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Heavy Metal Effects on Cellular Shape Changes, Cleavage, and Larval Development of the Marine Gastropod Mollusk, (*Ilyanassa obsoleta* Say)

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The spawning areas for many marine invertebrates are in intertidal zones which can be exposed to surface water run-off containing heavy metals. The fertilized eggs of the gastropod mollusk, Ilyanassa obsoleta Say, are deposited on the eel-grass of Atlantic intertidal mudflats. The cellular shape changes and cleavage patterns of Ilyanassa embryos greatly resemble those of bivalve mollusks, such as Mytilus edulis, that occur in the same intertidal areas. Determining the concentrations of heavy metals tolerated by the molluscan embryos inhabiting such clam and mussel beds therefore is of some economic significance. Moreover, such research may provide data on the mechanism of heavy metal effects on the cytoskeleton.

There is increasing evidence that components of the cytoskeleton, directly or indirectly, are targets for toxic agents. Microtubules in living cells are depolymerized within minutes as methylmercury binds to tubulin monomers, inhibiting further microtubule polymerization (Sager and Syversen 1986). Microfilaments also may be targets for heavy metals (Strzelecka-Golaszewska et al. 1978), although the effects may be indirect: chromium binds to and activates calmodulin (MacNeil et al. 1987); silver (Salama and Abramson 1984; Gould et al. 1987) and copper (Akberali and Trueman 1985) both cause release of Ca^{2+} within cells. Increased cytosolic $[Ca^{2+}]_i$ and activated calmodulin are presumed to be involved in microfilament-dependent cellular shape changes, such as cytokinesis.

Polar lobe formation is a cellular shape change that resembles cytokinesis. It is seen in the fertilized eggs of many marine mollusks. Recent data with inorganic and organic Ca^{2+} antagonists suggest that both polar lobe formation and cytokinesis utilize Ca^{2+} released from sequestered, intracellular sites (Conrad et al. 1987). Both of these cellular constrictions are associated with microfilaments and are preceded by activation steps requiring microtubules. The data presented below suggest that several heavy metals affect the microfilament-dependent steps.

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MATERIALS AND METHODS

Ilyanassa obsoleta were collected from mudflats on the coast of Mt. Desert Island, Maine, and maintained in aquaria of fresh running sea water from Frenchman Bay (12-18°C, pH 8.0, 923 mosmol/L), with daily feeding of minced mussels. Under these conditions, a single batch of snails lays capsules of fertilized eggs on aquarium walls steadily for at least 2 months. Fertilized eggs were removed from capsules and placed in 0.45 μm pore size Millipore-filtered natural sea water containing 50 μg/mL gentamycin sulfate (MFSW-G). Such cells were then dispensed into control and experimental solutions. This generally occurred immediately after formation of the second polar body, resorption of the second polar lobe, and resumption of spherical shape (Fig. 1). Fertilized eggs were dispensed (∿ 15 embryos/mL) into 35-mm tissue culture-type plastic petri dishes containing 3 mL of MFSW-G containing one of five serial 1:10 dilutions of a single heavy metal. All solutions were made in and dispensed with plastic disposable tubes and pipettor tips. Dishes were incubated at 20 ± 1°C and photographed periodically with an inverted microscope using phase-contrast optics. All experiments were performed at least four times and numbers of embryos recorded. In control MFSW-G, fewer than 10% of the embryos showed abnormal development (suggested value for bivalve mollusk larvae bioassay controls (ASTM 1984)).

Fertilized eggs of the mudsnail, Ilyanassa obsoleta, undergo a series of cellular shape changes, called polar lobe formation, that resemble cytokinesis and yet are distinct from it (Fig. 1). Polar lobe neck constrictions resemble genuine cleavage furrows: (1) in general morphology, (2) in being dependent upon microtubules for stimulation (of at least Phase II) as judged by sensitivity to colchicine and nocodazole, and (3) in being associated with a band of actin-like microfilaments whose stability can be disrupted by cytochalasin B (Conrad and Williams 1974a; Conrad and Vernon 1986). Polar lobe neck constrictions are distinct from cleavage furrows: (1) in forming without obvious association with a mitotic apparatus, (2) in forming in a fixed position in the cell, (3) in not cleaving the cytoplasmic neck completely through and in thereafter totally relaxing the constriction, and (4) in constricting at two sharply different rates. Phase I is slower and occurs without microtubule involvement, whereas Phase II is faster and is dependent upon microtubules. This pattern of reversible cell constriction occurs during formation of each of the two polar bodies and during each of at least the first two cleavages.

When fertilized Ilyanassa eggs are dispensed into control and experimental solutions immediately after resorption of the second polar lobe at 20°C, they remain spherical for 58 ± 8 min and then begin Phase I of third polar lobe formation. Phase I lasts 50 ± 5 min; Phase II and first cleavage then begin simultaneously and last for 15 ± 2 min; cytokinesis per se is completed during Phase

III, which lasts 4 ± 1 min; Phase IV requires ∿ 15 min (Fig. 1) (Conrad and Vernon 1986; Conrad et al. 1987).

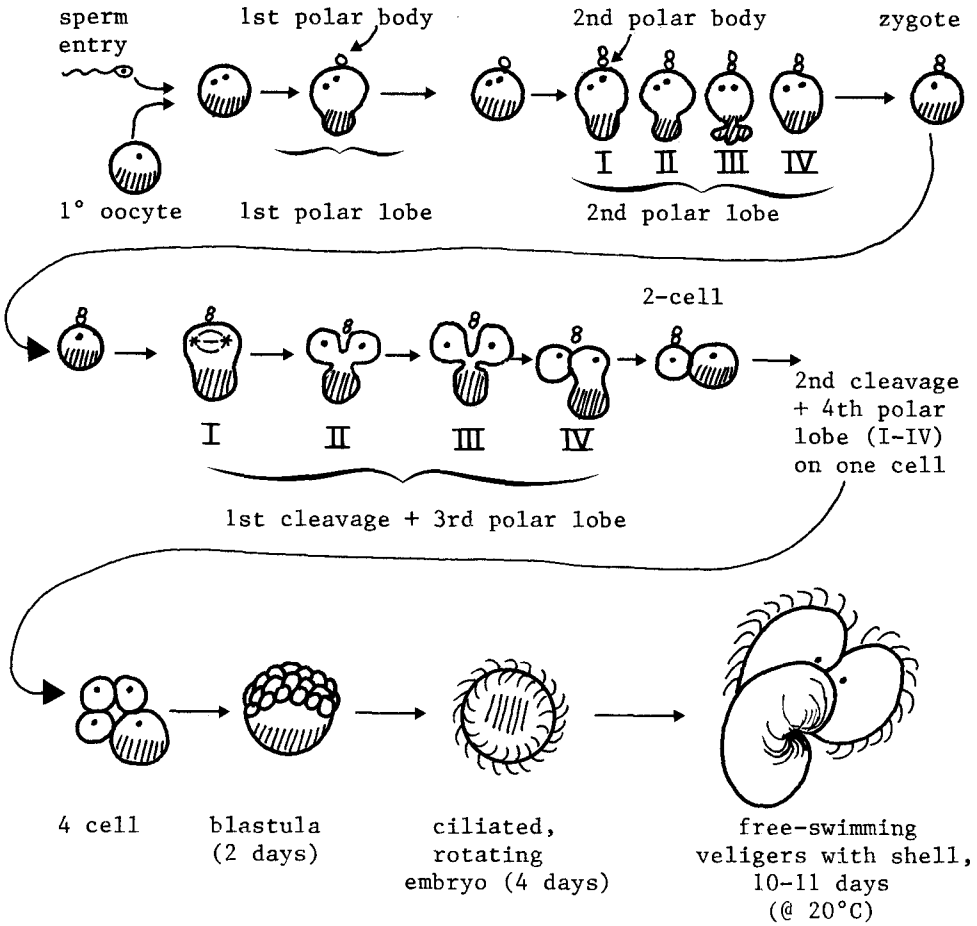
RESULTS AND DISCUSSION

The concentrations of seven metal salts that stopped polar lobe constrictions, stopped cleavage furrows, caused abnormal later cleavage, or caused abnormal veliger development are shown in Table 1. The concentrations of Ag^+ are given as dilutions of sea water saturated with $AgNO_3$ (precipitated as AgCl), with undiluted Ag^+-saturated sea water designated as "10^0" and a 1:10 dilution as "1 x 10^{-1}", etc. Concentrations also are given as molar concentrations calculated from the solubility product of AgCl (1.76×10^{-10} K_{sp}) and the total chloride concentration of sea water (0.502 M). The relative order of sensitivity was

$$Ag^+ > Hg^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Pb^{2+} > Cr^{3+}.$$

Of greatest interest from these experiments were the following observations: (1) 10^{-5} M Cu^{2+} caused early formation of a polar lobe constriction. Caffeine and divalent cation ionophores are the only other agents that cause early constrictions (Conrad et al. 1987). (2) "5-10 x 10^{-2}" dilutions of sea water saturated with AgCl, calculated to contain 1.8-3.5 x 10^{-11} M Ag^+, allowed third polar lobe neck constrictions and first cleavage furrows to begin on time, but caused the lobe constriction to become essentially a cleavage furrow by cleaving totally through the lobe neck rather than stopping for normal Phase III. (3) 1-2 x 10^{-3} M Cr^{3+} caused long delays in the starting times for third polar lobe neck constrictions and first cleavage furrows, and generally stopped the shape changes while a cleavage furrow and Phase II lobe constriction were in progress. Rather than becoming round, the cells remained constricted in Phase II by the polar lobe neck for hours, whereas the cleavage furrow relaxed. (4) The minimum concentrations needed to stop or significantly inhibit polar lobe formation and very early cleavage were far higher than those needed to cause abnormal later development. Concentrations sufficient to cause very abnormal differentiation of veliger larvae occurred over a 100-fold range for Pb^{2+} (10^{-5}-10^{-3}), but occurred at lowest concentrations in solutions of Ag^+ (7×10^{-12} M) and Hg^{2+} (10^{-7} M). Even considering the different concentrations at which each metal caused the inhibitions noted in Table 1, it appeared unlikely that all the metals were perturbing the same steps. Only Cu^{2+} caused early constrictions, only Ag^+ caused lobe necks to cleave through totally, and only Cr^{3+} stopped the lobe neck constrictions and "froze" them in a constricted state.

Data from earlier ultrastructural experiments on Ilyanassa indicate that normal and Ca^{2+}-induced polar lobe neck constrictions, as well as cleavage furrows, are associated with a band of F-actin microfilaments sensitive to cytochalasin B (Conrad and Williams 1974a,b; Schmidt et al. 1980). Phase I polar lobe neck constriction can occur in the absence of microtubules (Conrad and Williams 1974a). The effects of the metals observed in the



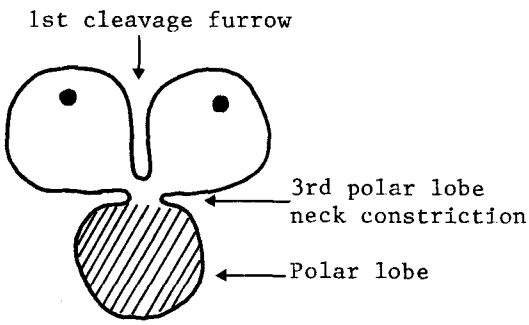


Figure 1. Polar lobe formation, cytokinesis, and early development of Ilyanassa obsoleta embryos. Roman numerals I-IV refer to the four phases of polar lobe neck constriction described by Conrad and Williams (1974a).

Table 1. Minimum molar concentrations of heavy metals that inhibit polar lobe formation, cytokinesis and early development of Ilyanassa obsoleta embryos

Metal salt	No apparent effect	Abnormal veliger development	Abnormal late cleavage	Stop early cleavage	Stop 1st cleavage and normal cell shape changes (polar lobe formation)
$HgCl_2$	10^{-9}-10^{-8}	10^{-7}	10^{-7}	10^{-6} @2-cell and 4-cell	10^{-5}-10^{-3}
$CuCl_2$	10^{-9}-10^{-7}	10^{-6}	10^{-6}	10^{-6} @4-cell	10^{-5}-10^{-3}
$ZnCl_2$	10^{-7}-10^{-6}	10^{-5}	10^{-4}-10^{-3}	None	10^{-2}
$CdCl_2$	10^{-6}	10^{-5}-10^{-4}	10^{-3}	10^{-3}	$5x10^{-3}$-10^{-2}
$Pb(NO_3)_2$	10^{-9}-10^{-6}	10^{-5}-10^{-3}	10^{-5}-10^{-3}	10^{-2}	None up to 10^{-2}
$CrCl_3$	10^{-6}-10^{-5}	10^{-4}	None	$1\text{-}2x10^{-3}$	$5x10^{-3}$-10^{-2}
AgCl: Relative dilutions of sat'd AgCl in SW*	"10^{-4}-10^{-2}"	"$2x10^{-2}$"	"$2x10^{-2}$"	"$5x10^{-2}$-$2x10^{-1}$"	"$5x10^{-1}$-10^{0}"
Calculated molar concs of Ag^+	$3.5x10^{-14}$-10^{-12}	$7.0x10^{-12}$	$7.0x10^{-12}$	$1.8\text{-}7.0x10^{-11}$	$1.8\text{-}3.5x10^{-10}$

* Dilutions of sea water saturated with $AgNO_3$ (precipitated as AgCl), with undiluted Ag^+-saturated sea water designated as "10^0" and a 1:10 dilution as "1 x 10^{-1}", etc.

present study therefore suggest several hypotheses to test. Cu^{2+} may induce the early appearance of a microfilament band and induce its precocious constriction. Ag^{+} may cause the microfilament band of late Phase II polar lobe necks to remain organized and constricting (rather than depolymerizing as usual), thereby cleaving the neck. Cr^{3+} may cause the microfilament band of late Phase II polar lobe necks to become stabilized in structure and stop constricting. Microtubule involvement is not suggested at this time in the effects of Cu^{2+}, Ag^{+}, and Cr^{3+} on these early cellular shape changes.

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