SCAC Generic Report of Work Accomplished (Summer Stipend)

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Department: Biological Sciences

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Original Title of Approved Project: *In situ* EDX and WDX Microanalysis of Cu in the Gill Epithelia of Molluscs exposed to Cu.

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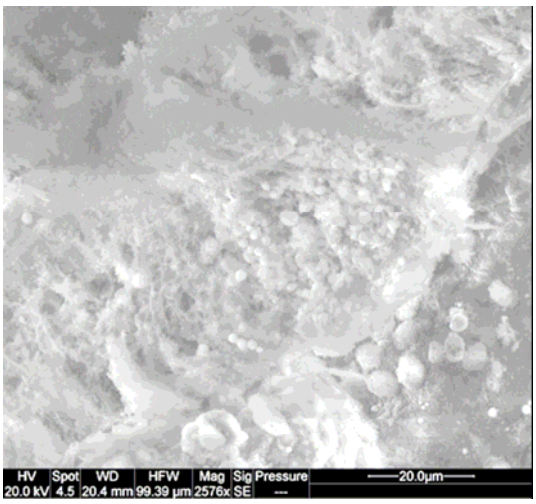
An empirical approach for understanding and differentiating between the beneficial and detrimental roles of metals is to undertake speciation studies and correlate the subcellular and molecular distributions of the metals with the physiological condition of the organism. In recent years, our laboratory has developed new technologies involving high performance liquid chromatography (HPLC) interfaced inductively coupled plasma mass spectrometry (ICP-MS) to study the speciation of metals in biological fluids and tissue extracts [1, 2, 3]. The major virtues of this hybrid technique for studying metal homeostasis and toxicity are its selectivity, sensitivity and multi-elemental capability. Also, because the interfacing of the two instruments is relatively simple, the technique is highly versatile and can be readily modified for specific applications. It can be used either qualitatively or quantitatively and increased selectivity can be achieved by the addition of multiple columns coupled in tandem prior to the ICP-MS [1, 4, 5]. The use of a mass selective detector also raises the possibility of using stable isotopic tracers to monitor temporal changes in the speciation of metals [6]. This feature is particularly useful for the kinetic analysis of Cu, and other metals, having radionuclides with short physical half-lives[1,7]. To illustrate the utility of this technique for studying Cu metabolism we have undertaken preliminary studies using a molluscan model system. Molluscs are particularly suitable for studying Cu homeostasis since they require substantial quantities of Cu for the synthesis of the Cu-containing respiratory pigment hemocyanin, but are nevertheless highly sensitive to elevated concentrations of the metal, experiencing toxicity at concentrations tolerated by most other invertebrates. Using this approach, we have shown have shown differential accumulation, turnover and loss of Cu in tissues and within specific cytosolic proteins over physiological time scales. These studies are the first to report pharmacokinetic tracer data on Cu beyond a week of study. While this new technique can provide valuable information on the biochemical speciation, turnover and distribution of cytosolic Cu and other metals, it cannot be used to study the relative importance of non-cytosolic metal-ligand pools in the overall cellular homeostatic process. This can be only accomplished using methodologies with capabilities for

probing the chemical constituents of cellular organelles and other cellular structures *in situ*.

To better understand the cellular mechanisms underlying these tissue-level responses observed in our directly coupled HPLC-ICPMS studies, this SCAC award was used to study the subcellular distribution of Cu and Cd in the supramolecular structures of the gills, kidney and visceral complex of animals exposed to elevated concentrations of these two metals. Analyses were conducted using an Oxford Inca energy dispersive X-ray microanalysis system (EDX) attached to our new FEI Quanta 200 Environmental Scanning Electron Microscope (ESEM). As indicated in the original proposal, animals were exposed for 8 weeks to filtered seawater (0.45µm Millipore, $35^{o}/_{oo}$ salinity) containing NTA ($5x10^{-6}$M) and $CuCl_2$ ($5x10^{-6}$M) to give a calculated (MINEQL+) free ion activity of Cu (II) of 10^{-11} M. For the Cd^{2+} treatment animals were exposed to seawater containing $1.0x10^{-4}$ M NTA and $2x10^{-6}$ M $CdCl_2$ to give a calculated free ion activity of Cd^{2+} of 10^{-7} M. These concentrations were chosen since prior studies had shown that these organisms could physiologically adapt to tolerate these elevated doses of metal without showing signs of overt toxicity. No deaths or morbidity was observed during the study. Unexposed animals, maintained in untreated seawater, were used as a control group. The media was replaced weekly and the animals were maintained, without feeding, at 15^{o}C on a 12-hour light/dark photoperiod. Food was withheld to ensure appropriate dosage and exposure.

Although originally it was proposed that animals would be euthanized weekly to allow the temporal sequence of metal accumulation to be followed, the protocol was modified such that the animals were only sampled at the completion of exposure. Organs of interest of three individuals from the control and metal exposed groups were removed by dissection and fixed for one hour at room temperature in a solution of 4% gluteraldehyde in filtered seawater containing 0.05 M sodium cacodylate buffer (pH = 7.2) that had been osmotically adjusted to1200 mosmoles using distilled water. The tissues were then washed 3 x in the same cacodylate buffer and dehydrated using a graded ethanol series before transfer into acetone for critical point drying. Drying was achieved using liquid CO_2 as a transitional fluid and subjecting the specimen to conditions above the critical temperature (35^{o}C) and pressure (1200 psi). The dried tissues were then submerged in liquid nitrogen and cryofractured to provide tangential breaks in the cell to reveal discernible intracellular features for EDX analysis. Typical scanning electron micrographs of the cryofractured plane of a basophil and a digestive cell from the visceral complex of control animals are shown in figures 1 and 2.

Elemental X-ray microanalysis of various subcellular features found in the cells showed distinct peaks for Cu $K\alpha_1$ X-rays at 8.046 KeV Cu associated with lysosomal vacuoles within the gill lamellae of Cu exposed individuals. Cu was not observed to accumulate to the same level of significance in the other organs. These observations confirm the findings of the previous HPLC-ICP-MS studies that a large proportion of the newly assimilated Cu in the gill is associated with non-cytosolic ligands. Partially mineralized lysosomal features of this type normally arise from the degradation of intracellular molecules and organelles that accumulate the metal and are presumably inactivated as a result of this binding. Rather than being targeted for subsequent ubiquination and proteosome degradation, these proteins appear to be subject to lysosomal accumulation by microautophagy. One explanation for the apparent susceptibility of the molecules in the gill to be specifically denatured by Cu may be the relatively high oxygen tension values found in these cells. Respiring tissues are known to produce a number of reactive oxygen species as a result of oxidative metabolism that are highly reactive and destructive to the cell causing protein oxidation and lipid



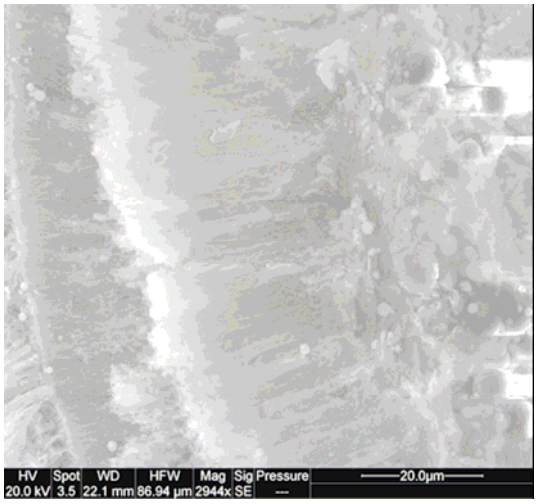


Figure 1 ***Figure 2***

Legend Figure 1. SEM micrograph of a cryofractured basophil cell showing Ca, Mg pyrophosphate concretions approximately 2µm in diameter found at the serosal portion of the cell. The primary function of this anabolic cell is to secrete digestive enzymes to effect extracellular digestion and subsequent endo-phagocytosis by the digestive cells of the gland. Scale bar represents 20µm, kV = 20.

Legend Figure 2. High power SEM micrograph of the microvillus brush border of a cryofractured digestive cell. The individual elements of the microvilli are clearly visible together with the supporting actin fiber cytoskeletal network Scale bar represents 20µm, kV = 20.

peroxidation. We hypothesize that this situation is exacerbated by the presence of the accumulated Cu, since this redox reactive transition metal has resting eV potentials that enables it to undergo redox cycling within cells via Fenton-like and Haber Weiss reactions by reacting with physiologically produced superoxide anions and peroxides generated during respiration. Cu (Me) catalyzed reactions with hydrogen peroxide and the superoxide radical would follow the generalized scheme thus

$Me^{n+} + H_2O_2 \rightarrow Me^{(n-1)+} + HO_2^* + H^+$

$2HO_2^* \rightarrow 2O_2^{*-} + 2H^+ \rightarrow H_2O_2 + O_2$

$O_2^{*-} + Me^{n+} \rightarrow O_2 + Me^{(n-1)+}$

The reduced Cu(I) metal ions can subsequently react with hydrogen peroxide generating hydroxyl radicals that promote cellular breakdown as shown by the reaction

$Me^{(n-1)+} + H_2O_2 \rightarrow Me^{n+} + OH^* + OH^-$

Recent studies indicate that the reaction shown in the last equation may not be a simple one-electron oxidation of the metal and it has been proposed that an intermediate high oxidation state form of the metal (Me^{2+}) may be formed in the reaction.

The data generated from the X-ray microanalyses of the Cd exposed specimens was disappointing in that no major peaks for Cd at 3.133KeV were noted. This implies that the majority of the accumulated metal occurs evenly distributed in cytoplasmic proteins. This dispersal essentially dilutes the relative concentration of the element

below 0.5 gravimetric % rendering it below the limit of detection of analysis by the instrument. This situation was not unexpected, in that our earlier HPLC-ICP-MS had shown that the majority of the accumulated Cd in the metabolically active tissue was associated with two major isoforms of a protein that had been tentatively identified as metallothionein[7]. A full-length cDNA of one isoform of the protein has been cloned during this project by RT-PCR from RNA from Cd exposed animals using degenerative primers (see below). The deduced sequence clearly illustrates an abundance of cysteines (30%), which is characteristic for this class of protein (figures 3 & 4).

Figure 3. Nuclotide sequence for full-length cDNA of a molluscan metallothionein gene.

TCTTCAGTTTTCGGAGCAGGATGCACGGACGTGTGCAAGCA
GACGCCATGCGGCTGTGCCACCTCGGGCTGTAACTGCACGG
ACGACTGCAAGTGTCAGTCATGCAAATACGGAGCGGGTTGC
ACGGACACATGCAAGCAGACACCATGTGGGTGTGGCAGCGG
GTGCAACTGTAAGGAGGACTGTCGCTGTCAGAGCTGTTCCAC
CGCCTGCAAGTGTGCGGCTGGAAGCTGCAAGTGCGGCAAGG
GATGCACAGGGCCAGACAGCTGCAAGTGTGACCGATCGTGC
TCCTGCAAATAA

Figure 4. Deduced amino acid sequence showing preponderance of cysteines arranged in C-x-C and C-x-x-C motifs characteristic for metallothioneins.

SSVFGAGCTDVCKQTPCGCATSGCNCTDDCKCQSCKYGAGCT
DTCKQTPCGCGSGCNCKEDCRCQSCSTACKCAAGSCKCGKG
CTGPDSCKCDRSCSCK

In the original SCAC proposal we had intended to increase the relative concentration of Cd in the ESEM samples above the detection limits using oxygen plasma incineration. This technique removes the organic bulk of the specimen as volatile oxides (CO_2, H_20, SO_2, NO_2), leaving the Cd *in situ* as non-volatile metal oxides and phosphates. The overall effect of this process is to increase the X-ray signal to noise ratio of the Cd in the remaining deposits by removing the *Bremmstralung* radiation arising from non-specific kinetic effects caused by the deceleration of the primary electron beam as it is passes the proximity of proton fields. Unfortunately this procedure was not possible because the sputter coater that was originally intended for conversion to produce an O_2 plasma was inoperable during the summer when the project was being conducted. These specimens have been archived and will be incinerated in the future once the opportunity arises.

In addition to undertaking the science to help complete a dataset for publication, one of the other primary goals of this SCAC award was to allow a full assessment of the capabilities, virtues and limitations of the new FEI ESEM and Oxford EDX instrumental software and hardware for teaching purposes. As stated in the original proposal, it was felt that this familiarization with the instrument would facilitate two important developments regarding the future use of the instrument in instruction and research. First, it would permit the design and development of an interdisciplinary upper division/graduate level analytical ESEM class for students in the natural and social sciences. This class reflects the diversity of the PI's on the original NSF award for the acquisition of the ESEM, who are from the Colleges of Natural Sciences and Mathematics (CNSM) and the College of Liberal Arts (CLA), and their objective of develop a student-based Interdisciplinary Institute that uses this, and other technologies,

for interdisciplinary research. While this SCAC award has made me reasonably conversant with the general operation and diagnosis of some of the idiosyncrasies of the instrument, I believe that following the vendor training workshop to be held in January 2005, I should have obtained sufficient additional technical expertise in operating, troubleshooting and maintaining the instrument to offer the class. The second training goal of this SCAC award was to assess the potential use of the ESEM in a GE class in nanotechnology. Nanotechnology is the next important growth area in bioengineering and a collaborative proposal involving myself, and Co-PI's from the Departments Physics and Engineering has been funded by NSF to develop undergraduate lower division and GE classes in this emerging discipline. In this respect, two specific aims were proposed to be addressed in the SCAC proposal: i) a determination of the spatial resolution of the instrument and ii) the linkage of software for remote access to the instrument. Some progress was made during this SCAC award with respect to both of these goals. First, it is now clear that the spatial resolution of the instrument resides outside of the nano-realm and its major use in this context will be to provide students a spatial bridge from the nano- to the more familiar micro-scale. Second, the instrument has been successfully fitted with PCI Quartz hardware and software and it is now possible to access the microscope remotely from a distance providing the client has Internet access and a web browser. This development allows the instrument to be a useful learning tool for distance learning, and collaborative demonstrations using this feature have been given to demonstrate the utility of this technology for large classes and for outreach. The software has been linked to a file server to allow clients to capture, store and retrieve messages. A search engine with an associated SQL server allows for data enquiry. Search terms include date, operator, specimen/file name and IP address. While the system is versatile and powerful, certain technical problems still exist with the hardware, including a substantial loss of resolution in the browser image on the client-side which compromises the ability to collaborate over the internet. PCI quartz was developed to accept video signals from a NTSC cable. Predictably, the FEI Quanta is the first generation all-digital microscope without any analog output, which was unknown at the time of purchase by any of the vendors. To solve the problem it will be necessary to retrofit the microscope with a digital to analog interface board to comply with 90's technology. The issues surrounding this retrofit without voiding the manufacturers extended warranty are currently being negotiated. Hopefully these problems can be corrected in the near future so that interactive sessions can be incorporated into the GE classes as indicated in the funded proposal.

Student Involvement: Two undergraduates, Saryna Bahadarakhann and Rachel Boornazian, have assisted with this project and have enrolled in Directed Research (Biology 496) to receive 3 units of class credit for their endeavors. To facilitate comprehension of the techniques and the project, a collaborative learning approach has been used to instruct the two students. The projects have been divided to be interdependent and complimentary with each student being responsible for one of the two treatments with metal. They have shown good collaboration and have been supportive in each other's endeavors. Together they have learnt the fundamentals of critical point drying, cryofracture, EDX, WDX and secondary and primary backscatter electron imaging. They are currently mastering micrograph interpretation and their directed research reports are due the end of this semester.

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