A ^{31}P-Nuclear Magnetic Resonance (NMR) Spectroscopic Study into the Sublethal Effects of Cadmium on the Red Swamp Crayfish (*Procambarus clarkii*)

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The use of cadmium in recent years has greatly contributed to the level of heavy metals in the environment. Elevated concentrations of cadmium in aquatic systems due to leaching from terrestrial landfills have become an environmental concern. The toxic effects of these contaminants on aquatic organisms have been extensively studied. Most of these studies, however, have concentrated on the effects of lethal concentrations of heavy metals on these organisms. Very little is understood of the physiological effects of such pollutants at sublethal concentrations. Nuclear magnetic resonance (NMR) spectroscopy has been demonstrated to show changes in stress related metabolites such as phosphoarginine, inorganic phosphorus, adenosine triphosphate, and nicotinamide adenine dinucleotide. Most of this research has concentrated on non-pollutant causes of stress such as hypoxia and anoxia. In this study, specimen of the red swamp crayfish (*Procambarus clarkii*) were subjected to acute exposures of cadmium at concentrations of 708 mg/L and 7700 mg/L while being simultaneously monitored by NMR spectroscopy. Fluctuations in levels of all associated metabolites were determined and correlations between physiological stress, cadmium-spiked water concentrations, and the cadmium concentrations of the specimen were examined. There was a significant increase in levels of inorganic phosphorus, with an associated decrease in phosphoarginine, at both cadmium concentrations. Additionally, there were trends in the decrease of ATP and inherent increase of ADP indicating possible inhibition of mitochondrial ATP production. Direct correlations were observed between the levels of cadmium in the water and the crayfish, and the response of measured high-energy phosphates.

Introduction

The need for increased energy supplies and industrial products worldwide has exacerbated the quantity of associated wastes and pollutants (Stigliani, *et al.* 1993). The direct effects of these pollutants on human health has become the focus of a large portion of recent environmental research (Nriagu 1990). Increased use of cadmium during the last several decades in industry, fertilizers, PVC plastics, as a pigment in paints, and most recently, in nickel-cadmium batteries has made this toxic metal more ubiquitous in the environment (Bergback, *et al.* 1994: Wu 1995; Park, *et al.* 1994). All of these sources have combined to elevate cadmium levels up to six times the naturally occurring levels in the environment (Nriagu 1990).

Little is known of the actions of cadmium at the molecular level. Recent studies indicate that cadmium may compete with zinc, an important cofactor in many enzymatic reactions, replacing it and effectively denaturing the enzyme (Torra, *et al.*, 1995; Goyner, *et al.* 1994; Tacnet, *et al.* 1991). Acute exposure to cadmium results in several effects at the cellular level: the disruption of protein synthesis and the breakdown of mitochondrial metabolism (Behra 1993). Interference with mitochondrial function may be related to the

inhibition of transmembrane calcium which, in turn, leads to depression of phosphorylation. Ultimately, there is an implied reduction in adenosine triphosphate (ATP), and a subsequent increase in adenosine diphosphate (ADP). In addition to mitochondrial effects, there have been observed decreases in hepatic microsomal cytochrome P450 (CY450) content, which may be linked to the inability of heme groups to take up essential iron (Goyner, *et al.* 1994). There is also some evidence of increased concentration of cadmium in red blood cells following acute and chronic exposure (Cortesi, et al. 1992).

Cadmium accumulates readily in fresh and saltwater systems (De Gregori, *et al.* 1994; Wu and Groves, 1995), and builds up quickly in sediments where it is easily ingested by bottom feeders, such as fish and invertebrates (Bou-olayan, *et al.* 1995; Sharif, *et al.* 1993; Petri and Zauke, 1993). Using aquatic organisms to monitor cadmium concentrations in environments enables a better understanding its toxicological effects. Invertebrates are excellent organisms with which to monitor cadmium uptake, concentration, and sublethal effects in freshwater and marine systems (Petri and Zauke, 1993). Many of these species show relatively high levels of cadmium accumulation, as well as exhibit a strong correlation between body and environmental concentration (Kilgour 1991). As such, these organisms work exceptionally well in monitoring the sublethal effects of cadmium levels. Unfortunately, little work has been done using macroinvertebrates as biomonitors of aquatic ecosystems (Maciorowski, et al. 1980). The red swamp crayfish (*Procambarus clarkii*) is an excellent biomonitor of freshwater systems. It inhabits watersheds that are frequently threatened by human actions. Agricultural runoff of pesticides and fertilizers drain into creeks, rivers, swamps, and lakes, and accumulate in these areas at relatively high rates. The diet of *P. clarkii* focuses on algae, other invertebrates, detritus, and growing vegetation (Clancy 1997). Several studies have been performed using *P. clarkii* (and other related species of crayfish) to determine bioaccumulation and tissue concentration of cadmium. The crayfish exhibits an unusually high resistance to cadmium. This is in spite of accumulation rates that exceed most other invertebrates. Del Ramo, *et al.* (1989) have found elevated amounts of a protein located in the mid-gut gland of the crayfish. This ability to withstand high cadmium exposures makes this species ideally suited for this experiment.

Actual metabolic fluctuations during the time of exposure must be determined in order to more fully understand the impacts of cadmium on an organism. Techniques previously used, such as in LD_{50} and LC_{50} analyses, typically monitor only the survivorship. Monitoring reproductive rates during long-term life cycle tests may be a good indicator of acute and chronic toxicity, but the high variability between organisms and the length of time necessary for analyses make them prohibitive (Pitt, *et al.* 1995). Blood chemistry analysis has also met with limited success, due to the inability to identify the chemical parameters as the common denominator between different types of stress (Knittel 1980).

A more suitable technique for monitoring the biochemical activity of organisms is *in vivo* nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy was originally designed for measuring samples of non-biotic homogenous content. It has recently, however, been adapted to monitor fluctuations in high-energy phosphates of living marine organisms and physiological stress due to the effects of pollutants on cellular respiration (Higashi, *et al.* 1989; Thillart, *et al.* 1989; Tjeerdema, *et al.* 1991). By measuring the relative proportions of phosphoarginine (phoshocreatine in vertebrates), inorganic phosphates , and adenosine triphosphate (ATP), adenosine diphosphate (ADP) and nicotinamide adenine dinucleotide (NAD), the total

stress on an organism may be determined. Most ^{31}P-NMR studies, however, have been limited to non-pollutant stressors, such as hypoxia and anoxia. Those that examined the effects of organic pollutants, such as the study performed by Tjeerdema, *et al.* using pentachlorophenol (PCP), have not been able to draw correlations between the limit of stress and tissue concentrations of the chemical in the sample organism. In this study, the sublethal effects of cadmium on P. clarkii were determined using ^{31}P-NMR spectroscopy. In addition, an evaluation of cadmium concentration in the tissues of the crayfish was performed using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) in order to ascertain any correlation.

Methods and Materials

Specimens of juvenile crayfish (*C. clarkii)* 1.5- to 2.0-cm long were obtained from a swamp area located on the west campus of Tulane University in Belle Chase, Louisiana. They were kept at approximately 25^{o} C in site water and the pH was maintained at approximately 6.5. The water was continuously aerated by a battery-powered portable aerator. Indirect, diffuse lighting was regulated in cycles of 12 light/12 dark throughout the time in the lab. The crayfish were fed with decaying organic debris also obtained from the sampling site. All analyses were performed within 72 hours of specimen capture.

A Bruker 200 MHz NMR spectrometer was fitted with a 10-mm vertical bore, multinuclear broad band probe and tuned for acquisition of ^{31}P spectra. Observed frequency was set to 97 MHz at a spectral width of 81 ppm. A 1 db high power, 15 μsec, and a 45° pulse were used for the experiment. A total of 500 scans with 1.969 hz/pt were taken for each spectra. Acquisition time was set to 0.508 sec with a one second delay, and a spectral width of 16k was employed. Line broadening was utilized and set to 30. Fourier transformation was performed on each spec following the scanning. Identification of the peaks representing inorganic phosphorus, phosphoarginine, NAD, ADP, and ATP was established and the individual peaks were then integrated as specified by the protocol for the Bruker 200. Phosphoric acid (H_3PO_4) was used as an external standard and all peaks were referenced to the inorganic phosphorus peak at 0 ppm.

Each specimen was inserted tail-first into a 10-ml NMR tube filled with 2 ml of H_2O. A 5-ml NMR test tube filled with D_2O was then inserted into the 10-ml test tube to provide a lock signal for the NMR. A small cotton swab was placed just above the crawfish to minimize upward movement and maintain the specimen's position within the test tube. The NMR test tube cap was modified by drilling two holes of approximately 3/16 inch diameter. A 20-foot length of flexible Teflon tubing with an inside diameter of 1/16 inch was inserted into one hole, the other was left open to allow excess air to escape (see Figure 1.1). Airflow, and the introduction of the $CdCl_2$ solution, into the test tube was regulated by a Manostat Simon Model peristaltic pump adjusted to a constant flow rate of approximately one ml per minute.

The crayfish were allowed a period of time to adjust to the environment of the test tube and the darkness of the NMR magnet. Solutions were prepared from distilled water and $CdCl_2$ at three concentrations of 0 (control), 700, and 7000 ppm. Six replicates were used at each concentration and specs of ‘before’, ‘during’ and ‘after’ exposure were obtained.

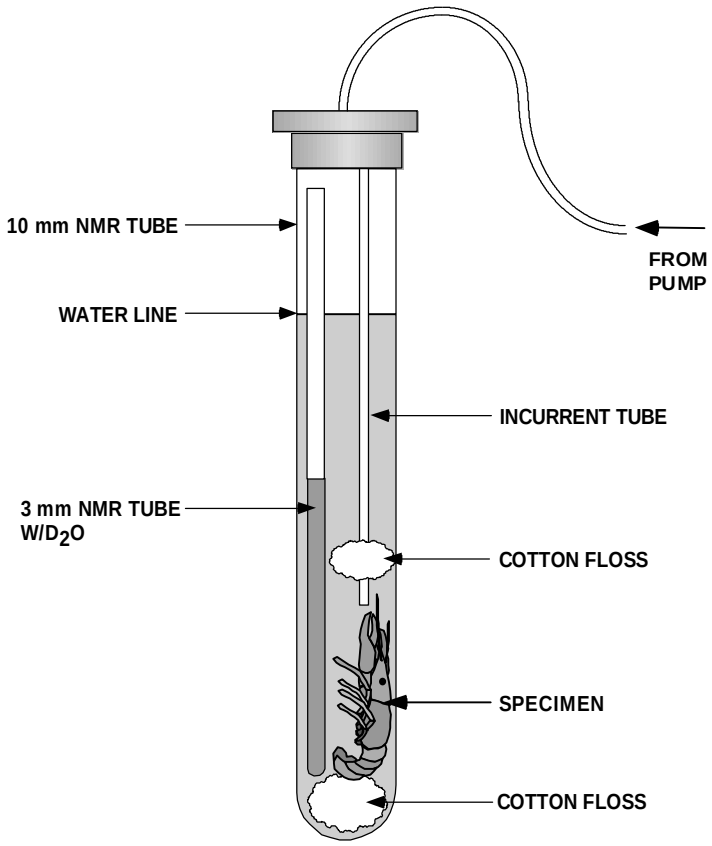

Figure 1. Diagram of NMR test tube showing the positioning of the crayfish, D_2O tube, and flexible tubing.

Each of the crayfish samples from the NMR tests and the 12-hour exposure study were frozen overnight to facilitate more efficient handling. They were each weighed, digested with 10 ml of 17.5 % nitric acid and placed in a microwave for 60 minutes. The system was ramped at 60% power through five stages at 12 minute intervals of 20, 40, 85, and 135 psi up to a final pressure of 175 psi. The samples were then brought to 30 ml with the addition of deionized water. The spiked water samples from the NMR test tubes were diluted to one part per thousand and all water samples, including the swamp water obtained from the sampling site, were increased to a final volume of 10 ml with 17.5 % Nitric acid. The analyses were performed on a Perkin-Elmer Optima 3000 ICP-AES equipped with a Spectro Sonic Nebulizer. The standard curve was established with standard solutions of 2.5, 5, 10, 25, 50, 100, and 2000 ppb obtained from High Purity of Charleston, South Carolina.

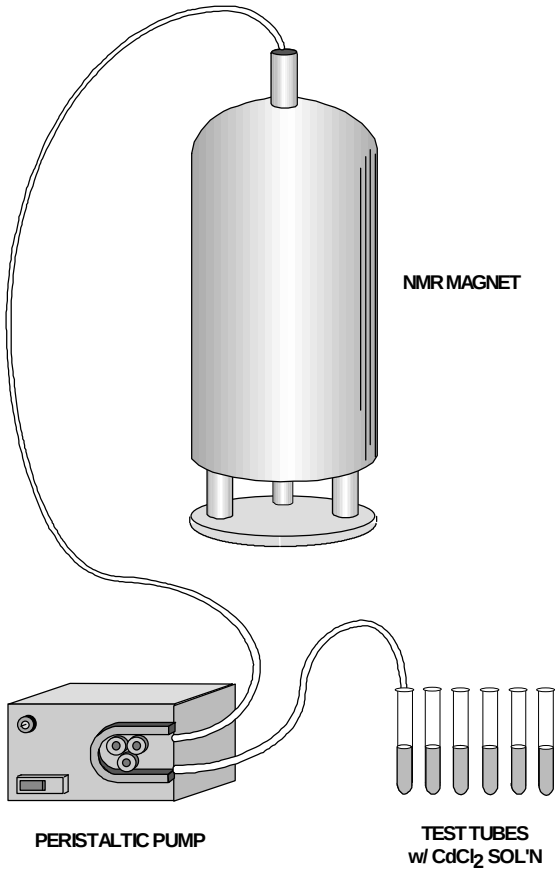

Figure 2. Illustration depicting arrangement of pump, $CdCl_2$ test tubes, tubing, and NMR magnet.

Results

^{31}P NMR Spectroscopy

Each metabolite concentration was normalized by expressing its units with respect to sum of the inorganic phosphorus and phosphoarginine levels. Each of the five peaks indicated in Figure 2.3 were identified per Tjeerdema, *et al*, as inorganic phosphate, phosphoarginine, and the nucleoside phosphates (NPs) γ-, α-, and β-NP. The NPs of γ-, α-, and β-NP have been previously identified as γATP + βADP, αATP + αADP + the phosphate moiety of NAD, and βATP, respectively. The T_1s of the NPs were similar, as per Tjeerdema, *et*

al, allowing for the conversion of each NP as follows: [ATP] = β, [ADP] = γ - β, and [NAD] = α - γ.

Statistical analyses were performed using a one-sided student's t-test. In order to determine if multiple use of the t-test would contribute to a possible Type I error, an ANOVA was also performed. No difference was determined in the values obtained from each test, and the t-test was used for each of the metabolite comparisons within each of the exposures.

Table 1. Results from ^{31}P-NMR analysis.

STATISTICAL ANALYSIS RESULTS						
		P_i	**P-Arg**	**ATP**	**ADP**	**NAD**
CONTROL	Initial Mean	0.389983	0.61002	0.37715	0.03797	0.085868
	Final Mean	0.365433	0.634567	0.38845	0.063482	0.081416
	t	0.511084	-0.51108	-0.21815	-0.46616	0.055041
	P-value	0.310193	0.31019	0.415851	0.32554	0.478595
708 PPM	Initial Mean	0.3776	0.6225	0.41891	-0.0494	0.128686
	Final Mean	0.43179	0.54037	0.28229	0.099365	0.066625
	t	-1.5541	2.169	2.0283	-2.0518	0.905
	P-value	0.0688	0.0219	0.0288	0.0275	0.1887
7700 PPM	Initial Mean	0.3853	0.614717	0.38945	-0.02707	0.047471
	Final Mean	0.605083	0.394917	0.305117	-0.11682	0.073995
	t	-5.5087	5.511	1.6366	1.5801	-0.3001
	P-value	0.0001	0.0001	0.0664	0.0726	0.3851

An ANOVA was used to determine if there was any difference in the levels of initial metabolite concentration between each of the three exposure tests. There were no significant differences found between the three exposures with $P > 0.05$ for all the metabolites.

Exposure to the NMR environment alone resulted in no significant change in any of the metabolites with P-values all greater than 0.05 (refer to Table 1). Definable trends, however, indicated a slight increase in phosphoarginine and a corresponding drop in inorganic phosphorus of approximately two percent. Elevations in ADP and ATP were also indicated after the one hour period with an increase of one percent for ATP. No significant difference was found in the level of inorganic phosphorus at the 708 ppm exposure level. There was, however, an identifiable trend in the increase of this metabolite with levels dropping 14 % below the initial value during the time of exposure. A corresponding decrease in phosphoarginine was found as significant, with elevated levels of 13 %. The level changes in ATP and ADP were also inversely related and significant in this test. Initial levels of ADP at negative values indicates levels too small for accurate measurement with this technique as may be calculated as zero. Therefore, as ATP decreased 33 % below initial levels, there was an increase in ADP of approximately 100 % over baseline measurements. There was no significant difference found in concentrations of NAD between initial and final values.

Phosphoarginine levels at exposures at 7700 ppm decreased dramatically, dropping 36 % over the one hour dosage interval. Inorganic phosphorus values were elevated approximately 57 % over initial levels. The statistical analysis indicated no significant difference between the initial and final levels of ATP, ADP and NAD. There was, however, a reduction in levels of ATP and ADP of 22 % and 300 %, respectively. There was also a marked trend towards decreasing values for both ATP and ADP. The slight increase in AND indicated in the graph is offset by the error bars and has been disregarded.

ICP-AES Analysis

The results of the ICP-AES analysis are shown in Table 2. Cadmium concentrations in the crayfish are shown in units of dry weight. Water concentrations are shown in the accepted units of parts per million. The use here of different units does not affect final analysis of observed trends in concentration fluctuation. The control in this test represents the crayfish and swamp water obtained from the collection site. No NMR exposure was administered to these samples. The extremely low levels of cadmium in both the crayfish and the water are consistent with previous research. No replicates were consequently used since the object of this study was to show NMR responses at three extreme levels. This also accounts for the absence of both standard error and standard deviation data.

There was a marked increase in cadmium concentration within the crayfish as levels of cadmium in the water were increased. At 708 ppm the cadmium levels had increased by a factor of 50 to a tissue concentration of 166.8 µg/g dry weight. The cadmium uptake by the crayfish during this test occurred over the longer exposure time period of six hours. Depressed levels of iron were also found with a mean value of 72.85 µg/g versus 117 µg/g in the control specimen.

In initial dose/response testing, increasing the level of cadmium exposure to higher than 6000 ppm resulted in high mortality. This indicated the need for shortened time intervals for this test. Increasing the $[Cd^{2+}]$ of the water 10-fold to 7757 ppm was performed over a one-hour time period and resulted in a mean crayfish concentration of 501 µg/g. Iron concentrations were again lower in the crayfish exposed to cadmium with a mean value of 82.31 µg/g with several values below 50 µg/g.

Table 2. Cadmium concentrations from crayfish indicating mean, standard error, and standard deviation.

ICP-AES ANALYSIS RESULTS								
	Control		**708 ppm**		**3000 ppm**		**7700 ppm**	
	Crayfish (µg/g)	Water (ppm)	Crayfish (µg/g)	Water (ppm)	Crayfish (µg/g)	Water (ppm)	Crayfish (µg/g)	Water (ppm)
Mean	3.42	0	166.8	708	963	2869	501.17	7756.6
Standard Error	N/A	N/A	22.89	N/A	136.90	N/A	111.36	N/A
Standard Deviation	N/A	N/A	72.37	N/A	335.33	N/A	272.77	N/A

The twelve-hour exposure at 2869 ppm performed during the dose/response tests resulted in a considerably lowered mortality level. This allowed for the ICP-AES analysis at this concentration, although no NMR data was collected at this time. There was an increase in uptake resulting in cadmium concentrations rising accordingly to 963 µg/g. Iron values ranged from 62 to 90.4 µg/g with a mean value of 77.48 µg/g.

Discussion

The results of the NMR analyses indicate a definite disturbance of the high-energy phosphates during exposure to cadmium. The degree to which this occurs is dependent on the cadmium concentration, as well as the length of time of exposure. The results of the analysis using zero concentration reflect the adaptation of the crayfish to the test tube/NMR environment. This is in keeping with the inherent tendency of crayfish to back into small, dark crevices to achieve security. The slight trend of increased phosphoarginine and ATP suggest this relaxed state as well. This also allowed the determination of a baseline against which the other concentration results were measured.

The depletion of phosphoarginine, and subsequent increase of inorganic phosphorus, during exposure to elevated cadmium concentrations is in agreement with similar tests involving environmental stresses on aquatic organisms. Stress causes a rise in metabolic rate, requiring increased usage of energy. This energy is found initially in storage units such as phosphoarginine and ATP. Since phosphoarginine acts as a buffer against ATP depletion it is typically the first to be decreased. This appears to be true in short-term/high-concentration events. At 7700 ppm the phosphoarginine has been reduced significantly, allowing ATP to be somewhat protected from energy demands. Conversely, at the lower 708 ppm concentration the initial decrease in phosphoarginine was insufficient for the energy demands over a longer time period of six hours. This decline in ATP, however, may also be due to the effects of cadmium on mitochondrial ATP production. In addition, the longer the exposure period, the more time the toxic effects have on enzyme function breaking down the important reactions necessary for phosphylation to take place.

The ICP-AES results indicated a definite trend in the increase of cadmium in crayfish tissues when subjected to elevated cadmium levels. The mechanism behind this uptake is most likely through the gills, with concentration in the hepatopancreatic (midgut) gland. There was no linear association found between the environmental and tissue concentrations. A more detailed analysis looking at these two concentrations should establish this relationship. The coupling of the NMR data with the ICP-AES data revealed a close relationship between tissue concentration and stress. Again, more research is necessary to determine an exact correlation.

The use of ^{31}P-NMR spectroscopy has thus far been confined to non-environmental research. In the future, its role in biochemical and metabolic analyses should be joined with contamination and toxicological studies. The flexibility and non-invasive qualities of this technique is useful for defining and monitoring trends as they occur. Although this research was confined to cadmium, further studies should concentrate on other lethal metals such as trimethyl tin and organolead.

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