molecular weight markers are designated on the left. Arrow on the right denote stress proteins of 20, 30, and 70 kD.

^{35}S-methionine/cysteine into each of the stress proteins increased with time of exposure. Densitometric analysis indicated that maximum incorporation of radiolabel into all three proteins occurred at 12h exposure. Thus, a 12-h exposure was deemed to be an appropriate sampling time for the concentration response study. Our goal was to correlate incorporation of radiolabel into stress proteins to an eventual effect, namely percent mortality.

A qualitative description of one of the replicate fluorograms with gill tissue shows that radiolabel incorporation into proteins of 20, 30, and 70 kD did not increase with increased chromate concentration (Figure 10A). However, statistical analysis of densitometric scans over all treatments showed that chromate concentration significantly affected isotope incorporation into stress protein 70 (Figure 10B; Kruskal-Wallis, p=0.04). A Dunnett's T test performed on ranked data showed significantly increased radiolabel incorporation above control rates at 75.4 mg Cr/L, however, chromate treatments did not significantly affect synthetic rates of the other two stress proteins (20 and 30). As found in gill, three stress proteins of 20, 30, and 70 kD were observed in striated muscle (Figure 11A). Increased incorporation of ^{35}S-methionine/cysteine into stress protein 30 occurred from 49.7 to 104.9 mg Cr/L. The incorporation of radiolabel into stress protein 20 was greater than controls at chromate concentrations of 104.9 and 159.4 mg Cr/L. No discernable differences over all chromate treatments were observed for the 70 kD protein.

Chromate treatments significantly affected the synthesis and accumulation of only stress protein 20 in the striated muscle (Figure 3B; Kruskal-Wallis, p=0.04).

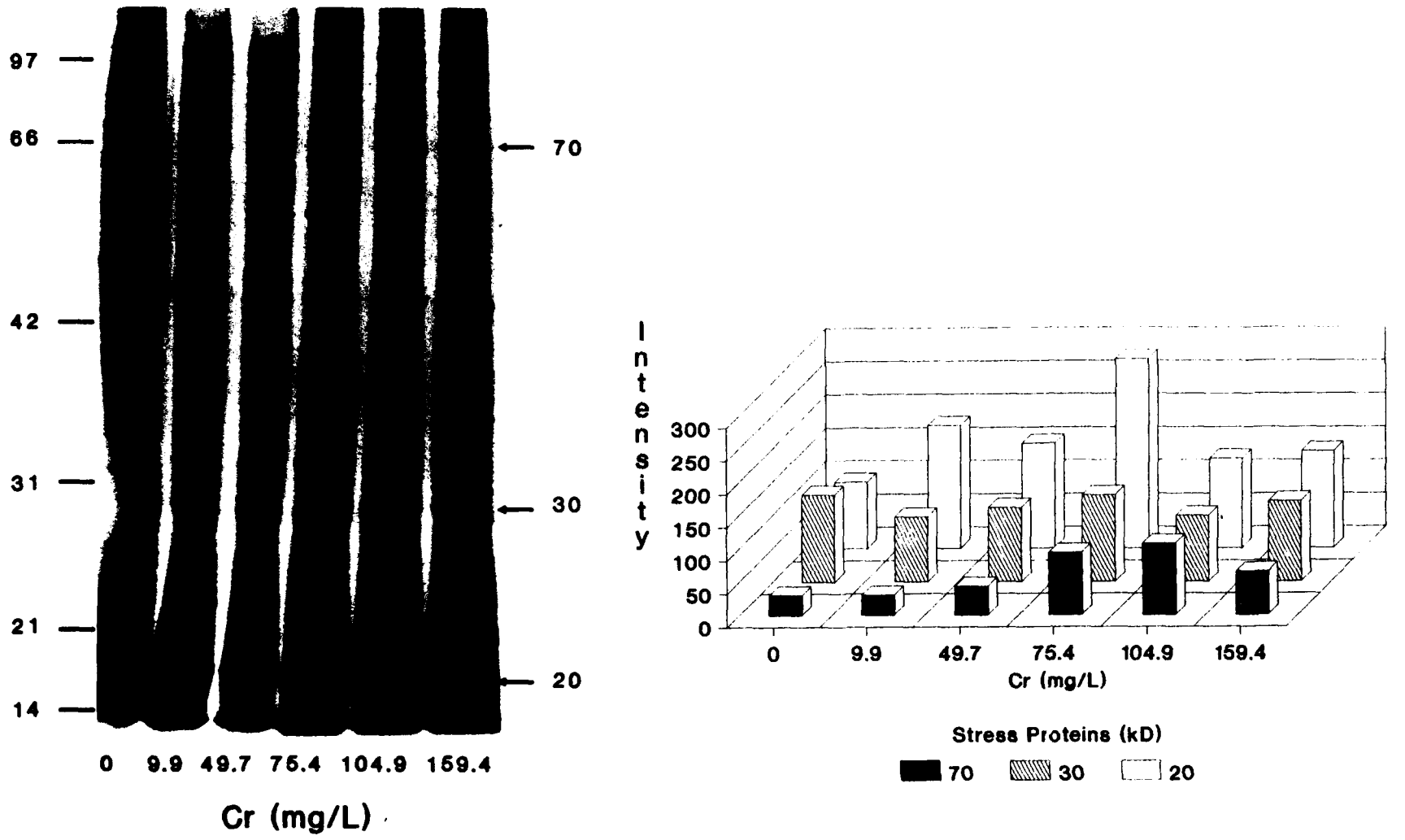

Figure 10. A. A fluorgram of gill tissue proteins from fish exposed to 0, 9.9, 49.7, 75.4, 104.9, and 159.4 mg Cr/L for 12h. Molecular weight markers are designated on the left. Arrows on the right denote stress proteins of 20, 30, and 70 kD. B. Median intensities of 20-, 30-, 70-kD proteins in fluorograms from replicate samples (n = 5) of experiment described in A.

Significantly increased radiolabel incorporation occurred at 49.7, 75.4, and 159.4 mg Cr/L (Dunnett's T test, $p \leq 0.05$.). Significance of treatment levels on stress protein 30 synthesis and accumulation was compromised due to high variance.

Intensities from replicate fluorograms from both gill and striated muscle were correlated to 96-h mortalities for each respective treatment replicate. In gill, incorporation of radiolabel into stress protein 70 was highly significantly correlated with mortality (Spearman correlation, $r=0.54$, $p=0.0026$). The other two stress proteins were not significantly correlated with mortality. Stress protein 20 striated muscle was highly significantly correlated with mortality ($r = 0.50$, $p = 0.0086$). In contrast, stress proteins 30 and 70 were not found to be correlated with mortality. Gill and striated muscle protein of 96-h survivors were immunoblotted with an antibody to hsp 72/73 (Figure 12). Two bands with apparent molecular weights of 70 and 68 kD were observed in gill whereas only one band, 70 kD, was found in the striated muscle. The number of micrograms of total protein required for minimum detection decreased markedly with increased chromate concentration for the 68-kD band in the gill. In comparison, detection levels for the 70-kD bands of both gill and muscle tissues were only slightly lower in treated fish versus control fish. Parametric analysis of variance showed that chromate concentrations had no significant effect on the 70 kD band protein levels in both gill and muscle. Contrastingly, the 68-kD band of the gill was highly significantly affected by treatment levels (ANOVA, $p=0.0001$). Dunnett's T test performed on raw data showed that minimum detection levels for the 68-kD protein from treated fish were significantly less than those from controls

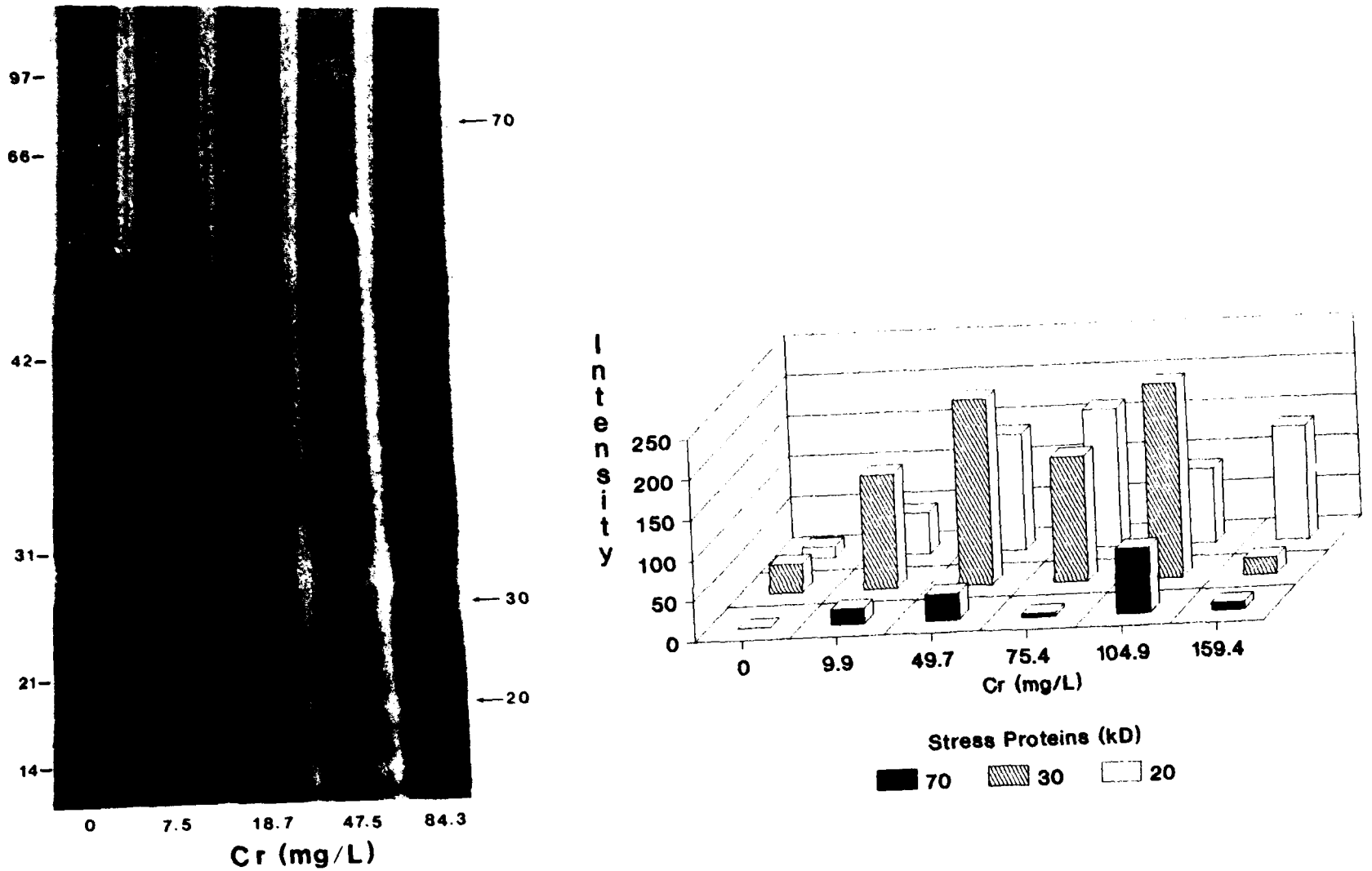

Figure 11. A. A fluorgram of striated muscle tissue proteins from fish exposed to 0, 9.9, 49.7, 75.4, 104.9, and 159.4 mg Cr/L for 12h. Molecular weight markers are designated on the left. Arrows on the right denote stress proteins of 20, 30, and 70 kD. B. Median intensities of 20-, 30-, 70-kD proteins in fluorograms from replicate samples (n = 5) of experiment described in A.

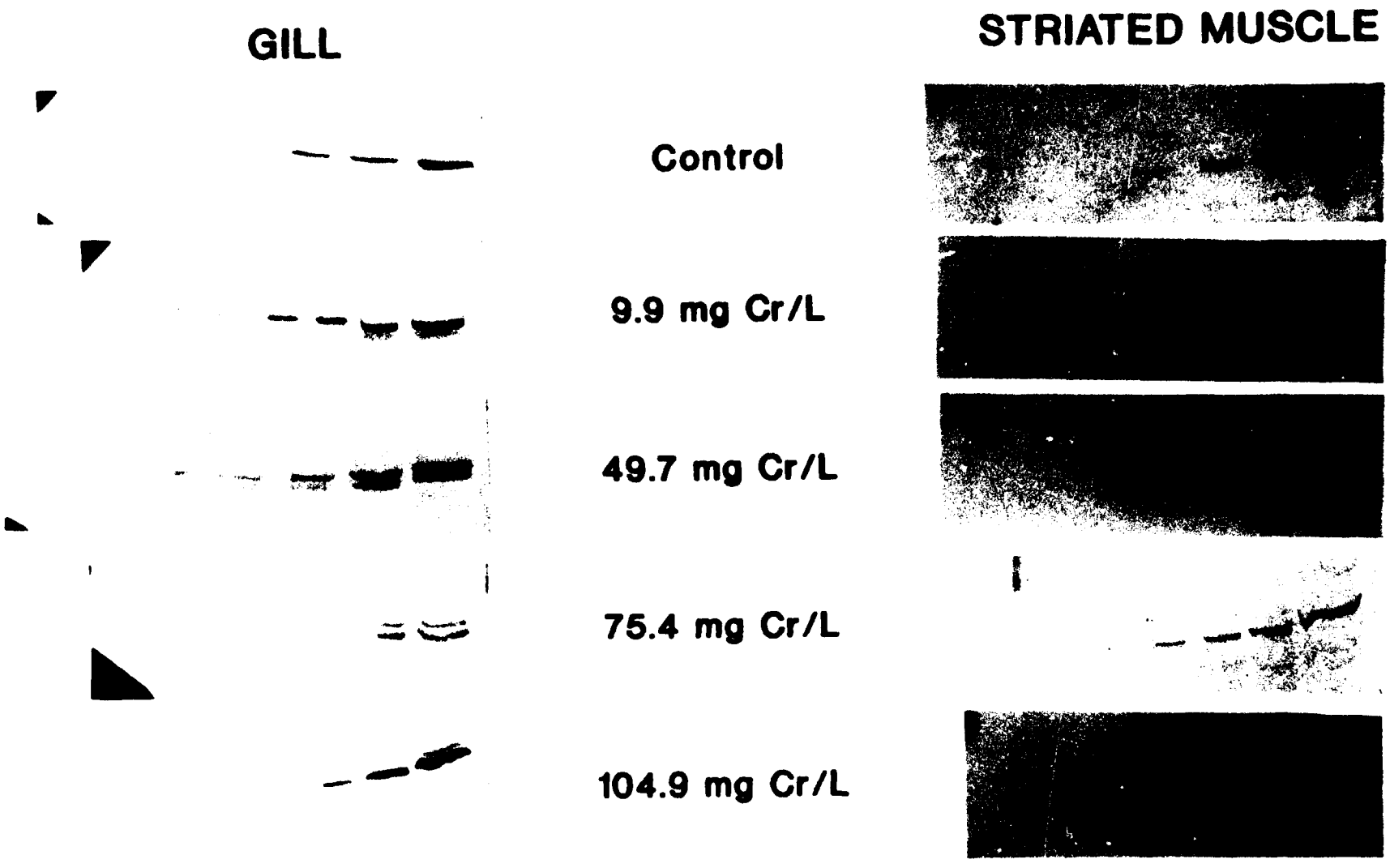

Figure 12. Quantitative immunoblots of gill and striated muscle tissue of fish exposed to 0, 9.9, 49.7, 75,4, and 104.9 mg Cr/L for 96h using an antibody raised against hsp70. Blots with gill protein are designated on the left, while blots with striated muscle are on the right.

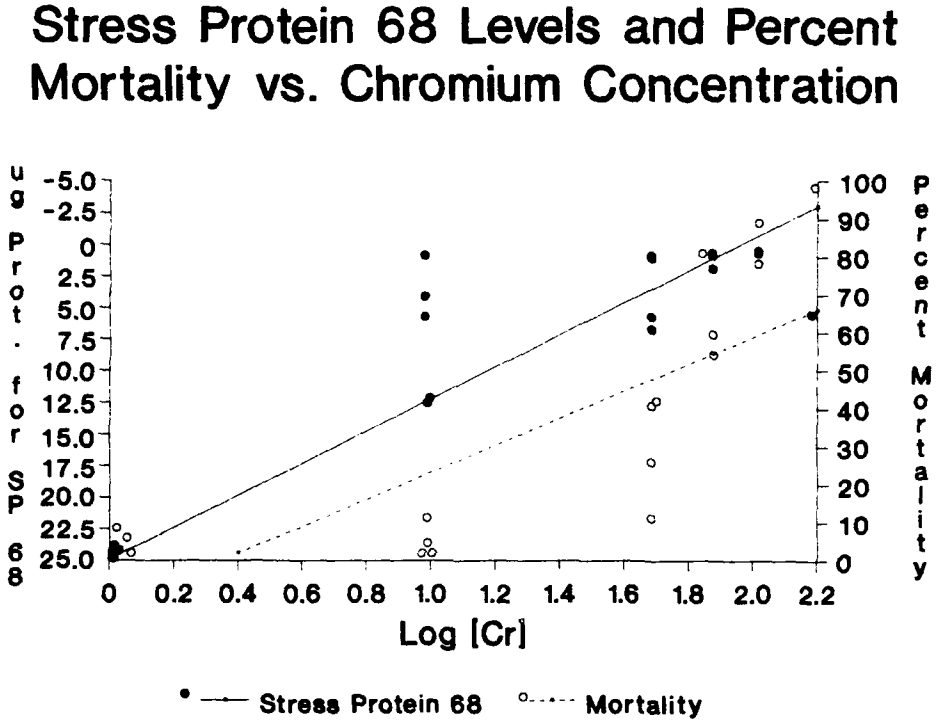

Figure 13. A graphic that displays the relationships between accumulation of the 68 kD protein, as determined via quantitative immunoblots, and percent mortality of fish exposed to different chromium concentrations for 96h. Solid dots correspond to micrograms of total protein required for minimum stress protein detection. The solid line represents the regression between detection levels of the 68 kD protein and log [Cr]. Open dots correspond to percent mortality. The dotted line respresents the regression between 96h percent mortality and log [Cr]. Regression equations for both lines are described in the results.

($p \leq 0.05$). Regression analysis showed that there was a highly significant relationship between 68 kD protein detection levels and log [Cr] (Figure 13; $p=0.0001$, $r^2=0.65$, Detection level = 25.0 - 12.8 (log[Cr])). In addition, 68-kD levels were highly significantly correlated to percent mortality (Spearman correlation, $p = 0.0002$, $r = -0.74$.

III. C. 3. Discussion

The translation pattern in gill of fathead minnows was found to be affected upon acute exposure to 75 mg Cr/L. By 12h, synthesis and accumulation of stress proteins 20, 30, and 70 had increased beyond 0-h levels. Similarly, synthesis of the stress proteins in mouse fibroblasts has been shown to be induced by 0.2 mM K_2CrO_4 within 2h (Hiwasa and Sakiyama, 1986). Gill and striated muscle from fish exposed to a range of chromium concentrations yielded similar translational patterns. Both tissues elicited three stress proteins of 20, 30 and 70 kD. Significant concentration response relationships were determined for synthesis and accumulation of the 70 kD protein in gill and the 20 kD protein in the striated muscle. Incorporation of radiolabel into both proteins, as mentioned, were also significantly correlated with percent mortality. Significant concentration-response relationships for the 20 kD protein of the gill and the 30 kD protein found in the muscle were compromised due to high variance within the controls.

Gill and striated muscle protein from 96-h survivors were quantitatively immunoblotted with a monoclonal hsp 72/73 Ab. Both tissues synthesized a 70-kD

whose levels did not change significantly with Cr concentration. However, the gill also synthesized a 68 kD protein whose levels accumulated and were significantly correlated to Cr concentration and percent mortality. A comparison between accumulation of the 68-kD protein and percent mortality values showed that significantly increased accumulations of the 68 kD protein occurred at concentrations of 9.9 mg Cr/L and greater whereas significant increases in mortality occurred at 49.7 mg Cr/L and greater. Thus, accumulation of the 68-kD protein was five-fold more sensitive than 96-h mortality.

Potential use of immunoblot technology to ascertain chronic toxicant exposure appears to have great potential. For example, significantly increased 68- kD protein accumulation occurred at a concentration nearly half that of the LC1, 18.1 mg Cr/L. In addition, significant accumulations at 9.9 mg Cr/L correspond well with the MATC for fathead minnows, 2.3 mg Cr/L (Spehar and Fiandt, 1986). Furthermore, Sanders et al., (in press) has recently shown mantle tissue of *Mytilus edulis* to be an order of magnitude more sensitive than scope for growth, a whole organism index of health. In contrast, significantly increased radiolabel incorporation into stress proteins 70 (gill) and 20 (striated muscle) was first determined at concentrations in which acute mortality resulted. This comparison illustrates the marked difference in the sensitivity of the two methods employed to examine the stress protein response, that of radiolabel incorporation into proteins (less sensitive) vs. stress protein accumulation as determined by quantitative western blots (more sensitive).

Toxicity of chromium is dependent on chemical speciation and subsequent

associated health effects are, thus, influenced by the chemical form of the exposure (Nieboer and Jusys, 1988). In general, chromium toxicity has been attributed to two species; Cr VI, such as sodium chromate, and Cr III. In biologic systems, Cr VI has been found to oxidize low-molecular-weight reductants (i.e cysteine, glutathione, lipoic acid), proteins, and DNA (Ono, 1988; Nieboer and Jusys, 1988; Nieboer and Shaw, 1988). At physiologic pH's, Cr VI is rapidly reduced to CrIII (Nieboer and Jusys, 1988). This species (CrIII) has been shown to exhibit a high affinity for nucleotides and nucleic acids (NAS, 1974, Nieboer and Jusys, 1988) The binding of CrIII to nucleic acids and nucleotides, results in the displacement of MgII as the major counterion. This ion swap alters the three-dimensional geometry of RNA and DNA. Thus it is not surprising that DNA replication and protein translation become impaired and result in mutagenesis and carcinogenesis (Ono, 1988; Nieboer and Shaw, 1988; Yassi and Nieboer, 1988) as well as the potential production of aberrant proteins.

Chromium VI exerts its toxicity in fish in a manner different from most heavy metals. Whereas cationic metals such as Zn and Cd have been shown to accumulate in the gill and drastically alter lamellar structure and function (Skidmore and Tovell, 1972; Olsson and Hogsatrand, 1987), CrVI exists as an oxo-anion which allows it to pass readily through gill membranes and accumulate within internal organs (Van der Putt et al., 1981; Van der Putt and Part, 1982). It has been postulated that at exposure pH's of 7.8 and greater that CrVI induced toxicity is caused by pathological changes within internal organs (Van Der Putt et al, 1981). This has also been

supported by the lack of response from various enzymes in gill versus those in other tissues (Kuhnert et al., 1976; Buhler et al, 1977). Even so, chromium has repeated been shown to accumulate rapidly in gill and localize in microsomal and soluble fractions (Kuhnert et al, 1976; Buhler et al, 1977; Van der Putt et al, 1981).

Causal factors that relate chromium exposure and induction of the stress proteins have thus far been ignored. Our study showed that in both gill and muscle tissues three stress proteins were synthesized, 20-, 30- and 70-kD. In regard to function, much is known of the 70- and 30-kD proteins in cellular metabolism but not as much is known about the 20-kD protein. Stress protein 70 has been shown to have at least three functions, multimeric protein assembly, unfolding, for translocation, and disaggregation of protein aggregated (Craig, 1989; Rothman, 1989; Welch, 1990; Schlesinger, 1990). These functions are thought to facilitate repair of proteins and proteins complexes associated with common and critical metabolic processes and thus protect cells from stressor induced damage.

Recent research has shown that structurally altered proteins serve as a signal for the induction of the stress protein 70 family (Munro and Pelham; 1986; Ananthan et al., 1986; Beckman, et al., 1990). Denaturation of proteins due to CrVI oxidation and/or aberrant translation of proteins caused by CrIII's action on nucleic acid tertiary structure may explain the induction of the 70-kD protein.

A 32-kD stress protein known to be induced by arsenite, chromium, cationic heavy metals, and oxidizing agents has recently been identified to be heme oxygenase (Goering et al., 1987; Shibahara et al., 1987; Keyse and Tyrell, 1989). This is an

essential enzyme in heme catabolism where it cleaves heme to form biliverdin. Biliverdin is subsequently converted to bilirubin via biliverdin reductase. These heme products have been shown to be efficient peroxyl radical scavengers as well as CrVI reductants (Stocker et al. 1987a; Stocker at al. 1987b; Nieboer and Jusys, 1988). Thus, it has been suggested that induction of heme oxygenase may serve as a protective response against oxidative damage (Goering et al, 1987; Keyse and Tyreli, 1989).

Little is known of the function of stress protein 20. Several proteins in the 20-27 kD range observed in a variety of cell cultures have been shown to be phosphorylated during cell stress (Kim et al., 1984; Duncan, and Hershey, 1987; Regazzi et al., 1988). These phosphorylated proteins accumulate in the nucleus and other large sedimenting organelles where it is suggested that they perform their protective roles (Shuman et al., 1984; Duband et al., 1986; Arrigo, 1987).

As to which spieces of chromium may be most responsible for the stress response, our data suggest that CrIII is the likely candidate. The lack of a major induction of stress proteins at 12h, as measured by radiolabel incorporation, and a highly significant response at 96h, as determined via quantitative immunoblots, coincides well with that of the rapid reduction of CrVI to CrIII (Niebor and Jusys, 1988). Thus, oxidative damage of proteins was minimized but synthesis of aberrant proteins increased, due to CrIII's interaction with nucleic acids.

Results of this study as well as another with sodium arsenite that we have performed (Dyer et al., in preparation) demonstrate that the stress protein response

has potential to serve as a biochemical indicator of cellular and organismal stress. Future research with emphasis on immunological techniques and their calibration to chronic effects is needed to fully understand the limits of this cellular protective mechanism. Several investigations have shown that different suites of proteins are induced upon exposure to different stressors (Courgeon et al., 1984; Honda et al., 1986; Shelton et al., 1986). Thus, additional studies utilizing antibodies to other stress proteins may reveal other relevant bioindicators of stress as well as help to further elucidate toxicant induced effects and mode of action.

III. D. LINDANE EXPOSURES

III. D. 1. Materials and Methods

Organisms

Ninety-day-old fathead minnows (*Pimephales promelas*) were obtained from a culture located at the University of North Texas. Fish used for the study were acclimated to 25°C and a 16L:8D photoperiod for at least 7d and were fed frozen brine shrimp twice daily to satiation.

Exposure System

Prior to each test, a fresh solution of lindane (Supelco, Inc. Bellefonte, PA) was prepared in certified grade DMF. The toxicant was metered into a modified Mount-Brungs diluter via a Beckman syringe pump. All exposures were flow-through where water volumes in each 6L exposure chamber were exchanged 5-6 times daily.

Experimental Design

Range find experiments were carried out to determine a range of lindane concentrations suitable for a definitive 96h toxicity test (LC50) and to determine times to first signs of intoxication. Fish were exposed to lindane concentrations ranging from 6.6 to 214.7 ug/L. Minnows exposed to concentrations ranging from 88.4 to 214.7 ug/L displayed first signs of intoxication within 6h and persisted for another 12 to 24h. Behavioral signs of toxicity followed a progression where fish first displayed extreme hyperactivity, periodic muscle spasms alternating with lethargy, and

lastly whole body spasms and loss of equilibrium.

In order to determine the length of time required for stress protein induction, three replicates of three fish each were exposed to 220.0 $\pm$ 48.4 ug lindane/L and sampled at 0, 2, 4, 6, 8, 10, and 12h. Immediately after sampling, fish were sacrificed and brain, gill, and striated muscle tissues were disected and respectively pooled within each treatment replicate. Tissues were metabolically labeled for 2h with ^{35}S-methionine/cysteine. Incorporation of radiolabel into proteins was examined by SDS-PAGE and fluorography. Fluorograms showed that a 70-kD band was maximally induced in all three tissues at 12h. Thus, 12h was determined to be the appropriate sampling time in the subsequent concentration-response experiment.

To ascertain the concentration range in which gill, brain, and striated muscle tissue synthesize stress proteins, fish were exposed to five different lindane concentrations (7.5 $\pm$ 2.5; 18.7$\pm$ 2.8; 47.5 $\pm$ 9.2; 84.3 $\pm$ 19.9; 180.9 $\pm$ 13.0 ug lindane/L), plus control, and sampled at 12h. Each treatment was replicated four times. Thirteen fish were placed into each treatment replicate. Three fish were subsampled at 12h and the remaining fish were exposed for 96h. Brain, gill and striated muscle tissues of subsampled fish were treated as in the induction study. Time to mortality was recorded every 24h. Metabolic labeling, sample preparation, SDS-PAGE, and quantitation were performed as stated in the heat-shock experiment materials and methods. Quantitative immunoblotting with brain, gill, and striated muscle tissues from 96-h toxicity test survivors was performed using the protocol outlined in the chromate experiments.

Water Chemistry

All exposures used dechlorinated tap water. Ranges of alkalinity, hardness, and conductivity were 80-120 mg CaCO3/L, 100-140 mg CaCO3/L, and 270-310 umhos, respectively. Mean dissolved oxygen and pH measured were 5.1 $\pm$ 1.2 mg O2/L and 7.70 $\pm$ 0.02, respectively. Lindane concentrations were determined using a Tracor 560 Gas Chromatograph fitted with an electron capture detector and a SPB-5 (Supelco, Inc.) capillary column.

III. D. 2. Results

Lindane treatments caused significant changes in mortality (Kruskal-Wallis,$p \leq 0.05$). Percent mortality ranged from 2.5% in controls, 7.5 and 18.7 μg lindane/L, to 100% at 180.9 μg lindane/L. Dunnett's T tests revealed mortality was significantly greater in 47.5, 84.3, and 180.9 ug lindane/L treatments compared to controls. Probit analysis showed the LC50 to be 43.8 μg lindane/L and the LC1 to be 12.6 μg Lindane/L.

The stress protein response was elicited slightly in brain, gill, and muscle tissue exposed to 219.3 μg Lindane/L. A stress protein with an apparent molecular weight of 70 kD was observed in all three tissues and an additional 72 kD protein was found in the gill (Figure X). Incorporation of ^{35}S-methionine/cysteine into the 70kD protein increased with time such that maximal levels were achieved at 12-h exposure. Thus, a 12-h exposure was deemed to be an appropriate sampling time for the

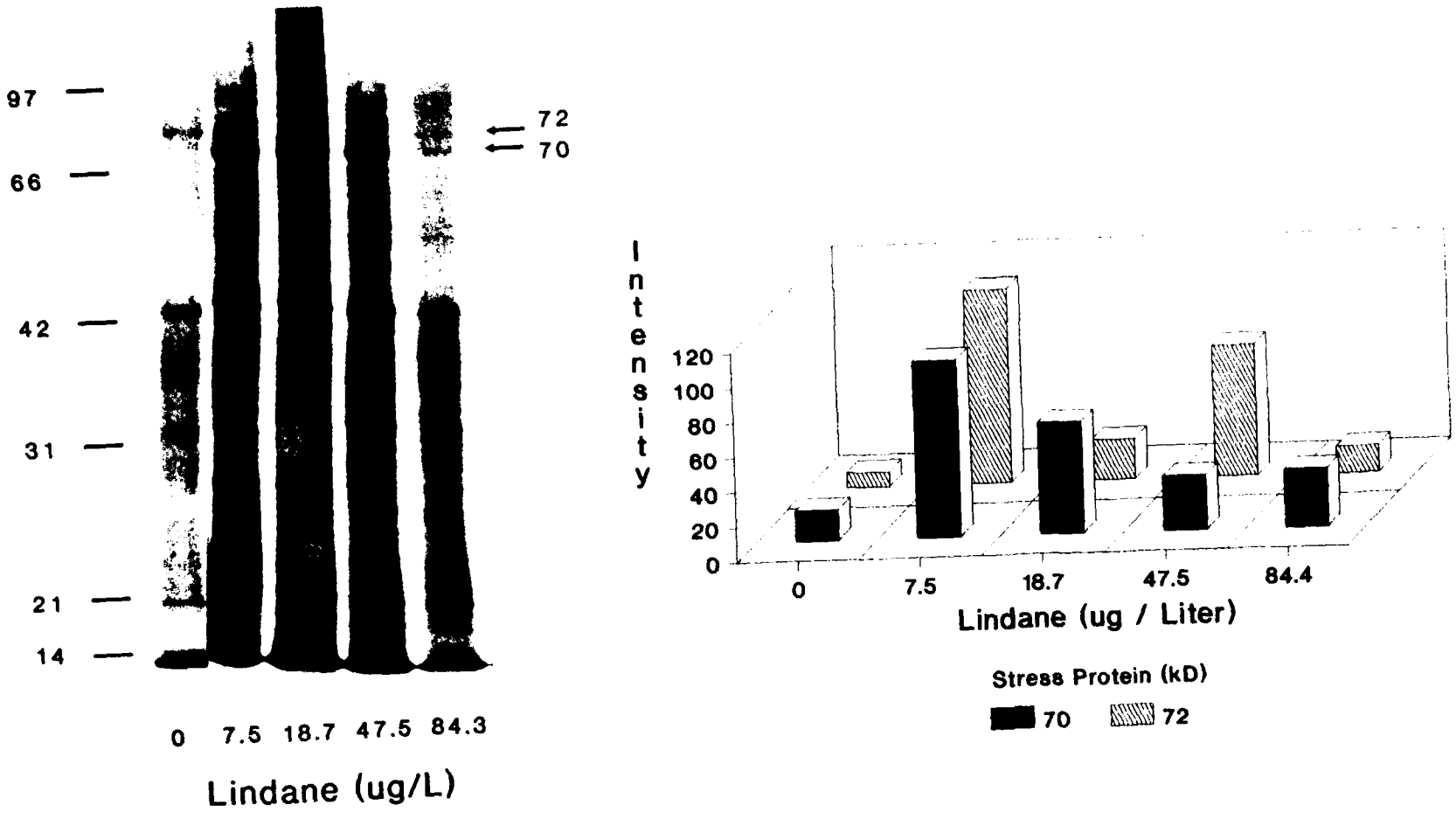

Figure 14. A. A fluorgram of gill tissue protein from fish exposed to 0, 7.5, 18.7, 47.5, and 84.3 ug lindane/L for 12h. Molecular weight markers are designated on the left. Arrows on the right denote stress proteins of 70 and 72 kD. B. Median intensities of 70- and 72-kD proteins in fluorograms from replicate samples (n = 4) of experiment described in A.

concentration response study. Our goal was to correlate incorporation of radiolabel into stress proteins to an eventual effect, namely percent mortality.

Gill tissue of fathead minnows exposed to 7.5 μg lindane/L responded by eliciting two proteins (70- and 72-kD) at incorporation rates significantly greater than controls (Figures 14A and 14B; Dunnett's T test, $p < 0.05$). However, over all other treatments, lindane did not significantly induce a stress response (Kruskal-Wallis, $p = 0.14$). Similarly, increased radiolabel incorporation into a 70 kD protein was evident at 18.7 and 47.5 μg lindane/L in the brain (Figure 15A and 15B). However, due to high variance at these concentrations there were no significant differences between controls. Nonparametric analysis of variance indicated that lindane had no effect on the stress protein response (Kruskal-Wallis, $p = 0.19$). In addition, Lindane treatments were not found to affect ^{35}S-label incorporation into a 70-kD protein observed in the striated muscle (Figure 16A and 16B; Kruskal-Wallis, $p = 0.51$). Brain, gill, and striated muscle protein of 96-h survivors were immunoblotted with an antibody to hsp 72/73. One band, with an apparent molecular weight of 70-kD, was found in each of the tissues. The mean number of micrograms of total protein required for minimum detection decreased in all lindane treatments for each of the three tissues, as compared to control levels. Parametric analysis of variance showed that lindane concentrations significantly affected 70-kD protein levels in gill ($p = 0.0008$) but not, however, in brain tissue and striated muscle.

Dunnett's T test performed on raw data from the gill showed that lindane significantly decreased the minimum detection level for the 70-kD protein at all

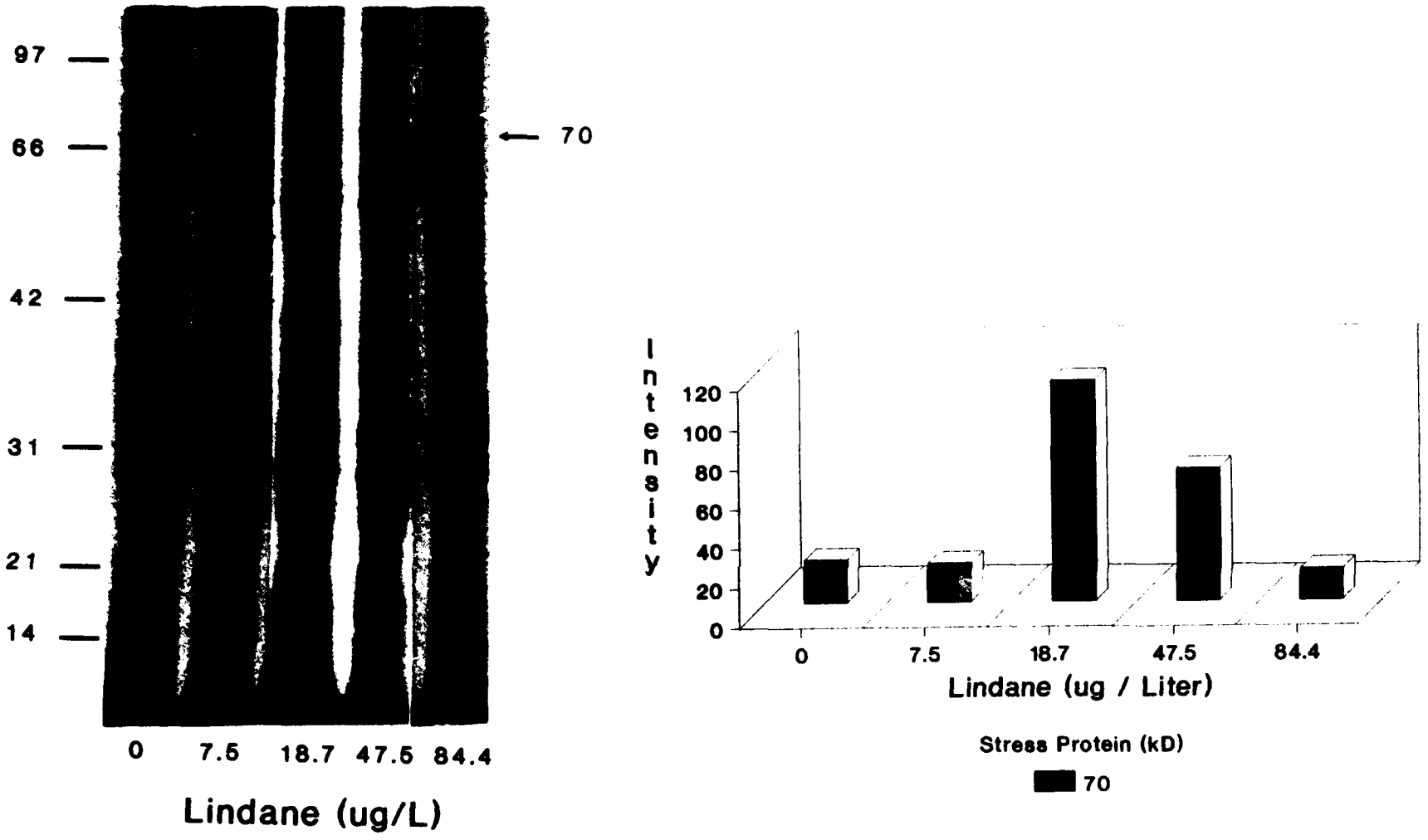

Figure 15. **A.** A fluorgram of brain tissue protein from fish exposed to 0, 7.5, 18.7, 47.5, and 84.3 ug lindane/L for 12h. Molecular weight markers are designated on the left. The arrow on the right denotes a stress protein of 70 kD. **B.** Median intensities of the 70-kD protein in fluorograms from replicate samples (n = 4) of experiment described in **A.**

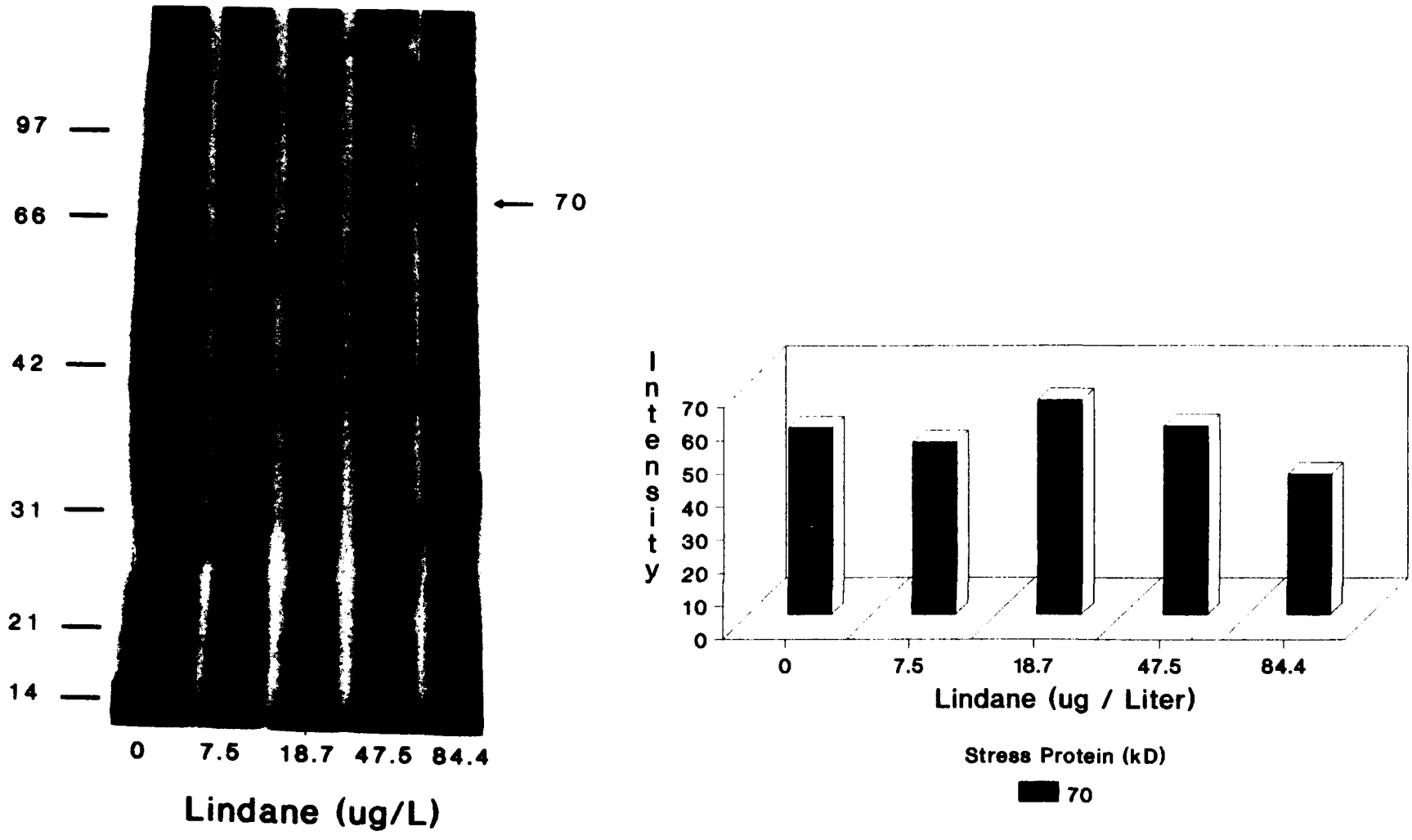

Figure 16. A. A fluorgram of striated muscle tissue protein from fish exposed to 0, 7.5, 18.7, 47.5, and 84.3 ug lindane/L for 12h. Molecular weight markers are designated on the left. The arrow on the right denotes a stress protein of 70 kD. B. Median intensities of the 70-kD protein in fluorograms from replicate samples (n = 4) of experiment described in A.

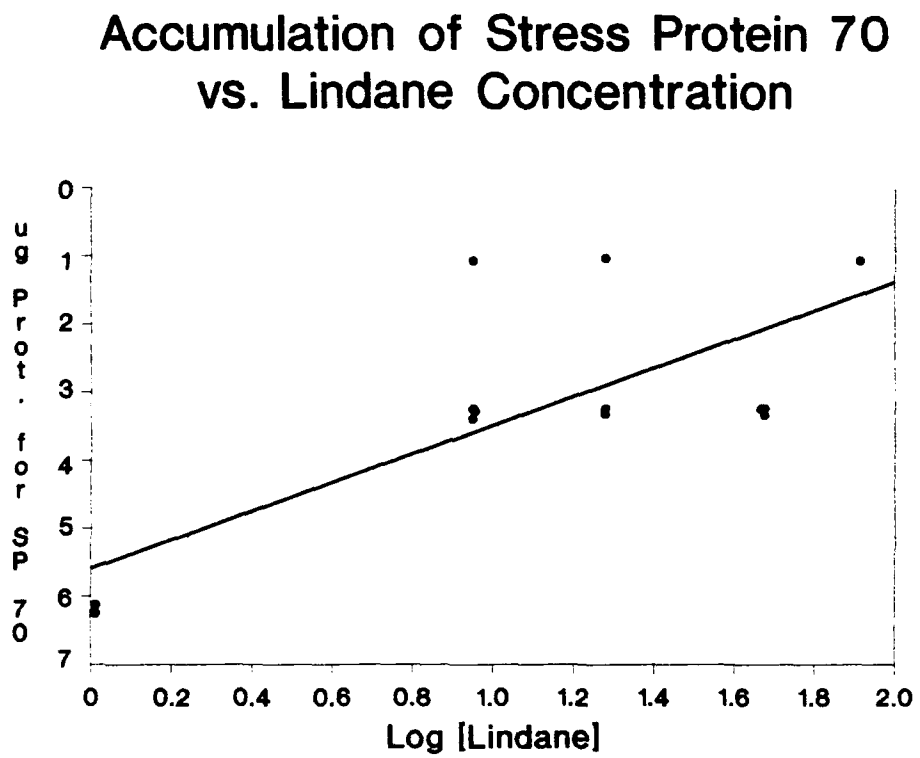

Figure 17. A scatter diagram of the minimum detection levels for the 70 kD protein, as determined via quantitative immumoblots, versus different lindane concentrations to which fish were exposed for 96h. The regression equation as stated in the results is represented by the solid line.

treatment levels. Regression analysis showed that there was significant relationship between the 70-kD detection levels and log [lindane] (Figure 17; $p = 0.0009$, $r^2 = 0.62$, Detection level = 5.6 - 2.1(log [lindane]). However, 70-kD protein levels were not significantly correlated with 96-h mortality (Pearson correlation, $p = 0.22$, $r = -0.35$).

III. D. 3. Discussion

The translation patterns in three tissues (brain, gill, and striated muscle) of the fathead minnow were found to be affected upon acute exposure to 219.3 μg lindane/L. By 12h, synthesis and accumulation of stress protein 70 (and 72 as also found in the gill) had increased beyond O-h levels. Brain, gill, and striated muscle of fish exposed to a range of lindane concentrations elicited similar translational patterns. Both brain and striated muscle elicited a 70-kD protein, while gill elicited a 70 as well as a 72-kD protein. Statistically, lindane did not affect radiolabel incorporation into the above mentioned proteins in any of the tissues.

Blots with protein from brain, gill, and striated muscle of 96-h toxicity test survivors revealed a single band with an apparent molecular weight of 70 kD. Only levels found in the gill were significantly affected by lindane concentration. Accumulation of the 70 kD protein observed in gill was significantly affected by lindane at all treatment levels. Mortality, however, was significantly greater than controls at lindane concentrations of 47.5 ug/L and greater. Thus, a comparison

between stress protein 70 accumulation and mortality revealed that accumulation was six-fold more sensitive than 96-h percent mortality.

Future use of quantitative immunoblots to ascertain chronic toxicant exposure appears to have great potential. For example, significantly increased 70-kD protein accumulation occurred at a concentration below that of the LC1, 12.6 μg lindane/L. In addition, Sanders et al., (in press) has shown that mantle tissue of the mollusc *Mytilus edulis* is an order of magnitude more sensitive than scope for growth, a whole organism index of health.

At present, relationships of the stress protein response and insecticide exposure have thus far been ignored. Our study demonstrated the presence of a 70-kD protein in brain, gill, and strated muscle tissues. In regard to its function, much is known about the 70-kD protein. This stress protein has been shown to have at least three functions, multimeric protein assembly, unfolding for translocation and disaggregation of protein aggregates (Craig, 1989; Rothman 1989; Welch, 1990; Schlesinger, 1990). These functions are thought to facilitate repair of proteins and protein complexes associated with common and critical metabolic processes and thus protect cells from stressor induced damage. In addition, it has been shown that this 70 kD protein interacts with proteins involved with cytoskeletal structure and function (Clark and Brown, 1986; Guidon and Hightower, 1986; Whatley et al., 1986; Green and Liem, 1989). Several studies have shown that structurally altered proteins serve as a signal for the induction of stress protein 70 (Munro and Pelham, 1986; Ananthan et al., 1986; Beckman et al., 1990). However, how this relates to the accumulation

of stress protein 70 in the gill and striated muscle and the mode of action of lindane is not obvious.

It is generally accepted that the toxicity of lindane is a result of its inhibition of the GABA - activated chloride channel in the nerve (Matsumura and Ghiasuddin, 1983; Woolley and Zimmer, 1986; Fishman and Gianutsos, 1987; Joy et al., 1987; Ogata et al., 1988). Organochlorine insecticides such as lindane, cyclodienes, and toxaphene inhibit GABAergic transmission by binding to a site associated with the GABA-Cl channel complex. GABA inhibition results in the presynaptic release of neurotransmitter (Shankland, 1982; Woolley and Zimmer, 1986; Joy et al., 1987). Postsynaptically, lindane has been found to reduce sensitivity of the end plate to acetylcholine (Woolley and Zimmer, 1986).

In aquatic organisms, lindane has been shown to: disrupt osmoregulatory mechanisms, particularly chloride regulation, in the crab *Eurypanopeus depressus* (Shirley and McKenney, 1987), induce hypoglycenia and alter the membrane lipidic matrix in carp *Cyprinus carpio* (Demael et al., 1987); and inhibit protein synthesis in *Tetrahymena pyriformis* (Al-Chalabi and Al-Khayat, 1989). In addition, neonates of *Daphnia magna* exposed to different concentrations of chlordecone, a cyclodiene insecticide, showed decreased protein/organism with increased exposure concentration (McKee and Knowles, 1986).

Given the affinity of stress protein 70 for denatured proteins and protein repair, including those involved in cytoskeletal structure, and lindane's effects on membrane processes, leads one to suggest that the lindane-induced stress response

is due to membrane associated aberrations.

III. E. DIAZINON EXPOSURES

III. E. 1. Materials and Methods

Exposure System

Stock concentrations of Diazinon (Supelco, Inc., Bellefonte, PA) were prepared in certified grade DMF. All exposures were static/renewal. Appropriate volumes of stock solution were pipetted into 19L (stock) tanks, filled with 18L of dechlorinated tap water, and subsequently gently mixed with a glass rod. The contents of the stock tanks were transferred into four 6L glass aquaria, each fitted with a center drain pipe such that the overflow volume was 5L. Due to high volatilization, 90% of the exposure volume was renewed every 12h.

Experimental Design

A range find experiment was performed to determine a range of diazinon concentrations suitable for a definitive LC50 test and to determine times to first signs of intoxication. Five diazinon concentrations (2, 4, 6, 8, and 10 mg/L, nominal) were used. Each treatment was replicated twice. Four to six fish were placed into each treatment replicate and observed for onset of intoxication. By 4h, fish in all treatments showed varying degrees of intoxication. Fish in the 2 and 4 mg Diazinon/L treatments were lethargic and located on the bottom of the aquaria. Contrastingly, 6,8, and 10 mg/L exposed fish were near the surface operculating rapidly and displayed erratic swimming, including lazy 'S' curves. By 24h, percent mortality was 33% and 67% in 8 and 10 mg/L exposed fish, respectively, however,

none had died in the other treatments.

To determine the length of time required for induction of the stress proteins, two replicates of three fish each were exposed to 8 mg/L (nominal) and sampled at 0, 4, 8, 12, and 24h. Immediately after sampling, fish were sacrificed. Gill (37.1 $\pm$ 8.0), and striated muscle tissue (36.3 $\pm$ 6.2) were excised and respectively pooled within each treatment replicate. Tissues were metabolically labeled for 2h with ^{35}S-methionine/cysteine (Trans ^{35}S-label, ICN). Incorporation of label into proteins was examined by SDS-PAGE and fluorography. Resulting fluorograms showed that all stress proteins observed within the 24-h treatment period were maximally induced at 12h. Thus, 12h was selected as the sampling time to examine the stress protein response in the subsequent 96-h acute toxicity test.

The range of concentrations in which the gill and striated muscle elicit the stress protein response was determined by exposing fish to five different diazinon concentrations (1.44 $\pm$ 0.21; 2.46 $\pm$ 0.51; 3.45 $\pm$ 0.80; 3.79 $\pm$ 0.49; 5.59 $\pm$ 0.50 mg Diazinon/L). plus control, and sampled at 12h. Each treatment was replicated four times. Thirteen fish were placed into each treatment replicate. Three were subsampled at 12h and the remaining fish were exposed for 96h. Tissues of subsampled fish (gill 40.6 $\pm$ 6.3; striated muscle 44.6 $\pm$ 7.1 mg) destined for metabolic labeling were treated as mentioned in the induction study. Time to mortality was recorded every 24h. Metabolic labeling, sample preparation, SDS-PAGE, and quantitation of fluorograms was conducted using protocols described in the heat shock experiment's materials and methods.

Water Chemistry

All exposures used dechlorinated tap water that had the following mean characteristics: alkalinity 100 mg $CaCO_3$/L; hardness 38 mg $CaCO_3$/L; and conductivity 300 umhos. Dissolved oxygen (4.2 $\pm$ 0.8 mg O_2/L) and pH (7.2 $\pm$ 0.2) were measured at the end of each renewal. Diazinon water concentrations were determined using a Tracor 560 Gas Chromatograph fitted with an electron capture detector and SPB-5 capillary column (Supelco, Inc.).

III. E. 2. RESULTS

A range of diazinon concentrations caused significant changes in mortality (Kruskal-Wallis, $p \leq 0.05$). Percent mortality ranged from 5.0% in controls to 75% at 5.6 mg/L. A Dunnett's T test revealed that 5.6 mg/L had significantly greater mortalities compared to controls ($p \leq 0.05$). Probit analysis determined the LC50 to be 4.74 mg/L and the LC1 to be 2.38 mg/L.

Gill tissue of fathead minnows exposed to 8 mg Diazinon/L elicited the stress response. Five stress proteins with apparent molecular weights of 32, 70, 78, 90, and 96 kD were observed. Radiolabeled methionine/cysteine incorporation into all stress proteins increased beyond 0-h rates by 8h exposure and reached maximum rates at 12h. Compared to 12-h rates, there was a decrease in label incorporation at 24h. Similar kinetics of stress protein induction was observed in striated muscle. Ten stress proteins (27, 32, 33, 42, 52, 55, 60, 70, 90, and 96 kD) were observed.

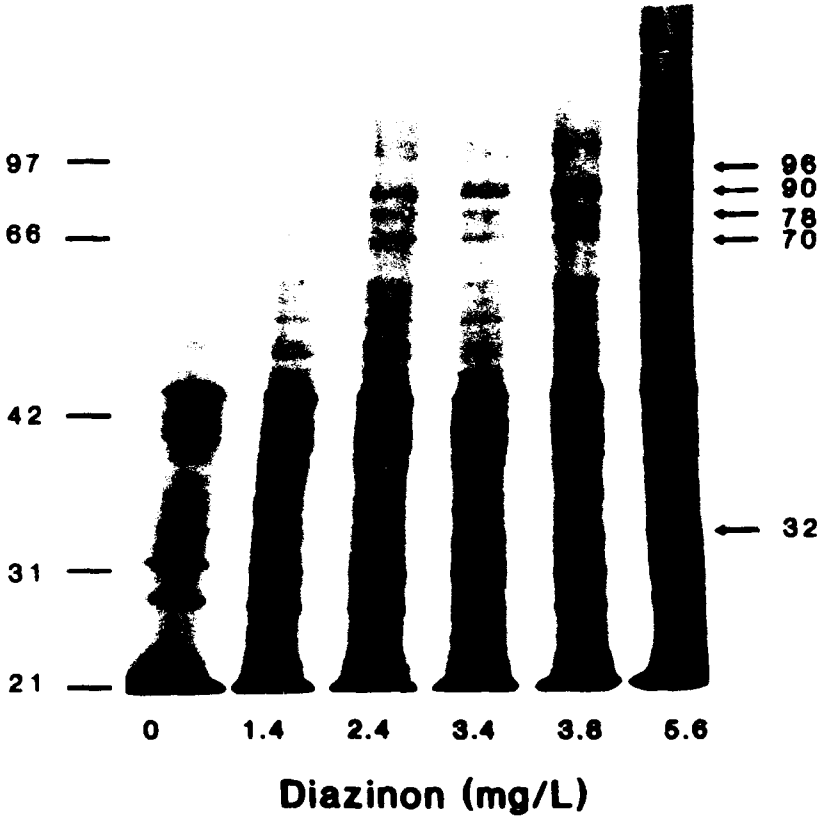

Figure 18. A fluorgram of gill tissue protein from fish exposed to 0, 1.4, 2.4, 3.4, 3.8 and 5.6 mg diazinon/L for 12h. Molecular weight markers are designated on the left. The arrows on the right denote stress proteins of 32, 70, 78, 90, and 96 kD.

As found with gill, maximum incorporation of ^{35}S-methionine/cysteine occurred at 12h, with an apparent decrease at 24h. Therefore, a 12-h exposure was selected to be the sampling time for the concentration-response study.

The stress protein response was found to be highly tissue specific in fish exposed to a range of diazinon concentrations. Total numbers and molecular weights of stress proteins observed differed markedly between the gill and striated muscle tissues. In addition, the range of concentrations required for the increased incorporation of radiolabel into proteins of similar molecular weight was also tissue specific.

Five stress proteins were observed in the gill (Figure X). A visual inspection of one of the replicate fluorograms shows that ^{35}S-methionine/cysteine incorporation into proteins of 70 and 78 kD increased above controls from 1.4 to 5.6 mg/L. A 32 kD protein showed an increase from 3.4 to 5.6 mg/L whereas, the 96-kD protein appeared only at the highest concentration. In contrast, the 90-kD protein did not appear to be induced above controls at any diazinon concentration. Kruskal-Wallis analysis of variance of densitometric scans of over all four replicates showed that diazinon did not significantly affect radiolabel incorporation into any of the stress proteins. Nevertheless, significantly increased incorporation rates above controls were found at 3.8 and 5.6 mg/L for the 32-kD protein and 5.6 mg/L for stress protein 70 (Dunnett T test, $p \leq 0.05$).

A strikingly different stress protein pattern was found in the striated muscle (Figure 19). Seventeen different proteins were observed with apparent molecular

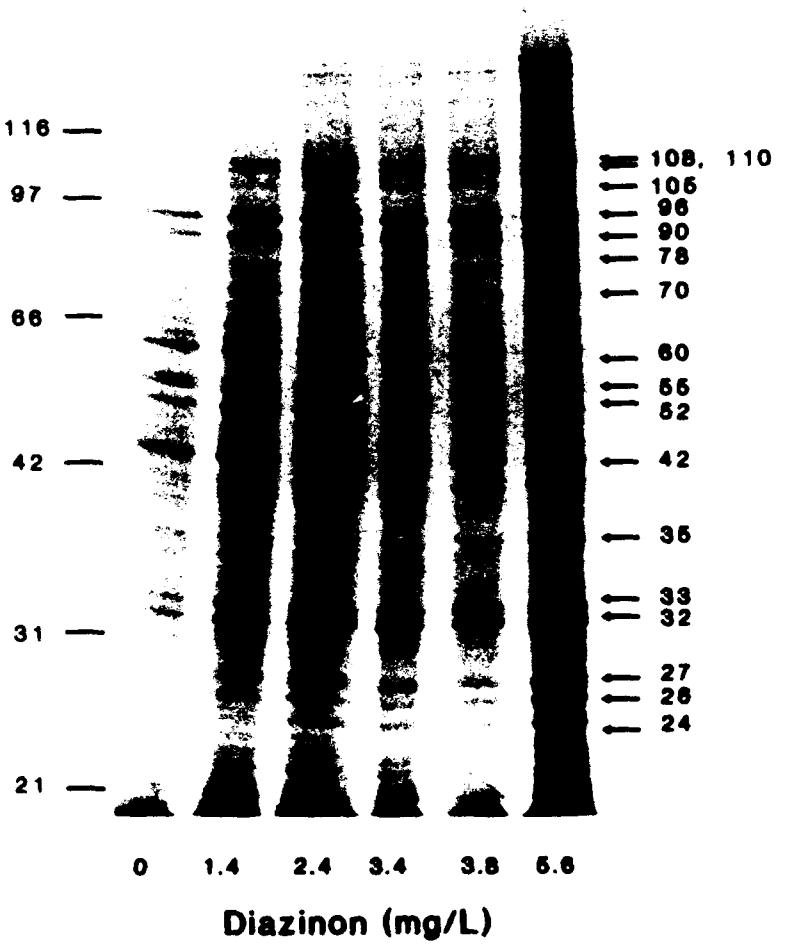

Figure 19. A fluorgram of striated muslce tissue protein from fish exposed to 0, 1.4, 2.4, 3.4, 3.8 and 5.6 mg diazinon/L for 12h. Molecular weight markers are designated on the left. The arrows on the right denote stress proteins of 24, 26, 27, 32, 33, 35, 42, 52, 55, 60, 70, 78, 90, 96, 105, 108, and 110 kD.

Table 2. Mean and standard deviations (in parentheses) of stress protein from striated muscle of fathead minnows exposed to different diazinon concentrations for 12 hours. * denote significant differences from control (Dunnett's T test, $p \leq 0.05$).

TREATMENT	24	26	27	32	33	35	42	52	55
CONTROL	0 (0)	0 (0)	0 (0)	23.8 (24.8)	25.3 (22.2)	13.0 (10.6)	44.8 (37.0)	21.0 (17.6)	36.6 (21.4)
1.4 mg/L	2.5 (5.5)	26.0 (29.6)	22.0 (44.0)	117.1 (135.4)	101.6 (121.6)	42.3 (40.7)	144.1 (105.0)	62.4* (1.8)	99.1* (80.0)
2.4 mg/L	0 (0)	19.8 (18.1)	56.8* (34.2)	136.3* (40.5)	132.5* (66.9)	47.5* (19.8)	190.2* (69.1)	96.2* (36.6)	118.0* (37.2)
3.4 mg/L	11.3 (13.1)	24.2 (28.8)	66.6* (66.3)	163.8 (142.7	) 161.5 (132.4)	34.9* (24.7)	178.1* (100.1)	86.4 (58.6)	116.3* (69.2)
3.8 mg/L	3.6 (7.2)	16.2 (18.0)	31.9 (31.1)	107.7 (45.3)	94.0 (55.2)	21.4 (13.8)	148.6 (102.8)	75.2* (27.5)	98.9* (47.2)
5.6 mg/L	17.1* (13.0)	56.0* (27.7)	74.7* (34.3)	188.0* (98.8)	203.5* (49.0)	59.6* (10.4)	287.9* (96.5)1	75.0* (63.4)	208.5* (70.3)
k-W PROB. P =	.09	0.07	0.04	0.07	0.08	0.04	.02	.01	.02

TREATMENT	60	70	78	90	96	105	108	110
CONTROL	33.4 (18.9)	4.1 (8.3)	3.7 (7.4)	19.6 (21.7)	33.2 (24.7)	2.3 (4.6)	10.1 (13.3)	2.9 (5.8)
1.4 mg/L	68.2 (31.5)	16.1 (19.8)	13.6 (10.6)	63.3 (51.8)	94.6 (88.9)	8.2 (16.4)	37.4 (18.6)	26.9 (28.7)
2.4 mg/L	92.9* (31.5)	27.4 (8.9)	29.9* (10.4)	66.9* (9.3)	98.0 (7.8)	15.1 (7.5)	24.5 (12.3)	32.8 (14.4)
3.4 mg/L	87.9* (32.8)	38.9* (21.1)	29.6* (21.1)	66.8 (37.6)	100.1 (56.4)	8.7 (10.3)	31.6 (15.4)	18.9 (17.2)
3.8 mg/L	74.0 (39.4)	26.9 (10.4)	25.2 (12.6)	61.5 (29.2)	88.6 (34.5)	6.1 (7.9)	23.3 (11.9)	19.2 (8.2)
5.6 mg/L	161.1 (71.8)	53.8 (22.4)	36.0 (15.5)	126.2 (38.3)	180.7 (55.6)	15.8 (10.6)	54.5 (7.7)	32.7 (18.6)
k-W PROB. P =	0.02	.02	.04	.02	.04	0.29	.02	.09

weights of 24, 26, 27, 32, 33, 35, 42, 52, 55, 60, 70, 78, 90, 96, 105, 108, and 110 kD. All but the 24, 26, and 27 kD proteins were observed in the controls. However, radiolabel incorporation into all observed stress proteins increased above controls at all diazinon concentrations.

Diazinon treatments significantly affected the incorporation of label into eleven out of the seventeen proteins quantitated in the striated muscle (Kruskal-Wallis, $p \leq 0.05$; Table 2). Significantly altered incorporations were determined for proteins of 27, 35, 42, 52, 55, 60, 70, 78, 90, 96, and 108 kD. Dunnett's T tests performed on ranked data showed a significantly increased radiolabel incorporation into 52 and 108 kD proteins at 1.4 mg/L. At 2.4 mg/L eleven proteins (27, 32, 33, 35, 42, 52, 55, 60, 78, 90, and 110 kD) were significantly induced. At 3.4 mg/L, seven (27, 42, 52, 55, 60, 70, and 78 kD) were induced. Large variability in the 3.8 mg/L replicates compromised significance such that only the 52-kD protein showed a significantly increased incorporation rate. However, sixteen of the seventeen quantitated proteins showed significant increases in label incorporation at the highest concentration, 5.6 mg/L.

III. E. 3. Discussion

The acute exposure of fathead minnows to 8 mg/L resulted in the elicitation of the stress protein response in both gill and striated muscle tissues. By 12h, incorporation of ^{35}S-methionine/cysteine into stress proteins was maximum in both

tissues. However, the numbers and sizes of stress proteins induced were tissue specific.

Markedly different translational patterns were observed in the gill and striated muscle tissue of fish exposed to different diazinon concentrations. Striated muscle synthesized and accumulated more stress proteins (seventeen) than gill (five) and required a lower concentration of diazinon to induce the stress response. Statistically, diazinon did not induce the stress response in the gill whereas eleven out of the seventeen observed, and quantitated, proteins in the striated muscle were found to be significantly affected by diazinon concentration. Diazinon's mode of action may partially explain the drastic tissue specific translational differences.

The classical definition of organophosphate insecticide mode of action is that they are acetylcholinesterase inhibitors (Matsumura, 1985). Inhibition of this enzyme results in the accumulation of acetylcholine in nerve tissue and effect on organs with consequent signs and symptoms that mimic muscarinic, nicotinic, and central nervous system actions of acetylcholine (Murphy, 1986). Muscarinic effects are found primarily in smooth muscle, the heart, and exocrine glands (Durham, 1987; Murphy, 1986). Nicotinic effects occur primarily at neuro-muscular junctions and antonoic ganglia (Murphy, 1986; Durham, 1987). In summary, toxicity of diazinon most likely involves respiratory distress (Murphy, 1986) caused by bronchial constriction and increased lamellar secretions, and muscular fatigue (Smith, 1984) caused by overstimulation of the post synaptic neuromuscular junction.

Therefore, a plausible explanation for the lack of stress response in gill might

be due to respiratory failure and the increased stress response in the muscle due to stimulation of muscle cells.

In support of this explanation, Vijayalakshmi (1980) found that sumithion exposed fish resulted in greater inhibition of oxygen consumption and succinate dehydrogenase activity in the gill versus the striated muscle. In contrast, acetylcholinesterase activity was inhibited more in the muscle compared to gill tissue. Exposure of *Tilapia mossambica* to organophosphates resulted in the inhibition of oxydative enzymes (Rao and Rao, 1979; Basha et al., 1984) and increased levels of total protein and free amino acids in muscle and gill tissue (Rao and Rao, 1979).

The use of acetylcholinesterase inhibition as a biochemical indicator of organophosphate and carbamate insecticide exposure has been proposed (William and Sova, 1966; Coppage, 1972; Coppage et al., 1975; Coppage, 1977; Ansari and Kumar, 1984). Unfortunately, acetylcholinesterase inhibition has been shown to be markedly affected by season and temperature and is not significantly correlated to mortality (Thiruganonam and Forgash, 1975; Mayer, 1983). In addition, Olson and Christensen (1980) showed that fish acetylcholinesterase was inhibited 156 fold greater by arsenite compared to diazinon. Thus, use of this enzyme's inhibition as a biomarker of organophosphate exposure is doubtful.

Our study, on the other hand, showed that a significant stress response occurred at concentrations below the LC1, an estimator of chronic toxicity. Therefore, the stress protein response has potential to serve as a biochemical indicator of organophosphate exposure. Furthermore, in comparing the stress

responses of gill and muscle from diazinon exposed fish versus responses in the same tissues from arsenite exposed fish (Dyer et al., in preparation), we propose that the translational pattern may be diagnostic of toxicant specific stress.

IV. PRELIMINARY FIELD VALIDATION

IV. A. Introduction

Biological effects from toxicant exposure are manifest first at the molecular/cellular level and then proceeds through increasing levels of biological organization (i.e. organism, population, community, etc). Thus, use of biochemical parameters to assess biotic stress has the potential to be more sensitive than higher order indicators and may indeed serve as an early warning of impending doom for higher levels of organization.

Several studies performed in our laboratory with fathead minnows have shown that stress protein 70 is induced under a variety of exposures, such as heat shock, sodium arsenite, sodium chromate, lindane, and diazinon. The sum of these studies advocates the potential use of stress protein 70 as a general stress indicator. In particular, quantitative methods using immunological techniques have proven to be highly sensitive, especially in gill tissue.

To test the efficacy of stress protein 70 induction as a potential biomonitoring tool, we performed a preliminary field assessment by exposing laboratory reared fish in cages to an acute to chronic toxicity gradient. This study was conducted in Soldier Creek, Midwest City, Oklahoma. Soldier Creek orginates as an industrial effluent from Tinker Air Force Base. The creek flows approximately five miles north-northwest before its confluence with Crutcho Creek. Crutcho Creek flows approximately another 2 miles until its confluence with the North Canadian River.

Historically, Soldier Creek has been shown to be acutely toxic throughout its entire length and contain few fish (Simpson et al., 1987; Ontech, Inc. and Trac Laboratory, Inc., 1987). However, reconnaisance visits by members of our laboratory during the summers of 1989 and 1990 found viable fish communities in the middle and lower reaches of the creek, whereas the upper reach, toward Tinker Air Force Base, contained little or no fish. Thus, there was an acute-chronic toxicity gradient within the creek. The purpose of this study was to compare the induction of stress protein 70 to percent mortality and *in situ* fish species richness.

IV. B. Materials and Methods

Organisms

Ninety- to 120-day-old fathead minnows (*Pimphales promelas*) were obtained from a culture located at the University of North Texas. Fish used for the study were acclimated to 25°C for at least 7 days and a 16L:8D photoperiod and were fed frozen brine shrimp twice daily to satiation.

Cages

Cages consist of 12 inch long, 6 inch diameter heavy PVC pipe with 40-52 one inch dia. holes drilled over the pipe length. Fiberglass window screen was wrapped around the outside of the pipe and fitted with adhesive tape and 6 inch stainless steel ring clamps.

Exposure

Three to four fathead minnows were placed into each cage. One cage each was placed at six different sites along Soldier Creek and one in the North Canadian River (Figure 20). Fish were exposed 4d, from 7/3/90 through 7/6/90. After the exposure period, fish were sacrificed, wrapped in aluminum foil, and then placed in liquid nitrogen. Gill tissue destined for stress protein 70 analysis was disected in our laboratory.

Fish Collections

Fish were collected from all Soldier Creek and North Canadian River stations on 9/14/90. Three ten meter seine hauls, using a twenty foot quarter-inch mesh seine, were conducted at each site.

Quantitative Immunoblots

Protein from gill tissue of caged fish was assayed for stress protein levels using the methodology described in the chromate and lindane experiments. Gill protein from non-exposed cultured fish was used as a performance control.

Statistics

A spearman correlation was used to assess the significance of the relationship between minimum detection levels for stress protein 70 and species richness.

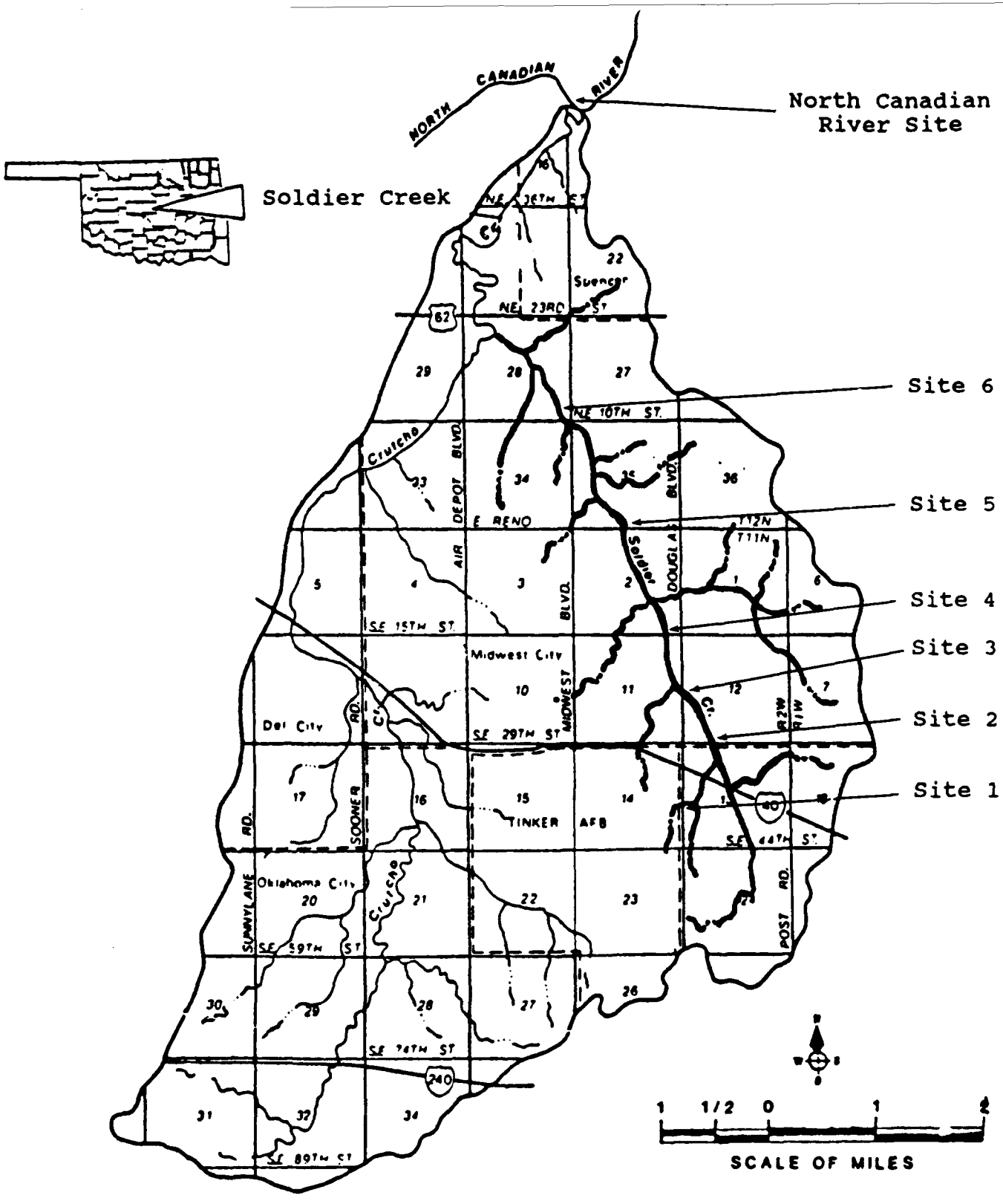

Figure 20. Locations of fish cages and *in situ* fish collection sites on Soldier Creek and North Canadian River during 7/90 and 9/90.

IV. C. Results

Proceeding from upstream to downstream, toxicity (percent mortality) decreased while fish numbers and species richness increased (Figure 21; Table 3). From sites 3 through 6 on Soldier Creek and the North Canadian River site, caged fish stress protein minimum detection levels were highly significantly correlated to species richness (Spearman correlation, $r = 0.95$, $p \leq 0.001$). Minimum detection levels of stress protein 70 in North Canadian River fish (6.25 ug total protein) was the same as that found in reference controls.

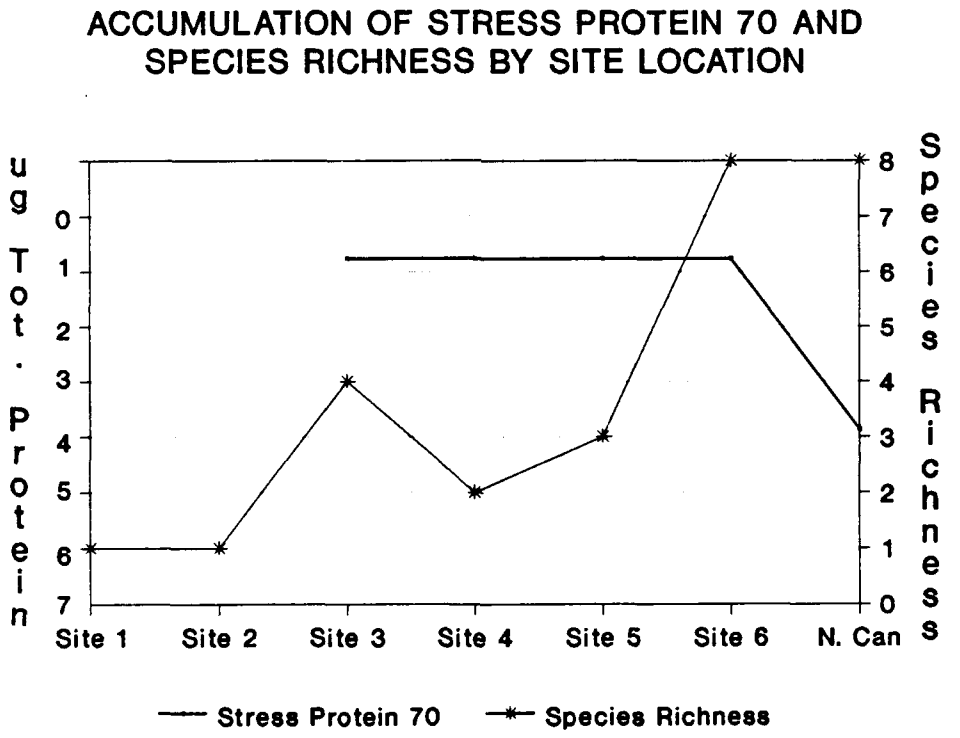

Figure 21. Line diagram that illustrates the relationships between accumulation of stress protein 70 in gill tissue of caged fish and *in situ* fish species richness to site location.

Table 3. Minimum detection levels for stress protein 70, percent mortality of caged fish, species, and numbers of individuals collected in Soldier Creek and North Canadian River, by site.

Site	sp70 Level	Percent Mortality	Species	Numbers of Individuals
1	NA	1.0	fathead minnow (*Pimephales promelas*)	1
2	NA	1.0	red shiner (*Notropis lutrensis*)	6
3	3.125	0.0	mosquitofish (*Gambusia affinis*)	330
			red shiner (*Notropis lutrensis*)	21
			fathead minnow (*Pimephales promelas*)	3
			redfin shiner (*Notropis umbratilis*)	2
4	3.125	0.0	red shiner (*Notropis lutrensis*)	105
			mosquitofish (*Gambusia affinis*)	12
5	3.125	0.0	red shiner (*Notropis lutrensis*)	374
			mosquitofish (*Gambusia affinis*)	92
			green sunfish (*Lepomis cyanellus*)	13
6	3.125	0.0	red shiner (*Notropis lutrensis*)	716
			mosquitofish (*Gambusia affinis*)	384
			fathead minnow (*Pimephales promelas*)	81
			green sunfish (*Lepomis cyanellus*)	43
			sand shiner (*Notropis stramineus*)	38
			bigmouth shiner (*Notropis dorsalis*)	19
			suckermouth minnow (*Phenacobius mirabilis*)	1
			common carp (*Cyprinus carpio*)	1
No. Can. Riv.	6.250	0.0	red shiner (*Notropis lutrensis*)	1867
			mosquitofish (*Gambusia affinis*)	99
			bullhead minnow (*Pimephales vigilax*)	49
			fathead minnow (*Pimephales promelas*)	4
			green sunfish (*Lepomis cyanellus*)	2
			bigmouth shiner (*Notropis dorsalis*)	1
			river carpsucker (*Carpiodes carpio*)	1
			gizzard shad (*Dorosoma cepedianum*)	1

NA = Not applicable since no fish tissue was available

IV. D. Discussion

This preliminary test indicated that use of stress protein 70 as a general indicator of stress has applicability. Continued field research coupled with extensive water chemistry and *in situ* biotic assessments is required to fully evaluate the efficacy of the stress proteins as a biomonitoring tool. Moreover, investigations using antibodies to other stress proteins, such as stress proteins 20, 60, and 90, are warranted since previous studies in our laboratory have indicated the positive potential of teasing out chemicals or classes of chemicals responsible for biotic aberrations observed in the environment.

V. CONCLUSIONS

The objective of this research, as stated in our proposal, was to evaluate the phenotypic expression of the stress proteins as indicators of biotic stress with the potential for adapting the response as a rapid, water quality biomonitoring method. The approach used for assessing the stress protein response (SPR) as a potential biomonitoring tool consisted of laboratory and *in situ* (field) exposures. To accomplish the proposed objective, we first characterized the SPR in fathead minnows (*Pimephales promelas*) via laboratory experiments by exposing fish to a variety of stressors, including chemicals with different modes of action. The goal of the laboratory studies was to determine relationships between the types and concentrations of stressors and the SPR in selected tissues. In these studies we: 1) identified stress protein patterns in gill and striated muscle of fathead minnows; 2) compared stress protein responses elicited by a variety of stressors (heat shock, sodium arsenite, sodium chromate, lindane, and diazinon); and 3) correlated (calibrated) the SPR to conventional toxicity endpoints such as effects on survival.

Secondly, we attempted to validate the SPR as a potential biomonitoring tool by: 1) exposing caged laboratory raised fathead minnows *in situ* to an acute-chronic toxicity gradient; 2) evaluating the SPR in gill tissue of fish placed at selected locations in relation to the toxicity gradient; and 3) correlating the SPR to *in situ* fish species richness.

The following paragraphs describe the salient features and conclusions we have made based on the performed research.

Potential Application for Biomonitoring

Characteristics of an ideal biochemical indicator of stress include that it: 1) be equivelent or more sensitive than existing indices of stress, such as mortality; 2) be significantly correlated to chemical exposure and/or effects; and 3) ideally, give information on the type(s) of stressors encountered. Our study has shown that the stress protein response fulfills many of these characteristics and, thus, has potential to serve as a biomonitoring tool.

Rapid, Sensitive, and Relevant

We have shown that the SPR is a rapid response. Significantly increased synthetic rates of stress proteins were observed within hours of stressor exposure. In addition, significantly increased accumulations were determined by four days of exposure. Most promising is the sensitivity of the SPR to sublethal toxicant and heat shock exposures. Both synthetic rates and accumulation levels of stress proteins were found to be significantly increased at toxicant concentrations that did not cause significant changes in mortality by. Moreover, increased synthesis and accumulation of the stress proteins were significantly correlated to toxicant concentration, percent mortality, and species richness via field studies.

Indicators of General and Toxicant Specific Stress

Fish exposed to toxicants of different modes of action elicited toxicant-specific translation patterns. All stressors induced a 70 kD protein (stress protein 70) thus

this protein appeared to be indicative of general stress. This was verified in our preliminary field validation test where an increase in stress protein 70 accumulation was observed in fish exposed to an *in situ* toxicity gradient in Soldier Creek, Midwest City, Oklahoma. Proteins of other molecular weights may be indicative of chemical class or mode-of-action. For example, stress proteins of 20 and 30 kD were found only in tissues of metal exposed fish.

Comparisons of Different Methodological Techniques

Due to the recent availability of a stress protein 70 antibody, we were able to compare sensitivities of the radiolabel and immunological methodsfor assessing the SPR in fish. The incorporation of radiolabeled amino acids into proteins allowed us to examine the translation patterns as related to toxicant type and concentration. However, to adequately induce the SPR for densitometric quantitation, acutely toxic concentrations were required. Via quantitative western blots using the stress protein 70 antibody we were able to determine significantly increased accumulations of stress protein 70 at sublethal toxicant concentrations. Thus, the immunological methodology was several fold more sensitive. However, our investigations using this technique were limited to just the one stress protein due to the limited availability of other stress protein antibodies. Therefore, development of fish antibodies to other stress proteins is a future direction of this laboratory.

Future Research Needs

In view of the results presented, several future research initiatives are warranted. Field validation studies where acute to chronic or sub-chronic gradients

exist should be undertaken. Comparisons of the SPR to extensive water and sediment chemistry data, as well as, biotic indices are necessary to fully understand the efficacy and limitations of this biochemical approach to assess *in situ* stress. Moreover, investigations using antibodies to other stress proteins such as 20, 60, and 90 are needed since our study has indicated the positive potential of the SPR as a diagnostic tool to identify chemicals or chemical classes responsible for biotic aberrations. In addition, continued laboratory assessments of the SPR's efficacy focusing on translation patterns in response to a wide array of chemicals is needed. With such research endevours, it may be possible to use protein patterns to indicate chemical class and/or mode-of-action.

Research on the SPR in aquatic species other than fish is also necessary. Due to the highly conserved nature of the SPR, techniques explored during this research project should be applicable to other life forms. Interpretation of cross species stress responses would give valuable information on the fate and effects of environmental contaminants and could be incorporated into ecological risk assessments.

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