8-8-1985

UPTAKE OF ZN^{65} AND MN^{54} INTO BODY TISSUES AND RENAL GRANULES BY THE SOUTHERN QUAHOG, *MERCENARIA CAMPECHIENSIS*

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by

William Lynn Miller, Jr.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Marine Science in the University of South Florida

December, 1985

Major Professor: Dr. Norman J. Blake

Graduate Council
University of South Florida
Tampa, Florida

CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's Thesis of

William Lynn Miller, Jr.

with a major in Marine Science
has been approved by the Examining Committee
on August 8, 1985, as satisfactory for the
thesis requirement for the Master of Science
degree.

Thesis Committee:

Major Professor: Norman J. Blake

Member: Dr. Robert H. Byrne

Member: Dr. Joseph J. Torres

ACKNOWLEDGEMENTS

I would like to thank my committee, N.J. Blake, R.H. Byrne and J.J. Torres, for valuable input into the research and writing contained in this thesis. I am also grateful to R.H. Byrne for guidance into new and interesting research directions and to my major professor, N.J. Blake, for sticking with me during a time of personal development which I am sure, at times, seemed unproductive with respect to this thesis.

Special thanks also go to my friend Tom Matteucci for aid in clam collection and for the use of his light utility vehicle, "The LUV Truck." I also would like to thank my family for their patience and continued support. I am especially grateful to my wife, Joby, who has shared both my mundane chores and my future dreams. She is my constant support and my steady friend.

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UPTAKE OF ZN^{65} AND MN^{54} INTO BODY TISSUES AND RENAL GRANULES BY THE SOUTHERN QUAHOG, *MERCENARIA CAMPECHIENSIS*

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An Abstract

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A study was conducted to examine the influence of elevated inorganic phosphate and trace metal concentrations on the uptake and distribution of Zn^{65} and Mn^{54} into the body tissue and phosphoritic renal concretions of a subtropical estuarine bivalve, Mercenaria campechiensis. Chronological sampling of gills, mantle, adductor muscle, viscera, kidneys, and renal granules using gamma ray spectrometry allowed examination of both tissue distribution and accumulation patterns over time. The kidney was the principal site of Mn^{54} accumulation whereas Zn^{65} was concentrated in both the gill and the kidney. The gill initially accumulated Zn^{65} three times faster than the kidney and reached a steady state concentration after about five days. The kidney exhibited a linear accumulation throughout the ten day study for both Zn^{65} and Mn^{54} without obtaining a steady state concentration.

Elevated total Zn and Mn concentrations caused increased accumulation levels to the kidney and stimulated general metal uptake and distribution rates. Elevated dissolved phosphate seemed to increase the metal excretion rate by increasing the excretion of metal containing phosphoritic renal granules. Large concretions (up to 400 microns) occur in the kidney lumen of this bivalve and are

thought to result from the aggregation of smaller intracellular granules. Results from this study suggest that renal concretions function as both a long and short term detoxification response to elevations in the concentration of potentially toxic metals. Evidence also suggests that much of the metal metabolism involving the kidney, and consequently the general tissue distribution of metals, depends on the processes which control formation and excretion of these concretions.

Abstract approved: _____

Major Professor: Norman J. Blake

Professor of Marine Science

8/20/85

Date of Approval

CHAPTER 1: INTRODUCTION

Marine bivalves concentrate metals from their environment (Brooks and Rumsby, 1965; Segar et al., 1971). This capacity combined with their relative inability to escape contaminated conditions makes them useful as biological indicators of metal contamination in the marine environment (Goldberg, 1975; Schulz-Baldes, 1974). The successful use of bivalves as indicators requires an understanding of the relationship between tissue metal levels and environmental metal levels. Knowledge of the cellular processes and kinetics involved in the uptake and excretion of trace metals is essential to the understanding of this relationship.

Although much research has addressed the issue of trace metal uptake and excretion in marine bivalves, few studies have been devoted to tropical and subtropical species under typical temperature and salinity regimes. Even though mechanistic models for the processes involved in trace metal accumulation have been presented for some bivalve species (George and Pirie, 1980; Simkiss, 1981; George, 1982), there are fundamental aspects of the models that remain unclear. This is especially true of the time course of uptake into selected tissues and phosphoritic

granules which occur in the kidneys of marine bivalves (Carmichael et al., 1980). Better definition of metal accumulation models is needed along with a close examination of the usefulness of these models in application to varied bivalve species from differing environments.

This study is intended to contribute in three areas to the current knowledge on metal accumulation and detoxification in marine bivalves: 1) Limited metal accumulation research has been directed at subtropical bivalve species. Using radiotracers to obtain time series measurements, this study examines the general incorporation and tissue distribution of zinc and manganese in the southern quahog, Mercenaria campechiensis under conditions representative of its suptropical environment. 2) Dissolved phosphate and metal concentrations can show substantial temporal fluctuations in estuarine environments impacted by anthropogenic activities. An attempt is made to identify deviations from generalized accumulation patterns resulting from exposure to varied phosphate and total metal concentrations. 3) Although inorganic renal granules constitute a major metal detoxification mechanism for many bivalves, very little kinetic information on granule formation or trace metal incorporation is available. This study uses histological and radiochemical techniques to examine both of these processes.

CHAPTER 2: LITERATURE REVIEW

The scientific literature contains numerous articles concerning the relationship between marine bivalve molluscs and potentially toxic metals in their environment. Research has covered most areas of this relationship from the simple observation of elevated metal levels in bivalve tissue (Brooks and Rumsby, 1965; Seger et al., 1971) to the careful investigation of cellular mechanisms of metal uptake, transport, storage, and elimination. Several very good reviews exist which cover both general and specific aspects of the subject (Phillips, 1977; Cunningham, 1979; Eisler, 1979; George, 1982; Lucas and Hignette, 1983)

Present research interests seem directed at understanding the mechanisms which allow bivalves to accumulate potentially toxic metals far above environmental levels with no apparent detrimental effects to the individual. This includes both direct study of cellular pathways and study of intrinsic and extrinsic factors which influence cellular pathways. This review will concentrate on selected areas that seem particularly applicable to the present study.

Intrinsic Factors Affecting Metal Accumulation

Several authors have noted the relationships between age, size and weight, and accumulation of metals from seawater by marine bivalves. Schulz-Baldes (1973, 1974) noted that younger *Mytilus edulis* contain more lead per gram than larger individuals collected from the same area. It was also observed in the laboratory that the rates of uptake and loss in large mussels (shell length = 45-55 mm, average dry weight = 750 mg) were less than those in small mussels (shell length = 19-21 mm, average dry weight = 30 mg). Phillips (1976) has shown that seasonal fluctuations in the concentration of zinc (Zn), cadmium (Cd), and copper (Cu) in *M. edulis* are due, at least in part, to variations in wet weight. It is suggested from this that the use of mussels as bioindicator organisms of metal pollution be restricted to individuals of similar shell length and wet weight. Cossa *et al*. (1980), studying the environmental distribution of Zn, Cd, Cu, iron (Fe), manganese (Mn), and nickel (Ni) in *M. edulis*, noted that most of the variance in his data could be attributed to weight variation. Presumably, the increased metabolic and growth rates of younger (i.e. smaller, lighter) bivalves (Dame, 1972; Kennedy and Mihursky, 1972) contribute to metal concentration differences reported for bivalves of different size classes.

Another intrinsic factor which has been shown to affect metal accumulation is the sex and reproductive

condition of the study organism. This factor has received relatively little attention and effects seem to depend on the bivalve species and particular metal being investigated. Early work by Galstoff (1964) showed that Mn concentrations in female oysters were fifteen times higher than those in males. However, this was not the case for Fe, Cu, or Zn. Alexander and Young (1976) found no difference for lead (Pb), Cu, chromium (Cr), silver (Ag), or Zn in the mussel Mytilus californianus with regard to sex. For the mussel Chloromytilus sp., Watling and Watling (1976) found concentrations of Zn, Cu, and Mn to be greater in females while males showed greater concentrations of Pb and bismuth (Bi). These differences were most pronounced during the reproductive period. Lowe and Moore (1979) proposed that the higher Zn levels they found in the kidney of male mussels was a result of Zn in females being divided between the kidney and oocytes. Donax trunculus females examined by Mauri and Orlando (1983) exhibited higher levels of Mn and Zn than males with females showing much greater variation in Mn concentrations than males.

Knowledge of the reproductive condition of the organism has also proved important when interpreting metal accumulation data. Bryan (1973) noted large seasonal metal concentration variations for the scallops Pecten maximus and Chlamys opercularis. He attributed part of this variation to the seasonal changes of the

reproductive cycle. Delbaye and Cornet (1975) observed that Cu toxicity could be linked indirectly to reproductive condition due to alterations in metabolic rates during spawning. Similarily, Frazier (1975, 1976) showed Cd, Zn, and Cu tissue concentrations to follow gonadal development and spawning in the Chesapeake Bay oyster Crassostrea virginica. Few laboratory metal accumulation studies report the reproductive condition of the bivalve under investigation unless the study is aimed at this factor in particular. This seems an important consideration, especially in long term metal turnover studies where reproductive changes and the accompanying metabolic changes have been demonstrated to affect metal accumulation.

Extrinsic Factors Affecting Metal Accumulation

Factors such as temperature and salinity also play a role in the accumulation of metals by marine bivalves. Temperature affects metabolism (measured by pumping rates) in various bivalves (Loosanoff, 1939; Cole and Hepper, 1954; Feng, 1965; Kennedy and Mihursky, 1972) which in turn affects metal turnover rates. Cunningham and Tripp (1975) found the biological half life of mercury (Hg) in oysters to be shorter at higher summer temperatures (25 °C) than during a declining temperature regime which represented the change from autumn to winter (25-5 °C). A study involving Crassostrea virginica by Zaroogian (1980) showed that

temperature is also significantly related to accumulation of Cd.

Salinity effects on metal accumulation are also reported. Schulz-Baldes (1973) suggests that gradients of Pb concentration in M. edulis upstream in the Weser Estuary, Germany may either result from dilution of a polluted river by seawater or from the direct effect of salinity on uptake of Pb. George et al., (1977) showed Cd accumulation both in vivo and in vitro for M. edulis to be increased by increasing dilution of seawater. This was shown to be a function of the corresponding osmolarity that occurs with changing salinity.

Chemical form and concentration can affect metal uptake rates and fates in marine bivalves. Schulz-Baldes (1974) showed M. edulis to exhibit a constant rate of Pb uptake into tissue soft parts that depended on the total concentration of Pb in the uptake media. Other studies (George and Pirie, 1980; Zaroogian, 1980) have shown similar relationships between total metal concentration and accumulation for both Cd and Zn. Borchardt (1983) has examined the relative importance of metal association with food as it pertains to uptake of Cd into M. edulis. Mussels were exposed simultaneously to algae labeled with Cd^{109} and seawater labeled with Cd^{115}. It was determined from this study that Cd incorporated from food accounted for only 0.2 - 0.5 percent of the total Cd body

burden. This further emphasized the great importance, at least for Cd, of dissolved metals accumulated directly from seawater in the overall accumulation of metals by bivalves.

Closer examination of factors affecting metal uptake directly from seawater have shown contrasting results. Coombs (1977), while investigating Pb accumulation by M. edulis, found that when lead is complexed with various organic ligands such as humic acid, alginic acid, pectin, and citrate, both the rate of uptake and the total concentration of accumulated Pb increases as much as three to four times that of controls. George and Coombs (1977) showed a similar increase, about two times that of controls, due to chelation with EDTA, humic acid, alginic acids, and pecten. Work using model chelate buffer systems on the uptake of metals into marine bacteria, algae (Sunda and Lewis, 1978; Sunda and Gillespie, 1979; Sunda and Huntsman, 1985), and phytoplankton (Sunda and Guillard, 1976) indicated that the free metal ion is the chemical species which affects metal accumulation and toxicity. This suggests that complexation would reduce accumulation of a particular metal by reducing its free metal ion concentration.

Zamuda and Sunda (1982) were first to apply these techniques to bivalves. They used a seawater system buffered with nitrilotriacetic acid (NTA) to study the accumulation of Cu by the oyster C. virginica and found that for bivalves also, metal uptake was directly related

to the free Cu ion concentration and not to the concentration of chelated copper. The apparent discrepency of this finding with those reporting increased uptake with increased chelation (i.e. decrease in free ion concentration) was explained as resulting from decreased competition from other unmeasured metals for uptake sites. Because of a greater capacity of other metals to complex with the organic chelators, the availability of the measured metal to uptake sites is effectively raised relative to other metals present. This would explain the observed rise in uptake rates and accumulation for 'complexed' metals. Engel et al. (1981) pointed out that while a free ion model of accumulation has been found to be valid in many cases, it does not appear to apply to all metals and/or organisms.

Tissue Distribution and Cellular Mechanisms

Studies of the fate of metals in marine bivalves reveal considerable tissue specificity with regard to metal concentrations. Bryan (1973), studying the tissue distribution of metals in Pecten maximus and Chlamys opercularis, found the kidney to be a major site for Mn, Zn, and Pb accumulation with the digestive gland containing most of the Cd, Fe, Cu, Cr, Al, and Ag. Numerous studies of the tissue distribution of metals in M. edulis (Schulz-Baldes, 1974, 1977; Coombs, 1977; Coombs and George, 1977; George and Coombs, 1977; George and Pirie,

1980; Nolan and Duke, 1983) have shown the kidney to contain the highest concentrations of Pb, Cd, Cu, and Zn. Mercenaria mercenaria also accumulates Zn and Mn predominently in the kidneys (Carmichael et al., 1980). Distribution studies of Zn^{65} in M. edulis by George and Pirie (1980) showed the initial distribution to be predominantly in the viscera and mantle. But after equilibrium was established (about 25 days), the kidney again was seen to be the largest single compartment for Zn. It seems that even though specific metals and organs show differences in distribution patterns (especially between oysters and other bivalves), the kidney plays a large role in metabolism of most metals that are accumulated by marine bivalves.

Reports of differential accumulation and redistribution of metals among various bivalve tissues has stimulated investigation of cellular and subcellular mechanisms which may explain reported results. Combined analytical, ultrastructural, and biochemical studies have shown that marine bivalves appear to possess a variety of mechanisms which effectivly accumulate and remove potentially toxic metals from biochemical pathways essential to normal cellular function.

One of the more intensely studied areas is the role of metal binding proteins in metal detoxification and accumulation. Review articles by Coombs (1979), Roesijadi (1980), George (1980), and Luk'yanova and Evtushenko (1982)

have covered the literature in this area in great detail. Briefly, Cd, Cu, Zn, Mn, and Hg have all been found associated with metal binding proteins in various marine bivalves (Casterline and Yip, 1975; Talbot and Magee, 1978; George et al., 1979; Ridlington and Fowler, 1979; Carmichael et al., 1980; Frankenne et al., 1980; Viarengo et al., 1980; Carmichael and Fowler, 1981; Roesijadi et al., 1981). Engel and Brouwer (1982) have shown that for C. virginica, production of cadmium-binding proteins can be induced in the laboratory from a population showing no evidence of these specific proteins in the natural environment. These studies suggest that for the wide variety of shellfish investigated, metal-binding proteins play a significant role in uptake and inter-organ distribution of both essential and potentially toxic metals.

The eventual fate of metals in marine molluscs seems to be compartmentalization within subcellular organelles, a process which effectively removes excess or toxic metals from biochemical processes (George, 1982). The occurrence of metal-containing granules and vesicles in invertebrate tissues is well documented in reviews by Simkiss (1976), Coombs and George (1978), Brown (1982), George (1982), and Lucas and Hignette (1983). Three such compartments that seem to be of considerable importance with regard to metal metabolism in marine bivalves are granular amoebocytes, tertiary lysosomes, and inorganic calcium granules.

The haemolymph of Ostrea edulis possesses granular amoebocytes which contain Zn in membrane-limited vesicles within the cell (George et al., 1978). Pirie et al. (1984) has reported that O. edulis possesses not only granular haemolymph amoebocytes which specifically accumulate Zn but also amoebocytes specific for Cu accumulation. O. edulis, O. angasi, and C. virginica were also shown to possess granular cells containing both Cu and Zn. Thomson et al. (1985) also found blood amoebocytes in C. gigas which selectively accumulated Cu and Zn into membrane bound vesicles. In the oyster species mentioned above, the haemolymph amoebocytes play an important role in metal compartmentalization. In some cases amoebocytes contain greater than 90 percent of the whole body Cu and Zn (Thomson et al., 1985).

Another way in which marine bivalves sequester metals is through the lysosomal system. Lysosomal cells occur in many molluscan tissue types (Eble and Tripp, 1969; Moore, 1976, 1977; Moore and Lowe, 1977). Mytilus edulis concentrates metals in the tertiary lysosomes of the kidney (George et al., 1976, 1982; Moore, 1977; Schulz-Baldes, 1977; Lowe and Moore, 1979). Recent work by Thomson et al. (1985) showed Crassostrea gigas to sequester Ca, Fe, Cu, and Zn in tertiary lysosomes in all tissues examined except the reproductive tissues. George (1982) suggests that since lysosomal bodies often contain membraneous remnants, they may accumulate metals by a

'dustbin' mechanism which collects indigestible remains from lysosomal activity. Analysis of granules from the kidney of Pecten maximus by Overnell (1981) shows the presence of oxalate and protein, with amino acid analysis showing included protein to be very similar to erythrocyte membrane protein.

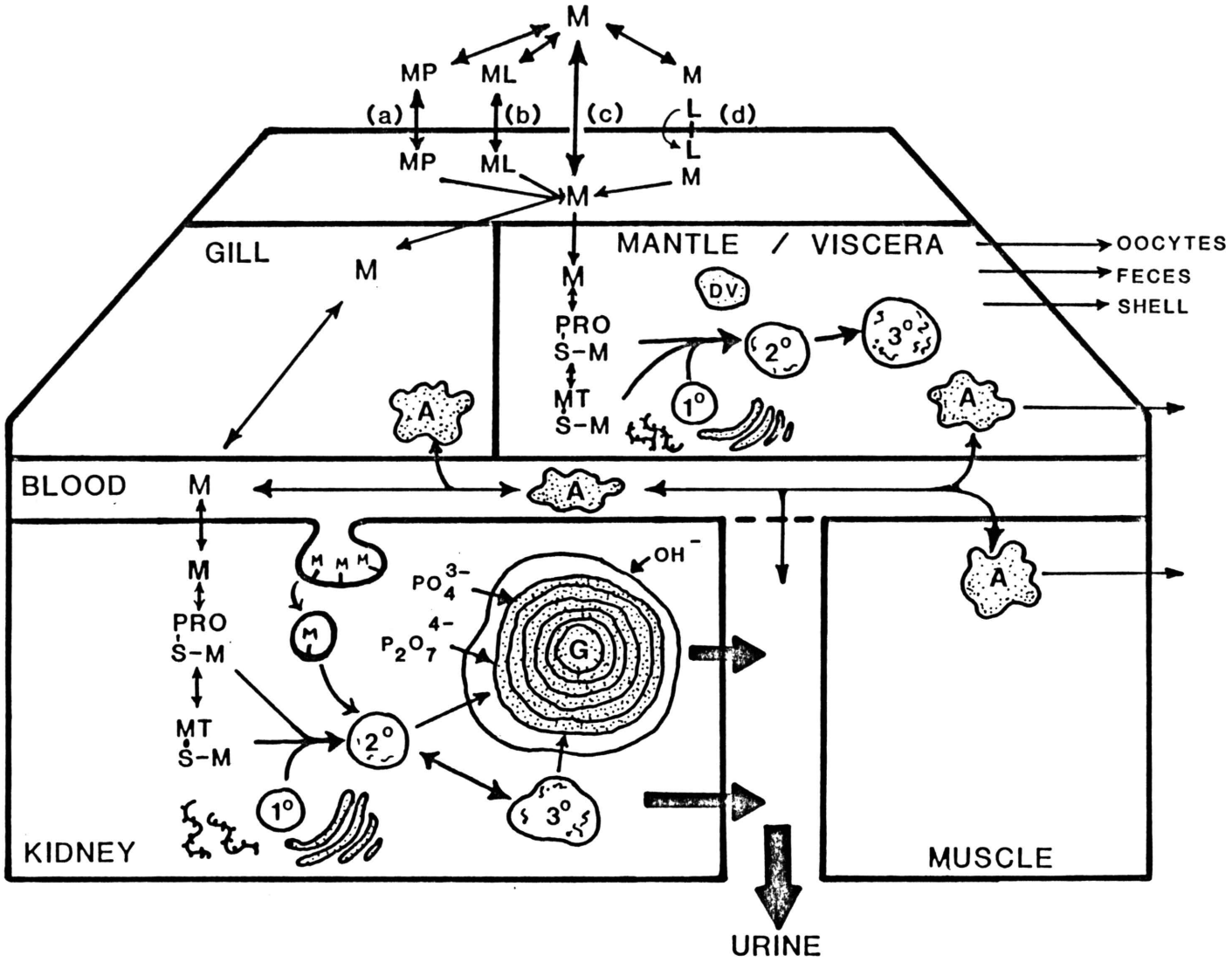
Inorganic granules are also used by marine bivalves to isolate excess or potentially toxic metals from biochemical pathways. These calcium granules, not to be confused with the pure calcium carbonate granules used for calcium storage, have been noted to occur in all phyla (Simkiss, 1976; Coombs and George, 1978). They are highly insoluble in saline solutions and are composed primarily of calcium and magnesium phosphates, but may contain lesser amounts of Al, Ag, Ba, Co, Cu, Fe, Mn, Pb, Sn, and Zn (Simkiss, 1976; Doyle et al., 1978; George, 1980, 1982). George (1982) reviews the occurrence of intracellular calcium phosphate-based granules and shows that all such granules in Lamellibranchia occur in the kidney. Lucas and Hignette (1983) cite 32 literature references to renal 'concretions' in marine bivalves dating back to 1791 (Poli, 1791). Although the chemical composition reported in these citations is variable, most of those analyzed were composed primarily of calcium and phosphorus. Simkiss (1981) has shown that some granules contain phosphorus not only as orthophosphate but as pyrophosphate which would tend to further decrease their solubility. By virtue of occurring

primarily in the kidney and being insoluble and therefore nonmetabolizable, these inorganic granules are generally considered to be excretory in nature and the last step in the removal of 'unwanted' or 'unneeded' metal ions (George and Pirie, 1979,1980; George et al., 1980; Simkiss, 1981).

Inorganic granules, along with haemolymph amoebocytes, metal binding proteins, and the lysosomal-vacuolar system comprise the major means by which marine bivalves metabolize potentially toxic metals. Each system alone is complex and not entirely understood. An attempt to integrate various mechanisms into a single useful model that deals with the entire organism becomes even more complex. Coombs and George (1977), George and Pirie (1980), Simkiss (1981), and George (1982) have all presented models for various components of this complex detoxification system. Figure 1 in this review is based on these four publications and combines them into one model.

Initial uptake of metals across the membrane into the gill, mantle, and digestive gland is proposed to have four possible routes. Figure 1(a) shows the 'pore' theory in which ions are channeled through an opening in the membrane with a shape that may impart some ion specificity. Figure 1(b) illustrates uptake of the metal after prior complexation with a ligand which neutralizes ionic charge, allowing easier penetration of the uncharged membrane. The third possible pathway, shown in figure 1(c), is carrier

Figure 1. Composite model for metal metabolism in marine molluscs. Based on Coombs and George (1977), George and Pirie (1980), Simkiss (1981), and George (1982). M = unbound metal ion; L = ligand; P = pollutant; PRO-S = protein with sulfhydral group; A = amoebocyte; MT-S = metallothionein; G = inorganic granule; DV = digestive vacuole; 1°, 2°, 3° = primary, secondary, and tertiary lysosomes.


M
MP
ML
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(a)
(b)
(c)
L
(d)
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M
GILL
M
MANTLE / VISCERA
OOCYTES
FECES
SHELL
M
DV
PRO
S-M
2°
3°
MT
S-M
1°
A
A
BLOOD
M
A
A
M
M M M
M
PRO
S-M
MT
S-M
2°
1°
PO4 3-
P2O7 4-
OH -
G
3°
KIDNEY
MUSCLE
URINE

mediated transport of metal ions across the cell membrane by attachment to a membrane bound ligand which passes the metal into the cellular cytoplasm by means of a conformational change in the membrane. This pathway is potentially very ion specific. Figure 1(d) represents the possible complexation of the metal with an organic contaminent prior to transfer across the membrane, altering the normal pathway. Once across the cell membrane, the metal is distributed throughout the organism by means of passive or active transport across cell membranes, or by granular amoebocytes in the haemolymph. Metal-binding proteins and/or metallothionein-like proteins bind metals (for Lamellabranchia, proteins have been reported to bind Cd, Cu, Zn, Hg, and Mn) and are eventually incorporated into the lysosomal system. Tertiary lysosomes have been reported for Lamellabranch molluscs in the gut, mantle, and kidney which contain Cd, Fe, Zn, and/or Pb. Metals which are transported to the kidney (the major portion for many metals and bivalve species) can then be incorporated into inorganic phosphoritic granules. The formation of these granules seems to involve a non-specific Ca pump with a supply of ortho- and pyrophosphate (Simkiss, 1981). Hydroxide ions may also be supplied in order to maintain the pH at a value high enough for precipitation to occur. Phosphate granules and tertiary lysosomes seem to be the major excretory routes for excess and/or toxic metals. Some additional loss of metals results from spawning, fecal

processes, and shell production.

This model is a framework for further definition and elaboration. It is presented as a generalized picture of the types of mechanisms active in the detoxification of metals by marine bivalve molluscs. The study which follows was designed to contribute needed data to this model.

CHAPTER 3: MATERIALS AND METHODS

Collection and Experimental Configuration

Specimens of *Mercenaria campechiensis* measuring from 15 to 30 centimeters in length were collected in September of 1983 near the mouth of Tampa Bay, Florida, U. S. A. Individuals were scrubbed with a stiff brush to remove epiphytic growth and mud. They were held a minimum of 48 hours in 17 ppt, 25 °C seawater. This allowed metabolic equilibration to experimental conditions and clearing of gut contents prior to use in this study.

Exposure tanks were arranged as in figure 2. All seawater used in this study was filtered through a 0.45 micron (μm) Gelman filter. Each tank contained 80 liters of filtered seawater (17 ppt, 27.5 °C) with 40.0 microcuries (μCi) of added Mn^{54} and 200.0 μCi of added Zn^{65}. Low metal concentrations (tanks 1 and 3) were due to environmental levels plus added radionuclides. Lower bounds (i.e. added metals) for Zn and Mn were 1.5×10^{-8} moles/liter and 1.3×10^{-10} moles/liter respectively. High metal concentrations (tanks 2 and 4) were initially adjusted with stable zinc and manganese chlorides to 0.2 mg/liter (Zn = 3.1×10^{-3} moles/liter; Mn = 3.6×10^{-3} moles/liter). Phosphate levels were

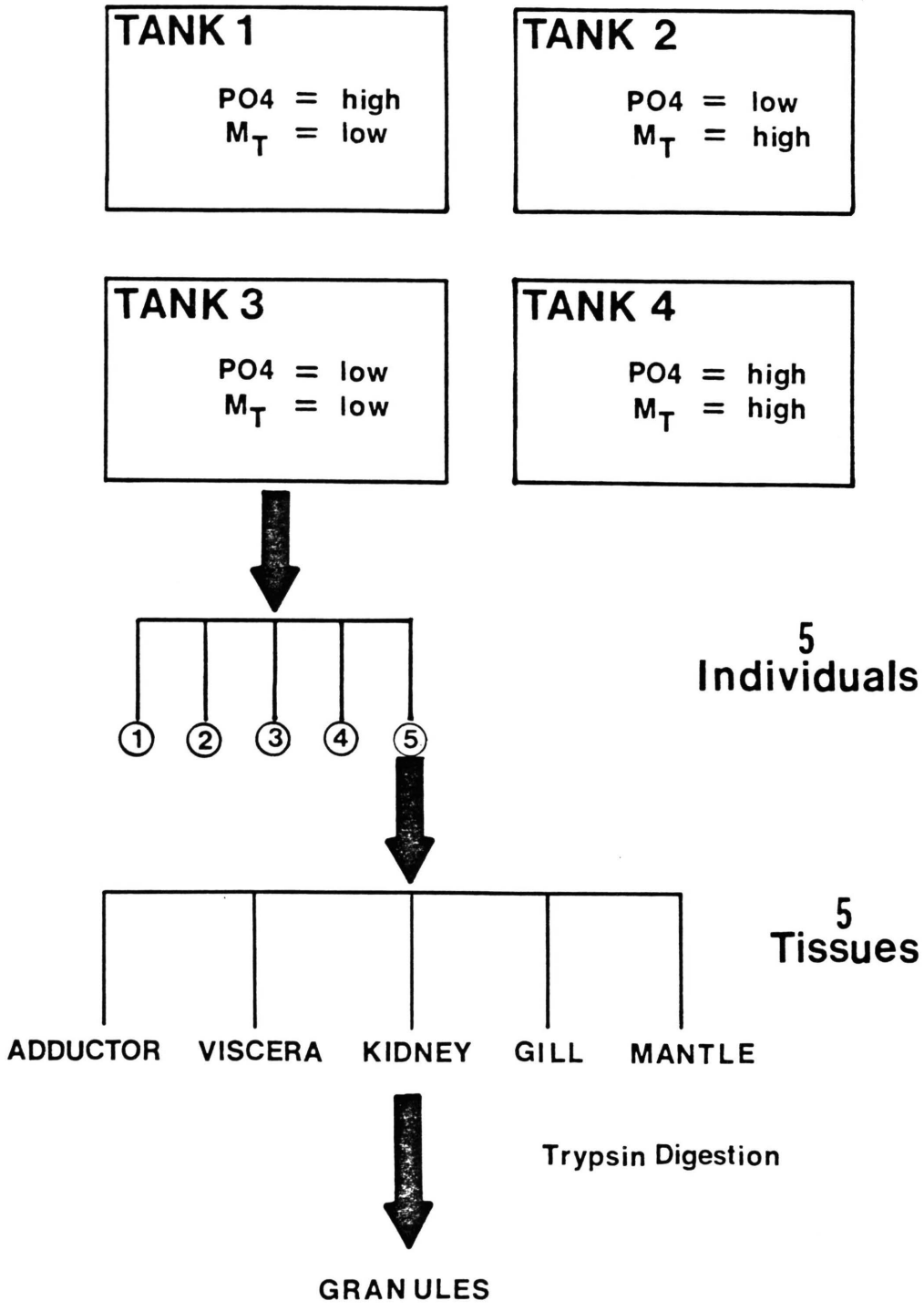


Figure 2. Schematic of experimental configuration.

initially adjusted with sodium phosphate and were measured daily thereafter. Orthophosphate concentrations were determined using spectrophotometric measurements of molybdate complexation with phosphate (Strickland and Parsons, 1968). Averages and standard deviations for measurements taken over the duration of this study were as follows: tank 1 = 29.0 ± 2.0 µM, tank 2 = 21.3 ± 2.3 µM, tank 3 = 17.9 ± 1.2 µM, tank 4 = 41.4 ± 3.9 µM. Tanks were aerated throughout the exposure period and clams were not fed.

Sampling and Data Analysis

At intervals of 1, 2, 4, and 10 days five clams were removed from each tank. The left mantle, the posterior adductor muscle, the left gill filaments, a cross section of the visceral mass dorsal to the foot (containing digestive diverticula and gonadal material), and both kidneys were removed from each individual (Figure 2). Tissues were blotted dry, placed into acid cleaned, preweighed glass vials (for wet weight determination) and frozen with liquid nitrogen. They were stored frozen until analysis. After initial measurements of whole kidneys, phosphoritic kidney granules were isolated by digestion of whole, analyzed kidneys with the filtrate of a 2.5 percent aqueous suspension of trypsin (Gold et al., 1979). Granules were collected by sedimentation, washed with deionized water, air dried, and weighed on a Mettler

balance to the nearest microgram. Granules isolated by this method showed no visible difference from granules isolated manually.

Gamma ray spectrometry employed a Bicron model TA-1161, 2" by 2" well type sodium iodide crystal detector connected to a Tracor Northern model TN-1710 multichannel analyzer. All tissues were counted whole in the glass vials in which they were stored. Each gamma peak was allowed to achieve a minimum of 10,000 counts in order to achieve statistical counting errors of less than or equal to one percent. All gamma spectra were stored on a Digital PDP-11 computer and analyzed using a Fortran program modified from Savitzky and Golay (1964). The program subtracted background counts, applied a 5 point smoothing routine, located peak positions, and summed gamma counts from 50 consecutive channels centered at each peak. Mn^{54} peaks were corrected for Zn^{65} interference using an equation of the form

$$CPM = y\ (A + Bx + Cx^2)$$

where CPM is the counts per minute in channel x, and y is the computer determined counts per minute for the Zn^{65} peak. Constants A, B, and C were determined by application of SAS (Helwig and Council, 1979) nonlinear least squares analysis to 10 seperate gamma spectra containing only Zn^{65} of varying activities. This allowed a mathematical description of the Zn^{65} count spectrum in

the region coincident with the Mn^{54} peak as a function of the amount of Zn^{65} present in each sample.

Histology

Clams were collected at 0, 4, and 10 days exposure for histological examination. Individuals were fixed with Helly's fixative made with zinc chloride (Barszcz and Yevich, 1975). After rinsing, clams were dissected into duplicate samples of the gill, viscera, and kidney and placed in cassettes for storage in a dehydrating agent (S-29) until processing. An Autotechnicon tissue processing system was used to automatically dehydrate (S-29), clear (UC-670), and embed in parafin (melting point 57-58 °C) the tissues prior to sectioning. Ten micron thick sections were cut from embedded tissue using a rotary microtome. These were mounted on slides and stained with Hemotoxylin and Eosin (Luna, 1968). Sample tissues were examined microscopically with regard to physiological state. Gonadal sections were examined to determine sex and the state of reproductive development. Representative kidney sections were examined with particular attention to inorganic phosphate granule formation.

Statistical Treatment

Data were analyzed using SAS graduated linear methods (Helwig and Council, 1979) allowing identification of significant differences between tissues and treatments. A

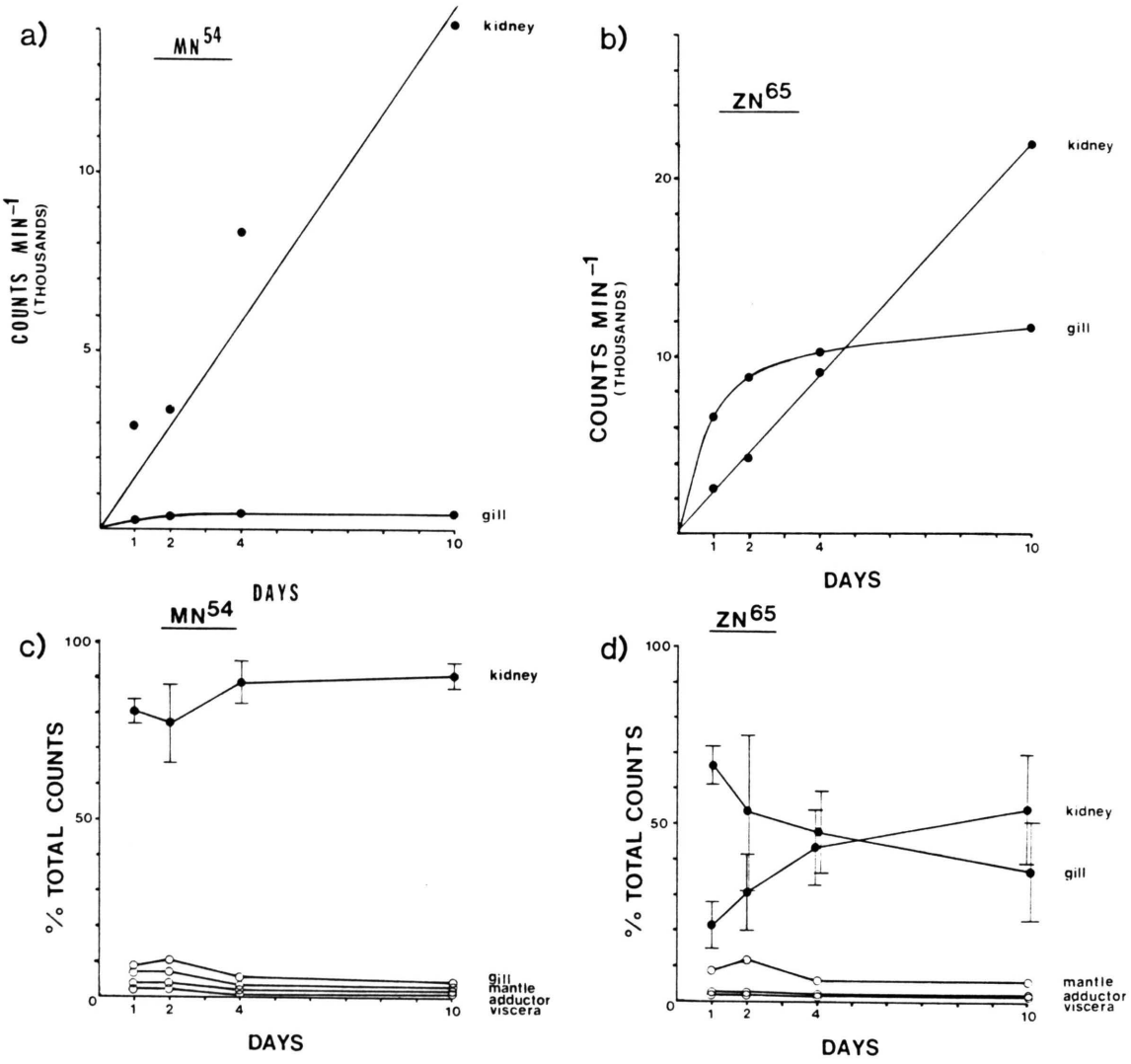
significant result was chosen to be $P \leq 0.050$. Means, standard deviations, range, and standard errors were also determined with SAS packaged programs.

CHAPTER 4: GENERAL UPTAKE OF Mn^{54} AND Zn^{65}

The southern hard clam, Mercenaria campechiensis, constitutes a significant recreational fishery in the Gulf of Mexico. In Tampa Bay, Florida, U. S. A., it is abundant and is harvested extensively for home use (Sims and Stokes, 1967; Menzel, 1976). Mercenaria sp. occurs in estuarine environments near dense population centers where contamination by heavy metals would seem likely. Relatively little detailed data exists on the genus Mercenaria concerning its accumulation of trace metals from seawater. There is even less direct information on the southern species, M. campechiensis, which may, due to environmental differences and increased growth rate (Saloman and Taylor, 1969), exhibit metal metabolism that is different from that of the northern species, M. mercenaria.

Mercenaria mercenaria has been shown to accumulate Zn and Mn from seawater under both natural (Shuster and Pringle, 1968; Segar, et al., 1971) and laboratory conditions (Carmichael et al., 1980). Accumulation of these two metals in both conditions shows the same high degree of organ specificity that has been observed in other marine bivalves (Brooks and Rumsby, 1965; Segar et al.,

Figure 3. General accumulation and tissue distribution patterns for Mn^{54} and Zn^{65} metabolism by Mercenaria campechiensis (control tank, tank number three). Average counts per minute (n=5) in (a) and (b) are normalized to wet weight. Mantle, viscera, and adductor were all much lower than the gill and kidney for both metals and are omitted from the graphs. Tissue distribution is shown in (c) and (d) (mean ± 1 s.d., n=5).


a)
MN54
kidney
gill
COUNTS MIN-1
(THOUSANDS)
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1
2
4
10
DAYS
b)
ZN65
kidney
gill
COUNTS MIN-1
(THOUSANDS)
20
10
1
2
4
10
DAYS
c)
MN54
100
50
0
kidney
gill
mantle
adductor
viscera
% TOTAL COUNTS
1
2
4
10
DAYS
d)
ZN65
100
50
0
kidney
gill
mantle
adductor
viscera
% TOTAL COUNTS
1
2
4
10
DAYS

1971). Even though both Zn and Mn show differential tissue distributions, evidence exists that their distributions are not the same (Larsen, 1979; Carmichael et al., 1980). Examination of the tissue distribution of these metals in Mercenaria sp. with respect to time is important for comparison of differing metabolism of Zn and Mn.

In this chapter, data from the control tank (tank 3) is used to examine the general tissue distribution of Zn^{65} and Mn^{54} in the southern quahog, Mercenaria campechiensis. It provides a time series for metal uptake and distribution by monitoring the relative concentrations of Mn^{54} and Zn^{65} in five tissues using gamma ray spectrometry. Similarities and differences between the distribution of Zn and Mn in these five tissues are examined to provide information on the role that each organ plays in the metabolism of these two metals.

Results

Accumulation curves showing gamma counts for these two metals (Figures 3(a) and 3(b)) cannot be compared quantitatively in this experiment due to differences in initial exposure activity levels for Mn^{54} and Zn^{65}. However, uptake trends and general accumulation patterns can be examined. To allow a more direct comparison of the tissue distribution of these two metals, count data was converted to percent composition by dividing counts per minute per gram wet weight (cpm/gww) for individual tissues

by the total cpm/gww summed for all tissues counted (Figures 3(c) and 3(d)).

The relative tissue concentrations of Zn^{65} and Mn^{54} in this bivalve exhibited similarities when examined over a period of ten days. For both metals, after 10 days, the kidney contained the greatest percentage of total radioactivity. Examination of count data shows that accumulation curves for the kidney are nearly linear (Zn, $r = 0.9998$; Mn, $r = 0.9806$) while the gill exhibits curves that approach a constant count rate with time. This indicates the establishment of an equilibrium in the gill with respect to Mn^{54} and especially Zn^{65} within the 10 days of this study. No such equilibrium value is reached for either metal in the kidney. The adductor muscle, mantle and viscera, while perhaps functioning to some degree in metal uptake and transport, do not play a large part in the storage and concentration of either Mn or Zn in M. campechiensis.

Examination of relative tissue concentration curves (Figures 3(c) and 3(d)) shows that Mn and Zn exhibit distribution differences which can be considered individually. Mn^{54} was concentrated rapidly in the kidneys (<1 day) where it maintained a relatively constant percent distribution throughout the study period (Figure 3(d)). The kidney is by far the largest storage compartment for Mn in this bivalve, accounting for approximately 90 percent of all Mn^{54} counted. At the

end of 10 days, all other tissues added together accounted for only 6.5 percent of the total gamma radiation per gram due to Mn^{54} with the kidney showing 30 times more activity per gram than the next most active tissue. Although the relative distribution of Mn^{54} stabilized rapidly, the absolute concentration of Mn^{54} in the kidney continued to rise even after 10 days.

The organ distribution of Zn^{65} showed a marked difference from that of Mn^{54}. At the end of the 10 day exposure Zn, like Mn, was present predominantly in the kidney, but at only 1.9 times more activity than the next most active tissue, the gill. Zn initially showed the greatest concentrations in the gill (2.6 times larger than the kidney) but gradually became more concentrated in the kidney as can be seen by the steady rise in the percent counts in the kidney with a consequent decline in the percent counts in the gill. This appears to result from a larger concentration capacity for Zn in the kidney which is not reached by the end of 10 days while the gill appears to approach an equilibrium concentration after 5 or 6 days (Figure 3(b)).

Histological examination of representative samples of the gill and viscera in this study showed no evidence of cytological damage due to metal exposure. Tissues appeared healthy when compared to those of freshly collected clams. Some areas in the kidneys isolated from clams exposed to Zn and Mn showed the apical portion of the columnar epithelial

cells to be disrupted. This is thought to be part of the natural metal detoxification mechanism in some bivalves (Carmichael and Fowler, 1981) and will be discussed in greater detail later in this paper. Examination of reproductive tissue showed males to be in various stages of reproductive development, ranging from early gonadal development to ripe conditions. Females examined were mostly classified as spawned or indifferent (Keck et al., 1975) with only residual oocytes scattered among generally disrupted reproductive cells.

Discussion

The results of this study show that Mercenaria campechiensis rapidly accumulates Zn^{65} and Mn^{54} from seawater (Table 1). The subsequent distribution throughout the tissues differs depending on the metal. Carmichael et al. (1980) showed very similar tissue distributions for Mn and Zn in M. mercenaria. Direct comparison to the present data is difficult since the activity for both radionuclides and the total metal concentrations in our experimental conditions were considerably different from those used by Carmichael et al. Transformation of the data of Carmichael et al. (1980) to percent total counts as detailed previously (cpm/gww for individual tissue ÷ sum cpm/gww for kidney, mantle, gill, muscle and viscera) gives results (Table 2) that are very similar to those obtained here (Table 1).

Table 1. Accumulation of Zn^{65} and Mn^{54} by *M. campechiensis* in counts per minute per gram wet weight (first listing) and percent total counts (second listing) (± 1 s.d.).

	Mn^{54}			
Tissue	1 day	2 days	4 days	10 days
kidney	2905 ± 1828 80.3 ± 3.8	3373 ± 1665 77.2 ± 11.3	8315 ± 2783 89.0 ± 6.1	14115 ± 19199 90.7 ± 3.4
gill	281 ± 142 8.4 ± 3.0	403 ± 237 10.0 ± 7.2	444 ± 395 5.3 ± 5.7	467 ± 442 4.0 ± 1.3
mantle	198 ± 60 6.3 ± 1.9	264 ± 73 7.0 ± 3.4	275 ± 61 3.1 ± 0.9	283 ± 157 3.0 ± 1.4
viscera	47 ± 16 1.5 ± 0.7	77 ± 19 2.0 ± 0.9	53 ± 8 0.6 ± 0.2	66 ± 13 0.8 ± 0.4
muscle	123 ± 70 3.5 ± 0.4	155 ± 61 3.8 ± 1.0	181 ± 47 2.0 ± 0.7	162 ± 126 1.5 ± 0.7

Table 1. (Cont'd.)

	Zn^{65}			
Tissue	1 day	2 days	4 days	10 days
kidney	2549 ± 2585 21.4 ± 6.8	4353 ± 2823 30.7 ± 10.6	9142 ± 3481 43.5 ± 10.4	22044 ± 16349 54.3 ± 16.6
gill	6614 ± 3564 66.5 ± 5.6	8862 ± 5099 53.6 ± 21.8	10382 ± 4039 48.0 ± 11.7	11779 ± 4317 36.9 ± 14.0
mantle	802 ± 278 8.7 ± 2.4	1217 ± 348 11.7 ± 10.8	1214 ± 190 5.9 ± 1.4	1738 ± 529 5.4 ± 1.9
viscera	130 ± 96 1.2 ± 0.2	188 ± 59 1.7 ± 1.3	210 ± 76 1.0 ± 0.2	460 ± 147 1.4 ± 0.4
muscle	250 ± 209 2.3 ± 0.3	267 ± 53 2.3 ± 1.5	316 ± 63 1.6 ± 0.5	688 ± 379 2.0 ± 0.7

Table 2. Relative tissue distribution of Mn^{54} and Zn^{65} for Mercenaria mercenaria determined from 3 day exposure data presented in Carmichael et al., (1980). Percent counts equals counts for each individual tissue divided by total counts for all tissues counted (x 100).

Tissue	% Zn	% Mn
Kidney	32.2	77.8
Gill	42.9	11.5
Mantle	11.2	3.1
Viscera	10.6	6.4
Muscle	3.1	1.1

The somewhat higher values for viscera may be related to reproductive condition. Lowe and Moore (1979) have noted elevated metal concentrations in the oocytes of Mytilus edulis. The clams used by Carmichael et al., while not examined for reproductive condition, may have exhibited more fully developed oocytes than the mostly spawned females used in the present study.

It appears from comparison with the work of Carmichael et al. (1980) that Zn and Mn tissue distribution for southern quahogs exposed to a temperature representative of their subtropical environment is not significantly different over short exposure times from those observed in M. mercenaria. To determine whether this similarity extends to accumulation rates and/or metal concentrations at equilibrium, side by side experiments which expose both species to identical metal concentrations must be performed.

Observed Zn distribution can be misleading when only one determination is made far in advance of distribution equilibrium. Carmichael et al. (1980) reported that after 3 days of exposure, Zn was more evenly distributed than Mn with the majority occurring in the gill. The present study demonstrates that a redistribution of concentration occurs with respect to Zn^{65} in M. campechiensis with the kidney becoming the tissue compartment most highly concentrated in Zn^{65} by the end of 10 days. A similar redistribution of Zn was seen in

Mytilus edulis (George and Pirie, 1980) where the kidney gradually becomes the major zinc-containing tissue, and equilibrium in the kidney is achieved only after about 25 days. It seems reasonable that the same sort of redistribution of concentration is occurring in *M. campechiensis*. Conclusions concerning metal tissue distribution should be based on chronological studies of metal accumulation and equilibrium. Single measurements may reflect only a transient condition.

Observed differences in tissue concentration distributions between Zn^{65} and Mn^{54} result mainly from a Zn^{65} accumulation into the gills that is larger, relative to other tissues, than that of Mn^{54} (Figure 3(a)). Carmichael *et al*. (1980) mentioned zinc's role as a constituent of many essential enzymes as a factor which could account for its more 'even' distribution throughout the tissues. Even though over one hundred Zn metallo-enzymes have been identified (Lehninger, 1982), only four have been isolated from the marine bivalves *Ostrea edulis* (Coombs, 1972) or *Crassostrea virginica* (Wolfe, 1970). Of these, carbonic anhydrase is reasonable to consider as an identifiable storage compartment for Zn in the gills. It has been isolated from the blood cells of many mammals as well as from the gills of the mollusc *Sepia officinalis* (Addink, 1968) where it presumably participates in the exchange of carbon dioxide from the blood (Lehninger, 1982). Other Zn enzymes

not yet identified in molluscs may also constitute compartments for Zn accumulation and thereby influence Zn distribution. But as calculations on zinc requirements in oysters have shown (Pequegnat et al.,1969), only about 0.1 percent of the total Zn concentration can be attributed to zinc-enzymes. While this could vary according to species, it seems to be only a small Zn-containing compartment when considering the overall distribution of Zn in bivalve tissues.

By assuming a small storage compartment for zinc in the gill, possibly in zinc enzymes, a simple conceptual model for accumulation into the gill and transfer to the kidney can be presented (Figure 4) which explains the direct count data and concentration distribution curves observed for both Zn^{65} and Mn^{54} in this study. Both metals show uptake and steady transfer to the kidneys (shown by arrows 1 through 3). By examination of accumulation curves, it seems that Zn is present in the gill in two compartments (labeled mobile and bound) which exhibit differential rates of concentration. Transfer of Zn^{65} into the 'bound' compartment during the first day of exposure occurs at a rate about three times faster than the concentration of Zn^{65} into the kidney, probably due in part to the kidney being physically remote from the sites of initial uptake. Before, during, and after the saturation or equilibration of the 'bound' compartment with the 'mobile' compartment (about 4 days), no change is seen

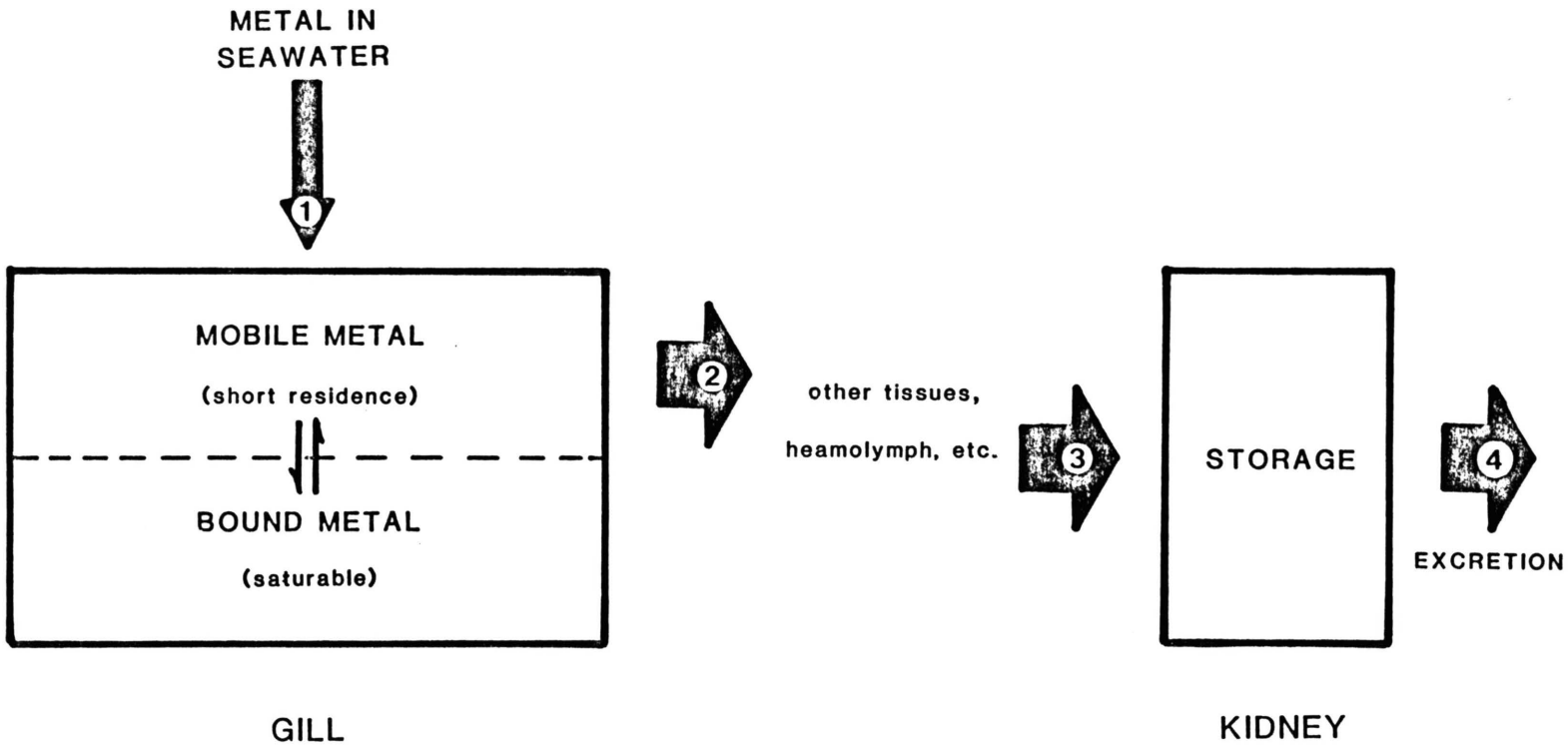


Figure 4. Simple conceptual model for Zn^{65} and Mn^{54} uptake and transfer by Mercenaria campechiensis.

in accumulation of Zn by the kidney. This indicates a transfer of Zn to the kidney which does not depend on the 'bound' constituent in the gills for the bulk of Zn transport. Mn shows little or no evidence of a significant 'bound' component in the gills and thus its accumulation and tissue concentration distibution is dominated by transport and storage in the kidney.

The concurrent study of the accumulation of more than one metal over the course of time aids in identification of differences in metal transport and compartmentalization in bivalves such as M. campechiensis. This can help in the understanding of observed environmental differences in metal tissue distributions. It can also point out tissues within the organism in which particular trace metals are concentrated, aiding in identification of cellular systems that are dependent on, or are impacted by various metals.

CHAPTER 5: PHOSPHATE AND METAL EFFECTS

The uptake of metals by marine bivalve molluscs has received considerable attention in hopes of using these sessile estuarine organisms as indicators of pollution by potentially toxic metals. Studies attempting to characterize the relationship between bivalves and metals in the natural environment have revealed that innocuous fluctuations of physical and chemical parameters such as temperature, salinity, and metal complexation can have significant effects on observed metal accumulation rates and natural concentrations in molluscs (Cunningham, 1979; Engel et al., 1981).

Phosphate, an essential part of normal cellular activity, is also a major constituent of metal containing, inorganic granules found in the molluscan kidney. Since these granules constitute a large metal detoxification sink for marine bivalves (Brown, 1982; George, 1982), the cellular pathways responsible for metal metabolism may be influenced by phosphate availability. The effects of dissolved phosphate on metal accumulation in marine molluscs are previously undiscribed.

Tampa Bay, Florida, periodically exhibits extremely high dissolved phosphate levels (Fanning and Bell, in

press) due largely to local phosphate deposits and mining activity. This study investigates the effects that phosphate levels comparable to those measured in Tampa Bay could have on the accumulation and tissue distribution of Zn^{65} and Mn^{54} in the southern hard clam, Mercenaria campechiensis. The effects of elevated total Zn and Mn, both with and without elevated phosphate, were also examined.

Results

Gamma ray spectrometry data for all tissues and all tanks are presented in appendix 1. Included data represents the result of a SAS (Helwig and Council, 1979) means procedure run on both count data and percent total count data. Percent total count data were calculated by division of total counts per gram wet weight (cpm/gww) for an individual tissue by the sum cpm/gww for all tissues counted for an individual clam. This allowed direct comparison between tanks containing different total and specific activity for both Mn^{54} and Zn^{65}. It also allowed tanks with a far lower specific activity due to added stable metals (tanks 2 and 4) to be compared to those without added nonradioactive metals (tanks 1 and 3). Figures 5 through 10 are graphic representations of the data listed in appendix 1. Only data for the kidney and gill are included and error bars listed in appendix 1 are omitted for visual clarity.

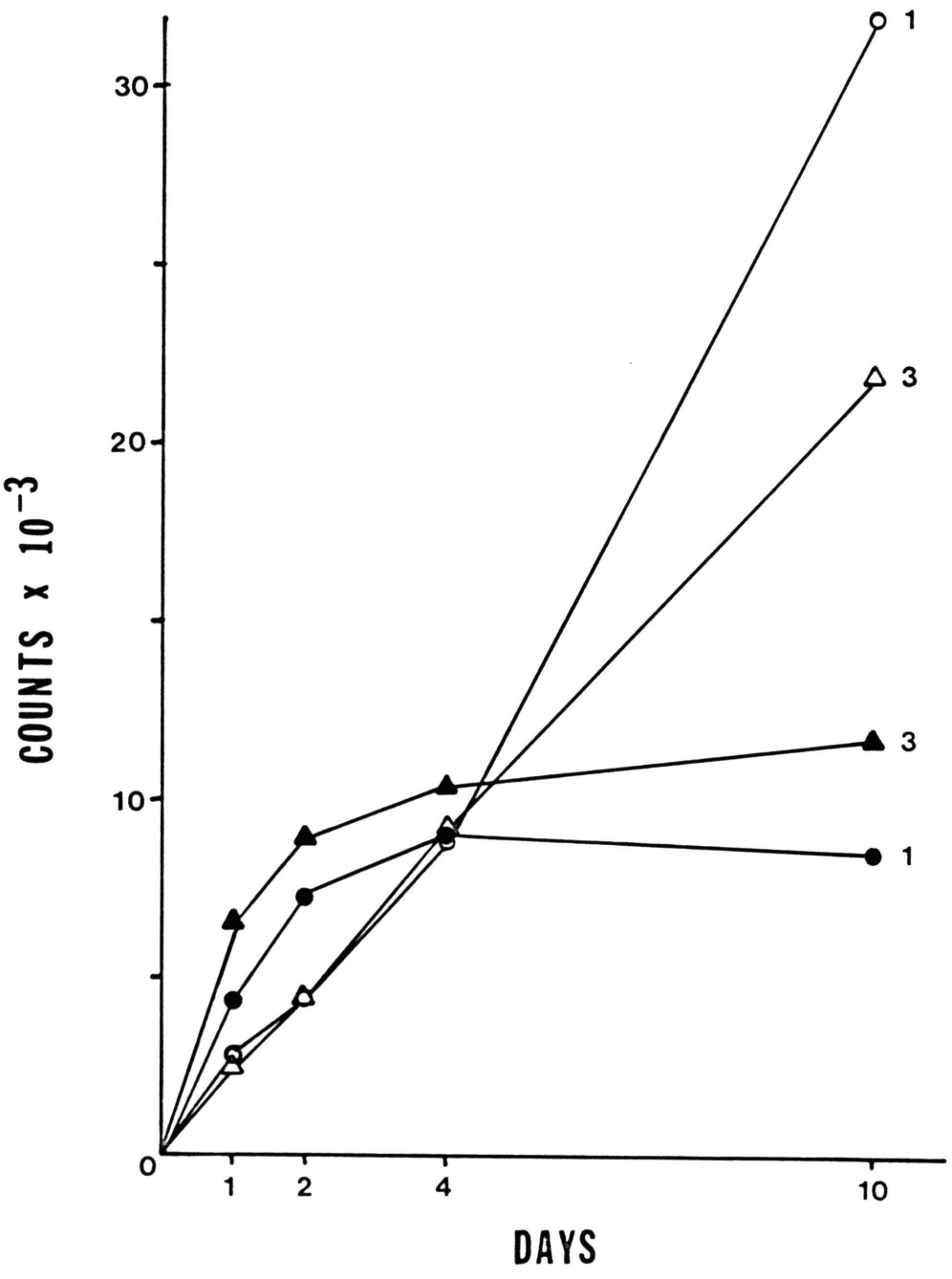


Figure 5. Average count data for Zn^{65} accumulation over ten days. ○ = kidney, tank 1; ● = gill, tank 1; △ = kidney, tank 3 ; ▲ = gill, tank 3.

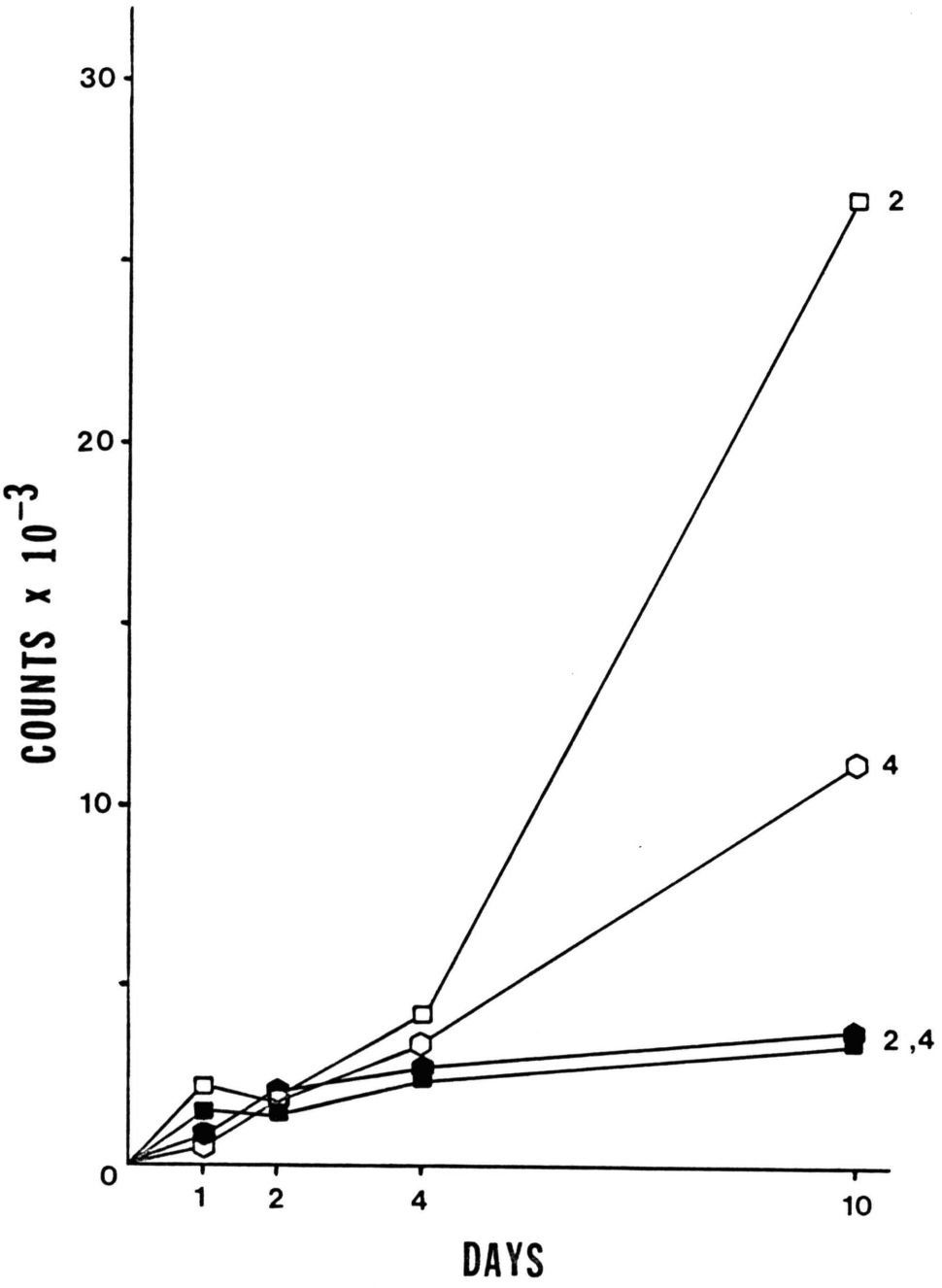


Figure 6. Average count data for Zn^{65} accumulation over ten days. □ = kidney, tank 2; ■ = gill, tank 2; ⬡ = kidney, tank 4 ; ⬢ = gill, tank 4.

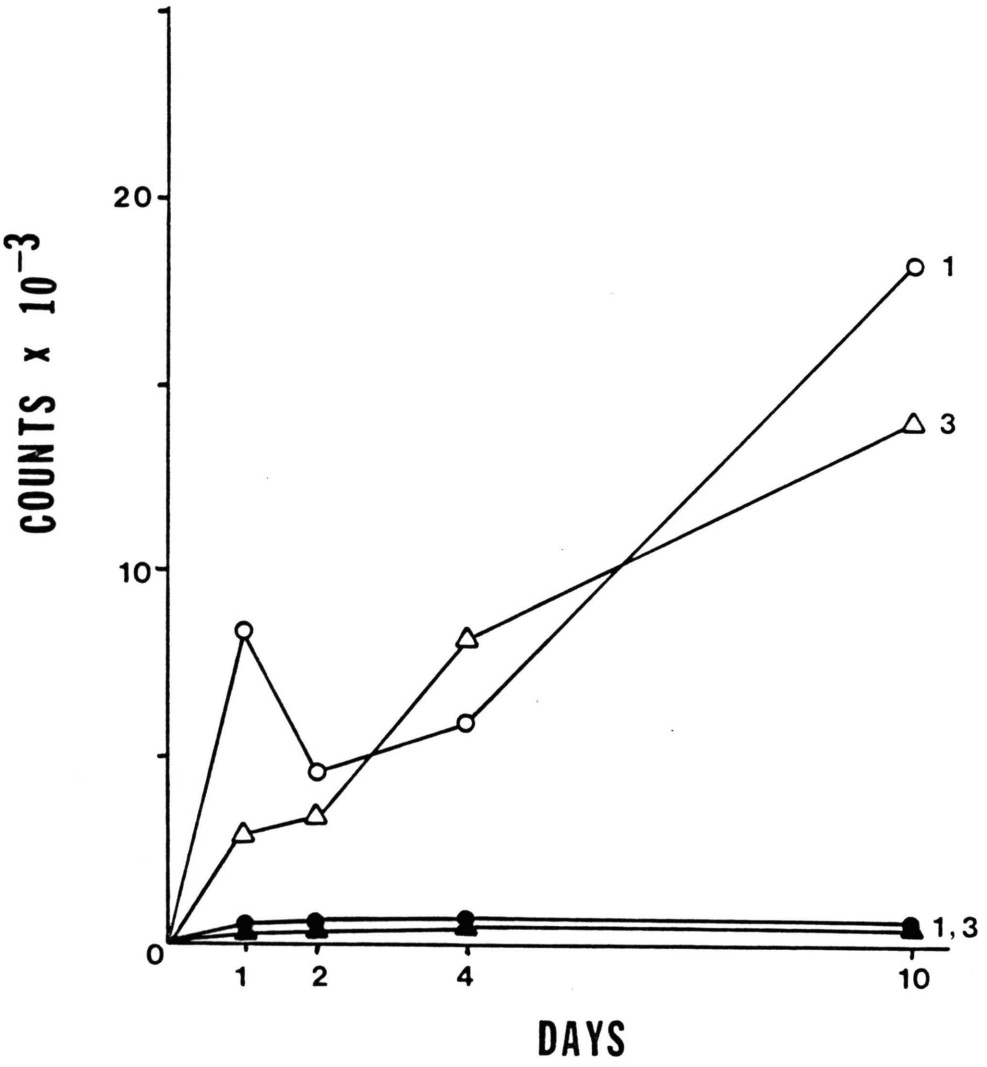


Figure 7. Average count data for Mn^{54} accumulation over ten days. ○ = kidney, tank 1; ● = gill, tank 1; △ = kidney, tank 3 ; ▲ = gill, tank 3.

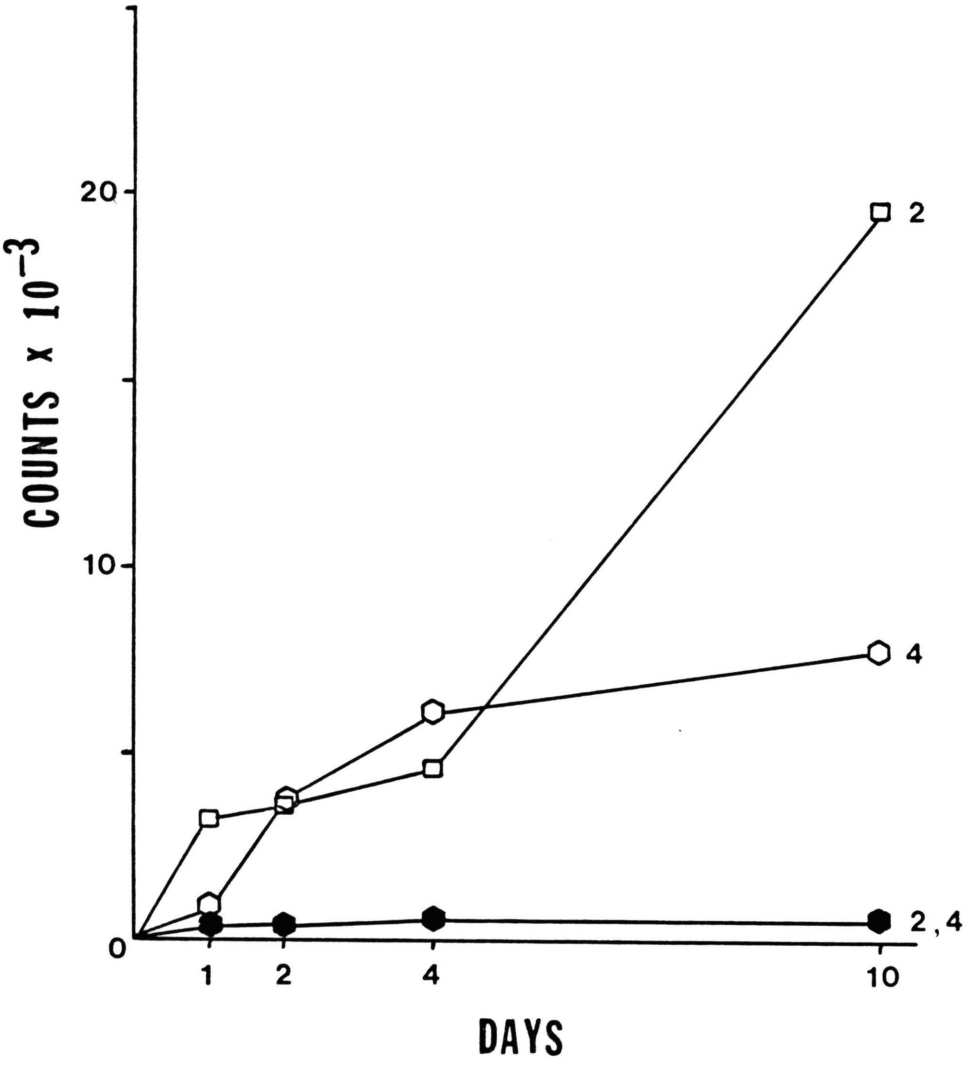


Figure 8. Average count data for Mn^{54} accumulation over ten days. □ = kidney, tank 2; ■ = gill, tank 2; ⬡ = kidney, tank 4 ; ⬢ = gill, tank 4.

Figure 9. Tissue distribution of Mn^{54} for ten day accumulation study. Kidney percent distribution, ○ = tank 1, □ = tank 2, △ = tank 3, ⬡ = tank 4. Gill percent distribution, ● = tank 1, ■ = tank 2, ▲ = tank 3, ⬢ = tank 4.

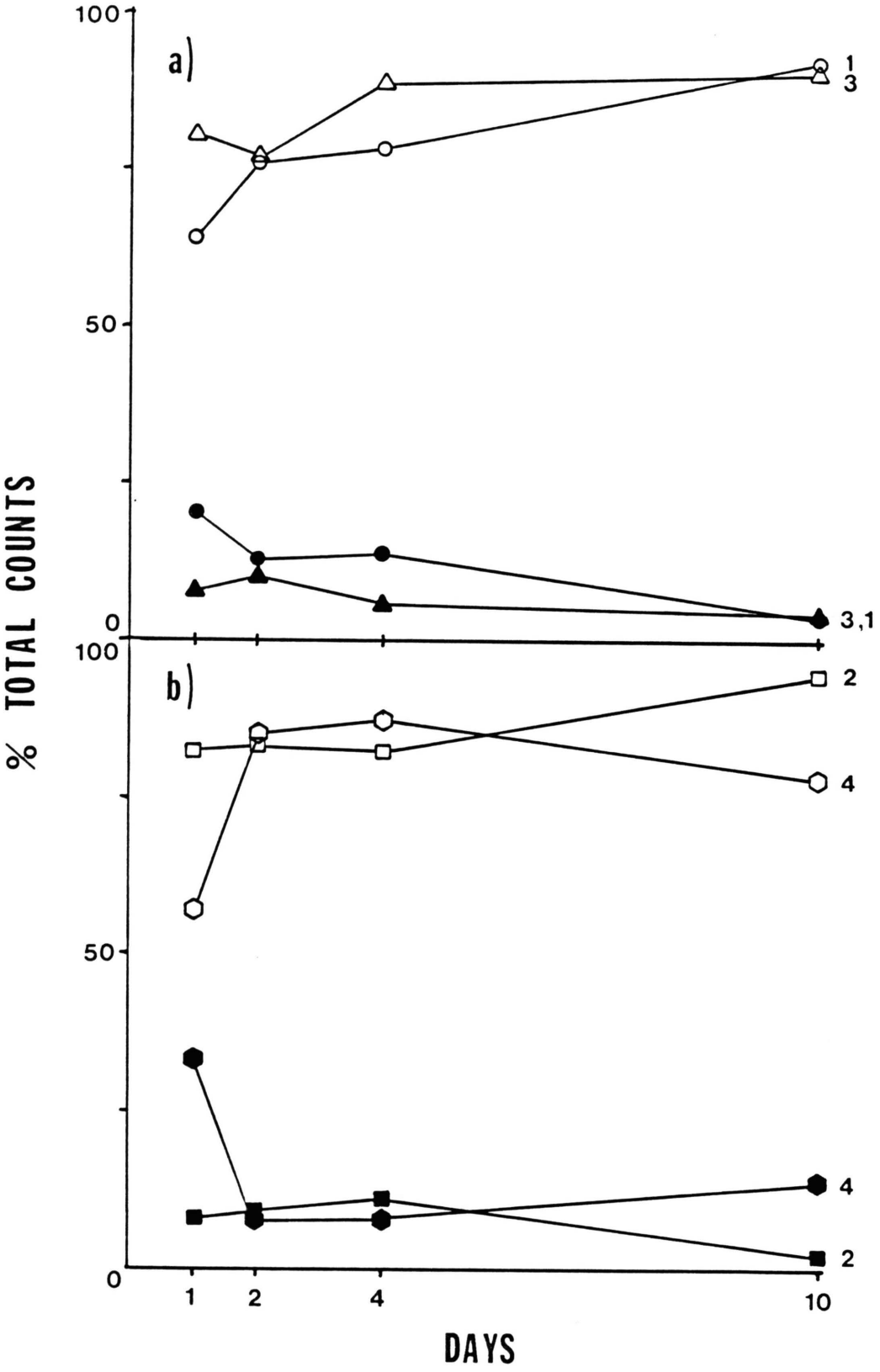
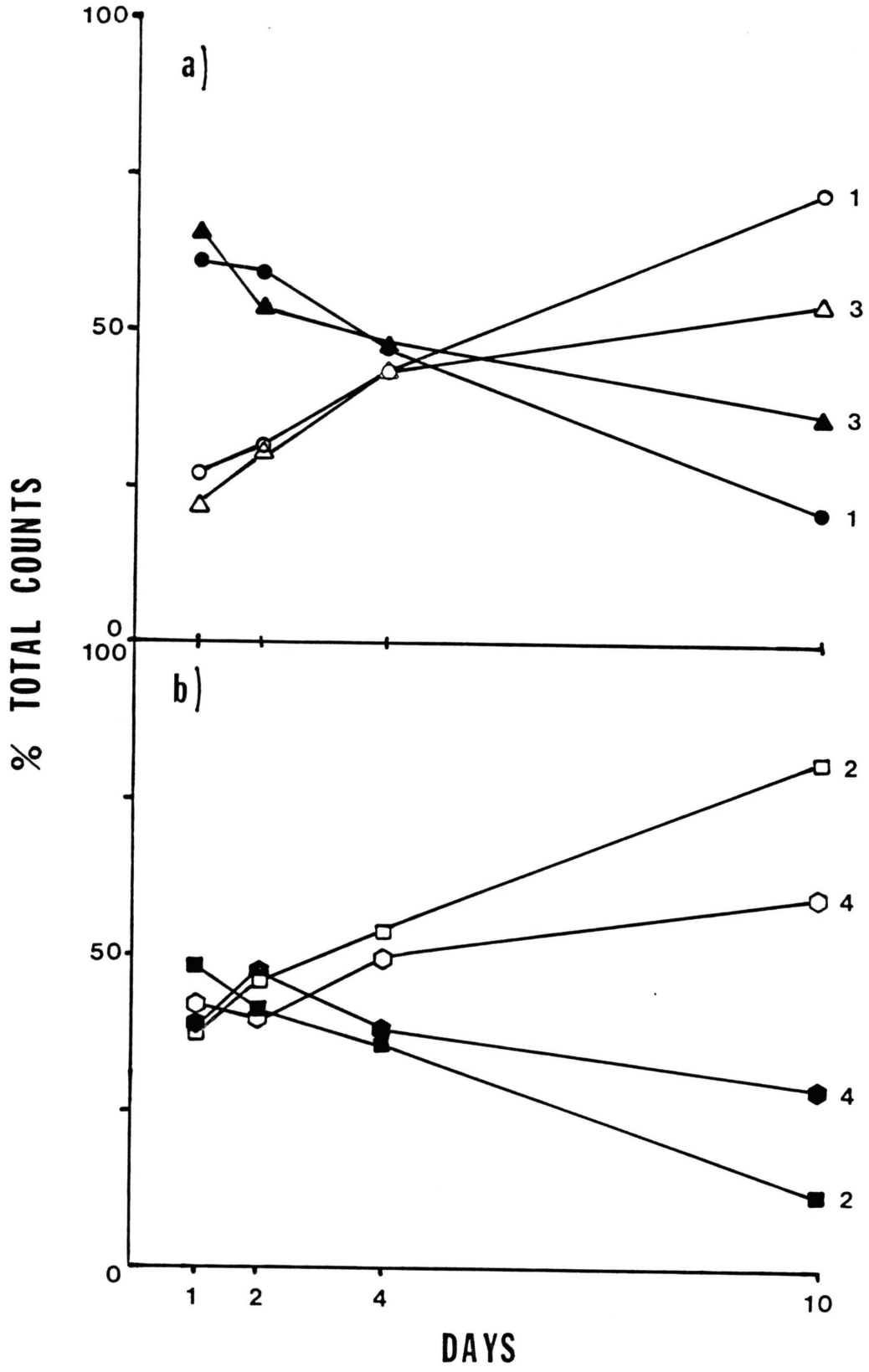

100
a)
1
3
50
0
3,1
100
b)
2
4
50
4
2
0
1
2
4
10
DAYS
% TOTAL COUNTS

Figure 10. Tissue distribution of Zn^{65} for ten day accumulation study. Kidney percent distribution, ○ = tank 1, □ = tank 2, △ = tank 3, ⬡ = tank 4. Gill percent distribution, ● = tank 1, ■ = tank 2, ▲ = tank 3, ⬢ = tank 4.


100
a)
50
0
1
3
3
1
100
b)
50
0
2
4
4
2
1
2
4
10
DAYS
% TOTAL COUNTS

Even though figures 5 through 8 show apparent accumulation differences (between treatments) for Mn^{54} and Zn^{65}, the count data proved too variable to demonstrate statistical differences at the $P \leq 0.05$ level. The observed variability could have several sources. Differences in individual uptake were somewhat minimized by using clams of similar size collected together from the same area. However, this probably is still the largest single contribution to data variability. The use of radionuclides necessitated the use of a closed seawater exposure system which exhibited a change over time with respect to Zn^{65} and Mn^{54} activity. An overall decrease in activity for both metals over the course of exposure can be attributed to adsorptive processes, uptake by Mercenaria, and, for Mn^{54}, perhaps some oxidation of the Mn^{54} ion to form insoluble MnO_2. Considering these probable sources of variation, direct statistical comparison for count data is difficult.

Improved exposure methods may be capable of reducing this source of data variability. Recent studies conducted on the accumulation of Mn by Donax variabilis (Appendix 2) have shown that seawater buffered with an ion exchange resin maintains stable metal chemistry thus eliminating many of the sources of data variation found in this study.

Data converted to percent total counts proved less variable and allowed statistical demonstration of accumulation differences. As shown in figures 7, 8, and 9,

both count data and percent total count data for Mn^{54} accumulation showed considerable cohesiveness. Elevated total Zn and Mn and/or orthophosphate concentration caused only marginal differences from controls ($0.050 \leq P \leq 0.100$). Both direct count accumulation curves (Figures 7 and 8) and tissue concentration distribution curves show similar results for all tanks examined.

$Zinc^{65}$ tissue concentration distribution after 10 days showed more clearly significant relationships ($P \leq 0.050$). Comparison of tanks 1 and 3 (Figure 10(a)) shows that the kidney contains a larger percent tissue concentration in the presence of elevated phosphate than the control with a corresponding lowered percentage in the gills. Elevated total Zn and Mn also causes significantly higher percent tissue concentrations in the kidney (Figures 10(a) and 10(b)). Tank 4, which contained the highest concentration of orthophosphate along with an elevated total Zn and Mn concentration, did not show a significant difference in kidney or gill percent tissue concentrations when compared to the control tank. Reasons for this apparent antagonistic effect on Zn^{65} tissue distribution between elevated metal and phosphate concentrations will be discussed later in this chapter.

Figure 10(a) shows that accumulation of Zn^{65} from both low metal tanks 1 and 3 is very similar until sometime after day 4. The gill is initially more concentrated with respect to Zn^{65} than the kidney in both tank 1 and 3

with the same slow redistribution of concentration observed that was discussed in chapter 4. The point at which the kidney obtains a percent distribution equal to that of the gill occur between 4 and 6 days after initial exposure. Elevated total Zn and Mn concentrations (tanks 2 and 4) move the point of equal distribution back to around 1 or 2 days after initial exposure (Figure 10(b)). If the gill ever contains the dominant percent distribution in tanks 2 or 4, it is prior to the first group sampled in this study (<1 day). $Zinc^{65}$ seems to be more rapidly concentrated in the kidney when clams are exposed to elevated metal concentrations.

Discussion

The same general accumulation and tissue distribution patterns observed for the control tank (presented in detail in chapter 4) are observed in clams exposed to elevated phosphate. $Manganese^{54}$ shows no statistically significant effects in tissue distribution due to phosphate for any sample period during this study. $Zinc^{65}$, while exhibiting little effect in the first few days of exposure, shows a significantly higher percent concentration in the kidneys from clams exposed to elevated phosphate after 10 days. This may be related to the natural formation of large numbers of metal containing phosphoritic kidney granules in this species (Gold et al., 1979; Reinberger et al., 1979). Simkiss (1981) suggested that both

ortho- and pyrophosphate are important in the formation of the intracellular granules which may act as biochemical 'dustbins' for cellular wastes. Excess phosphate may influence granule formation and the concomitant incorporation of available trace metals.

Zinc^{65} in the kidneys of clams exposed to elevated total Zn and Mn showed a significant increase in percent concentration distribution ($P \leq 0.010$). Doyle et al. (1978) have suggested that environmental stress may play a part in the formation of renal concretions in bivalves. While kidney granules in Mercenaria campechiensis appear to be a natural phenomenon (Carmichael et al., 1980; Reinberger et al., 1979), the process of granule formation may be enhanced by elevated metal concentrations. If this is the case, the observed increase of percent tissue distribution for Zn^{65} in the kidneys of clams exposed to elevated metal concentrations in this study may also depend on mechanisms of granule formation.

Although Mn^{54} shows only marginal statistical significance in percent tissue distribution due to elevated metal concentration, examination of count data supports evidence of an effect similar to that seen for Zn^{65}. Tank 2 contained Mn^{54} at a total activity equivalent to the control tank 3, but at a greatly reduced specific activity due to added stable Mn. Assuming that Mn^{54} and stable Mn are accumulated nondiscriminently, equal

incorporation of Mn in tanks 3 and 2 would result in a far smaller count rate for tissues from tank 2. This is not observed here. The kidneys from clams in tank 2, after 10 days showed more activity due to Mn^{54} than those from tank 3. This indicates an elevated rate of Mn incorporation into the kidneys of clams exposed to higher total Mn and Zn concentrations. Again, as for Zn^{65}, it is possible that enhanced formation of phosphoritic renal granules may be responsible for this effect.

An interesting result from the present study is the apparent antagonistic relationship between elevated phosphate and total Zn and Mn concentrations. Comparison between metal uptake in tissues from clams exposed to elevated Zn and Mn both with and without elevated phosphate showed similar effects for both metals. Examination of figures 5 and 7 show that count data obtained throughout the study for both Zn^{65} and Mn^{54} in the gills from clams in tanks 2 and 4 are almost identical. The differences in count data, and consequent differences in percent tissue distribution, appear in the kidney after 10 days of exposure. For both Zn and Mn, the presence of elevated phosphate concentrations in a high metal environment results in kidney accumulation that is significantly lower than that observed for exposure to elevated metals alone.

Since 'accumulation' is a term referring to the net result of uptake and excretory processes, both processes

must be considered when interpreting the accumulation of Zn and Mn into the kidney. The oyster, Crassostrea virginica, has been shown to accumulate dissolved phosphate directly from seawater (Pomeroy and Haskin, 1954). Consequently, elevated phosphate could conceivably interact with metal uptake mechanisms. Since Pomeroy and Haskin (1954) observed that phosphate uptake was most significant in the gill, one could expect the greatest potential metal uptake interference to occur there. The present study showed no such evidence of Zn^{65} and Mn^{54} accumulation differences in the gill due to elevated phosphate and metal concentrations.

It seems likely that depressed Zn^{65} and Mn^{54} concentrations in the kidney exposed to elevated phosphate concentrations at elevated metal concentrations (Figure 10(b)) does not result from inhibited metal uptake, but rather from enhanced metal excretion. Carmichael and Fowler (1981) reported the massive extrusion of metal bearing renal granules after 5 days of exposure to cadium in seawater. The slow release of these granules under natural conditions is believed to be a part of the normal response of bivalves to excess or toxic metals. Histological examination of kidney tissues from this study shows a similar extrusion in kidneys exposed to elevated concentrations of metal and phosphate. Data presented in chapter 6 (Figure 18) indicate that excretion of metals through kidney granules may indeed be enhanced in the

presence of elevated phosphate.

Data examined here indicate that the kidney and gill of Mercenaria campechiensis show Zn^{65} and Mn^{54} accumulation differences resulting from exposure to varied concentrations of dissolved orthophosphate and total Zn and Mn. These effects seem to be associated with the mechanisms controlling formation and excretion of phosphoritic kidney granules. The presence of elevated phosphate alone results in a small increase in the accumulation of Zn^{65} into the kidney by the end of 10 days exposure. Elevated metals increase the rate of transport of Zn^{65} and Mn^{54} to the kidney. Concurrent elevation of metal and phosphate show the same increase in transport rate of metals to the kidney but results in a significantly smaller 10 day accumulation factor than observed with elevated metals alone. Since there is no similar difference in the gill and the effect only appears after 4 days, this effect is likely to be a result of increased excretion of metals rather than inhibited uptake.

Increased excretion of accumulated metals by clams in 'high phosphate / high metal' environments may represent a natural detoxification response to periodic increases in seawater metal concentrations. This could complicate the use of this bivalve, and perhaps others, as indicators of metal contamination since the observed accumulation response is influenced by an unquantified factor not

directly related to metal availability.

CHAPTER 6: METAL CONTAINING RENAL GRANULES

Metal-containing inorganic granules have been noted to occur in almost every phylum (Coombs and George, 1978; Simkiss, 1978). They are often present both intra- and extracellularly in the tissues of marine bivalve molluscs (Brown, 1982; Lucas and Hignette, 1983). Because of their ability to concentrate trace metals to a high degree in bivalve kidneys, renal granules have been suggested as bioindicators for trace metal contamination in estuarine habitats (Doyle et al., 1978; Carmichael et al., 1979; Reinberger et al., 1979). Renal calculi in Macrocallista nimbosa have also been suggested as model systems for the study of urolithiasis in the human health sciences (Tiffany, 1979). For either of these applications, a detailed knowledge of the mechanisms affecting the general formation and the metal content of the renal granules in marine bivalves is required.

Most recent work has generated data on the elemental composition of renal granules. The general composition of these insoluble granules is amorphous calcium and phosphate, both ortho- and pyrophosphate (Howard et al., 1981; Simkiss, 1981), along with a suite of trace metals (Doyle et al., 1978; George et al., 1980).

Granules have also been reported to contain oxalate, protein, sulphate, and lipofushin (Hignette, 1978; Overnell, 1981; Reid et al., 1984). Histological and ultrastructural examination has allowed description of, and speculation on important aspects regarding granule function and formation (George et al., 1978; George and Pirie, 1980) but many areas remain unclear. There is very little kinetic information regarding the methods and rates of renal granule formation. Direct examination of the time frame of incorporation of trace elements into granules is severely lacking.

This study uses the southern quahog, Mercenaria campechiensis, to examine general formation processes and incorporation of Mn^{54} and Zn^{65} into renal granules. The granules in this southern species, like those described for M. nimbosa (Tiffany et al., 1980), are unusually large and suggest that mechanisms in addition to those reported for other bivalves may contribute to granule formation and metal accumulation in this species. The effect of metal related stress on the formation of renal granules was also examined.

Results

Visual inspection during dissection showed the kidney of many individuals, both freshly collected and exposed to Zn and Mn in the laboratory, to be filled with dark brown/black particles. This imparts a similar color to the

entire kidney. Tears in the tissue covering the kidney resulted in these particles spilling out in large numbers.

Histological examination of freshly collected specimens showed the kidney tubules to be composed of a single layer of columnar epithelial cells which lie along a basement membrane surrounded by flattened connective cells. There is typically a basal nuclei and a single apical, membrane bound, darkly staining granule from 5 to 20 µm in diameter (Figure 11). Some areas of the kidney were observed which contained nearly identical cells with no apical granule. This does not seem to be an artifact of fixation or processing procedures since cells both with and without apical granules were otherwise healthy and normal in appearance. Examination of the gill and viscera from both fresh and exposed individuals also showed healthy, normal cells. The kidney tissue from exposed clams, however, showed regions in which the apical portion of the columnar epithelial cells appeared to be torn apart. Both these regions and those exhibiting intact columnar cells showed no evidence of the small intracellular granules observed in freshly collected individuals. This may be indicative of an extrusion of granules from epithelial cells similar to that reported by Carmichael and Fowler (1981) for Argopecten after exposure to elevated metal concentrations

Large granules are also observed within the lumen of renal tubules. Sizes ranged from about 10 to 350 µm at the

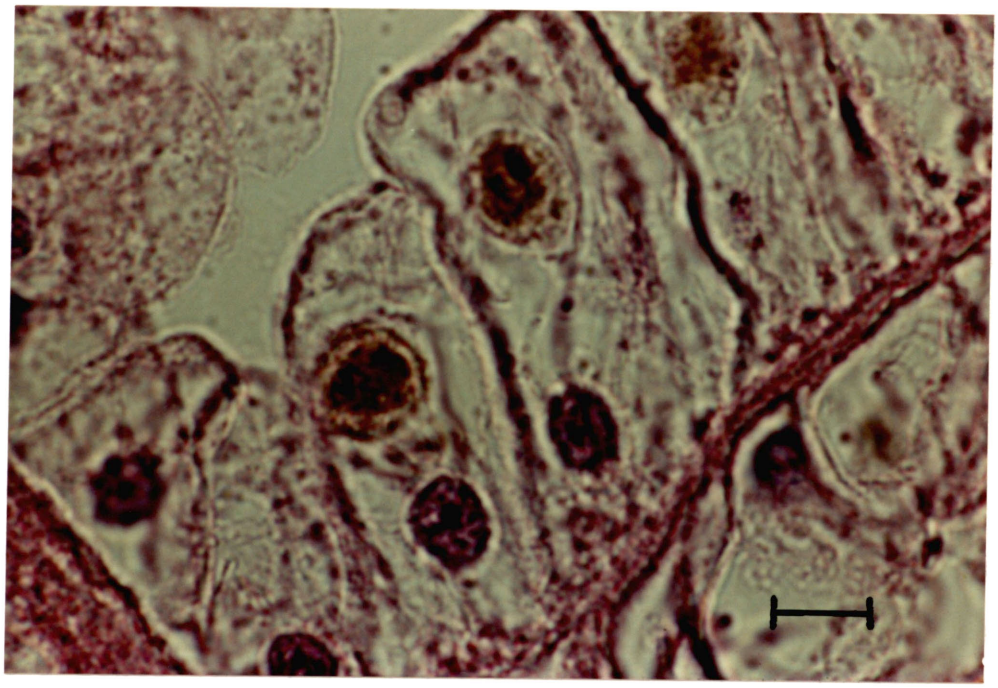


Figure 11. Typical columnar epithelial cell from the kidney of Mercenaria campechiensis. Note single apical granule. Bar equals 10 microns.

point of largest cross section. Large and intermediate sizes appeared to be composed of smaller granules which aggregate and assume a shape outlined by the lumenal walls surrounding them (Figures 12, 13, and 14). The very large lumenal granules showed a tendency to shatter during sectioning while the small and intermediate sizes did not, suggesting a secondary hardening of larger granules. Most granules stained darkly with Hemotoxylin and only limited internal structure could be observed in the sections examined. Granules showed differing opacity, ranging from completely opaque to semitransparent with evidence of concentric and/or globular internal structure.

Sedimentation of trypsin digested kidneys produced predominently large intercellular type granules. Examination of representative granules by scanning electron microscopy (SEM) showed variable exterior surfaces ranging from a rough microbotryoidal texture as shown in figure 15 to the smoother, polished texture seen in figure 16. SEM examination also suggested that larger granules may be composed of smaller particles as demonstrated by multiple centers of growth and spherical protrusions on the granule surface (Figure 17).

Data depicting the relationship between total granule weight and orthophosphate and total metal exposure are presented in figure 18. Air dried kidney granule weights were divided by the total dry weight of the kidneys from which the granules were pooled to determine percent weight

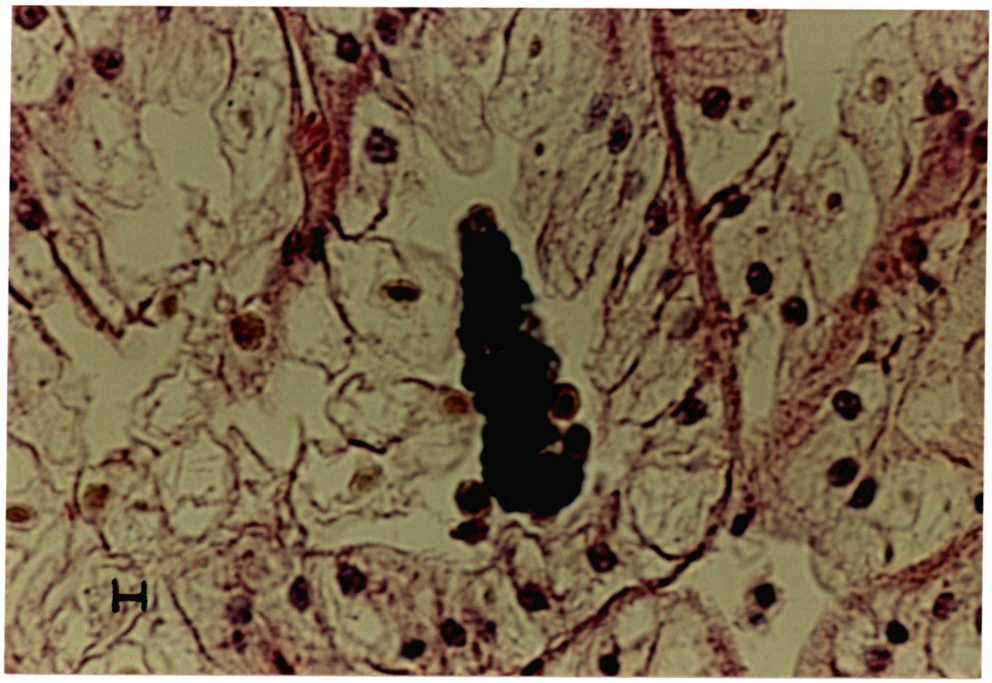


Figure 12. Representative intercellular phosphoritic granule. Early stage of formation. Bar equals 10 microns.

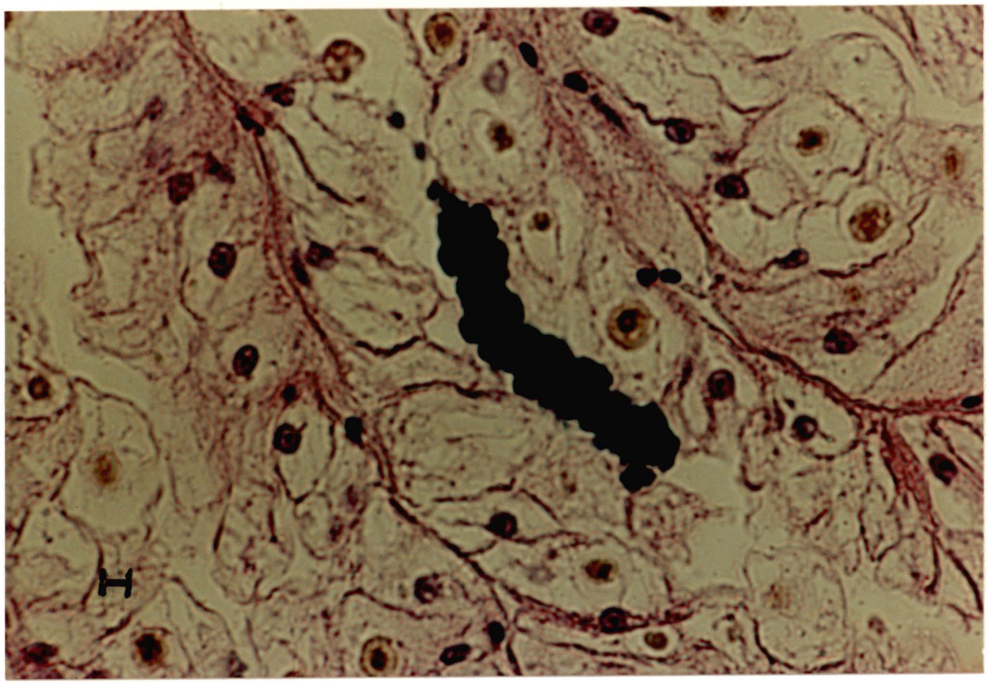


Figure 13. Representative intercellular phosphoritic granule. Apparent formation by accretion of smaller granules. Bar equals 10 microns.

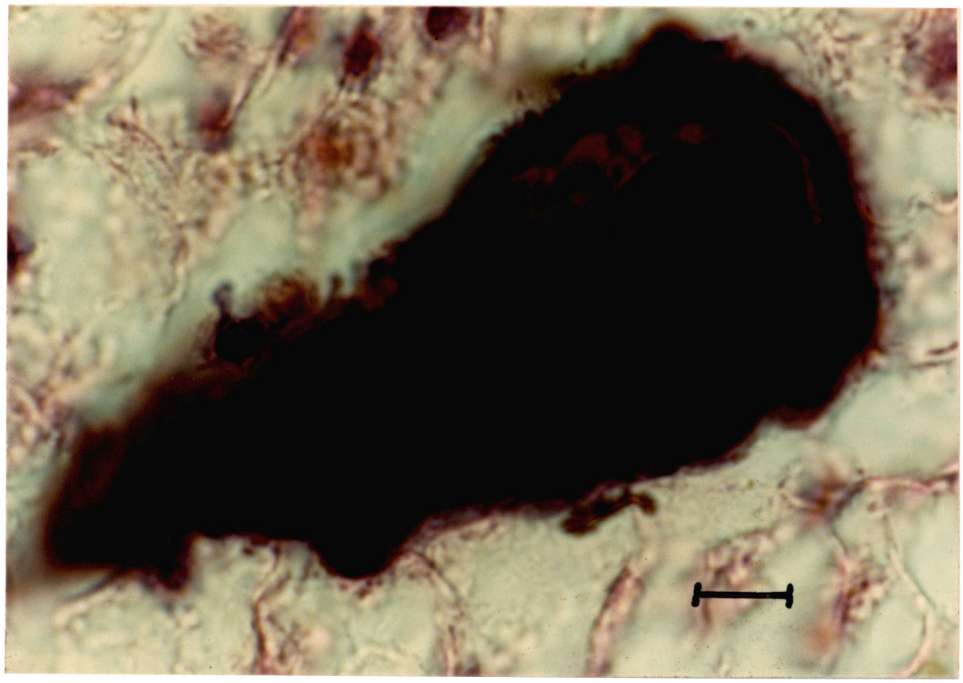


Figure 14. Representative intercellular phosphoritic granule. Apparent formation by accretion of smaller granules. Bar equals 10 microns.

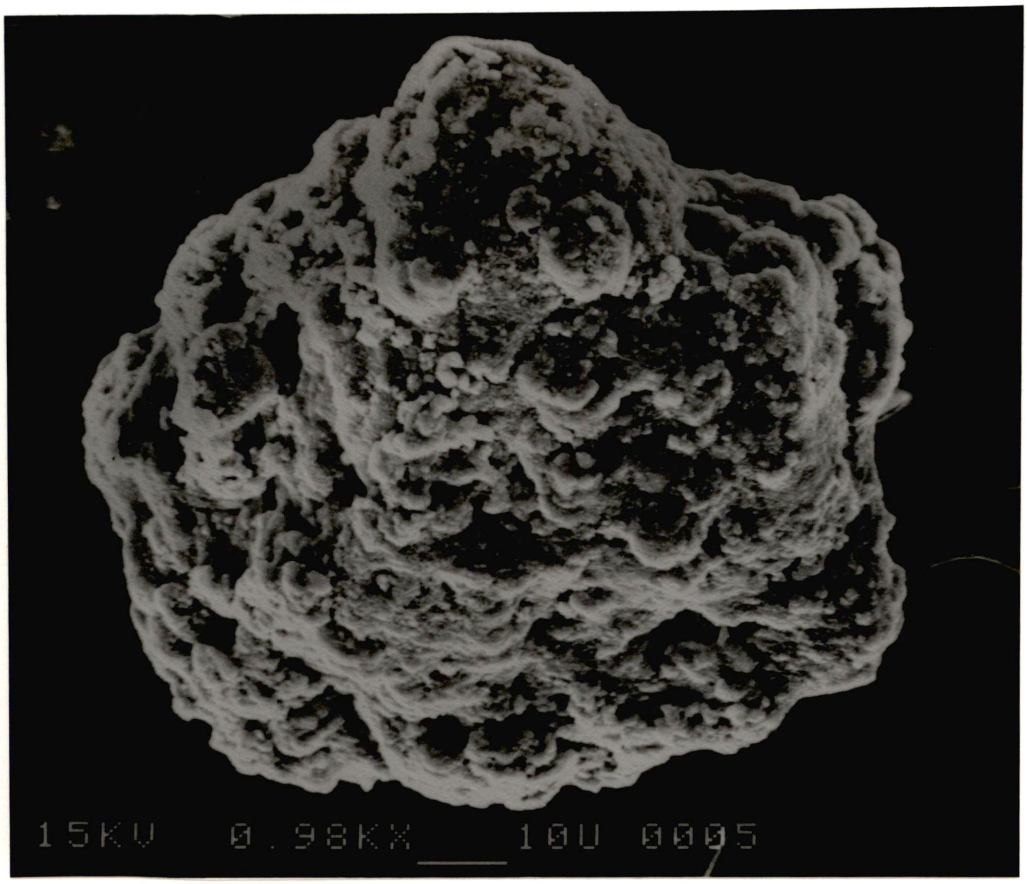


Figure 15. Electron micrograph of isolated granule. Note rough exterior texture.

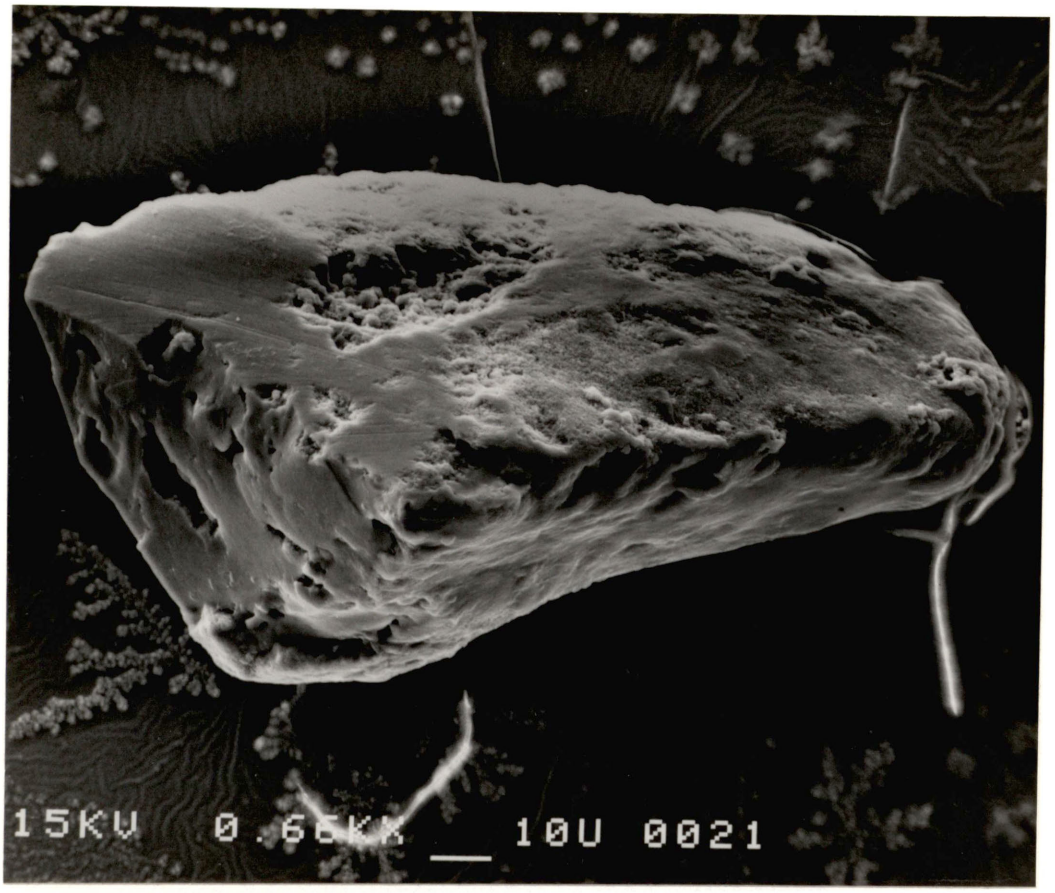


Figure 16. Electron micrograph of isolated granule. Note smooth exterior texture.

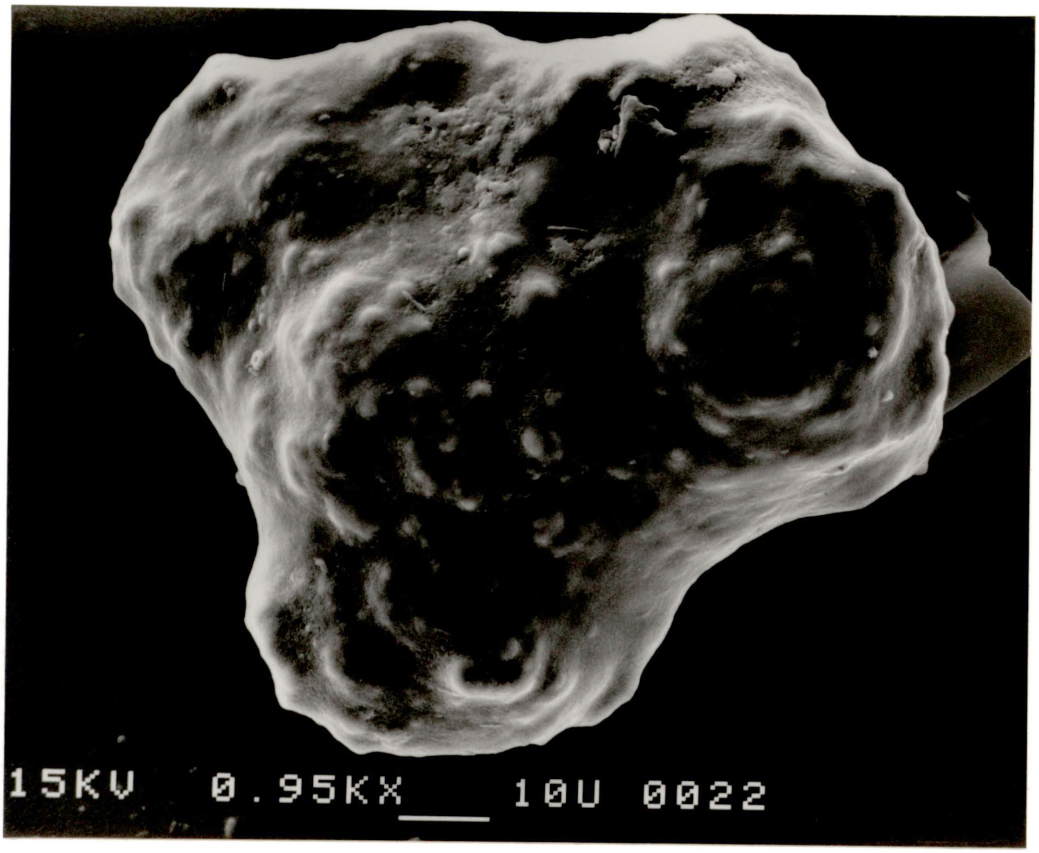


Figure 17. Electron micrograph of isolated granule. Note apparent multiple centers of growth.

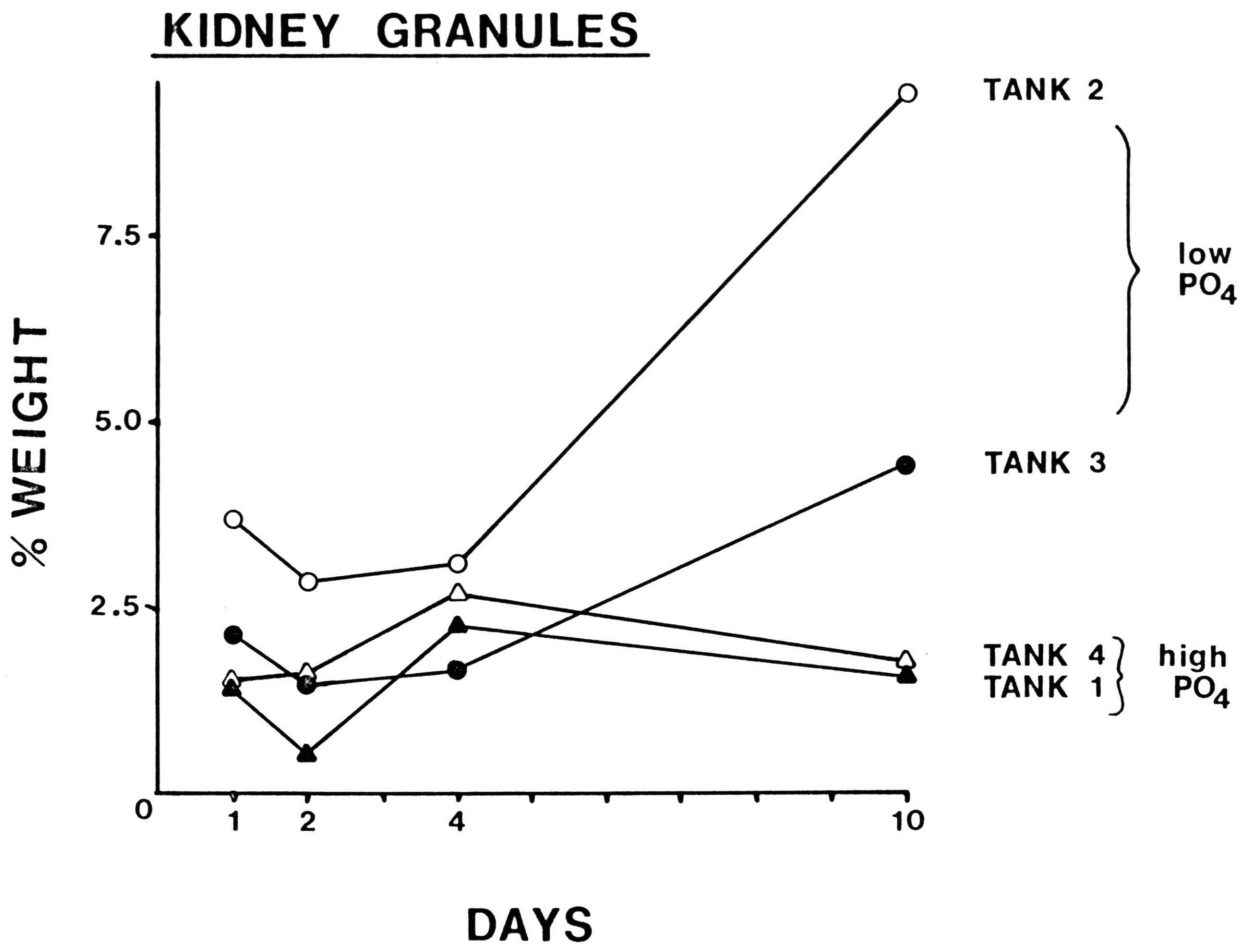


Figure 18. Relative weight of isolated kidney granules measured throughout the ten day accumulation study. Percent weight = (grams dry weight granules / grams dry weight kidney) x 100.

in figure 18. As a result of assuming a dry to wet weight ratio of 0.2 (Pequegnat et al., 1969), the scale only approximately reflects the true weight significance that granules represent in the kidney. Regardless, the curves can still be used to show general trends. In most cases there is an initial decline in the percent granule weight. Clams from the 'low phosphate' tanks show higher percent granule weight after 10 days than those exposed to elevated phosphate levels with tank 2 ('high' metal, 'low' phosphate) showing the greatest mass of granules per kidney. The kidneys from clams exposed to elevated phosphate show stabilized granule weights at the end of 10 days exposure. Because of the sampling scheme it is impossible to determine from these data whether the granule percent weight from the 'low' phosphate kidneys has stabilized at an elevated level or is still increasing after 10 days. Extended sampling would be necessary to identify the equilibrium levels for these particular conditions.

Results from gamma ray spectrometry performed on granules isolated from clams exposed for 1, 2, 4, and 10 days to Mn^{54} and Zn^{65} in seawater are shown in table 3. Results are presented as counts per minute per gram of dried granules. Each value represents the results of granules pooled from five kidneys except for day 10, tank 4 which represents four kidneys. Some of the curves presented in figures 19 and 20 show indications of a two

Table 3. Accumulation of Mn^{54} and Zn^{65} by renal granules. Percent weight equals gms dry weight granules divided by gms wet weight kidneys. Percent counts equals counts for granules divided by counts for whole kidneys.

			Counts x 10^{-3}		% Counts	
Days	Tank	% Wt	Zn	Mn	Zn	Mn
1	1	0.28	58.7	128.3	6.8	5.3
2	1	0.10	72.1	211.1	1.5	4.2
4	1	0.45	65.9	120.2	3.3	10.1
10	1	0.32	261.0	399.1	2.5	6.4
1	2	0.74	17.7	72.5	5.8	14.7
2	2	0.57	15.1	62.0	5.5	9.8
4	2	0.62	17.6	73.0	2.5	9.4
10	2	1.88	118.2	209.4	9.7	21.8
1	3	0.43	25.0	69.9	4.6	10.3
2	3	0.29	25.5	94.0	1.8	7.2
4	3	0.33	75.5	125.3	2.7	4.6
10	3	0.88	115.4	108.0	4.6	7.9
1	4	0.30	4.1	36.3	2.2	11.3
2	4	0.32	18.2	96.0	3.4	8.0
4	4	0.54	31.6	106.0	4.1	9.1
10	4	0.36	56.4	107.3	2.3	9.1

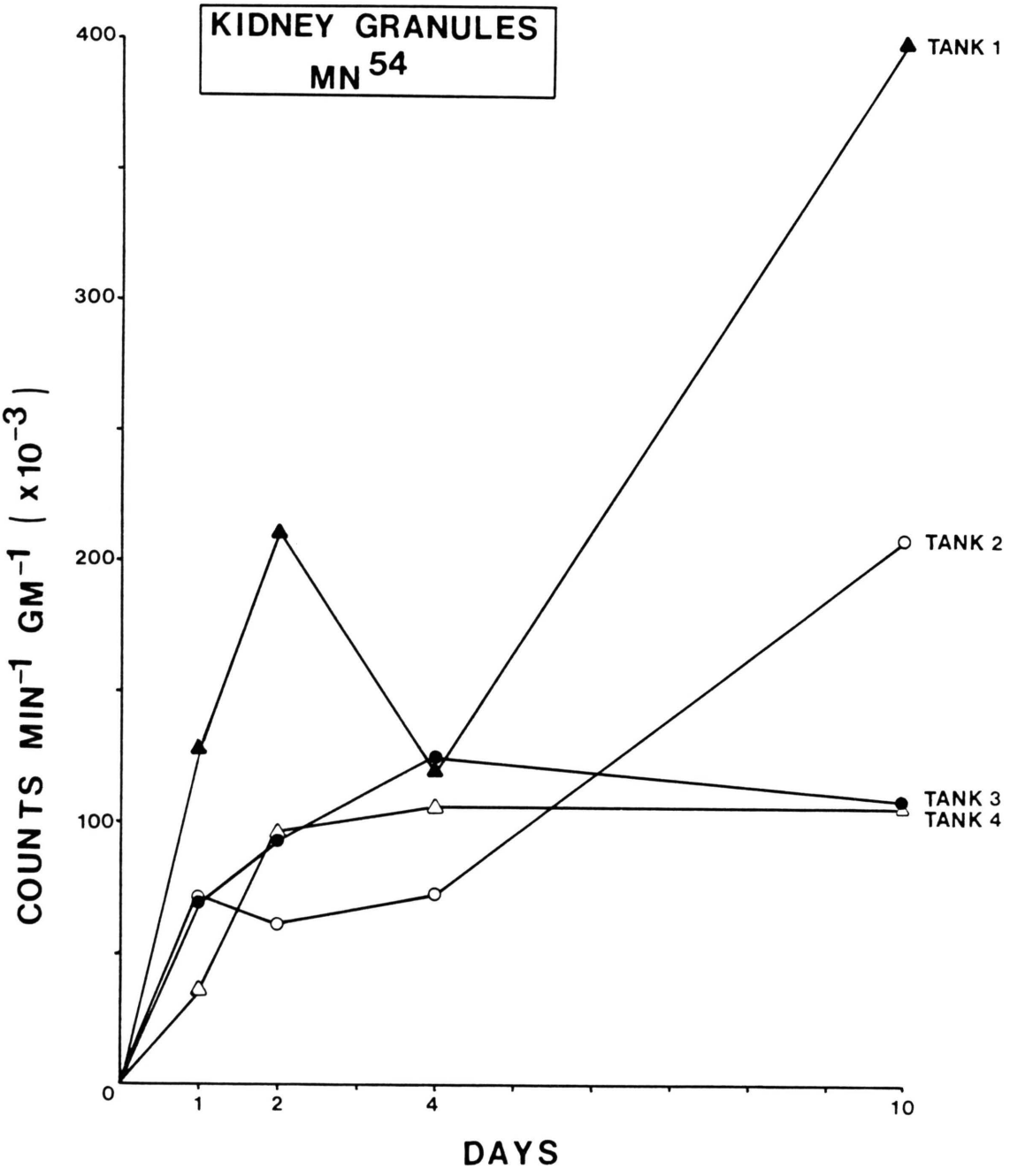


Figure 19. Mn^{54} incorporation into isolated kidney granules. ▲ = tank 1, ○ = tank 2, ● = tank 3, △ = tank 4.

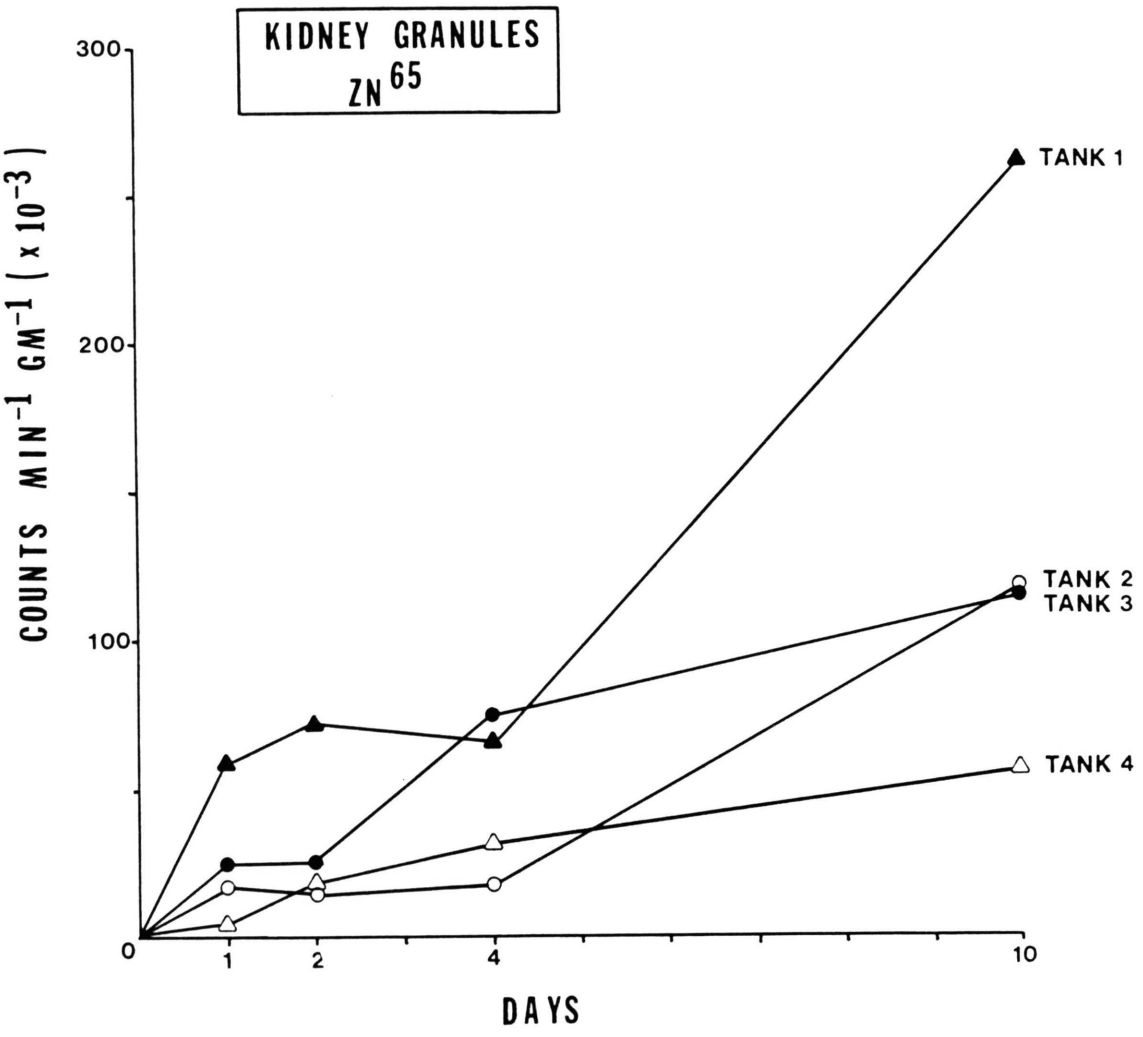


Figure 20. Zn^{65} incorporation into isolated kidney granules. ▲ = tank 1, ○ = tank 2, ● = tank 3, △ = tank 4.

stage uptake. The first two to four days of uptake appears as a saturation type curve followed by a large increase in concentration that occurs somewhere between 4 and 10 days. Results proved to be variable, and while there is some evidence for a phosphate effect (Figures 19 and 20), the necessity of pooling samples did not allow the identification of statistically well defined Zn^{65} or Mn^{54} incorporation effects resulting from either elevated phosphate or metal concentrations. It is interesting to note that Mn^{54} in kidney granules, while accumulated from a solution having considerably less specific activity for Mn^{54} than for Zn^{65}, still showed higher activity per gram of granule than did Zn^{65}.

The percentage of gamma counts in the kidney attributable to Zn^{65} and Mn^{54} in the renal granules are presented in figures 21 and 22. Both metals show curves that seem largely dependent on the mass of granules present in the kidneys and accordingly show curves similar in shape to those of figure 18. Of the radionuclides accumulated by the kidney, Mn^{54} is present to a greater degree in the granules than is Zn^{65}. This is consistent with the greater Mn^{54} activity demonstrated in figures 19 and 20.

Discussion

The intracellular granules examined here in the kidney

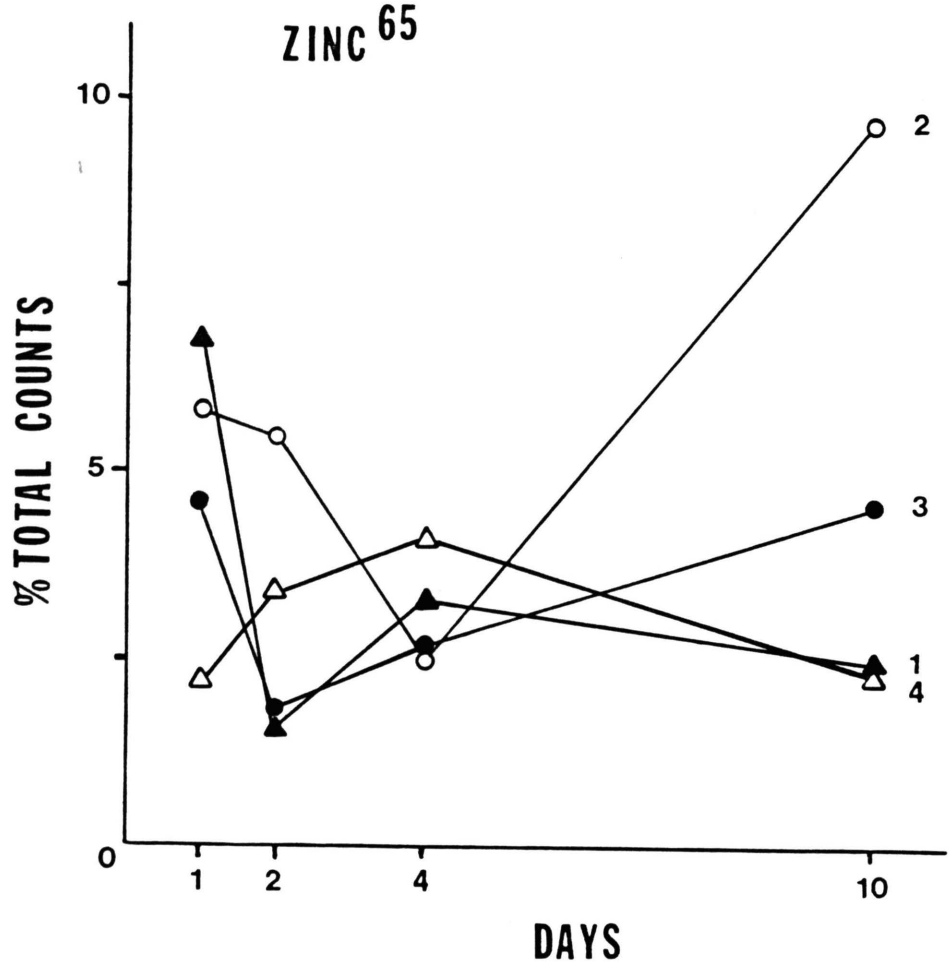


Figure 21. Percent of total Zn^{65} counts in kidney found in isolated kidney granules.

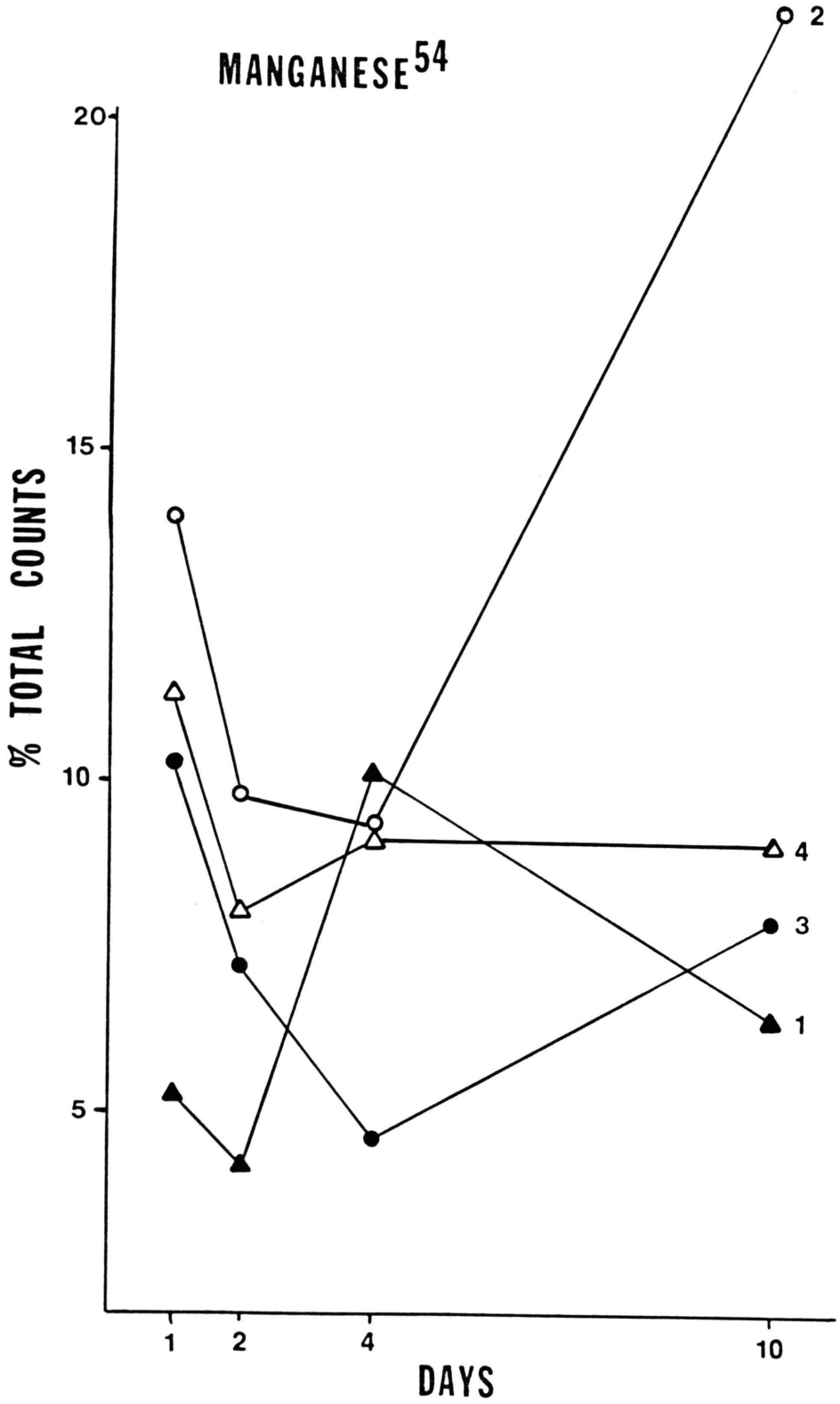


Figure 22. Percent of total Mn^{54} counts in kidney found in isolated kidney granules.

of M. campechiensis appear to be similar to those described for many other marine bivalves. The size, shape and staining characteristics show no reason to suspect a formation process very different from the generalized ones presented in reviews by Simkiss (1981), Brown (1982) and George (1982) involving metallo-proteins, lysosomes, and a possible non specific calcium ion pump. The occurrence of only one apical granule per cell is unlike Mytilus edulis which has numerous granules occurring throughout the kidney epithelial cell (Pirie and George, 1979). It is similar to Pinna nobilis (Hignette, 1978), Argopecten sp. (Carmichael et al., 1979; Carmichael and Fowler, 1981), and Macrocallista nimbosa (Tiffany et al., 1980) which contain either one or several granules in apical vesicles.

The extracellular granules which occur in the lumen of the kidney in M. campechiensis, seem to represent a relatively unexamined metal compartmentalization process for marine bivalves. While small (approximately 10 μm) intracellular renal granules have been noted to occur in almost all marine bivalve species (Lucas and Hignette, 1983), relatively few species have been shown to also possess very large (greater than 100 μm) lumenal concretions. For these large extracellular granules, only limited information exists regarding formation processes. This study suggests that M. campechiensis forms lumenal concretions as an aggregation of smaller granules,

presumably originating in the columnar epithelial cells of the kidney. This process of lumenal accretion of intracellular granules also has been proposed for concretion formation in Macrocallista nimbosa (Tiffany et al., 1980), Donax trunculus (Mauri and Orlando, 1982), and Pinna nobilis (Hignette, 1978, 1979). Expulsion of apical intracellular granules into the lumen is common, occurring in Mytilus edulis (Pirie and George, 1979) and Argopecten (Carmichael and Fowler, 1981). Reasons for secondary aggregation into larger concretions in selected species are only speculative at this point.

Electron micrographs of isolated granules support the above formation process and occasionally show a shape and texture strikingly similar to those observed by Mauri and Orlando (1982) in Donax trunculus. These highly textured concretions (Figure 15) appear to be made up of numerous spherical granules of roughly the same diameter as those found inside apical vacuoles in kidney columnal cells. Other lumenal concretions examined exhibit a smoother exterior surface (Figure 16). Ultrastructural and histochemical evidence suggests that secondary growth of lumenal concretions occurs by epitaxy (Doyle et al., 1978; Tiffany et al., 1980). Epitaxial growth has been used to explain the existence of what appear to be concentric growth rings observed in many phosphoritic granules. This process would tend to create the observed

smooth exterior surface if it occurs after aggregation. Also, Brown (1982) suggests that the ultimate size obtained by granules may be related to the rate of turnover in that tissue. If this is the case, the fact that lumenal concretions in Mercenaria are among the largest phosphoritic granules found in any invertebrate tissue (Brown, 1982) would suggest a lengthy residence time within the kidney tubules. This may result in a weathering of the exterior surface by movement against other concretions and the lumenal walls. It seems likely that both of these processes occur in M. campechiensis

Granule residence time and kinetic information on the formation, excretion, and metal incorporation rates of bivalve kidney granules is scarce. This study apparantly represents a first in vivo attempt to monitor both the granule weight equilibrium (resulting from formation and excretion) and trace metal accumulation over time. While results were highly variable, they do provide insight into the kinetics of these processes.

Both Mn^{54} and Zn^{65} occurred in isolated granules (predominantly lumenal concretions) after only one day. While perhaps possible for metals to traverse the entire uptake, transport and extracellular granule formation processes in less than one day, it seems more likely that the initial presence of Zn^{65} and Mn^{54} in isolated concretions results from direct adsorption onto pre-existing lumenal concretions. Similarly, Carmichael

and Bondy (1981) have demonstrated *in vitro* the existence of saturable, medium affinity metal binding sites on concretions isolated from *Mercenaria*. The *in vivo* accumulation curves obtained in this study (Figures 19 and 20) for Mn^{54} and Zn^{65} also suggest a saturation type uptake occurring at least over the first four days. The sharp increase in Zn^{65} and Mn^{54} concentrations observed in some of the accumulation curves after four days may reflect the completion of metal transport and intracellular granule formation processes during that time. If this is the case, the time period needed for cellular processes to sequester metals into the final excretory compartment for this species appears to be around 10 days.

Concretions, while commonly considered as long term metal sinks, are shown here to also participate in a short term response to introduced metals. The natural occurrence of large phosphoritic granules in the lumen of the kidney in this species may help provide a short term buffer against periodic increases in potentially toxic metals. The release of intracellular granules into the kidney lumen which was observed here could further increase this buffer capacity and may be an effective cellular response to rapid increases of toxic metals. Although it appears that tank 1 ('high phosphate') and tank 2 ('high metals') showed larger increases in concentrations between day 4 and day 10 for both Mn^{54} and Zn^{65}, possibly indicating an

increased rate of cellular processing, differences in uptake media and decreased statistical definition due to pooling make such a speculation unwise for this particular study. Additional research is needed to clearly identify any trace metal accumulation effects that may result from elevated phosphate and total metal concentrations.

Examination of changes in the concretion weights as a percentage of the total kidney weight (Figure 18) allows speculation on granule formation and excretion kinetics. Freshly collected clams from lower Tampa Bay were determined to have granules accounting for approximately 5.0 percent of the total weight of the kidneys. The initial drop below natural levels observed in all tanks could result from a process similar to the one described by Carmichael and Fowler (1981) for Argopecten irradians where cadmium exposure elicited a massive extrusion of concretions from the kidney epithelial cells. Histological examination of the kidneys from clams used in this study showed a similar extrusion of intracellular granules. $Zinc^{65}$ and Mn^{54} measurements from the present study suggests that such an extrusion may be extended to include excretion of granules from the organism. At the end of 10 days the control tank (number 3) had re-established a weight percentage very near natural levels. High phosphate tanks, both with and without elevated Zn and Mn concentrations, possibly remain lower than natural levels not due to decreased concretion formation but rather

continued stimulation of granule excretion rates. As suggested in previous literature (Doyle et al., 1978), elevated metal concentrations seem to stimulate granule formation. Tank 2 shows almost two times more percent granule weight than occurs naturally or in control clams. Although tank 4 also contained elevated metal concentrations, the expected increase in granule formation is not observed. It seems that the stimulation of granule excretion caused by elevated phosphate outweighs any increase in formation due to elevated metal concentrations.

This study contributes new histologic and kinetic information concerning the formation, excretion and trace metal incorporation characteristics of phosphoritic kidney granules in Mercenaria campechiensis. A more complete understanding of this system will only result from further investigation into trace metal incorporation and excretion processes. Such studies may show a direct relationship between equilibrium metal concentrations in kidney concretions and environmental metal levels. Only then can granules be reliably used as indicators of metal contamination.

CHAPTER 7: CONCLUSIONS

This study presents information in three basic areas concerning metal accumulation by the subtropical bivalve Mercenaria campechiensis: 1) General metal uptake and tissue distribution, 2) Zn and Mn accumulation responses to elevated dissolved phosphate and metal concentrations, and 3) the formation of, and metal incorporation into phosphoritic renal granules. While each area has been presented individually, they are interelated and cannot be separated when considering the overall process of metal accumulation by marine molluscs.

The data generated in this study, when compared to previous literature, suggests that the general accumulation of metals by M. campechiensis is comparable to that observed for other bivalves. There are several similarities between the present results and those of previous studies. The observance of the largest Zn and Mn accumulation in the kidney for the present study has also been noted in other molluscs and for other metals (Bryan, 1973; Schulz-Baldes, 1974; Carmichael et al., 1980; Nolan and Duke, 1983). In the kidney, toxic and excess metals have previously been observed to accumulate into phosphoritic renal granules (Simkiss, 1976; Doyle et

al., 1978; George, 1982) as is the case here for Zn and Mn. Several other studies have shown the same positive correlation between metal exposure concentrations and uptake rates and concentration factors (Schulz-Baldes, 1974; George and Pirie, 1980; Zaroogian, 1980) as is seen here.

While there are similarities between observations found in this and other investigations, several new considerations arise from observations in this study. Zn^{65} and Mn^{54} exhibited very different accumulation behavior. Mn^{54} is primarily distributed to the kidney and kidney granules while Zn^{65} exhibits a distribution to both the kidney and the gill within one day. Extended exposure results in a steady state concentration in the gills (at about 5 days) with continued concentration into the kidney. Both uptake rates and distribution patterns vary between these two metals. This supports the idea that the time frame of uptake and tissue distribution (and presumably possible tissue impact) can vary greatly between various metal ions. Consequently, studies of lethal and sublethal effects of metals on marine molluscs, as well as studies which use molluscs as indicators of metal pollution, must consider metals individually.

Phosphate is observed here to be an additional environmental factor which affects the accumulation of metals in bivalve molluscs. Examination of general tissue distribution and analysis of kidney granules show

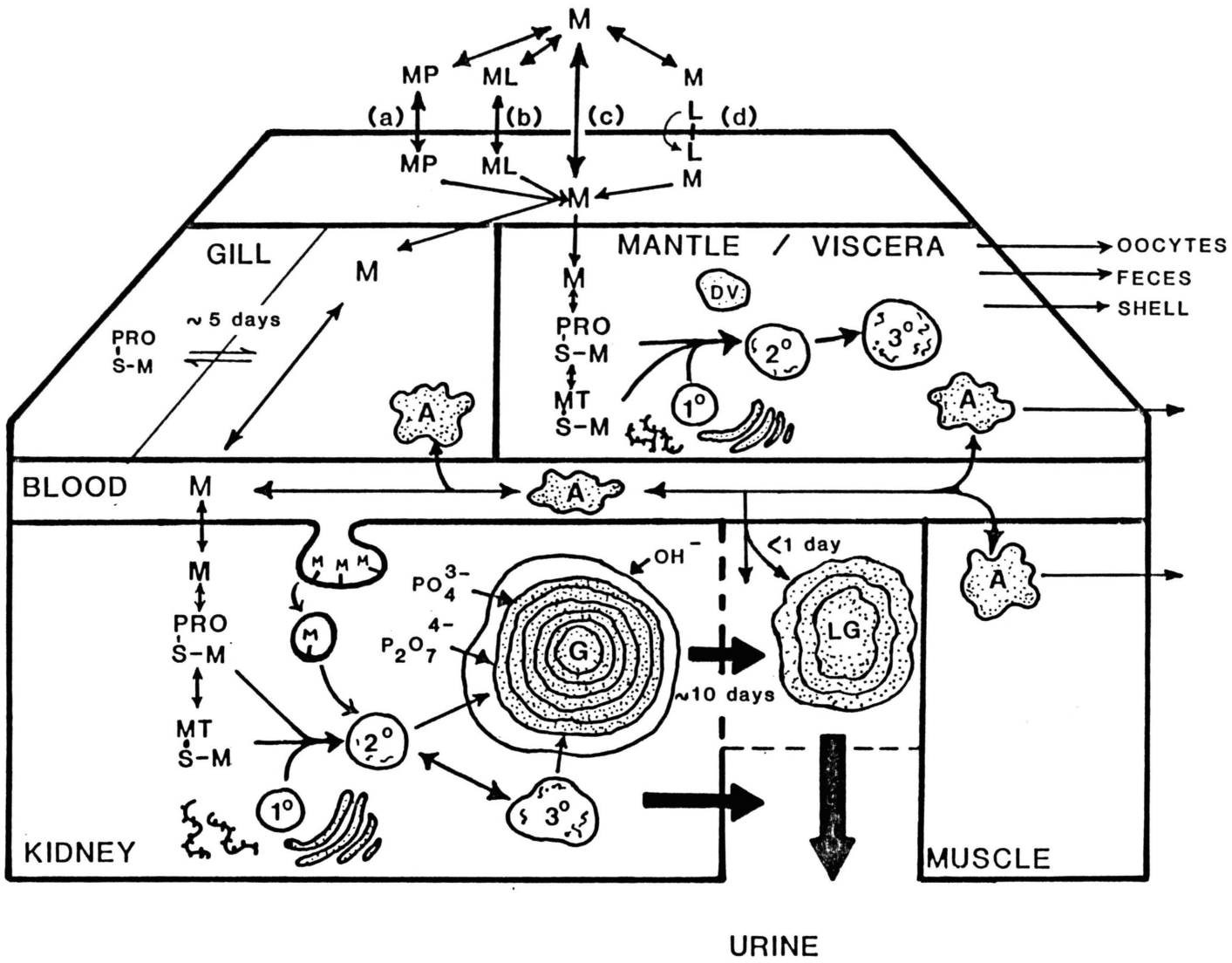
variations in the accumulation of Zn^{65} and Mn^{54} resulting from variations in dissolved phosphate concentrations. Unlike other environmental factors such as salinity and the presence of metal chelators, phosphate does not seem to affect the availability of these metals but rather influences the metabolic pathways by which Mercenaria accumulates and excretes Zn and Mn. The effects in tissue distribution and overall accumulation of metals observed in this study seem to result from changes in the processes involved in metal detoxification, particularily the formation and excretion of kidney granules. While the chemical form and general availabilty of metals in seawater have been shown to be very important to metal accumulation by molluscs, significant accumulation changes can also result from changes in metabolism caused by chemical constituents other than those which directly affect metal chemistry.

In previous studies, molluscan inorganic kidney granules have been postulated as deposition sites for excess and toxic metals. This effectively removes potentially toxic metals from cellular processes by incorporating them into insoluble granules prior to excretion. In the case of Mercenaria, it seems that renal granules, particularly large lumenal granules, not only serve as a long term repository for metals but also function as a short term detoxification response to periodic elevations in the environmental levels of

potentially toxic metals. These studies suggest that existing granules remove metals from metabolic pathways by direct adsorption onto their surfaces. Elevated metal concentrations seem to stimulate accumulation of metals into granules and elevated dissolved phosphate stimulates excretion of both inter- and intracellular granules. Although renal granules are generally acknowledged as participating in the detoxification of metals, results from this study indicate that they may play a larger role than previously assumed. In fact, the major tissue distribution patterns and uptake rates observed in this and other metal uptake studies may result largely from a dependance on the processes which control granule formation and excretion.

With information obtained in the present study, the general model for metal accumulation in bivalves presented in chapter 2 (Figure 1) can be modified to include several additional items observed in Mercenaria campechiensis. Figure 24 includes large intercellular granules which occur in the lumen of the kidney (LG). Histological examination and SEM studies support a method of formation involving accretion of smaller intracellular granules (G). Radiotracer studies indicate that short term adsorption of metals occurs in less than one day while incorporation into lumenal granules by way of cellular pathways (i.e. binding to metallo-proteins, lysosomal mechanisms, release and aggregation of intracellular granules) may occur in about ten days. Figure 23 also includes the observed Zn

Figure 23. Composite model for metal metabolism in Mercenaria campechiensis. Based on Coombs and George (1977), George and Pirie (1980), Simkiss (1981), George (1982) and the present study. M = unbound metal ion; L = ligand; P = pollutant; PRO-S = protein with sulfhydral group; A = amoebocyte; MT-S = metallothionein; G = inorganic granule; LG = lumenal granule; DV = digestive vacuole; 1°, 2°, 3° = primary, secondary, and tertiary lysosomes.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accumulation compartment in the gills where a steady state concentration is obtained after about five days. While not identified directly, Zn containing proteins, perhaps a Zn enzyme such as carbonic anhydrase, are thought to account for the observed accumulation.

Although many of the details concerning metal metabolism by marine molluscs have been identified, there are still many details which are not fully defined. Additional studies are needed which compare the uptake kinetics and tissue distribution of different metals under controlled conditions. Such studies could help explain observed differences between metals with regard to both lethal and sublethal effects. The exact role of inorganic kidney granules (both intra- and intercellular granules) in metal detoxification remains poorly understood. Future research aimed directly at such basic characteristics as granule solubility and formation kinetics may help clarify the cellular processes and environmental conditions which regulate this major metal detoxification mechanism.

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APPENDIXES

APPENDIX 1: DATA SUMMARY

Table 4. Summary of gamma ray count data. Tissue 1 = mantle, tissue 2 = gill, tissue 3 = viscera, tissue 4 = kidney, tissue 5 = adductor. Percent Zn and Mn represents total gamma counts per minute per gram wet weight for the individual tissue divided by total gamma counts for all tissues counted multiplied by 100. Cnt. = gamma counts per minute per gram wet weight of tissue. MIN. = minimum value, MAX. = maximum value, STD. DEV. = standard deviation, STD. ERR. = standard error of the mean. For all statistics, n = 5.

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=1, tank=1,	tissue=1	----------	----------	----------
% Zn	8.26	2.13	6.64	11.94	0.95
% Mn	8.58	7.87	1.20	21.12	3.52
Zn Cnt.	656.62	374.18	42.70	972.10	167.34
Mn Cnt.	264.22	151.97	38.00	388.80	67.96
----------	day=1, tank=1,	tissue=2	----------	----------	----------
% Zn	61.00	14.57	45.78	82.72	6.51
% Mn	20.31	23.65	0.49	58.98	10.58
Zn Cnt.	4346.20	2420.59	532.40	6276.90	1082.52
Mn Cnt.	465.98	376.37	33.10	865.70	168.32
----------	day=1, tank=1,	tissue=3	----------	----------	----------
% Zn	1.65	1.20	0.44	3.66	0.54
% Mn	2.47	3.09	0.33	7.84	1.38
Zn Cnt.	136.90	86.14	2.80	214.00	38.52
Mn Cnt.	60.12	37.92	14.10	106.50	16.96
----------	day=1, tank=1,	tissue=4	----------	----------	----------
% Zn	26.94	14.26	10.04	42.98	6.38
% Mn	64.26	37.16	4.34	95.43	16.62
Zn Cnt.	2788.78	2541.34	64.60	5845.40	1136.52
Mn Cnt.	8428.86	12628.85	7.80	30640.90	5647.79

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
		day=1, tank=1, tissue=5			
% Zn	2.15	1.42	0.17	4.13	0.63
% Mn	4.37	3.17	1.20	7.72	1.42
Zn Cnt.	197.36	129.39	1.10	341.70	57.86
Mn Cnt.	191.36	154.72	13.90	391.80	69.19
		day=1, tank=2, tissue=1			
% Zn	7.62	1.37	5.80	9.53	0.61
% Mn	4.68	2.68	1.87	9.00	1.20
Zn Cnt.	301.30	52.11	238.50	377.70	23.30
Mn Cnt.	145.00	21.10	116.20	165.80	9.43
		day=1, tank=2, tissue=2			
% Zn	38.27	6.98	31.02	47.03	3.12
% Mn	8.80	6.11	3.76	19.06	2.73
Zn Cnt.	1505.54	190.91	1177.00	1655.40	85.38
Mn Cnt.	264.04	53.84	223.50	350.50	24.08
		day=1, tank=2, tissue=3			
% Zn	2.02	0.42	1.53	2.59	0.19
% Mn	1.36	0.65	0.53	2.17	0.29
Zn Cnt.	79.46	14.57	64.80	99.10	6.52
Mn Cnt.	43.30	9.40	33.10	57.60	4.20
		day=1, tank=2, tissue=4			
% Zn	48.48	8.70	37.09	58.89	3.89
% Mn	82.61	10.47	65.60	92.96	4.68
Zn Cnt.	2063.90	891.77	928.30	3116.20	398.81
Mn Cnt.	3248.08	1714.20	1205.90	5779.60	766.61
		day=1, tank=2, tissue=5			
% Zn	3.61	0.73	2.50	4.53	0.33
% Mn	2.55	1.23	0.88	4.17	0.55
Zn Cnt.	146.56	50.64	94.10	231.00	22.65
Mn Cnt.	80.52	16.11	54.50	95.10	7.21

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=1, tank=3, tissue=1	----------	----------	----------	----------
% Zn	8.66	2.43	5.58	12.10	1.09
% Mn	6.26	1.87	3.48	8.51	0.84
Zn Cnt.	802.14	277.71	508.30	1239.50	124.19
Mn Cnt.	197.54	60.39	130.70	270.80	27.01
----------	day=1, tank=3, tissue=2	----------	----------	----------	----------
% Zn	66.48	5.59	58.26	73.58	2.50
% Mn	8.43	3.01	6.38	13.69	1.35
Zn Cnt.	6614.18	3564.24	4496.20	12944.10	1593.98
Mn Cnt.	280.60	142.30	147.70	518.80	63.64
----------	day=1, tank=3, tissue=3	----------	----------	----------	----------
% Zn	1.21	0.22	0.95	1.45	0.10
% Mn	1.55	0.73	0.88	2.69	0.33
Zn Cnt.	130.02	95.88	61.20	295.70	42.88
Mn Cnt.	47.36	15.74	28.20	62.10	7.04
----------	day=1, tank=3, tissue=4	----------	----------	----------	----------
% Zn	21.39	6.83	14.88	32.03	3.06
% Mn	80.31	3.76	74.74	85.00	1.68
Zn Cnt.	2549.28	2584.67	909.20	7116.30	1155.90
Mn Cnt.	2905.22	1828.29	1403.50	6021.30	817.64
----------	day=1, tank=3, tissue=5	----------	----------	----------	----------
% Zn	2.26	0.32	2.04	2.80	0.14
% Mn	3.46	0.43	3.11	4.13	0.19
Zn Cnt.	249.80	209.45	135.50	623.20	93.67
Mn Cnt.	123.38	70.27	58.40	235.30	31.43
----------	day=1, tank=4, tissue=1	----------	----------	----------	----------
% Zn	11.85	4.08	8.04	17.59	1.82
% Mn	11.86	9.07	3.61	26.80	4.05
Zn Cnt.	188.62	139.15	4.10	340.50	62.23
Mn Cnt.	88.62	42.60	39.80	131.10	19.05

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=1, tank=4,	tissue=2	----------	----------	----------
% Zn	38.99	20.07	11.76	57.01	8.97
% Mn	33.18	28.39	10.40	76.74	12.70
Zn Cnt.	768.48	719.13	6.00	1694.60	321.60
Mn Cnt.	235.28	102.80	70.20	330.60	45.97
----------	day=1, tank=4,	tissue=3	----------	----------	----------
% Zn	2.79	2.36	0.38	6.74	1.06
% Mn	3.71	3.46	1.68	9.83	1.55
Zn Cnt.	35.02	30.68	1.00	73.70	13.72
Mn Cnt.	27.86	15.61	11.60	49.70	6.98
----------	day=1, tank=4,	tissue=4	----------	----------	----------
% Zn	42.37	22.63	25.01	75.10	10.12
% Mn	46.86	38.16	4.95	82.25	17.07
Zn Cnt.	587.38	523.02	38.30	1327.50	233.90
Mn Cnt.	922.50	941.15	8.70	2158.30	420.90
----------	day=1, tank=4,	tissue=5	----------	----------	----------
% Zn	4.00	1.70	2.32	6.23	0.76
% Mn	4.38	3.40	1.84	10.24	1.52
Zn Cnt.	63.50	59.59	1.60	160.10	26.65
Mn Cnt.	40.86	32.18	9.20	90.10	14.39
----------	day=2, tank=1,	tissue=1	----------	----------	----------
% Zn	10.76	6.86	6.91	22.94	3.07
% Mn	7.12	3.09	3.89	11.80	1.38
Zn Cnt.	1165.08	363.37	906.90	1791.20	162.50
Mn Cnt.	314.66	219.01	158.40	675.40	97.94
----------	day=2, tank=1,	tissue=2	----------	----------	----------
% Zn	54.35	11.83	36.36	62.66	5.29
% Mn	12.85	17.42	0.20	43.16	7.79
Zn Cnt.	7346.32	3659.67	1845.10	12076.30	1636.66
Mn Cnt.	588.10	656.92	5.70	1356.30	293.79

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=2, tank=1, tissue=3	----------	----------	----------	----------
% Zn	1.44	0.77	0.76	2.74	0.34
% Mn	1.78	0.92	0.68	3.18	0.41
Zn Cnt.	168.50	86.03	100.20	316.90	38.47
Mn Cnt.	68.26	30.25	37.70	117.60	13.53
----------	day=2, tank=1, tissue=4	----------	----------	----------	----------
% Zn	31.22	6.16	25.09	40.74	2.75
% Mn	75.99	21.16	39.84	91.50	9.46
Zn Cnt.	4390.02	3367.19	1702.90	10240.70	1505.86
Mn Cnt.	4647.72	5859.96	1251.90	15078.30	2620.65
----------	day=2, tank=1, tissue=5	----------	----------	----------	----------
% Zn	2.22	1.41	0.97	4.40	0.63
% Mn	2.25	1.12	0.77	3.44	0.50
Zn Cnt.	280.24	244.14	126.80	712.40	109.18
Mn Cnt.	104.24	83.23	21.90	242.10	37.22
----------	day=2, tank=2, tissue=1	----------	----------	----------	----------
% Zn	8.67	2.30	6.86	12.68	1.03
% Mn	3.87	2.30	1.21	6.59	1.03
Zn Cnt.	292.12	80.31	164.90	365.30	35.92
Mn Cnt.	111.54	19.53	81.20	130.50	8.74
----------	day=2, tank=2, tissue=2	----------	----------	----------	----------
% Zn	41.38	14.66	15.37	50.44	6.56
% Mn	9.71	3.83	3.62	13.60	1.71
Zn Cnt.	1547.96	822.80	323.50	2228.70	367.97
Mn Cnt.	317.46	116.19	168.40	485.40	51.96
----------	day=2, tank=2, tissue=3	----------	----------	----------	----------
% Zn	1.53	0.47	11.03	2.11	0.21
% Mn	1.10	0.64	0.41	2.13	0.29
Zn Cnt.	55.64	30.67	23.20	97.30	13.72
Mn Cnt.	33.18	8.54	24.20	46.80	3.82

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=2, tank=2,	tissue=4	----------	----------	----------
% Zn	46.04	12.18	38.55	67.67	5.45
% Mn	83.54	7.05	76.21	93.69	3.15
Zn Cnt.	1562.06	451.39	827.50	1892.60	201.87
Mn Cnt.	3615.60	2790.26	1067.20	8059.70	1247.84
----------	day=2, tank=2,	tissue=5	----------	----------	----------
% Zn	2.38	0.67	1.80	3.40	0.30
% Mn	1.79	0.56	1.07	2.38	0.25
Zn Cnt.	84.08	43.13	48.20	156.90	19.29
Mn Cnt.	62.98	28.21	31.00	93.10	12.62
----------	day=2, tank=3,	tissue=1	----------	----------	----------
% Zn	11.69	10.79	5.73	30.84	4.83
% Mn	6.99	3.40	3.82	11.93	1.52
Zn Cnt.	1216.54	347.61	831.30	1674.80	155.45
Mn Cnt.	264.08	72.40	156.60	334.30	32.83
----------	day=2, tank=3,	tissue=2	----------	----------	----------
% Zn	53.57	21.75	16.44	71.65	9.73
% Mn	10.01	7.22	0.75	19.35	3.23
Zn Cnt.	8862.10	5098.94	667.20	13213.80	2280.31
Mn Cnt.	403.08	237.42	21.70	632.20	106.18
----------	day=2, tank=3,	tissue=3	----------	----------	----------
% Zn	1.71	1.33	0.77	4.05	0.60
% Mn	2.04	0.93	1.12	3.19	0.42
Zn Cnt.	188.10	59.19	141.90	291.70	26.47
Mn Cnt.	76.9	19.32	48.80	94.30	8.64
----------	day=2, tank=3,	tissue=4	----------	----------	----------
% Zn	30.71	10.58	17.10	43.80	4.73
% Mn	77.17	11.34	62.13	87.77	5.07
Zn Cnt.	4353.36	2822.56	1777.30	8956.8	1262.29
Mn Cnt.	3372.82	1664.85	1741.10	5295.10	744.54

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=2, tank=3, tissue=5	----------			
% Zn	2.32	1.46	1.39	4.86	0.65
% Mn	3.80	1.02	2.34	5.05	0.46
Zn Cnt.	266.86	53.35	197.20	346.70	23.86
Mn Cnt.	155.32	60.89	95.40	254.40	27.23
----------	day=2, tank=4, tissue=1	----------			
% Zn	7.64	2.81	3.38	11.07	1.25
% Mn	3.91	1.77	2.57	6.99	0.79
Zn Cnt.	334.00	136.22	128.90	454.70	60.92
Mn Cnt.	153.82	16.43	133.50	166.50	7.35
----------	day=2, tank=4, tissue=2	----------			
% Zn	47.18	6.42	40.20	54.42	2.87
% Mn	8.87	4.71	5.34	17.12	2.11
Zn Cnt.	2007.82	192.34	1678.60	2168.70	86.02
Mn Cnt.	342.26	47.01	278.60	407.60	21.01
----------	day=2, tank=4, tissue=3	----------			
% Zn	2.17	0.33	1.63	2.50	0.15
% Mn	1.34	0.78	0.52	2.63	0.35
Zn Cnt.	93.76	23.38	73.10	133.50	10.46
Mn Cnt.	53.04	21.86	23.70	82.90	9.78
----------	day=2, tank=4, tissue=4	----------			
% Zn	40.34	6.54	34.34	49.55	2.92
% Mn	83.97	7.92	69.92	88.96	3.54
Zn Cnt.	1795.1	647.77	1181.60	2593.90	289.69
Mn Cnt.	3778.32	1494.87	1664.70	5767.30	668.52
----------	day=2, tank=4, tissue=5	----------			
% Zn	2.67	0.73	1.63	3.61	0.33
% Mn	1.92	0.85	1.13	3.32	0.38
Zn Cnt.	115.40	43.78	85.30	192.80	19.58
Mn Cnt.	77.86	25.41	54.60	120.30	11.36

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=4, tank=1, tissue=1				
% Zn	6.62	1.19	5.22	8.38	0.53
% Mn	4.81	1.65	3.69	7.67	0.74
Zn Cnt.	1248.62	454.53	818.00	1955.70	203.27
Mn Cnt.	319.04	166.46	124.10	524.30	74.44
----------	day=4, tank=1, tissue=2				
% Zn	46.76	7.02	35.54	52.87	3.14
% Mn	13.05	12.86	6.35	36.02	5.75
Zn Cnt.	8897.10	3629.96	6184.50	15023.60	1623.37
Mn Cnt.	744.42	466.79	217.30	1367.20	208.76
----------	day=4, tank=1, tissue=3				
% Zn	1.04	0.32	0.56	1.42	0.14
% Mn	1.02	0.38	0.64	1.61	0.17
Zn Cnt.	202.26	99.43	83.20	335.20	44.47
Mn Cnt.	62.72	22.81	31.70	90.80	10.20
----------	day=4, tank=1, tissue=4				
% Zn	43.22	7.85	35.83	55.84	3.51
% Mn	78.46	15.34	51.09	87.21	6.86
Zn Cnt.	8876.52	5160.77	4537.20	14772.10	2307.97
Mn Cnt.	5872.92	4046.49	1939.10	10324.60	1809.64
----------	day=4, tank=1, tissue=5				
% Zn	2.35	0.55	1.87	3.23	0.24
% Mn	2.66	1.00	1.62	3.78	0.45
Zn Cnt.	499.80	352.13	224.00	1070.40	157.48
Mn Cnt.	205.2	164.92	44.10	446.70	73.75
----------	day=4, tank=2, tissue=1				
% Zn	6.04	2.13	4.06	9.59	0.95
% Mn	3.52	2.04	1.51	6.88	0.91
Zn Cnt.	411.60	105.95	295.90	542.70	47.38
Mn Cnt.	167.54	68.43	77.40	253.70	30.60

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=4, tank=2, tissue=2				
% Zn	35.20	11.91	25.97	50.75	5.33
% Mn	11.05	8.37	4.53	25.65	3.74
Zn Cnt.	2456.26	918.19	1592.20	3934.50	410.63
Mn Cnt.	483.54	181.50	306.00	776.90	81.17
----------	day=4, tank=2, tissue=3				
% Zn	1.88	0.42	1.60	2.61	0.19
% Mn	1.38	0.78	0.39	2.21	0.35
Zn Cnt.	129.58	23.56	99.10	157.30	10.54
Mn Cnt.	60.10	15.05	33.20	67.40	6.73
----------	day=4, tank=2, tissue=4				
% Zn	54.14	14.33	32.48	65.16	6.41
% Mn	82.03	11.95	61.84	93.45	5.35
Zn Cnt.	4114.10	1842.78	1232.50	6098.30	824.12
Mn Cnt.	4635.44	2528.99	1873.30	7949.50	1131.00
----------	day=4, tank=2, tissue=5				
% Zn	2.74	1.05	1.94	4.57	0.47
% Mn	2.02	1.31	0.12	3.43	0.58
Zn Cnt.	186.00	45.33	118.10	228.20	20.27
Mn Cnt.	86.08	47.81	10.20	140.30	21.38
----------	day=4, tank=3, tissue=1				
% Zn	5.94	1.14	4.79	7.86	0.51
% Mn	3.11	0.88	1.90	4.24	0.39
Zn Cnt.	1213.74	190.40	1086.40	1539.70	85.15
Mn Cnt.	275.58	60.56	213.90	361.90	27.08
----------	day=4, tank=3, tissue=2				
% Zn	48.01	11.67	31.43	62.66	5.22
% Mn	6.27	5.67	1.10	14.92	2.54
Zn Cnt.	10382.46	4038.94	4505.90	14674.60	1806.28
Mn Cnt.	443.64	394.76	94.40	1074.10	176.54

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=4, tank=3, tissue=3	----------	----------	----------	----------
% Zn	1.00	0.23	0.73	1.35	0.10
% Mn	0.60	0.16	0.34	0.73	0.07
Zn Cnt.	210.32	76.31	145.70	342.70	34.13
Mn Cnt.	52.98	8.32	44.10	62.50	3.72
----------	day=4, tank=3, tissue=4	----------	----------	----------	----------
% Zn	43.48	10.39	29.39	56.94	4.65
% Mn	88.97	6.12	79.25	93.58	2.74
Zn Cnt.	9142.24	3481.33	6245.70	15163.80	1556.90
Mn Cnt.	8315.24	2783.20	5703.30	13057.90	1244.68
----------	day=4, tank=3, tissue=5	----------	----------	----------	----------
% Zn	1.57	0.49	1.26	2.43	0.22
% Mn	2.04	0.66	1.25	2.82	0.29
Zn Cnt.	315.72	62.61	250.40	408.40	28.00
Mn Cnt.	180.94	47.36	119.40	240.20	21.18
----------	day=4, tank=4, tissue=1	----------	----------	----------	----------
% Zn	7.74	2.26	5.22	11.32	1.01
% Mn	3.56	2.32	2.24	7.70	1.04
Zn Cnt.	59.50	141.64	420.40	767.20	63.34
Mn Cnt.	251.38	180.11	147.60	572.50	80.55
----------	day=4, tank=4, tissue=2	----------	----------	----------	----------
% Zn	38.77	11.47	24.15	49.28	5.13
% Mn	6.50	4.18	3.71	13.84	1.87
Zn Cnt.	2620.38	568.26	1781.20	3339.00	254.13
Mn Cnt.	456.88	322.98	257.80	1029.50	144.44
----------	day=4, tank=4, tissue=3	----------	----------	----------	----------
% Zn	1.88	0.89	1.08	3.26	0.40
% Mn	0.88	0.51	0.57	1.77	0.23
Zn Cnt.	130.42	59.80	61.20	221.10	26.74
Mn Cnt.	62.88	40.74	36.20	131.70	18.22

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=4, tank=4, tissue=4	----------	----------	----------	----------
% Zn	49.36	14.47	32.61	67.57	6.47
% Mn	87.80	7.50	74.47	91.95	3.35
Zn Cnt.	3638.48	2046.78	2209.80	7135.10	915.35
Mn Cnt.	6079.50	932.07	5272.60	7643.20	416.83
----------	day=4, tank=4, tissue=5	----------	----------	----------	----------
% Zn	2.24	0.74	1.76	3.52	0.33
% Mn	1.25	0.57	0.71	2.21	0.26
Zn Cnt.	156.84	56.60	99.10	238.50	25.31
Mn Cnt.	89.36	48.03	40.90	164.60	21.48
----------	day=10, tank=1, tissue=1	----------	----------	----------	----------
% Zn	4.48	0.97	3.38	5.71	0.43
% Mn	2.57	1.20	1.19	4.08	0.54
Zn Cnt.	1881.86	590.33	1199.40	2791.20	264.00
Mn Cnt.	429.58	121.65	285.60	600.10	54.40
----------	day=10, tank=1, tissue=2	----------	----------	----------	----------
% Zn	20.62	3.37	15.53	23.67	1.51
% Mn	3.46	1.59	2.15	5.21	0.71
Zn Cnt.	8558.32	1970.70	6806.20	11927.90	881.32
Mn Cnt.	588.28	153.56	373.40	767.50	68.67
----------	day=10, tank=1, tissue=3	----------	----------	----------	----------
% Zn	1.29	0.33	0.88	1.79	0.15
% Mn	0.66	0.55	0.27	1.62	0.25
Zn Cnt.	561.56	263.59	313.50	984.30	117.88
Mn Cnt.	109.12	74.58	46.30	238.50	33.35
----------	day=10, tank=1, tissue=4	----------	----------	----------	----------
% Zn	72.22	4.89	68.57	79.30	2.19
% Mn	92.19	3.27	87.90	95.42	1.46
Zn Cnt.	32055.20	16167.83	24010.30	60905.90	7230.47
Mn Cnt.	18276.74	8932.85	8692.90	27976.10	3994.89

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=10, tank=1,	tissue=5	----------	----------	----------
% Zn	1.38	0.82	0.25	2.48	0.37
% Mn	1.12	0.28	0.92	1.58	0.12
Zn Cnt.	508.32	256.67	191.20	885.00	114.77
Mn Cnt.	205.18	64.88	147.40	284.10	29.02
----------	day=10, tank=2,	tissue=1	----------	----------	----------
% Zn	3.49	1.43	1.96	5.28	0.64
% Mn	1.64	0.84	0.92	3.08	0.37
Zn Cnt.	1013.14	184.47	831.60	1229.50	82.50
Mn Cnt.	283.08	93.24	194.70	384.80	41.70
----------	day=10, tank=2,	tissue=2	----------	----------	----------
% Zn	12.00	4.80	6.77	17.50	2.14
% Mn	2.31	1.36	1.40	4.71	0.61
Zn Cnt.	3466.56	451.34	3002.00	4129.30	201.85
Mn Cnt.	380.24	68.56	298.00	443.50	30.66
----------	day=10, tank=2,	tissue=3	----------	----------	----------
% Zn	1.15	0.43	0.70	1.79	0.19
% Mn	0.47	0.31	0.16	0.96	0.14
Zn Cnt.	338.42	53.79	268.70	418.30	24.06
Mn Cnt.	77.36	41.79	42.70	147.20	18.69
----------	day=10, tank=2,	tissue=4	----------	----------	----------
% Zn	81.43	6.97	74.52	89.27	3.12
% Mn	94.79	2.82	89.85	96.69	1.26
Zn Cnt.	26814.76	11545.55	17383.40	43148.80	5163.32
Mn Cnt.	19683.32	9413.08	5678.90	28308.90	4209.66
----------	day=10, tank=2,	tissue=5	----------	----------	----------
% Zn	1.92	0.66	1.11	2.50	0.30
% Mn	0.79	0.36	0.49	1.40	0.16
Zn Cnt.	562.72	75.08	435.40	627.10	33.57
Mn Cnt.	137.62	36.21	88.70	182.00	16.20

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
----------	day=10, tank=3,	tissue=1	----------	----------	----------
% Zn	5.40	1.87	3.43	7.87	0.84
% Mn	3.01	1.39	1.05	4.96	0.62
Zn Cnt.	1738.34	529.30	1289.60	2354.80	236.71
Mn Cnt.	282.76	157.29	149.50	531.00	70.34
----------	day=10, tank=3,	tissue=2	----------	----------	----------
% Zn	36.92	14.01	18.24	52.79	6.27
% Mn	3.95	1.26	2.45	5.67	0.56
Zn Cnt.	11778.84	4317.06	8608.70	19135.80	1930.65
Mn Cnt.	467.26	441.95	166.90	1243.60	197.65
----------	day=10, tank=3,	tissue=3	----------	----------	----------
% Zn	1.41	0.44	0.96	1.90	0.19
% Mn	0.81	0.36	0.17	1.06	0.16
Zn Cnt.	460.34	146.89	288.00	627.30	65.69
Mn Cnt.	65.64	12.96	57.00	88.20	5.80
----------	day=10, tank=3,	tissue=4	----------	----------	----------
% Zn	54.33	16.60	35.09	75.49	7.42
% Mn	90.69	3.45	86.15	95.58	1.54
Zn Cnt.	22044.20	16349.15	5747.20	46692.00	7311.56
Mn Cnt.	14114.86	19199.43	4987.80	48452.20	8586.25
----------	day=10, tank=3,	tissue=5	----------	----------	----------
% Zn	1.95	0.67	1.33	2.84	0.30
% Mn	1.53	0.70	0.74	2.33	0.31
Zn Cnt.	688.04	378.72	327.00	1228.40	169.37
Mn Cnt.	162.26	126.05	62.60	376.20	56.37
----------	day=10, tank=4,	tissue=1	----------	----------	----------
% Zn	6.52	3.61	3.91	11.82	1.81
% Mn	8.91	12.78	1.37	27.95	6.39
Zn Cnt.	933.75	456.86	490.50	1525.10	228.43
Mn Cnt.	220.08	70.84	140.30	300.60	35.42

Table 4. (Cont'd)

VARIABLE	MEAN	STD. DEV.	MIN.	MAX.	STD. ERR.
		day=10, tank=4, tissue=2			
% Zn	28.32	11.84	17.57	42.31	5.92
% Mn	14.56	20.66	2.05	45.19	10.33
Zn Cnt.	3948.23	1257.95	2924.70	5767.30	628.96
Mn Cnt.	358.63	165.10	180.40	573.90	82.55
		day=10, tank=4, tissue=3			
% Zn	1.71	0.86	1.17	3.00	0.43
% Mn	1.59	1.98	0.44	4.54	0.99
Zn Cnt.	257.43	135.18	100.90	429.80	67.59
Mn Cnt.	53.85	25.22	30.30	81.60	12.61
		day=10, tank=4, tissue=4			
% Zn	59.73	18.60	34.60	74.15	9.30
% Mn	70.84	41.47	9.08	95.81	20.73
Zn Cnt.	11237.00	9674.01	3060.60	24337.30	4837.00
Mn Cnt.	7753.18	7373.62	60.60	17697.40	3686.81
		day=10, tank=4, tissue=5			
% Zn	3.73	3.04	1.75	8.26	1.52
% Mn	4.11	6.11	0.33	13.23	3.05
Zn Cnt.	504.28	282.13	222.40	762.60	141.06
Mn Cnt.	89.40	20.78	60.50	106.50	10.39

APPENDIX 2: ACCUMULATION OF Mn^{54} BY *DONAX*

The seawater chemistry of potentially toxic metals can affect their availability to marine organisms. Investigation of the relationship between metal chemistry and metal bioavailability has progressed slowly due to difficulties in controlling and measuring metal speciation in uptake media. Recent work with strong metal chelators such as NTA and EDTA has allowed a closer examination of how metal chemistry relates to biological accumulation and toxicity (Sunda and Guillard, 1976; Zamuda and Sunda, 1982; Oakden *et al.*,1984). However, the presence of a strong chelator at membrane transport sites and the possible alteration of microenvironments by strong chelators could create unnatural uptake behavior. This study presents another method for stabilizing metal chemistry in accumulation experiments. A cation exchange resin was used to study Mn^{54} accumulation by a small bivalve, *Donax variabilus*. The resin proved an effective method for buffering manganese chemistry in seawater and could provide a useful tool to look for subtle effects present in other metal buffered seawater systems.

Donax variabilus, periodically the dominant macroinvertebrate inhabiting sandy beach surf zones in

Florida, U. S. A. (Mikkelsen, 1981), was collected by hand and acclimated to 35 ppt seawater at 25 °C. This small intertidal bivalve has a relatively thin shell, can tolerate limited air exposure, and has been shown to accumulate manganese (Mauri and Orlando, 1982; Watling and Watling, 1983). These attributes allowed direct application of gamma ray spectrometry to live individuals at specific time intervals over the course of Mn^{54} exposure. The exposure system was designed as shown in figure 24. Both the cation exchange resin and the teflon exposure chamber were maintained at 25.0 ± 0.1 °C. The strong acid cation exchange resin, BioRad AG 50W-X8, was converted to the 'seawater' form by repeated equilibration with fresh 35 ppt seawater. Resin was then equilibrated with seawater having a specific activity for Mn^{54} of 1.07×10^{-2} mCi mg^{-1}.

The accumulation of Mn^{54} from our resin buffered system by Donax progressed at a steady rate over the course of four days. The ability to monitor clams without sacrificing the individual proved useful in identifying noncharacteristic behavior (ex. extended nonfiltering). Clams which exhibited such behavior were removed from analysis. This allowed better statistical definition of accumulation curves.

Monitoring of our uptake medium for Mn^{54} throughout the study showed constant total concentrations with fluctuations of only 1.6 percent (Figure 25). The

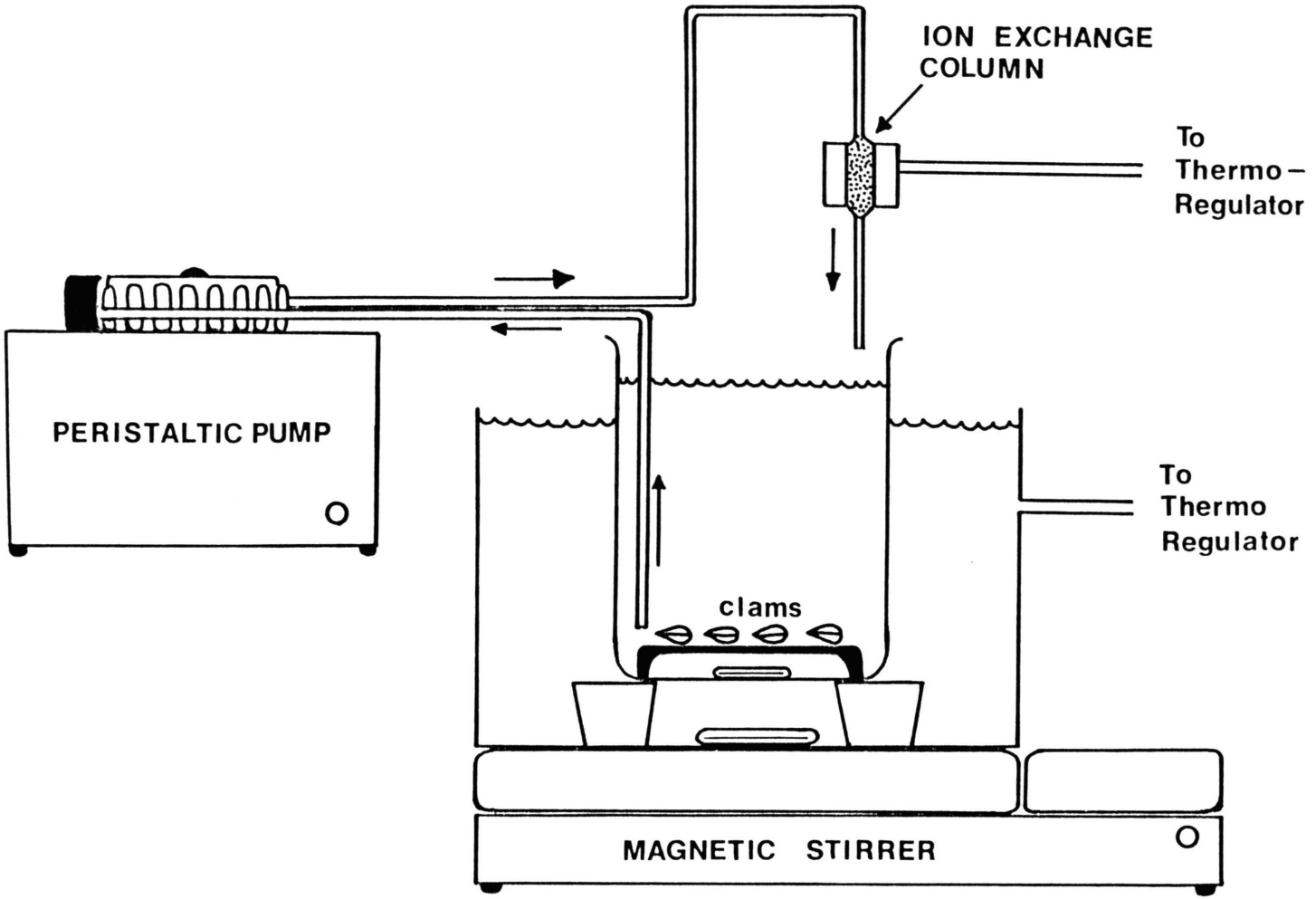
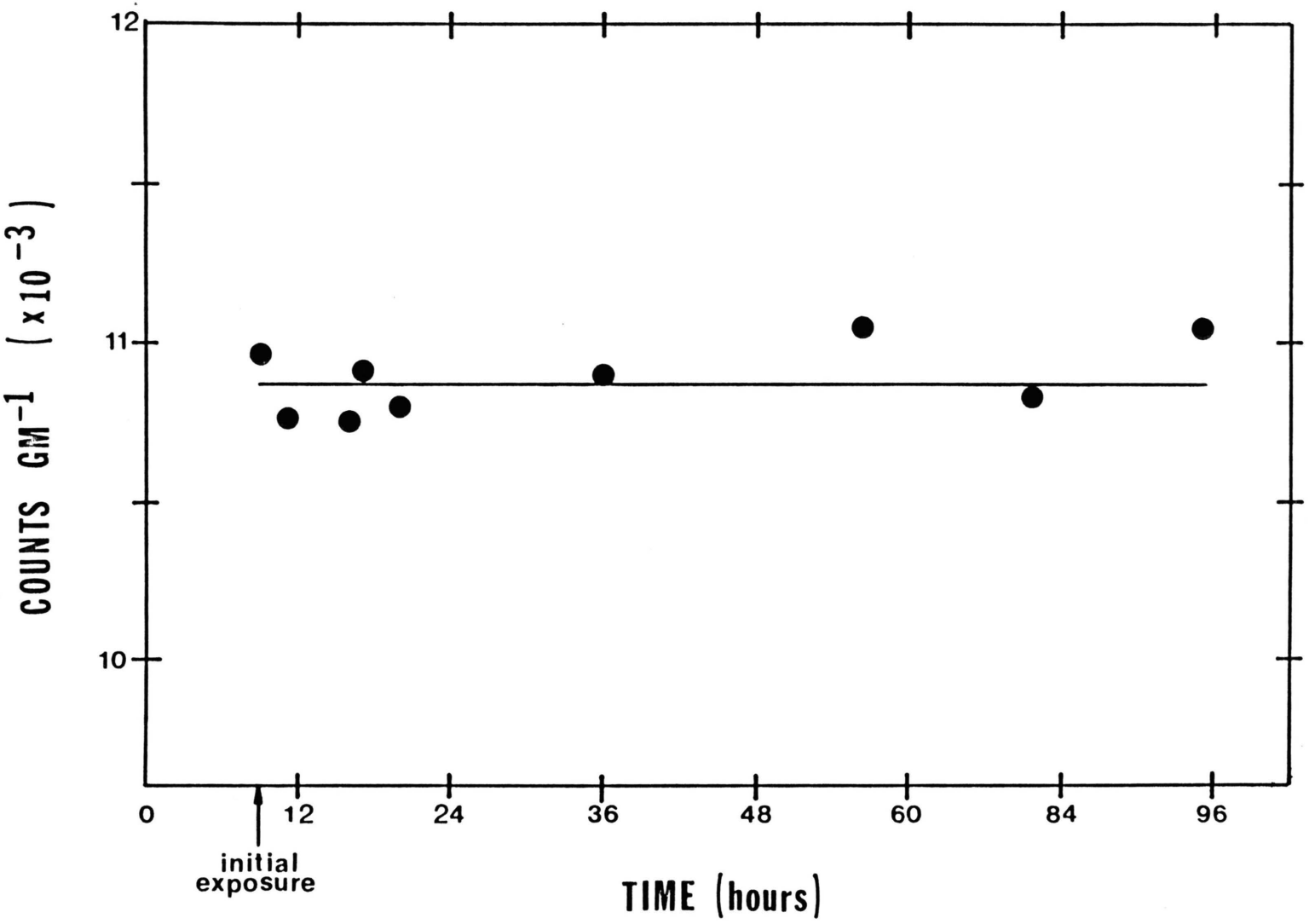


Figure 24. Exposure system. Ion exchange resin used is BioRad AG 50-W8. Salinity equals 35 ppt. Temperature equals 25.0 ± 0.1 °C.

Figure 25. Stability of total Mn^{54} in uptake medium. Each point indicates the average of five seperate determinations. Straight line indicates average value for total gamma counts due to Mn^{54} over the course of the experiment.


12
11
10
COUNTS GM^{-1} (x10^{-3})
0
12
24
36
48
60
84
96
initial exposure
TIME (hours)

equilibrium of the resin bound functional group (SO_3^-) primarily with the free manganese ion (Mn^{2+}) seems to maintain stable manganese speciation in this particular medium. Decrease of Mn^{2+} in solution due to uptake by clams, adsorption, or increased chemical complexation results in release of Mn^{2+} from the resin to maintain equilibrium. This restores a constant free manganese concentration. Increased complexation of manganese with organic or inorganic ligands can hence be observed as an increase in total gamma radiation due to Mn^{54} in solution. In this study, no alteration in total Mn^{54} was observed that would indicate a significant change in manganese complexation.

Control of Mn^{2+} concentrations with this technique requires prior knowledge of distribution coefficients (metal per gram of resin at equilibrium / metal per gram of solution at equilibrium) for the metals and media to which it is applied. Predetermination of appropriate distribution coefficients (D), resin and solution weights (gms_{res} & gms_{sol}), and total metal present in the system (M_{tot}) allows straightforward calculations of the total metal concentrations in the resin (M_{res}) and solution (M_{sol}) using the following equations:

$$M_{tot} = M_{res} + M_{sol};$$

$$M_{res} = (D \times gms_{sol} / gms_{res})\, M_{sol};$$

$$M_{sol} = M_{tot} / (1 + D \times gms_{sol} / gms_{res}).$$

Accepted metal-ion stability constants appropriate to the exposure media (Smith and Martell, 1961; Sillen and Martell, 1964) can then be used to determine free metal ion concentrations. Work is presently underway to more clearly define the specific metal chemistry in seawater equilibrated with this cation exchange resin. In our present study, exchange resins allowed controlled free metal ion concentrations in seawater while exposing organisms to a system which is representative of natural conditions. When carefully applied, this technique holds promise for further clarification of the effects of metal chemistry on biological systems.