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Lysosomal membrane stability, histopathology, and serum enzyme activities as sublethal bioindicators of xenobiotic exposure in the bluegill sunfish (Lepomis macrochirus Rafinesque)

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LYSOSOMAL MEMBRANE STABILITY, HISTOPATHOLOGY, AND SERUM ENZYME ACTIVITIES AS SUBLETHAL BIOINDICATORS OF XENOBIOTIC EXPOSURE IN THE BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS* RAFINESQUE)

By

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ABSTRACT

LYSOSOMAL MEMBRANE STABILITY, HISTOPATHOLOGY, AND SERUM ENZYME ACTIVITIES AS SUBLETHAL BIOINDICATORS OF XENOBIOTIC EXPOSURE IN THE BLUEGILL SUNFISH (LEPOMIS MACROCHIRUS RAFINESQUE)

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Lysosomal enzyme release (LERA), histopathology, and serum enzyme activities were investigated to determine the relative sensitivities of these measures to toxicant-induced changes in tissues of the bluegill sunfish (Lepomis macrochirus). Biotic factors were studied to understand the natural variability in LERA and serum enzymes for bluegill sunfish. LERA, histopathology, and serum enzyme activities were investigated during acute CCl_4 and short-term cadmium exposures in an attempt to develop these techniques as useful indicators of toxicity. The effects of cadmium on the ecologically relevant parameters of growth and survival were determined in chronic exposures and compared to effects on LERA, histopathology, and serum enzyme activities.

The effects of the biotic factors sex, sexual maturity, and body size on LERA and serum enzyme activities were studied. Mean labilization indices (LI) and enzyme activities for N-acetyl-β-D-glucosaminidase (NAG) and acid phosphatase (ACP) in liver were not different between the sexes. Sexual maturity did not affect the LI or

enzyme activities of NAG or ACP. Size (weight) was negatively correlated with the LI for ACP.

Carbon tetrachloride (2.0 ml/kg, ip.) caused significant changes in histopathological and biochemical indicators of toxicity. Activities of the enzymes NAG, ACP, lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT) in serum were elevated after one day of exposure, while only ACP remained elevated three and seven days after injection. As indicated by both NAG and ACP, lysosomal membranes were destabilized one day after injection with CCl_4. Lysosomes in treated fish were more stable after three days, but were destabilized again seven days after treatment.

Cadmium concentrations of 3.9 and 12.7 mg Cd/l caused decreased growth in a chronic 163 d exposure. The greater concentration also caused decreased survival. Histopathological lesions occurred at both concentrations in gill tissue only. No other organs displayed degenerative lesions due to cadmium exposure. Exposure to 12.9 mg Cd/l caused elevations in serum NAG and ACP activities after 32 d of exposure. Lysosomal membranes were destabilized after 8, 16, and 32 d of exposure to 12.9 mg Cd/l.

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INTRODUCTION

Study of xenobiotic effects at the biochemical, physiological, and histological levels of organization can be useful in developing improved toxicological test protocols and in understanding the effects of xenobiotics on aquatic organisms. Important, ecologically relevant adverse effects of toxicants on the organism are the culmination of effects on biochemical and physiological processes. Measurement of effects at the biochemical or physiological level of organization allow early detection of adverse effects on the organism and give insight into the toxicologic site and mode of action. Establishing environmentally safe concentrations for the many new and existing pollutants will require shortening chronic exposure studies and a thorough understanding of the toxic effects of these pollutants.

Xenobiotic-induced alterations at the suborganismal level of organization cannot be considered ecologically relevant unless the changes are not fully compensated for by the organism, resulting in reduced fitness (Livingstone, 1982) or unless the results of tests at the suborganismal level are correlated with population-level effects (Mehrle and Mayer, 1980). Such a correlation would allow these tests to be used as predictors of chronic toxicity, thereby reducing the time necessary to test a xenobiotic for sub-lethal effects on aquatic organisms. Because of the relative ease and rapidity of conducting

some clinical tests and the fact that general indicators of xenobiotic stress integrate all of the stressors to which an organism is exposed, these procedures have been proposed as useful in determining synergism or antagonism of xenobiotics and the influence of accessory environmental factors on toxicity.

A problem in studying the effects of xenobiotics on the biochemistry, physiology, and histology of fish is the lack of basic information in these disciplines. Detailed understanding of the basic biology of fish is being generated through aquatic toxicology because there is a need to understand the biochemical, physiological, and histological systems in fish and the effects of xenobiotics on these systems.

Research into the basic biochemistry and physiology of fish and the effects of two xenobiotics on these systems was undertaken to contribute to this area of science. Furthermore, this research is an attempt to develop a general indicator of sublethal xenobiotic stress in fish. The primary areas which this research addresses are lysosomal membrane stability, serum enzyme activities, and histopathology.

Lysosomal Enzyme Release Assay (LERA)

Lysosomes are a morphologically heterogeneous group of membrane-bound subcellular organelles, containing acid hydrolases and ranging in size from 250 Å to 1 μm diameter (Karp, 1979). Lysosomal hydrolases are produced in the endoplasmic reticulum and placed in membranes by the Golgi complex or Golgi endoplasmic reticulum lysosome (GERL) (Cohn and Fedorko, 1969). The structures produced are referred to as lysosomes or dense bodies (Figure 1). Lysosomes fuse with membrane

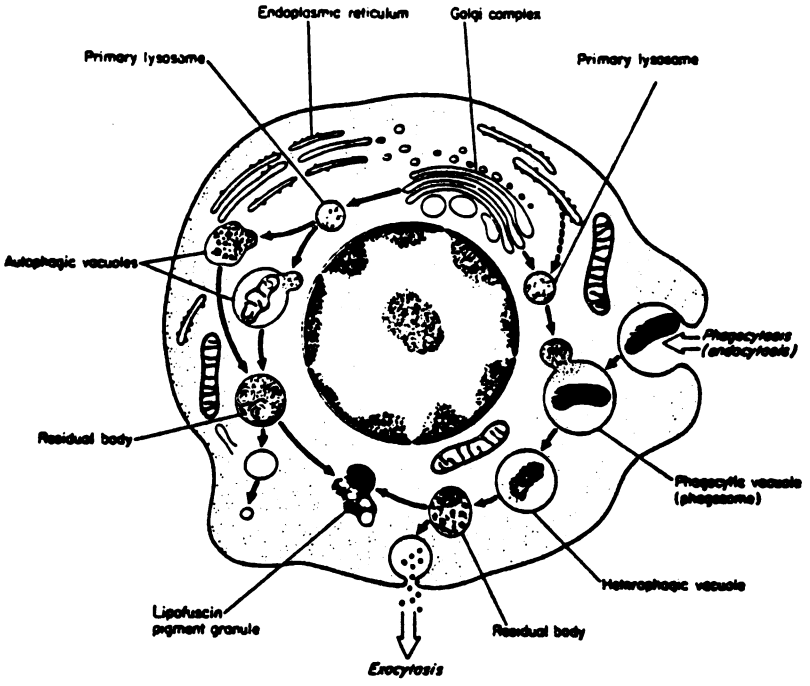


Figure 1. Schematic representation of the biogenesis, function, and fate of a lysosome in a vertebrate cell. (Modified from Bloom and Fawcett (1975))

bound vesicles containing intracellular macromolecules or extracellular materials which have been endocytosed into phagolysosomes. The fusion of one or more primary lysosomes with membrane bound vesicles or other organelles (eg. mitochondria) form secondary lysosomes. An autophagic vacuole results from the fusion of primary lysosomes with intracellular material. Phagolysosomes are formed by fusion of primary lysosomes with phagocytic vacuoles. Both autophagic vacuoles and phagolysosomes are secondary lysosomes (or heterolysosomes).

The lysosomal membrane release assay (LERA) is a measure of the stability of the lysosomal membrane. In stable lysosomes, hydrolases are prevented from reacting with substrate by an intact membrane. Theoretically, membrane stability decreases in response to stress and enzyme activity increases as membrane permeability increases. Lysosomal membrane stability has been demonstrated to be a useful measure of environmental stressors in aquatic organisms (Moore and Stebbing, 1976; Mensi et al. 1982).

The two LERA techniques, a histochemical and a cytochemical/biochemical technique, both measure the functional integrity of the lysosomal membrane (Table 1). In the histochemical LERA procedure, unfixed frozen sections are exposed to a low pH buffer. The incubation time required for staining is proportional to the susceptibility of the lysosomal membrane to the pH shock, and is referred to as the latency period (Baccino and Zuretti, 1975). The longer the latency period, the more stable the lysosomal membrane is to low pH. This technique has been most successfully applied in describing environmental pollution effects on the marine mussel,

Table 1. A summary of the literature concerning lysosomes and stressors.

Organism	Stressor	Method	Effect	Reference
		Invertebrates		
Mytilus edulis	WAF-oil	Cytochemistry/ stereology	Increased lysosome size, decreased number	Lowe et al. 1981
M. edulis	Salinity	Histochemical	Lysosomal destabilization altered enzyme activity	Moore et al. 1980
M. edulis	Anthracene (injected)	Histochemical	Lysosomal destabilization	Moore et al. 1978a
Littorina littorea	Oil (*in situ*)	Histochemical	Lysosomal destabilization	Moore et al. 1982
M. edulis	Cortisol (injected)	Histochemical	Lysosomal stabilization	Moore et al. 1978b
Campanularia flexuosa	Copper, cadmium, mercury	Histochemical	Increased enzyme activity	Moore and Stebbing, 1976
M. edulis	WAF-oil	Histochemical	Increased lysosomal size, decreased number	Moore and Clarke, 1982
M. edulis	Temperature	Histochemical	Lysosomal destabilization	Moore 1976
M. edulis	WAF-oil	Histochemical	Lysosomal destabilization	Widdows et al. 1982
M. edulis	Copper	Histochemical	Lysosomal destabilization	Harrison and Berger, 1982
M. galloprovincialis	Copper	Histochemical	Lysosomal destabilization	Viarengo et al. 1981

Table 1 Continued:

Organism	Stressor	Method	Effect	Reference
		Fish		
Pimelodus maculatus	Cadmium	Histochemistry	Increased lysosome number	Ferri and Macha, 1980
Salmo gairdneri and *S. trutta*	Copper	Electron microscopy	Increased lysosomal size and number	Leland 1983
S. gairdneri	NO_2	Biochemical	Lysosomal destabilization decreased enzyme activity	Mensi et al. 1982
S. gairdneri	NH_4	Biochemical	Lysosomal destabilization increased enzyme activity	Arillo et al. 1981
		Mammals		
Rat	Starvation	Biochemical	Lysosomal destabilization	Bird 1975
Guinea Pig and Rat	Physical Stressors Hypothermia Swimming Gravitational Emotional	Histochemical	Lysosomal destabilization	Gabrielescu 1970

Mytilus edulis. Lysosomal membranes are destabilized in hepatopancreas cells of marine mollusks exposed *in situ* to the water soluble fraction of crude oil (Moore et al. 1982) and in the laboratory (Widdows et al. 1982). Copper also destabilizes lysosomes during acute (6 d) (Viarengo et al. 1981) and chronic (76 d) (Harrison and Berger, 1982) exposure of the marine mussel. In addition to the effects of xenobiotics on lysosomal membrane stability, a variety of other stressors affect membrane stability in mussels and in mammals. Exposure to salinity (Moore et al. 1980) and thermal stressors (Moore, 1976) destabilize mussel hepatopancreas lysosomal membranes. Rats and guinea pigs subjected to hypothermia, swimming, gravitational, and emotional stresses display reduced lysosomal latency in neurons (Gabrielescu, 1970).

The histochemical LERA technique has several limitations. Quantification of staining is difficult, time consuming, and relies on an expensive microscope-densitometer. Therefore, the technique is difficult to perform on many organisms at one time. This decreases the sample size, reduces replication and decreases the statistical degrees of freedom.

The cytochemical LERA technique involves tissue homogenization and differential centrifugation to produce an enriched lysosomal fraction. The quantity of available enzyme which is released by *in vitro* hyposomotic shock is then quantified by the determination of enzyme activity. The more unstable the lysosomal membrane the greater the release of lysosomal enzymes and the greater the enzyme activity. This technique has been used in fish and rats to determine the effects of stressors on membrane stability (Table 1). Bird (1975) observed destabilization of rat muscle lysosomal membranes following five and

six days of starvation as determined by three lysosomal enzymes, aryl sulfatase, cathepsin D, and RNAase. Increased lysosomal fragility was believed to be due to secondary lysosome formation. In toxicity studies with the rainbow trout, Salmo gairdneri, acutely toxic concentrations of nitrite (Mensi et al. 1982) and ammonia (Arillo et al. 1981) resulted in lysosomal membrane destabilization in hepatocytes.

One drawback of the cytochemical technique is that an unknown number of lysosomes are destroyed in the isolation procedure. This reduces the sensitivity of this assay to toxicant effects, since these lysosomes may be the most seriously affected by the stressor being tested. LERA was selected to evaluate the effects of xenobiotics on aquatic organisms due to the important role lysosomes play in tissue injury and the successful application of LERA in other studies.

Serum Enzyme Activities

Serum enzyme activities have been used extensively to provide simple accurate measures of organ dysfunction in mammals, and recently, they have received increased attention from aquatic toxicologists (Table 2). Elevated serum enzyme concentrations can result from:

1) enzyme leakage from a cell with a damaged cell membrane,
2) increased enzyme production, and leakage from the cell,
3) or decreased enzyme clearance from the blood.

Currently it is not known how serum enzyme activities increase, however, it is agreed that it is due to, and diagnostic of, tissue damage (Galen, 1975).

Table 2. A summary of the literature concerning the effects of stressors on serum enzyme activities.

Organism	Stressor	Enzyme	Activity	Comment	Reference
			Mammals		
Human	Disease Cardiac failure Hepatic failure Bone cancer	NAG	Elevated	Abnormal isozyme patterns	Tucker et al. 1980
Human	Disease Chronic liver disease (fibrosis)	NAG	Elevated	NAG important in the breakdown of connective tissue	Ackerman et al. 1981
Human	Aniline denatured rapeseed oil ingestion within past 10 months	NAG	Elevated		Cabezas-Delamare et al. 1983
Human	Prostrate cancer	ACP	Elevated	Most common use of this enzyme in man	Sullivan et al. 1942
Rat	Cadmium (2 mg/kg; sc.)	ASAT	Elevated	Vitamin B Complex reduces the effect	Flora and Tandon, 1983
		ALAT	Elevated	Vitamin B Complex reduces the effect	
Beagle	Paraquat (25 mg/kg; iv.)	LDH	Elevated	Effect observed at 0.5, 1.5, 3, 6, 12, 24, and 36 hours	Giri et al. 1982
		ASAT	Elevated	Effect observed at 6, 12, 24, and 36 hours	
Rabbit	CCl_4 vapors (6 ppm; 6 h)	ASAT	Elevated	Effect observed at 8, 24 and 48 hours	Dinman et al. 1962
		ALAT	Elevated	Effect observed at 8, 24, 48 and 72 hours	
		LDH	Elevated	Effect observed at 8 and 24 hours	

Table 2 Continued:

Organism	Stressor	Enzyme	Activity	Comment	Reference
---------- Mammals Continued ----------					
Human	Disease Acute infectious hepatitis	ASAT/ALAT	Elevated		Wroblewski and LaDue, 1956
	Cirrhosis	ASAT/ALAT	Elevated		
	Myocardial infarction	ASAT/ALAT	Elevated		
	Angina pectoris	ASAT/ALAT	Elevated		
Human	Myocardial infarction	LDH	Elevated	Effect observed during first 3 days post infraction, normal levels after 5 days	Hsieh and Blumenthal, 1956
	Liver carcinoma	LDH	Elevated		
	Leukemia	LDH	Elevated		
---------- Fish ----------					
S. gairdneri	Dehydroabietic acid (DHAA) (1.2 mg/l; 4 days)	ASAT	No effect		Oikari et al. 1983
		ALAT	No effect		
		LDH	No effect		
	DHAA (20 µg/l, 30 days)	ASAT	No effect		
		ALAT	No effect		
		LDH	No effect		
Oncorhynchus nerka	CCL_4/bromobenzene (1; 1 mixture; 900 mg/kg ip.)	ASAT	Elevated		Bell 1968
	Kidney disease	ASAT	Elevated		

Table 2 Continued:

Organism	Stressor	Enzyme	Activity	Comment	Reference
---------- Fish Continued ----------					
Notopterus notopterus	Mercury (.04 of the 96 h LC_{50}; 60 d)	ASAT	Elevated		Verma et al. 1984
	(.05 of the 96 h LC_{50}; 50 d)	ALAT	Elevated		
Salvelinus fontinalis	Copper (38.2 µg/l; 21 d)	ASAT	Elevated	Effect observed at 6 and 21 d	McKim et al. 1970
	(17.4 & 32.5 µg/l; 337 d)	ASAT	Depressed		
Parophrys vetulus	CCL_4 (3.0 mg/kg; ip)	ASAT	Elevated	Effect observed at 4, 8, 16, 24, and 48 hours	Casillas et al. 1983
		ALAT	Elevated	Effect observed at 8, 16, 24, and 48 hours	
S. gairdneri	Oil (IMOL S-140) (0.9 mg/l; 127 d)	ASAT	Elevated	Effect observed at 16 and 127 days	Lockhart et al., 1975
		LDH	Elevated	Effect observed at 16 and 127 days	
S. gairdneri	Sewage treatment plant effluent (in situ)	ASAT	Elevated	Correlation existed between proximity to the outfall and serum levels	Wieser and Hinterleitner, 1980
		ALAT	Elevated	Correlation existed between proximity to the outfall and serum levels	

Table 2 Continued:

Organism	Stressor	Enzyme	Activity	Comment	Reference
----------	----------	Fish Continued	----------	----------	----------
S. gairdneri	CCl_4 (1.0 mg/kg; ip)	ASAT	Elevated	Effect observed at 2, 4, 8, and 72 h	Statham et al, 1978
		ALAT	Elevated	Effect observed at 2, 4, 8, and 72 h	
	CCl_4 (80 mg/l; 337 h)	ASAT	No effect	No effect at 2, 4, 8, or 336 h	
		ALAT	No effect	No effect at 2, 4, 8, or 336 h	
S. gairdneri	CCl_4 (1.33 mg/kg; ip)	ASAT	Elevated	Effect observed at 6, 12, 18, and 24 h	Racicot et al, 1975
		ALAT	Elevated	Effect observed at 6, 12, 18, and 24 h	
		LDH	Elevated	Effect observed at 6, 12, 18, and 24 h	
	Aeromonas infection	ASAT	Elevated	Correlation between severity and serum levels	
		ALAT	Elevated	Correlation between severity and serum levels	
		LDH	Elevated	Correlation between severity and serum levels, LDH showed best relationship	
S. gairdneri	CCl_4 (2.0 mg/kg; ip)	ALAT	Elevated	Effect observed at 3, 6, 12, 18, and 24 h. Normal at 36 h	Gingerich and Weber, 1979
	Monochlorobenzene (2.6 mg/l; 15 d)	ALAT	Elevated	Normal after 30 days, water exposure	

Certain serum enzymes, by virtue of their elevated concentrations in a specific tissue, are indicative of cellular damage or dysfunction in that tissue (Table 2). Acid phosphatase (ACP) is present at elevated concentrations in the mammalian prostate. Elevated serum activities in males is a specific indicator of prostate cancer (Sullivan et al. 1942). Elevated serum activities of the transaminases, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) are diagnostic of heart and/or liver dysfunction. Elevated serum N-acetyl-β-D-glucosaminidase (NAG) activities (Ackerman et al. 1981) and altered serum isozyme patterns (Tucker et al. 1980) are indicative of a variety of diseases in humans. In fish, ASAT, ALAT, and lactate dehydrogenase (LDH) serum activities have been used to determine the effects of a number of xenobiotics (Table 2).

Concentrations of ASAT, ALAT, LDH, NAG, and LDH in serum were determined in my work to complement histology, the visual detection of tissue injury. ASAT, ALAT, and LDH may be indicative of damage to one or several organs while the lysosomal enzymes, ACP and NAG, are found at similar levels in many organs and therefore may be useful as indicators of a whole body response to a toxicant. A similar approach has been suggested for leucyl aminopeptidase (Bouck, 1980). Finally, if lysosomal membranes are destabilized within the body, leakage of lysosomal hydrolases from the cell may result. Serum lysosomal enzyme measurements may enable the researcher to determine the extent of this leakage as a non-specific indicator of tissue damage. In addition, research into isozyme patterns in blood and tissues may enable one to determine specific organs which are affected.

Histopathology

Histopathology is the microscopic study of tissues with the intention of understanding the effects of a disease or toxicant on the tissue. Histopathology is potentially useful in identifying the site of toxic action of a xenobiotic, and in estimating the amount of damage produced by the toxicant. In mammals, histopathology has been useful in identifying the kidney and specifically the proximal tubular epithelium as the site of cadmium's chronic toxicity (Axelsson et al. 1968; Itokawa et al. 1974). This information allowed the development of non-invasive techniques for the diagnosis and monitoring of chronic cadmium poisoning (Lauwerys et al. 1974).

Histopathological assessment of xenobiotic exposed fish has been used primarily following acute exposure regimens (Gardner and Yevich, 1970; Sangalang and O'Halloran, 1973; Kendall, 1977; Hawkins et al. 1980; Stromberg et al. 1983). In a chronic exposure of the rainbow trout to sublethal concentrations of lead, no histopathological lesions were observed in the organs studied (Sippel et al. 1983).

Model Toxicants

Two model toxicants, cadmium (Cd) and carbon tetrachloride (CCl_4) were selected to investigate the utility of these measures of toxicant stress to aquatic toxicologists. CCl_4 was selected because it is a liver toxicant, which causes a well known pathology and alterations in serum enzyme activities. Cd was chosen because it is an important water pollutant, toxic to fish, easily measured, and its site and mode of toxic action are not known in fish.

Cadmium

Cadmium is a group IIB transition element of atomic weight 112.4 capable of losing 2 electrons in a chemical reaction (Sienko and Plane, 1966). Cadmium occurs naturally in zinc bearing ores and is present at an average concentration of 0.2 µg Cd/g in igneous rocks (Peterson and Alloway, 1979). Cadmium is used in a variety of industrial processes, but its entry into the environment occurs largely from pigment, smelting, mining, battery, and electroplating industries (Aylett, 1979).

Cadmium is highly toxic to aquatic organisms (Biesinger and Christensen, 1972; Giesy et al. 1977). The toxicity is dependent on a constituent of water hardness (Pickering and Henderson, 1966; McCarty et al. 1978) probably calcium (Carroll et al. 1979; Wright and Frain, 1981a). For the fathead minnow (Pimephales promelas), the acute 96 h LC_{50} values were 0.63 mg Cd/l in soft (20 mg/l as $CaCO_3$) and 72.6 mg Cd/l in hard (360 mg/l as $CaCO_3$) water (Pickering and Henderson, 1966). The 96 h LC_{50} values for the goldfish (Carassius auratus) were 2.13 mg Cd/l in soft water (20 mg/l as $CaCO_3$) and 46.8 mg Cd/l in hard water (140 mg/l as $CaCO_3$) (McCarty et al. 1978).

Cadmium adversely affects reproductive success during chronic life cycle exposures. In the bluegill sunfish, 239 µg Cd/l reduced embryo survival while 80 µg Cd/l reduced larval growth and survival (water hardness, 200 mg/l as $CaCO_3$) (Eaton, 1974). Egg production was reduced at 57 µg Cd/l while 110 µg Cd/l caused elevated larval mortality in fathead minnows (water hardness, 200 mg/l as $CaCO_3$) (Pickering and Gast, 1972). In the flagfish (Jordanella floridae), spawnings per female and egg production per female were the most sensitive measures

of cadmium exposure. These parameters were affected by 8.1 µg Cd/l at a water hardness of 44 mg/l as $CaCO_3$ (Spehar, 1976). In contrast, survival and growth were demonstrated to be the most sensitive manifestations of toxicity in the brook trout (Salvelinus fontinalis) exposed to cadmium in a multigeneration study (Benoit et al. 1976).

The biochemical effects of cadmium on fish are not well known. Despite research on enzyme activities in a variety of organs (Jackim et al. 1970; Christensen et al. 1977; MacInnes et al. 1977; Dawson et al. 1977; Roberts et al. 1979) and other biomolecules within fish (Schreck and Lorz, 1978; Gill and Pant, 1983) a good biochemical measure of cadmium toxicity has not been developed.

Carbon Tetrachloride

Carbon tetrachloride was chosen as a model toxicant for this study because it is a relatively specific hepatic toxicant in mammals. It causes a suite of well studied histological and biochemical effects on coldwater fish and mammals. It was utilized during methods development to validate the procedures, and generate information necessary to formulate a hypothesis concerning lysosomal membrane alterations during xenobiotic exposure.

Carbon tetrachloride is a widely used organic solvent of molecular weight 153.8. Its toxicity depends on free radical formation ($CCl_3^{\cdot}$) by metabolic enzymes which result in lipid peroxidation and membrane alterations (Rechnagel and Glende, 1973). Carbon tetrachloride is not generally considered an important aquatic toxicant, however, it has been studied extensively because it is a good model toxicant for hepatotoxicity and there exists a need to develop procedures which are diagnostic of tissue dysfunction.

The use of CCl_4 as a model toxicant in aquatic toxicology is predominantly based on its toxicity following intraperitoneal administration, since it has not been demonstrated to be toxic following water-borne exposure. The failure to achieve adequate tissue CCl_4 concentrations is believed to be the reason for the lack of effects during water-borne CCl_4 exposure in the rainbow trout (Statham et al. 1978).

Intraperitoneal (ip.) injection of undiluted CCl_4 is toxic to fish. The 96 h LD_{50} value for the English sole (Parophrys vetulus) and the rainbow trout were 4.8 (Casillas et al. 1983) and 4.75 ml CCl_4/kg (Gingerich and Weber, 1979) respectively. CCl_4 (3.0 ml/kg, ip.) produced liver and kidney histopathology and elevated serum ASAT and ALAT activities, which were maximal at 24 h in the sole (Casillas et al. 1983). Administration of 1.33 ml CCl_4/kg to the rainbow trout produced maximal increases in serum ASAT, ALAT and LDH activities at 6 to 8 h following exposure (Racicot et al 1975). Plasma bromosulfophthalein clearance was maximally inhibited at 48 h and remained inhibited at 120 h following a 2.0 ml CCl_4/kg dose in the rainbow trout (Gingerich and Weber, 1979).

Experimental Organisms

The bluegill sunfish, Lepomis macrochirus, was chosen as the experimental organism due to its distribution and abundance in the United States, identification by the U.S. EPA as a bioassay organism, importance as a sport fish, and the need for further research on warm water fish.

Objectives

This research was undertaken with the following general objectives:

1) to develop procedures for the lysosomal enzyme release assay (LERA) and serum enzyme activities with the warm water bluegill sunfish,

2) to determine the effects of a number of biotic factors on LERA and serum enzyme activities,

3) to determine the utility of histopathology, serum enzyme activities, and LERA in understanding the toxicity of carbon tetrachloride,

4) to determine the effects of chronic exposure to cadmium in local water on the ecologically relevant parameters of growth and survival,

5) to determine the utility of histopathology, serum enzyme activities, and LERA in understanding the toxicity of cadmium,

6) to correlate the chronic, ecologically relevant effects of cadmium on survival and growth with biochemical and histological effects, so that the future utility of these indices of xenobiotic stress can be assessed, and

7) to determine the relative sensitivities of organismal, histological, and biochemical indicators of chronic cadmium effects.

This dissertation is divided into three Chapters. Chapter 1 reports the methods development and an analysis of some of the factors which may affect the measures of stress and toxicant damage reported in the remainder of the dissertation. Chapter 2 reports the sensitivity of the methodology used to detect xenobiotic exposure. Carbon tetrachloride is used as the model toxicant. Chapter 3 reports the results of several exposures of the bluegill sunfish to cadmium. The first exposure was a 163 day chronic exposure to cadmium. During this exposure, survival and growth were monitored and tissue samples were collected for histopathology. Chapter 3 also reports the effects of

several subchronic cadmium exposures on the LERA and serum enzyme concentrations, and investigates the factors which may cause the observed alterations in lysosomal membrane stability.

several subchronic cadmium exposures on the LERA and serum enzyme concentrations, and investigates the factors which may cause the observed alterations in lysosomal membrane stability.

CHAPTER 1

DEVELOPMENT OF SERUM ENZYME AND LYSOSOMAL MEMBRANE STABILITY ASSAYS AND THE EFFECTS OF FISH SEX, SIZE, AND SEXUAL MATURITY OF THE BLUEGILL SUNFISH ON THE ASSAYS

INTRODUCTION

The development of indicators of toxicant stress in aquatic organisms may:

1) facilitate the determination of the mode of action of some toxicants,

2) aid in shortening long-term toxicity tests, and

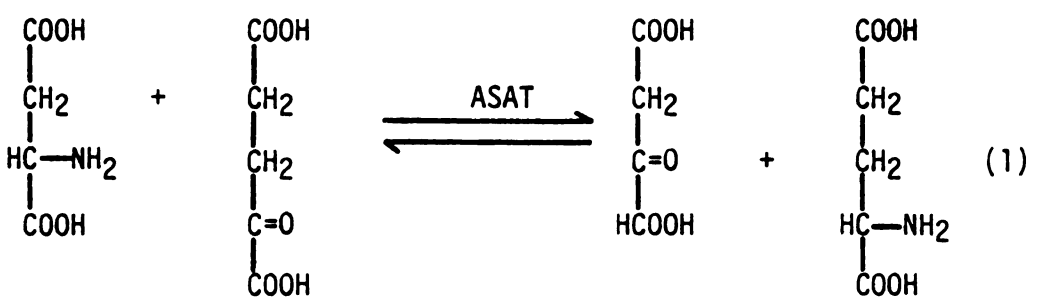
3) improve the extrapolation of data collected from acute toxicity studies to safe concentrations of environmental toxicants.

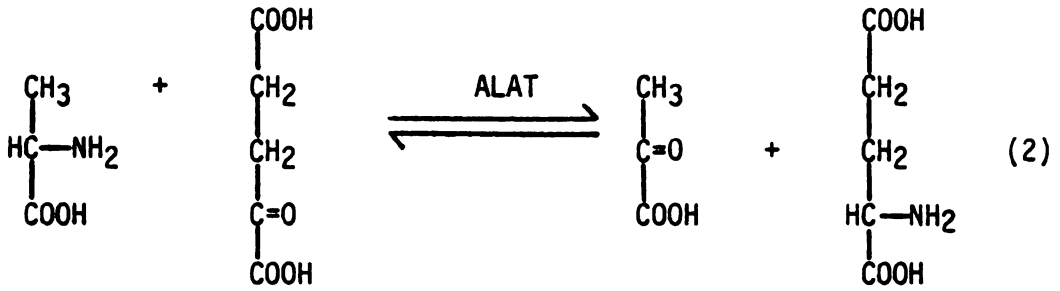
Chronic studies are expensive due to substantial personnel, equipment, and overhead costs. Given the increasingly large number of pollutants to which aquatic organisms are exposed, short-term toxicity tests capable of predicting toxicity in the long-term must, if possible, be developed. Development of these tests will require correlations between the indicators of toxicity and the ecologically relevant parameters such as survival, growth, and reproduction that one is attempting to predict and subsequently preserve. Test development will require properly controlled chronic studies on a variety of compounds in which the appropriate assays are conducted.

I have selected LERA and serum enzyme activities for study to garner information required to develop an environmentally meaningful short-term toxicity test and to better understand fish-toxicant interactions. Even though the LERA has been demonstrated to be useful as a sensitive indicator of xenobiotic exposure in fish (Arillo et al. 1981; Mensi et al. 1982) the relationship between biotic variables such as sex, size, age, water temperature, and season, and the assay, are not well understood.

The serum enzymes, which were investigated in this study, were selected either to provide information concerning the status of lysosomal enzymes (NAG and ACP) and lysosomes in the body, or because of their reported utility in understanding the toxicology of xenobiotics, ASAT, ALAT, LDH (Table 2). The serum enzymes, NAG and ACP, have not received much applicable research attention in mammalian or aquatic toxicology, so the information provided here will be one of the first attempts to understand the biology and toxicological utility of these enzymes.

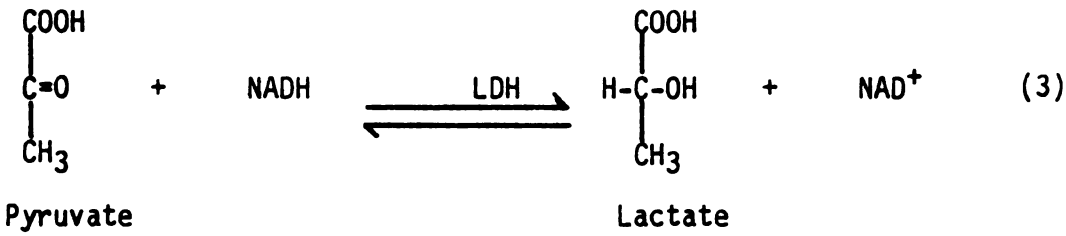
The transaminases, ASAT and ALAT, catalyze the reversible transfer of an alpha amino group from an amino acid to a keto acid (Henry, 1979).





These pathways are important in the biosynthesis and oxidative degradation of amino acids (Lehninger, 1975). ASAT and ALAT occur in the mitochondria and cytosol and their presence at elevated concentrations in serum is indicative of a variety of liver and heart disorders (Henry, 1979).

LDH is located in the cytoplasm of cells in most tissues and catalyzes the reaction:



Serum LDH activity is frequently utilized along with serum transaminase activities to aid in the diagnosis of myocardial infarction. However, serum LDH activities alone have reduced clinical importance in mammals due to the variety of pathological conditions which cause elevated levels (Erickson and Morales, 1961; Henry 1979). Differential serum isozyme patterns, however, can be useful in identifying the affected organ (Henry, 1979).

The development of diagnostic procedures, such as serum enzyme activities, for use in aquatic toxicology require research in two areas, understanding the basic biochemical and physiological systems in these organisms, and understanding the impact of xenobiotics on these systems (Mehrle and Mayer, 1980). Although some information exists concerning the specific organ distribution of ASAT, ALAT, and LDH in trout, little, if any information exists for NAG and ACP in other fish species. In addition, the development of diagnostic tests based on these enzymes requires research on the optimal assay conditions and the effects of biotic factors on serum and tissue levels. This information will increase our understanding of the basic biology of fish and enable interpretation of laboratory and field experiments using these procedures.

The objectives of this study were to develop the lysosomal enzyme release assay, determine the tissue and serum activities of ASAT, ALAT, LDH, NAG, and ACP, and determine the effects of size, sex, and reproductive status on these parameters in the bluegill sunfish (Lepomis macrochirus).

METHODS AND MATERIALS

Experimental Organisms

Adult bluegill sunfish were collected by hook and line from a pond located adjacent to the R.M. Fink Manufacturing Company property, Wiliamston, Michigan. The pond is spring fed except for several effluents from the manufacturing plant. The effluents are composed of well water used to cool equipment within the plant. The fish were maintained in a glass house laboratory with a continuous supply of

freshwater for a minimum of three weeks prior to beginning an experiment. Within one week of capture, hook injuries were healed. The fish were accepting food, and were accustomed to movement in the laboratory. Fish were fed a moist pelleted diet (Bioproducts, Inc.) to satiation and their general condition monitored daily. Tanks were siphoned to remove uneaten food and feces biweekly and scrubbed to remove organisms attached to the walls monthly. Some of the fish had minor trematode infections (blackspot) located primarily on the opercular flap and head. These fish were not treated as the degree of infection would have little or no adverse effect on the fish (Allison et al. 1977).

Exposure Water

Michigan State University tap water was passed through a rust and dirt filter and two charcoal filters to remove large particulates and chlorine. Water was heated to 21 C and delivered to an elevated fiberglass constant head tank where the water was continuously aerated. Water was gravity fed via stainless steel tubing and metering valves into five, three liter glass mixing tanks. Each mixing tank overflowed into a 244 x 61 x 46 cm fiberglass lined exposure tank. Water flow into each tank was maintained at 78 l/hr which gave approximately 3.9 turnovers per day. Water quality parameters were monitored with standard procedures (Table 3) (A.P.H.A., 1976).

Preparation of Serum, Tissues, and the Lysosomal Fraction

Fish were removed from the tanks and bled by cardiac puncture using a 2.5 cm, 22 gauge thin walled needle and three ml untreated

Table 3. Chemical Characteristics of Exposure Water.

Parameter	Concentration
Dissolved Oxygen	> 6.0 mg/l
pH	7.6
Hardness	363 mg $CaCO_3$/l
Alkalinity	322 mg $CaCO_3$/l
Ca^{++}	78.5 mg/l
Na^{+}	15.4 mg/l
K^{+}	2.1 mg/l
Mn^{++}	0.1 mg/l
Fe^{+++}	3.0 mg/l
Mg^{++}	28.9 mg/l
Zn^{++}	0.18 mg/l
Cl^{-}	10.5 mg/l
$SO_4^{=}$	52.8 mg/l
Cl_2^{o}	< 0.03 mg/l

vacutainer. This is the method of choice in sampling blood for the determination of enzyme activities (Gaudet et al. 1975). Care was taken to insure minimal contamination of the blood with muscle cellular fluid since elevated concentrations of certain enzymes are contained in muscle (Oikari et al. 1983). If difficulty was encountered in puncturing the heart and removing blood, the sample was discarded after sampling, since damage to the heart was observed to affect the activities of blood enzymes.

Blood was placed on ice immediately after sampling and allowed to clot. Within three hours, blood was centrifuged at 2000 x gravity (g) for 10 minutes and the serum removed for analyses. All serum enzyme assays were completed within 30 hours.

Following exsanguination, the liver was removed and placed in ice-cold 0.25 M sucrose. During the dissection, care was taken to avoid rupturing the gall bladder, stomach, and intestines. The tissue temperature was maintained near 0^{o} C from the time the livers were removed from the fish until enzyme assays were performed later that day. Livers were weighed (mean weight 0.7 g), minced with scissors, and then homogenized in 10 volumes of 0.25 M sucrose with four strokes of a loose fitting teflon-glass homogenizer. The teflon pestle was rotating at a maximum of 1225 r.p.m. and the total homogenization time was seven seconds.

The lysosomally enriched pellet was prepared by the method of Chvapil et al. (1972). The homogenate was centrifuged at 700 x g for 10 minutes to remove nuclei and unbroken cells. The supernatant was centrifuged at 15,000 x g for 20 minutes. The resulting pellet was washed once with 10 volumes of 0.25 M sucrose and resuspended in 10 volumes of 0.25 M sucrose. The pellet was dispersed by repeatedly

aspirating it through a narrow bore pipette. This suspension contains large quantities of lysosomes and mitochondria (Sawant et al. 1964), however, it has been demonstrated that ACP (Shibko and Tappel, 1963) and NAG (Sellinger et al. 1960; Barrett and Heath, 1977) are located almost exclusively in the lysosomes, therefore, the contamination by mitochondria had little effect on the lability assay.

Osmotic shock was used to determine the status of the lysosomal membrane by procedures similar to those described by Arillo et al. (1981). Following isolation, aliquots of the lysosomal suspension were incubated at nominal sucrose concentrations of 0.25, 0.17, and 0.12 M sucrose at 0°C. After 40 minutes, concentrated sucrose was added to adjust the sucrose concentration to 0.25 M, which was the concentration at which the enzyme assays were performed. A fourth aliquot was treated with 0.1% Triton^{R} X-100[1] to solubilize the lysosomal membrane and enable total activities of the enzymes to be measured. The treated lysosomes were centrifuged at 15,000 x g for 20 minutes and the supernatant collected for enzyme assays. This final supernatant was always clear. The supernatant contained the lysosomal enzymes which had been released to a variable extent by the osmotic treatments or detergent from 0.0294 grams of original tissue per ml.

Stability of lysosomal membranes is determined by comparing the quantity of enzyme released from the lysosomal pool with the total

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[1]Triton is a registered trade mark of the Rohm and Haas Company.

enzyme available in the pool. The labilization index (LI) is:

$$LI\ (\%) = \frac{\text{enzyme activity at a given osmotic shock level}}{\text{total enzyme activity}} \times 100 \quad (4)$$

The LI can then be compared among fish to determine the effects of treatments on lysosomal membrane stability. In fish with destabilized lysosomes, the LI will be greater than normal.

Enzyme Assays

The temperature, pH, and assay duration which yielded maximal activity in the 0.1% Triton X-100 treated bluegill sunfish lysosomal fraction were determined by individually varying one of these parameters. The following procedures are based on the results of this optimization and were used in all subsequent assays.

N-acetyl-β-D-glucosaminidase (NAG) (E.C. 3.2.1.30) activity was measured according to the procedures of Ockerman (1968) with the modifications of Barrett and Heath (1977). The incubation mixture consisted of equal volumes of 8 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co.) in distilled water, 0.3 M citrate buffer, pH 4.8, and the lysosomal enzyme supernatant (final volume 0.3 ml). The mixture was incubated for 60 minutes at 28 C, then stopped with a glycine-sodium hydroxide buffer, pH 10.7. Activity was quantified by measuring the absorbance of p-nitrophenol at 420 nm against reagent blank and comparison with standards. All spectrophotometric analyses were conducted on a Varian model 630, double-beam, UV-Visable spectrophotometer.

The lysosomal acid phosphatase (ACP) (E.C. 3.1.3.2) assay was modified from the procedures of Gianetto and Duve (1955). The

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The lysosomal acid phosphatase (ACP) (E.C. 3.1.3.2) assay was modified from the procedures of Gianetto and Duve (1955). The

incubation mixture consisted of equal volumes of 0.05 M β-glycerophosphate (Sigma Chemical Co.) dissolved in 0.1 M acetate buffer, pH 5.0, and the lysosomal enzyme supernatant (final volume 1.0 ml). The reaction was stopped with 0.5 ml of 10% trichloroacetic acid. After 10 minutes, the mixture was centrifuged at 10,000 x g for 10 minutes and the supernatant removed. The phosphate (PO_4) liberated from the substrate by the enzyme was quantified in the supernatant by the method of Lowry and Lopez (1946). Tissue and reagent blanks were treated similarly. Detergent (Triton X-100) treated samples were diluted to less than 0.01% Triton X-100 to eliminate interferences which were observed at greater concentrations.

Serum aspartate aminotransferase (ASAT) (E.C. 2.6.1.2) and alanine aminotransferase (ALAT) (E.C. 2.6.1.1) were determined by the method of Reitman and Frankel (1957). The substrate consisted of 1.8 mM α-ketoglutarate and 0.2 ml of either DL-aspartate for ASAT or DL-alanine for ALAT in a phosphate buffer, pH 7.5. To start the reaction, 0.1 ml of serum was added to the 0.5 ml of substrate and the reaction was incubated at 28 C for 60 (ASAT) or 30 (ALAT) minutes. The reaction was stopped with 0.5 ml of 0.02% 2,4-dinitrophenylhydrazine. The reaction products were developed with 5 ml of 0.4 N NaOH and the absorbance of the resulting phenylhydrazone was determined at 505 nm. Activity was determined by comparing the absorbance with pyruvate standards.

Serum lactate dehydrogenase (LDH) (E.C. 1.1.1.27) was determined by the kinetic method of Wroblewski and LaDue (1955). Serum (0.05 ml) was incubated with 2.85 ml of 0.07 mg/ml β-NADH (Sigma Chemical Co.) in 0.1 M phosphate buffer, pH 7.4, for 20 minutes at room temperature. Fifty microliters of 0.02 M pyruvate were added and the decrease in

absorbance recorded at 340 nm. Activity quantification was based on the oxidation of NADH during the initial linear portion of the absorbance versus time plot.

Serum acid phosphatase was measured by incubating 0.05 ml of serum with 0.1 ml of 0.02 M p-nitrophenyl phosphate in 0.1 M acetate buffer, pH 5.0 for 30 minutes at 28 C. The reaction was stopped with 2.0 ml of the glycine-hydroxide buffer, pH 10.7, and the absorbance determined at 420 nm. Serum NAG was determined using 0.05 ml of serum in a procedure identical to the determination of the lysosomal enzyme. Throughout this manuscript, I have used the convention that activity expressed on a per gram basis refers to original liver tissue, activity expressed on a per milligram basis refers to protein in the assay, and activity expressed on a per milliliter basis refers to the volume of serum.

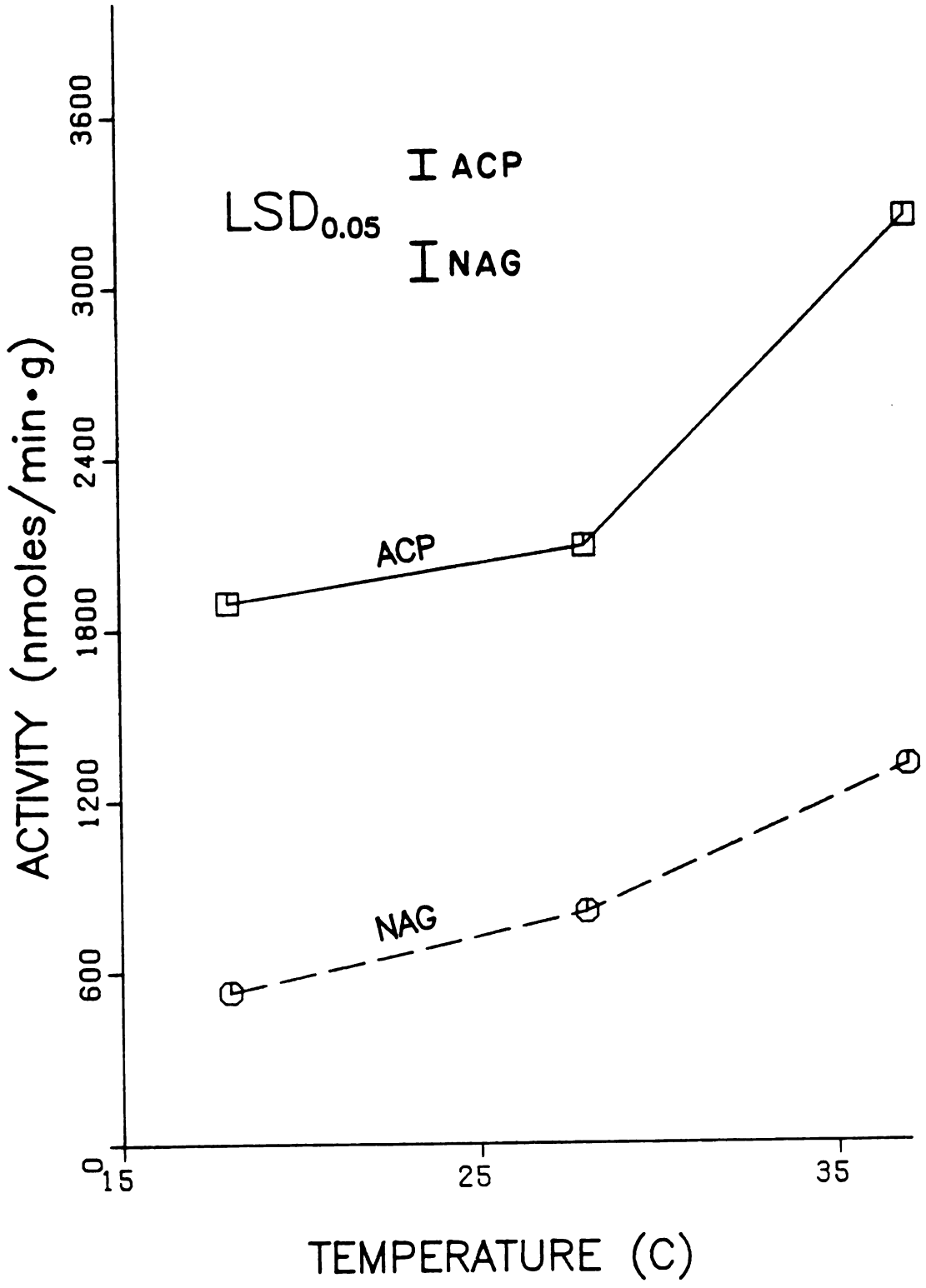
Statistical Analyses

The LI was calculated throughout these experiments according to equation 4. Means were compared with each other using the Student's t-test. Statistical comparisons of tissue enzyme activities were made by the Kruskal-Wallis test (McClave and Dietrich, 1979). This test involves ranking the activities obtained and conducting a one-way analysis of variance and Duncan's multiple range test to determine differences between means (SAS, 1982). The nonparametric procedure was necessary since the assumption of homogeneity of variance among tissues was not met.

RESULTS

Triton X-100 treated lysosomal fractions were assayed for NAG and ACP activity at 18, 28, and 37 C to determine the effects of these temperatures on enzyme activity (Figure 2). NAG and ACP activity were observed at all temperatures, and were positively related to temperature. The greatest increases in activity occurred between 28 and 37 C for both enzymes. NAG activity increased 53% with a temperature increase from 18 to 28 C and 63% with an increase to 37 C. For ACP, the activities were 1895.0 and 2092.0 nmoles/min·g at 18 and 28 C and not significantly different. At 37 C, the activity was increased to 3242.0 nmoles/min·g. The incubation temperature of 28 C was selected for all future assays based on four criteria. First, appreciable measurable activity is obtained at this temperature for both enzymes. Second, this temperature is within the normal thermal range for this species. Utilizing an incubation temperature of 28 C will reduce adverse thermal effects on enzyme functionality, not necessarily for these lysosomal enzymes but for other enzymes investigated in these studies. Third, this temperature is readily maintained in the laboratory, as opposed to 18 C. Fourth, the results can be expressed in rates which are biologically realistic for the fish.

Figure 2. Effect of temperature on the activity of ACP and NAG in lysosomally enriched fractions of bluegill sunfish liver. Values represent means, n=2. Multiple range test least significant difference (LSD) for a type I error of 0.05 is presented for ACP and NAG.


ACTIVITY (nmoles/min•g)
0
600
1200
1800
2400
3000
3600
15
25
35
TEMPERATURE (C)
LSD0.05
I ACP
I NAG
ACP
NAG

Temporal changes in enzyme activity were determined to identify the optimal duration for the assays, and to determine if the enzymes would degrade at the incubation temperature. The product produced due to NAG and ACP activity increased linearly with time up to 120 and 80 minutes, respectively (Figure 3). These results indicate that NAG and ACP do not lose activity in the buffers selected at the assay temperature of 28 C. In addition, the results indicate that the substrate concentrations selected did not limit enzyme activity.

Activity versus pH profiles for the two lysosomal enzymes produced distinct activity patterns and optimal pH values. ACP had a broad pH range (pH 4.2 to 5.4) over which it was most active (Figure 4). Above a pH of 5.4, the enzyme activity decreased sharply. The pH selected for ACP assays was 5.0 because this was the pH at which optimal activity was obtained, and small deviations from this pH would have little effect on the activity of the enzyme. The NAG activity-pH profile was biphasic with activity maxima at pH 4.4 and 5.2 (Figure 4). The pH selected for assays was 4.8, because it lies between the 2 pH optima. This will minimize the effects of small shifts in pH optima, which may occur seasonally or following xenobiotic stress.

The labilization index (LI) measures the stability of the lysosomal membrane by determining the quantity of enzyme released from the lysosome over a period of time. In the assay which I developed, this release can be enhanced by incubating the lysosomes in hyposomotic sucrose. The lysosomal membrane lability, as measured by the proportion of total enzyme activity released, is directly proportional to osmolarity of the incubation medium. In the bluegill sunfish, 1.4%

Figure 3. Time versus activity plot for the enzymes NAG and ACP from lysosomally enriched fraction of bluegill sunfish liver. Values represent means, n=2. LSD for ACP and NAG are given.

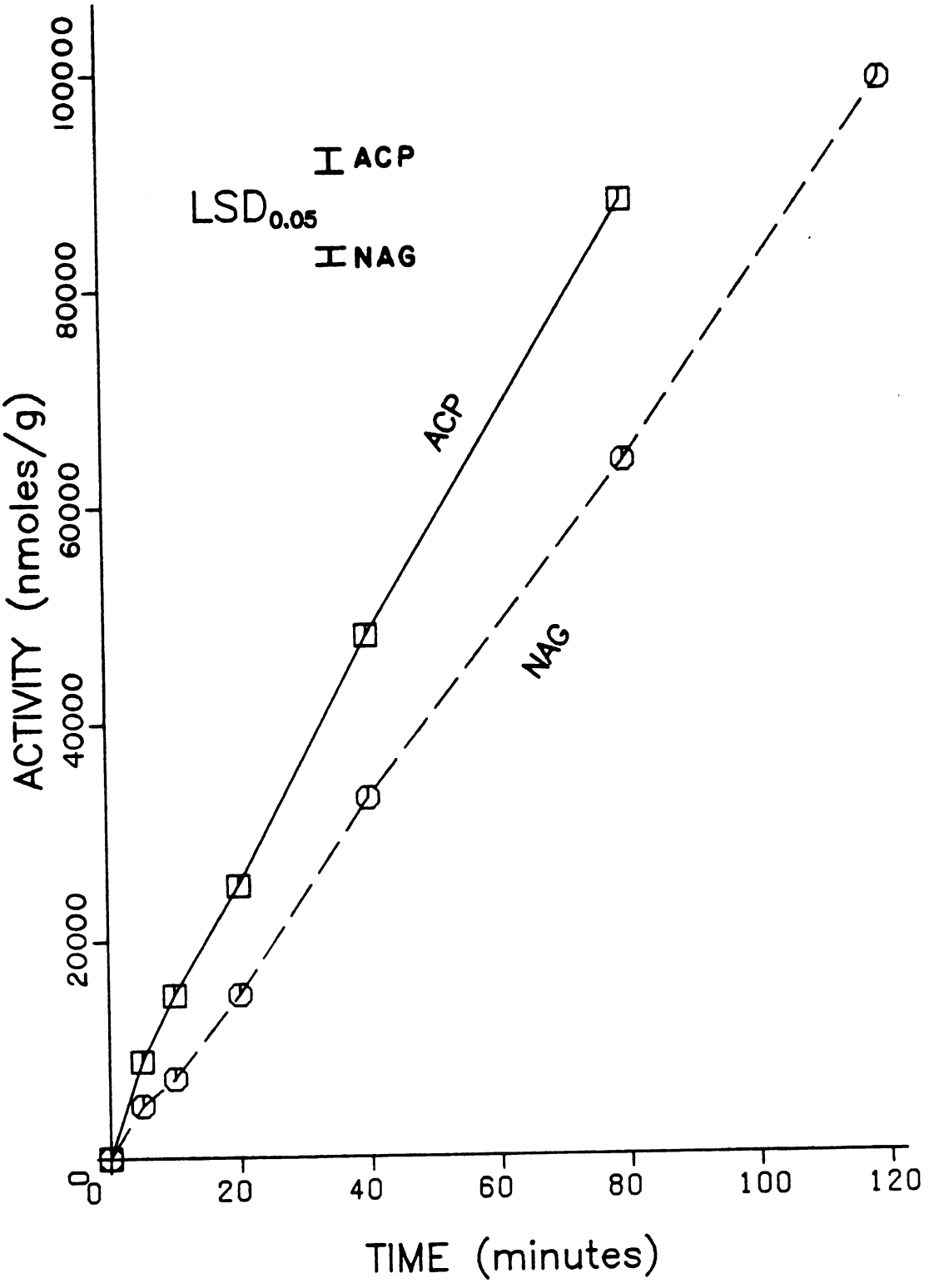
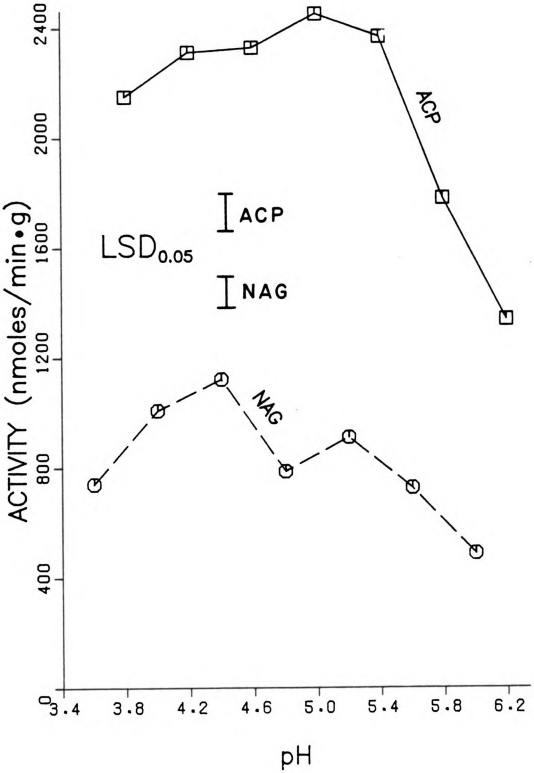

ACTIVITY (nmoles/g)
TIME (minutes)
LSD0.05
ACP
NAG
ACP
NAG
0
20000
40000
60000
80000
100000
0
20
40
60
80
100
120

Figure 4. Activity versus pH plots for the enzymes NAG and ACP from lysosomally enriched fractions of bluegill sunfish liver. Values represent means, n=2. LSD for ACP and NAG are given.


ACTIVITY (nmoles/min•g)
0
400
800
1200
1600
2000
2400
3.4
3.8
4.2
4.6
5.0
5.4
5.8
6.2
pH
ACP
NAG
LSD0.05
ACP
NAG

of the available ACP is released into the surrounding media during a 45 minute incubation at 0 C in 0.25 M sucrose. At 0.17 and 0.12 M sucrose 6.3% and 19.4% of the available ACP is released, respectively (Figure 5). All species investigated demonstrated a graded release of lysosomal enzyme inversely related to the osmolarity of the incubation media. For ACP, lake trout (Salmo salar) and rainbow trout lysosomes were more sensitive to hyposomolar sucrose than were bluegill sunfish or rat liver lysosomes, which had similar LI for both enzymes. For NAG, LI were similar among fish species (Figure 2).

The effects of sex, sexual maturity, and size on the lysosomal enzyme release assay (LERA) and serum enzyme activities were studied to understand and control variability in the toxicological studies which follow. To increase the number of fish which could be studied, only one osmotic incubation concentration, 0.17 M sucrose, was selected for study. The mean liver lysosomal LI and enzyme activity for all fish studied were 13.2% and 930.3 nmoles/(min·g) respectively for NAG (Table 4). The mean liver lysosomal LI and enzyme activity for ACP were 10.3% and 1203.0 nmoles/(min·g) respectively. Neither the LI nor enzyme activities for ACP and NAG were significantly different between males and females, however, total ACP activity was 34% greater in males than females (Table 4).

The gonad somatic index (GSI) was correlated against the LI and enzyme activity for NAG and ACP to determine if a relationship between sexual maturity and LERA existed. The GSI for males and females ranged from 0.11 to 11.8%, respectively. GSI were measured for 24 fish for which LERA was also determined. No correlation between sexual maturity

Figure 5. A comparison of the labilization indices (%) for ACP and NAG at three sucrose osmolarities for △ rainbow trout, ○ lake trout, ✡ freshwater clam, □ rat, and + bluegill sunfish.

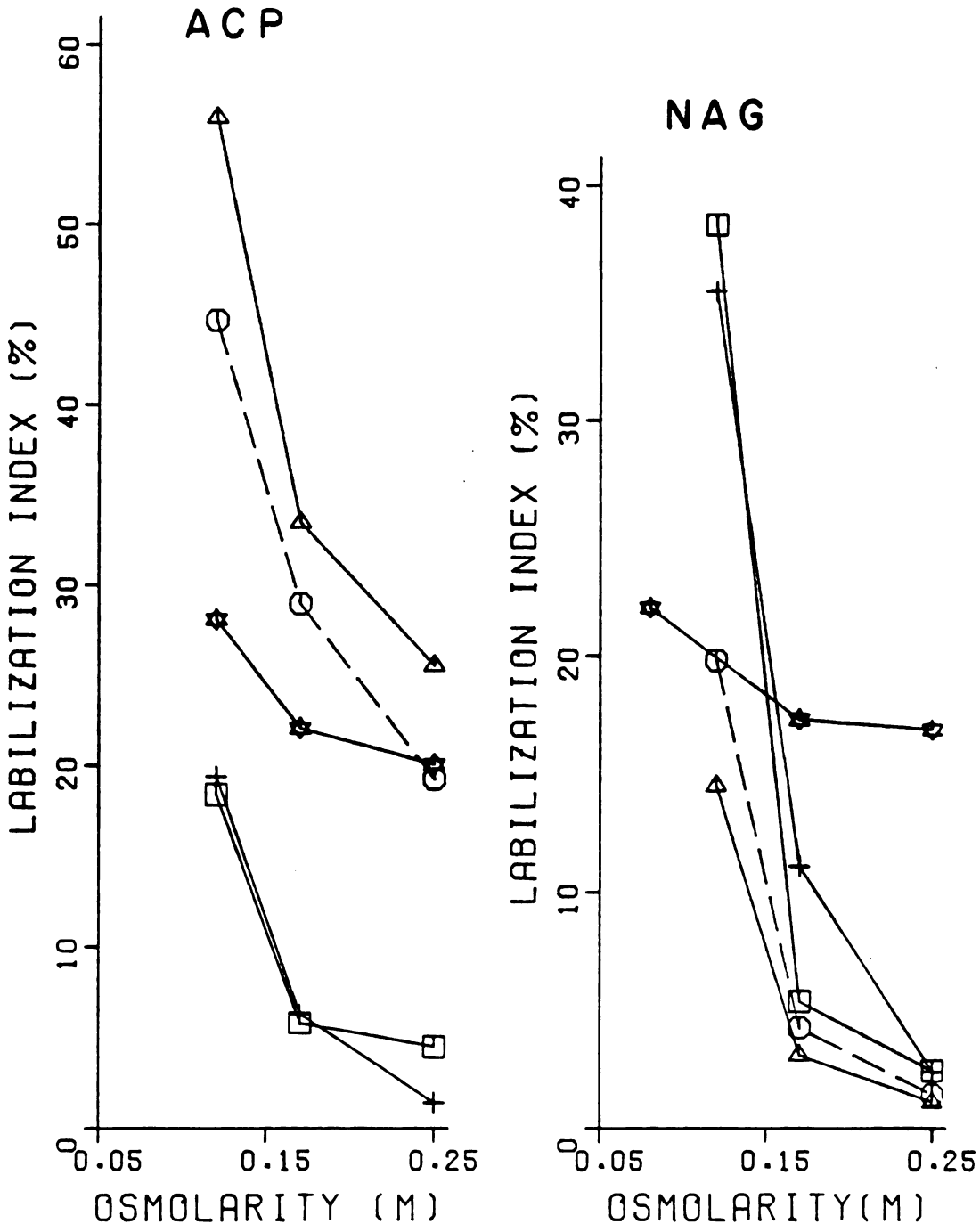

ACP
LABILIZATION INDEX (%)
0
10
20
30
40
50
60
0.05
0.15
0.25
OSMOLARITY (M)
NAG
LABILIZATION INDEX (%)
0
10
20
30
40
0.05
0.15
0.25
OSMOLARITY(M)

Table 4. Comparison of mean liver lysosomal enzyme activities and labilization indices (LI, %) for male and female bluegill sunfish. $\bar{X}$ (SD). n below. Total enzyme activities are reported as nmoles/(min·g, wet wt.).

	All Fish	Males	Females
LI			
NAG	13.2 (3.98) 42	13.4 (2.95) 19	13.1 (4.66) 23
ACP	10.3 (4.68) 42	9.75 (2.76) 16	10.7 (3.51) 23
Activity			
NAG	930.3 (411.90) 43	937.8 (335.1) 20	965.4 (449.04) 23
ACP	1203.0 (518.97) 33	1388.4 (557.01) 16	1028.6 (425.81) 23

of 10 males and 14 females and the LI or enzyme activity was observed (Table 5).

Fish size was negatively correlated with LI ($P < 0.01$) for ACP (Table 5). In subsequent experiments, this variability was minimized by constraining the variability in fish size. Neither the LI of NAG nor NAG and ACP activities in isolated lysosomal fractions were significantly correlated with fish size.

With the exception of LDH, mean serum enzyme activities (NAG, ACP, ASAT, ALAT) were always greater in males than in females, however, the differences were not statistically significant (Table 6). Serum NAG ($P < 0.05$) activities were inversely correlated with sexual maturity in male fish (Table 6). ACP, LDH, and ALAT were not correlated with sexual maturity in males. None of the enzymes were significantly correlated with sexual maturity in females (Table 7).

Serum NAG activity in all fish was inversely correlated ($P < 0.01$) but ACP, LDH, ASAT, and ALAT were not significantly correlated with size (Table 7).

Tissue activities of NAG, LDH, ASAT, and ALAT were not significantly different between the sexes. ACP activity, however, was significantly (ANOVA, $P < 0.001$) greater over all tissues. There was no significant difference within tissues due to sex, however, in every organ, except the stomach, ACP activity was 9 to 30% greater in the male.

Activities of the lysosomal enzymes differed among tissues. The intestine, spleen, and liver exhibited the greatest NAG activity while ACP was greatest in the spleen. Spleen concentrations of ACP were approximately twice the activity of other tissues (Table 8). LDH was greatest in dorsal muscle and heart tissue which had activities

Table 5. Correlations of liver lysosomal enzyme activities and labilization indices (LI) versus sexual maturity (GSI) and size. r(n). Pearson's product-moment correlation coefficient (r) is presented.

	Sexual Maturity[a]		
	Males	Females	Size
LI			
NAG	-0.55 (10)	0.43 (14)	-0.18 (42)
ACP	0.18 (10)	-0.43 (6)	-0.40 (42)**
Activity			
NAG	0.27 (10)	-0.28 (14)	0.03 (43)
ACP	-0.14 (10)	0.02 (8)	0.15 (33)

[a]Significant correlation between the variables, **P < 0.01.

Table 6. Comparison of serum enzyme activities for male and female bluegill sunfish. $\bar{X}$ (SD), n below. Total enzyme activities are reported as nmoles/(min·g, wet wt.).

	All Fish	Males	Females
NAG	9.43 (4.39) 41	8.81 (3.42) 26	10.6 (5.58) 15
ACP	37.3 (13.93) 41	35.3 (10.80) 27	41.4 (18.39) 14
LDH	317.3 (222.29) 36	320.2 (228.9) 24	311.5 (218.29) 12
ASAT	33.5 (20.88) 37	32.7 (21.10) 24	34.8 (21.24) 13
ALAT	20.1 (20.75) 37	16.9 (18.68) 23	25.4 (23.52) 14

Table 7. Correlations of serum enzyme activities versus sexual maturity (GSI) and size. r (n). Pearson's product-moment correlation coefficient (r) is presented.

	Sexual Maturity[a]		
	Males	Females	Size
NAG	-0.68 (25)***	-0.14 (18)	-0.47 (41)**
ACP	-0.21 (24)	-0.14 (17)	0.06 (41)
LDH	-0.31 (20)	-0.24 (15)	-0.20 (36)
ASAT	-0.50 (24)*	-0.20 (16)	-0.23 (37)
ALAT	-0.34 (23)	-0.32 (17)	-0.05 (37)

[a]Significant correlation between the variables, *P < 0.05, **P < 0.01, ***P < 0.001.

approximately ten times greater than the other tissues. LDH activity was lowest in the liver. ASAT activity was greatest in heart tissue, while ALAT activity was greatest in liver tissue. Among the organs, only the liver had greater ASAT activities than ALAT with an ASAT:ALAT ratio of 0.68. Due to the elevated concentrations of ASAT in the heart, the ASAT:ALAT ratio was 14.9, the greatest among the tissues investigated (Table 8).

DISCUSSION

In this study, NAG and ACP activities were measured in the mitochondrial fraction of bluegill sunfish liver homogenates. This fraction is primarily composed of mitochondria and lysosomes (Chvapil et al. 1972). The observation that these enzymes display latency, have acid pH optima, and are associated almost exclusively with lysosomes in other animals (Sellinger et al. 1960; Shibko and Tappel, 1963; Conchie and Hay, 1963) indicates that the source of both NAG and ACP is lysosomal.

Incubation of mitochondrial-lysosomal fractions isolated from a number of different species in hyposmotic concentrations of sucrose causes a graded release of lysosomal enzymes which is inversely proportional to the osmolarity of the media.

Comparing LI profiles among species reveal differences. Clam (Eliptio sp.) hepatopancreas lysosomes are not as sensitive to osmotic shock as the other species tested, which suggests a greater osmotic protection of lysosomes in hepatopancreas of clams relative to analogous tissues in other species (Figure 5). For ACP, profiles for

Table 8. Total activities of NAG, ACP, LDH, ASAT, and ALAT and mean ASAT:ALAT ratios in internal organs of bluegill sunfish. $\bar{X}$, n=7 (SD). Total enzyme activities are reported as nmoles/(min·mg (Protein)).

	Liver	Spleen	Heart	Stomach	Kidney	Intestine	Dorsal Muscle
NAG	26.3 (12.38)[B]	30.8 (13.24)[B]	17.4 (6.56)[C]	6.37 (1.78)[D]	17.8 (5.35)[C]	71.2 (24.38)[A]	2.94 (2.13)[D]
ACP	66.0 (8.22)[C]	122.2 (22.74)[A]	58.5 (4.07)[C]	35.2 (7.01)[D]	86.4 (25.52)[B]	74.8 (10.51)[B]	12.2 (4.62)[E]
LDH	31.8 (21.10)[D]	386.3 (230.44)[C]	2507.6 (1070.3)[B,2]	503.1 (338.04)[C]	525.3 (126.62)[C,2]	572.3 (27.67)[C,2]	7253.3 (3674.04)[A,2]
ASAT	86.2 (14.52)[A,2]	25.5 (5.78)[C,2]	441.9 (365.25)[A]	34.4 (27.00)[C]	54.3 (21.44)[B]	26.8 (11.62)[C]	40.8 (21.18)[B]
ALAT	127.0 (31.14)[A]	22.2 (5.923)[B]	37.0 (20.23)[B]	5.04 (3.07)[C]	23.9 (15.08)[B]	10.4 (2.92)[C]	12.2 (7.60)[C]
ASAT:ALAT	0.75 (0.22)[D,2]	1.17 (0.15)[D,2]	17.76 (14.22)[A]	4.31 (2.36)[B,2]	2.07 (0.65)[C,2]	2.70 (1.05)[B]	4.55 (2.57)[B]

[1]Values with the same letter are not significantly different within enzyme, Kruskal-Wallis test, $P < 0.05$.

[2]n=6.

the bluegill and rat are similar but distinct from several trout species. For NAG, the same relationship exists between osmolarity of the incubation media and LI.

As previously stated, the subcellular tissue fractions utilized in this research contain mitochondria, however, the degree of contamination of the lysosomal enzyme pool by mitochondrial enzymes can be investigated through the property of latency. The percentage of NAG (2.4%) and ACP (1.4%) in the non-latent form at 0.25 M sucrose indicates that the maximum contamination of non-lysosomal enzymes is low (Figure 5). Based on the results of kinetic and inhibition studies, it has been suggested that these non-latent enzymes are also of lysosomal origin, which are released during isolation (Shibko and Tappel, 1963). The treatments of lysosomes which have been shown to labilize lysosomal membranes include incubation in hyposomotic media, high speed blending, sonication, heat (37 C), detergent, low pH, freeze-thawing, and incubation with a number of compounds in vitro (Berthet and Duve, 1951; Gianetto and Duve, 1955; Applemans et al. 1955; Applemans and Duve, 1955; Wattiaux and Duve, 1956; Verity and Reith, 1967; Ignarro et al. 1973; Moore et al. 1978b).

Lysosomes function cellularly in catabolizing organelles and macromolecules. ACP cleaves a phosphate molecule from a variety of substrates (Barrett and Heath, 1977). ACP was the first enzyme to be demonstrated to be associated with the lysosome. Initially, ACP was believed to be a mitochondrial enzyme (Berthet and Duve, 1951), however, the property of latency and its association with other latent acid hydrolases led to the theory of a separate particle (Applemans et al. 1955). Both β-glycerophosphate and p-nitrophenol are hydrolyzed by ACP in rat liver, although lysosomal activity of the p-nitrophenol is

30 to 70% greater. The pH optima (5.2 to 5.8) were similar for the two substrates (Shibko and Tappel, 1963).

NAG cleaves the glucose-amine bond and is important in the hydrolytic degradation of chitin, glycoproteins, mucopolysaccharides, and glycolipids (Robinson and Stirling, 1968). In mammals, NAG has been shown to have two to three multiple forms in internal organs (Robinson and Stirling, 1968; Wetmore and Verpoorte, 1972) and approximately five forms separable by DEAE-cellulose chromatography in body fluids (Tucker et al. 1980). The two major peaks obtained from tissues, peaks A and B, contain the majority of the NAG activity and have almost identical amino acid composition (Wetmore and Verpoorte, 1972). Form A is less stable to heat and with pH treatment appears to be converted to form B with the removal of sialic acid residues by neuramidase (Robinson and Stirling, 1968; Tucker et al. 1980). Both forms display similar pH-activity profiles and have two pH optima at 4.0 to 4.2 and 4.6 to 4.8.

Enzyme activity profiles from synovial and seminal fluid demonstrated 4 forms of NAG when chromatographed on DEAE-cellulose. Those forms are: A^S, a more rapidly eluting form of NAG-A, I_1 and I_2 intermediate forms, and B. During pathologic states in the human, the relative amounts of the intermediate and NAG-B forms of the enzyme recovered in serum and urine are increased (Tucker et al. 1980).

The relative activities of NAG in porcine tissue were reported as kidney > spleen > liver (Findlay and Levvy, 1960). Normal serum activities of NAG and ACP in the human are reported as 9.3 nmoles/(min·ml) and 3.0 nmoles/(min·ml) respectively (Cabezas-Delmare et al. 1983). In the bluegill sunfish, spleen and liver NAG activities

were approximately equal and had approximately twice the activity of the kidney. Lower activities in kidneys of fish, relative to that of human, may be due to the functional differences in the kidneys of these two species.

This study is one of the first to relate lysosomal LI, lysosomal enzyme activity, and serum enzyme activities to sex, sexual maturity, and size in fish. Hence, no definitive explanations can be given for the inverse relationship noted between size and LI, and size and serum activity (Tables 4 and 5). However, RNA concentrations and RNA:DNA ratios are elevated in smaller, more rapidly growing fish (Bulow, 1970). This suggests that lysosomal labilization may be greater in smaller fish, especially in the liver, due to the need to generate the necessary biomolecules for growth. The facts that strong correlations between LI and size were not observed and that this correlation was not observed for all lysosomal enzymes, indicates that reasonable care in the design of experiments will enable the researcher to avoid the complications that these factors might present.

The lability of lysosomal membranes increased in the liver, spleen, and kidney of female lake trout during sexual maturation (Sidorov et al. 1980). These effects were attributed to either a general stress response, the need to mobilize stored metabolites for incorporation into the eggs, or the effects of starvation. I did not observe an effect of sexual maturity on lysosomal membrane stability. This may have been due to the fact that none of the fish examined were fully mature sexually, the lack of a heightened stress level, or the lack of starvation. This analysis of the effect of sexual maturity on the lability of lysosomal enzymes indicates that small differences in gonadal maturation among individuals will not affect the LERA.

Additional research will have to be conducted to determine the effects of full sexual maturity on lysosomal membrane lability in the bluegill sunfish.

The transaminases and LDH were selected for study due to their cytosolic location and their usefulness in the diagnosis of tissue damage. Elevated serum transaminase and/or LDH activity is thought to indicate disruption of the plasma membrane and subsequent cytosolic enzyme leakage (Chenery et al. 1981). However, elevated cellular production of these enzymes has also been suggested as a causative process (Pappas et al. 1984). Elevated serum lysosomal enzymes may result from increased lysosomal-cell membrane interaction due to either increased pinocytosis or exocytosis or to cell necrosis and release of cellular contents. I have determined the activities of several enzymes located in the cytosol and lysosomes of the bluegill sunfish to enable the interpretation of elevated serum enzyme activities in this species.

The lysosomal enzymes were selected due to their importance in metabolic and pathologic processes and their recent use in toxicological investigations (Sunderman and Horak, 1981). Serum and tissue activities of the lysosomal enzymes have received little attention in fish research, however, the available studies indicate their utility in understanding the effects of detergent, pesticides (Gupta and Dhillon, 1983), and metals (Jackim et al. 1970) on fish.

Unlike the lysosomal enzymes, activities of LDH, ASAT, and ALAT have been determined in the tissues of a number of fish species. Unfortunately, comparison of results among studies is complicated by different tissue extraction procedures, assay methods, and incubation

temperatures. However, some comparisons can be made. D'Appollonia and Anderson (1980) optimized the transaminase procedure of Bergmeyer and Bernt (1974) and obtained serum and liver ASAT:ALAT ratios of 12.0 and 1.2 respectively, in the rainbow trout. In the bluegill sunfish, the ASAT:ALAT ratios were 1.67 in serum and 0.68 in liver. The optimum conditions used by D'Appollonia and Anderson (1980) included: pH 7.2, aspartate 125 mM or alanine 80 mM, and α-ketoglutarate 1.5 mM. The assay conditions used for bluegill sunfish were not optimized, however, they are relatively similar.

Wilson (1973) determined ASAT and ALAT in tissues of the channel catfish (Ictalurus punctatus). Tissue ASAT activities were greatest in heart tissue, followed by liver then kidney tissues. This order is in agreement with that observed in the bluegill sunfish, where ALAT activities were greatest in liver and kidney tissues. ASAT:ALAT ratios were 1.33 in liver, 1.64 in kidney, 7.97 in heart and 2.47 in spleen. Tissue transaminase ratios are potentially useful in the identification of the organ which releases these enzymes into serum. For example, an increase in the serum ASAT:ALAT ratio from 1.5 to over 3.0 would suggest heart damage. Tissues were sonicated in Wilson's study, releasing a portion of the mitochondrially bound transaminases, thus possibly altering the amount and proportion of the enzymes assayed. Bell (1968) measured ASAT activity in sockeye and coho salmon (Oncorhynchus nerka and O. kisutch, respectively). Enzyme activities were similar in the heart, liver, and kidney, and were 10 times as great as those in muscle tissue. In the rainbow trout, ASAT activity was greater in liver than heart tissue, which was approximately equal to that in muscle (Oikari et al. 1983). Rao and Rao (1984) determined

relative specific activities of the transaminases in Tilapia mossambia and observed the greatest ASAT and ALAT activities in muscle and liver, which had ASAT:ALAT ratios of 0.39 and 0.54, respectively.

Transaminase activities of the tissues of bluegill sunfish were different from other fish in four ways: 1) the ASAT activities in heart were greater than those observed for other species, 2) the greater ASAT:ALAT ratio; 3) the great ALAT activity in liver tissue, and 4) the small ASAT:ALAT ratio in liver tissue. The only other fish which has been found to have an ASAT:ALAT ratio in liver tissue less than one is the Tilapia species.

LDH activity in fish occurs at greater concentrations in muscle tissue than in other tissues. Oikari et al. (1983) observed relative LDH activities of muscle > liver > kidney > serum in rainbow trout. This is in contrast to the bluegill sunfish in which dorsal muscle and kidney tissue LDH activities are greater than in liver.

There are significant differences in enzyme activities among species. Some of the differences are due to variations in tissue homogenization and enzyme assay procedures, however, some of the differences are real and indicate large interspecies differences. Until procedures are standardized, or unequivocal results are obtained in different laboratories working on the same species, interpretation of serum activities following xenobiotic exposure will be complicated.

CONCLUSIONS

Optimal conditions of pH and temperature were identified for NAG and ACP in the liver mitochondrial-lysosomal subcellular fraction from bluegill sunfish. A biochemical/cytochemical procedure (LERA) to determine the effects of hyposmotic sucrose concentrations on lysosomal enzyme release has been developed. I demonstrated lysosomal membrane lability in livers of rat, lake trout, rainbow trout, and bluegill sunfish, and hepatopancreas of clam. In bluegill sunfish, liver lysosomal enzyme activities and labilization indices were not affected by sex or sexual maturity.

The enzymes NAG, ACP, LDH, ASAT, and ALAT were detected in serum and internal organs of bluegill sunfish. Mean serum enzyme activities were not affected by sex, however, sexual maturity and size were correlated with activities of certain serum enzymes. LERA and serum enzyme activities will be used to investigate xenobiotic effects on lysosomal membrane stability (Chapters 2 and 3).

CHAPTER 2

EFFECTS OF CARBON TETRACHLORIDE ON THE HISTOLOGY, SERUM ENZYME ACTIVITIES AND LYSOSOMAL MEMBRANE STABILITY OF THE BLUEGILL SUNFISH

INTRODUCTION

Chemical diagnostic procedures are routinely utilized to detect disease and toxicant damage in mammals (Erickson and Morales, 1961). The degree of organ damage has been correlated with the concentrations of certain serum enzymes (Casillas et al. 1983). Elevated concentrations of serum ASAT and ALAT generally indicate heart or liver damage, while elevated LDH activities are diagnostic of heart, liver and blood disorders (Hsieh and Blumenthal, 1956; Erickson and Morales, 1961). The development of these tools in aquatic toxicology will provide a valuable tool for the determination and quantification of organ damage in the laboratory and field. Serum ASAT and ALAT have been utilized to quantify the effects of temperature (Sauer and Haider, 1977), sewage effluents (Wieser and Hinterleitner, 1980), and a variety of toxicants in fish (Lockhart et al. 1975; Casillas et al. 1983; Rao et al. 1983). Statham et al. (1978) reported that serum ASAT and ALAT activities remain elevated for 24 hours following a single injection of 1.0 ml/kg CCl_4 in rainbow trout. Fat and liver contained the highest concentrations of CCl_4 during an environmental exposure. The half-time

for elimination of the radiolabelled CCl_4 from liver was 39 hours, which indicates that tissue recovery was able to occur despite the presence of CCl_4 in the tissue. The observed decrease in serum enzyme activities following CCl_4 administration indicates rapid tissue recovery. However, impaired hepatocyte function may persist. Gingerich and Weber (1979) have demonstrated that bromosulfothalein (BSP) clearance from rainbow plasma is impaired for 120 hours following a 2.0 mg/kg CCl_4 dose. BSP is removed from the blood by the liver. Thus, BSP clearance is an excellent indicator of cell function since it integrates the hepatocyte functions of BSP uptake, conjugation, and excretion in bile. The lysosomal enzyme release assay (LERA) is another assay which has been developed for use in aquatic organisms. LERA assesses the metabolic status of the cell by investigating the status of the lysosomal membranes. Since it was hoped that the LERA would be a useful indicator of toxicant stress, I decided to use CCl_4 as a model stressor. CCl_4 is a known liver toxicant which has toxic effects on many of the membrane systems of the hepatocyte. In mammals, CCl_4 elicits elevated serum transaminase and LDH activity, and causes histopathological effects in a dose and time dependent manner. For these reasons, CCl_4 was utilized as the stressor. If alterations in histopathology, serum enzyme activity, and LERA were not elicited by CCl_4 further testing with other toxicants would have been contraindicated. Thus the objectives of the studies reported in this chapter were to:

1) determine if LERA, as it has been developed, is sensitive to the effects of a specific liver toxicant,

2) determine if the serum enzymes ASAT, ALAT, LDH, NAG, and ACP are sensitive indicators of CCl_4-induced cell damage in the bluegill sunfish, and

3) provide information on the effects of CCl_4 for longer durations of time than have been previously studied in fish.

METHODS AND MATERIALS

Bluegill sunfish (60-100 grams) were obtained from the field, and housed and acclimated to laboratory conditions as described in Chapter 1. Fish were randomly selected from the acclimation tank and injected intraperitoneally with 2.0 ml/kg carbon tetrachloride (CCl_4) or saline (0.15 M NaCl). Tissue samples were taken at 24, 72, and 168 h as previously described.

Lysosomal membrane integrity was determined as previously described, with the exception that lysosomal ACP was determined with p-nitrophenyl phosphate as the substrate instead of β-glycerophosphate. This procedure is similar to that used for determining ACP in serum, except that 0.1 ml of the liver lysosomal suspension was used in the assay in place of serum. The activities observed when using the two substrates, β-glycerophosphate and p-nitrophenyl phosphate, are different which indicates that the substrates are acted upon by different enzymes. The comparison of the labilization indices measured using these two substrates is valid since these enzymes are used merely as indicators of lysosomal membrane integrity. Although the situation is not clear, some of the same enzymes are active on both

substrates. β-glycerophosphate use was discontinued because several lots were observed to give high blank readings and variable enzyme activities.

Discontinuous slab polyacrylamide gel electrophoresis (Disc-PAGE) was conducted on serum, liver, heart and muscle from control (uninjected) and CCl_4 treated (2.0 ml CCl_4/kg, ip.) bluegill sunfish in an attempt to identify the organ or organs releasing LDH into the serum. The basic procedures of Dietz and Lubrano (1967) were followed. A 7.5% running gel and a 5.5% stacking gel of acrylamide-N,N' methylenebisacrylamide (wt:wt, 37.5:1) were crosslinked with ammonium persulfate and TMED (N,N,N',N'-tetramethylethylenediamine) (Biorad Products). Fresh tissue was diluted appropriately with cold 0.1 N potassium phosphate buffer (pH 7.5) and homogenized in a teflon-glass homogenizer at 4 C. Serum and tissue homogenates were diluted with an equal volume of 20% sucrose and 0.001% bromphenol blue solution and 50 µl of this solution were applied to the stacking gel. Slab gel electrophoresis was run at 60 V and 30 A for 8 h at 7.5 C. Gels were stained for 30 minutes at 25 C with an LDH specific stain as described in Dietz and Lubrano (1967). Non-specific background staining was determined by incubating a replicate gel in the stain solution without lactate.

RESULTS

All of the fish injected with saline or CCl_4 survived the exposure. Internal organs were all intact and had no gross lesions except for the discoloration in the liver of fish treated with CCl_4. One day after

injection with CCl_4, livers were very pale. In some fish only a fringe of the liver was affected. Livers removed three and seven days after CCl_4 injection were still slightly pale in coloration.

Histopathological assessment of liver, heart, and kidney of CCl_4 injected fish revealed significant toxicant related tissue destruction in the liver only. Livers observed one day after injection with CCl_4 exhibited extensive subcapsular coagulative necrosis. In the affected region, cell structure and liver architecture were completely absent, nuclei were pyknotic, and the tissue matrix was acidophilic (Figures 6 and 9). The remainder of the hepatocytes appeared normal in size and location. There was no evidence of extensive fatty accumulation. Two of the eight fish examined after one day of exposure to CCl_4 had moderate degrees of biliary hyperplasia. After three days of CCl_4 exposure, areas of coagulative necrosis were still evident near liver lobule borders, however, the extent of these areas was greatly reduced. Seven days after injection, there was no evidence of liver tissue necrosis. Fish livers assessed three and seven days after CCl_4 injection did not display biliary hyperplasia.

After one day of exposure, the kidneys of CCl_4-injected fish did not display any degenerative changes, although the glomeruli were shrunken and the glomerular capillaries constricted (Figures 7 and 10). No degenerative changes were observed in the heart after CCl_4 injection (Figures 8 and 11).

Carbon tetrachloride exposure produced statistically significant alterations in all serum enzyme activities measured one day after injection. Serum LDH was increased to the greatest extent relative to control of all the serum enzyme activities measured. Serum LDH activity was increased to 1652.0 nmoles/min·ml, 500% over controls

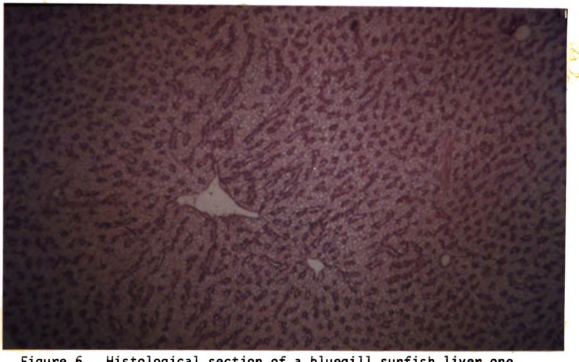


Figure 6. Histological section of a bluegill sunfish liver one day after an intraperitoneal saline (2.0 ml/kg) injection (200x).

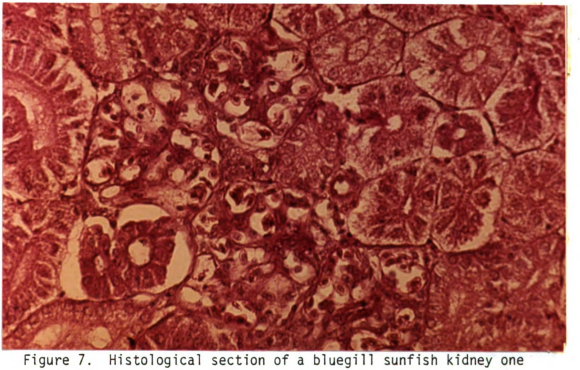


Figure 7. Histological section of a bluegill sunfish kidney one day after an intraperitoneal saline (2.0 ml/kg) injection (500x).

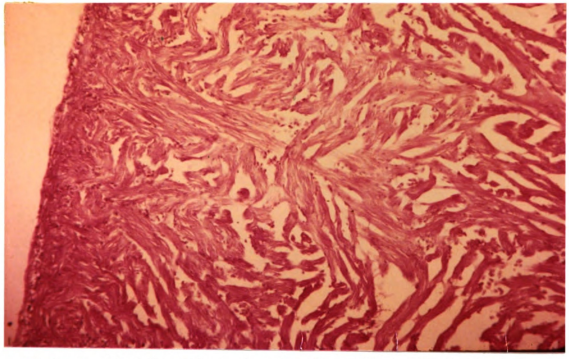


Figure 8. Histological section of a bluegill sunfish heart one day after an intraperitoneal saline (2.0 ml/kg) injection (200x).

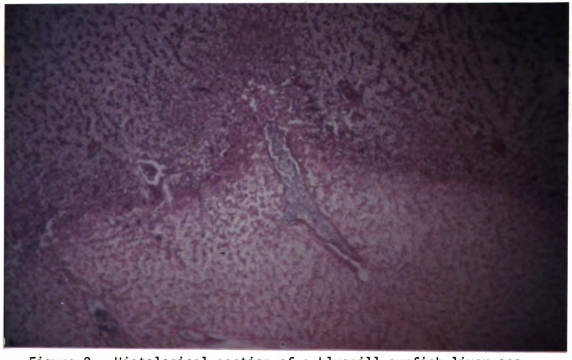


Figure 9. Histological section of a bluegill sunfish liver one day after an intraperitoneal CCl_4 (2.0 ml/kg) injection (200x).

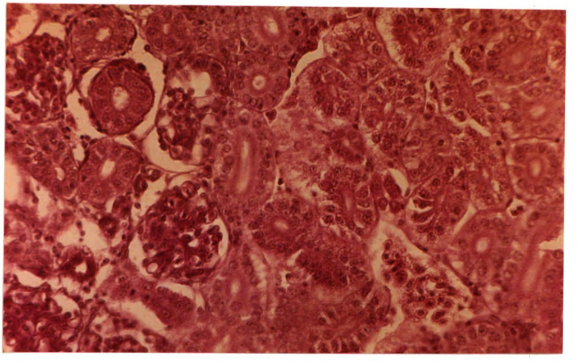


Figure 10. Histological section of a bluegill sunfish kidney one day after an intraperitoneal CCl_4 (2.0 ml/kg) injection (500x).

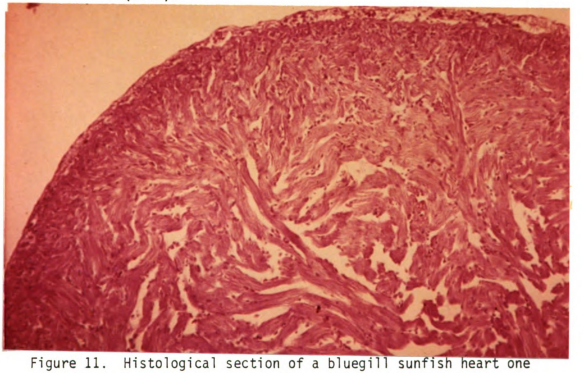


Figure 11. Histological section of a bluegill sunfish heart one day after an intraperitoneal CCl_4 (2.0 ml/kg) injection (200x).

(Figure 12). Three and seven days after injection with CCl_4, serum LDH activities were not significantly different from controls (Figure 9).

Electrophoresis revealed four isozymes of LDH in the untreated bluegill sunfish, two rapidly migrating and two slowly migrating isozymes (Figure 13). Muscle contained approximately equal quantities of the rapidly migrating LDH isozymes while heart and liver contained the two more slowly migrating LDH isozymes. Enzyme concentrations in the liver were lower than in heart. Serum contained all four LDH isozymes. This same pattern of LDH isozymes appeared in bluegill sunfish tissues one day after a 2.0 ml CCl_4/kg dose. The serum from the treated fish contained greater quantities of the LDH isozymes, however, no single organ could be identified as the source of the LDH in serum (Figure 13).

In control animals, the mean ratio of the transaminases, ASAT:ALAT in serum, was 1.7, while one day after CCl_4 injection, the ratio was 3.1, which indicates selective release of ASAT over ALAT from the affected organs. One day after injection with CCl_4, ASAT was increased 280% above control activities to 113.1 nmoles/min·ml (Figure 14). Similarly, ALAT was increased 100% over control levels to an activity of 36.2 nmoles/min·ml. Three and seven days after injection, ASAT in treated fish was not significantly elevated above control activities. ALAT, however, was decreased approximately 40% below control activities three and seven days after exposure. These decreases were not statistically significant (Figure 14).

NAG and ACP activities were determined in serum to ascertain the status of circulating lysosomal enzymes after CCl_4 injection. One day after injection, both NAG and ACP activities were greater than

Figure 12. Effect of CCl_4 exposure (2.0 ml/kg, ip.) on serum LDH activity of the bluegill sunfish. Bars represent mean $\pm$ standard error (S.E.), n=30 control, n=10 treated. ***Means significantly different from control $P < 0.001$ (Student's t-test).

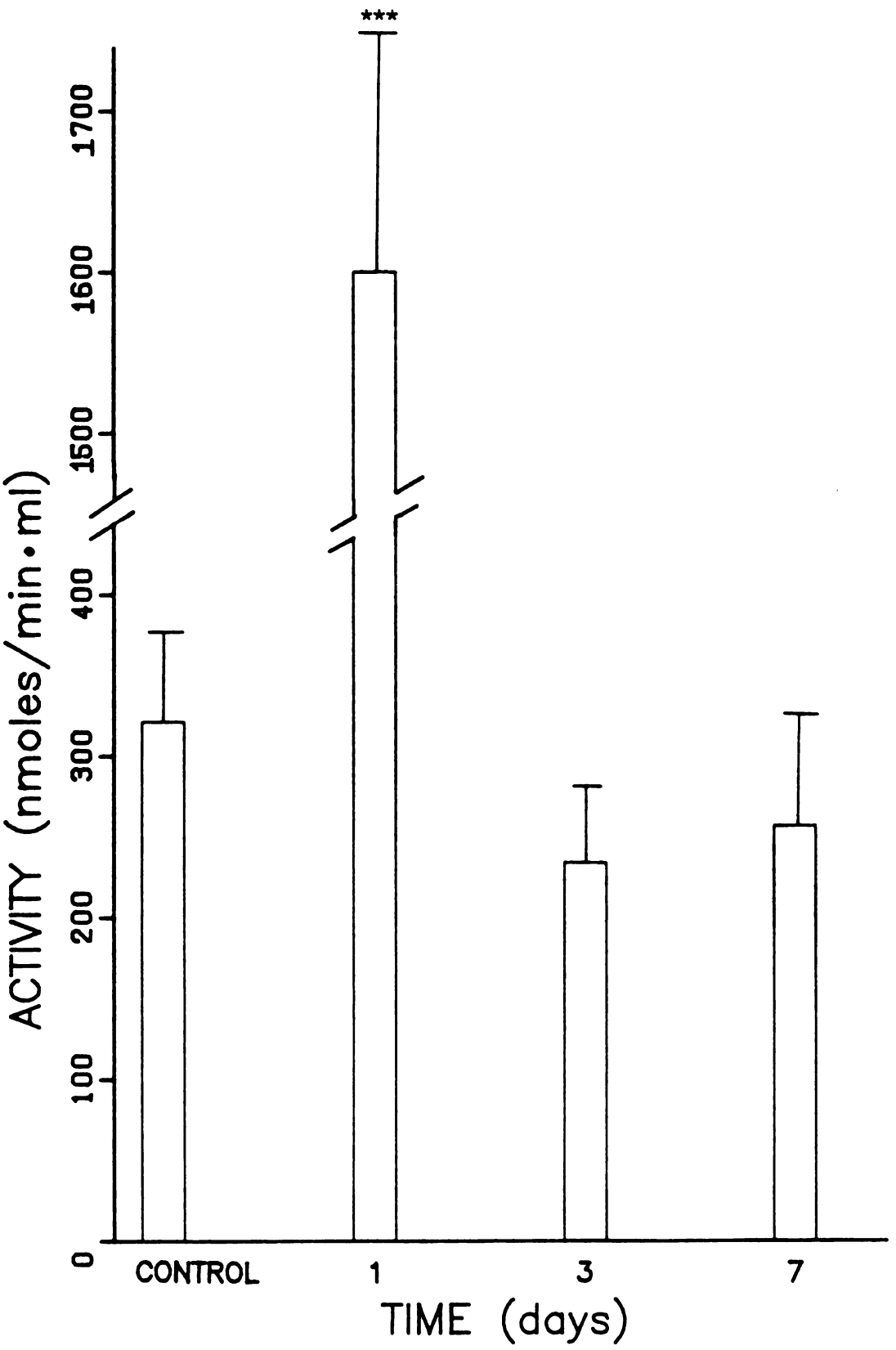

***
ACTIVITY (nmoles/min•ml)
0
100
200
300
400
1500
1600
1700
CONTROL
1
3
7
TIME (days)

Figure 12. Effect of CCl_4 exposure (2.0 ml/kg, ip.) on serum LDH activity of the bluegill sunfish. Bars represent mean $\pm$ standard error (S.E.), n=30 control, n=10 treated. ***Means significantly different from control $P < 0.001$ (Student's t-test).

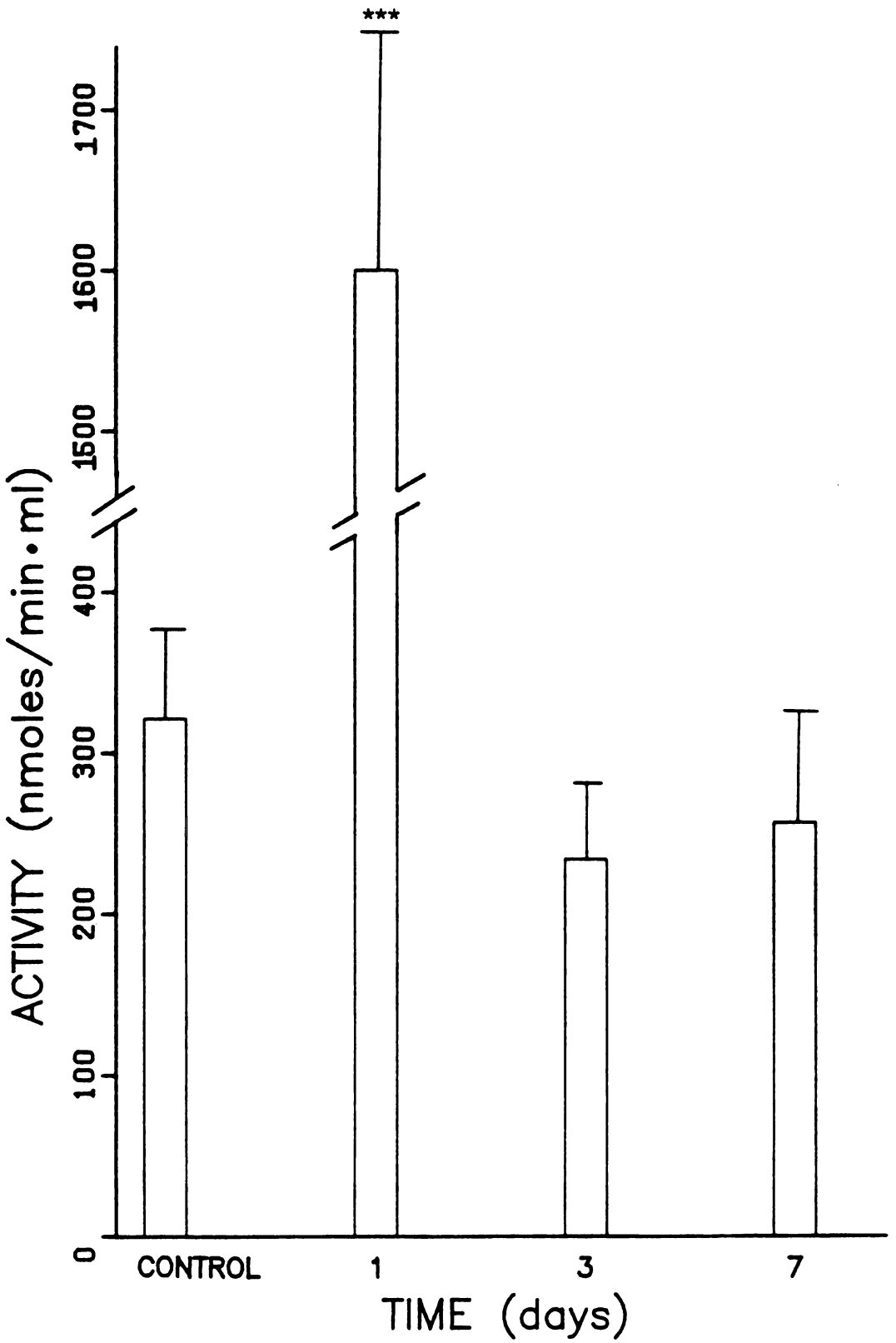
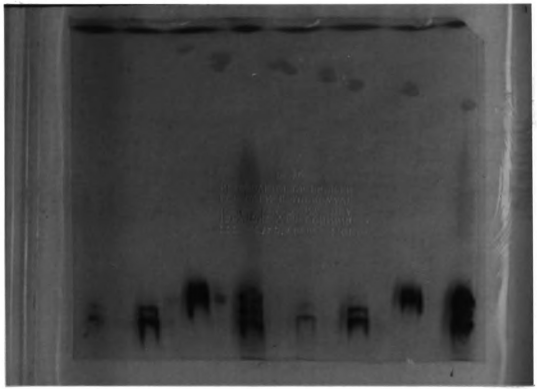

***
ACTIVITY (nmoles/min • ml)
0
100
200
300
400
1500
1600
1700
CONTROL
1
3
7
TIME (days)

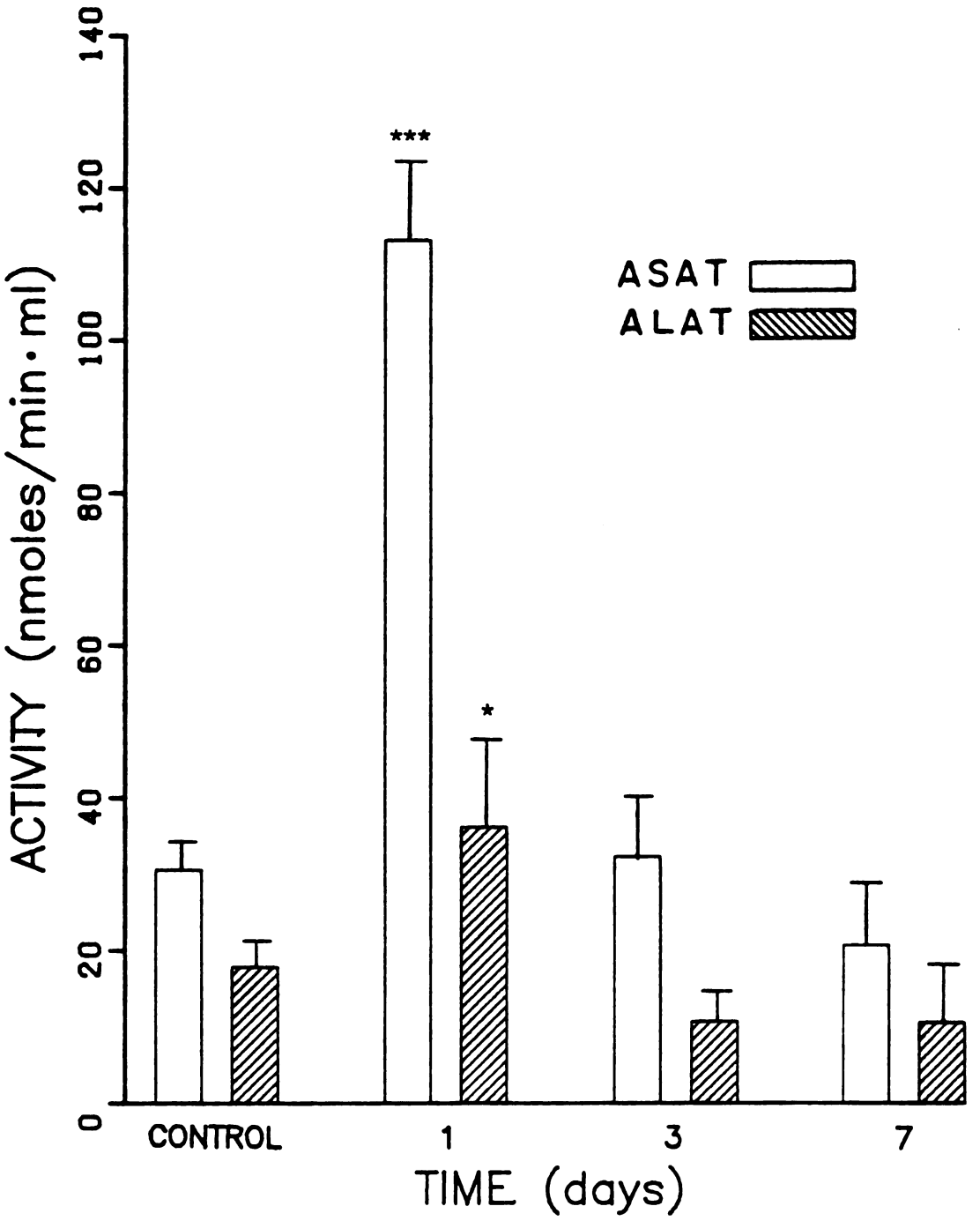
Figure 13. Electrophoretically separated LDH isozymes from the S serum; M muscle; H heart, and L liver of control and CCl_4 intraperitoneally injected (2.0 ml/kg) bluegill sunfish.



L H M S L H M S

CONTROL CCl_4

Figure 14. Effect of CCl_4 exposure (2.0 ml/kg, ip.) on the serum ASAT and ALAT activities of the bluegill sunfish. Bars represent mean $\pm$ S.E., n=30 control, n=10 treated. *Means significantly different from control $P < 0.05$, ***$P < 0.001$ (Student's t-test).


ACTIVITY (nmoles/min·ml)
0
20
40
60
80
100
120
140
***
*
ASAT
ALAT
CONTROL
1
3
7
TIME (days)

controls, which indicates either tissue damage or increased lysosomal involvement in the restructuring of damaged tissue (Figure 15). Three and seven days following CCl_4 injection, serum NAG activity had returned to near control values. Serum ACP activities in treated fish decreased from their peak of 58.6 nmoles/min·ml one day after injection, but remained significantly elevated over control values three and seven days after CCl_4 injection.

The lysosomal enzyme release assay was used to assess lysosomal membrane labilization in bluegill sunfish hepatocytes. The labilization indices (LI) for both NAG and ACP were significantly increased one day after CCl_4 treatment (Figure 16). Three days after injection, the LI for NAG was not significantly different from the control value, while the labilization index for ACP was significantly less than the control. This indicated a stabilization of the lysosomal membrane soon after injection. Seven days after CCl_4 injection, the labilization indices of both NAG and ACP were greater than controls, however, only the increase in ACP LI was statistically significant. Total activities of NAG and ACP in the lysosomal fractions of CCl_4-treated animals were not significantly different from control fish at any time during the exposure.

The coefficient of variation (CV) of the transaminases and LDH are in the 60 to 80% range in control animals. The CV's of the lysosomal enzymes in serum are approximately 45%. The CV's for the LI are approximately 30%. In experimental animals the CV's are always greater, but maintain the same relationship to each other. This is in part due to the variability in the assays, which are based on different techniques but a case can be made that lysosomal enzymes in serum as

Figure 15. Effect of CCl_4 exposure (2.0 ml/kg, ip.) on the serum NAG and ACP activities of the bluegill sunfish. Bars represent mean $\pm$ S.E, n=30 control, n=10 treated. **Means significantly different from control $P < 0.01$, ***$P < 0.001$ (Student's t-test).

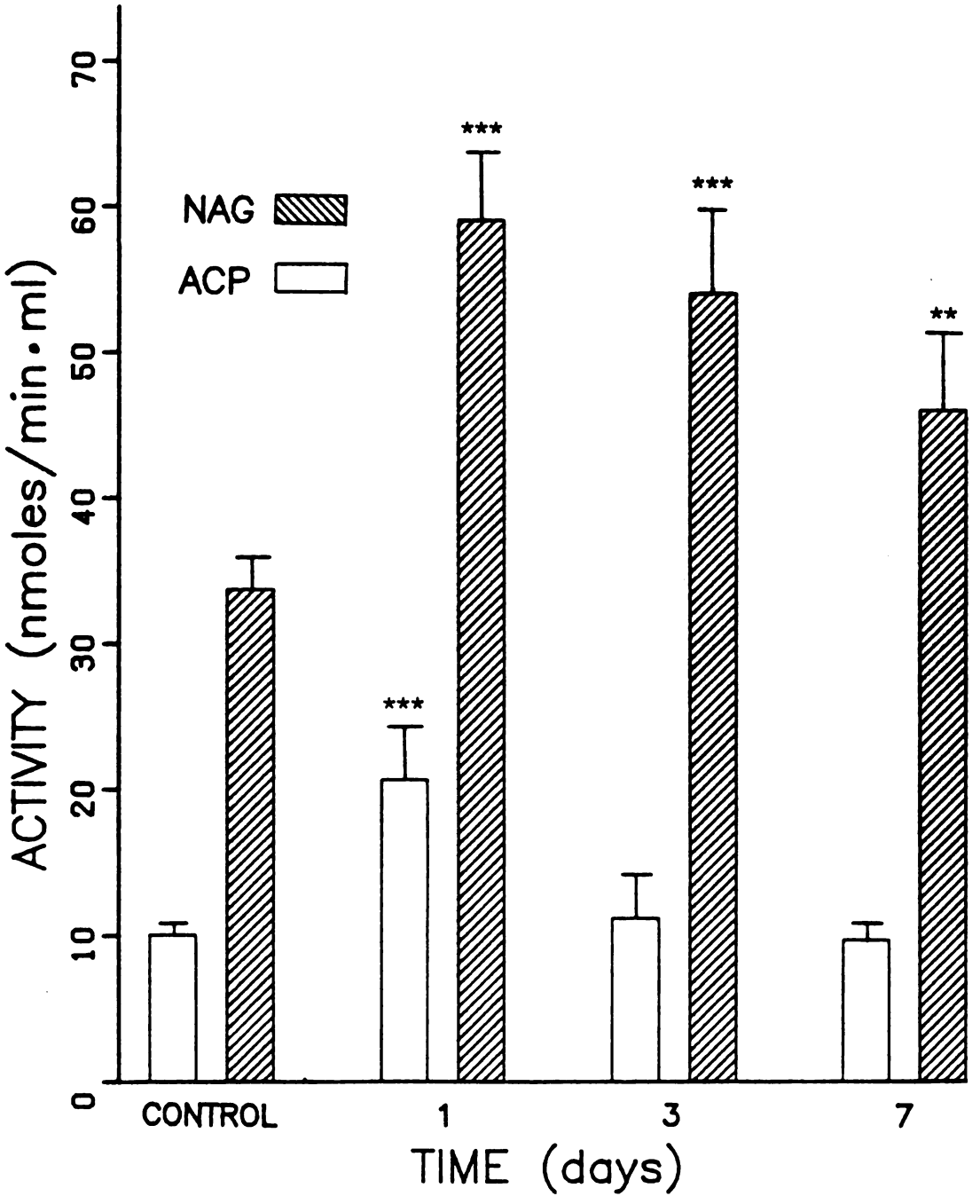
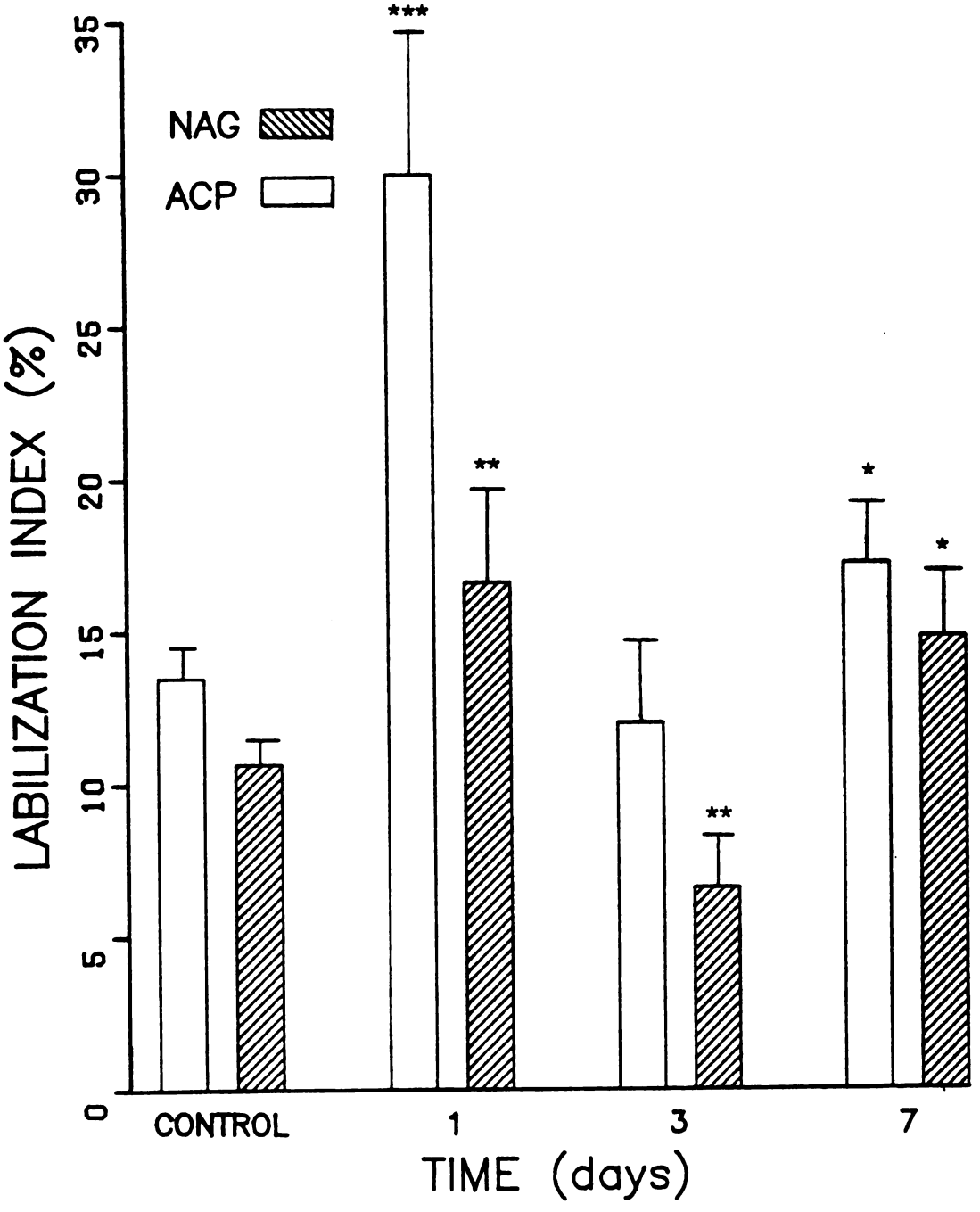

ACTIVITY (nmoles/min • ml)
0
10
20
30
40
50
60
70
NAG
ACP
***
***
***
**
CONTROL
1
3
7
TIME (days)

Figure 16. Effect of CCl_4 exposure (2.0 ml/kg ip.) on the liver lysosomal labilization indices (%) for NAG and ACP of the bluegill sunfish. Bars represent mean $\pm$ S.E., n=30 control, n=10 treated. *Mean significantly different from control $P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's t-test).


LABILIZATION INDEX (%)
0
5
10
15
20
25
30
35
NAG
ACP
***
**
**
*
*
CONTROL
1
3
7
TIME (days)

well as LERA may be better indicators due to their lower natural variability than transaminases.

DISCUSSION

Carbon tetrachloride was used as a model toxicant. Its relative specificity for the liver is due to the necessity for molecular activation (lethal cleavage) by the cytochrome P_{450} monoxygenase system, which occurs at the greatest activity in the liver (Rechnagel and Glende, 1973). The cytochrome P_{450} monoxygenase catalyzed reaction produces the free radical $CCl_3\cdot$ which is believed to initiate lipid peroxidation (Rechnagel and Glende, 1973) or binding to cellular components (Diaz-Gomez et al. 1975). CCl_4 toxicity results in reduced metabolic enzyme activity, inhibition of the cytochrome P_{450} monoxygenase system, increased permeability of the mitochondrial, lysosomal, and cell membrane, and an increase in hepatocyte fat content due primarily to reduced lipoprotein secretory mechanisms (Cornish, 1980).

Recent evidence indicates a dependence of CCl_4 toxicity on extracellular calcium and elevated phospholipid degradation. In isolated rat hepatocytes, CCl_4 toxicity is abolished in the absence of calcium even though CCl_4 binding to cell constituents is not affected. Cell death has been ascribed to a breakdown in cellular calcium regulatory mechanisms and calcium toxicity (Casini and Farber, 1981; Chenery et al. 1981). In freshly isolated hepatocytes, CCl_4 was more toxic in the absence of extracellular calcium (Smith et al. 1981). Lamb et al. (1984) were able to demonstrate the requirement for calcium

in the toxicity of CCl_4 to cultured rat hepotocytes and primary cultures of cells. CCl_4 caused the activation of the calcium-dependent enzyme phospholipase C and a decrease in the activity of sn-glycerol-3-phosphate acyltransferase. These effects increased phospholipid degradation and decreased formation of phosphatidic acid, a key intermediate in phospholipid biosynthesis. Agents which block the $CCl_4^{\cdot}$ dependent increase in phospholipase C activity, reduce the effects of CCl_4 on the functional integrity of the cell.

The disposition, kinetics and effects of CCl_4 on fish, although more heavily influenced by exposure temperature, are expected to be similar to those observed in mammals, due to the well developed monoxygenase systems in fish (Gooch and Matsumura, 1983). Intraperitoneal administration of CCl_4 to rainbow trout results in elevated concentrations in adipose tissue, brain, liver, and spleen. Half-times for elimination are two to three hours in all organs except the liver which has the longest half-life, 38.9 hours (Statham et al. 1978).

The effects of CCl_4 on serum enzymes of the bluegill sunfish were similar to those observed in mammals (Dinman et al. 1962) and other fish species (Racicot et al. 1975; Statham et al. 1978; Casillas et al. 1983). CCl_4 (2.0 ml/kg, ip.) caused dramatic increases in all serum enzymes one day after injection of bluegill sunfish. Three days (72 h) after injection, all serum enzymes measured, except ACP, had returned to normal. CCl_4 treatment (1.0 ml/kg, ip.) of rainbow trout at cooler temperatures resulted in elevated transaminase activities at two to 72 h post injection (Statham et al. 1978). The English sole, exposed to 3.0 ml/kg at 11 C, displayed significantly elevated serum ASAT and ALAT for up to 48 hours (Casillas et al. 1983). Elevated serum transaminase

activities are usually associated with increased tissue leakage of the enzymes, however, elevated tranaminase synthesis also appears to be important (Pappas et al. 1984).

Serum ASAT:ALAT ratios in the bluegill sunfish increased after CCl_4 treatment. The greater increase in serum ASAT than in serum ALAT suggests that either the liver with its ASAT:ALAT ratio of 0.68 was not the only organ leaking these enzymes or that ASAT is released to a greater degree than ALAT from liver. The heart, with a relatively high ASAT content, could provide a significant portion of the serum ASAT. In rainbow trout, serum ASAT:ALAT ratios do not change upon CCl_4 treatment (Racicot et al. 1975; Statham et al. 1978), however, in the English sole, the serum ratio increases from 0.3 to 5.4 upon CCl_4 treatment (Casillos et al. 1983). The involvement of the heart in CCl_4 toxicity in the bluegill sunfish is further indicated by the rise in serum LDH from 321.2 to 1652.0 nmoles/min·ml.

The LDH protein is a tetramer composed of H (heart) and M (muscle) subunits. In general, it has five isozymes in birds and mammals corresponding to the five possible arrangements of the subunits (Bailey and Wilson, 1968). The H_4 (LDH_1) is the most negatively, and the M_4 (LDH_5) the least negatively charged isozyme. Liver LDH is most similar in composition to skeletal muscle LDH (Galen, 1975).

LDH in fish differs in a number of ways even though genetic control is similar to mammals. Generally, fish have between one and five LDH isozymes with the muscle type being more negatively charged and liver LDH being similar to heart type, electrophoretically (Markert and Faulhaber, 1965; Bailey and Wilson, 1968).

Bluegill sunfish were found to have four LDH isozymes. In other organisms a fifth isozyme is sometimes present but difficult to separate (Dietz and Lubrano, 1967). Muscle-type LDH is more negatively charged than heart-type. Liver LDH isozymes are electrophoretically similar to heart LDH isozymes. All LDH isozymes appear in serum. CCl_4 treatment does not cause a preferential increase of any specific LDH isozymes in serum which indicates that the toxicity of CCl_4 is not specific to the liver. Both heart and skeletal muscle LDH isozymes occurred at elevated activities in the blood after CCl_4 injection. IMOL S-140, a tri-aryl phosphate oil produces similar results (Lockhart et al. 1975). Exposed fish had elevated serum ASAT and LDH activities. The elevation in serum LDH was due to release of both muscle and heart-type LDH isozymes.

Another method for differentiating LDH isozymes is based on the different reaction kinetics of the isozymes with pyruvate (Gaudet et al. 1975). Racicot et al. (1975) used the pyruvate saturation test and Michaelis-Menton kinetics (Km) to determine that the increase in rainbow trout serum LDH after CCl_4 exposure was due primarily to liver LDH isozymes. This technique was not used in my experiments because this technique has not given unequivocal results in experiments with rainbow trout nor are the effects of pyruvate concentration on bluegill sunfish isozymes well understood.

Serum NAG and ACP activities were both elevated one day after bluegill sunfish were injected with CCl_4. Three and seven days post injection, NAG was similar to control activities while ACP remained elevated. Lysosomal enzymes are released from macrophages, leukocytes, osteoclasts, and fibroblasts (Ignarro and Columbo, 1973; Davies and

Allison, 1976). Currently it is not known if release is a normal function of healthy, organ related cells (Davies and Allison, 1976), however, comparison of tissue and serum enzyme forms indicates this possibility (Tucker et al. 1980). Additional research is needed to determine if CCl_4 causes elevated serum lysosomal enzyme activities by increasing lysosomal enzyme leakage or altering the function of the lysosome. Although care was taken to reduce the activity of non-lysosomal acid phosphatase in serum, p-nitrophenol is not a specific substrate for lysosomal acid phosphatase (Neil and Horner, 1964). The observation that aldrin exposure causes elevated serum alkaline, acid, and glucose-6-phosphatases suggests caution in ascribing alterations in acid phosphatase in my study to lysosomal effects (Gupta and Dhillon, 1983). The duration of the effects of CCl_4 on ACP however, indicates its potential use in the future as an indicator of toxicant exposure.

Histopathological assessment of CCl_4 exposed fish have confirmed the occurrence of tissue damage. Six hours following injection of rainbow trout with 1.0 ml CCl_4/kg, Statham et al. (1978) observed elevated hepatocyte vacuolation and focal and laminar (subcapsular) necrosis of the liver. In a similar study with rainbow trout, Racicot et al. 1975, observed extensive hepatocyte vacuolation 6 and 12 h after exposure. After 18 h some necrotic areas were observed in the liver, but by 24 h the cell vacuolation was reduced and necrosis was not observed in the organs studied. Exposure of the English sole to 3.0 ml CCl_4/kg by intraperitoneal injection produced a variety of liver and renal histopathological effects. Livers displayed both a central and subcapsular coagulation necrosis, sinusoidal congestion and fatty

[illegible]ltration throughout the 48 h sampling period. Kidneys were not as

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cell degeneration and necrosis with pyknotic nuclei and a degree of glomerular and hematopoietic tissue congestion. These renal effects were also present, but to a reduced extent, 48 h after injection. In fish injected with 0.2 and 1.0 ml CCl_4/kg, ip., fatty infiltration and subcapsular coagulation necrosis were evident to a lesser extent in the liver. A degree of proximal tubule degeneration and necrosis also occurred in the kidney 24 h after injection.

Lysosomes displayed increased membrane lability one and seven days after CCl_4 injection into the bluegill sunfish. Three days after injection, the LI was significantly decreased, which indicates stabilization of the lysosomal membranes. The decreased production of phospholipids and lipid peroxidative destruction of lysosomal membranes caused by CCl_4 may explain the initial decrease in lysosomal membrane stability. Exposure of lysosomes to irradiation results in a similar reduction of membrane stability by a lipid peroxidative mechanism (Wills and Wilkerson, 1966). The free radical oxygen lipid peroxidation mechanism is indicated by the reduction in labilization by vitamin E or an N_2 atmosphere. The formation of lipid peroxidase was also directly related to lysosomal enzyme release.

The stabilization of the lysosomal membrane three days after CCl_4-injection into bluegill sunfish suggests formation of a different pool of lysosomes, perhaps primary lysosomes. If CCl_4 had destroyed a large number of cells or lysosomes, new cells or lysosomes would be produced. The newly formed lysosomes in existing cells or those in newly formed cells, would be primary lysosomes. These lysosomes would have less surface area, more homogeneous membranes and would be less susceptible to osmotic shock than secondary lysosomes. Seven days after CCl_4

injection, the LI for ACP was greater than in controls which indicates continued lysosomal membrane toxicity or continued formation of secondary lysosomes.

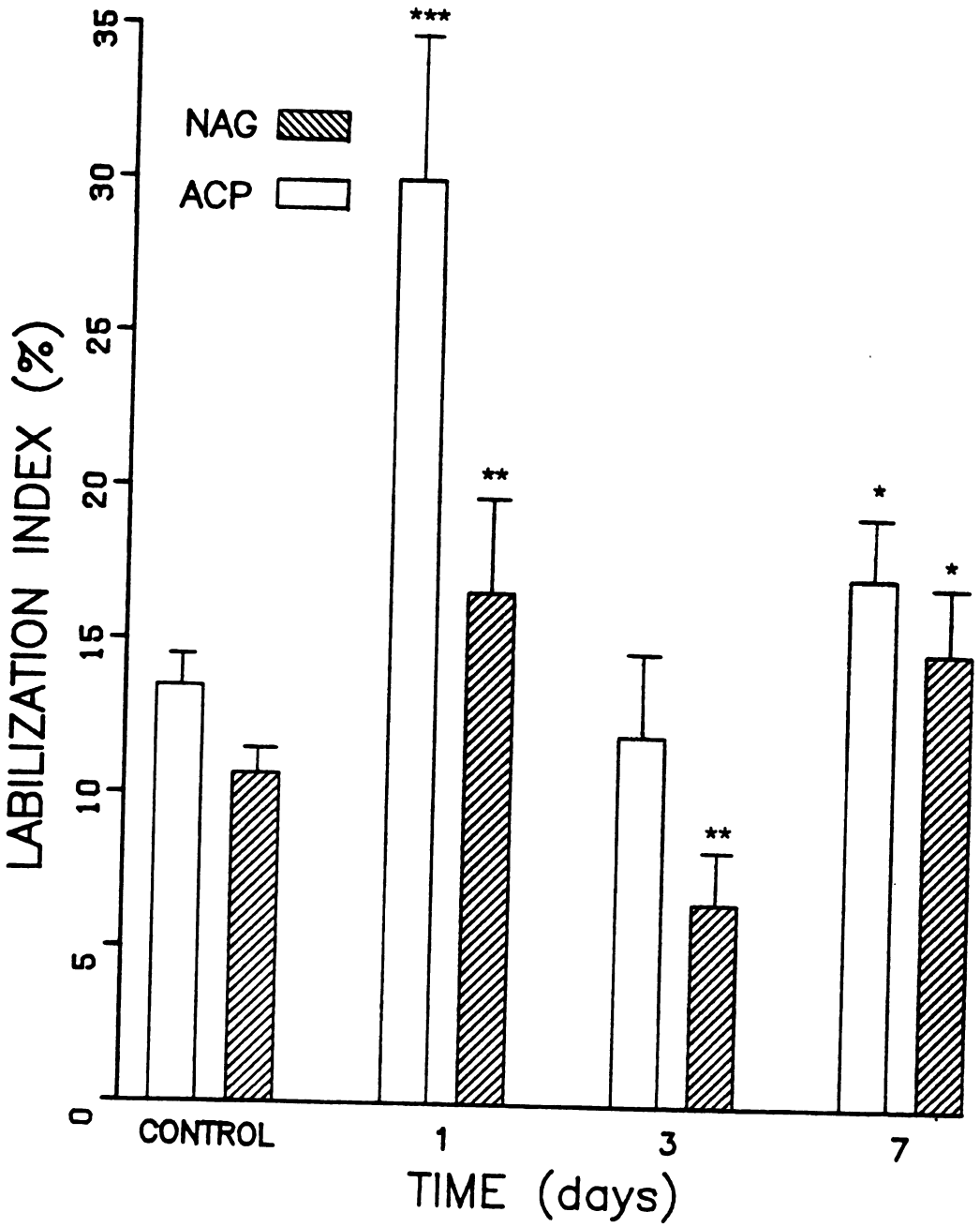
In aquatic organisms, lysosomal enzyme release has been used to describe acute and chronic toxicity. Using a histochemical assay to determine lysosomal membrane stability, Moore et al. (1978) demonstrated a decreased stability 24 h after anthracene injection into a marine mussel. This response of the lysosomal membrane of the mussel appears to be a general response to stressors such as chemical (oil), thermal, nutritional, and salinity, which all induce the same type of response (Bayne et al. 1976; Moore, 1976; Widdows et al. 1982). An alteration in lysosomal membrane stability may also be a general response to stress in fish. I have demonstrated this response in the bluegill sunfish following CCl_4 treatment and Arillo et al. (1981) and Mensi et al. (1982) have demonstrated this response in rainbow trout exposed to ammonia and nitrate respectively for 24 to 48 h.

CONCLUSIONS

Histopathological and biochemical analyses demonstrate significant hepatocyte damage only during the first day following injection of the bluegill sunfish with 2.0 ml CCl_4/kg, ip. CCl_4 exposure caused elevated ASAT, ALAT, and LDH in serum. This indicates their use as specific indicators of organ related toxicity in bluegill sunfish. Evidence from electrophoretic analyses of LDH isozymes in serum and serum ASAT:ALAT ratios indicates that muscle and heart tissue were damaged by CCl_4 during the first day of exposure. CCl_4 exposure resulted in elevated serum NAG and ACP activities. LERA, however, demonstrated

lysosomal membrane destabilization on days one and seven of exposure and membrane stabilization on day three. The successful utilization of these procedures indicates the potential use of histological and biochemical measures of xenobiotic stress in fish.

Figure 16. Effect of CCl_4 exposure (2.0 ml/kg ip.) on the liver lysosomal labilization indices (%) for NAG and ACP of the bluegill sunfish. Bars represent mean $\pm$ S.E., n=30 control, n=10 treated. *Mean significantly different from control $P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's t-test).


LABILIZATION INDEX (%)
0
5
10
15
20
25
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35
NAG
ACP
***
**
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CONTROL
1
3
7
TIME (days)

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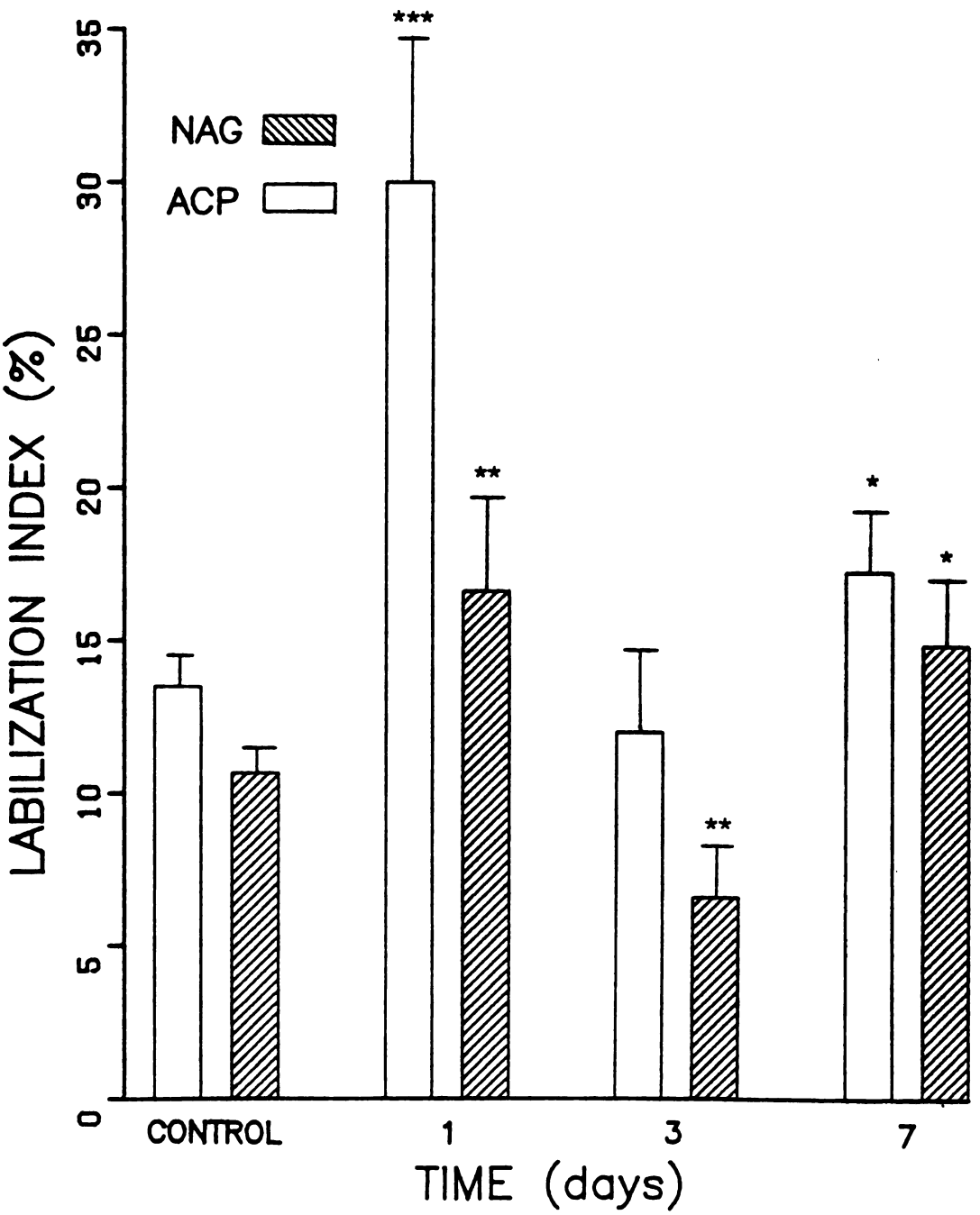
LABILIZATION INDEX (%)
0
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well as LERA may be better indicators due to their lower natural variability than transaminases.

DISCUSSION

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lysosomal membrane destabilization on days one and seven of exposure and membrane stabilization on day three. The successful utilization of these procedures indicates the potential use of histological and biochemical measures of xenobiotic stress in fish.

CHAPTER 3

EFFECTS OF CADMIUM ON THE GROWTH, SURVIVAL, HISTOLOGY, SERUM ENZYME ACTIVITIES AND LYSOSOMAL MEMBRANE STABILITY OF THE BLUEGILL SUNFISH

INTRODUCTION

Carbon tetrachloride, a specific liver toxicant, has been shown to cause biochemical and histological alterations in the liver of bluegill sunfish (Chapter 2). These results indicate that these methods are useful to understand liver toxicity. However, the general utility of these measures of stress needs to be established, especially for LERA. CCl_4 initiates lipid peroxidation, which results in membrane effects, thus, the effect on LERA was expected.

Biochemical and histopathological techniques have been used to some extent to study cadmium (Cd) toxicity. Serum transaminase and LDH activity have been useful as measures of Cd toxicity in mammals (Chapatwala et al. 1982; Flora and Tandon, 1983), however, their use in fish have been unequivocal (Christensen et al. 1977). Increases in serum NAG activity after CCl_4 injection followed a time course similar to the transaminases and LDH, which indicates the potential of NAG as a specific indicator of liver damage. Histopathological studies of the effects of Cd on fish have primarily utilized acute exposures (Sangalang and O'Halloran, 1973; Stromberg et al. 1983), which leaves in doubt the utility of histopathology for understanding chronic exposures.

Xenobiotic-induced alterations at the suborganismal level of organization, ie. biochemical and histological, cannot be considered ecologically relevant unless the alterations observed are correlated with population-level effects. Use of this type of approach may enable short-term indicators of toxicity to be utilized to establish safe concentrations of environmental contaminants.

Therefore, this Chapter reports the results of experiments designed to determine:

1) the chronic effects of Cd on the ecologically relevant parameters growth and survival,
2) the utility of histopathological tissue analyses during chronic Cd exposure,
3) if serum enzyme activities of the enzymes demonstrated to be useful for understanding CCl_4 toxicity are useful for understanding Cd toxicity,
4) if LERA of gill and liver tissue is a potentially useful indicator of subchronic Cd exposure, and finally
5) to gain a better understanding of the effects of stressors on the lysosomal membrane stability in fish.

METHODS AND MATERIALS

Adult bluegill sunfish were obtained and maintained in the laboratory as previously described (Chapter 1). A number of separate exposures were conducted.

Chronic Study

A 163 d chronic exposure of the bluegill sunfish to five concentrations of cadmium was conducted. During the exposure, growth

and survival were monitored, and tissue samples were removed for histopathological examination. In this study, fish were separated into three size classes, then randomly assigned from each of the size classes into each of the five exposure tanks one week prior to initiating the exposure. This procedure resulted in 25 fish in each tank and assured approximately equal size distribution in each treatment. Fish weights and lengths were determined after 78 d and at the conclusion of the experiment (163 d).

Water flow was maintained at 78 liters/hour which yielded 3.8 turnovers per day. Cadmium as $CdCl_2 \cdot 2\ 1/2H_2O$ (Mallincrodt; Baker) was metered into each mixing tank by a multichannel peristaltic pump. Water flow rate was adjusted daily to maintain nominal cadmium concentrations of 0.0 (control), 0.3, 1.0, 3.5, and 12.0 mg Cd/l. The exposure system was housed in a greenhouse to provide a natural photoperiod to stimulate gonadal development. Water temperature was gradually increased from 17 C to 28 C between March 17 and June 1, then maintained at 28 C until the end of the exposure.

On days 14, 35, 64, 91, and 163 d of the chronic exposure, two fish were removed from each of the five exposure tanks and dissected. The gill, eye, liver, stomach, intestine, spleen, and gonads were fixed in Bouin's fixative for at least 48 h. Following routine histological procedures, sections five µm in thickness were stained with hematoxylin and eosin and examined by light microscopy. This exposure allowed comparison of the sensitivity to Cd exposure of toxicity indicators at the tissue and organismal levels of organization. In addition, histologic examination was used to obtain insight into the site of toxic action.

Liver LERA Experiments

Two experiments of nine and 22 d duration, were conducted in which bluegill sunfish were exposed to 12.0 mg Cd/l (nominal) to determine the effect of Cd on the LERA of liver. The procedures used for the exposure have been described above and in Chapter 1.

Gill LERA Experiment

A 16 d exposure of bluegill sunfish to 12.0 mg Cd/l (nominal) was conducted to determine the effects of cadmium on the LERA of gill tissue. Following the exposure, gill arches were removed and placed in cold 0.25 M sucrose. The filaments were cut from the arch and LERA was determined as described previously. This step was taken to reduce the amount of cartilage in the homogenization.

Cd Time Course Experiment

A 32 d exposure of bluegill sunfish to 12.0 mg Cd/l was conducted to determine the effects of Cd on LERA and serum enzyme activities over time. On days 4, 8, 16, and 32 of the exposure, blood and liver samples were removed for biochemical analyses. Serum enzymes analyzed were ASAT, ALAT, LDH, NAG, and ACP. To determine LERA in liver lysosomes, NAG and ACP were used as marker enzymes. The procedures have been previously presented.

<u>In Vitro</u> Cd Exposure

This experiment was undertaken to determine if Cd-induced alterations in the lysosmal enzyme release assay were mediated by a

direct effect on the lysosomal membrane. The lysosomal fraction, centrifugally isolated from untreated fish, was exposed to 0, 10, 100, and 1000 µM Cd for 20 minutes *in vitro*. The exposure was ended by adding 1 mM EDTA to complex Cd and render it nontoxic. The lysosomal fractions were then exposed to osmotic shock and enzyme assays were conducted as described previously.

Physical Stress Experiments

The following experiments were conducted to determine if factors related to the exposure but not a direct effect of Cd toxicity, were affecting the stability of the lysosomal membranes. To determine if the effects of toxicants on lysosomal membrane stability were due to a general stress response, fish were crowded in an exposure tank by lowering the water level to six cm. LERA was conducted as usual on day seven.

Fish exposed to Cd reduced, but did not completely eliminate, their food consumption. To determine if this lack of food affects LERA, fish were not fed for seven days. LERA was then conducted as described.

Experimental Design and Data Analysis

A one-way analysis of variance and subsequent Duncan's multiple range tests were used to determine differences in fish length and weight among treatments. Biochemical parameters were compared to control values by Student's t-test. Type I error of significance is 0.05 unless otherwise stated.

In the liver LERA experiment, lysosomal pellets from six fish per treatment were individually subjected to three sucrose concentrations

or 0.1% Triton X-100. This was the maximum number of assays which could be conducted. The results of this study were subjected to both a power analysis (Kirk, 1968), and a profile analysis (Morrison, 1967). The power analysis enabled me to determine the sample size required in future experiments to demonstrate significant differences between treatment means, based on my initial estimates of the standard deviation for the labilization index or total enzyme activities. The nonparametric profile analysis was used to examine the relationship among osmotic shock treatments and between treatments to determine if the different osmotic concentrations were supplying redundant information. I used Wilk's criterion and Hotellings criterion to examine the profile and main treatment effects, respectively (MANOVA, SAS, 1982).

RESULTS

Chronic Study

The computer model Geochem (Sposito and Mattigod, 1979) was utilized to determine the speciation of Cd in local waters. These simulations indicated that approximately 90 percent of the total Cd was available as the divalent Cd ion, Cd^{2+} (Table 9). The remainder was bound to sulfates, chlorides and hydroxides. Except where stated, the concentrations referred to in this paper refer to the total Cd.

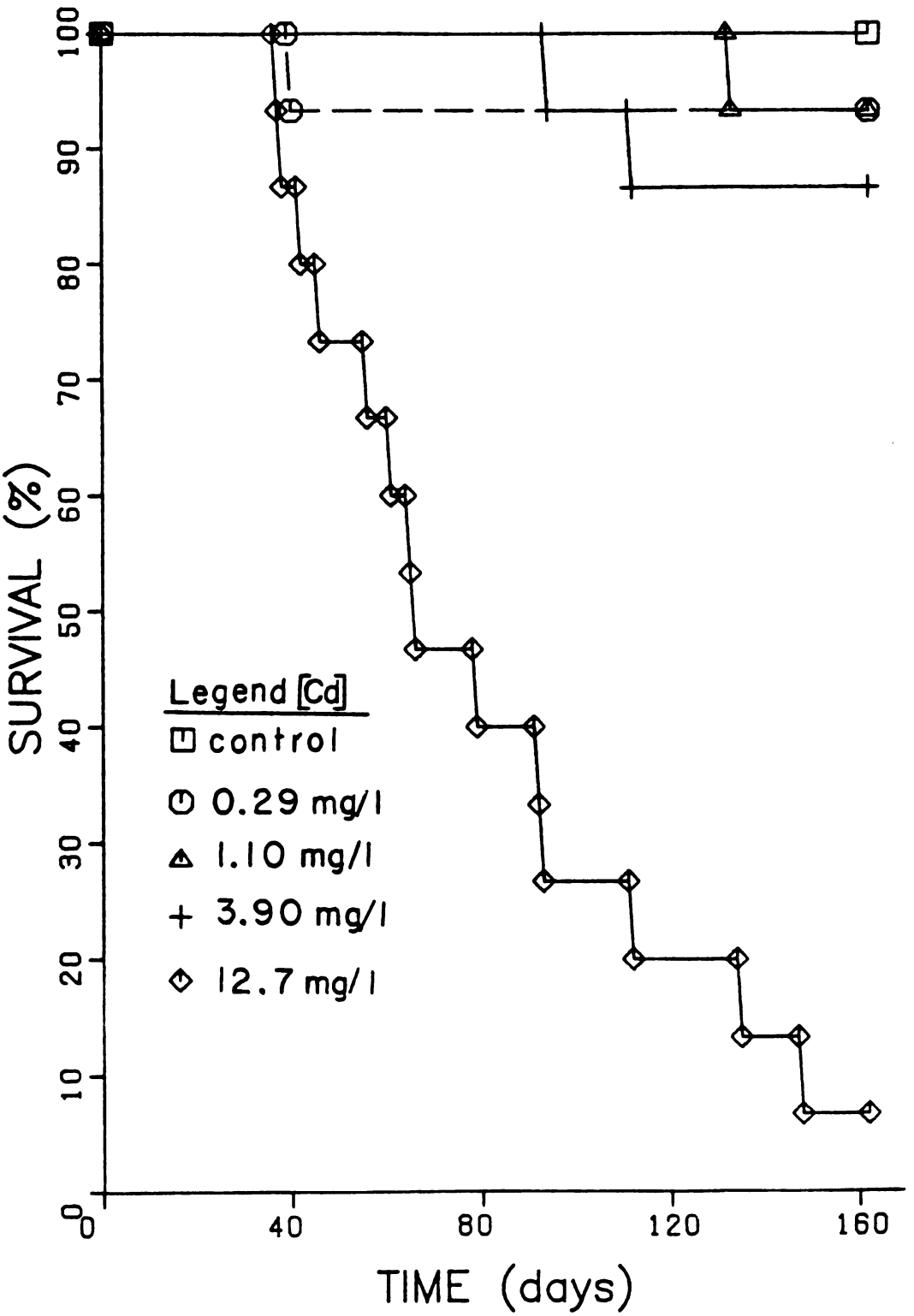
During the chronic exposure, the greatest concentration of Cd tested, 12.7 mg Cd/l, caused significantly greater mortality than the other treatments (Figure 17). The first death occurred after 21 d and the mortalities continued for 144 d. The median lethal time was

Table 9. Speciation of cadmium in test waters as determined by the geochemical simulation model GEOCHEM.

Form	% Total	Stability Constant (log K)
Cd^{2+}	92.7	-
$Cd\ SO_4^{2-}$	5.5	2.5
$Cd\ (SO_4)_s$	< 0.1	-1.6[a]
$Cd\ (SO_4)_2^{6-}$	< 0.1	2.9
$CdCl^+$	1.7	2.0
$CdCl_2$	< 0.2	2.6
$CdOH^+$	0.1	-10.1
$Cd\ (OH)_2$	< 0.1	-20.4
$Cd\ (OH)_{2\ (s)}$	< 0.1	-13.7[a]
$CdCO_3$	< 0.1	4.1
$Cd\ (CO_3)_2^{2-}$	< 0.1	-12.0[a]
$CdHCO_3^+$	< 0.1	12.4

[a]Solubility Product.

Figure 17. Effect of a 163 d exposure to five concentrations of Cd on survival of the bluegill sunfish. Values represent percentage surviving.


SURVIVAL (%)
100
90
80
70
60
50
40
30
20
10
0
0
40
80
120
160
TIME (days)
Legend [Cd]
control
0.29 mg/l
1.10 mg/l
3.90 mg/l
12.7 mg/l

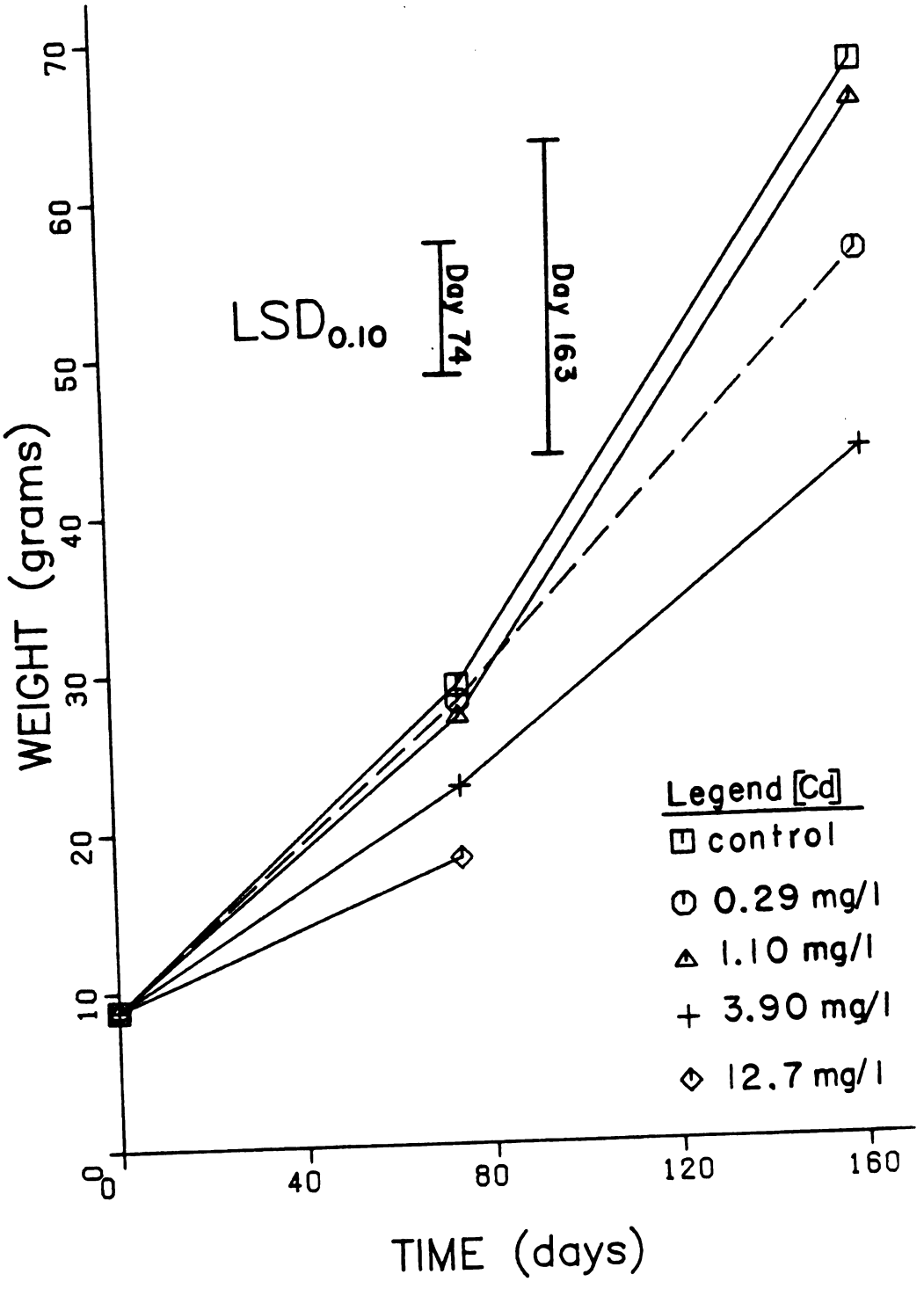
approximately 63 days at 12.7 mg Cd/l. Several days before death, fish stopped feeding and restricted their movements. Eventually the fish lost equilibrium, sank to the bottom and ceased ventilating. Signs of neurologic impairment such as erratic movement or tremors were not observed. Mortalities at Cd concentrations less than 12.7 mg Cd/l total Cd were not significantly greater than that of the control.

Decreased growth in exposed fish was noted after 74 d exposure to 12.7 mg Cd/l (Figure 18). Fish exposed to this concentration were both lighter and shorter than controls ($P < 0.05$). Lengths and weights of fish exposed to 0.29, 1.1 and 3.9 mg Cd/l were not significantly different from controls after 74 d (Figure 18). However, after 163 d, fish exposed to 3.9 mg Cd/l were significantly shorter and lighter than the control fish ($P < 0.10$). Due to the initial within-tank variability and the hierarchical social structures established by the fish, the among-treatment variability was great, which reduced the ability to demonstrate statistically significant differences among treatment means.

Fish exposed to 12.7 mg Cd/l developed opaque swollen corneas and dermal ulcerative lesions. These lesions were typically on the lateral surface posterior to the insertion of the pectoral fin, on the gular plate, and on the branchiostegal rays. These lesions were not observed at any of the other Cd concentrations.

Chronic exposure to Cd caused few discernable histological lesions in the internal tissues examined. The gills of fish exposed to 3.9 mg Cd/l and 12.7 mg Cd/l exhibited epithelial cell hyperplasia and hypertrophy which produced clubbed secondary lamellae (Figures 19 and 20). Clubbing of the gill lamellae was observed throughout the exposure period, however, since dying fish did not display signs of

Figure 18. Effect of a 163 d exposure to five concentrations of Cd on growth of the bluegill sunfish. Values represent the mean weight of surviving fish. LSD is given for 74 and 163 days.


WEIGHT (grams)
TIME (days)
LSD0.10
Day 74
Day 163
Legend [Cd]
control
0.29 mg/l
1.10 mg/l
3.90 mg/l
12.7 mg/l
0
10
20
30
40
50
60
70
0
40
80
120
160

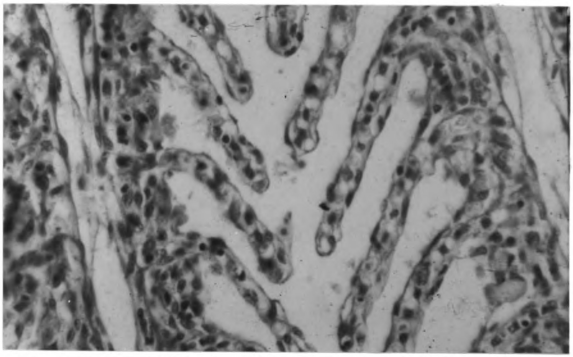


Figure 19. Histological section of a control bluegill sunfish gill (1000x).

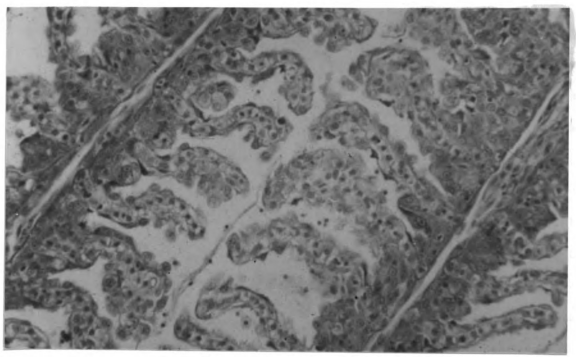


Figure 20. Histological section of a bluegill sunfish gill following 35 d exposure to 3.9 mg Cd/l (800x).

anoxia, gill function was not believed to have been seriously impaired. The spleen of fish exposed to 12.7 mg Cd/l had reduced white cord area on day 14 of exposure. Spleens examined during the remainder of the study appeared normal. The remainder of the organs examined displayed normal histology at all times in all Cd exposures.

Liver LERA Experiments

Bluegill sunfish exposed to 13.3 mg Cd/l began to develop external lesions where the posterior margins of the pectoral and dorsal fins rub against the body after 17 d. During these exposures, there were no deaths.

Incubating lysosomes from control fish livers in hypotonic sucrose concentrations of 0.17 and 0.12 M caused the release greater quantities of lysosomal enzymes than those incubated in isosomotic sucrose (Table 10). The labilization index for fish exposed to 13.3 mg Cd/l for 22 d was greater than controls at all osmotic shock intensities for both enzymes (Table 10). The greater release of NAG from liver lysosomes of bluegill sunfish exposed to Cd was statistically significant only at the most severe osmotic shock of 0.12 M sucrose. Cadmium exposure caused significantly greater ACP release from liver lysosomes in 0.17 M sucrose as well as in the absence of osmotic shock (0.25 M sucrose). A profile analysis of the data revealed a significant increase in the LI, due to Cd across all sucrose concentrations (MANOVA; $P < 0.05$). The fact that the mean LI of lysosomes isolated from treated fish was always greater than control fish indicated that the use of multiple sucrose incubation concentrations was generating redundant information regarding the

Table 10. Labilization indices (%) at three osmolarities and total activities of NAG and ACP for lysosomes isolated from livers of control bluegill sunfish and those exposed to 13.3 mg Cd/l for 22 d. $\bar{X}$, n=6 (SD). Total enzyme activities reported as nmoles/(min·g, wet wt.) after treatment with Triton X-100.

Enzyme	Osmolarity (M)	Treatment[a] Control		Cadmium
NAG	0.25	2.4 (0.73)	NS	4.1 (2.69)
	0.17	11.1 (2.17)	NS	22.5 (15.73)
	0.12	35.6 (6.88)	**	59.2 (21.83)
	Total Activity	892.3 (290.7)	**	1518.2 (225.1)
ACP	0.25	1.4 (0.62)	**	4.0 (2.78)
	0.17	6.3 (1.70)	*	11.7 (6.56)
	0.12	19.4 (4.18)	NS	26.2 (10.66)
	Total Activity	1653.9 (135.9)	NS	1796.5 (552.7)

[a]NS means not significantly different, t-test, $P > 0.10$, *Means significantly different, $P < 0.10$, ** $P < 0.05$.

stability of the lysosomal membrane following Cd exposure.

Exposure to Cd for 22 d induced significantly greater total activity of NAG but not ACP in liver tissue (Table 10). Specific activities of enzymes were always more variable than activity reported on a per gram liver weight basis due to the relatively large and variable amount of non-lysosomal protein associated with the suspensions (data not shown).

The variability of, and differences between, means observed in the first Cd exposure, were subjected to a power analysis to not only eliminate the redundancy of exposing lysosomes to three sucrose concentrations but also enable me to increase the sample size. I found that a sample size of $n = 12$ would be required to demonstrate a difference between means as small as $1.2\hat{\sigma}$ with a probability of type I error of 0.5 and type II error of 0.20. Thus, in subsequent exposures, only one sucrose concentration was used, which allowed me to increase the number of fish assayed.

Using this experimental protocol, I conducted a second Cd exposure to determine if lysosomal membrane alterations could be detected following 10 d Cd exposure. Exposing bluegill sunfish to 16.4 mg Cd/l for 10 d resulted in significantly ($P < 0.05$) greater lability of the lysosomal membranes at 0.17 M sucrose as measured by both NAG and ACP activities (Table 11). However, the total enzyme activity of both NAG and ACP were significantly less in fish exposed to Cd for 10 d exposure period.

Table 11. Labilization indices (%) at an osmolarity of 0.17 M and total activities of NAG and ACP for lysosomes isolated from livers of control bluegill sunfish and those exposed to 16.4 mg Cd/l for 10 d. $\bar{X}$, n=12, (SD). Total enzyme activities reported as nmoles substrates converted/(min·g, wet wt.) after treatment with Triton X-100.

Enzyme		Treatment[a]		
		Control		Cadmium
NAG	0.17 M Sucrose	10.6 (5.8)	**	38.0 (18.4)
	Total Activity	752.3 (198.0)	***	558.4 (160.2)
ACP	0.17 M Sucrose	7.0 (5.7)	**	18.5 (5.9)
	Total Activity	1268.0 (375.0)	**	860.4 (234.9)

[a]Treatment mean significantly different from control mean, t-test, **$P < 0.05$, ***$P < 0.01$.

Gill LERA Experiment

The LI of lysosomes isolated from the gill of bluegill sunfish exposed to 12.1 mg Cd/l for 15 d were not significantly different from those of controls (Table 12). In addition, no change in total activity due to Cd exposure was observed in NAG or ACP. LI for lysosomes from gill were significantly greater ($P < 0.05$) than those from liver for both ACP and NAG at 0.25 M sucrose. At 0.17 M sucrose, the labilization of gill lysosomes was significantly greater for ACP but not NAG. The LI in gill and liver at 0.12 M sucrose were not significantly different for either enzyme (Tables 10 and 12).

Cd Time Course Experiments

During the 32 d subchronic exposure, fish exposed to 12.9 mg Cd/l demonstrated significant changes in the activities of several serum enzymes. After 16 and 32 d, serum ACP activity was approximately 40% greater than the control activities (Figure 21). After 32 d, serum NAG activity in exposed fish was 250% greater than control, but at all other times, there were no significant differences. Activities of serum ASAT, ALAT, and LDH were not significantly different from control values at any time during the exposure (Table 13).

Lysosomal membrane integrity, as measured by lysosomal enzyme release, indicated substantial alteration in the function of this organelle during Cd exposure. The greater the LI, the lower the functional stability of the lysosomal membrane. Following 8 d of exposure to 12.9 mg Cd/l, the LI was significantly greater than that of controls (Figure 22). During the remainder of the exposure the LI

Table 12. Labilization indices (%) at three osmolarities and total activities of NAG and ACP for lysosomes isolated from gills of control bluegill sunfish and those exposed to 12.1 mg Cd/l for 15 d. $\bar{X}$, n=6, (SD). Total enzyme activities reported as nmoles substrate converted/(min·g, wet wt.) after treatment with Triton X-100.

Enzyme	Osmolarity (M)	Treatment[a] Control		Cadmium
NAG	0.25	6.3 (3.11)	NS	7.4 (4.3)
	0.17	9.0 (3.76)	NS	9.5 (3.99)
	0.12	27.5 (6.22)	NS	27.1 (5.20)
	Total Activity	622.2 (72.2)	NS	546.6 (142.6)
ACP	0.25	12.2 (2.70)	NS	13.2 (5.98)
	0.17	14.0 (4.16)	NS	15.3 (7.42)
	0.12	22.7 (5.48)	NS	27.2 (16.40)
	Total Activity	1413.0 (550.5)	NS	1574.6 (944.8)

[a]NS no significant difference between control and Cd-Treated, t-test, $P > 0.10$.

Figure 21. Effect of a 12.9 mg Cd/l exposure on the serum NAG and ACP activities of the bluegill sunfish. Bars represent mean ± S.E., n=17 control, n=5 treated. *Means significantly different from control $P < 0.05$, **$P < 0.01$ (Student's t-test).

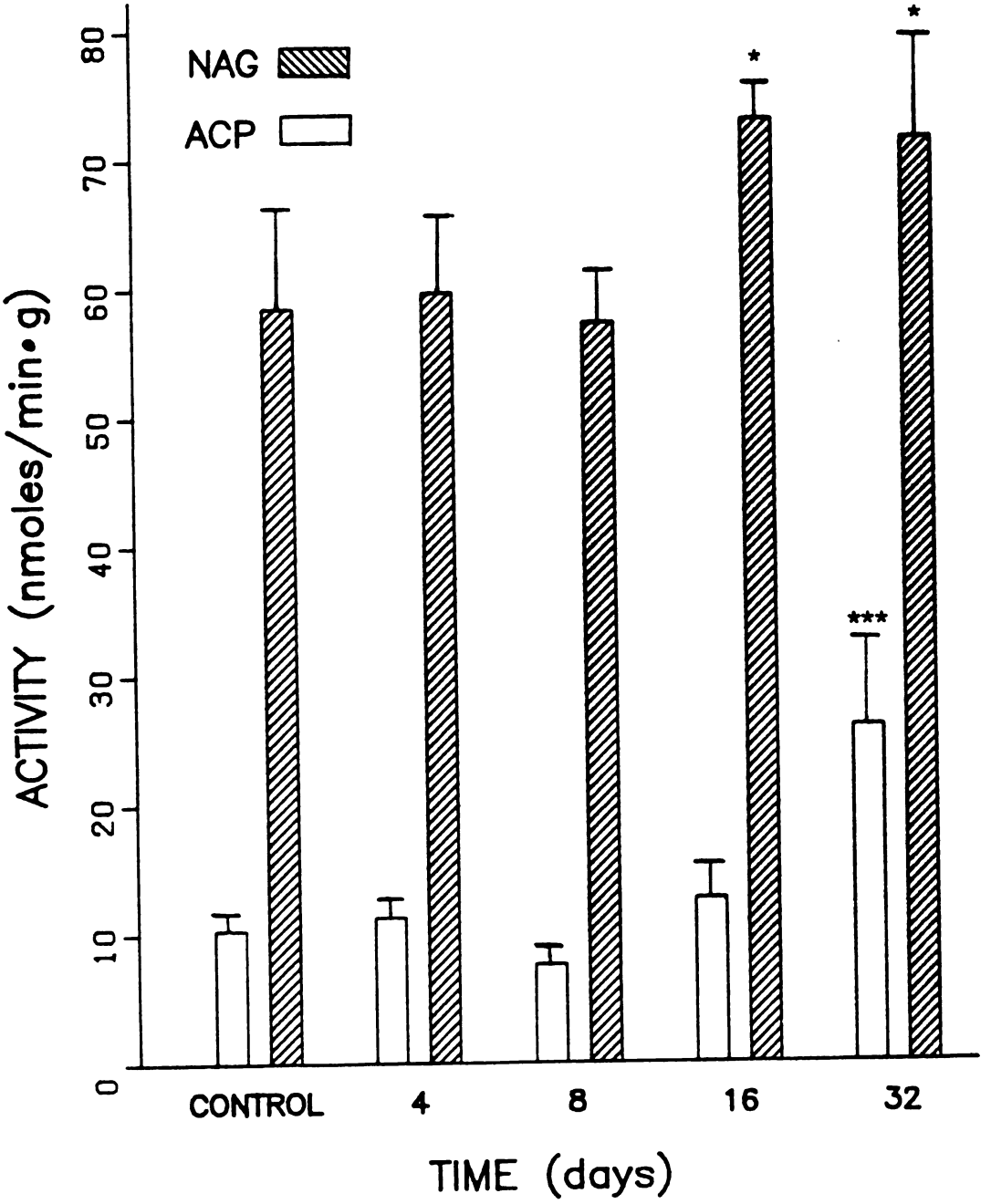
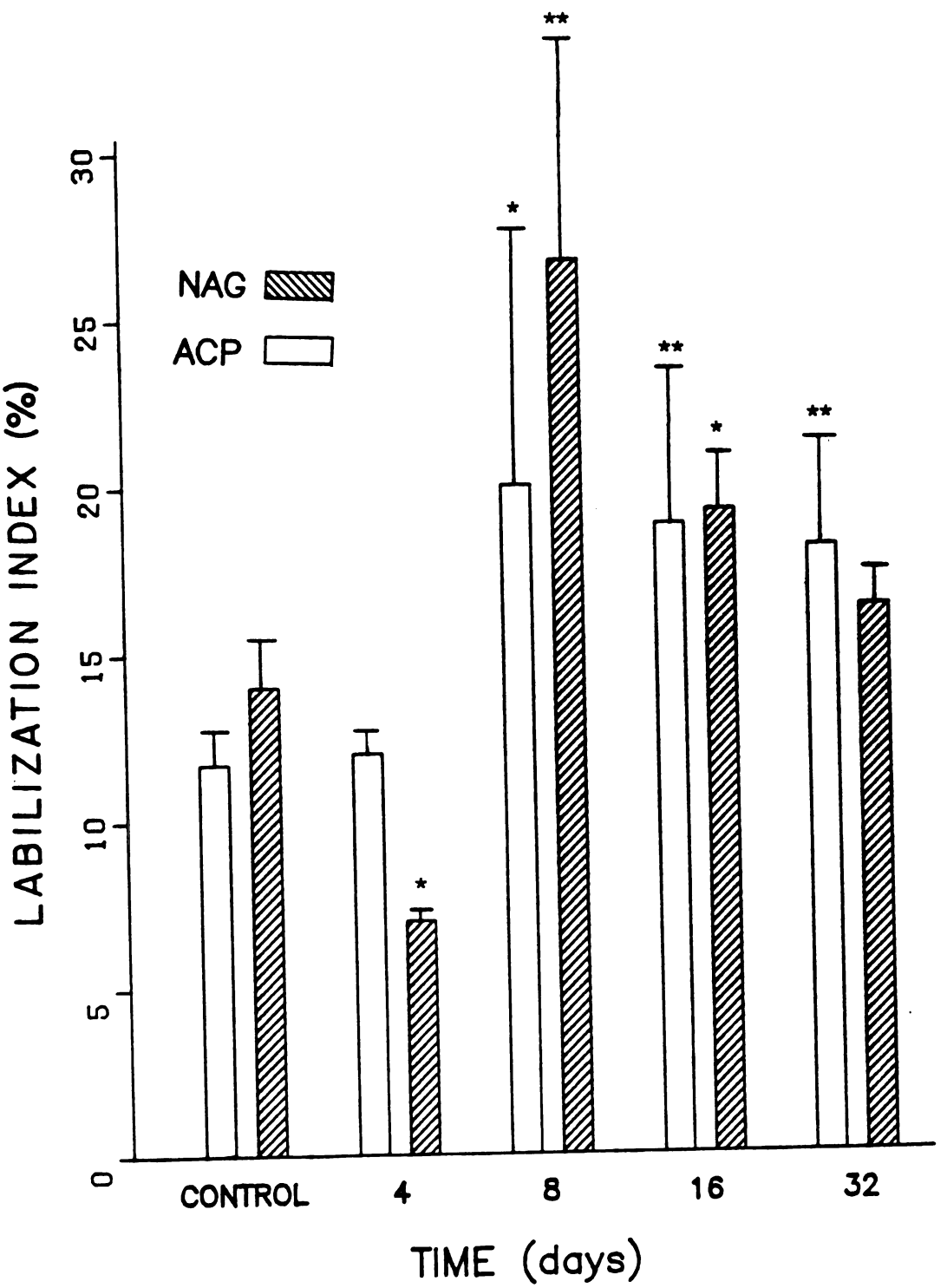

NAG
ACP
ACTIVITY (nmoles/min•g)
0
10
20
30
40
50
60
70
80
*
*
***
CONTROL
4
8
16
32
TIME (days)

Table 13. Total enzyme activities of ASAT, ALAT, and LDH in serum of control bluegill sunfish and those exposed to 12.9 mg Cd/l for 32 d. $\bar{X}$, (SD). n=17 control, n=5 treated. Total enzyme activities reported as nmoles/(min·ml).

	Control	Cadmium			
		Day 4	8	16	32
LDH	402.3 (507.97)	ND	375.8 (247.91)	353.6 (424.77)	505.5 (437.36)
ASAT	43.0 (48.38)	ND	17.8 (11.68)	46.2 (21.73)	51.7 (23.73)
ALAT	28.26 (35.23)	9.1 (4.71)	7.0 (4.05)	17.3 (23.73)	26.0 (17.13)

Figure 22. Effect of a 12.9 mg Cd/l exposure on the liver lysosomal labilization indices (%) for NAG and ACP of the bluegill sunfish. Bars represent mean $\pm$ S.E., n=17 control, n=5 treated. *Means significantly different from control $P < 0.05$, ***$P < 0.001$ (Student's t-test).


LABILIZATION INDEX (%)
0
5
10
15
20
25
30
NAG
ACP
*
**
**
*
**
CONTROL
4
8
16
32
TIME (days)

remained constant and significantly greater than that of controls. The LI of ACP represents an interesting contrast. Following four days of exposure, while the NAG LI was not significantly different from controls, the ACP LI was 7.0% which was significantly less than the control value of 14.0%. After 8 d of exposure to Cd the ACP LI was significantly greater than that of controls. During the remainder of the exposure, the ACP LI in Cd treated fish decreased so that after 32 d, the ACP LI, although elevated above controls, was no longer significantly greater than that of controls (Figure 22).

In Vitro Cd Exposure

The lability of the lysosomal enzyme NAG in liver tissue exposed to Cd in vitro was not significantly affected (Table 14). In vitro Cd exposure had a greater effect on the LI of ACP. At the greatest concentration tested, 1000 µm Cd, the LI was increased over control at 0.17 and 0.12 M sucrose. The LI was greater in liver lysosomes exposed to 100 µM Cd only following the 0.12 M sucrose incubation. The total activity of NAG was unaffected by in vitro exposure to Cd. However, ACP activity was less at all Cd concentrations.

Physical Stress Experiments

Since the fish reduced their food consumption when exposed to Cd, I conducted a fasting study to determine if reduced food consumption alone affects either total enzyme activity or LI. The seven day fasting resulted in small but significant increases in the labilization indices of both enzymes (Table 15). However, total activity of neither enzyme was affected by fasting.

Table 14. Labilization indices (%) at three osmolarities and total activities of NAG and ACP for lysosomes isolated from liver tissue and exposed in vitro to cadmium. $\bar{X}$, n=4. Total enzyme activities are reported as nmoles substrate converted/(min·g, wet wt.) after treatment with Triton X-100.

Enzyme	Osmolarity (M)	Cadmium (μM)			
		0	10	100	1000
NAG	0.25	2.1	2.4	2.2	8.1***
	0.17	16.4	15.3	13.1	18.2
	0.12	43.5	42.4	40.3	43.6
	Total Activity	871.4	853.0	832.6	892.5
ACP	0.25	1.4	1.2	1.2	2.2
	0.17	4.6	4.6	6.2	8.7**
	0.12	12.4	13.4	21.4**	22.7**
	Total Activity	541.5	459.4	186.4***	184.9***

Treatment means significantly different from control (0 μM Cd) mean, t-test, **P < 0.05, ***P < 0.01.

Table 15. Labilization indices (%) at three osmolarities and total activities of NAG and ACP for lysosomes isolated from livers of control bluegill sunfish and those which were fasted for 7 d. $\bar{X}$, n=3, (SD). Total enzyme activity reported as nmoles substrate converted/(min·g, wet wt.) after treatment with Triton X-100.

Enzyme	Osmolarity (M)	Treatment		
		Control		Fasted
NAG	0.25	2.6 (1.1)	NS	2.5 (0.2)
	0.17	5.1 (0.2)	**	7.9 (2.1)
	0.12	24.1 (3.84)	*	36.5 (7.0)
	Total Activity	744.7 (150.1)	NS	761.2 (100.0)
ACP	0.25	4.7 (2.5)	NS	5.7 (1,8)
	0.17	7.5 (1,1)	NS	7.4 (2.2)
	0.12	11.9 (1.1)	**	19.3 (3.8)
	Total Activity	793.4 (153.7)	NS	882.0 (202.8)

NS means not significantly different, t-test, $P > 0.10$, *means significantly different $P < 0.10$, **$P < 0.05$.

The "crowding stress" caused by low water levels did not increase labilization indices for either NAG or ACP (Table 16). The activities of neither ACP nor NAG were affected by the 10 day "crowding stress." During the exposure, the fish reduced their feeding and would crowd into a corner attempting to use other fish as cover. When approached by a person, the fish would swim rapidly about frequently running into the sides of the tank.

DISCUSSION

Cadmium has a relatively low toxicity in the waters used in this study. The smallest concentration producing an effect during the 163 d exposure was 3.9 mg Cd/l. The toxicity of Cd to aquatic organisms is inversely proportional to water hardness (Pickering and Henderson, 1966; Sauter et al. 1976; McCarty et al. 1978). Eaton (1974) reported adult bluegill sunfish mortality at a Cd concentration 250 times smaller than those used in this study at a water hardness of 200 mg/l as $CaCO_3$.

Water hardness is a measure of the total multivalent metal ions, primarily calcium and magnesium in solution. In general, metals are less toxic to fish in hard water, due to the binding of the free metal with carbonate, the primary anion associated with constituents of hardness (Andrews, 1976). Simulations with GEOCHEM demonstrated that Cd is not bound to any great extent by CO_3 under the conditions of these experiments, thus some other factor related to hardness other than reduced Cd^{2+} concentrations must be responsible for this effect of hardness on Cd toxicity.

Table 16. Labilization indices (%) at an osmolarity of 0.17 M sucrose and total activities of NAG and ACP for lysosomes isolated from livers of control bluegill sunfish and those maintained in low water levels for 10 d. $\bar{X}$, n=12, (SD). Total enzyme activities reported as nmoles of substrate converted/(min·g, wet wt.) after treatment with Triton X-100.

		Treatment[a]		
		Control		Low Water Stressed
NAG	0.17 M Sucrose	14.2 (6.2)	NS	13.1 (2.5)
	Total Activity	891.6 (268.3)	NS	844.0 (386.1)
ACP	0.17 M Sucrose	8.9 (2.4)	NS	12.3 (6.00)
	Total Activity	1141.0 (507.8)	NS	1109.5 (261.5)

[a]NS means of "stressed" not significantly different from "control", t-test, $P > 0.10$.

That component of water hardness which modulates Cd toxicity is calcium (Carrol et al. 1979; Wright and Frain, 1981a; Wright and Frain, 1981b). In invertebrates, an increase in calcium concentrations results in a concomitant decrease in Cd body burdens suggesting a competition between these two divalent cations for binding sites (Wright, 1977; Wright and Frain, 1981a). It is still unknown if this competition is at a gill membrane transport site or at an internal receptor site or both. Pagenkopf (1983) has developed and successfully utilized a gill surface-metal interaction model which predicts heavy metal acute toxicity. The model assumes that calcium and magnesium compete with divalent heavy metals for gill surface interaction sites (ie. sites of toxicity). However, a number of divalent metal ions decrease Cd toxicity to isolated rat hepatocytes while increasing Cd uptake (Stacey and Klassen, 1981), which indicates that metal interactions at internal receptors decrease Cd toxicity in the liver.

In waters softer than those used in this study, chronic Cd-induced mortality was associated with neurologic involvement (Eaton, 1974; Benoit et al. 1976). In this study, where the water was much harder, indicating greater calcium and magnesium concentrations, lethal Cd exposure resulted in dermal and corneal lesions and death was not associated with erratic swimming or tetany. These observations indicate that water composition may not only influence mortality but may alter the mode and site of Cd toxicity to fish. Calcium may protect a neurological site from Cd toxicity, allowing internal Cd concentrations at a specific receptor to be increased and this allows the secondary site to be adversely affected. While this discussion is

highly speculative, it is important to note the great degree of variability in the toxicity of Cd observed among studies.

Exposure to 12.7 mg Cd/l was lethal while 3.9 mg Cd/l reduced growth in bluegill sunfish during the 163 day exposure. Mortality at the greater Cd concentration was not believed to be due to gill damage, since death was not associated with signs of respiratory distress. During chronic exposure, Cd has been shown to accumulate in the liver, kidney, and gut of bluegill sunfish (Mount and Stephan, 1967; Eaton 1974). Since chronic exposure to Cd produces histological lesions in mammals (Axelsson et al. 1968; Itokawa et al. 1974) and acute exposure to Cd produces histological effects in fish (Gardner and Yevich, 1970; Sangalang and O'Halloran, 1973; Hawkins et al. 1980; Stromberg et al. 1983), I expected to be able to observe histological lesions in these organs and possibly gain insight into the site and mode of chronic Cd toxicity.

The histological consequences of Cd exposure are well documented in mammals. Axelsson et al. (1968) injected rabbits daily with 0.15 mg Cd/kg for 29 weeks and observed renal lesions predominantly in the proximal tubular epithelium but involving other tubule segments and the glomeruli at longer exposure durations. Itokawa et al. (1974) administered 50 mg Cd/l to rats in drinking water and observed similar tubular epithelial and glomerular degeneration. These renal changes, following chronic administration, explain the proteinuria observed in humans (Lauwerys et al. 1974). Muller et al. (1979) reported decreased white pulp in the spleen of Cd exposed mice in association with decreased immune response in exposed individuals. However, Ohsawa et al. (1983) found increased numbers of lymphocytes in the spleen and

decreased blood lymphocytes following Cd injection and feeding. The mild lymphopenia which was observed in mice was not attributed to lymphocyte destruction by Cd but to a redistribution of the lymphocytes among lymphoid organs.

Histological studies of the effects of Cd on fish have concentrated on acute exposures. Brook trout exposed to 25 µg Cd/l for 24 h in soft water have shown marked alterations in the testes. Blood vessels were dilated and ruptured, leading to infiltration of the testes lobules with erythrocytes. There was extensive necrosis and lobule boundary cell nuclei were pyknotic. Gardner and Yevich (1970) reported lesions in the intestine, kidney and gill of killifish (Fundulus heteroclitus), exposed to 50 mg Cd/l for up to 48 h. Intestinal columnar epithelial cells and submucosal cells were necrotic while kidney proximal tubule cells showed some degree of degeneration. Gills displayed hyperplasia of the respiratory and interlamellar filament epithelium. The respiratory epithelial cells were also hypertrophic. In a 48 h exposure of the spot (Leiostomus xanthrus) to up to 25 mg Cd/l, Hawkins et al. (1980) reported vacuolation and degeneration of renal and proximal tubule cells. The glomeruli were swollen and contained debris. Ultrastructurally the proximal tubule cells contained increased number of lipid droplets, autophagic vacuoles, and degenerating mitochondria. The authors also noted differentiating cells indicate of active tissue repair. Fathead minnows exposed to 12 mg Cd/l for 96 h had widespread epithelial cell necroses. The lesions were severe in the gill, epidermis, olfactory epithelium, kidney, ureter, urinary bladder and hematopoetic tissue.

The only histological effect of Cd on an internal organ of the bluegill sunfish was a decrease in spleenic white cord area. This

effect has also been observed in mice exposed to Cd (Muller et al. 1979), however, this effect has been attributed to lymphocyte redistribution in the organism and may not represent a deleterious effect (Ohsawa et al. 1983). As demonstrated in my study, chronic Cd exposure to fish does not produce lesions similar to those produced by chronic exposure in mammals or acute exposure in fish. This suggests that chronic Cd toxicity has different modes of toxicity in fish and mammals and that histopathology will have limited utility in understanding the chronic toxicity of Cd. My observations, in agreement with those of others (Sippel et al. 1983), indicate that histopathological assessment of chronic metal toxicity is not a sensitive indicator of metal induced internal tissue damage.

I initially hypothesized that LERA would indicate tissue damage. Therefore, I selected a toxicant, Cd, which was known to accumulate in liver tissue and cause biochemical (Jackim et al. 1970) and histological (Stromberg et al. 1983) effects in fish. Using a histochemical procedure to determine lysosomal membrane latency, Moore and Stebbing (1976) observed greater labilization of lysosomes in the hydroid, Campanularia flexuosa, following exposure to Cd, copper, and mercury at concentrations less than those which affected colonial growth rates (r). Similar effects on liver lysosomal lability have been observed in vertebrates acutely exposed to xenobiotics. Several lysosomal marker enzymes were used to demonstrate increased lysosomal membrane fragility during osmotic shock following 48 h exposures of rainbow trout to 20 µg N/l of unionized ammonia (Arillo et al. 1981) and 450 µg NO_2-N/l (Mensi et al. 1982). Oral administration of dieldrin to rats resulted in increased LI at 24 h as measured by acid

phosphatase, cathepsin and acid ribonuclease (Kohli et al. 1977). These studies along with the results of my experiments on the effects of Cd on liver LI in bluegill sunfish indicate a general effect of toxicants on lysosomal membrane stability.

The effects of toxicants on lysosomal enzyme activity have been dependent on the toxicant and the duration of exposure. Exposure of the freshwater murrel, Channa punctatus, to 50 µg Cd/l resulted in elevated liver ACP activities during a 35 d exposure (Dubale and Shah, 1981). Arillo et al. (1981) observed an increase in total proteolytic activity following a 48 h exposure of the rainbow trout to 20 µg N/l of unionized ammonia. Following a 72 h exposure of the rainbow trout to 450 µg NO_2-N/l, total protease activity and the activities of the lysosomal enzymes leucylaminopeptidase, cathepsin B and cathepsin C were decreased (Mensi et al. 1982). In my study, the activity of NAG was decreased following a 10 d exposure to 16.4 mg Cd/l but increased following a 22 d exposure to 13.3 mg Cd/l.

Arillo et al. (1981) have proposed three hypotheses for the lysosomotropic action of ammonia in trout liver tissue: 1) cationic groups of lysosomal enzymes form electrostatic bonds with the intralysosomal matrix. Exogenous cations, such as Cd, compete for anionic sites, displacing the enzymes and increasing the ease with which they pass through the lysosomal membrane; 2) toxicant accumulation in the lysosomes results in an osmotic gradient, leading to lysosomal swelling which results in increased lysosomal fragility; and 3) endogenous hormones elicited by the general stress response increase lysosomal membrane fragility.

The cationic competitive displacement hypothesis does not explain the fact that nitrite caused destabilization of lysosomal membranes in

fish (Mensi et al. 1982) or that temperature (Moore, 1976), salinity (Moore et al., 1980) or exposure to a water soluble hydrocarbon fraction (Widdows et al. 1982; Moore and Clarke, 1982) all decreased lysosomal latency in Mytilus edulis.

The cationic displacement and xenobiotic induced swelling hypotheses have been investigated by in vitro incubations of isolated lysosomes with metals. Mercury causes greater lability of lysosomal membranes in in vitro exposures of isolated mouse lysosomes (Verity and Reith, 1967). Copper and mercury increased lysosomal fragility in in vitro incubations of isolated rat livers, while zinc, Cd and lead decreased lysosomal fragility (Chvapil et al. 1972). These authors suggested that metals which form redox systems may catalyze lipid peroxidation and cause membrane damage. These results are consistent with the fact that I did not observe in vitro effects of Cd on lysosomal lability, based on NAG release from lysosomes. I did however, observe some in vitro effects on labilization, as measured by the relative amount of free ACP at the two greatest Cd concentrations. However, the unrealistically elevated concentrations of ionic Cd used in these in vitro exposures, and the small effect Cd had on the ACP and NAG LI, indicate that direct Cd interactions with the enzyme or lysosomal membrane were not responsible for the in vivo effects on LI.

The hypothesis that changes in lysosomal latency are hormone mediated responses is not supported by the study of Schreck and Lorz (1978) since they were unable to induce an increase in plasma cortisol concentrations by exposing coho salmon (Oncorhynchus kisutch) to Cd. These authors suggested that Cd does not elicit a general stress response. The lack of effects on lysosomal membrane labilization in my "low-water" stress experiment further supports the conclusion that

the general stress response does not alter lysosomal membrane fragility or enzyme activities. In fact, incubation of isolated rabbit lysosomes with cortisol, or cortisone, decreased the release of ACP relative to control lysosomes (Bangham et al. 1965). Furthermore, in the hydroid (C. flexuosa), incubation of tissue sections with hydrocortisone decreased the labilizing effects of copper on lysosomal enzymes (Moore and Stebbing, 1976). Cortisol also stabilized lysosomal membranes in the digestive gland of the marine mussel (Moore et al. 1978b). Conversely, Gabrielescu (1970) used a histochemical procedure to demonstrate increased lysosomal labilization in neurons due to a variety of short-term stressors in the rat.

Starvation causes a similar alteration in lysosomal membranes. In starved rats, there is an increase in the percent of free lysosomal enzymes at 0.25 M sucrose (Bird, 1975). I believe that these alterations in the lysosomal membrane are evidence of increased autophagy and secondary lysosome formation in response to the altered demands of the cell. Biochemical adaptation of the cell, necessitated by a toxicant or starvation, requires additional raw materials, e.g., amino acids, nucleic acids, triglycerides, etc. to counteract the effect of the stressor. This cellular adaptation would result in altered phagocytic and lysosomal activities, possibly necessitating cell and lysosomal membrane alterations which are measured by the LERA.

With this understanding of the response of liver lysosomes to Cd exposure, I decided to conduct a time-course experiment in an attempt to determine the time-course of LERA changes and to investigate the use of serum enzyme activities as diagnostic aids in Cd toxicity.

Alterations in biochemical parameters during Cd exposure have been demonstrated at Cd concentrations which did not result in significant histological alterations. Serum NAG and ACP activities were elevated over control levels during the exposure to Cd. At the end of the exposure serum enzyme activities of both lysosomal enzymes were elevated which indicates their potential usefulness in Cd exposures of longer duration. Cadmium may have its site of toxic action in the liver, spleen or intestine as ACP and NAG activities are elevated in these organs of bluegill sunfish (Chapter 1). This suggests that Cd is damaging one of these organs. In agreement with the results of Roberts et al. (1979), who exposed the rainbow and brown trout (S. gairdneri and S. trutta) to sublethal Cd concentrations, I was unable to observe alterations in the serum transaminases or LDH activities of exposed fish. In previous work, sublethal Cd exposure has been associated with elevated serum LDH in the brook trout, (Christensen et al. 1977), and elevated serum transaminases in rats (Chapatwala et al. 1982; Flora and Tandon, 1983).

Cadmium caused maximal lysosomal membrane labilization after eight days of exposure. The stabilization of the lysosomal membrane caused by four days of Cd exposure has been observed during the tissue repair following CCl_4 induced hepatocyte destruction in the bluegill sunfish. The elevated NAG LI at the end of the 32 d exposure indicates that Cd toxicity was ongoing and demonstrated the potential of LERA as an indicator of toxicity during longer Cd exposure durations.

I have biochemically detected Cd induced tissue damage during an exposure regime which failed to produce histopathological effects on internal organs. These effects appear to be due to Cd induced damage

to the liver, spleen or intestine and changes in cellular autophagy. Further research concerning the relationship between short-term specific and nonspecific indicators of stress and long-term effects on growth, reproduction and survival need to be conducted to further develop short-term indicators of chronic toxicity.

CONCLUSIONS

Because fish gill tissue is known to be a primary site of damage with exposure to Cd (Stromberg et al. 1983), I examined the effect of Cd on LERA of gill tissue. I was unable to demonstrate a statistically significant effect of Cd on the LI. This may have been due to the presence of cartilage in the tissue. The cartilage would have altered the homogenization characteristics of the tissue, possibly disrupting the lysosomes which were affected by Cd exposure. However, a large osmotically sensitive pool of lysosomes still existed which suggests that these lysosomes do not respond to Cd exposure as detected by this assay. Use of the histochemical techniques described by Moore and Stebbing (1976) may be useful in understanding the effects of cadmium on the gill lysosome.

I conclude that the lysosomal enzyme release assay holds promise as a useful indicator of stress and will be particularly useful in field situations. Presently, it appears that the LERA is most useful as a measure of biochemical alterations in specific tissues which are known to be affected by the stressor of interest. However, the response is not due entirely to tissue destruction and may be representative of toxicant induced alterations within the cell.

In addition to understanding the effects of xenobiotics on the lysosomal system it is necessary to understand the effects of other factors which may effect the LERA such as sex, reproductive status, water temperature, nutritional status and crowding (social stress). In my experiments, size and sex did not significantly affect the LI, relative to the effects of Cd. Also, the low-water level study indicated that short-term stressors (e.g., capture) do not influence the LI as they do in the case of some other chemical measures such as blood cortisol concentration, adenylate concentrations and some specific enzyme activities. Additional research concerning the interaction between nutrition and Cd, with regard to LERA should be conducted.

Chronic exposure (163 d) of the bluegill sunfish to 3.9 mg Cd/l reduces growth while 12.7 mg Cd/l reduced survival and growth. Cd induced histological lesions occurred at 3.9 and 12.7 mg Cd/l only in the gill. Exposure of fish to Cd at concentrations which reduce growth and survival caused elevations in serum NAG and ACP. Serum transaminase and LDH activities were not affected by Cd exposure which indicates limited damage of heart and liver tissues. LERA demonstrated Cd induced destabilization of lysosomal membranes.

Lysosomal membrane destabilization was not due to a direct effect of Cd on the membrane since in vitro Cd exposure did not result in elevated membrane lability. Physical stress was also ruled out as causing the lysosomal membrane destabilization. It is suggested that Cd exposure alters the function of lysosomes within the cell by causing intracellular protein and lipid damage, which results in reduced lysosomal membrane stability.

The results of the effect of feeding on membrane lability indicates that further research concerning the interaction between nutrition and Cd, with regard to LERA, be conducted.

I conclude that measurement of serum enzyme levels and the use of the lysosomal membrane release assay hold promise as useful indicators of xenobiotic stress. I propose that they will be particularly useful in field situations. Presently, it appears that LERA is most useful as a measure of biochemical alterations in specific tissues, which are known to be affected by the xenobiotic of interest. Additional research relating the concentrations of xenobiotics causing chronic toxic effects with those resulting in biochemical, histological, or physiological effects is recommended.

GENERAL DISCUSSION

This research has concentrated on the development and use of techniques to understand the effects of xenobiotics on bluegill sunfish. Short term, subchronic indicators of toxic effects, capable of predicting chronic toxicity, must be developed if toxicological information on the vast number of pollutants is to be obtained. The biochemical, histological, and physiological indicators of toxicity developed will enable more rapid promulgation of water quality criteria and standards which will be protective of aquatic life.

I have successfully demonstrated that Cd and CCl_4 alter the stability of lysosomal membranes. LERA was not exceptionally sensitive to Cd toxicity; however, it was at least as sensitive as serum enzyme activities to CCl_4 toxicity. These results, as well as those of other researchers, indicate a wide susceptibility of lysosomes, as detected by LERA, to toxicants. Additional experiments with LERA need to be conducted to increase this assay's sensitivity and determine if LERA is indicative of toxicity from a wide variety of toxicants.

My current understanding of LERA and the interaction between xenobiotics and lysosomes indicates that the assay can be improved and utilized successfully as an indicator of xenobiotic stress. Xenobiotics appear to induce secondary lysosome formation, however, the assay has not been optimized to measure alterations in the membrane stability of these lysosomes. In fact, secondary lysosomes are

destroyed in the current procedure. To optimize the assay for investigation of the membrane properties of secondary lysosomes, the homogenization procedure must either be utilized to investigate the occurrence of secondary lysosomes, or eliminated. Two approaches which more directly measure secondary lysosomal membrane stability are: a) use of tissue slices or b) the methods of Bird (1975). Incubation of tissue slices in a buffered solution and examination of the time course of lysosomal enzyme release would simulate the technique refined by Moore and coworkers, however, as enzyme quantification would be biochemical instead of histochemical, the quantification problems would be avoided. The technique of Bird (1975) is a direct measure of the proportion of the lysosomal pool comprised of secondary lysosomes. In this technique, enzyme release is quantified after homogenization. The quantity of the enzymes released by homogenization is compared with total enzyme activity quantified following Triton X-100 treatment, to determine the status of lysosomes in the tissue. This procedure measures the proportion of secondary lysosomes since, theoretically, only the larger secondary lysosomes are destroyed during homogenization. The smaller primary lysosomes contribute to the total pool of lysosomes. The purported induction of secondary lysosomes during xenobiotic stress could be further investigated by electron microscopy (Leland, 1983). Use of tissue slices or freshly homogenized tissues would have the added benefit of reducing the time necessary to conduct the assay. This would enable a greater number of samples to be analyzed at a time. Finally, with both of these new approaches, samples could be prepared and frozen for determination of enzyme activity at a later date. I believe these suggestions will enable further development and enhance the sensitivity of LERA as an indicator

of sublethal toxic effects on fish.

The work conducted on serum lysosomal enzymes represents one of the first uses of NAG and nonprostatic ACP in describing toxicological effects. The results are presently difficult to assess toxicologically, but these enzymes hold promise as useful indicators of toxicity in fish. With increased understanding of isozyme patterns in serum and tissues, and distribution of enzyme activity among organs, these enzymes have potential to be either organ specific or general indicators of toxicity.

Serum transaminase and LDH activities were successfully utilized in this research to demonstrate the organ related toxicity of Cd and CCl_4. As specific indicators of toxicity, these enzymes will have limited utility in integrating all of the stressors which impinge on an organism. However, these enzymes are extremely useful to assess the health of an organism. Further development of these enzymes as tools for use in this field requires a thorough understanding of the biology of these enzymes in fish and the effects of xenobiotics on them.

Other measures of sublethal xenobiotic stress which are currently being investigated in aquatic organisms include serum cortisol concentrations (Schreck and Lorz, 1978), adenylate energy charge (Ivanovici, 1979; Dickson and Giesy, 1982), RNA to DNA ratios (Barron and Adelman, 1984), glucose and glycogen status (Zandee et al. 1980), and oxygen consumption (Darville and Wilhm, 1984). These methods, and those presented in this dissertation, can be utilized as indicators to prioritize pollutants, assess the health of a group of organisms, develop structure activity relationships among a group of chemicals, and determine toxicant effects in field situations. These methods are

able to integrate the effects of natural stressors into a comprehensive health assessment, and have been proposed as short cut methods for predicting chronic toxicity. However, there are a few problems with these methods which must be considered. Short-term effects of a toxicant, which these indicators measure, are not necessarily biologically linked with chronic toxicity, unless the specific indicator is matched to the toxin. This weakens the cause and effect relationship between the subchronic effect on the indicator and the eventual chronic effect on survival, growth or reproduction. Organisms have a large capacity to maintain homeostasis, which makes it difficult to distinguish between a physiological response to a toxicant and a pathological, destructive effect. These problems with the indicator approach can be partially overcome by conducting correlative studies between the effects of a compound on ecologically relevant parameters and effects on the specific assay. Although the predictability of a correlative approach linking biochemical and population effects will not be precise, the error produced may be acceptable. This raises an important societal question; that is, how precise must our estimates of safe concentrations in the environment be? If precision is required, at what cost will these estimates be obtained? The cost will be measured not only in dollars and cents, but also in environmental degradation, since the research time and effort expended to develop precise estimates will hamper and deter equally important research on other potentially toxic chemicals.

Although this research has not answered all of the questions concerning indicators of sublethal stress in aquatic organisms, it has accomplished its general purpose of contributing to the body of knowledge in this area of science. The tests developed in my research

will hopefully provide another tool for aquatic toxicologists to use in understanding the effects of toxicants on fish. In addition, the information presented here will contribute to future research into the development of procedures used by aquatic toxicologists to answer the many questions concerning pollutants in the environment.

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