THE EFFECTS OF EDTA (ETHYLENEDINITROTETRAACETIC ACID) ON THE SURVIVAL AND DEVELOPMENT OF SHRIMP NAUPLII (Penaeus stylirostris STIMPSON) AND THE INTERACTIONS OF EDTA WITH THE TOXICITIES OF CADMIUM, CALCIUM, AND PHENOL

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ABSTRACT

To examine the hypothesis that EDTA increases hatching rates and survival of penaeid shrimp larvae by decreasing the toxicities of heavy metals through chelation, the toxicity of cadmium, a highly toxic metal, was compared to the toxicities of calcium and phenol in both the presence and absence of EDTA. In addition, the toxicity of EDTA at higher concentrations was examined.

The toxicities of EDTA, cadmium, calcium, and phenol were evaluated in terms of the percentage of nauplii surviving after 24 hours of exposure and the percentage of nauplii which metamorphosed to protozoa. The toxicities of cadmium, calcium, and phenol were also determined in the presence of EDTA.

EDTA concentrations of 1.34 mM were lethal to nauplii. At 0.67 mM, EDTA reduced the percentage of nauplii which metamorphosed to protozoa but below 0.3 mM neither survival nor metamorphosis were affected.

Cadmium, phenol, and calcium were lethal to nauplii at concentrations of 20 μM, 7 mM, and 400 mM, respectively. Metamorphosis was blocked by concentrations of 1 μM, 0.9 mM, and 200 mM, respectively. However, in the presence of 0.3 mM EDTA, the toxicities of cadmium and calcium were reduced. Cadmium concentrations of 20 μM did not affect either survival or metamorphosis in the presence of EDTA. In calcium concentrations of 50 and 100 mM, the percentage of nauplii that metamorphosed to protozoa was increased by the addition of EDTA. The interaction of EDTA and phenol toxicity was not significant.

INTRODUCTION

Ethylendiaminetetraacetic acid (EDTA) is used in the intensive culture of penaeid shrimp larvae to increase both hatching rates (Cook 1969) and survival of larvae (Cook and Murphy 1966; Cook 1969). Despite the widespread use of EDTA in larval culture, the mechanism of its bene-
ficial effect to penaeid shrimp is poorly understood.

EDTA is also extensively used in the culture media of unicellular algae (Spenser 1958; Johnston 1964). Culture assays indicate that both EDTA and EDTA-chelated trace metals enhance the growth of phytoplankton in sea water (Johnston 1964). Two mechanisms have been proposed for the enhancement of algal growth by chelating agents such as EDTA. Johnston (1964) suggested that EDTA increases the solubility and thus the availability of trace metals that are necessary for growth. However, an alternative hypothesis is that chelating agents reduce the availability of metals to phytoplankton by lowering the concentration of free metal ions (Sunda and Guillard 1976). In this hypothesis, enhanced algal growth in the presence of EDTA is explained by reduced inhibition by toxic metals which are present in sea water (Davey et al. 1973; Sunda and Guillard 1976; Ragan et al. 1980).

Enhancement of survival similar to that observed in unicellular algae has also been reported in the calanoid copepod Euchaeta japonica. Survival of prefeeding naupliar stages is increased in natural sea water by the addition of EDTA (Lewis and Ramnarine 1969; Lewis et al. 1971) and in copper enriched sea water by the addition of either EDTA or naturally occurring chelators (Lewis et al. 1972, 1973). Lewis et al. (1971) suggested that EDTA reduces inhibition of naupliar development by reducing the availability of metallic cations in the sea water.

In penaeid shrimp, acute toxicity tests indicate that the toxicities of copper and manganese to larvae are reduced in the presence of EDTA (Lawrence et al. 1981). Lawrence et al. (1981) suggested that the chelation by EDTA lowers the concentration of free copper and manganese ions. To test the hypothesis that the mode of action of EDTA is reduction of toxicity by chelation, this study compares the acute toxicity of cadmium to the acute toxicities of calcium and phenol in both the presence and absence of EDTA. In sea water, cadmium and calcium are both divalent metal cations but vary enormously in toxicity to marine animals. Phenol, which is highly toxic to marine animals, is a non-metal. If the beneficial effect of EDTA is due to the reduction of toxicity by chelation, EDTA would not be expected to have any effect on phenol toxicity.

Cadmium is a highly toxic metal that enters the marine environment via fresh water effluents and atmospheric fallout. In adult crustaceans, acute cadmium toxicity levels have been determined for the calanoid copepod Acartia tonsa (Gosnowski and Gentile 1978), the crabs Pangurus longicarpus (Eisler 1971) and Callinectes sapidus (Frank and Robertson 1979), and the shrimps Penaeus duorarum, Palaemonetes vulgaris (Nimmo et al. 1977), Palaemonetes pugio (Engel and Fowler 1979), and Crangon septemspinosa (Eisler 1971). In crustacean larvae, acute toxicities have been determined for the harpacticoid copepod Tigriopus japonicus (D'Agostino and Finney 1974), the lobster Homarus americanus (Johnson and Gentile 1979), and the shrimp Palaemonetes pugio (Middaugh and Floyd 1978). In general, larvae are more susceptible to heavy metal toxicity than adults. Crangon crangon larvae are 570 times more sensitive to mercury and 89 times more sensitive to copper than are adults (Connor 1972). Histopathological effects of cadmium associated with shrimp are lesions of the gills, cuticle, and midgut (Nimmo et al. 1977). Studies with other crustaceans indicate that respiration, osmoregulation, reproduction, and enzyme activity are also affected (D'Agostino and Finney 1974; Thurberg et al. 1973, 1977; Paffenhöfer and Knowles 1978).
In contrast to cadmium, calcium is a relatively non-toxic metal that is a normal constituent of sea water. The concentration of calcium in sea water is about 10 mM, which is equivalent to 410 ppm (Barnes 1954). In marine crustaceans, internal calcium concentrations vary with the stage of the molt cycle but are generally higher than the concentrations in sea water. Calcium concentrations in the hemolymph of penaeid shrimp range from 21 to 68 mM (McFarland and Lee 1963). In Homarus vulgaris, calcium concentrations in the hepatopancreas, which stores calcium in premolt, range from 65 mg/g dry weight in intermolt to 326 mg/g dry weight in newly molted animals (Glynn 1968). After taking into account the water content of the hepatopancreas at the two molt stages, converting the concentrations onto a wet weight basis gives concentrations of 24,000 and 146,000 ppm, respectively, which illustrates that high internal calcium concentrations are not detrimental in this organ.

Phenol is a highly toxic hydrocarbon which disrupts epithelial integrity. The most obviously affected tissue is the gill. In the gills of fish (Hodgins et al. 1977) phenol causes sloughing of epithelial cells, inflammation, discharge of mucus glands, and lesions. Other tissues that are damaged are blood cells, skin, liver, spleen and kidney.

Although EDTA is used in shrimp larval culture at low concentrations, the effect of higher EDTA concentrations has not been examined. If EDTA can be used as either a trace metal buffer (Spenser 1958) or as a protection against heavy metal toxicity, it would be desirable to determine the toxicity of higher concentrations of EDTA. This study evaluates the toxicity of EDTA to penaeid shrimp larvae by testing the effect of EDTA concentration on the survival of nauplii and the metamorphosis of nauplii to protozoa. To explain the beneficial effects of EDTA at low concentrations, this study compares the effects of cadmium, calcium, and phenol concentrations in the presence and absence of EDTA on survival and metamorphosis.

MATERIALS AND METHODS

Third stage nauplii of Penaeus stylirostris Stimpson were obtained from the Texas A&M Mariculture Facility in Corpus Christi, Texas, and acclimated to a salinity of 28 ppt artificial sea water (Instant Ocean) at room temperature (23°C). Artificial sea water was used to avoid the possible presence of natural chelating agents present in sea water. Although Instant Ocean contains an EDTA concentration of 0.05 mg/liter (0.0015 mM), the amount is not significant when compared to either the amount in the experimental EDTA solutions or the amount of natural chelators present in sea water. Polyphenols, which are natural chelating agents released by brown algae, have been reported in concentrations of up to 2.5 mg/liter in nearshore waters (Sieburth and Jensen 1968).

Experimental EDTA solutions were prepared with artificial sea water from the disodium salt of EDTA to give concentrations of 0.03, 0.17, 0.33, 0.67, and 1.34 mM. Cadmium, calcium, and phenol experimental solutions were prepared from cadmium chloride, calcium chloride, and liquid phenol (88% phenol in aqueous solution) in both artificial sea water containing 0.03 mM EDTA and artificial sea water containing no additional EDTA. In the intensive culture of shrimp larvae, 0.03 mM (10 mg/liter) is a commonly used concentration of EDTA (Mock and Murphy 1970). The concentrations of the test substances in the experimental solutions were 0.05, 0.2, 1, 5, and 20 \( \mu \)M for cadmium, 33, 58, 108, 208, and 408 mM for...
calcium, and 0.5, 0.9, 1.8, 3.6, and 7.1 mM for phenol. Artificial sea water at the same salinity, 28 ppt, was used for the control group.

The 208 and 408 mM calcium solutions were filtered to remove a precipitate which formed in the solution. Measurements of the calcium and magnesium concentrations by the Texas Agricultural Extension Service Soil Testing Laboratory indicated that the measured calcium concentrations were within 3% of calculated concentrations and that the precipitate is probably a magnesium salt. Magnesium concentrations in the 208 and 408 mM calcium solutions were 76% and 73%, respectively, of the magnesium concentrations in the control and other calcium solutions.

The concentration of cadmium in the 20 µM cadmium solutions, which was determined by the Texas A&M University Agricultural Analytical Services, was within 16% of the calculated concentration. Unless stated otherwise, all concentrations subsequently reported in this study are the calculated rather than measured concentrations.

The toxicity tests were initiated by pouring the nauplii onto a 175 µ mesh filter, inverting the filter, and rinsing the nauplii off the filter with 4 ml of the control or one of the experimental solutions into a 35 x 10 mm plastic Petri dish. The mean number of animals per Petri dish was 6.1 with a standard deviation of 2.0 (360 determinations). Ten replicates were used for each treatment. Toxicity was evaluated in terms of the percentage of nauplii that were alive after 24 hours of exposure (percent survival) and in terms of the percentage of nauplii that metamorphose to protozoa (percent metamorphosis).

The effect of the concentration of EDTA on percent survival and percent metamorphosis was analyzed by single-classification analysis of variance (ANOVA). For cadmium, calcium, and phenol toxicities, the effects of concentration and the presence or absence of EDTA on percent survival and percent metamorphosis were analyzed by two-way ANOVA. Interactions and main effects were tested for significance with F-tests. Where the interactions of the effects of EDTA and the concentration of the test substance were significant, differences in survival and metamorphosis due to the presence or absence of EDTA were evaluated separately at each concentration. In a similar manner, comparisons of differences due to the concentration of the test substance were qualified by specifying the presence or absence of EDTA. A sum of squares simultaneous test procedure (SS-STP) was used for a posteriori comparisons of differences due to the concentration of the test substance. Prior to statistical analysis, the arcsine transformation was applied to the percent survival and percent metamorphosis to make the data normally distributed.

RESULTS

The percent survival and percent metamorphosis of nauplii exposed to EDTA are shown in Figure 1. At concentrations of 1.34 mM, EDTA is toxic to nauplii with 100% mortality occurring within 12 hours of exposure. At concentrations of 0.67 mM and below, there is no significant (0.25<P<0.50) mortality after 24 hours. However, at 0.67 mM, the percent metamorphosis is significantly (P<0.001) reduced. At concentrations of 0.33 mM and below, all nauplii metamorphose to protozoa.
Survival and metamorphosis of nauplii exposed to cadmium in the presence and absence of EDTA are shown in Figure 2. Two-way ANOVA of the data in Figure 2 indicates that for both survival and metamorphosis, there is a significant (P<0.001) interaction between the presence of EDTA and the concentration of cadmium. In the absence of EDTA, cadmium significantly (P<0.01) reduces survival at a concentration of 5 μM and causes 100% mortality at 20 μM. Metamorphosis is completely blocked at concentrations of 1 μM and above. In the presence of 0.03 mM EDTA, neither survival nor metamorphosis are affected by cadmium concentrations up to 20 μM. Thus, differences due to the presence of EDTA are significant (P<0.01) for percent survival at cadmium concentrations of 5 and 20 μM and for percent metamorphosis at concentrations of 1, 5, and 20 μM.

In contrast to cadmium, the calcium solutions are toxic only at very high concentrations (Fig. 3). Two-way ANOVA indicates that for percent survival there is no significant (0.50<P<0.75) interaction between the presence of EDTA and the concentration of calcium. In both the presence and absence of EDTA, there is no mortality after 24 hours at concentrations of 108 mM and below, and complete mortality at the 208 and 408 mM concentrations. There is no significant (0.05<P<0.10) effect of EDTA on survival. For percent metamorphosis, the interaction of EDTA and the concentration of calcium is significant (P<0.01). In the presence of EDTA, metamorphosis is not affected by the 33 and 58 mM calcium solutions. However, in the 108 mM calcium solution, metamorphosis is significantly (P<0.001) reduced. Similarly, in the absence of EDTA, the
percent metamorphosis is significantly ($P<0.001$) lower in the 108 mM calcium solution than in the 33 and 58 mM solutions, in which the percentages do not differ. However, metamorphosis is significantly ($P<0.05$) lower in the 58 and 108 mM calcium solutions without EDTA than in those containing EDTA.

Survival and metamorphosis of nauplii exposed to cadmium in the presence and absence of EDTA are shown in Figure 2. Two-way ANOVA indicates that for percent survival, the interaction between the presence of EDTA and the concentration of phenol is not significant ($0.75<P<1.00$). In both the presence and absence of EDTA, there is complete mortality at the phenol concentration of 7.1 mM. At concentrations of 3.6 mM and below, survival is not significantly ($0.25<P<0.50$) affected by the concentration of phenol. Differences in survival due to the presence or absence of EDTA are not significant ($0.05<P<0.10$). Metamorphosis is completely blocked by phenol concentrations of 0.9 to 7.1 mM in both the presence and absence of EDTA. At 0.5 mM, phenol severely inhibits metamorphosis. Where metamorphosis does occur, it is delayed by 24 hours compared to animals exposed to the control and other experimental solutions. Differences in metamorphosis due to the presence or absence of EDTA are not significant ($0.05<P<0.10$).
Figure 3. Survival and metamorphosis of nauplii exposed to calcium in the presence and absence of EDTA.

Figure 4. Survival and metamorphosis of nauplii exposed to phenol in the presence and absence of EDTA.
DISCUSSION

The results of this study demonstrate that cadmium, EDTA, and phenol are toxic to larval shrimp at low concentrations with cadmium being much more toxic than EDTA and phenol. On the basis of molarity, EDTA is more toxic to nauplii than phenol. However, metamorphosis to protozoa is more sensitive to phenol which either reduces or blocks metamorphosis at all of the phenol concentrations tested.

With calcium, survival is not affected by concentration $5.4 \times 10^3$ times greater than cadmium concentrations which cause 100% mortality. Although mortality and blocked metamorphosis in the more concentrated calcium solutions may reflect toxic effects of calcium, it is more likely that osmotic stress is responsible. Exposure to the 208 mM calcium solution represents an acute osmotic shock of approximately 600 mOsm/kg. It is remarkable that nauplii in the 108 mM calcium solution tolerate an osmotic shock of 300 mOsm/kg with no mortality and that 27% metamorphose to protozoa. Another factor that may contribute to the toxicities of the 208 and 408 mM calcium solutions is the reduced magnesium concentrations.

Cadmium reduces the 24-hour survival of P. stylirostris nauplii at concentrations of 5 μM (1.14 mg/liter) and blocks metamorphosis at 1 μM (0.23 mg/liter). These concentrations are lower than the 96-hour LC50 (mean lethal concentration) of 3.5 mg/liter and the 30-day LC50 of 0.72 mg/liter reported for juvenile and subadult Penaeus duorarum (Bahner and Nimmo 1975, cited by Nimmo et al. 1977; Nimmo and Bahner in press, cited by Nimmo et al. 1977). More comparable to P. stylirostris nauplii are the 96-hour LC50 of 0.76 mg/liter reported for adult Palaemonetes vulgaris (Nimmo et al. 1977) and the 96-hour LC50 of 0.32 mg/liter reported for Crangon septemspinosa (Eisler 1971).

Less information is available concerning the sensitivity of larval crustaceans to cadmium. Johnson and Gentile (1979) reported that in larvae of Homarus americanus, the 90-hour LC50 is 0.078 mg/liter. In newly hatched larvae of Palaemonetes pugio exposed to the cadmium concentration of 0.3 mg/liter, survival is decreased after 6 days of exposure at salinities of 10 and 15 ppt but not after exposure at 30 ppt (Middaugh and Floyd 1978).

In oceanic waters, cadmium is present at very low concentrations. Riley and Taylor (1972) reported ranges of 0.07 to 0.71 μg/liter with a mean concentration of 0.11 μg/liter in the tropical northeast Atlantic Ocean. Preston (1973), in reviewing the distribution and concentration of cadmium in British waters, reported oceanic concentrations of 0.01 μg/liter and offshore concentrations of 0.01 to 0.1 μg/liter. Although the concentrations in offshore waters are too low to affect penaeid shrimp, cadmium concentrations measured in some estuaries approach the concentrations toxic to shrimp. In Corpus Christi Bay, Texas, water concentrations of up to 78 μg/liter and sediment concentrations up to 720 μg/liter have been reported (Holmes et al. 1974, cited by Nimmo et al. 1977). If estuarine water is used for the production and intensive culture of penaeid larvae, the toxic effects of cadmium as well as other heavy metals is a potential problem.

Another problem in analyzing the potential of heavy metal toxicity is that acute toxicity determinations underestimate the effect of sustained exposure during the entire life cycle of the animals. In Homarus
*Americanus* larvae, the 24-hour $LC_{50}$ for cadmium is 13 times the 96-hour $LC_{50}$ (Johnson and Gentile 1979). Although in *Tigripus japonicus* nauplii are not acutely affected by 0.438 mg/liter cadmium and develop to adults, the development of ovigerous females is inhibited by concentrations down to 0.0438 mg/liter (D'Agostino and Finney 1974). Thus deleterious effects due to cadmium toxicity may also be present in penaeid shrimp at much lower concentrations than the levels which reduce survival and block metamorphosis.

The results of this study indicate that EDTA ameliorates survival and metamorphosis in the presence of cadmium. Similar results have been reported (Lawrence et al. 1981) for copper and manganese toxicities to *P. stylirostris* larvae. The reductions of the toxicities of cadmium, copper, and manganese by EDTA suggest that EDTA increases survival and metamorphosis of larvae by chelating toxic ions and thus reducing the concentrations of free ions. The increased toxicities of cadmium to *Callinectes sapidus* (Frank and Robertson 1979) and to *Palaemonetes pugio* (Middaugh and Floyd 1978; Engel and Fowler 1979) at decreased salinities probably reflect higher concentrations of free cadmium ions (activity) at decreased salinities. Rainbow et al. (1980) related both the accumulation and the toxicity of cadmium to the concentration of free cadmium ions. They concluded that the reduced accumulation of cadmium by the barnacle *Semibalanus balanoides* resulted from decreased levels of free cadmium ions due to the formation of EDTA-cadmium complexes. However, in contrast to Rainbow et al., George and Coombs (1977) reported that in the mollusc, *Mytilus edulis*, EDTA and other chelating agents increase rather than decrease the accumulation of cadmium.

Additional support of the hypothesis that EDTA reduces toxicity by chelation is provided by the results of this study for animals exposed to phenol. The absence of any significant differences in survival and metamorphosis due to either the presence of EDTA or an interaction between the presence of EDTA and phenol concentration indicates that EDTA does not affect the toxicity of phenol.

If the effects of the concentration of the calcium solutions are due to osmotic stress rather than calcium toxicity, the increase in metamorphosis in the 50 and 108 mM calcium solutions due to the presence of EDTA suggests that EDTA protects nauplii from osmotic stress. Further investigation on the effects of EDTA on osmotic stress are warranted.

The results of this study demonstrate that EDTA provides limited protection from cadmium toxicity to *P. stylirostris* nauplii. Thus, the use of EDTA in the intensive culture of penaeid larvae would be beneficial where the potential for trace metal toxicity is present.

Even with the use of EDTA, the presence of trace metals may remain a problem because of the sensitivity of penaeid larvae to trace metals. The use of higher levels of EDTA in larval culture is possible but is limited by the toxicity of EDTA. Although this study indicates that 24-hour survival and metamorphosis of nauplii are not affected by EDTA concentrations up to 0.33 mM, detrimental effects of chronic exposure to concentrations above 0.03 mM may occur.
SUMMARY

This study demonstrates that cadmium, EDTA, and phenol are toxic to larval shrimp at low concentrations. On the basis of molarity, cadmium is much more toxic than EDTA and phenol. Calcium is toxic at high concentrations but the observed mortality may be due to osmotic stress rather than calcium toxicity. The beneficial effect of EDTA in reducing the toxicity of cadmium but not phenol, supports the hypothesis that the reduction is due to chelation of divalent ions by EDTA. Although EDTA is not acutely toxic at concentrations up to 0.33 mM, detrimental effects of chronic exposure may occur.

ACKNOWLEDGMENTS

This work is a result of a research program sponsored in part by Texas A&M University Sea Grant College Program, supported by the National Oceanic and Atmospheric Administration, Office of Sea Grant, U.S. Department of Commerce under Grant #4-7-158-44105 and a grant from the Caesar Kleberg Foundation for Conservation of Wildlife to Texas A&M University, Addison L. Lawrence, principal investigator. The authors wish to express their appreciation to Rhonda Beesley and Karen Hall for their technical assistance and to Tommy Crumbley and Ginny Mitchell for their assistance in the preparation of this manuscript.

LITERATURE CITED


