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UNIVERSITY OF CALIFORNIA

SANTA CRUZ

APPLICATIONS OF ^{31}P-NMR SPECTROSCOPY FOR THE ASSESSMENT OF SUBLETHAL TOXICITY IN MARINE ORGANISMS

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Scott Leigh Shofer

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The dissertation of Scott Leigh Shofer is approved:

Dean of Graduate Studies and Research

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APPLICATIONS OF ^{31}P-NMR SPECTROSCOPY FOR THE ASSESSMENT OF SUBLETHAL TOXICITY IN MARINE ORGANISMS

Scott Leigh Shofer

ABSTRACT

Many important marine species are suffering declines. Release of pollutants into aquatic environments are likely contributors to this decline, and so biochemical methods to study the effects of sublethal toxicant exposure in marine organisms are urgently needed. The goal of this dissertation has been to develop non-invasive ^{31}P-nuclear magnetic resonance (NMR) techniques to evaluate the effects of toxicant exposure on energy metabolism in marine invertebrates. Red abalone (*Haliotis rufescens*), the basis for an important commercial and sport fishery were chosen to serve as a model for other invertebrates due to the general physiological adaptations, such as their ability to survive extended periods of hypoxia, possessed by these animals, as well as their sedentary nature and large homogeneous foot muscle, which makes them ideal organisms for *in vivo* NMR spectroscopy.

Initial studies examined changes in adenosine triphosphate (ATP), phosphoarginine (PA), inorganic phosphate, intracellular pH, and intracellular free Mg^{2+} in response to exposure to the mitochondrial stressors hypoxia, pentachlorophenol (PCP), and sodium azide. These studies were also adapted for use with a larval abalone stage, and the effects of PCP exposure was examined in these animals.

Further studies refined these techniques to examine the rate of ATP formation from PA via the arginine kinase reaction using a magnetization transfer approach. Stress application increased rates of ATP formation, in contrast to mammalian systems, where declines in ATP formation are generally noted.

In the final study the sensitivity of the classical biochemical parameter adenylate energy charge (AEC) was compared with NMR derived measurements of cytosolic adenosine diphosphate (ADP) in response to the three stressors. Cytosolic ADP increased two-three fold in response to stress application while AEC showed little or no change. The effect of increasing ADP on arginine kinase flux is also discussed.

The work provided in this dissertation demonstrates some of the utility of *in vivo* approaches in evaluating the effects of sublethal stress exposure, and suggests future environmental applications in identifying currently unrecognized populations of marine organisms subject to toxicant induced stress.

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Many others have contributed their time and expertise to various portions of this work. In particular I would like to recognize Rob Franks for his willingness to always take the time to share his knowledge of analytical chemistry at a moments notice, and Jim Loo who has spent many hours helping me to understand the mechanics of a tempermental spectrometer. In addition, Dr. James "Beau" Willis has been instrumental in helping develop both the work contained here as well as my abilities as a scientist. Finally, I would like to thank my wife Sharon whose support and patience has been essential to the completion of this degree.

CHAPTER 1

ASSESSMENT OF TOXICITY IN MARINE ORGANISMS BY IN VIVO NMR: INTRODUCTION

Many marine species are suffering population declines that have significant ecologic, economic and recreational consequences. Though not fully understood, one potential factor for these declines results from the toxicological risks of sublethal chemical exposure. An organism's growth rate, fecundity, ability to defend against predators, as well as resistance to variable environmental conditions can be compromised by environmental conditions (Calow, 1991). The response of an organism to chemical toxicity is a function of specific biochemical actions and the activity of inherent detoxification mechanisms, yet *in vivo* descriptions of toxicological risks of chemical agents to marine organisms are generally described by gross behavioral or morphological responses (Parish, 1985; McKim, 1985; Luoma and Carter, 1993). These approaches are able to identify obviously harmful toxicant concentrations, but provide little information of the mechanism

of toxic action and generally do not have the sensitivity to detect sublethal stress. Conversely, description of the biochemical actions of toxicants in marine organisms has always involved invasive *in vitro* techniques which invariably fail to measure whole organism responses of the interaction of natural stress factors (Mehrle and Mayer, 1985). Therefore, the specific effects of potentially dangerous sublethal exposures on in vivo biochemical homeostasis are often unknown.

Descriptions based on sensitive, physiologically relevant, biochemical markers are urgently needed to assess the realistic long-term effects of known pollutants, detect sublethal effects of presently unrecognized chemical hazards and understand how important marine species respond to environmental stress. One technique which suggests a practical application able to fulfill these requirements is *in vivo* ^{31}P-NMR spectroscopy which provides a sensitive, non-invasive methodology to describe cellular biochemistry and sublethal toxic effects in intact living marine organisms. Biochemical processes can be measured in 'real-time' as they occur, advancing our understanding of both toxic mechanisms of action and the interactive effects of environmental stress factors.

NMR is a non-invasive technique which can detect nuclei possessing a magnetic moment (^{1}H, ^{13}C, ^{15}N, ^{31}P). When placed in a strong magnetic field, these nuclei populate varying energy states as determined by the Boltzman distribution resulting in a slight deviation from unity within the energy states of the sample, and therefore a net magnetic moment aligned with the applied magnetic field. This net magnetization may be perturbed through the application of electromagnetic radiation (1-750 MHz), with spectra acquired during the return of the magnetization to its equilibrium state. The resonant frequency may be determined using equation (1):

$$\omega=\gamma B_0 \qquad (1)$$

in which ω is the angular frequency, γ is the nucleus gyromagnetic ratio, and B_0 is the applied magnetic field. The low radiofrequency energy associated with the NMR

experiment permits the nondestructive evaluation of suitable concentrations of sensitive nuclei within living organisms, while variations in electron density surrounding nuclei of different molecules results in alteration of the observed B_0 values at the nucleus, and so different resonant frequencies permitting molecular identification. For a more complete discussion of the NMR phenomenon the reader is referred to the work of Farrar and Becker (1971).

The work described in this dissertation has focused on developing applications of ^{31}P-NMR for use with abalone as a model marine organism. Phosphorus NMR is particularly well suited for toxicological studies due to the central role of ATP as the energy-currency of cellular metabolism in all living organisms (Atkinson, 1977). Consequently, virtually any type of toxicant induced perturbation would result in the increase in activity of an organism's homeostatic mechanisms with a subsequent increase in energy utilization, observable via the appropriate ^{31}P-NMR technique.

Abalone occur throughout the world's oceans, with five species present along the California coast. Red abalone (*Haliotis rufescens*), the largest abalone species, are found from the low intertidal to subtidally (180 m), ranging from Sunset Bay, Oregon to Bahia de Tortugas, Baja Mexico (Abbott and Haderlie, 1980). Adult animals occur as separate sexes, and reproduction is achieved through the release of gametes into the water column where fertilization occurs. Swimming trochophore larvae hatch from fertilized eggs after 12-24 hrs depending on water temperature, and then develop into the characteristic shelled veliger larvae which remain swimming for an additional 4-14 days until settlement and metamorphosis occur (Abbott and Haderlie, 1980). Larval abalone are thought to be highly sensitive to environmental conditions and toxicants, and so have been used in California state mandated toxicity tests for effluent discharges.

The abalone fishery has been in decline in California since the peak catch of 4,964,000 lbs in 1966, down to current catch of 265,147 lbs in 1995 (CDFG, 1997). Declines in catch size have fostered the growth of abalone mariculture, with current production (256,582 lbs in 1994) roughly equivalent to fished abalone landings for the same year (CDFG, 1996). Abalone mariculture is a growing industry with sales increasing from $1,976,000 in 1992 to $3,851,251 in 1995, and so represents an important sustainable industry for California.

Declining catch is primarily attributed to overfishing of these slow-growing molluscs which has resulted in the near complete stripping of fishing grounds, particularly in the more productive waters south of San Francisco to southern California. Consequently, the state legislature is currently considering an abalone fishing moratorium in this area. However, abalone populations have also suffered from a pair of troublesome events which have recently occurred in southern California which may prove to be the final demise of abalone in that region. During the late 1980's, black abalone (*H. cracherodii*) in the Channel Islands began to suffer mass mortality associated with a wasting of tissue mass known as withering syndrome which continues presently, and has resulted in 90% population declines in effected areas (Richards and Davis, 1993) The syndrome has been associated with a prokaryote pathogen (Gardner et al., 1995) although others have suggested toxicant contamination as a possible contributing factor (Vanblaricom et al., 1993). The ultimate cause of withering syndrome is still unknown. In more recent years sex ratios of remaining wild abalone have become severely skewed with the virtual disappearance of male animals throughout the state (P. Haaker, personal communication). This phenomenon has been previously observed by mariculturists in the late 1960's (D. Ebert, personal communication) to be associated with unusually heavy rainfall in the previous winter, leading to one working hypothesis that the additional rainfall moves terrestrial toxicants with estrogenic effects into the coastal ocean inducing sex change in

male animals (P. Haaker, personal communication). A better understanding of abalone response to toxicants in necessary to answer these important questions.

As intertidal organisms, abalone may be subject to periods of hypoxia due to immersion at low tide, and, like many molluscs, have a well developed capacity to maintain anaerobic respiration during these periods. In the foot muscle (up to 50% of body mass) ATP concentrations are maintained by utilization of the high-energy phosphagen phosphoarginine (PA) resulting in the increase in inorganic phosphate (P_i) concentrations (Tjeerdema et al., 1991) during hypoxia. This system is only able to maintain ATP concentrations for brief periods, and so longer bouts of hypoxia require glycolytic energy production, resulting in intracellular acidification and the accumulation of lactate and tauropine (a conjugation product of taurine and pyruvate which is thought to be a functional equivalent to lactate). In addition longer periods of hypoxia may also induce metabolic suppression as seen in other molluscs (Storey, 1993) although this response has not been characterized in abalone.

The mode of action of many pollutants is the disruption of mitochondrial oxidative phosphorylation through the inhibition of electron transport (hydrogen sulfide, tributyltin, rotenone; Corbett, 1984) or by uncoupling ATP formation from the proton gradient generated by electron transport (pentachlorophenol, *p*-nitrophenol, arsenic; Corbett et al., 1984). These compounds cause oxidative ATP formation to be less efficient resulting in an energetic debt within the organisms which if not replaced, results in death of the organism (Tjeerdema et al., 1991). Therefore, the effects of an oxidative phosphorylation inhibitor and uncoupler (to be used as models of environmentally significant pollutants) on energetic metabolism were examined. Sodium azide blocks electron transport at cytochrome oxidase and so should act in a similar manner as hypoxia, while the model uncoupler pentachlorophenol shuttles protons across the inner mitochondrial membrane, resulting in a reduction of the gradient necessary to drive ATP synthesis (Stryer, 1988; Corbett et al.,

1984). The overall goal of this dissertation has been to examine the mechanisms whereby mitochondrial poisons induce sublethal toxicity in abalone. In light of the extensive anaerobic capacity of abalone (from 12-48 hrs if kept cool; Bowen, 1987) why do these type of compounds cause mortality in abalone after periods which are much shorter than the sustainable hypoxic period.

The specific objectives of these studies were to develop a system suitable for use in commonly available vertical bore spectrometers, to assess the relevant biochemical changes in response to toxicant or hypoxia exposure in living abalone using ^{31}P-NMR spectroscopy. This system is described in Chapter 2, and then adapted in Chapter 3 for use with larval animals. In Chapter 4 the experiments were further refined through the use of the saturation transfer technique to examine the effects of the stressors on rates of arginine kinase mediated ATP formation, and finally in Chapter 5 the effects of the stressors on a classical biochemical parameter, adenylate energy charge, are compared with the results obtained by *in vivo* NMR.

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CHAPTER 2

EFFECTS OF HYPOXIA AND TOXICANT EXPOSURE ON PHOSPHOARGININE, INTRACELLULAR pH, AND FREE Mg^{2+} IN ABALONE AS MEASURED BY ^{31}P-NMR

INTRODUCTION

Many toxicants found in aquatic systems act by disturbing energetic metabolism within an organism (Calow, 1991). Mechanistic studies of these compounds have focused on cell cultures or subcellular organelles, and so the biochemical effects of sublethal toxicant exposures in whole organisms is often unknown. One technique which has proved valuable in studying energy metabolism in whole tissues and organisms is ^{31}P nuclear magnetic resonance spectroscopy (NMR), which permits the non-invasive evaluation of high-energy phosphorylated compounds such as adenosine triphosphate (ATP), ortho-phosphate (P_i), and phosphocreatine or phosphoarginine (an invertebrate analog of phosphocreatine; PA), as well as the determination of intracellular pH (pH_i; Gadian, 1982); it is the method of choice for the evaluation of intracellular free Mg^{2+} (Mg_{free}; Gupta et al., 1978; Gupta and Moore, 1980; Williams and Smith, 1995). Intracellular pH declines with anaerobic respiration (Hochachka and Somero, 1984; Portner, 1993), and so serves as a qualitative measure of the extra-mitochondrial contribution to energy metabolism. In

addition, declines in pH effect glycolysis as well as other regulatory functions in the cell (Portner, 1993), and so a better understanding of the effects of xenobiotics on pH_i will help to elucidate the role of these compounds in disrupting cellular function. Magnesium is of primary importance in cellular functions because of the complexation with adenine nucleotides, where the Mg^{2+}-adenylate complex forms the true substrate with most kinases and phosphotransferase enzymes (Aikawa, 1981). In addition, Mg^{2+} also has been implicated in regulation of pH_i (Hochachka and Mommsen, 1983; Russell and Brodwick, 1988), as well as control of mitochondrial metabolism and transport reactions (Jung and Brierly, 1994).

Abalone, marine gastropod molluscs, represent an important commercial and recreational fishery, and are ideal organisms for *in vivo* NMR due to their large, homogeneous foot muscle and sedentary nature. Previous studies using aquatic organisms have demonstrated the use of ^{31}P NMR to evaluate the effects of hypersalinity (Higashi, et al., 1988; Tjeerdema et al., 1996), hypoxia/anoxia (Tjeerdema et al., 1991b), temperature changes (Tjeerdema et al., 1993), toxicant exposure (Aunaas et al., 1991; Tjeerdema et al., 1991a), and the action of multiple stresses (Tjeerdema et al., 1991c). NMR studies of hypoxia by emergence in red abalone (*Haliotis rufescens*) resulted in declines in PA and pH_i, coincident with increases in P_i (Tjeerdema et al., 1991b), while studies of the in vivo effect of pentachlorophenol (PCP) exposure in abalone resulted in greater declines in PA, ATP and pH_i, along with increases in P_i, and the TCA cycle intermediates succinate, malate, citrate, as well as lactate (Tjeerdema et al., 1991a)

The goal of the present study was to evaluate the differences in biochemical response (as measured by changes in concentration of high-energy phosphagens, pH_i, and Mg_{free}) of red abalone, exposed to several stresses with well-defined biochemical mechanisms of action. The three stresses to be evaluated were hypoxia, sodium azide (NaN_3), an inhibitor of mitochondrial electron transport (Smith and Wilcox, 1994), and

PCP, an uncoupler of mitochondrial oxidative phosphorylation (Weinbach and Nolan, 1956). Through a better understanding of the response of the integrated organism to these toxicants it may be possible to identify the modes of action of other, not as well understood, xenobiotics. In addition, this study demonstrates a modification of the previously described exposure system and surface probe (Tjeerdema et al., 1991a) which permits the use of vertical bore NMR spectrometers, present at most research univeristies, as opposed to previous studies which required less common specialized large-bore horizontal magnets.

MATERIALS AND METHODS

Chemicals

Methanol, NaN_3, $MgCl_2$, HCl, and K_2CO_3 were purchased from Fisher Scientific Inc., while PCP (sodium salt) and perchloric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI), and ATP was purchased from Sigma (St. Louis, MO). Filtered seawater (0.2 μm) was obtained from the UCSC Long Marine Laboratory and used within 6 hrs.

Animals

Red abalone (averaging 2 yrs and 40 mm maximum shell width) were obtained from U.S. Abalone (Davenport, CA) and maintained in fiberglass aquaria with flowing seawater for a minimum of 2 weeks prior to use. They were fed giant kelp (*Macrocystis pyrifera*) *ad libitum* and acclimated at ambient seawater temperatures.

Exposure System

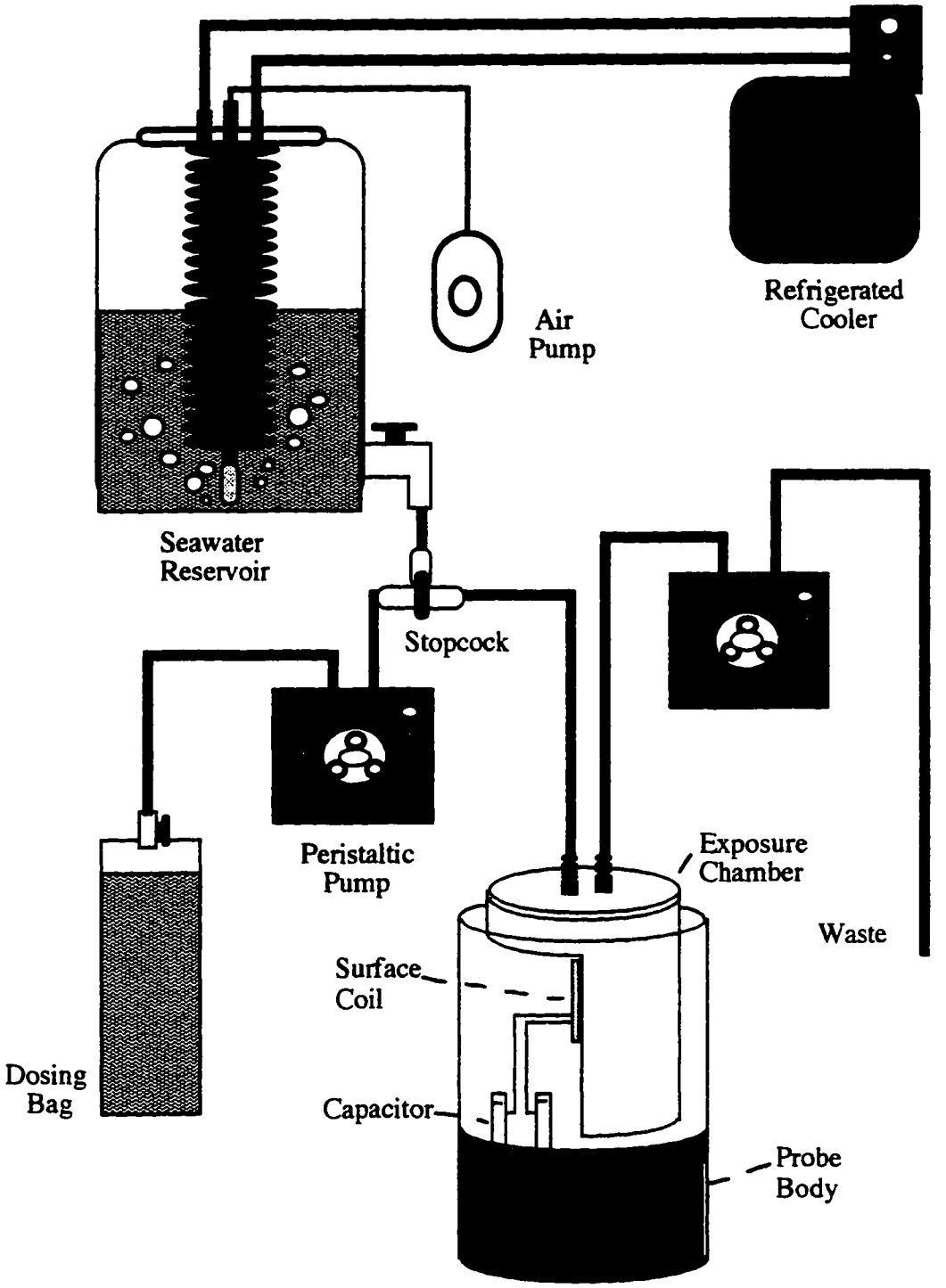
The flow-through exposure system previously described by Tjeerdema et al. (1991a) has been modified for use with vertical-bore spectrometers. The exposure chamber

(Fig. 1) was milled from a solid Teflon® core (diameter, 6.64 cm; height, 9.39 cm; chamber volume, 90 ml) and sealed with an O-ring mounted on a threaded Teflon® lid fitted with fluoropolymer nipples (0.90 cm thread O.D.) to permit the passage of seawater through the chamber. An animal was placed within the chamber in the vertical position, locating the foot muscle in the proper orientation for observation with the surface coil. Water was drawn (20 ml min^{-1}) from a chilled, aerated seawater reservoir, through silicon tubing (size 16, Cole-Parmer, Chicago) via a single peristaltic pump (Masterflex model 7523-00) equipped with a Quick-load pump head. A second peristaltic pump metered in a concentrated toxicant solution stored in a chilled Tedlar® fluoropolymer bag (AeroVironment, Monrovia, CA).

NMR spectroscopy

A 2-cm, 2-turn photoetched (Fan and Higashi, 1989) spiral surface coil was employed for all animal studies, utilizing a 35 μs pulse width, ± 5,000 Hz sweep, and a 250 ms delay for the acquisition of ^{31}P spectra at 121.48 MHz on a 7.05 Tesla, General Electric GN-300 spectrometer equipped with a Nicolet 1280 computer. Each spectral point is composed of 1,000 acquisitions accumulated in 6.11 min, with 2K zero filling to 4,096 data points, and 20 Hz exponential multiplication applied prior to Fourier transformation. The proton resonance from water was used for magnetic field (B_0) optimization, where ^{1}H spectra were acquired without retuning of the probe (Griffiths et al., 1981). Spectra for the pH and Mg^{2+} calibrations were acquired using a conventional 12 mm probe, 17.5 μs pulse width, 250 ms delay, 4,096 data points, ±5,500 Hz sweep width, and 10 Hz exponential multiplication prior to Fourier transformation.

Figure 1. The flow-through exposure apparatus used for in vivo NMR studies of red abalone with a vertical-bore spectrometer. The surface probe is constructed using a conventional probe body with the saddle coil removed.



Magnesium and pH calibration

Magnesium calibration solutions were prepared using abalone foot muscle which was extracted by homogenization using a Waring blender with addition of perchloric acid (1 ml 0.4 M PCA g^{-1} tissue), pelleted by centrifugation, adjusted to pH 6.0 with 1.0 M K_2CO_3, and repelleted, while an additional set of tissue extracts was prepared to determine the resting concentrations of PA and ATP using the high pressure liquid chromatography (HPLC) method described below. The supernatant was frozen on dry ice and lyophilized, then returned to physiological concentration by adjusting the extract volume until $[Mg^{2+}]$ was equivalent to homogenate values, as determined by atomic absorption (AA) flame photometry (1 M KCl used as diluent and for calibration curve generation). Magnesium was removed from the reconstituted extract using Chelex-100 (Biorad, Richmond, CA) batchwise at pH 8.8 until $[Mg^{2+}]$ was at background levels as determined by flame AA.

ATP concentrations of the solution were determined by HPLC (Ally and Park, 1992). Briefly, chromatography was conducted using a C_{22} column (ES Industries Berlin, NJ) equipped with a C_{22} guard column, and two mobile phases: A; 35mM KH_2PO_4, 6 mM tetrabutyl ammonium chloride, pH 6.0, and B; 50% A and 50% MeOH, pH 6.0. The gradient program was as follows; 100% A for the first three minutes followed by a 3-min linear ramp to 50% A, 50% B which was held for 14 min and returned to 100% A using a 4-min linear ramp, flow rate 1 ml min^{-1} with UV detection at 214 nm. After ATP quantification, the solution was brought to 5 mM. The solution was aliquoted in small volumes at nine pH's ranging from 4.5 to 8.0, and spectra were acquired as each solution was serially titrated with concentrated $MgCl_2$ (1.10, 2.20, 4.40, 8.80, 16.59, 32.14, 63.24, and 125.44 mM Mg^{2+}, respectively). The following relationship was used to evaluate Mg_{free} (Fig. 2):

$$[Mg_{free}] = K_d \frac{\delta_{ATP} - \delta_{obs}}{\delta_{obs} - \delta_{MgATP}} \quad (1)$$

Where Mg_{free} is the concentration of intracellular free magnesium, K_d is MgATP binding constant, δ_{ATP} is the chemical shift difference between the α and β ATP resonances in the absence of Mg^{2+}, δ_{obs} is the observed $\alpha\beta$ chemical shift difference, while δ_{MgATP} is the $\alpha\beta$ chemical shift difference in the presence of excess Mg^{2+} (Williams et al., 1993). The K_d of MgATP was derived as a function of pH according to Bock *et al.* (1985):

$$K_d(pHx) = \frac{1 + 10^{pKa - pHx}}{1 + 10^{pKa - pHy}} K_d(pHy) \quad (2)$$

Where K_d(pHx) is the K_d value at the pH of interest, pKa is the negative log of the Ka of ATP at physiologic Mg^{2+} concentrations, and K_d(pHy) is the K_d value determined at a particular pH. A K_d value of 169.70 μM at pH 7.4 was used for all calculations (Doumen and Ellington, 1992). The $\delta_{\alpha\beta}$ values from the calibration curve for δ_{ATP} (0 mM Mg^{2+}) and δ_{MgATP} (32.14 mM Mg^{2+}) were fit to the Henderson-Hasselbach equation (Williams et al., 1993):

$$pH = pKa + \log \frac{\delta_{acid} - \delta_{obs}}{\delta_{obs} - \delta_{base}} \quad (3)$$

where δ_{acid}, δ_{obs}, and δ_{base} are the differences in the chemical shifts at acidic, observed, and basic pH of the compound of interest, respectively.

Intracellular pH was determined from chemical shift difference between the PA and P_i resonances (Gadian, 1982). A calibration solution was constructed by powdering abalone foot muscle frozen in liquid N_2 in a mortar and pestle, then centrifuging (30,000x

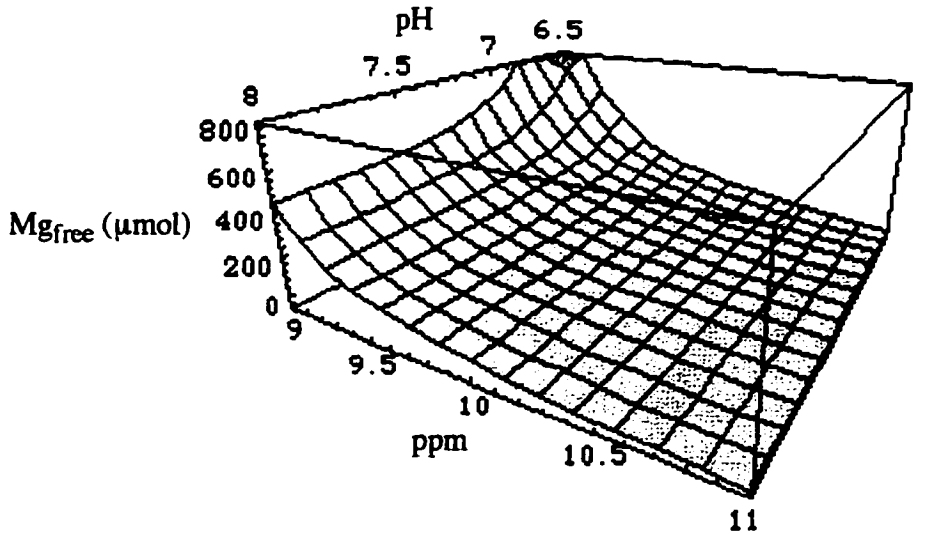


Figure 2. Calculated surface for changes in intracellular free Mg^{2+} with pH and chemical shift difference between α and β ATP resonances. See text for details of the calculations.

g, 45 min) the thawed tissue to extract the intracellular fluid. The supernatant protein was precipitated with a minimal volume of 6 N HCl and pelleted, followed by addition of PA standard. The pH of the solution was then adjusted and spectra were acquired for nine pH values ranging from 5.5 to 8.1. The data from the calibration solution was fit to equation 3 using MacCurve Fit (Raner Software, Australia) and δ_{acid}, δ_{base}, and P_i p*K*a determined to be 3.84 ± 0.22 ppm, 6.56 ± 0.11 ppm, and 6.70 ± 0.20 respectively (R^2 = .931).

Experimental Procedure and Data analysis

Animals were individually exposed to each type of stress (N=5), and were only subjected to a single experimental period. All animals were placed in the exposure chamber with recirculating seawater 12 hrs prior to the beginning of each experiment to allow acclimation. Reference spectra were collected for 1 hr (10 data points) prior to toxicant

exposure. Stock chemical solutions (4.8 mg PCP L^{-1} dissolved in 250 µl MeOH carrier, or 200 mg NaN_3 L^{-1}) were mixed in 1 L of filtered seawater within the toxicant reservoir, and peristaltically pumped at 5 ml min^{-1} into the clean seawater flow (20 ml min^{-1}) to achieve a 4-fold dilution of the concentrated stock, while hypoxia studies were conducted using Tygon® vinyl tubing (replacing the O_2-permeable silicon tubing) and a chilled N_2-bubbled seawater reservoir, during the 2-hr exposure periods. Oxygen concentrations were monitored at the waste water outlet using a dissolved O_2 electrode (Orion Instruments Cambridge, Mass) and were maintained $\leq$ 1.0 ppm O_2 throughout the hypoxia exposure period. Chemical and hypoxia-exposed animals were allowed to recover in clean flowing seawater for 6 or 3 hrs respectively.

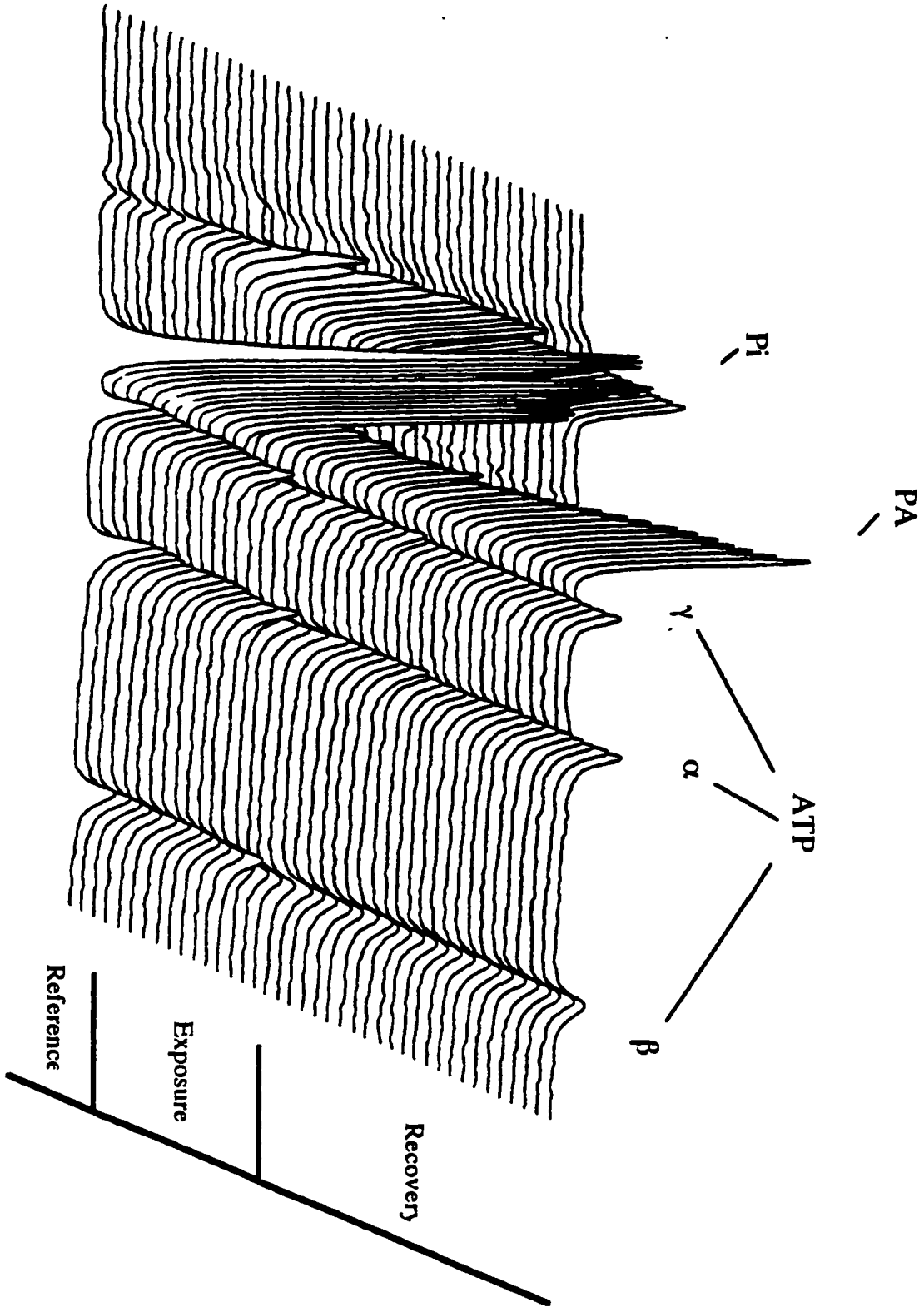
Changes in phosphagen resonance intensity were used to evaluate the effects of toxicant exposure, where reference period intensities for P_i, PA, and β-ATP resonances were averaged, and each data point intensity was then divided by the average reference intensity, and pH_i and Mg_{free} were determined as described above. Statistical tests were conducted by one-way ANOVA or Student-Neuman-Keuls *t*-Test as appropriate (Glantz, 1987).

RESULTS

^{31}P NMR Spectroscopy

A series of representative spectra are shown in Figure 3. Peak assignments were guided by comparison of spectra of PCA extracts with known standards. Resonances of interest include orthophosphate (P_i), phosphoarginine (PA), and the three nucleoside triphosphate resonances which, based on HPLC analysis of PCA extracts, are predominantly attributable to the α, β, and γ resonances of ATP. Resting foot muscle

Figure 3. Representative time course sequence of ^{31}P NMR spectra taken from an individual abalone exposed to 1.2 ppm PCP. Resonances of interest include orthophosphate (P_i), phosphoarginine (PA), and the α, β, γ resonances of adenosine triphosphate (ATP), which also include the α and β resonances of adenosine diphosphate at the ATP α and γ positions respectively, while reference, exposure, and recovery periods are 1, 2, and 6 hrs respectively. Each spectrum represents 1000 aquisitions averaged over 6.11 min.



metabolite concentrations were 9.5 and 2.0 mM for PA and ATP respectively. All quantification was based on resonance intensity, normalized to average resonance intensity during the reference period for each individual. Chemical shifts for determination of pH_i and Mg_{free} were taken from the resonance apex.

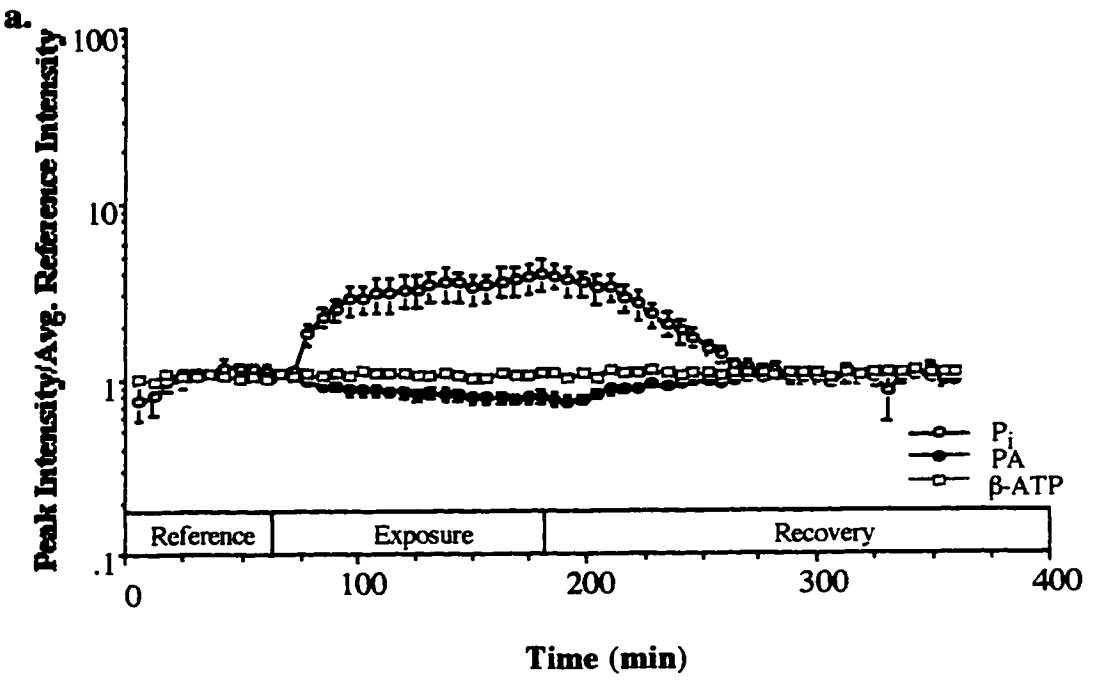
Hypoxia-Exposed Abalone

Hypoxic (1.0 ppm O_2) abalone displayed moderate response as measured by changes in high-energy phosphorylated compounds and pH_i (Fig. 4). Onset of effect was rapid, and stabilized after 40 min of exposure. Ortho-phosphate peak intensity increased 3.98 over average reference P_i intensity, the PA minimum was 0.73, and time to recovery (determined as the return of P_i to $\leq$ 1.1 of the reference intensity) averaged 4.77 hrs (1.77 hrs after the end of the exposure period). Intracellular pH and Mg_{free} showed no significant changes ($p > 0.05$; Table 1) throughout the exposure and recovery periods, although declines in Mg_{free} from a reference period average of 739 μM to 640 μM during 2.5 to 3.5 hrs following the start of the experiment were detected (Fig. 8a), followed by recovery to reference values in the final hour of the experiment (Table 1). No changes were observed in β-ATP resonance intensities throughout the entire course of all exposures. The large variability in all exposures in pH_i during the reference and recovery periods is likely due to the small signal/noise ratio in the P_i resonance, which causes large variability in the observed P_i resonant frequency. Calculations of Mg_{free} are also effected due to the pH dependence of K_d, δ_{ATP}, and δ_{MgATP} values.

Azide-Exposed Abalone

Azide exposure resulted in a more severe response, with maximum P_i increase of 10.66, while PA levels declined to 0.53 (Table 1). The phosphagen response showed rapid

Figure 4. Phosphagen (a) and intracellular pH (b) response in $\leq$ 1.0 ppm O_2 exposed abalone. Phosphagen resonance intensities are plotted proportional to average reference period intensities for a given compound ± SEM. Intracellular pH was determined as described in the text. Each point represents 1000 spectra averaged over 6.11 minutes, and N = 5 for all points.



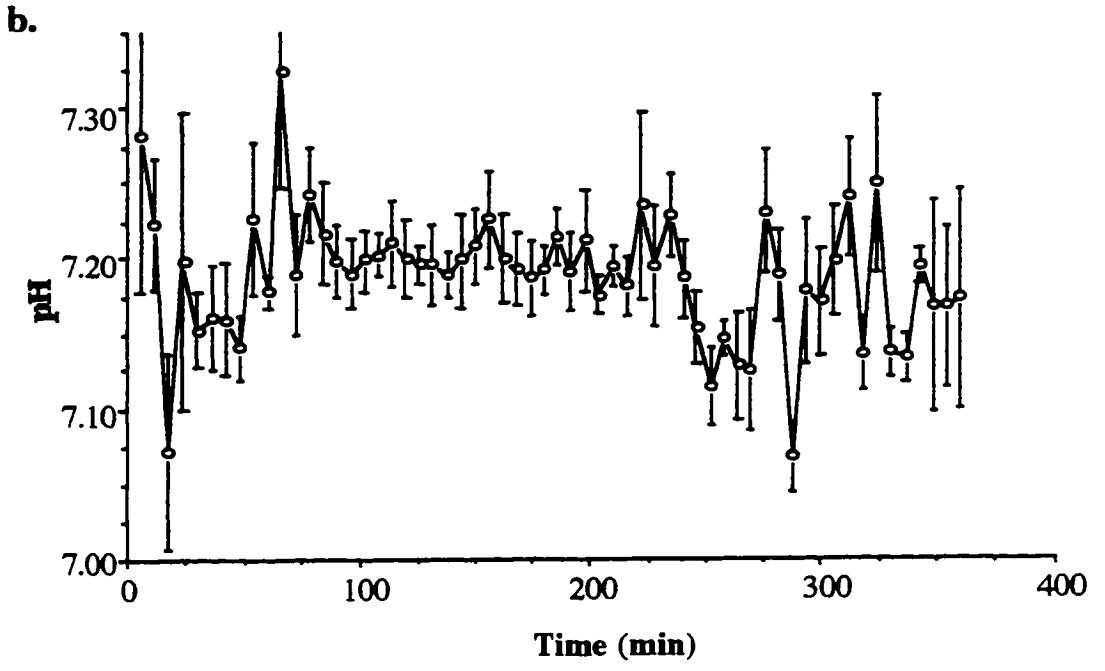


Table 1. Changes in pH_i, Mg_{free}, and phosphagens under the three exposure conditions.

	Hypoxia	50 ppm Sodium Azide	1.2 ppm PCP[c]
pH_i[a]			
Reference	7.19 ± 0.04	7.17 ± 0.04	7.18 ± 0.05 {7.16 ± 0.03}
Exposure	7.20 ± 0.02	7.06 ± 0.02[d]	7.14 ± 0.02 {7.13 ± 0.02}
Recovery	7.19 ± 0.04	7.13 ± 0.01	7.13 ± 0.02 {7.05 ± 0.02}[d]
Mg_{free} (μM)[a]			
Reference	739 ± 59	854 ± 123	867 ± 171 {928 ± 13}
Exposure	640 ± 57	832 ± 110	775 ± 121 {935 ± 44}
Recovery	742 ± 74	1008 ± 148	892 ± 147 {918 ± 23}
Phosphagen Changes[b]			
P_i Maximum	3.98 ± 0.77	10.66 ± 2.71	6.56 ± 1.48 {15.33 ± 1.73}
PA Minimum	0.73 ± 0.05	0.53 ± 0.06	0.65 ± 0.05 {0.50 ± 0.06}
Time to Recovery (hrs)	4.77 ± 0.17	6.56 ± 0.44	7.83 ± 0.69 {NA}

[a] Intracellular pH and Mg_{free} values ± SEM, are the average of 10 data points at reference (0-60 min), exposure (150-210 min), and recovery (480-540 min) periods.

[b] Maximum changes in Pi and PA intensity ± SEM were averaged for each exposure type, and did not necessarily occur at the same time point for each animal. Time to recovery as determined by the return of P_i to 1.1 reference average value.

[c] PCP values from abalone 1-3 and {4, 5} respectively.

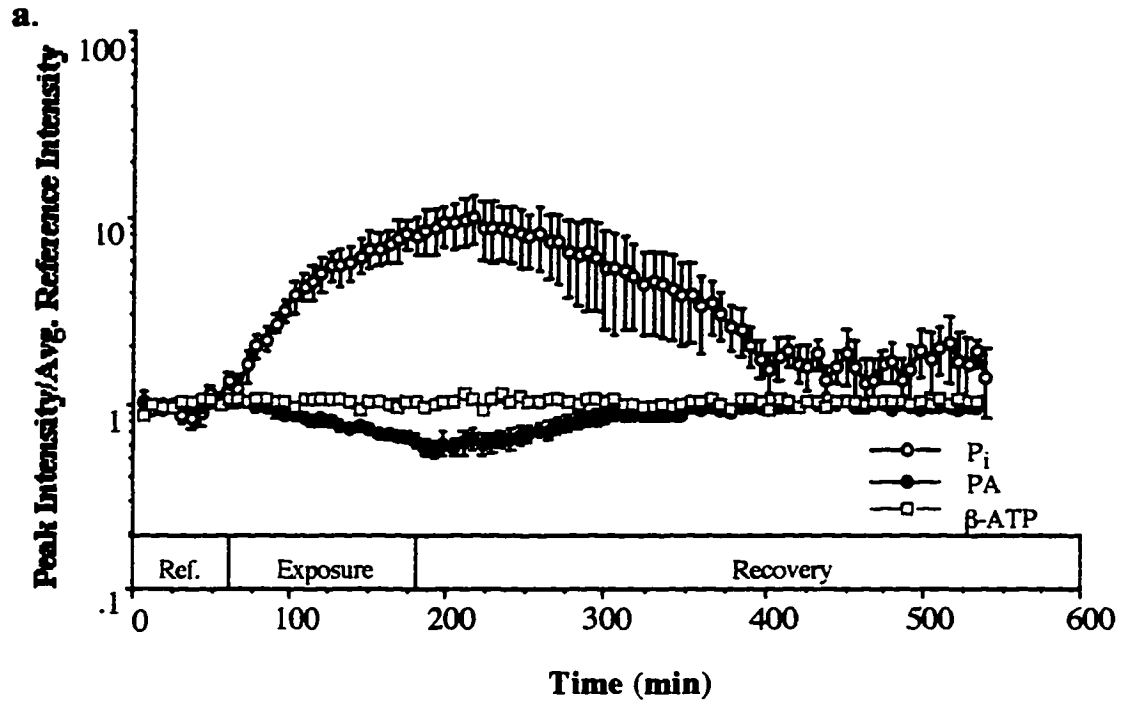
[d] $p < 0.05$

onset of effects, beginning almost immediately after start of exposure, and peaking just after the end of the exposure period, followed by initiation of recovery, without an intervening stabilization in concentration of phosphorylated compounds (Fig. 5). Recovery times were 6.56 hrs although, interestingly, three of the five animals displayed increases in Pi intensities following the initial recovery of P_i to reference levels, which is reflected at 400 min by the leveling off of P_i intensity at roughly twice the reference average, despite the complete recovery of PA concentrations. Intracellular pH significantly declined from an average of 7.17 to 7.06 ($p < 0.05$) at 2.5-3.5 hrs into the study, and then returned to 7.14 (no significant difference when compared with reference values) in the final hour of recovery. Average Mg_{free} showed little change between reference and exposure periods (854 μM and 832 μM respectively; Fig. 8b), however, values had increased, although not significantly, to 1009 μM ($P > 0.05$) by the final hour of recovery.

PCP-Exposed Abalone

PCP-exposed animals all displayed similar severity of response, but a bimodal recovery, and so are presented separately (Figs. 6 and 7). Three animals (Fig. 6) displayed response and recovery patterns similar to those described above, with increases in P_i to 6.53, declines in PA to 0.65 (Table 1), and average recovery times of 7.98 hrs from the start of the experiment. Increases in P_i did not begin to occur for approximately 1 hr after start of exposure, and stabilized just after the end of the exposure period. Phosphagen concentrations remained essentially stable from 200-300 min, and gradually recovered to reference values. Intracellular pH displayed surprisingly moderate changes, declining from 7.18 during the reference period to 7.14, and 7.13 during the exposure period and final hour of recovery respectively. No noteworthy changes in Mg_{free} were observed throughout the experiments (Fig 8c).

Figure 5. Phosphagen (a) and intracellular pH (b) response in 50 ppm sodium azide exposed abalone. Phosphagen resonance intensities are plotted proportional to average reference period intensities for a given compound ± SEM. Intracellular pH was determined as described in the text. Each point represents 1000 spectra averaged over 6.11 minutes, and N = 5 for all points.



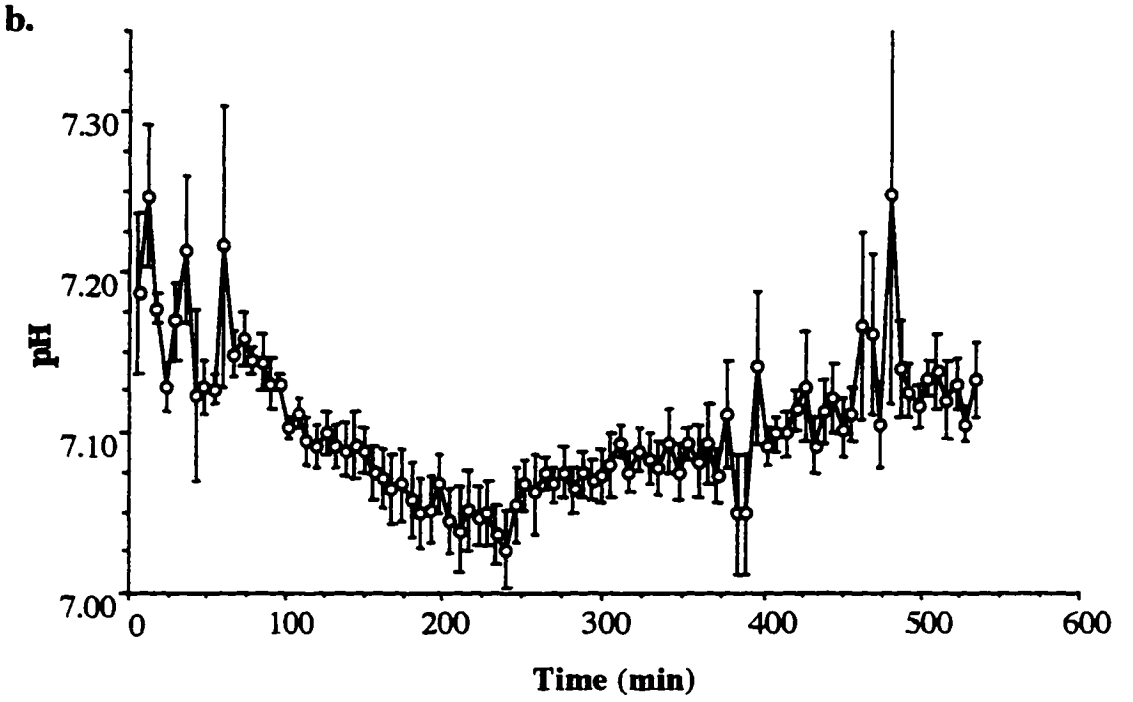
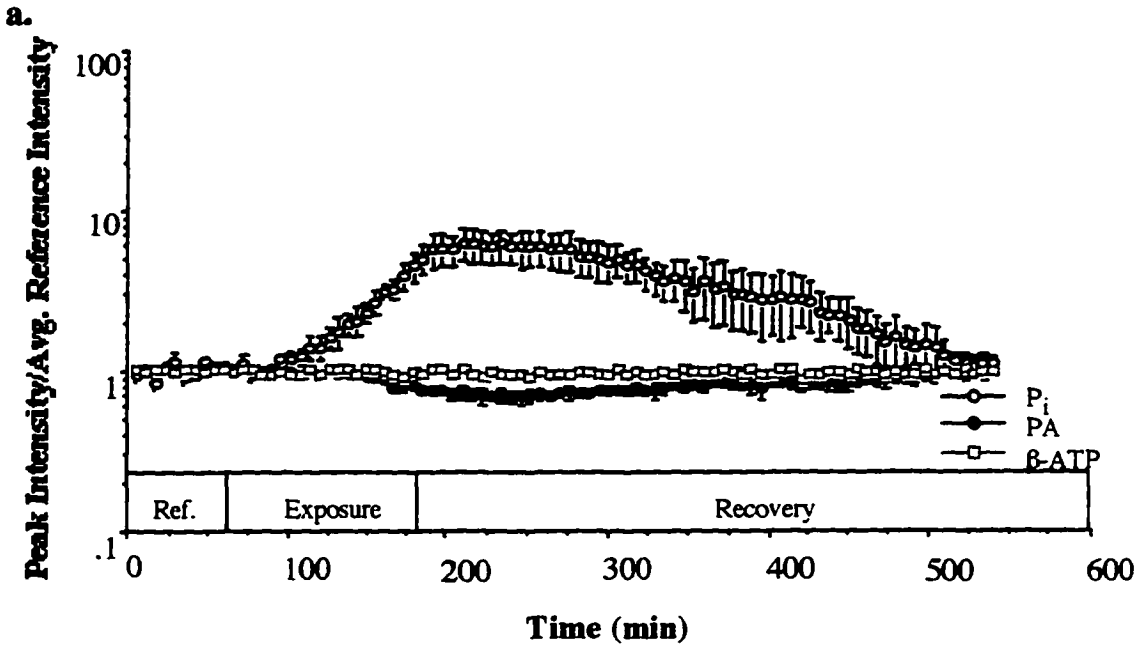


Figure 6. Phosphagen (a) and intracellular pH (b) response in 1.2 ppm pentachlorophenol exposed individuals 1-3. Phosphagen resonance intensities are plotted proportional to average reference period intensities for a given compound ± SEM. Intracellular pH was determined as described in the text. Each point represents 1000 spectra averaged over 6.11 minutes, and N = 3 for all points.



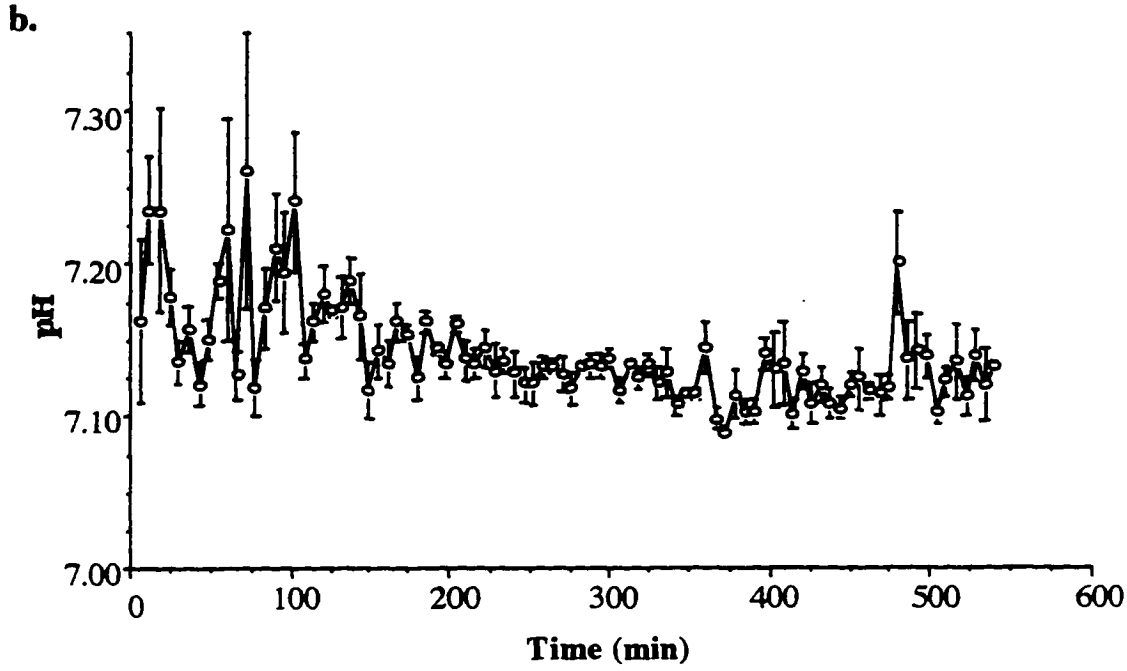
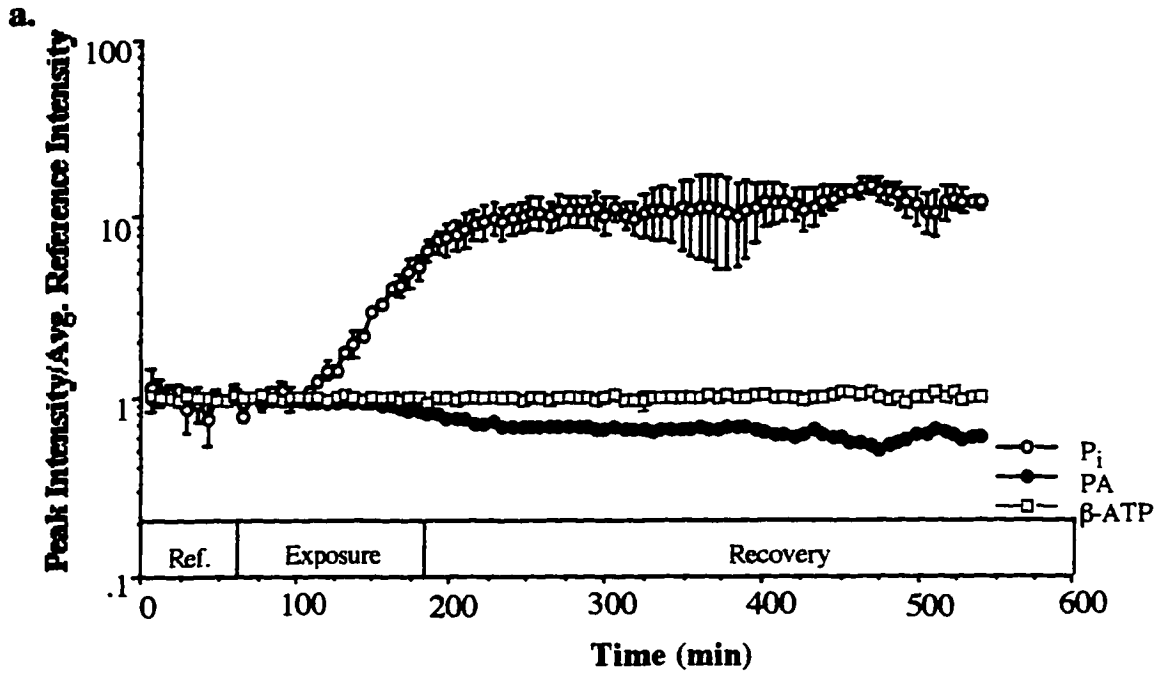
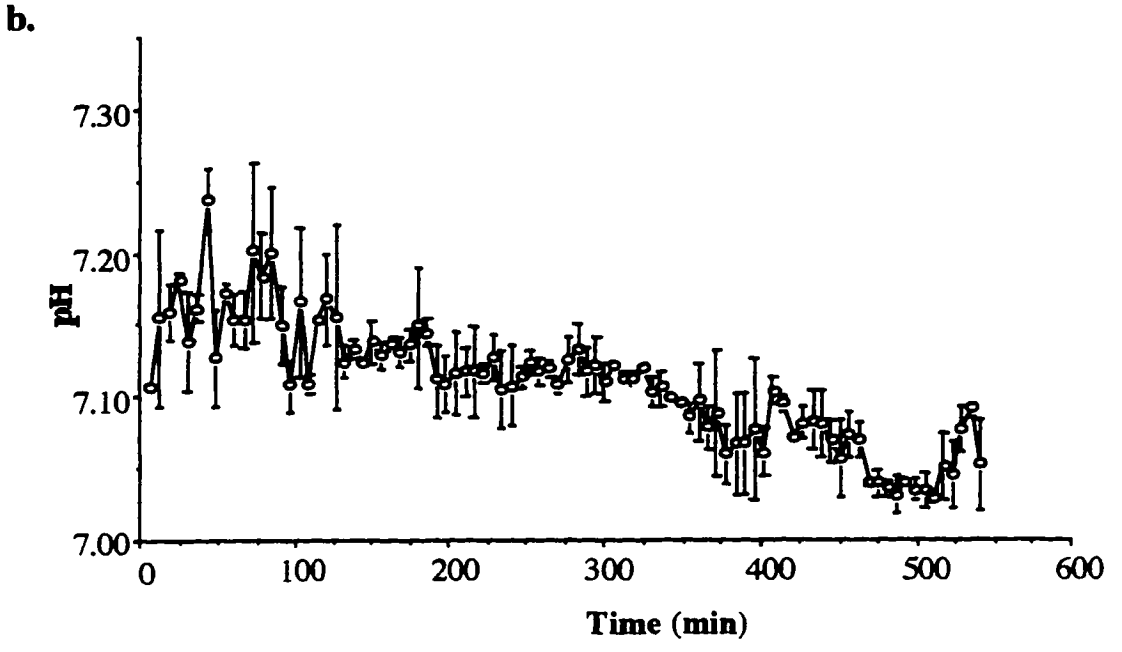


Figure 7. Phosphagen (a) and intracellular pH (b) response in 1.2 ppm pentachlorophenol exposed individuals 4, 5. Phosphagen resonance intensities are plotted proportional to average reference period intensities for a given compound ± SEM. Intracellular pH was determined as described in the text. Each point represents 1000 spectra averaged over 6.11 minutes, and N = 2 for all points.





Two other individuals displayed more severe responses to 1.2 ppm PCP exposure, with P_i levels rising to 15.33, while PA concentrations dipped to 0.50 (Fig. 7), and recovery did not occur within the 6 hr observation period. Initial response was similar to the animals described above, although P_i stabilized 10-fold higher (compared with 6.53) than reference values, but was followed at 425 -525 min by further increases/decreases of Pi/PA to their extreme values respectively. Intracellular pH showed only small but significant changes from reference values during the exposure period (7.16 to 7.13, $p < 0.05$), but declined dramatically to 7.05 ($p < 0.05$) during the final hour of the recovery period. Again no significant changes were observed in Mg_{free}.

DISCUSSION

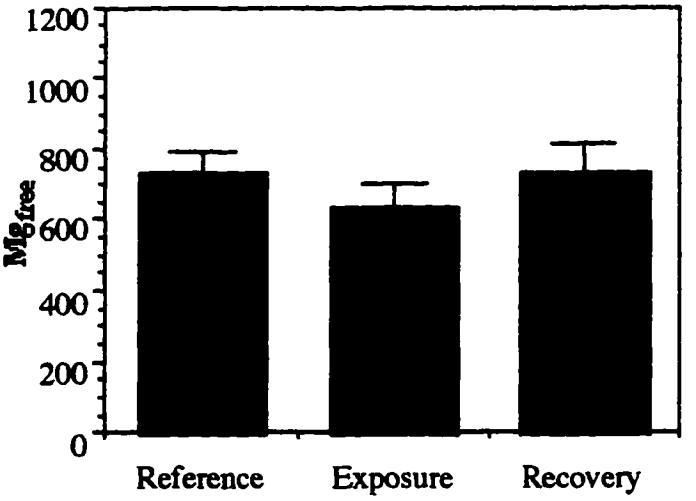
Mg_{free} Determinations

Intracellular Mg_{free} concentrations have not previously been described for any intact marine organisms. Average Mg_{free} were 830 ± 225 μM Mg^{2+} (combined reference period average for all three exposures; N = 15), which correlates well with values previously determined using ^{31}P NMR, which range from 336 to 1,200 μM Mg_{free} for rat heart and brain, frog leg muscle, and horseshoe crab muscle (Gupta and Moore, 1980, Murphy et al., 1989; Kirkels et al., 1989; Doumen and Ellington, 1992; Williams and Smith, 1995). Changes in Mg_{free} reflect changes in [MgATP], where only MgATP is thought to be active in most ATP utilizing reactions (Aikawa, 1981), and so toxicant induced changes in Mg_{free} may effect ATP utilization, without altering absolute ATP concentration. Resting values of free ATP ranged from 15-20%, although values as high/low as 25%/5% were seen in hypoxic and azide exposed animals respectively.

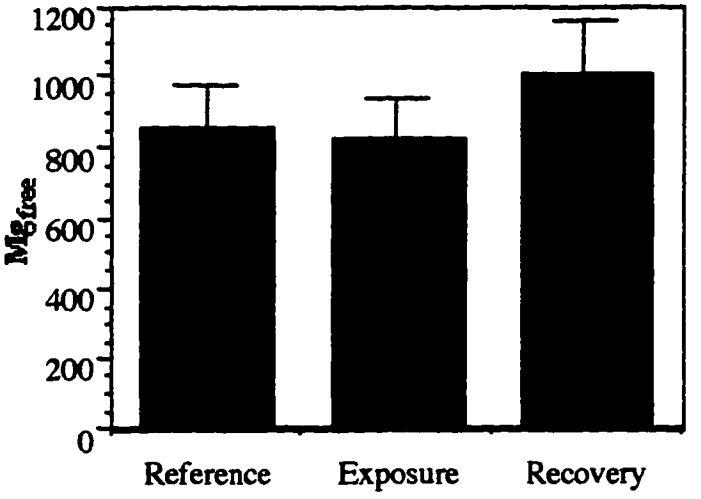
Accuracy of Mg_{free} determinations are subject to accuracy of pH_i determinations, chemical shift values for free and Mg^{2+} bound ATP, and variability in determination of *in*

Figure 8. Changes in free Mg^{2+} for hypoxia (a), azide (b), and PCP (c) exposed animals ± SEM. Values shown are averages of 10 data points for all animals within an exposure group taken from reference (0-60 min), exposure (150-210 min) and recovery periods (480-540 min), respectively. Values for both groups of PCP exposed animals are given on chart c.

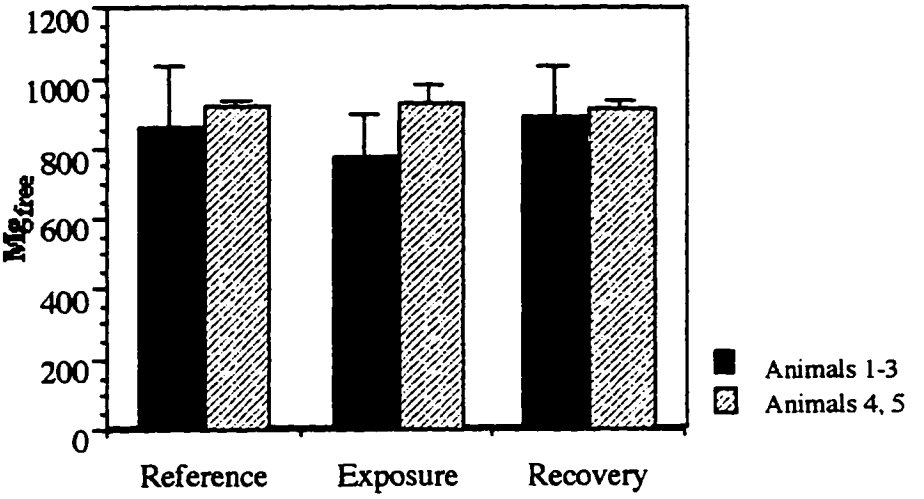
a.



b.



c.



vivo ATP chemical shifts which are broad due to short T_2 effects and peak multiplicity within particular resonances. In addition, inhomogeneous tissue response to toxicant, possible intracellular compartmentation, and accuracy of K_d values all contribute to the high error involved with *in vivo* NMR determinations of Mg_{free} (Williams and Smith, 1995). Consequently, the high variability in Mg_{free} determinations are inherent in the method and similar to previous findings (Doumen and Ellington, 1992; Williams and Smith, 1995).

Hypoxia

In general, the response of abalone to hypoxia in this study was moderate and similar to responses seen in abalone exposed to 6 hr of anoxia (Gade, 1988) or 1 hr air emergence (Tjeerdema et al., 1991b). Many molluscs are well adapted to resist long periods of hypoxia/anoxia primarily through reductions in metabolic rate, and extensive capacities for anaerobic respiration (Hochachka and Somero, 1984; de Zwaan and Putzer, 1985). Abalone have been shown to survive emerged for several days, as long as heating and desiccation are checked (Bowen, 1987). This adaptive response is reflected by the small changes in P_i and PA, which occurred early during hypoxic exposure and remained stable for the exposure duration, which indicates the induction of some compensitory mechanism permitting the maintenance of homeostasis with minimal physiological perturbation. Intracellular pH did not change significantly in this study, although pH_i changes were previously observed in red abalone exposed to air for 1 hr (Tjeerdema et al., 1991b), which may be attributable to increases in temperature that occurred during emergence, but avoided in the present study by maintaining the organism in chilled de-oxygenated water.

Reductions in Mg_{free} during hypoxia are similar to changes seen in Doumen and Ellington (1992), where induction of metabolic acidosis in perfused muscle preparations of the horseshoe crab *Limulus polyemus* showed decline in Mg_{free} with pH_i. Russell and

Brodwick (1988) have shown that increases in Mg_{free} competitively inhibit Cl^-/H^+ transport in barnacle muscle fibers. Consequently, Mg_{free} decreases may act to enhance net H^+ transport, helping to maintain pH_i. The majority of investigations on effects of hypoxia/ischemia find large elevations of Mg_{free} (Kirkels et al., 1989; Murphy et al., 1989; Williams and Smith, 1995) during the ischemic period, which primarily reflects release of bound Mg^{2+} due to the hydrolysis of ATP. In this study, ATP concentration was unchanged over the total observation period, and so could not serve as a source of Mg^{2+}.

Sodium Azide Exposure

The comparison of hypoxic response with NaN_3 exposure is instructive, since both stresses are thought to act via a similar mechanism (inhibition of mitochondrial electron transport), however, the response to azide is more severe, as determined by all parameters, and shows no period of stabilization. There are several possibilities which may explain these results. First, azide exposure may fail to trigger the metabolic suppression responsible for the moderate hypoxia response, although parallel studies using saturation transfer NMR to evaluate rates of ATP formation via the arginine kinase (AK) reaction, show the lowest levels of AK activity for NaN_3 exposures relative to hypoxia and PCP studies (Shofer et al., 1997), which suggests that azide may suppress metabolic activity in a manner similar to hypoxia. Secondly hypoxia, as conducted in this study, may not be sufficient to completely inhibit all electron transport, while azide exposure may do so. However, studies of anoxia in a related abalone species (*Haliotis lamellosa*) resulted in changes in PA and ATP concentrations at 2 hrs which are qualitatively similar to those displayed in the present study (Gade, 1988), indicating similar levels of electron transport inhibition. Finally, NaN_3 may act via more than one mechanism of action. Nitric oxide (NO) has been reported to be produced from azide in exposed cell cultures (Smith et al., 1991; Markert et al., 1994; Smith and Wilcox, 1994) and has been proposed by Smith and

Wilcox (1994) to be the primary cause of toxicity in azide exposure via neuroexcitation. The effects of NO are not well described for invertebrate exposures, but in vertebrates NO is an endogenously produced compound involved in modulation of blood pressure, peripheral and central nervous transmission, and insulin release (Smith and Wilcox, 1994). Nitric oxide has also been shown to be a potent multisite inhibitor of mitochondrial electron transport (Schweizer and Richter, 1994; Poderoso et al., 1996), whose action is enhanced in the presence of NaN_3 which prevents mitochondrial reduction of NO (Borutaite and Brown, 1996). Consequently, the results of azide exposure may reflect a combination of electron transport inhibition and NO effects.

PCP Exposure

The results of the PCP exposures were similar to those previously described (Tjeerdema et al., 1991a; Tjeerdema et al., 1993). Comparing the response of pH_i and P_i between NaN_3 and PCP exposures, it becomes apparent that in azide-exposed animals changes in P_i and pH_i occur simultaneously, with declines in pH_i mirroring increases in P_i. However, a weaker association of these two parameters is observed in PCP-exposed animals as displayed by the small changes in pH_i (0.03 pH points, abalone 3,4; Table 1) at levels of P_i increase which were associated with pH_i declines of 0.11 in azide-exposed animals. Large pH_i declines in PCP-exposed abalone did not occur for several hours into the recovery period, which may reflect incomplete uncoupling of mitochondrial oxidative phosphorylation. The correlation of changes in pH_i and phosphagen concentration may be a useful parameter in evaluating the mitochondrial effects of toxicants for future studies.

Variability in abalone response to PCP, compared with NaN_3, may be caused by variability in lipid content between individuals. PCP is a moderately hydrophobic molecule and so is bioconcentrated in animal lipid stores (5 h TCF of 16-63 in abalone; Tjeerdema and Crosby, 1992; Shofer and Tjeerdema, 1993). Increases in lipid content results in

greater toxicant accumulation and retention, reflected in increased toxicant effects. Similar variability was not seen in azide-exposed abalone due to the high water solubility of NaN_3 (41.7% at 17°; Budavari et al., 1989), which results in minimizing the effects of variations in lipid content between animals.

General Conclusions

1) NMR spectroscopy is an effective tool for identifying the sublethal effects of toxicants in medium-sized (up to 50 mm width) intact molluscs.

2) Toxicant induced changes in Mg_{free} are modest although all animals displayed declines during periods of stress exposure, followed by returns to control (hypoxia and PCP exposures) or elevated (NaN_3 exposures) values during recovery periods

3) The correlation of changes between pH_i and high energy phosphorylated compounds provides a sensitive marker for evaluating the effects of toxicants on mitochondrial energy production in molluscs.

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CHAPTER 3

SUBLETHAL EFFECTS OF PENTACHLOROPHENOL IN ABALONE (*HALIOTIS RUFESCENS*) VELIGER LARVAE AS MEASURED BY ^{31}P-NMR

INTRODUCTION

Toxicity studies involving the early lifestages of aquatic organisms are commonly used as monitoring tools by scientific and regulatory agencies (Anderson et al., 1994). The sensitivity of their developmental endpoints, as well as their short exposure periods and responsiveness to a wide variety of toxicants, make them attractive alternatives to toxicity testing with adult organisms (Hunt and Anderson, 1989; Anderson et al., 1994). However, because similar developmental perturbations occur in response to exposure to dissimilar chemicals (Hunt and Anderson, 1989), it is generally difficult to determine the toxic action mechanisms associated with these tests; consequently, the predictive value of toxicant effects on advanced lifestages of the test species, or other species, is poor. In order to enhance the applicability of information derived from these studies, the biochemical effects of toxicant exposure must be understood.

One method which has proved successful in observing the biochemical effects of stressful perturbations in intact marine organisms is *in vivo* nuclear magnetic resonance spectroscopy (NMR). While inherently insensitive, it non-invasively permits observation of the actions of chemicals on energy production and utilization in living organisms through

the application of strong magnetic fields and low-energy radio-frequency irradiation. A number of studies have previously employed ^{31}P NMR, which permits the observation of high energy phosphates such as nucleoside triphosphates (NTP), phosphoarginine (PA; an invertebrate analog of phosphocreatine) and inorganic phosphate (P_i), on a wide variety of aquatic organisms. ^{31}P NMR has previously demonstrated the biochemical perturbations associated with hypoxia (Tjeerdema et al., 1991b), changes in salinity (Higashi et al., 1988; Tjeerdema et al., 1996), and toxicant exposure in abalone (Tjeerdema et al., 1991a; Shofer et al., 1997a), and mussels (Aunass et al., 1991), as well as increases in rates of arginine kinase mediated exchange due to several types of stress in abalone (Shofer et al., 1997b).

Developmental toxicity tests utilizing red abalone (*Haliotis rufescens*) larvae are currently mandated by the State of California for use by effluent producers (Anderson et al., 1990). The biochemical actions of these effluents in larva are generally unknown. Consequently, it was the goal of this study to adapt previously demonstrated in vivo ^{31}P NMR techniques for use with abalone veligers (a free-swimming early developmental lifestage) to describe the actions of the oxidative phosphorylation uncoupler pentachlorophenol (PCP: Weinbach and Nolan, 1956. These experiments were designed to parallel previous studies conducted on adult abalone (Shofer et al. 1997a) to elucidate potential differences in toxicant response between the two distinct lifestages.

MATERIALS AND METHODS

Chemicals

Tris-Base was purchased from Sigma, H_2O_2 (30%) from Fisher Scientific, and PCP (sodium salt) from Aldrich Chemicals. Filtered seawater (0.2mm) was obtained from the UCSC Long Marine Laboratory and used within 6 hrs.

Animals

Gravid male and female adult abalone were maintained in flowing seawater and fed giant kelp (*Macrocystis* spp.) *ad libitum*. Spawning was induced according to the California Fish and Game protocol (Anderson et al. 1990). Briefly, gravid male and female abalone were separately placed in 6 L of filtered seawater, and spawning was induced by the addition of freshly prepared tris-base and H_2O_2 to a concentration of 8.3 mM and 0.025%, respectively. Animals were incubated for 2.5 hrs, or less if spawning occurred, followed by replacement of the tris-H_2O_2 solution with clean seawater. Gametes were collected separately and pooled in 3-L flasks to initiate fertilization. Embryos and larvae were maintained in 3-L glass flasks at ambient seawater temperatures with water replacement every 24 hrs, and larvae were used for experiments upon reaching the veliger stage (approximately 60 hrs after fertilization and 250 μM in diameter), as determined by the presence of a complete shell and developed velum on the animals.

Exposure System

The exposure system (Fig. 1) was similar to one previously used with adult animals (Shofer et al., 1997a) with the primary difference being the construction of a perfusion chamber for larvae. It consisted of two modified NMR tubes (10 mm and 12 mm; Wilmad Glass, Buena, NJ) concentrically oriented. The closed end of the 10 mm tube was replaced with a fine glass frit (30μm: Ace glass, Vineland, NJ), while the open end was attached to a glass nipple which passed through a ground-glass plug. A second nipple was also attached off center of the ground glass plug to permit water to exit the chamber. The larger tube had a ground-glass fitting annealed to the open end to facilitate a water-tight seal when the glass plug was fixed in place using silicone aquarium cement. Chilled aerated seawater was drawn (2 mL min^{-1}) through silicon tubing (Size 16, with size 13 tubing located in the pump heads; Cole parmer, Chicago, IL) via peristaltic pump (Masterflex model 7523-00)

fitted with a quick-load pump head, while a concentrated toxicant solution (4.8 mg L^{-1}) was pumped from a chilled Tedlar® (AeroVironment, Monrovia, CA) storage reservoir via a second peristaltic pump at 0.5 mL min^{-1} to achieve a four-fold dilution. Waste toxicant was removed at the water outflow using a packed column of Amberlite® XAD-4 resin (Rohm and Haas, Philadelphia, PA).

NMR Spectroscopy

All experiments were conducted in a 7.05 Tesla, General Electric GN-300, wide-bore spectrometer, equipped with a Nicolet 1280 computer control system and using a 12-mm broad-band probe. B_0 field optimization was conducted using the ^1H resonance of water, achieving typical peak widths of 5 Hz. Spectral acquisition parameters were as follows: ^{31}P-spectrometer frequency, 121.48 MHz; sweep width ± 5000 Hz; pulse width, 8.5 μs; 2048 data points; acquisition time, 0.205 s; interpulse delay, 300 ms; 30 Hz exponential multiplication; and zero filling to 4096 data points, while 3000 transients (20 min per data point) were acquired prior to Fourier transformation of the FIDs.

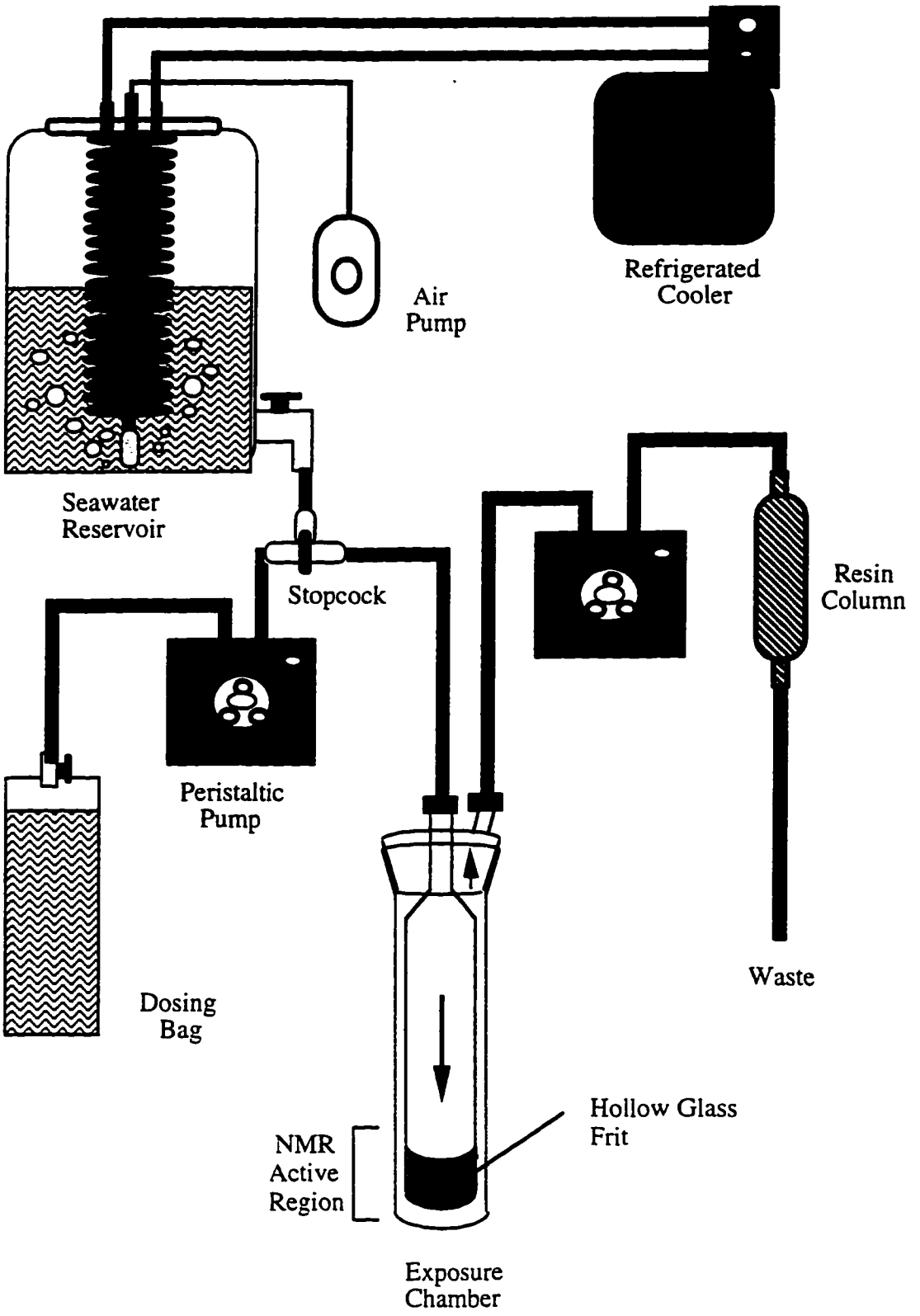
Intracellular pH Determination

Intracellular pH (pH_i) was determined using the difference in chemical shift between PA and P_i resonances and the following form of the Henderson-Hasselbach equation:

$$pH = pK_a + \log \frac{\delta_{acid} - \delta_{obs}}{\delta_{obs} - \delta_{base}} \qquad (1)$$

where δ_{acid} and δ_{base} are the chemical shift values of the fully protonated and unprotonated forms of P_i respectively, δ_{obs} is the observed chemical shift of P_i, and pK_a is the negative

Figure 1. The flow-through exposure system for use with veliger larvae.



log of the K_a of P_i. The values of δ_{acid}, δ_{base}, and pK_a were determined as previously described (Shofer et al., 1997a) to be 3.84 ± 0.22 ppm, 6.56 ± 0.11 ppm, and 6.70 ± 0.20, respectively.

Experimental Procedure and Data Analysis

Three replicates of approximately 400 mg wet weight of veliger larvae were placed within the exposure chamber and allowed to acclimate for 1 hr, followed by an additional hour of reference spectra acquisition. Animals were then exposed for 2 hrs at 15°C to stock chemical solutions (4.8 mg PCP L^{-1} dissolved in 250 µL MeOH carrier) which were mixed in 1 L of filtered seawater within the toxicant reservoir and pumped at 0.5 mL min^{-1} into the clean seawater flow (2.0 mL min^{-1}) to achieve a four-fold dilution of the concentrated stock to 1.2 mg L^{-1}, followed by a 2 hr recovery period in clean flowing seawater. Direction of water flow was reversed every 2 hrs for 5 min through the use of a timer attached to the pumps to permit redistribution of the veligers in the exposure chamber.

Phosphagen peak intensities were determined for each spectrum using the peak height function provided in the computer program Nuts® (Acorn Software, Fremont, CA). Reference period peak intensities (three time points) were averaged within a replicate for each resonance of interest, while intensity values for each individual time point were reported proportional to this average reference period intensity. Statistical tests were conducted by one-way ANOVA or Student-Neuman-Keuls *t*-Test as appropriate (Glantz, 1987).

RESULTS

NMR Spectroscopy

Spectra from veliger larvae showed significant differences from those taken from adult abalone (Fig. 2). The primary differences include the low concentration of PA relative to NTP peak intensities and a large sugar phosphate resonance which is not apparent in the foot muscle spectra of adults. They are due to the whole-body nature of the larval spectra, which include contributions from non-muscular tissues which tend to possess higher concentrations of sugar phosphates, as opposed to previous studies which have focused on adult foot muscle. In addition, the low PA concentrations which appear to be roughly equivalent to NTP concentrations may reflect the juvenile energetic status of the velum and cilia.

Exposure conditions were not ideal, as the low signal inherent to individual larva required packing of the perfusion cell with high larval densities. In addition, upon reversal of the water flow spectra displayed degraded response. Thus, some of the variability in peak intensity is due to changes in perfusion conditions and does not reflect toxicant-induced perturbations. Nevertheless, acquired spectra were stable in the absence of toxicant, and remained so for periods up to 18 hrs

Phosphagen Response

In general, veliger response to PCP was similar to previously described responses in adults (Tjeerdema et al., 1991a: Shofer et al., 1997a). Significant changes in P_i, PA, and

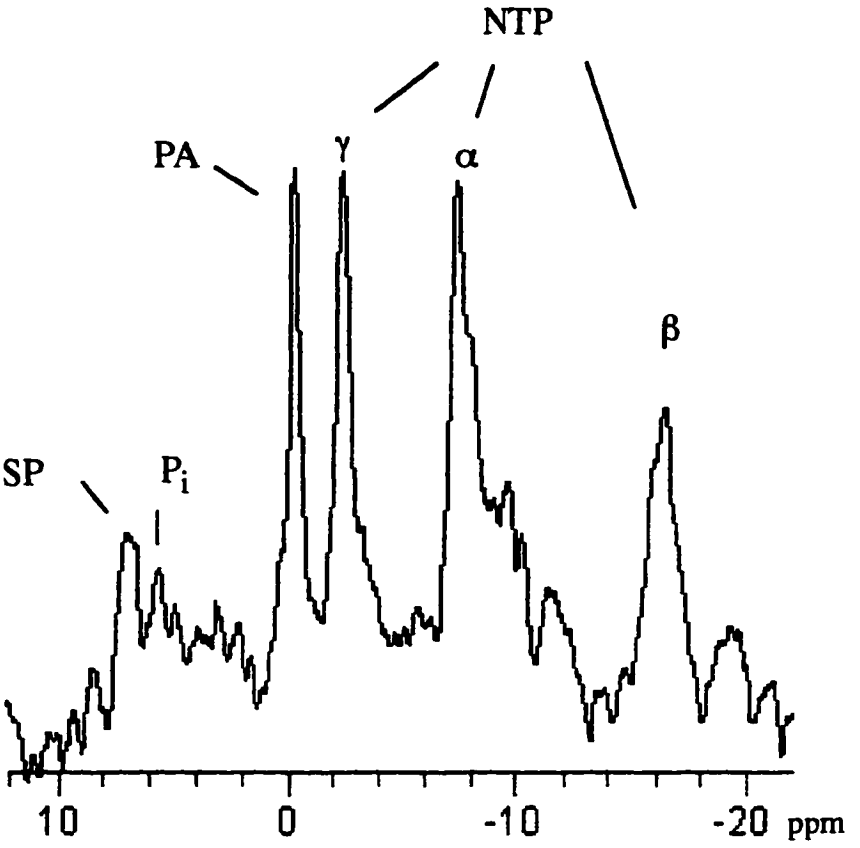


Figure 2. Representative spectra from veligers (approximately 400 mg wet weight) in the resting state. Peak labels are as follows: sugar phosphates (SP), inorganic phosphate (P_i), phosphoarginine (PA), and the nucleoside triphosphate (NTP) γ, α, β resonances respectively. The chemical shift of PA has been offset to 0 ppm. See text for description of spectral parameters.

NTPs were observed (Fig. 3a), with P_i increasing to 3.02 ± 0.63 (± SEM for all values; $p < 0.01$) over average reference values, while PA and NTPs declined to 0.38 ± 0.10 ($p <$

0.01) and 0.61 ± 0.16 ($p < 0.05$) reference values, respectively. Sugar phosphates also displayed increases to 1.44 ± 0.24 times reference values during the second hour of the recovery period, although these changes were not significant ($p > 0.05$). Larva displayed near complete recovery by the end of 2 hrs as displayed by the return of peak intensities to 1.96 ± 0.53, 0.89 ± 0.12, and 0.89 ± 0.13 times reference intensities for P_i, PA, and NTPs, respectively ($p > 0.05$ for all values).

Intracellular pH (Fig. 3b) was also monitored in this study. Values, while fluctuating, did decline from a reference period average pH_i of 7.12 ± 0.03 to 7.04 ± 0.04 which did not significantly differ from reference pH_i ($p > 0.05$), and then returned to 7.08 ± 0.11 by the end of the recovery period.

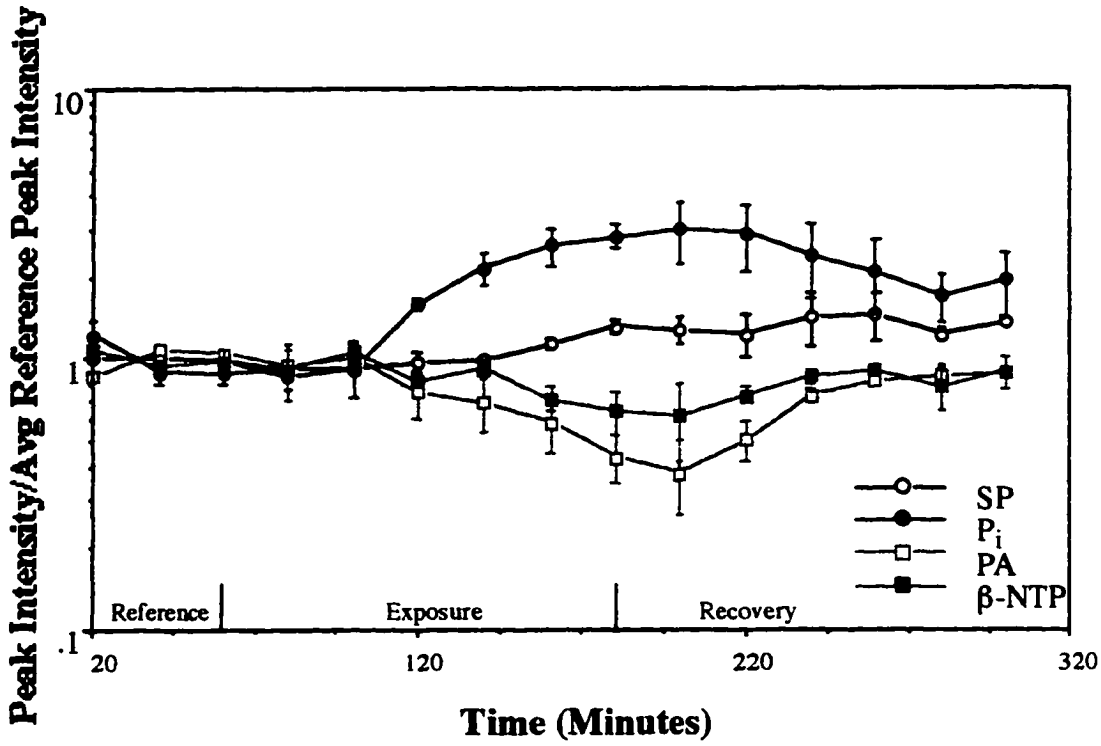
DISCUSSION

The responses of veliger stage abalone larvae to 1.2 mg L^{-1} PCP are similar to changes in phosphagen concentrations previously seen in adult animals, although differences were observed in changes in pH_i and in time to recovery. Adult animals exposed to 1.2 mg L^{-1} PCP displayed greater increases when compared with veliger animals in P_i, which ranged from 6.56 to 15.33 fold increases over reference values, more moderate declines in PA and pH_i ranging from 0.50 to 0.65 of reference values and 0.03 pH units respectively, and no observed changes in adenosine triphosphate (ATP) concentrations (represented by NTPs in veliger spectra). Adults also took longer to recover from PCP exposure averaging 6 hrs for return of phosphagen concentrations and pH_i to reference values (Shofer et al., 1997a) as opposed to only 2 hrs in larvae.

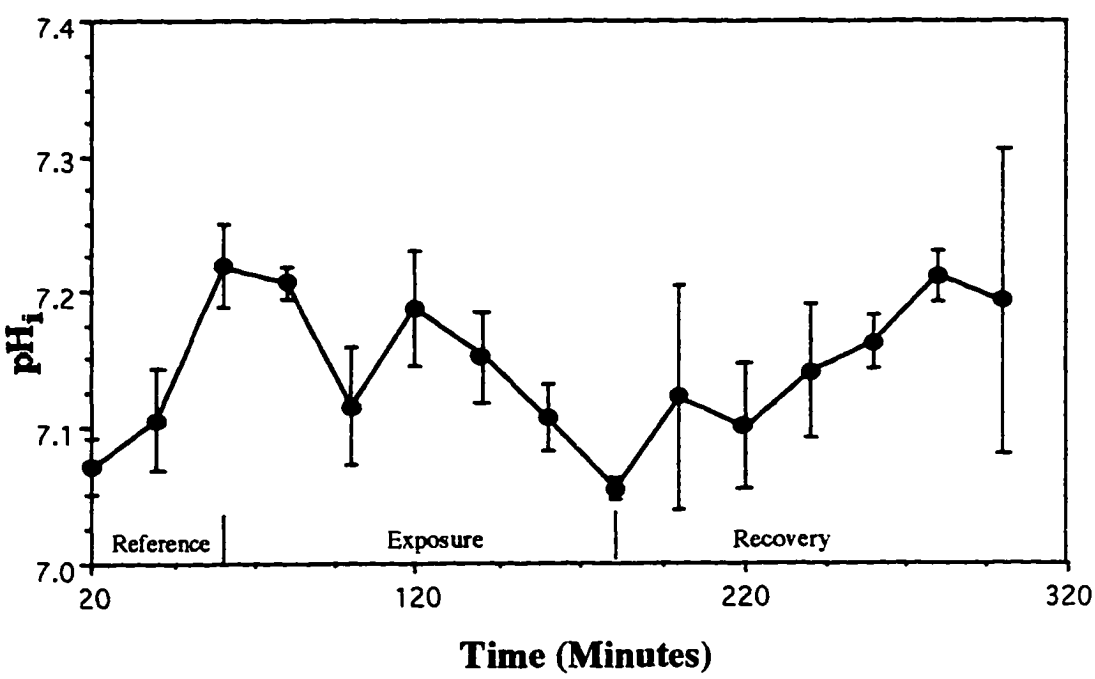
The small increases of P_i in veligers (Fig. 3a) was likely a reflection of the limited pool of PA (roughly equivalent to NTP concentrations), which is closer to 5 times ATP concentrations in adult animals. Consequently, adult animals are better able to buffer ATP energy potential by utilizing PA with the resulting buildup of P_i while veligers, with a

Figure 3. Phosphagen (a) and intracellular pH (b) response in 1.2 mg L^{-1} PCP exposed veligers. Phosphagen resonance intensities are plotted proportional to average reference period intensities for a given compound ± SEM. Intracellular pH was determined as described in the text. Each point represents 3000 acquisitions averaged over 20 minutes, N = 3 for all points.

a.



b.



limited PA pool, are unable to buffer NTP energy potential at reference levels under these exposure conditions and consequently display a more limited increase in P_i concentration as well as declines in NTP. As may be expected, due to the large surface to volume ratio in larvae, one would assume a greater toxic response when compared with an adults subjected to similar dosing regimes simply due to differences in toxicokinetics. Utilizing similar reasoning, the rapid recovery from toxic insult was expected due to limited toxicant storage and rapid diffusion possible in a low volume lifestage. Interestingly, although adults and veligers demonstrate similar effects of PCP, their LC_{50}s are very different. A PCP static 2 hr LC_{50} of 1.6 mg L^{-1} was observed in adult abalone, while veligers displayed no mortality in similar exposures of up to 10 mg L^{-1} PCP.

Veligers displayed more severe declines in pH_i than did adults which may be due to differences in physiological adaptation. Veliger larvae are a particularly active stage in abalone life-history as they continuously swim for periods up to 14 days prior to metamorphosis (Hahn, 1989). Consequently, the velum likely contains large concentrations of mitochondria as seen in similar active ciliated tissue such as mytilus gill (Doeller et al., 1993), and so relies almost exclusively on aerobic mechanisms for ATP production which would result in a limited adaptation for anaerobic energy synthesis. In contrast, adult abalone foot muscle is relatively low in mitochondrial density (unpublished observations) and adapted to periods of anaerobiosis (Bowen, 1987) which may result in greater resistance to PCP induced toxicity. The greater change in pH_i (0.08 ± 0.04 units) relative to adult animals (0.03 ± 0.02 pH units) supports the presence of a limited capacity and/or adaptation for anaerobic respiration in larval animals in response to PCP exposure.

Few studies have previously looked at the biochemical effects of toxicant exposure in larval organisms and, to our knowledge, this is the first report of the use of in vivo NMR to observe viable invertebrate marine larva. In the case of abalone larval toxicity tests, the primary toxic endpoint is generally the development of shell deformations which

is often associated with low toxicant concentrations (Anderson and Hunt, 1989; Anderson et al., 1990). The biochemistry associated with developmental perturbations in abalone has not been investigated, and the applications of the techniques presented here may prove useful to that end. In conclusion, this study has demonstrated the in vivo observation of high energy phosphorylated compounds in abalone veliger larvae, providing the ability to obtain information which would be difficult or impossible to do by other methods. In addition, veliger larvae display significant differences in toxicant response to PCP when compared with adult animals both in extent of perturbations in phosphorylated compounds and pH_i as well as in time to recovery of steady-state phosphagen concentrations.

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CHAPTER 4

EFFECTS OF HYPOXIA AND TOXICANT EXPOSURE ON ARGININE KINASE FUNCTION AS MEASURED BY ^{31}P-NMR MAGNETIZATION TRANSFER IN LIVING ABALONE

INTRODUCTION

Many toxicants released into aquatic environments act on organisms by perturbing mitochondrial energy production, either as electron transport inhibitors (rotenone; Rach and Gingerich, 1986), or uncouplers (pentachlorophenol; Weinbach and Nolan, 1956). While lethal concentrations of these compounds have well understood effects, sublethal exposures, and the associated toxicant-induced perturbations of homeostasis within the exposed organism, are not as clearly understood. Often, no observed effect concentrations (NOECs) are used as guides to determine the point at which toxicity begins; however many studies have demonstrated that significant effects on bioenergetic metabolism do occur at or below NOEC concentrations of xenobiotics (Shofer et al., 1996; Shofer et al., 1997; Tjeerdema et al., 1991a; Tjeerdema et al., 1991b; Tjeerdema et al., 1993)

Arginine kinase (AK), analogous to creatine kinase (CK) in vertebrates, catalyzes the reversible exchange of phosphate from phosphoarginine (PA) to adenosine diphosphate (ADP) forming adenosine triphosphate (ATP), and is thought to be an important spatial and

temporal energy buffering system in tissues with high energy demands (Walliman et al., 1992). Previous studies have employed in vivo saturation transfer NMR to determine rates of CK or AK-mediated ATP formation (Matthews et al., 1982; Kupriyanov et al., 1984; Shoubridge et al., 1984; Graham et al., 1986; Bittl et al., 1987ab; Yoshizaki et al., 1987; Fan et al., 1989). This technique, which relies on the chemical transfer of a perturbation in magnetization which is introduced into the sample via a low-power, narrow-frequency irradiation, permits the determination of psuedo-first order rate constants for forward and reverse reaction directions forming arginine and ATP or PA and ADP respectively. If reactant concentrations are known, flux may also be determined by taking the product of the rate and appropriate reactant concentration.

Previous studies have shown that the CK rate (Kupriyanov et al., 1984) equation is a good predictor of both flux and rate constants in normoxic tissue (Bittl et al., 1987b), with the low concentration substrates ADP and creatine (which normally occur near K_m for these compounds) acting as limiting reagents controlling the forward and reverse reaction rates, respectively. However, under non-physiological conditions such as hypoxia or ischemia, rates of product formation fail to respond proportionally to increases in low concentration substrates for reasons which are unclear (Bittl et al., 1987a; Cox et al., 1988; Neubauer et al., 1988; Yoshizaki et al., 1991). Many of these studies require the use of isolated perfused tissues or surgical manipulation in order to achieve the appropriate conditions for utilizing NMR, and so the tissues suffer varing degrees of damage as shown in their elevated resting levels of inorganic phosphate (P_i). Further introduction of a hypoxic or ischemic insult acts to cause tissue damage, which may result in the failure of CK reaction rates to respond to changes in limiting reactant concentrations as occurs in normoxic tissue.

The goal of the present study was to use non-invasive techniques to evaluate the effects of hypoxia and toxicant exposure on AK-mediated ATP formation in live intact

marine organisms. Toxicants were selected which would alter mitochondrial function, and so mimic hypoxic effects, to determine the role of suppression of mitochondrial function and resulting changes in concentration of AK substrates on forward reaction rates (k_{for}) and flux. In addition, NOEC toxicant levels were used to evaluate the relationship between biochemical effects and morphologic effects of toxicant exposure. Red abalone (*Haliotis rufescens*) were chosen for these studies due to their large homogenous foot muscle and sedentary nature, which makes them ideal organisms for conducting *in vivo* NMR without the need for surgery or tissue isolation. The toxicants chosen were sodium azide (NaN_3), thought to act by inhibiting electron transport at cytochrome c oxidase (Smith and Wilcox, 1994), and pentachlorophenol (PCP), an uncoupler of mitochondrial oxidative phosphorylation (Weinbach and Nolan, 1956).

MATERIALS AND METHODS

Chemicals

Methanol and sodium azide were purchased from Fisher Scientific Inc., while PCP (sodium salt) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Filtered seawater (0.2 μm) was obtained from the UCSC Long Marine Laboratory and used within 6 hrs.

Animals

Red abalone (averaging 2 yrs of age and 40 mm maximum shell width) were obtained from U.S. Abalone (Davenport, CA) and maintained in fiberglass aquaria with flowing seawater for a minimun of 2 weeks prior to use. Animals were fed giant kelp (*Macrocystis pyrifera*) *ad libitum* and acclimated at ambient seawater temperatures.

Exposure System

The flow-through exposure system has been previously described (Shofer et al., 1997). Briefly, the exposure chamber was milled from a solid Teflon® core and sealed with an O-ring mounted on a threaded teflon lid fitted with fluoropolymer nipples to permit the passage of seawater. The animal was placed within the chamber in the vertical position, locating the foot muscle in the proper orientation for observation with the surface coil. Water was drawn (20 ml min^{-1}) from a chilled and aerated seawater reservoir, via silicon tubing (size 16, Cole-Parmer, Chicago) and a single peristaltic pump (Masterflex model 7523-00 equipped with a Quick-load pump head). A second peristaltic pump metered in a concentrated toxicant solution stored in chilled Tedlar® fluoropolymer bag (AeroVironment, Monrovia, CA).

Toxicant concentrations were determined previously (Shofer et al., 1997) with the NOEC evaluated by the ability of the animal to maintain a firm grip with the foot muscle on the side of the exposure chamber.

NMR spectroscopy

All experiments were conducted in a 7.05 Tesla, General Electric GN-300, wide bore spectrometer, equipped with a Nicolet 1280 computer control system and using a 2-cm, 2-turn photoetched (Fan and Higashi, 1989) spiral surface coil for all animal studies. Spectral acquisition parameters were as follows: ^{31}P spectrometer frequency, 121.48 MHz; sweep width ± 5500 Hz; 35 μs pulse width; 1024 data points; aquisition time .092 s; 10 s interpulse delay; 20 Hz exponential multiplication; and zero filling to 2048 data points were applied prior to fourier transformation of the FIDs. Saturation rates were determined using the relationship developed by Forsen and Hoffman (1963):

$$M^* = M_0/(1+k_{for}T_1) \qquad (1)$$

Where:

M^* = the observed PA magnetization.

M_0 = the steady state magnetization in the absence of γ-ATP irradiation.

k_{for} = psuedo-first order rate constant for the forward (ATP forming) AK reaction.

T_1 = The PA relaxation rate constant in the abscence of exchange.

Steady-state saturation transfer measurements were made for the forward direction by setting the spectrometer frequency to match the γ-ATP frequency and applying a low power irradiation continuously except during application of the high power pulse and signal acquisition. Control spectra were also collected where an identical low power irradiation was applied in a position symmetrically downfield across the PA peak to correct for any off-resonance saturation effects. In addition, because the PA peak displayed small decreases in intensity during control irradiation, spectra were also accumulated without any saturation, and these spectra were used for determination of steady-state magnetization peak intensities. Spectral acquisition was interleaved in blocks of 12 accumulations for each of the three sets of spectra, requiring 1 hr for a total of 120 acquisitions per set.

Previous studies (Bittl et al., 1987a,b; Ugurbil et al., 1986) employed a form of the saturation transfer experiment which permitted the determination of T_1 values simultaneously with exchange rate constant measurements; however, we felt the additional time required, and thus the loss of resolution in rate changes, made the application of this technique undesirable. Consequently, T_1 measurements were made during the 12 hr acclimation period by the saturation recovery technique (Matson et al., 1984). The γ-ATP resonance was continuously saturated during T_1 measurements in order to prevent cross

relaxation between the PA and ATP. Two measurements were performed, and only animals in which the consecutive values fell within 100 ms were used for saturation transfer determinations.

Experimental Procedure and Data analysis

Animals were exposed to the three stresses (hypoxia, PCP, azide) as previously described (Shofer et al., 1997). Briefly, animals in the perfusion cell were placed in the magnet 12 h prior to the beginning of exposures and T_1 determinations were made in the presence of γ-ATP saturation at this time. Two measurements of k_{for} were performed on each animal prior to the initiation of toxicant/hypoxia exposure. Animals were exposed for 2 h at 15°C to stock chemical solutions (4.8 mg PCP L^{-1} dissolved in 250 µl MeOH carrier, or 200 mg NaN_3 L^{-1}) which were mixed in 1 L of filtered seawater within the toxicant reservoir and pumped at 5 ml min^{-1} into the clean seawater flow (20 ml min^{-1}) to acheive a 4-fold dilution of the concentrated stock. Hypoxia studies were similarly conducted but using Tygon© vinyl tubing (replacing the O_2 permeable silicon tubing) and a chilled N_2 bubbled seawater reservoir. Oxygen concentrations were monitored at the waste water outlet using a dissolved O_2 electrode (Orion) and were maintained $\leq$ 1.0 ppm O_2 throughout the hypoxia exposure period. Chemical-and hypoxia-exposed animals were allowed to recover in clean flowing seawater for 6 hrs.

Total phosphagen peak area was determined for each spectra taken in the absence of saturation irradiation using the integration function provided in the computer program Nuts® (Acorn Software, Fremont, CA), and normalized to reference measurements. Flux determinations were made by taking the product of k_{for} and PA concentration assuming an initial starting concentration of 9.5 mM PA for each animal represented by the normalized peak area for the two reference rate determinations, while ATP values were determined in a similar fashion using 2.0 mM as the steady state ATP concentration (Shofer et al., 1997).

Statistical tests were conducted by one-way ANOVA or Student-Neuman-Keuls *t*-Test as appropriate (Glantz, 1987).

RESULTS

NMR Spectroscopy

Five animals were monitored for 2 hrs followed by a 2 hr exposure to hypoxia (< 1.0 ppm O_2), NaN_3 (50 mg L^{-1}), or PCP (1.2 mg L^{-1}), and then 6 hrs of clean seawater exposure all while saturation transfer measurements were conducted (each complete measurement required 1 hr for completion). Measurements were also made on an additional five animals which were not exposed to any stress to determine basal k_{for} and flux values. No effect was observed due to T_1 or saturation transfer measurements, while animals maintained within the perfusion chamber displayed no significant changes in phosphagen concentration or k_{for} rates for periods up to 4 days. Complete γ-ATP saturation was achieved using a low-power pulse (Fig 1.). However in control spectra (saturating irradiation placed at a position symmetric with the γ-ATP resonance downfield of the PA resonance) small decreases in PA peak intensity were observed, consequently an additional set of spectra were used for determination of PA steady-state peak intensity which were collected in the absence of saturating irradiation.

T_1 Determinations

The saturation recovery method proved an effective method for measuring accurate T_1 values employing a surface coil (Fig. 2). Relaxation rate (T_1) constants for PA in the presence of γ-ATP saturation averaged 1.49 ± 0.16 s (42 measurements ± SD). Consecutive T_1 measurements were made for each animal, which were initiated immediately after loading the abalone into the magnet, while still suffering from handling

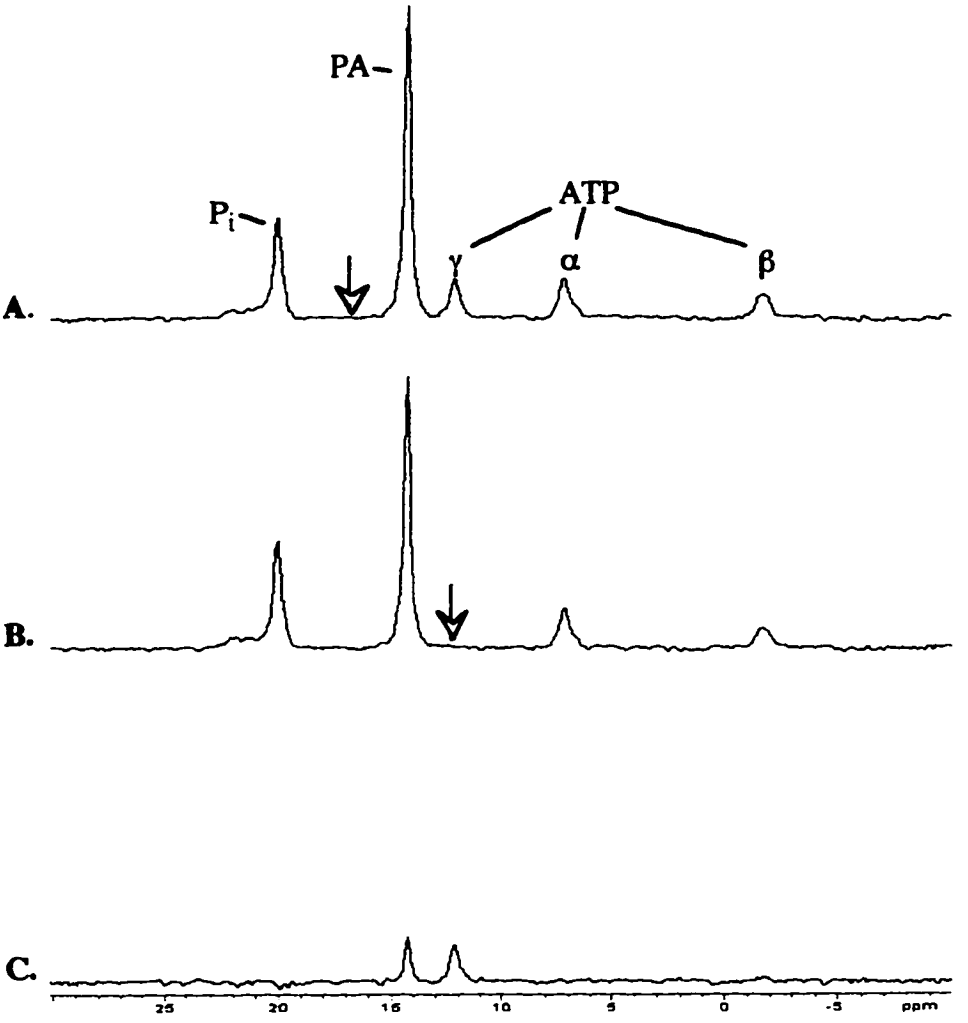

Figure 1. Representative spectra presenting the saturation transfer technique; arrows indicate placement of saturating irradiation. (A) control saturation, while (B) is γ-ATP saturation, and (C) is the difference spectrum showing transfer of saturation to the PA resonance (B), and the γ-ATP resonance (A). Other resonances cancel each other. Peak labels are as follows: inorganic phosphate (P_i), phosphoarginine (PA) and the γ, α, and β resonances of adenosine triphosphate (ATP) respectively. NMR spectral acquisition parameters are described in the text.

stress (as demonstrated by elevated P_i levels), and a second 6 hrs later after P_i levels had returned to normal resting values. In every case both measured values fell within 100 ms for each animal, which indicates a minimal role for stress-induced changes of T_1 values (Neubauer et al., 1988; Yoshizaki et al., 1991), thus permitting the use of predetermined T_1 values for later k_{for} determinations.

Phosphagen Response

Phosphagen response in toxicant-and hypoxia-exposed animals was similar to previous studies (Shofer et al., 1997). Phosphoarginine declined to minimum values of 7.29 ± 0.83, 6.92 ± 0.44, and 5.11 ± 0.63 mM PA in NaN_3-, hypoxic-, and PCP (± SEM for these and all following values) exposed animals, respectively, while minimum observed values for basal animals were 9.27 ± 0.30 mM PA (Fig. 3). All stress-exposed animals showed significant differences in PA concentration from basal abalone ($p < 0.01$), but no differences between exposures ($p > 0.05$). Significant changes in ATP concentration were only observed in PCP-exposed animals which declined from a steady state concentration of 2.0 to 1.52 ± 0.05 mM ATP ($p < 0.05$), while ATP concentration in hypoxic animals fell to 1.70 ± 0.08 mM ATP, although they were not significant ($p > 0.05$). No changes were observed in NaN_3-exposed animals. Maximum changes in phosphagen concentration occured at the end of the 2 hr exposure period in hypoxia-and NaN_3-exposed animals, but they quickly returned to reference values upon perfusion with clean seawater. PCP-exposed abalone displayed maximum phosphagen changes during the first hour of clean seawater perfusion; they did not return to reference values until the end of the recovery period.

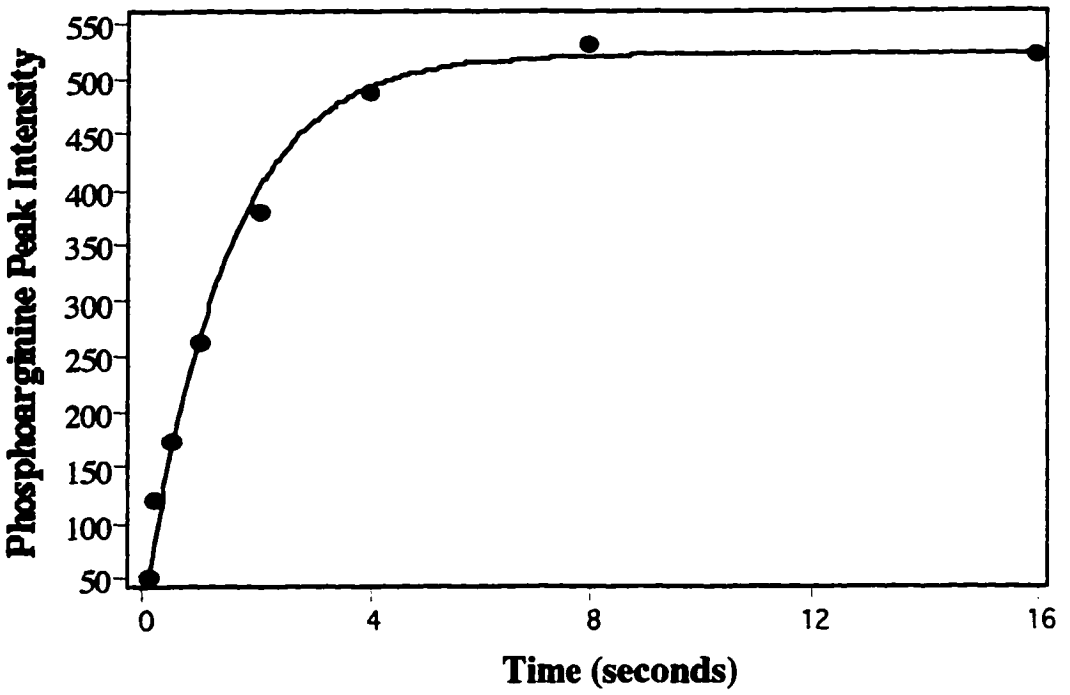

Figure 2. Representative data from a saturation recovery T_1 experiment, showing phosphoarginine peak intensity (arbitrary units) with varying repetition times. The solid line represents a two parameter non-linear least squares fit to these data. T_1 = 1.40 ± 0.09 s

k_{for} and Flux Determinations

Basal k_{for} rates averaged 0.025 ± 0.005 s^{-1} (10 measurements from 5 individuals, 50 total points), the calculated flux was 0.235 ± 0.049 mM ATP s^{-1}, and there were no observed trends in k_{for} or flux observed throughout the 10 hr experimental period. Increases in k_{for} were observed in all stress-exposed animals and were inversely proportional to declines in ATP but not PA. Maximal k_{for} values were 0.095 ± 0.016 s^{-1}, 0.114 ± 0.015 s^{-1}, and 0.126 ± 0.020 s^{-1} for NaN_3, hypoxia, and PCP exposures, respectively. All increases significantly differed ($p < 0.01$) from maximal basal rates, and

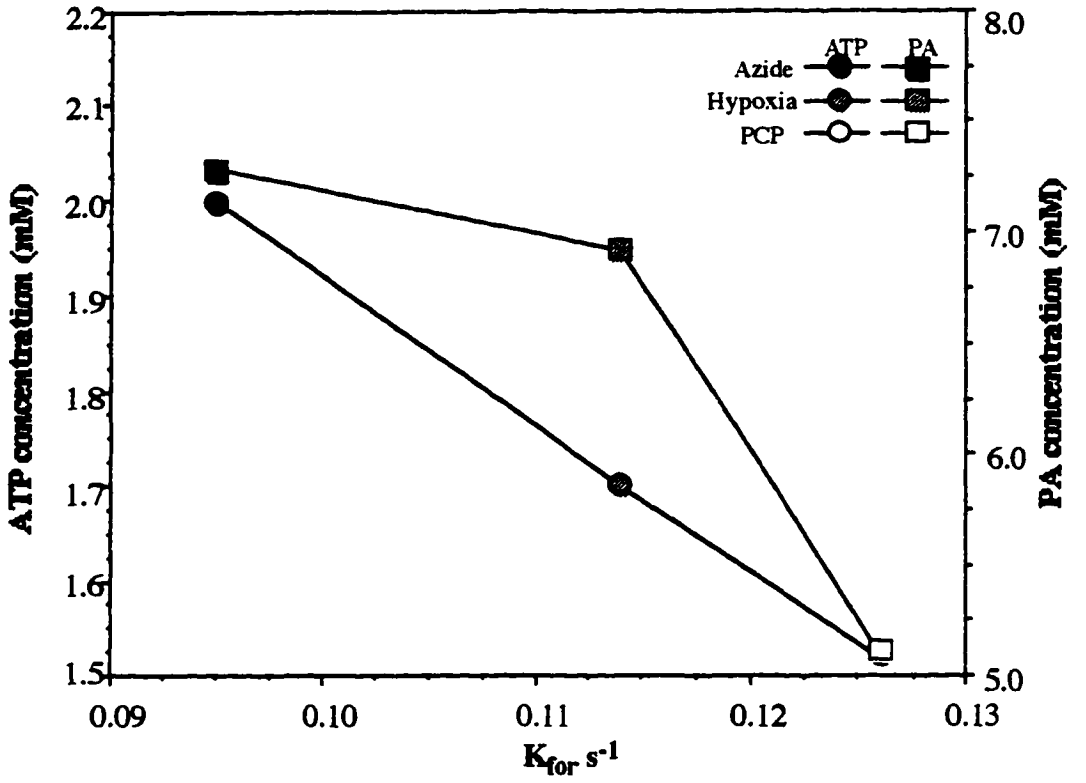

Figure 3. Declines in phosphoarginine peak intensity with application of various stresses. Absolute values ± sem are given in the text (n = 5 for each exposure).

although not significantly different from each other, the pattern observed for average maximal rates was the same for each individual exposure as well. The k_{for} values for hypoxia- and azide-exposed animals returned to reference rates within 3 hrs of the end of stress exposure, while in PCP-exposed animals k_{for} remained elevated throughout the recovery period, despite a return of PA and P_i concentrations to pre-exposure values.

Calculated rates of AK-catalyzed ATP formation (flux) between the three stresses appeared to converge on a common value (average, 0.700 mM ATP s^{-1}) which was not readily apparent from examining the k_{for} rates. Maximal flux values were 0.257 ± 0.026, 0.703 ± 0.115, 0.770 ± 0.064, and 0.627 ± 0.038 mM ATP s^{-1} for basal, NaN_3, hypoxia,

and PCP exposures respectively (Fig. 6). All stress responses were significantly different from basal flux rates ($p < 0.01$), but did not differ significantly between each other ($p > 0.05$). Maximal flux rates coincided with maximal k_{for} values for hypoxia and azide exposures, but occured in the hour following maximal k_{for} rates in PCP exposed abalone.

Total Phosphorus

Total NMR visible phosphorus was examined by calculating total phosphorus peak area for each control spectra. Total phosphorus values significantly declined in hypoxia- and PCP-exposed animals from reference period values to 91.5 ± 3.2% ($p < 0.05$) and 79.6 ± 3.0% ($p < 0.01$), respectively. These declines appear to be due to formation of NMR-invisible P_i, and maximal declines were inversely proportional to P_i concentration. T_1 discrimination (signal declines due to incomplete magnetization recovery between spectral acquisition) through the formation of P_i is not the cause of this decline since the 10-s presaturation period permitted near complete recovery of P_i magnetization (P_i T_1, 3.3 s). Previous reports have described transport of P_i into mitochondria, forming insoluble Ca_2PO_4 salts (Huston et al., 1992), which renders bound phosphate effectively NMR invisible due to the extremely broad linewidths resulting from the short T_2 values of bound metabolites.

DISCUSSION

Arginine kinase-catalyzed rates of ATP formation in abalone foot muscle were relatively low compared with vetebrate (CK) and other invertebrate measurements. Resting rates of k_{for} in excised scallop (*Argopecten irradians*) adductor muscle were 0.238 ± 0.032 s^{-1} (Graham et al., 1986) and 0.16 ± 0.01 in ridgeback prawn (*Sycionia ingentis*; Fan et al., 1992); both nearly twice the maximum rates measured in this study. Low k_{for} rates may be due to low abundance of AK (Bittl et al., 1987b), reflecting the primarily aerobic

function of abalone foot muscle, which is designed for long periods of isometric contraction to maintain a grip on a substrate (Gade, 1988). The equilibrium condition for AK exchange was achieved in this study, as flux rates exceeded rates of decline in PA by a factor of 1500 in PCP-exposed animals, which displayed the greatest PA declines. Complete saturation of γ-ATP was observed for each measurement (Fig. 1), and previous studies have demonstrated the validity of the two-site exchange assumption for k_{for} measurements in vertebrate (CK) systems (Kupriyanov et al., 1984; Ugurbil et al., 1986).

Of primary interest in this study was the adjustment of AK flux rates to a common value despite larger variations in k_{for} rates, which we interpret as a compensatory homeostatic mechanism responding to the application of three different types of stress. Previous studies on myocardial CK have shown that the rate equation for CK-mediated flux is a good predictor for reaction rates in normoxic tissues (Bittl et al., 1987b). In particular, these studies showed that CK activity was sensitive to ADP and creatine concentrations, essentially acting as the rate-limiting substrates for the forward and reverse directions, respectively. However, in hypoxic or ischemic tissues the rate equation does not predict flux accurately. While these tissues display increases in k_{for} inversely correlated to declines in phosphocreatine, i.e., proportional to calculated increases in ADP concentration derived from CK equilibrium constants as observed in normoxic tissue, the rates of flux remained static or decline with ensuing hypoxia (Bittl et al., 1987a; Cox et al., 1988; Neubauer et al., 1988; Yoshizaki et al., 1991).

In the present study, increases in k_{for} were exactly proportional to declines in ATP (Fig. 4) but not PA. Examination of the arginine kinase equilibrium equation (Ellington, 1989) shows that ADP concentrations are more sensitive to changes in PA than ATP, which indicates that ADP is probably not the critical substrate controlling k_{for} increases. In addition, unlike previous studies, flux rates all displayed an elevation from basal values, and flux rose to similar values for each stress regardless of changes in substrate

concentration. We interpret the similarity in flux between all three stressors to be due to similar metabolic effects of stress exposure, possibly the transition to anerobic respiration due to complete inhibitition of mitochondrial function, which in turn drives elevations in flux rates.

It is unclear what parameter controls the degree of flux elevation, but increases in flux may reflect similar elevations in ATP utilization, although several studies have demonstrated that at steady state CK flux is at least an order of magnitude above ATP production rates as determined by O_2 consumption (Neubauer et al., 1988; Dzeja et al., 1996). Thus, unless ATP consumption increased drastically, flux elevations would not necessarily be required to maintain ATP concentrations. Alternatively, AK flux values, which increased by 2.5 - 3 fold, may approach V_{max}, the maximal flux velocity which can be achieved in this tissue. Previous studies using perfused rat heart demonstrated a 1.5 - 2 fold increase CK-mediated flux during the transition from resting to maximal workload in these tissues, which was assumed to be the maximal attainable flux (Kupriyanov et al., 1984), although other studies have reported theoretical increases in CK activity ten times resting rates in similar tissue (Bittl et al., 1987b).

While the three stresses all invoked similar responses in AK-mediated flux, the effects on total ATP consumption were different, as shown by variations in stress-induced PA and ATP declines as well as the resulting increases in k_{for} rates. Although ATP production and demand were nearly at unity, inability of tissue ATP production to meet energy requirements were observed, as evidenced by the declines in PA and ATP concentrations, which were specific to the type of stress applied to the animals. Toxicant exposures were conducted at NOEC levels, yet this level of exposure appears to result in suppression of mitochondrial function equivalent to hypoxic exposure, as demonstrated by the similar increases in AK flux. Assuming complete, or near complete, mitochondrial inactivation, increases in toxicant dose should not have significant additional effects on

these animals, yet small increases in dose (PCP 2 hr LC_{50}, 1.6 mg L^{-1}; NaN_3 2 hr LC_{50}, 75 mg L^{-1}; unpublished observations) are lethal in some individuals. Therefore, we conclude that the ultimate modes of action of these compounds are not exclusively suppression of mitochondrial function, but additional effects on ATP production or utilization, either through inhibition of metabolic arrest or stimulation of ATP utilization, which cause the exposed organisms to succumb.

The declines in PA elicited by both compounds, as well as previously seen changes in intracellular pH and free Mg^{2+} (Shofer et al., 1997) argue for additional metabolic functions, particularly when compared with hypoxia, since all three compounds appear to be equally effective in inhibiting mitochondrial ATP formation. Sodium azide has been shown to produce nitric oxide *in vivo*; NO has been related to modulation of blood pressure, blood platelet aggregation, and nervous transmission in vertebrate systems (Smith and Wilcox, 1994). Recently, NO has also been shown to inhibit creatine kinase in rat heart (Gross et al., 1996), although no evidence for that effect was seen in this study. PCP has been shown to inhibit Na^+ transport (Quevado et al., 1992) and neurotransmitter release (Montoya and Quevado, 1992) possibly through a blockage of Ca^{2+} channels. It has previously been hypothesized that PCP may inhibit AK function (Tjeerdema et al., 1991a; Shofer et al, 1996); however, the present study does not support that assertion.

Changes in total NMR-visible phosphorus were also observed, which appeared to be related to increases in P_i and was most clearly observed PCP-exposed animals. The NMR invisibility of P_i has been previously observed (Neubauer et al., 1988; Hutson et al., 1992), and appears to be associated with its sequestration within the mitochondrial matrix as insoluble Ca_2PO_4 salts. The increased movement of P_i into mitochondria may effectively diminish the H^+ gradient across the mitochondrial inner membrane (Koike et al., 1991; Hutson et al., 1992), resulting in secondary effects of toxicant exposure. If in fact, the decrease in NMR visible phosphorus is due to mitochondrial P_i sequestration, it is not clear

if it is a toxicant-specific phenomenon. Hutson et al. (1992) observed P_i movement into isolated rat liver mitochondria as a function of P_i concentration, which may be the case in abalone, as both PCP and hypoxia caused declines in total phosphorus.

The findings of this study deviate from previous work in the correlation between ATP and k_{for} values, as well as elevations in flux upon hypoxic exposure. These differences in abalone response may be due to several factors. First, abalone have evolved mechanisms to survive long periods (by mammalian standards) of hypoxia, such as an extensive capacity for anaerobic metabolism and the ability to down-regulate energy demands through metabolic arrest (de Zwaan and Putzer, 1985; Bowen, 1987; Gade, 1988). This may be apparent in this study, as AK flux increased to a common value with all three stress exposures. Second, unlike previous *in vivo* studies of CK, this investigation was completely non-invasive and did not require the use of anesthesia or surgical modification. This permitted the response of a fully intact animal to be determined, and so higher order physiological functions may have influenced observed metabolic rates within the abalone foot muscle. Finally, this study again demonstrated that toxicant exposure at NOEC concentrations had effects on bioenergetic production similar to hypoxic exposure, and that the toxicants in question likely have additional mechanisms of action through which lethal effects may occur.

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CHAPTER 5

EFFECTS OF HYPOXIA AND TOXICANT EXPOSURE ON ADENYLATE ENERGY CHARGE AND CYTOSOLIC ADP CONCENTRATIONS IN ABALONE

INTRODUCTION

Many researchers have emphasized the need for physiologic-based toxicity testing to permit an adequate understanding of the effects of xenobiotics on marine organisms (Calow, 1985; Widdows and Donkin, 1991; Aunass et al., 1991). One model which has been proposed as a useful index of organism health is the adenylate energy charge (AEC) described by Atkinson (1977). A measure of the ratio of metabolic energy (ATP) to total adenylate concentration, which is linear and can range in value from 1 in the fully charged state to 0 in the fully discharged state. Some useful properties associated with AEC are: (1) generality of response in that virtually any type of stressor has the potential to disrupt energy homeostasis, (2) a universally applicable measurement, since all organisms use ATP as the primary currency of energy transduction, (3) integrated view of metabolism, because AEC is related to both anabolism and catabolism and so AEC values should be predictive for the extent of either activity within an organism, and (4) critical values

(between 0.3 and 0.5; Marazza et al., 1994) of AEC are thought to be predictive for mortality (Geisy, 1988).

Unfortunately, AEC has not realized its potential as a practical tool for the monitoring and evaluation of toxicity due to variability in correlation between AEC and an organism's condition, particularly as mortality occurred (Geisy et al., 1983; Zaroogian and Johnson, 1989). Other studies have shown contradictory responses in AEC, with low values associated with low toxicant concentrations, while higher values occurred with high toxicant concentrations (Marazza et al., 1994). Furthermore, a lack of correlation has been observed in AEC between tissues of the same animal, with high and low values simultaneously occurring (Geisy, 1988). Consequently, use of AEC as a physiological indicator has largely been abandoned until an explanation of the variability in results can be found (Mazarra et al., 1994).

It is thought that many important regulatory parameters in cellular energetics, such as ADP, AMP, and inorganic phosphate, occur either wholly or partially associated with large macromolecules (Cerdan and Seelig, 1990; Kamp et al., 1995; Graham et al., 1986), while only the cytosolic fractions are available to interact with cellular regulatory components (Kamp et al., 1995). AEC determines total adenylate pools within an organism, rather than the cytosolic component, which is more important from a regulatory standpoint (Kamp et al., 1995). Consequently, the variability observed in AEC and toxicant response in aquatic organisms may reflect a failure to detect toxicity at the level of regulation within effected animals. Cytosolic concentrations of ADP (ADP_{free}) may be calculated in abalone using equation 1 (below) and the arginine kinase (AK) equilibrium constant (van Ginneken, 1995), based on the assumption that the AK reaction is near equilibrium within the cell, one that has been validated for creatine kinase (CK) in mammals (Wiseman and Kushmerick, 1995). *In vivo* ^{31}P NMR provides a convenient method of determining the required values of phosphoarginine (PA), ATP, and intracellular

pH in an intact organism, although arginine (Arg) concentrations must be determined by another technique. The goal of this study was to compare the effects of sublethal exposures to toxicants, the mitochondrial poisons pentachlorophenol (PCP), and NaN_3, or hypoxia on AEC in red abalone (*Haliotis rufescens*) with changes in cytosolic concentrations of ADP_{free} and the physiologic consequences of that change as determined by a previously conducted ^{31}P-NMR saturation transfer study (Shofer et al., 1997b).

MATERIALS AND METHODS

Chemicals

Tetrabutylammonium chloride, ATP, ADP, AMP, adenosine, NAD, NADH, lactate dehydrogenase, alcohol dehydrogenase, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO), while potassium hydroxide, potassium phosphate (dibasic), acetonitrile, NaN_3, and ammonium sulfate were purchased from Fisher Scientific (Fair Lawn, NJ), and triethanolamine, pyruvate, PCP, and semicarbazide hydrochloride were obtained from Aldrich (Milwaukee, WI). Perchloric acid was purchased from Acros (NJ), and ethanol was purchased from the UCSC stockroom.

Animals

Red abalone (averaging 2 yrs of age and 40 mm maximum shell width) were purchased from U.S. Abalone (Davenport, CA) and housed in fiberglass aquaria with flowing seawater at the UCSC Long Marine Laboratory for a minimum of 2 weeks prior to use. Animals were fed giant kelp (*Macrocystis* spp.) *ad libitum* and acclimated at ambient seawater temperatures.

Exposure System and Experimental Procedure

Abalone exposures were conducted as previously described (Shofer et al. 1997a). Briefly, animals were exposed in a Teflon® flow-through (20 ml min^{-1}) perfusion cell. Seawater (15° C) was drawn from a chilled, aerated reservoir through the system by peristaltic pump, while concentrated solutions of toxicant or N_2-saturated seawater were mixed into the system via a second reservoir and pump.

Toxicant and hypoxic exposures were conducted as previously described (Shofer et al. 1997a). Animals were placed in the perfusion cell 12 hrs prior to start of exposure. Three animals were sacrificed after the acclimation period to determine resting metabolite concentrations, while other animals (n=3 for each time point) were sacrificed for an additional three sampling periods following 2 hrs of stress exposure (< 1 mg L^{-1} O_2, 1.2 mg L^{-1} PCP, or 50 mg L^{-1} NaN_3) and 1 and 6 hrs of recovery in clean seawater.

Tissue Extraction

Abalone were rapidly removed from the perfusion cell and tissue samples (0.25-in. diameter cores), which transected the foot muscle parallel to the dorsal-ventral plane from a region just peripheral to the base of the adductor muscle, were isolated and frozen in liquid N_2 (total time < 1 min). This area was chosen to represent the region of detection used in previous surface coil NMR experiments. Samples were stored at -70°C prior to analysis.

Samples were processed to a fine powder for extraction using a stainless steel tissue pulverizer (Fisher, Fair Lawn, NJ) followed by mortar and pestle, both cooled in liquid N_2. Perchloric acid (PCA) extraction was performed for analysis of adenylates and NAD. Briefly, 100-200 mg powdered tissue was added to a tared centrifuge tube containing 500 µL 0.4 M ice cold PCA. Samples were weighed and vortexed for 30 sec, followed by centrifugation for 5 min at 5000x g. The supernatant was removed and stored on ice during a second tissue extraction. Supernatants were pooled and neutralized (pH 7.0) with 100 µL

1.0 M K_2HPO_4 and 25% KOH and frozen for $\leq$ 1 hr prior to high performance liquid chromatography (HPLC) analysis.

NADH analysis was performed using an alkaline extract (Bergmeyer, 1974) by adding 100 - 200 mg tissue to a tared centrifuge tube containing 1 mL of 50% ethanol, 50% 0.5 N KOH solution which was weighed, vortexed for 30 sec and incubated for 5 min at 90°C in a shaking water bath. Following incubation, the solution was cooled on ice for 5 min and neutralized with 800 µL of 0.5 M K_2HPO_4, 0.5 M triethanolamine solution to pH 7.8 which was allowed to stand at room temperature for 10 min to flocculate the dissolved protein. The precipitate was pelleted by centrifugation (15,000x g, 5 min), and the supernatant was frozen for $\leq$ 1 hr prior to HPLC analysis.

HPLC Analysis

HPLC was employed to quantify tissue metabolites using a modification of the method of Ally and Park (1992). Chromatographic separation (Fig. 1) of the metabolites was achieved using a HP model 1090 HPLC (Hewlett-Packard; Palo Alto, CA) equipped with a UV/vis diode-array detector and a reverse-phase C_{18} column (25 cm x 4.6 mm, 4.6 µm particle size; Vydac; Hesperia, CA) fitted with a C_{18} guard cartridge (20 mm x 4.6 mm, 4.6 µm particle size). The two mobile phases were composed of buffer A: 35 mM K_2HPO_4, 6.0 mM tetrabutylammonium chloride pH 6.0, and buffer B: 50% buffer A and 50% Acetonitrile (Optima grade), pH 6.0. Elution conditions were as follows: 5 min of 98% buffer A, followed by a 10-min linear ramp to 55% buffer A, which was held steady for an additional 5 min and then returned to starting conditions over 5 min, flow rate 1 mL min^{-1}. Detector wavelength was initially set to 214 nm to permit detection of PA and arginine, then switched at 5 min to 260 nm for nucleotide detection. A 5-min delay was added between analyses to permit column equilibration.

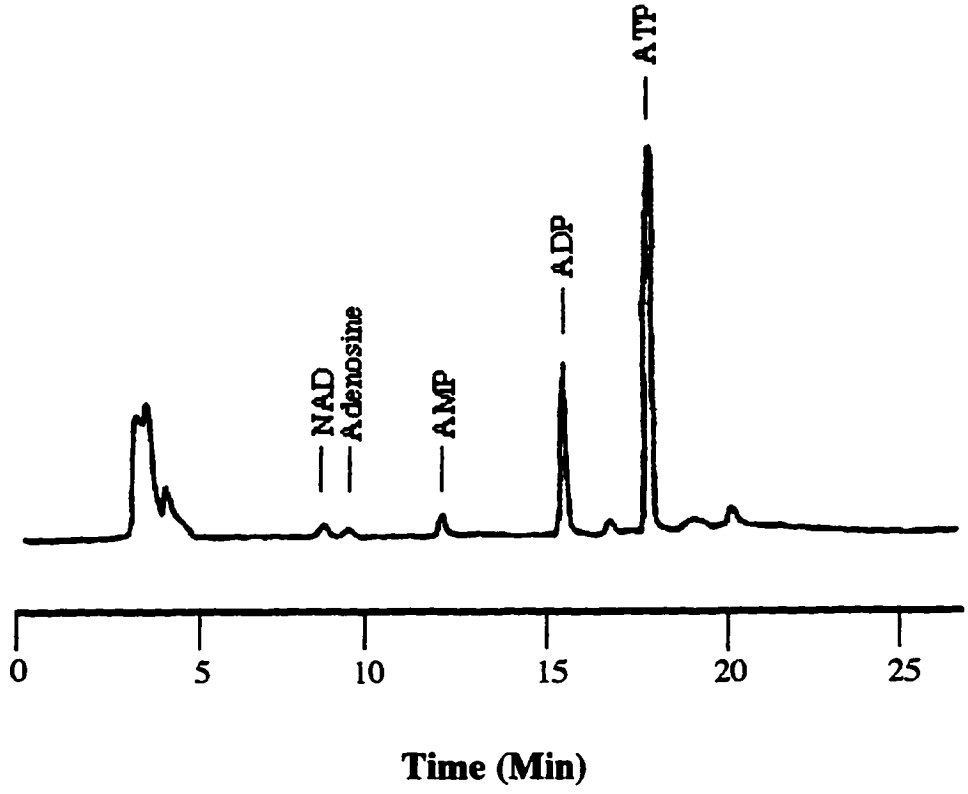


Figure 1. Chromatogram from a PCA extract of hypoxic abalone foot muscle. Chromatographic conditions are described in the text.

Metabolite identification and quantitation was achieved by comparison with known standards, while additional verification of ATP, ADP, NAD, and NADH by specific enzymatic conversions was conducted. The identities of both ATP and ADP were confirmed by the addition of 100 μg of creatine phosphokinase (250 units mg^{-1}) to a 100 μL aliquot of PCA-extracted tissue with creatine added to a concentration of 20 mM. This resulted in a decline in ATP peak height and an increase in ADP peak height, which was compared with a control aliquot which had an equivalent concentrations of BSA and creatine respectively. Lactate dehydrogenase and pyruvate were employed to oxidize

NADH in alkaline tissue extracts similar to the procedure described above, while alcohol dehydrogenase, ethanol and semicarbazide hydrochloride were used to reduce NAD to NADH in selected acid extracts (Bergmeyer, 1974). Unfortunately, PA and arginine separation were not reliable using the C_{18} column, and so no quantitation of these metabolites was performed. In a previous study (Shofer et al, 1997a), a reverse phase C_{22} column was employed which permitted sufficient separation of these two metabolites for quantitation and so steady-state PA concentration values were determined from that study.

Calculation of ADP_{free} and Adenylate Energy Charge

Concentrations of cytosolic ADP (ADP_{free}) were calculated using the following equation (Kamp et al., 1995; van Ginnekin et al., 1995):

$$[ADP_{free}] = \frac{[ATP]\,[Arg]}{[PA]\,[H^+]\,K_{eq}} \qquad (1)$$

Where ATP and PA concentrations were derived from previously determined data (Shofer et al., 1997b), and pH values were determined using the same data set according to protocol in Shofer et al. (1997a), while Arg concentration was determined assuming a resting cytosolic value of 8.0 μMol g^{-1} (Mai et al. 1994) and a K_{eq} value of $1.32 \cdot 10^8$ (Ellington, 1989). Adenylate energy charge (AEC) was determined according to Atkinson (1977) using the following:

$$AEC = \frac{[ATP]\,0.5[ADP]}{[ATP]\,[ADP]\,[AMP]} \qquad (2)$$

Adenylate concentrations were determined from PCA tissue extractions.

Data Analysis

One-way ANOVA and Student-Neuman-Keuls *t*-Tests were performed as appropriate with significance determined at $p < 0.05$ (Glantz, 1987).

RESULTS

Adenylate Nucleotides

Changes in adenylate concentrations and AEC in abalone were similar to previously reported values (Shofer et al, 1997b; Baldwin et al., 1992: Table 1). ATP concentrations displayed the greatest changes, particularly in PCP-exposed animals which occurred in the hour after toxicant exposure similar to previously observed responses (Shofer et al., 1997a, b) However, the observed decline of ATP in PCP-exposed abalone is primarily due to the response of a single individual, where the other two animals displayed declines similar to the hypoxia and NaN_3-exposed animals. Concentrations of ADP were essentially constant, while AMP displayed small increases coincident with declines in ATP. Small increases in ADP/ATP ratios were observed and were similar between exposures apart from the 1 hr recovery PCP animals, which showed large and variable increases in ADP/ATP ratios for reasons described above. Similar declines were also observed in AEC during stress exposure, although these changes were more modest than those observed for ATP concentrations or ADP/ATP ratios.

In contrast to PCA-extractable ADP, resting ADP_{free} concentrations were a third of the value of acid-extracted ADP and increased by a factor of 1.5-2 in response to stress exposure. In addition, ADP_{free} concentrations were well correlated with previously (Shofer et al., 1997b) determined AK psuedo-first order rate constants (k_{for}) and rates of flux (formation of ATP from ADP and PA; Fig. 2, 3), although PCP-exposed animals display reductions in flux rates at high ADP_{free} concentrations which may be due to depletion of PA concentrations in these animals.

Table 1. Effects of Stress Exposure on Foot Muscle Adenylate Concentrations.

	ATP[a]	ADP	AMP	AEC	ADP/ATP	Total Adenylate	ADP_{free}
Control	2.14 ± 0.31[b]	0.61 ± 0.14	0.11 ± 0.05	0.86 ± 0.03	0.29 ± 0.04	2.85 ± 0.47	0.17 ± 0.01[c]
1.2 mg L^{-1} PCP							
2 hr Exposure	1.85 ± 0.23	0.62 ± 0.06	0.10 ± 0.02	0.84 ± 0.02	0.34 ± 0.04	2.57 ± 0.27	0.29 ± 0.02[d]
1 hr Recovery	0.82 ± 0.45	0.53 ± 0.16	0.21 ± 0.12	0.71 ± 0.11	0.91 ± 0.47	1.56 ± 0.50	0.37 ± 0.04[d]
6 hr Recovery	1.77 ± 0.07	0.49 ± 0.01	0.05 ± 0.01	0.87 ± 0.01	0.28 ± 0.01	2.31 ± 0.07	0.21 ± 0.02
50 mg L^{-1} NaN_3							
2 hr Exposure	1.65 ± 0.13	0.65 ± 0.09	0.15 ± 0.04	0.81 ± 0.02	0.39 ± 0.03	2.44 ± 0.25	0.24 ± 0.03
1 hr Recovery	1.68 ± 0.19	0.56 ± 0.02	0.08 ± 0.01	0.84 ± 0.01	0.34 ± 0.04	2.31 ± 0.21	0.26 ± 0.04
2 hr Recovery	1.94 ± 0.16	0.54 ± 0.05	0.06 ± 0.02	0.87 ± 0.02	0.28 ± 0.04	2.53 ± 0.17	0.19 ± 0.01
Hypoxia							
2 hr Exposure	1.63 ± 0.14	0.57 ± 0.10	0.08 ± 0.03	0.84 ± 0.02	0.35 ± 0.06	2.28 ± 0.23	0.28 ± 0.03[d]
1 hr Recovery	2.04 ± 0.06	0.57 ± 0.05	0.06 ± 0.01	0.87 ± 0.01	0.28 ± 0.02	2.67 ± 0.11	0.22 ± 0.03
6 hr Recovery	1.93 ± 0.40)	0.45 ± 0.06	0.02 ± 0.01	0.89 ± 0.01	0.24 ± 0.02	2.40 ± 0.46	0.17 ± 0.01

[a]All values are reported in μMol g^{-1} wet weight of foot muscle ± SEM.

[b]N=3 for all values of ATP, ADP, AMP, AEC, ADP/ATP, and total adenylate.

[c]ADP_{free} values were calculated from Shofer et al.(1997b) using NMR determined values of PA and ATP and the arginine kinase equilibrium equation. N=40 for control value and N=5 for all other values.

[d]Significantly different from control values, $p < 0.01$.

Table 2. Effects of Stress Exposure on Nicotinamide Adenine Dinucleotide Concentrations.

	NADH[a,b]	NAD[a,b]	NADH/NAD
Control	19.3 ± 4.4	97.6 ± 14.7	0.198 ± 0.035
1.2 mg L^{-1} PCP			
2 hr Exposure	22.0 ± 3.8	97.2 ± 17.1	0.230 ± 0.025
1 hr Recovery	8.2 ± 2.3	64.7 ± 1.3[c]	0.127 ± 0.037
6 hr Recovery	16.5 ± 3.0	103.1 ± 3.1	0.159 ± 0.027
50 mg L^{-1} NaN_3			
2 hr Exposure	26.9 ± 9.2	118.0 ± 7.3	0.220 ± 0.069
1 hr Recovery	16.0 ± 1.6	113.6 ± 2.9	0.141 ± 0.016
2 hr Recovery	14.8 ± 3.2	117.6 ± 3.0	0.125 ± 0.025
Hypoxia			
2 hr Exposure	17.9 ± 7.2	85.9 ± 9.6	0.197 ± 0.077
1 hr Recovery	18.1 ± 8.2	111.8 ± 11.0	0.157 ± 0.063
6 hr Recovery	17.9 ± 7.2	105.2 ± 22.2	0.135 ± 0.054

[a]All values are reported in nMol g^{-1} wet weight of foot muscle ± SEM.
[b]N=3 for all values.
[c]Significantly different from control values, $p < 0.05$.

Nicotinamide Adenine Dinucleotides

The HPLC method proved adequate for the determination of NAD concentrations (Table 2); however, NADH concentrations were close to the limits of detection in some extractions, so the resulting determinations were hampered by a significant noise contribution. In addition, the separate extraction required for NADH determinations added variability to these values. Therefore, the resultant concentrations and NADH/NAD ratios display significant deviation. Nevertheless, NAD values did display the expected declines in PCP and hypoxia-exposed animals, indicating the use of anaerobic respiration for energy production. Surprisingly, NaN_3-exposed animals did not display declines in NAD as was expected, although NADH/NAD ratios did show a modest increase over control values.

DISCUSSION

Adenylate Concentrations

Responses of ATP to stress exposures in this study are similar to previously reported values as determined by NMR (Shofer et al., 1997b) and conventional biochemical methods in studies of hypoxic effects in other species of abalone (Gade, 1988; Baldwin et al., 1994). ATP is thought to occur primarily in a freely soluble form within the cytosol and so NMR visible (Cerdan and Seelig, 1990; Kamp et al., 1995), which is supported by the correspondence between NMR and conventional determinations of ATP in this investigation. In contrast, acid-extractable ADP concentrations are significantly higher then ADP_{free} concentrations (Table 1) by a factor of two as determined using the AK equilibrium equation. This may reflect the intracellular binding of ADP to macromolecules (Cerdan and Seelig, 1990), resulting in broad linewidths caused by short T_2 relaxation times, effectively rendering these compounds undetectable by NMR.

Acid-extractable ADP values measured in this study were similar to previously reported concentrations of 0.52 μmol g^{-1} (wet wt) in the scallop *Argopecten irradians*

Figure 2. Psuedo-first order rate constants (k_{for}) vs ADP_{free} for the forward (ATP forming) direction of the AK reaction as determined by saturation transfer NMR in living abalone exposed to 1.2 mg L^{-1} of PCP, 50 mg L^{-1} of NaN_3, or $\leq$ 1.0 mg L^{-1} of O_2. ADP_{free} concentrations are determined from the AK equilibrium equation in the same animals. Exposure procedure was conducted as described in the text. N=5 for each data point; data from Shofer et al.(1997b).

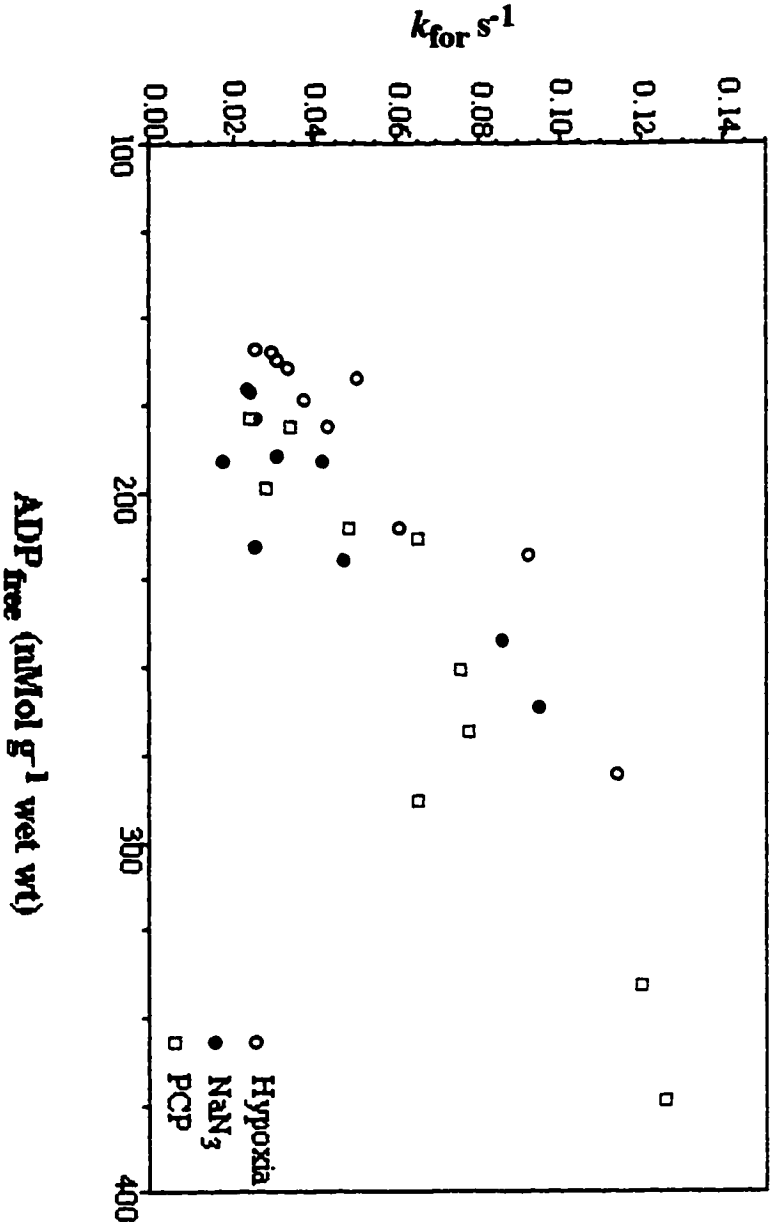
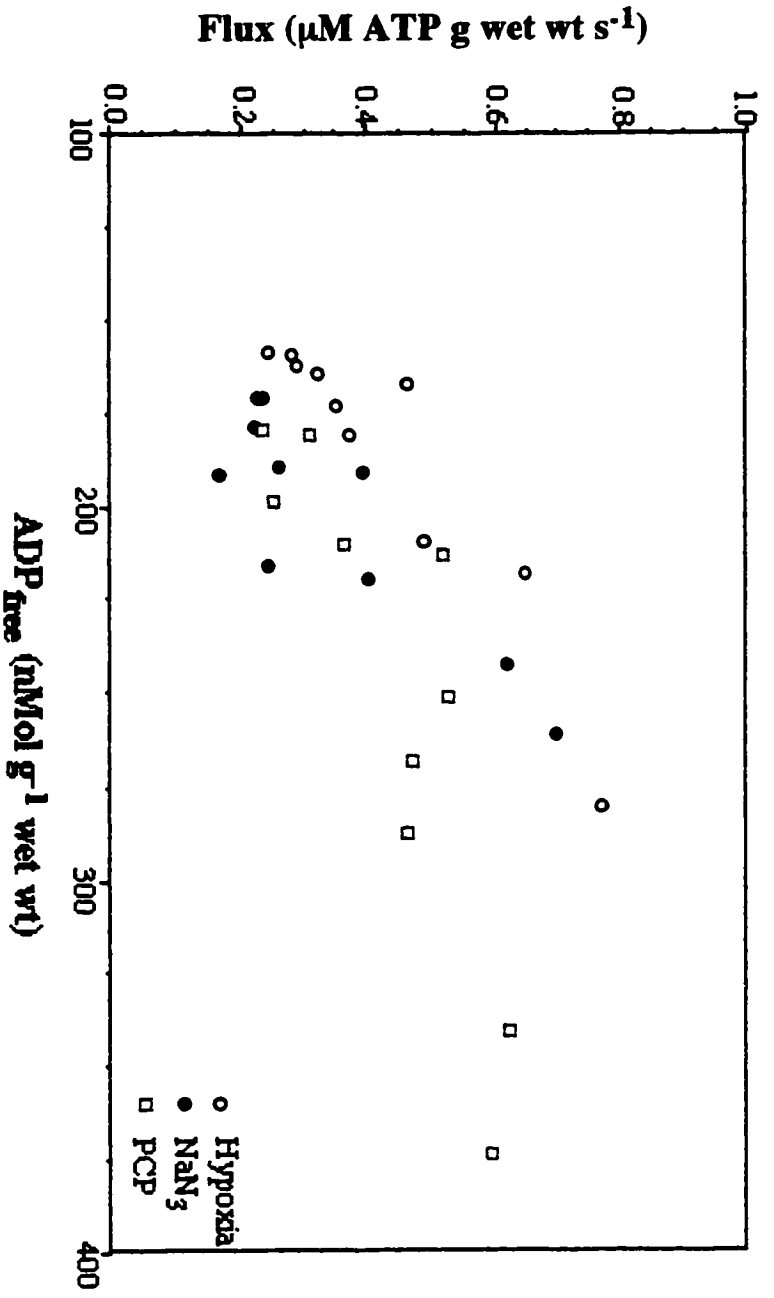


Figure 3. Flux (PA $*k_{for}$) vs ADP_{free}. Exposure conditions are described in Fig. 2. Note the decline in flux at high ADP_{free} concentrations in PCP exposed animals which may be due to inhibitory concentrations of ADP_{free} or declining levels of PA.



concentricus (Graham et al., 1986) and 0.37 μmol mL^{-1} (cellular water) in the lugworm *Arenicola marina* (Kamp et al., 1995). However the same studies reported ADP_{free} values of 0.051 μmol g^{-1} (wet wt) and 0.0026 μmol mL^{-1} (cellular water) respectively, much lower that those calculated here. This discrepancy may be due to the use of an Arg concentration of 8 μM g^{-1} (wet wt; Mai et al., 1994) in the ADP_{free} calculation; there was no indication whether the value had been corrected for PA hydrolysis. This Arg concentration is very close to resting concentrations of PA (9.5 μM g^{-1} wet wt), while Graham et al. (1986) reported free Arg concentrations in resting scallop muscle at 48% of PA concentrations. If a similar ratio of Arg/PA is used to calculate ADP_{free}, resting values are similar (0.085 μmol g^{-1} wet wt) to those of Graham et al. (1986).

ADP_{free} and Arginine Kinase

ADP is thought to play an important role in the regulation of mitochondrial oxidative phosphorylation (Chance et al., 1985; Jeneson et al., 1996), and in the regulation of glycolysis at the level of pyruvate kinase (Hochachka and Somero, 1984). In this investigation, increases in ADP_{free} correlate with increases in both AK k_{for} rates and flux, although PCP-exposed animals display deviations in linearity of flux at high ADP_{free} concentrations. In addition, strong inverse correlations also occur with declines in ATP concentrations (Shofer et al. 1997b). Previous *in vitro* (Kuprianov et al., 1984; van Dorsten et al., 1996) and *in vivo* (Bittl et al., 1987; Neubauer et al., 1988) studies have demonstrated that the creatine kinase (CK) rate equation accurately predicts reaction rate, and under physiological conditions ADP is the limiting substrate in setting forward reaction velocity in resting rat brain, heart, and skeletal muscle (Bittl et al., 1987). However, in perfused ferret heart recovering from ischemia, CK reaction velocity decreased and ADP was no longer rate controlling, although the rate equation was still predictive given

substrate pools which occurred following the ischemic insult (Neubauer et al., 1988). Unlike mammalian studies, abalone AK reaction velocities increase with the application of hypoxic or toxicant induced stress, and are well correlated with ADP_{free} concentration, suggesting that ADP_{free} may control AK forward reaction velocity as previously demonstrated for CK (Bittl et al., 1987)

The discrepancy in response of flux between previous mammalian studies and this investigation may be understood in light of the differences in anaerobic respiration capabilities and K_{eq} values of CK and AK between species. In mammals, anaerobic respiration capabilities are limited (Hochachka and Somero, 1984), and the K_{eq} of CK is the highest of any of the high-energy phosphagen storage compounds (ie., phosphocreatine, phosphoarginine, phosphotaurocyamine, phosphoglycocyamine: Ellington, 1989). Upon introduction of ischemia or hypoxia in mammalian tissues, which possess high concentrations of phosphocreatine (PCr), phosphorylation potential sharply declines (Neubauer et al., 1988; Hamaoka, et al., 1996). However, due to the high CK K_{eq} ATP, and therefore ADP, concentrations are maintained at normal physiological levels until approximately 95% of PC has been depleted (Fig. 4; Ellington, 1989). As ADP concentrations begin to rise, which would normally result in increases in CK reaction rates, PCr concentrations have fallen to values close to K_m, becoming important controlling elements in CK reaction velocity, and consequently the expected increases in rate are not observed (see Neubauer et al., 1988).

In abalone, hypoxia results in a decreased but stable phosphorylation potential as the animal begins to rely on glycolytic ATP production which, due to lower AK K_{eq}, results in declines in ATP (and increases in ADP_{free}) at modest decreases in PA concentration. This permits elevation of flux while PA concentrations are well above K_m (PA K_m = 3.5 mM for the squid *Symplectoteuthis oualaniensis*; Storey, 1978), resulting in the observed increases in reaction rate with increasing stress. Interestingly, reaction rates in

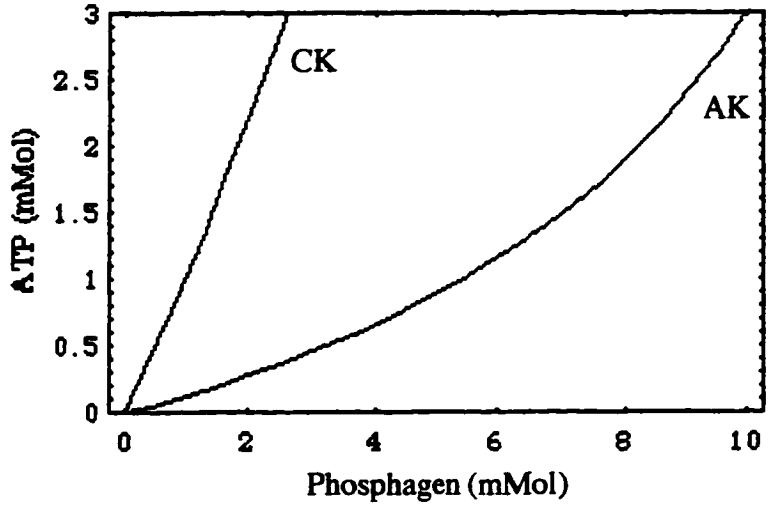


Figure 4. Calculated changes in ATP with declines in PA or PCr. Curves were calculated using equation 1 with ADP and pH set at fixed values of 0.17 mMol and 7 respectively. K_{eq} values of $1.32 \cdot 10^8$, and $1 \cdot 10^9$ were used for AK and CK respectively.

PCP-exposed animals appear to decline at the highest levels of ADP_{free} (Fig. 3), which may reflect either inhibitory actions of high ADP_{free} concentrations or low PA concentrations which occurred in these animals. This is similar to the action of ischemia on CK velocity in mammalian systems as described above.

Ellington (1989) discussed the possible advantages of low K_{eq} phosphogens (ie. PA) in organisms which frequently experience periods of hypoxia/anoxia. The low K_{eq} for compounds like PA permit the buffering of ATP concentrations over a greater range of phosphorylation potentials then would be achieved with a high K_{eq} phosphagen such as PCr. Increases in ADP and AMP enhance glycolytic flux (Lehninger, 1993), and so low

K_{eq} phosphagens permit the transition to anaerobic respiration while still maintaining an ATP buffering system.

Adenylate Energy Charge

Many authors have proposed the use of AEC as a sensitive, general physiological measure of stress imposed by toxicant or environmentally induced perturbations in energy production within an organism (Ivanovici, 1980; Giesy, 1988). Atkinson (1977) theorized that declining AEC values would reflect inhibition of ATP-utilizing processes such as those involved in growth and reproduction, with a simultaneous stimulation of ATP generating reactions which consume stored energy forms such as glycogen and lipids; declines in AEC below a critical value of 0.5 would result in death. In this investigation, AEC showed small, non-significant changes in response to hypoxia and NaN_3 exposure, while PCP-exposed animals displayed larger, but highly variable declines in AEC. Previous evaluations of AEC in abalone subjected to hypoxia also displayed similar small declines (Baldwin et al., 1992; Ryder et al., 1994). In contrast, ADP_{free} doubled in response to the same stresses, and previous studies have shown large increases in lactate and tauropine concentrations in response to hypoxia demonstrating a significant perturbation of energy homeostasis not reflected in AEC declines. This investigation demonstrates that the lack of sensitivity in AEC is due to the failure of AEC to measure changes in cytosolic adenylate concentrations which are the critical parameters involved in control of energetic homeostasis.

Measurement of AK-catalyzed exchange rates may be a useful tool for the assessment of physiological stress in marine invertebrates. Unlike AEC, AK exchange rates directly measure the cytosolic phosphorylation potential reflected in ADP_{free} concentrations. This may also provide a qualitative *in vivo* measure of important kinetic parameters of energy producing pathways because ADP is a substrate for several glycolytic

enzymes, and so changes in AK flux due to increases in ADP_{free} are also likely mirrored by enzymes such as phosphofructokinase and pyruvate kinase. In conclusion, this study has shown that *in vivo* NMR approaches are more sensitive to subtle but significant physiologic changes in abalone energy homeostasis which are not detected by monitoring of AEC.

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CHAPTER 6

ASSESSMENT OF TOXICITY IN MARINE ORGANISMS BY IN VIVO NMR: CONCLUSIONS

This dissertation documents the development of ^{31}P-NMR spectroscopy techniques for the evaluation of sublethal toxicity in fully intact marine organisms. These techniques permit the monitoring of several important physiological parameters in real time during periods of intoxication and recovery. In addition, classical biochemical parameters were also evaluated and compared with results from NMR experiments in order to emphasize the strengths and weaknesses inherent in the different approaches.

Each set of experiments compared physiological response of red abalone to PCP, NaN_3, or hypoxia using a flow-through perfusion system while a variety of information concerning energetic homeostasis of high-energy phosphates was collected. Animals displayed rapid onset of effect due to hypoxia and NaN_3 exposure, resulting in declines in PA, and subsequent increases in P_i. PCP resulted in a more gradual onset of physiological stress, but a longer time to recovery when compared with hypoxia and NaN_3 exposure, where animals recovered within several hours of the removal of the stressor. Changes in intracellular pH were not simply predicted by increases in P_i concentrations, but rather

responded in a toxicant specific manner, where NaN_3 produced sharp declines in pH_i which are likely due to increased anaerobic metabolism, while PCP exposed animals displayed moderate declines in pH_i at similar P_i concentrations which may reflect partial reliance on mitochondrial energy production. Metabolic suppression may also have contributed to mild intracellular acidification as was observed hypoxia exposed abalone which showed small declines in pH_i and PA. Intracellular free Mg^{2+} was also monitored using the α and β resonances of ATP. This showed small declines during all three stress exposures, but during recovery in NaN_3 exposed animals Mg_{free} increased above resting levels. The significance of this result is unclear, but may be indicative of mitochondrial damage resulting in the release of sequestered Mg^{2+} (Chapter 2).

An additional perfusion system was developed (Chapter 3) to permit the evaluation of changes in energetic status of abalone veliger larvae by ^{31}P-NMR in response to toxicant exposure. Veligers, which are thought to be more sensitive to toxicant exposure then adult animals, were exposed to PCP at concentrations similar to adult animals. Surprisingly, changes in PA and P_i were similar to adult animals, while total nucleotides declined, which was not observed in adults, although this is likely due to the limited pool of PA, and so limited nucleotide buffering, within veliger tissues. In contrast to adult exposures, veligers displayed a rapid onset of effect and recovery from PCP exposure which is likely due the high surface to volume ratio of the larvae resulting in rapid toxicokinetics. This study demonstrated that high sensitivity of abalone larva to toxicants is likely due to perturbations of development, rather than differences in biochemical response when compared with adult animals.

Saturation transfer techniques were employed to evaluate the formation of ATP via the arginine kinase reaction in response to stress exposure. Rates of flux were highly sensitive to toxicant or hypoxia exposure, increasing by roughly 10-fold over resting values (Chapter 4). This appears to be correlated with increases in cytosolic concentrations

of ADP (Chapter 5) which may act as the rate limiting substrate controlling the AK reaction while PA concentrations are high. These results were contrasted with the classical biochemical stress response of adenylate energy charge which displayed no significant declines during the imposition of stress. Consequently these studies have demonstrated the greater sensitivity of the NMR approach in evaluating the effects of toxicant or hypoxia induced disturbance of energetic homeostasis.

The original goal of this study was to evaluate the mechanisms whereby mitochondrial poisons cause toxicity in anaerobic tolerant organisms. The experiments presented here (Chapters 2-5) demonstrate that anaerobic respiration is adequate to maintain abalone during periods of mitochondrial shutdown. Toxicity is likely the result of additional mechanisms of action of PCP and NaN_3, either by preventing the reduction in ATP utilization, or by stimulating ATPases and so exceeding the capacity of glycolysis to maintain phosphorylation potential. Studies involving the environmental application of the techniques developed in this dissertation are already in progress, and will hopefully result in new methods for identifying unrecognized threats to the health of marine organisms early enough to avoid serious consequences.