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The Action of Heavy Metals on the Gametes of the Marine Mussel, *Mytilus edulis* (L.)—III. The Effect of Applied Copper and Zinc on Sperm Motility in Relation to Ultrastructural Damage and Intracellular Metal Localisation

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ABSTRACT

Treatment of Mytilus edulis *sperm with external concentrations of copper or zinc (0·1–3·3 mM) causes a decrease in motility in which zinc is more inhibitory than copper. Zinc also appeared to cause more extensive mitochondrial damage, as revealed by transmission electron microscopy, than did treatment with copper. The relationship between sperm motility and respiration in the presence of the various heavy metal concentrations used indicates that the depression of sperm motility can be explained largely on the basis of respiratory inhibition. However, zinc produces a less pronounced effect on sperm motility than on respiration.*

X-ray microanalysis of thick sections of fixed treated sperm showed that copper accumulation occurs in the acrosomes, mitochondria and nuclei, whereas zinc is found in the acrosomes and in mitochondrial granules in association with calcium and phosphorus. No evidence was obtained for zinc accumulation in the nuclei. Treatment with either copper or zinc resulted in considerable reductions of bound calcium and phosphorus in both the acrosomes and mitochondria. It is suggested that

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the heavy metal ions cause an increase in the permeability of the organelle membranes to calcium and phosphorus.

It is concluded that the less marked effect of zinc on sperm motility as compared to respiration may be due to an increase in the cytosolic free calcium concentration which, in turn, may stimulate the flagellar contractile apparatus.

INTRODUCTION

Marine molluscs are efficient accumulators of metal ions and emphasis has been placed on their use as indicators of environmental pollution (Phillips, 1977; Bryan, 1979). Exposure to heavy metal stress, however, produces a number of physiological effects (Akberali & Trueman, 1985). Siphon withdrawal followed by valve closure appear to be the initial responses of the estuarine bivalve molluscs *Mytilus edulis* and *Scrobicularia plana* when subjected to either copper or zinc (Davenport, 1977; Akberali & Black, 1980; Akberali *et al.*, 1981). Low concentrations of copper cause spontaneous contractions of the isolated siphon whereas zinc has no such effect (Akberali, 1981; Akberali *et al.*, 1981). The copper-induced contraction of the isolated siphon depends on the presence of external calcium (Akberali *et al.*, 1982) and subsequent work with isolated *Mytilus* mitochondria has produced a cellular model for the action of copper (Akberali & Earnshaw, 1982*b*; 1985). The addition of copper or zinc to mitochondria which have been pre-loaded with calcium produces a rapid calcium efflux but this effect is less marked with zinc. The model thus involves a copper-induced displacement of calcium from intracellular reservoirs which results in a stimulation of the nerve-muscle system leading to siphon contraction (Akberali & Earnshaw, 1982*b*, 1985).

More long-term effects of exposure to heavy metals inevitably include a reduction in reproductive potential. During gametogenesis in the American oyster, *Crassostrea virginica*, heavy metals are transferred from the gonadal tissue to the eggs (Greig *et al.*, 1975). In *Mytilus edulis* both copper and zinc suppress gametogenesis, with copper showing greater toxicity than zinc (Maung-Myint & Tyler, 1982). In addition, several studies have demonstrated that embryonic development and larval growth in marine bivalve molluscs are inhibited in the presence of heavy metals (e.g. Brereton *et al.*, 1973; Calabrese *et al.*, 1977).

The previous two papers in this series (Akberali *et al.*, 1984; 1985) have provided information on the mechanism of cellular action of copper and zinc on the gametes of *Mytilus edulis*. Uptake of metal ions is approximately three-fold greater into sperm than into eggs, and in both cases zinc uptake exceeds that of copper (Akberali *et al.*, 1985). Copper produces a respiratory stimulation in the eggs which appears to represent an uncoupling of oxidative phosphorylation (Akberali *et al.*, 1984, 1985). However, zinc produces a mild inhibition only of egg respiration. Both copper and zinc depress respiration in the sperm, but here zinc is the more effective inhibitor (Akberali *et al.*, 1985).

The application of heavy metals at concentrations $>10^{-4}$ M to sea urchin sperm decreases motility via an unknown mechanism with copper being more effective than zinc (Young & Nelson, 1974). Comparable data for marine bivalve molluscs are not available and it was clearly of interest to determine whether the inhibition of respiration in *M. edulis* sperm caused by copper and zinc (Akberali *et al.*, 1985) results in a reduction in sperm motility. This paper accordingly presents the results of a study on the effects of copper and zinc on the sperm of *M. edulis* in which motility effects have been assessed in relation to ultrastructural damage and intracellular metal ion localisation as determined by X-ray microanalysis.

MATERIALS AND METHODS

Gamete production

Adult specimens of *Mytilus edulis* were collected from Bangor, North Wales, Great Britain, during the spawning season of Spring, 1983. Electrical stimulation was used to induce spawning (Iwata, 1950; Sugiura, 1962) as described previously (Akberali *et al.*, 1984). The sperm suspension was filtered, concentrated by centrifugation at 10 000 $g \times$ 10 min at 2°C and the pellets made up in 1·2 ml of filtered, buffered seawater (pH 7·91) and stored on ice. In all experiments, the buffering capacity of filtered seawater was increased by the addition of 10 mM Tris-Tes, pH 7·91, in order to minimise the pH shift caused by heavy metal additions which is particularly pronounced in the case of copper (Akberali *et al.*, 1985).

Determination of sperm motility

Sperm motility was measured using an optical method in which sperm suspensions are pumped through a flow-cell positioned in a spectrophotometer which results in orientation of the sperm in the direction of the light path (Timourian & Watchmaker, 1970; Atherton, 1975; 1979). Upon cessation of flow the sperm assume a random orientation, at a rate proportional to their motility, which is followed by measuring the decrease in absorbance. In all experiments, the concentrated sperm suspension was added to 10 ml of ice-cold buffered seawater. For each determination, a 1·3 ml aliquot was diluted 20–40-fold in buffered seawater at 10°C to produce an initial absorbance of 0·7–0·9. The flow-cell (L.K.B. Type 10, 10 mm path length) had a volume of 0·075 ml and was positioned in the light path of an L.K.B. Ultraspec Model 4050 spectrophotometer fitted with a thermostated cell holder. The flow rate of the sperm suspension was controlled at 7 ml min^{-1} using an infusion pump (Harvard Instruments Model 935 syringe pump) equipped with a 10 ml glass syringe. The apparatus was positioned in a constant temperature room at 10°C and for each determination absorbance at 450 nm was monitored until a constant reading was achieved (*ca.* 30 s). The flow was then stopped and absorbance readings taken at 15-s intervals for 2·5 min. The percentage change in absorbance is expressed as the sperm motility index (SMI):

$$\text{SMI} = \frac{A_{t=0} - A_{t=2\cdot5}}{A_{t=0}} \times 100$$

The effectiveness of the spectrophotometric method for assessing motility of *M. edulis* sperm was tested by combining aliquots of active sperm and sperm inactivated by heating suspensions at 55°C for 15 min. Figure 1 shows that dilution of active sperm with the inactivated suspension results in a decrease of the SMI producing a comparable plot to previously published data for vertebrate sperm (Atherton, 1979).

Pre-treatment of the sperm suspension with heavy metals followed the regime described previously for our studies on metal uptake and respiration (Akberali *et al.*, 1985). Stock heavy metal solutions were 100 mM $Cu(NO_3)_2$—KOH, pH 7·0 and 100 mM $Zn(NO_3)_2$—KOH, pH 6·0. Following the addition of 1·2 ml of concentrated sperm suspension to 10·0 ml of ice-cold buffered seawater, 1·3 ml aliquots were withdrawn

at timed intervals and pre-treated with a range of heavy metal concentrations (0·1–3·3 mM) for 20 min at 0°C. At the end of the pre-treatment, each sample was diluted in buffered seawater at 10°C containing the same heavy metal concentrations and motility at 10°C determined as described above. Each sperm preparation was derived from several

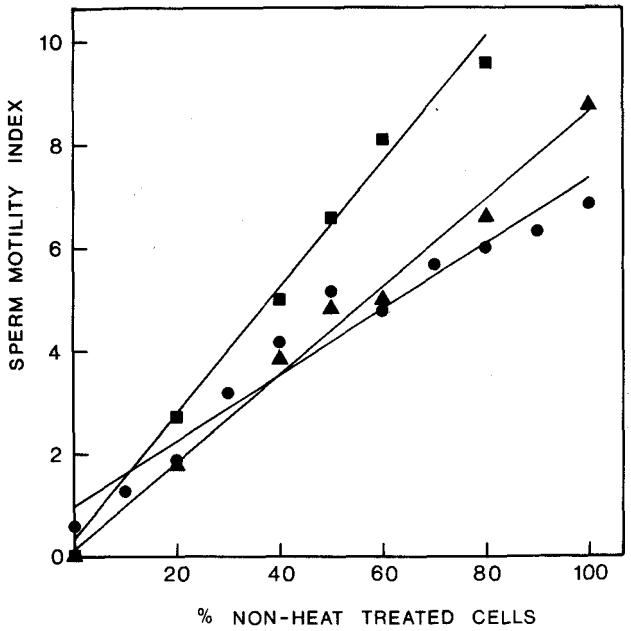

Fig. 1. The sperm motility index (SMI) as a function of the relative concentration of motile sperm of *M. edulis* (●). Previously published data (Atherton, 1979) for human (▲, $r^2 = 0{\cdot}99$) and rabbit (■, $r^2 = 0{\cdot}98$) sperm have been included for comparative purposes. The data for *M. edulis* represent the mean of three separate determinations. The lines were fitted using regression analysis ($r^2 = 0{\cdot}95$).

individual animals and was used to carry out a single concentration series as described above.

The heavy metal concentrations used in this study are clearly much higher than recorded environmental levels (Phillips, 1977). However, the gamete concentrations necessary to carry out biochemical measurements (Akberali *et al.*, 1984, 1985) are clearly many orders of magnitude higher than would be found under natural conditions. Moreover, the extreme

lability of gamete respiration reduced the pre-treatment period to only 20 min even at 0°C, thus necessitating the use of relatively high heavy metal concentrations (see Discussion in Akberali *et al.*, 1985).

Transmission electron microscopy

Sperm were treated $\pm 3{\cdot}3$ mM $Cu(NO_3)_2$ or $Zn(NO_3)_2$ for 20 min at 0°C and subsequently fixed in a final concentration of 2·25% glutaraldehyde solution in buffered seawater for 1 h at 0°C. The suspension was centrifuged for 3 min using a Beckman Microfuge B centrifuge and the resultant pellet washed with ice-cold, buffered seawater. The pellet was re-suspended and post-fixed for 1 h in a 1·0% solution of osmium tetroxide in seawater at room temperature. The pellet was dehydrated through an ethanol series and embedded in Spurr resin (Spurr, 1969). Thin sections were stained for 20 min in 2% uranyl acetate in 70% alcohol followed by 5 min in 0·4% lead citrate and examined in a Phillips 400T electron microscope. Images were recorded on Ilford cut film. Estimates of mitochondrial damage in controls and treated samples were derived by scoring 1000 mitochondria, 200 from each of five sections taken from separate blocks. There was minimal variation between the blocks.

X-Ray microanalysis

Sperm were fixed in glutaraldehyde as for transmission electron microscopy and embedded directly in resin without osmium tetroxide post-fixation. Thick (100 nm) sections were mounted on carbon-coated 240-mesh nylon grids. Sections were analysed unstained using an AEI CORA analytical electron microscope equipped with a Kevex-Link System energy dispersive detector. All observations were carried out at an accelerating voltage of 60 kV; beam current, $1{\cdot}9 \times 10^{-6}$ A; spot size 500 nm, and a count time of 240 s. Analyses were carried out of the acrosome, nucleus and mitochondrion of five separate untreated, copper-treated and zinc-treated sperm. Variation in specific peaks between different samples varied from 5·4% of the mean (copper, nuclei) to 27·4% (copper, mitochondria). Some of this variability would be attributable to variation in section thickness. The spectra illustrated (Fig. 8) represent typical examples of each treatment.

RESULTS

Sperm ultrastructure

The untreated sperm (Figs 2 and 3) shows an acrosome, a perforatorium penetrating a dense homogeneous nucleus, a mitochondrial ring surrounding a centriole and a tail with an axoneme of conventional structure. This structure conforms with the descriptions of previous authors (Niijima & Dan, 1965; Longo & Dornfield, 1967). In untreated sperm relatively few (10·5%) of the mitochondria show evidence of structural damage involving the outer membrane, swollen cristae or electron-transparent areas in the matrix (Fig. 3). However, whilst the incidence of mitochondrial damage in copper-treated sperm (Fig. 4) was little different from the control (14·1%), treatment with zinc (Fig. 5)

Fig. 2. Longitudinal section through a mature sperm of *M. edulis* showing acrosome (ac), perforatorium (p), nucleus (n), mitochondrial ring (m), centriole (c) and flagellum (f). X 18 000.

Fig. 3. Transverse section through mitochondrial ring in an untreated control cell. X 33 330.

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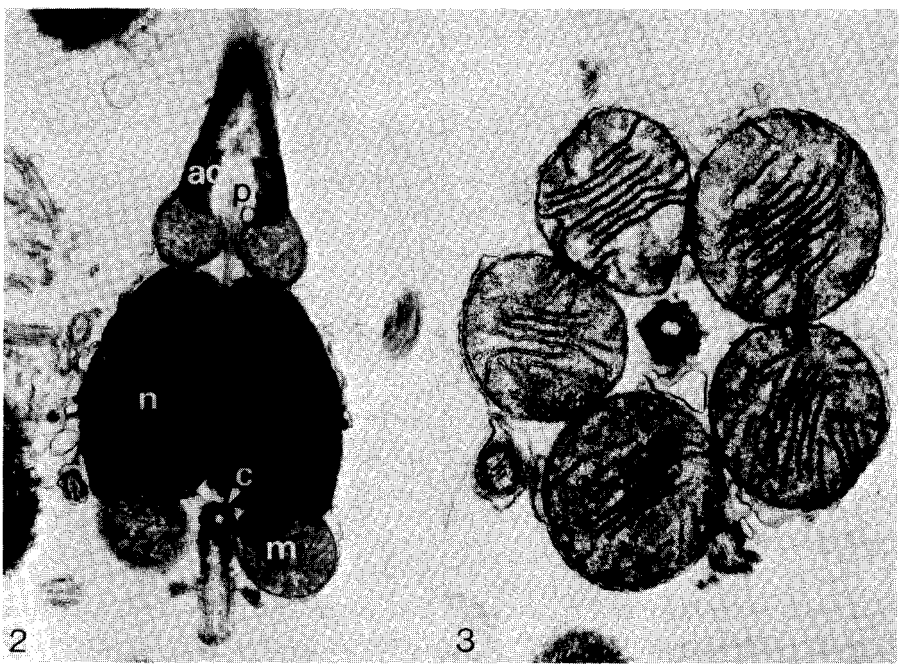

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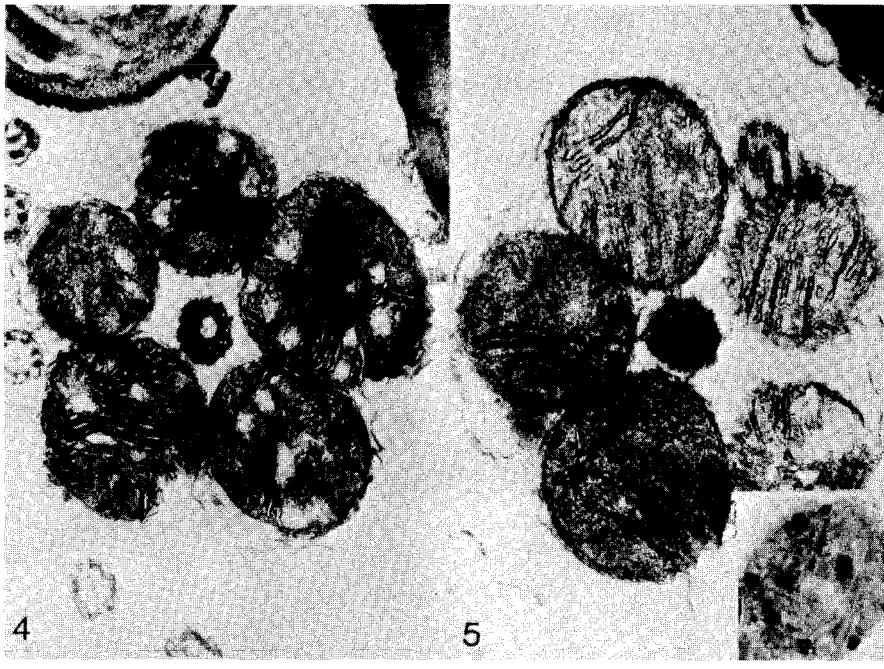

Fig. 4. Transverse section through mitochondrial ring following treatment with copper. X 37 330.

Fig. 5. Transverse section through mitochondrial ring following treatment with zinc. X 38 000. Inset: mitochondrial granules in material which had not been osmium-treated. X 31 330.

produced extensive structural damage (40·1%). In some instances granules were present in zinc-treated sperm, most obviously in material that had not been post-fixed (Fig. 5 inset).

Sperm motility

The data presented in Fig. 6 show that there is a slow decline in the SMI with storage time on ice. The experiments involving the effects of copper and zinc on motility were carried out so that the final motility measurement commenced 160 min after gamete release. Figure 7 illustrates plots of sperm motility as a function of metal ion concentration showing that zinc is the more effective inhibitor, producing a maximum inhibition of *ca.* 45% compared with *ca.* 20% for copper.

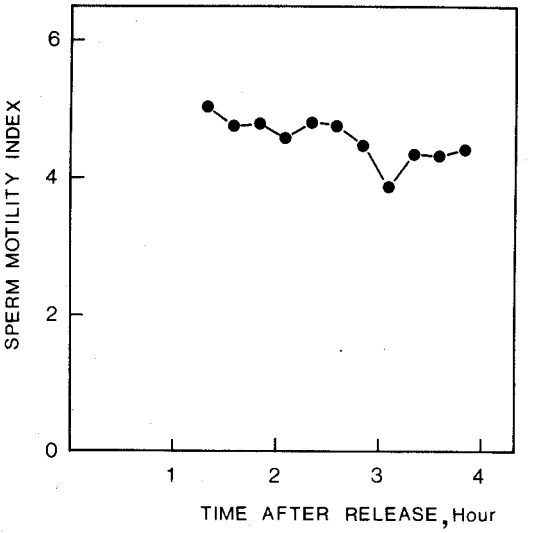

Fig. 6. The sperm motility index (SMI) determined at 10°C as a function of storage time on ice.

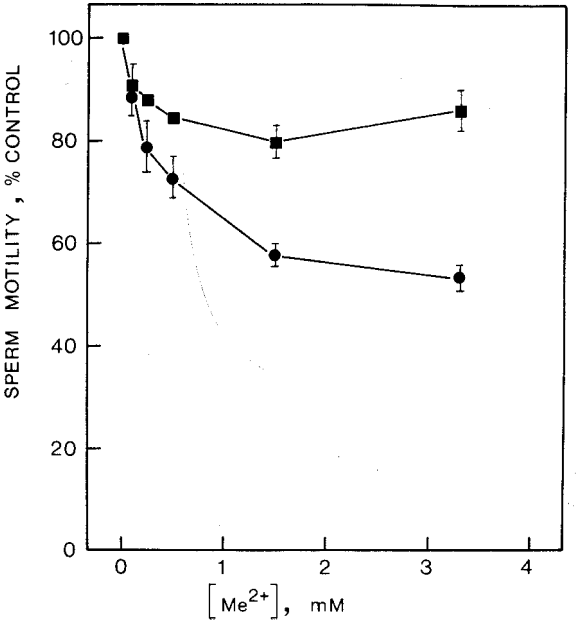

Fig. 7. The effect of copper (■) and zinc (●) concentrations on sperm motility. The data represent the mean of four determinations for each metal ion and are expressed ±SE. The mean control SMI (100%) was 6·17 ± 0·53.

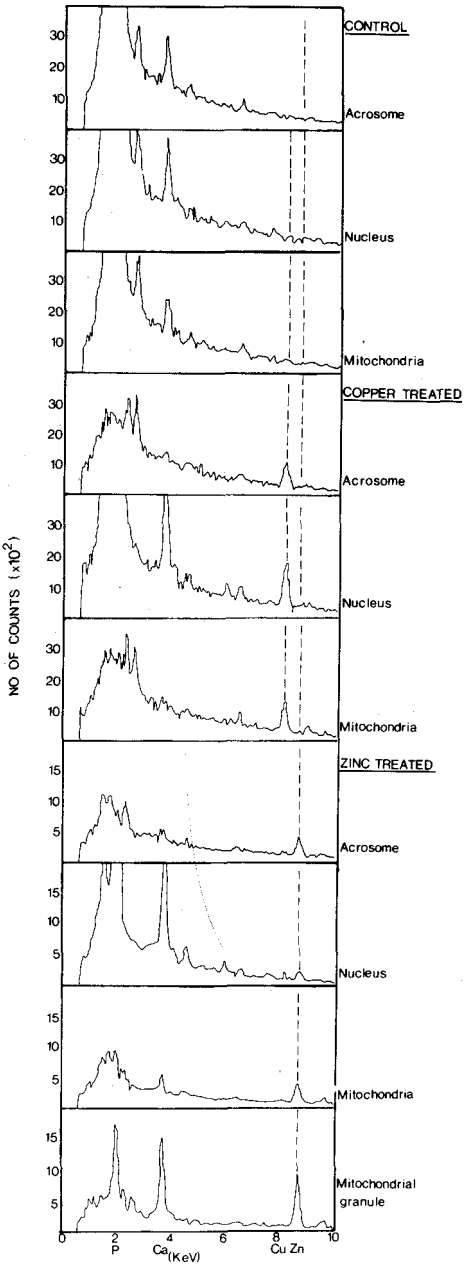

Fig. 8. X-ray emission spectra from an untreated control and cells treated with copper and zinc (see text).

X-Ray microanalysis

The spectra of untreated sperm show a high level of calcium in the nuclei with slightly lower levels in both acrosomes and mitochondria (Fig. 8). There was no detectable copper or zinc in any of the regions analysed in untreated sperm.

In copper-treated sperm, the acrosomes showed a detectable level of bound copper but the calcium and phosphorus evident in the control cells were no longer present (Fig. 8). The nuclei, on the other hand, showed high levels of accumulated copper but also high levels of calcium with respect to the control. The mitochondria produced spectra similar to those of the acrosomes, with a detectable level of copper but substantial reductions in both calcium and phosphorus (Fig. 8).

In zinc-treated sperm, the acrosomes showed much the same pattern as in copper-treated cells in having little calcium or phosphorus but here, of course, showing a detectable level of zinc (Fig. 8). The nuclei showed no accumulation of zinc but maintained the high levels of calcium and phosphorus as in the copper-treated sperm. Detectable levels of zinc occurred in the mitochondria associated with reduced amounts of calcium and phosphorus. However, these three elements appear to be concentrated mainly in intramitochondrial granules (Fig. 8).

DISCUSSION

Previous work has shown that in *M. edulis* sperm there is a greater uptake of zinc than copper from equivalent external concentrations, and that zinc produces a more substantial inhibition of respiration than does copper (Akberali *et al.*, 1985). However, the exact nature of this respiratory inhibition is unknown. The action of heavy metals on these mollusc gametes could be located at the level of glycolysis as carboxylase activity in yeast is inhibited by the binding of heavy metals to sulphydryl groups (Stoppani *et al.*, 1953). Alternatively, respiratory inhibition could involve mitochondria as the electron transport chain of isolated mitochondria is susceptible to inhibition by both zinc and copper (Kleiner, 1974; Zaba & Harris, 1976; Akberali & Earnshaw, 1982*a*,*b*; Akberali *et al.*, 1984). The present paper shows that application of zinc to *M. edulis* sperm clearly results in ultrastructural damage to the

mitochondria (Fig. 5) suggesting that the locus of respiratory inhibition may well be the mitochondria. Comparable mitochondrial damage has been shown to occur in the gill epithelial cells of the American oyster, *Crassostrea virginica* as a result of exposing the animals to copper for 14 days (Engel & Fowler, 1979).

The mechanism of inhibition of motility in sperm of the sea urchin, *Arbacia puntulata* by heavy metals is unknown but it has been suggested that binding to active sites on contractile proteins or inhibition of ATPase activity may be involved (Young & Nelson, 1974). Motility in the sperm of *M. edulis* is somewhat more resistant to zinc and much more resistant to copper than sperm motility in this sea urchin (compare Fig. 7, this paper with Young & Nelson, 1974). From our previous work, it seemed possible that the inhibition of motility in *M. edulis* sperm by copper and zinc (Fig. 7) could be due to an inhibition of respiration (Akberali *et al.*, 1985). Partial support for this view is obtained by plotting (Fig. 9) sperm motility at various levels of metal ions as a function of the respiration values published previously (Akberali *et al.*, 1985). A correlation clearly exists between reduction in motility and inhibition of respiration, suggesting that inhibition of oxidative

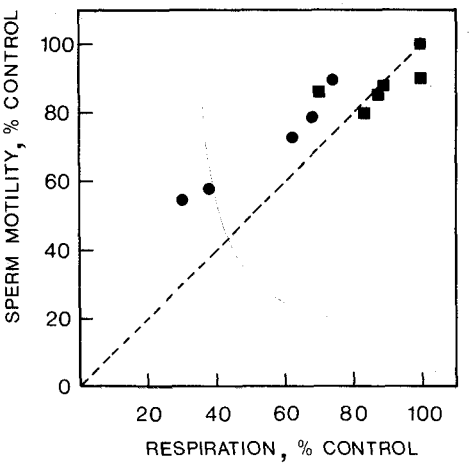

Fig. 9. Sperm motility as a function of respiration in the presence of a range of copper (■) and zinc (●) concentrations. The data for sperm motility are taken from Fig. 8, this paper and those for respiration from Akberali *et al.* (1985). The control respiration rate (100%) in the absence of heavy metal was 8·01 ± 1·21 nmoles O_2 mg protein^{-1} min^{-1}. The dotted line represents the plot anticipated if the reduction in sperm motility caused by heavy metals was directly correlated with inhibition of respiration.

phosphorylation by heavy metals reduces the ATP supply available for the contractile process. However, it is also clear that motility is less affected by zinc than would be predicted if the role of zinc was solely to act as an inhibitor of respiration.

The action of heavy metals on the motility and metabolism of sperm has been of interest for a long period and is patently a complex issue. Sperm of both vertebrate and invertebrate species tend to contain high levels of endogenous zinc located in the flagellar accessory fibres which may play a role in sperm maturation and motility (Baccetti *et al.*, 1976). The addition of chelating agents to sea urchin sperm prolongs their duration of motility and fertilising capacity (Tyler, 1953; Rothschild & Tyler, 1954). Recent work has suggested that chelation of endogenous heavy metals delays spontaneous acrosome reactions which eventually lead to loss of motility (Johnson & Epel, 1983). In apparent contradiction to the above, low concentrations of zinc (*ca.* 10^{-5} M) can cause a transitory stimulation of swimming speed in sea urchin sperm whereas higher concentrations of zinc inhibit motility (Young & Nelson, 1974). Clearly, the experimental effects of zinc are both time- and concentration-dependent; there is a role of endogenous zinc in the motility mechanism which can be augmented, at least temporarily, by the application of extracellular zinc. This stimulatory role of applied zinc may be responsible for motility in *M. edulis* sperm being somewhat greater than would be predicted from the effect of zinc on respiration (Fig. 9).

Analysis of the intracellular distribution of metal ions by X-ray microanalysis reveals that both copper and zinc are accumulated in the acrosomes (Fig. 8). However, whilst high bound copper concentrations are found in the nuclei with some accumulation in the mitochondria, zinc does not appear to be bound in the nuclei, but is found in the mitochondria (Fig. 8). Although the changes described were clear cut, these data should be interpreted with caution as the residual levels of bound cations in fixed material may not be proportional to the total *in vivo* amounts. The analysis of frozen sections, which would include unbound labile cations, was attempted but was unsuccessful due to poor resolution of the organelles.

In *M. edulis* sperm, mitochondrial zinc is found in granules located in the matrix which also contain calcium and phosphorus (Figs 5 and 8) but no evidence has been found for a similar localisation of copper. Isolated mitochondria are able to perform energy-driven transport of calcium and phosphate leading to deposition of matrix granules of

$Ca_3(PO_4)_2$ and comparable granules have been observed *in situ* in a range of mitochondrial types (Lehninger, 1970). It is possible that similar granules in sperm mitochondria possess a higher affinity for zinc than for copper and that this may explain the differential localisation of the heavy metal ions with respect to the nucleus (Fig. 8). Similar granules to those found in sperm mitochondria (Figs 5 and 8), consisting of a mixed phosphate of calcium and zinc, have been observed in membrane-limited vesicles in kidney cells of *M. edulis* following exposure of the animals to zinc (George & Pirie, 1980).

Both copper and zinc substantially decrease calcium and phosphorus levels in the acrosomes and mitochondria of *M. edulis* sperm (Fig. 8). Copper is able to bind to protein binding sites in the inner membrane of isolated beef heart mitochondria, producing increased passive permeability to both cations and anions, with zinc and lead being less effective but producing comparable effects (Hwang *et al.*, 1972). Heavy metals are also able to induce a rapid calcium release from sarcoplasmic reticulum vesicles isolated from mammalian skeletal muscle with copper being the most potent of the metal ions tested (Abramson *et al.*, 1983). Copper is similarly more effective than zinc in causing the release of pre-loaded calcium from the matrix of *M. edulis* mitochondria (Akberali & Earnshaw, 1982*b*). The decrease in bound calcium and phosphorus in the organelles of *M. edulis* sperm following treatment with heavy metals (Fig. 8) appears to be the first *in situ* demonstration of the ability of heavy metals to increase the ionic permeability of organelle membranes.

An important physiological consequence of this reaction of heavy metals with sperm organelle membranes is liable to be an increase in the concentration of free calcium in the cytosol. Calcium uptake into sperm appears to be regulated by calmodulin and is involved in both the acrosome reaction and the control of motility (Peterson *et al.*, 1983). Treatment of vertebrate sperm with high concentrations of the calcium ionophore A23187 produces a stimulation of motility which is associated with net uptake of external calcium. The transported calcium is largely confined to the extramitochondrial compartment and it is suggested that the increase in intracellular concentration of free calcium may directly affect the contractile apparatus of the sperm flagella (Babcock *et al.*, 1976; 1978). A similar increase in the cytosolic calcium concentration may well occur when *M. edulis* sperm are treated with copper and zinc (Fig. 8) which would potentially cause an increase in motility. However,

copper and zinc also inhibit sperm respiration (Akberali *et al.*, 1985) and these conflicting effects may explain why zinc is less inhibitory to motility than would be expected solely on the basis of inhibition of respiration (Fig. 9).

Finally, the observation that application of extracellular copper and zinc leads to depletion of organelle-bound calcium (Fig. 8) requires consideration in connection with our previous suggestions that the copper-induced contraction of the isolated mollusc siphon is due to release of calcium from intracellular stores (Akberali *et al.*, 1981; 1982; Akberali & Earnshaw, 1982*b*; 1985). The contraction of the isolated siphon is specific to low concentrations of copper (*ca.* 10 μM), a wide range of other metal ions producing no effect (Black, 1983). The X-ray microanalysis work in the present paper (Fig. 8) was carried out at an external metal ion concentration of 3·3 mM in order to relate to inhibitory effects on sperm motility (Fig. 7) and respiration (Akberali *et al.*, 1985). The lack of specificity in the heavy metal-induced depletion of organelle-bound calcium in *M. edulis* sperm (Fig. 8) is not surprising as a range of heavy metals are able to increase the membrane permeability of isolated organelles with copper being the most effective on a concentration basis (Hwang *et al.*, 1972; Akberali & Earnshaw, 1982*b*; Abramson *et al.*, 1983). Clearly, a further X-ray microanalysis study is required using the mollusc siphon in which determinations of intracellular calcium levels are made following treatment with much reduced external copper concentrations.

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