Involvement of Zinc in the Regulation of pHᵢ, Motility, and Acrosome Reactions in Sea Urchin Sperm

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ABSTRACT When sperm of Strongylocentrotus purpuratus or Lytechinus pictus are diluted into seawater, motility is initiated; and when exposed to egg jelly, an acrosome reaction is induced. In the presence of a variety of structurally different metal chelators (0.1–1 mM EDTA, EGTA, phenanthroline, dipyrrolidyl, cysteine, or dithiothreitol), motility initiation is delayed and the acrosome reaction is inhibited. Of the metals detected in the sperm of these two species, very low levels of Zn⁺² (0.1 μM free Zn⁺²) uniquely prevent this chelator inhibition. L. pictus sperm concentrate 65Zn⁺² from seawater, and EDTA removes 50% of the accumulated 65Zn⁺² by 5 min. Since both sperm motility and acrosome reactions are in part regulated by intracellular pH (pHᵢ), the effect of chelators on the sperm pHᵢ was examined by using the fluorescent pH sensitive probe, 9-aminoacridine. EDTA depresses sperm pHᵢ in both species, and 0.1 μM free Zn⁺² reverses this pHᵢ depression. When sperm are diluted into media that contain chelators, both NH₄Cl and monensin (a Na⁺/H⁺ ionophore) increase the sperm pHᵢ and reverse the chelator inhibition of sperm motility and acrosome reactions. The results of this study are consistent with the involvement of a trace metal (probably zinc) in the pHᵢ regulation of sea urchin sperm and indicate a likely mechanism for the previously observed effects of chelators on sperm motility and acrosome reactions.

Sea urchin spermatozoa are immotile in semen. The motility that ensues upon dilution into seawater requires an elevation of intracellular pH (pHᵢ) (see references 1, 2, and 3) and is accompanied by increased cAMP levels (4, 5). The acrosome reaction (induced by egg jelly) involves increases in both pHᵢ and intracellular Ca²⁺ as well as movements of Na⁺ and K⁺ (6–9).

Heavy metals are also somehow involved in sea urchin sperm motility and acrosome reactions. Metal chelators at concentrations that do not alter the Ca²⁺ or Mg²⁺ levels in the seawater prolong the fertilizable life of sea urchin spermatozoa (10, 11, 12), and a recent investigation of this phenomenon showed that this prolongation of sperm motility by metal chelators could be partially explained by a chelator inhibition of spontaneous (non-jelly initiated) acrosome reactions (12). It was also reported that these chelators would inhibit jelly-initiated acrosome reactions in the sperm of the sea urchin, Lytechinus pictus (12). Other reports have implicated Cu⁺² and/or Zn⁺² in the regulation of sea urchin sperm respiration and motility (13–18).

We have now studied this effect of chelators on both sperm motility and the acrosome reaction, and here report evidence that the effect is somehow related to a previously unsuspected role of a trace metal, most probably Zn⁺², in the regulation of pHᵢ. We show that when semen is diluted into seawater that contains metal chelators, motility initiation is delayed, and the jelly-initiated acrosome reactions are inhibited. Of the metals detected in sea urchin spermatozoa, only Zn⁺² reversed these inhibitions. Chelator addition also depressed the sperm pHᵢ, and Zn⁺² also reversed this effect. This chelator inhibition of motility and acrosome reactions was reversed by reagents that increased the sperm pHᵢ. Since motility and

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Abbreviations used in this paper: 9AA, 9-aminoacridine, a fluorescent amine; ASW, artificial seawater formulation consisting of 423 mM NaCl, 9 mM KCl, 9.27 mM CaCl₂, 22.94 mM MgCl₂, 25.5 mM MgSO₄, 2.15 mM NaHCO₃, and 10 mM Tris; EDTA-ASW, ASW to which 1 mM EDTA was added; OCaSW, ASW without CaCl₂; pHᵢ, intracellular pH.
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\[ \text{acrosome reactions are limited by pH, it appears} \]

\[ \text{and sperm samples were dried in platinum crucibles at 100°C, and then ashed} \]

\[ \text{for the spermatozoa (probably to DNA) in addition to being concentrated via its} \]

\[ \text{fucose content was determined according to the procedure of Spiro (19).} \]

\[ \text{Sperm Acrosome Reaction Assays: Unless indicated otherwise,} \]

\[ \text{acrosome reactions were initiated by the dilution of 0.6 μl semen into 250 μl} \]

\[ \text{of ASW (e.g. egg jelly, chelators, or other reagents). The egg jelly concentrations} \]

\[ \text{were mixed together, the pH was adjusted to 7.8 for} \]

\[ \text{S. purpuratus, L. pictus, and S. pictus were conducted at} \]

\[ \text{the pH gradient (1). In} \]

\[ \text{Chelators Inhibit Jelly-initiated Acrosome} \]

\[ \text{REACTIONS AND METHODs} \]

\[ \text{Animal Maintenance and Gamete Handling: Lytechinus pictus} \]

\[ \text{and Strongylocentrotus purpuratus were maintained in flow-through} \]

\[ \text{Seawater Formulations: Unless noted otherwise, the artificial} \]

\[ \text{water holding tanks, and gametes were collected by intracoelomic injection} \]

\[ \text{with 0.2-0.4 or 3-5 ml, respectively, of 0.5 M KCl. The semen was collected} \]

\[ \text{and the change in fluorescence was monitored as 9AA equilibrated across the} \]

\[ \text{Sperm, its application to} \]

\[ \text{Chemicals: Monensin was purchased from Calbiochem-Behring Corp. (La Jolla, CA). All other} \]

\[ \text{RESULTS} \]

\[ \text{Chelators Inhibit jelly-initiated Acrosome} \]

\[ \text{REACTIONS and fertilization} \]

\[ \text{RESULTS of this study confirm and extend the report of} \]

\[ \text{Johnson and Epel (12) and show that sea urchin acrosome} \]

\[ \text{REACTIONS are direct results of the chelator-produced pH, depression.} \]

\[ \text{MATERIALS AND METHODS} \]

\[ \text{pH}_{i} \text{,} \]

\[ \text{The fluorescent amine, 9-aminoacidine (9AA), was used to monitor changes in the pH of sea urchin spermatozoa. As with} \]

\[ \text{spermatozoa, and therefore the maximum free ion concentration was obtained} \]

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\[ \text{REACTIONS are inhibited by a variety of metal chelators. In} \]

\[ \text{triplicate experiments, dilution of} \]

\[ \text{L. pictus sperm into egg jelly that contained 0.1 mM EDTA, phenanthroline, diipyridyl, cysteine, or dithiothreitol produced only 3-7% acrosome} \]

\[ \text{REACTIONS (compared to 71% in controls with egg jelly that} \]

\[ \text{included reagents]. EDTA-buffered ASW formulations: To adjust free metal ion} \]

\[ \text{concentrations, 1 mM EDTA was added to ASW (EDTA-ASW), and varying} \]

\[ \text{levels of metal ions were added. The MINEQL computer program (21), which} \]

\[ \text{simultaneously calculates the concentrations of all programed EDTA-metal} \]

\[ \text{complexes and inorganic ion complexes, was used to determine both the} \]

\[ \text{maximum free concentration of each ion possible in ASW, and the total amount of each ion that had to be added to} \]

\[ \text{EDTA-ASW to produce the desired free ion concentrations. The EDTA stability constants for} \]

\[ \text{Cu}^{2+}, \text{Fe}^{2+}, \text{Mg}^{2+}, \text{Ni}^{2+}, \text{and} \]

\[ \text{Zn}^{2+} \text{were obtained from Anderegg (22), and constants for} \]

\[ \text{Cu}^{2+} \text{and Ti}^{4+} \text{were from the MINEQL program (21). Where applicable, both} \]

\[ \text{Cu}^{2+}-\text{EDTA and metal hydroxide-EDTA constants were utilized. The} \]

\[ \text{constants for complexes of metal ions with other inorganic ions (e.g.,} \]

\[ \text{ZnCO}, \text{FeOH}, \text{etc.} \text{are part of the basic MINEQL program. All constants were} \]

\[ \text{corrected to ionic strength 0.5 with MINEQL's activity correction subroutine.} \]

\[ \text{To compensate for pH, we used} \]

\[ \text{H}^{+}-\text{EDTA constants of 9.94 and 15.8 for complexes of} \]

\[ \text{1 and 2 M free Cu}^{2+} \text{were prepared by the addition of 884 and 986 μM, respectively, of} \]

\[ \text{CuCl} \text{to EDTA-ASW. For some experiments, 1.010 μM NiCl} \text{or CuCl} \text{was} \]

\[ \text{added to EDTA-ASW, thus assuring that the maximum solubility was exceeded} \]

\[ \text{and therefore the maximum free ion concentration was obtained} \]

\[ \text{(1.2 x 10^{-8}} \]

\[ \text{M free Cu}^{2+} \text{and 2.1 x 10^{-8}} \text{M free Ni}^{2+} \text{). After the ASW, EDTA, and metals} \]

\[ \text{were mixed together, the pH was adjusted to 7.8 for} \]

\[ \text{L. pictus experiments and 8.0 for} \]

\[ \text{S. purpuratus.} \]

\[ \text{Measurement of} \]

\[ \text{pH:} \]

\[ \text{The} \]

\[ \text{pH gradient between the spermatozoa and ASW (i.e., is proportional to} \]

\[ \text{ph for the egg fertilization experiment of} \]

\[ \text{S. purpuratus, L. pictus, and} \]

\[ \text{were conducted at 19°C and 12°C, respectively.} \]

\[ \text{RESULTS} \]

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\[ \text{REACTIONS (compared to 71% in controls with egg jelly that} \]

\[ \text{lacked chelators, and a background of 2.3% acrosome reac-} \]

\[ \text{these 60 elements, considering the small amount of sample available for analysis} \]
tions with chelators that lacked egg jelly). Chelator inhibition was also seen, albeit not as completely, in *S. purpuratus* sperm. In duplicate experiments, dilution of sperm into jelly that contained 1 mM EDTA, phenanthonine, or cysteine produced 28, 0.5, and 36% acrosome reactions, respectively (compared to 92% in controls with jelly that lacked chelators, and a background of 0% acrosome reactions with chelators that lacked jelly).

For the assessment of the effect of chelators on fertilization, sperm dilution versus percentage fertilization curves were compared in the presence and absence of EDTA. With *L. pictus* gametes, 10 times more sperm was required to produce 50% fertilization in the presence of 1 mM EDTA (a semen dilution of 1:10^6 was required in EDTA versus a 1:10^3 dilution in the controls).

The chelators used are structurally dissimilar: EDTA coordinates metals with carboxyl groups; phenanthonine (1,10-phenanthroline) and dipyridyl (α,α'-dipyridyl) use N atoms embedded in otherwise uncharged aromatic rings; and cysteine and dithiothreitol use sulphhydryl groups plus carboxyl or hydroxyl groups. Since these molecules, which chelate metals quite strongly (25), are structurally quite different, they most likely inhibit the acrosome reactions by removing metal ions. As the 0.1–1 mM chelator concentrations are too low to significantly alter the concentrations of Ca⁴⁺ or Mg⁴⁺ in ASW, as the 0.1–1 mM EDTA concentrations are too low to significantly alter the concentrations of Ca⁴⁺ or Mg⁴⁺ in ASW (9.3 and 48 mM, respectively), the chelator effect must be mediated by the removal of trace metals.

### Elemental Analysis of Sea Urchin Spermatozoa

A qualitative and semiquantitative analysis of the elements in spermatozoa was made by D.C. arc optical emission spectrometry. Table I lists the elements (Cu, Ni, and Zn) that were both detected in sperm or egg jelly and would also undergo free concentration changes upon addition of EDTA at the concentrations used. Additional elements detected that have stable cationic forms in ASW were: Na, K, Ba, Ca, Mg, Sr, Al, Fe, Sn, and Ti. Na⁺, K⁺, Ca⁴⁺, and Mg⁴⁺ were all present at concentrations at least ninefold greater than that of the chelators used and would therefore not undergo a significant concentration change upon addition of chelators. Ba²⁺ and Sr²⁺ have lower affinities to the chelators than do Ca⁴⁺ and Mg⁴⁺ and would also not undergo a significant concentration change. The remaining elements have higher affinities to EDTA than do Ca⁴⁺ and Mg⁴⁺, and therefore might be expected to undergo concentration changes upon addition of sperm to EDTA-ASW. However, Al, Fe, Ti, and Sn were not considered further since their most stable oxidation states in ASW [Al(III), Fe(III) and Ti(IV) (reference 26)] have very low solubilities (10⁻¹⁶ M or less) as computed by the MINEQL program, and therefore EDTA addition would not alter their free concentrations in ASW and would not remove them from spermatozoa or egg jelly. Stability constants were not available for Sn (IV), but it is apparently at least as insoluble in ASW as are Al(III), Fe(III), and Ti(IV) (reference 26). Also, the detected amounts of Al, Ti, and Sn were quite variable in the samples assayed and were most likely present as contaminants.

Only Zn²⁺ Reversed Chelator Inhibition of the Acrosome Reaction

To determine if Cu, Ni, or Zn was involved in the acrosome reaction, we utilized EDTA-metal buffers to individually control the metal ion concentrations and ascertain whether any of these metals would restore acrosome reactions in the presence of EDTA. The most stable oxidation states of these metals in ASW are Cu(II), Ni(II), and Zn(II) (26), and for each metal a series of concentrations was used with the highest concentration being that at which the metal precipitated in EDTA-ASW. As shown in Fig. 1 and Table II, only Zn²⁺ (10⁻⁷ M) reversed the EDTA inhibition, and this reversal was seen with sperm from both species of sea urchin.

As shown in Fig. 1, higher concentrations of Zn²⁺ initiated acrosome reactions of *L. pictus* sperm in the absence of egg jelly. This induction of the acrosome reactions by Zn²⁺ was not seen with *S. purpuratus* sperm. Indeed, in ASW (that lacked EDTA), Zn²⁺ above 1 μM inhibited jelly-initiated acrosome reactions in *S. purpuratus* (90% acrosome reactions at 1 μM added Zn²⁺, n = 2; and 2% acrosome reactions at 10–30 μM added Zn²⁺, n = 2); whereas in *L. pictus*, this level of Zn²⁺ (10–30 μM) initiated acrosome reactions in the absence of jelly (40%, n = 2). It should be noted that the maximum soluble concentration of Zn²⁺ in ASW is 10 μM total, of which 7.1 μM is free Zn²⁺.

### EDTA Removed ⁶⁵Zn from *L. pictus* Spermatozoa

As shown in Fig. 2, ⁶⁵Zn is rapidly concentrated from ASW by *L. pictus* spermatozoa, and 1 mM EDTA quickly removed much of the accumulated ⁶⁵Zn; EDTA removed 35% of the ⁶⁵Zn within 1-2 min and 50% by 5 min. (*L. pictus* spermatozoa suspended in OCaSW also concentrated ⁶⁵Zn²⁺, and 1 mM EDTA removed 50% of this sperm-associated ⁶⁵Zn²⁺ by 5 min.) Although this experiment does not distinguish between ⁶⁵Zn²⁺ being removed from external or internal sites, the results are consistent with the hypothesis that EDTA inhibits acrosome reactions by removing Zn²⁺ from these spermatozoa.

### Chelator Inhibition of Acrosome Reactions Involves an Event Associated with Dilution of Spermatozoa into ASW

The inhibition of acrosome reactions by EDTA described above is only seen if sperm in semen are diluted directly into ASW that contain both EDTA and jelly. If semen is first diluted into ASW (with or without the chelators), and 5 min

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**Table I**

| Elemental Analysis of Sea Urchin Spermatozoa and Egg Jelly |
|----------------------------------------------------------|
| **Element** | **Cu** | **Ni** | **Zn** |
|------------|-------|-------|-------|
| ASW        | 6     | 1     | <15*  |
| *L. pictus*|       |       |       |
| Semen      | 20    | <1*   | 20    |
| Sperm washed in ASW | 15    | <1    | <15   |
| Jelly in ASW | 5    |       | <15   |
| *S. purpuratus* |     |       |       |
| Semen      | 10    | 2     | 20    |
| Sperm washed in ASW | 20    | 2     | 15    |
| Jelly in ASW | 5    | <1    | <15   |

Samples were prepared and analyzed as indicated in Materials and Methods, and results are expressed as parts per million in ash. (1 ppm in ash corresponds to ~0.02 ppm in solution before ashing.) Egg jelly for *L. pictus* and *S. purpuratus* contained 126 and 43 μg fucose equivalents per milliliter, respectively. The ASW results are the average of two determinations; the other results represent single determinations.

* These are the detection limits of this method for these elements.
later this sperm is added to ASW that contains egg jelly plus a chelator, no inhibition of the acrosome reaction is observed (sperm of L. pictus or S. purpuratus). This result indicates that the spermatozoa must encounter both egg jelly and the chelator upon dilution in order for the chelator inhibition to be observed, and therefore that a sperm-dilution associated event is involved in the chelator inhibition of acrosome reactions. This result also implies that Zn$^{2+}$ may not be required for acrosome reactions, but may be involved in an earlier dilution-associated event; if the sperm get by this event, Zn$^{2+}$ is no longer required.

Chelators Also Delay the Initiation of Sperm Motility

Since the chelator inhibition of acrosome reactions was found to be associated with the dilution of semen, we examined another event that occurs upon dilution of semen into ASW—sperm motility initiation. When spermatozoa from either L. pictus or S. purpuratus were diluted into ASW, the initiation of motility was delayed if EDTA was present (Fig. 3). Zn$^{2+}$ alone reversed this inhibition of motility, and neither Cu$^{2+}$ (10$^{-8}$ M) nor Ni$^{2+}$ (10$^{-5.8}$ M) produced motility initiation different from that observed in EDTA-ASW. This result reinforces the concept that the Zn$^{2+}$ involvement is in a dilution-associated event.

EDTA Depresses the pH, of Sea Urchin Spermatozoa

In sea urchin sperm, pH, has previously been shown to increase upon sperm dilution into seawater (2), and this increased pH, is probably the trigger for sperm motility initiation (1, 2, 3). The following experiments tested the hypothesis that the chelators delay motility initiation (and perhaps prevent acrosome reactions) by preventing or delaying the dilution-associated increase in sperm pH.

Using 9AA to monitor the pH, we found that S. purpuratus sperm diluted into ASW that contained EDTA had a lower pH, than did spermatozoa diluted into media that lacked EDTA (Fig. 4.4). The pH, of sperm at 8 min after dilution into ASW or EDTA-ASW was calculated to be 7.32 and 7.08, respectively; therefore, EDTA depressed the sperm pH, by 0.24 pH unit. This pH, depression by EDTA was ~75% prevented by addition of Zn$^{2+}$.

In S. purpuratus, the effect of EDTA on pH, (and its reversal by 10$^{-9}$ M Zn$^{2+}$) is easily demonstrated in ASW (Fig. 4.4).

\[
\text{Table II}
\]

| Metal Ion | Acrosome Reactions % | SD | n |
|-----------|---------------------|----|---|
| Cu$^{2+}$ | -9                  | 1.3| 6.6| 5 |
| Cu$^{2+}$ | -8                  | 0.7| 1.2| 3 |
| Cu$^{2+}$ | -7.79*              | 0  | 0  | 3 |
| Ni$^{2+}$ | -8                  | 1.7| 1.5| 3 |
| Ni$^{2+}$ | -7                  | 3.3| 2.6| 3 |
| Ni$^{2+}$ | -5.68*              | 3.0| 2.6| 3 |
| Zn$^{2+}$ | -7                  | 71 | 5  | 3 |

The protocol is given in Materials and Methods and Fig. 1. In the absence of egg jelly (and with the exception of Zn$^{2+}$ added to L. pictus sperm), these metal ion formulations initiated 0-1% acrosome reactions. SD, standard deviation; -, not applicable.

* These are the maximum concentrations at which these ions are soluble in EDTA-ASW; at higher concentrations, both Cu$^{2+}$ and Ni$^{2+}$ precipitate as hydroxides. A sufficient quantity of each ion was added to assure that the maximum free concentration was exceeded.

* A larger range of Zn$^{2+}$ concentrations is shown in Fig. 1.
that was reversed by the subsequent addition of Zn$^{2+}$ at 15

sperm are diluted directly into seawater that contains

sperm diluted into EDTA-OCaSW that contained 10$^{-6}$ M free

the sperm passed through to form a pellet at the bottom of the centrifuge tubes. The ASW and disobutylphthalate were decanted, the bottom 4–5 mm of centrifuge tube was cut off, placed in 4 ml of Aquasol (New England Nuclear, Boston, MA), and counted in a liquid scintillation counter. For each data point, the counts from three pellets were averaged and corrected for extracellular $^{65}$Zn (14 cpm/pellet). Parallel experiments with $^3$H$_2$O and [3H]sorbitol were used to determine the extracellular volume in the sperm pellets. EDTA (1 mM) was added at the arrow marked EDTA. (The ASW was buffered with 10 mM Tris, and the EDTA addition produced a decrease in extracellular pH of 0.1 pH unit). At 30 min, a comparison of the $^{65}$Zn concentration in the sperm suspension to the $^{65}$Zn concentration in sperm pellets showed that $^{65}$Zn had been concentrated 225-fold by the spermatozoa (41.6 cpm $^{65}$Zn/μl of sperm suspension before centrifugation, and 9,350 cpm/μl of spermatozoa in the pellets). At 30 min, 16% acrosome reactions were present.

However, this effect is not as easily seen with $L$. pictus sperm in ASW, since these sperm undergo a rapid re-acidification after dilution (3), and little difference was seen in the presence and absence of EDTA by the time the 9AA distribution had equilibrated (> 4 min). However, if $L$. pictus sperm are diluted into OCaSW, the rate of re-acidification is slower. pH$_i$ values at 8 min after dilution of sperm into OCaSW or EDTA-OCaSW were 7.51 and 7.37, respectively, whereas $L$. pictus sperm diluted into EDTA-OCaSW that contained 10$^{-6}$ M free Zn$^{2+}$ had a pH$_i$ of 7.51. Therefore, Zn$^{2+}$ also abolished the suppression of pH$_i$ by EDTA in this species.

The acidification induced by EDTA can also occur after dilution, although the acidification may not be as great as when sperm are diluted directly into seawater that contains EDTA. This effect of EDTA is shown in the experiment depicted in Fig. 4B, in which $S$. purpuratus sperm were first diluted into ASW, and then 100 μM Ca-EDTA was added (at 8 min). This produced an intracellular acidification of 0.12 pH unit (the calculated pH$_i$ was 7.37 before EDTA addition) that was reversed by the subsequent addition of Zn$^{2+}$ at 15 min. [To prevent the EDTA addition from producing a significant change in the pH$_o$ of ASW, a lower concentration of EDTA (0.1 mM) was used; this could account for the lower magnitude of pH$_i$ depression in Fig. 4B as compared with Fig. 4A.]

Control experiments showed that neither EDTA (100 μM) nor Zn$^{2+}$ (65 μM) affected the relationship between 9AA fluorescence and sperm pH$_i$. No fluorescence change was observed if EDTA or Zn$^{2+}$ was added to either 9AA in ASW that lacked sperm or $S$. purpuratus sperm that had been loaded with 9AA but whose pH$_i$ had been equilibrated with the extracellular pH by addition of either monensin or Triton X-100. The latter experiment shows that EDTA and Zn$^{2+}$ do not affect the binding of 9AA to sperm and should neither alter the measured 9AA fluorescence through a non-pH-dependent mechanism nor affect the calculation of pH$_i$ values.

**NH$_4$Cl Reversed Chelator Inhibition of Sperm pH$_i$, Acrosome Reactions, and Motility**

The above results suggest the hypothesis that the chelator-induced depression of pH$_i$ slows down the initiation of motility and inhibits acrosome reactions. If so, then raising the pH$_i$ should reverse the inhibitory effects. The following experiments show that NH$_4$Cl and monensin raise the sperm pH$_i$, and reverse the effects of EDTA on both motility initiation and acrosome reactions. As shown in Fig. 4A, NH$_4$Cl (10 mM) produces a transient reversal of the EDTA depression of pH$_i$ in $S$. purpuratus spermatozoa. By use of a similar protocol with $L$. pictus, 10 mM NH$_4$Cl reversed ~50% of the

![Figure 2](image-url)  
**Figure 2** EDTA removes $^{65}$Zn from $L$. pictus spermatozoa. $^{65}$Zn (1 μCi/ml, 5 μM total Zn$^{2+}$, or 3.5 μM free Zn$^{2+}$) was added to a 1% sperm suspension in ASW (○). At the indicated times, triplicate 200-μl aliquots were removed and centrifuged (15,000 g, 45 s) through 300 μl disobutylphthalate to remove the extracellular $^{65}$Zn. With this procedure, the ASW (that contained the unbound $^{65}$Zn) stayed on top of disobutylphthalate, and the sperm passed through to form a pellet at the bottom of the centrifuge tubes. The ASW and disobutylphthalate were decanted, the bottom 4–5 mm of centrifuge tube was cut off, placed in 4 ml of Aquasol (New England Nuclear, Boston, MA), and counted in a liquid scintillation counter. For each data point, the counts from three pellets were averaged and corrected for extracellular $^{65}$Zn (14 cpm/pellet). Parallel experiments with $^3$H$_2$O and [3H]sorbitol were used to determine the extracellular volume in the sperm pellets. EDTA (1 mM) was added at the arrow marked EDTA. (The ASW was buffered with 10 mM Tris, and the EDTA addition produced a decrease in extracellular pH of 0.1 pH unit). At 30 min, a comparison of the $^{65}$Zn concentration in the sperm suspension to the $^{65}$Zn concentration in sperm pellets showed that $^{65}$Zn had been concentrated 225-fold by the spermatozoa (41.6 cpm $^{65}$Zn/μl of sperm suspension before centrifugation, and 9,350 cpm/μl of spermatozoa in the pellets). At 30 min, 16% acrosome reactions were present.

![Figure 3](image-url)  
**Figure 3** EDTA delays motility initiation in sea urchin spermatozoa. Semen (0.5 μl) was added to 500 μl of ASW or EDTA-ASW (± Zn$^{2+}$). Aliquots (15 μl) were removed at the indicated times and placed on a microscope slide (without a coverslip). The microscope was focused in the middle of the drop (to avoid surface effects) and viewed at 100X with a phase contrast microscope, and the percentage of motile sperm was estimated. Each point is the average of three determinations. (A) $P$. japonicus spermatozoa, ASW (○), EDTA-ASW (△). EDTA-ASW plus 0.95 mM Zn$^{2+}$ (1 μM free Zn$^{2+}$) ■. Results with 0.1 μM free Zn$^{2+}$ were the same as with 1 μM, except that 60% motility was observed at 15 sec (the first point). Similar results were obtained in OCaSW. (B) $S$. purpuratus spermatozoa. ASW (○), EDTA-ASW (△), EDTA-ASW plus 0.1 μM free Zn$^{2+}$ (■), EDTA-ASW plus 1 μM free Zn$^{2+}$ (□).
EDTA depression of pH, (data not shown) which was followed by a more rapid re-acidification than was observed with S. purpuratus. Monensin also alkalized spermatozoa of both species, but with slower kinetics (e.g., Fig. 4A).

Incubation in NH₄Cl also reversed the EDTA inhibition of jelly-induced acrosome reactions in both L. pictus and S. purpuratus (Table III). Monensin also reversed the EDTA inhibition in L. pictus, but could also initiate some acrosome reactions in the absence of egg jelly.

Both NH₄Cl and monensin reversed EDTA inhibition of sperm motility, with NH₄Cl acting more rapidly. S. purpuratus sperm diluted into EDTA-ASW produced ~5% motile sperm at 15 s and 20% at 1 min, whereas sperm diluted into EDTA-ASW that contained 10 mM NH₄Cl or 10 μM monensin produced respective motilities of 95 and 10% at 15 s, and 95 and 95% at 1 min. The greater effectiveness of NH₄Cl (than monensin) in reversing chelator inhibition of both motility and acrosome reactions could be due to its ability to more rapidly increase the sperm pH, (see, e.g., Fig. 4A).

**High Zn²⁺ Also Raises pH, in L. pictus But not in S. purpuratus**

As noted earlier, high Zn²⁺ (≥1 μM free Zn²⁺) in the absence of egg jelly could initiate acrosome reactions in L. pictus spermatozoa (Fig. 1) but not in S. purpuratus (Table II). In ASW (that lacked chelators), addition of 20 μM Zn²⁺ to spermatozoa also produced an increase in pH, in L. pictus (Fig. 5A), but not in S. purpuratus sperm (Fig. 5B). With L. pictus, Zn²⁺ alkalizes spermatozoa even in OCaSW (where no acrosome reactions occur because of the absence of Ca²⁺); this indicates that Zn²⁺ initiates changes that precede the Ca²⁺-dependent steps of the acrosome reaction.

**DISCUSSION**

The results presented in this paper reveal an unexpected involvement of trace metals in the regulation of pH, in sea urchin sperm, and via pH, a role in motility initiation and the acrosome reaction. The role of a trace metal was indicated by the observation that acrosome reactions are inhibited if the sperm in semen are diluted directly into ASW that contains three different types of chelators, and zinc was implicated since it was the only metal detected in sperm that reversed this inhibition. This is a dilution-associated event since once
sperm have been diluted for 5 min or longer, the chelators will no longer inhibit acrosome reactions. Sperm motility initiation, a well-characterized event associated with dilution, was also delayed by chelators, and again Zn\(^{2+}\) uniquely reversed this inhibition. Since pH\(_{i}\) is elevated during sperm acrosome reactions and motility initiation, the effects of chelators on sperm pH\(_{i}\) were investigated; EDTA depressed the sperm pH\(_{i}\), and Zn\(^{2+}\) reversed this depression.

Zn\(^{2+}\) is most likely the element physiologically involved in this pH\(_{i}\) regulation since (a) Zn\(^{2+}\) is present in sea urchin sperm and egg jelly, (b) EDTA removes Zn\(^{2+}\) from these sperm, (c) of the elements detected in sea urchin sperm, only Zn\(^{2+}\) reverses the chelator effects, and (d) the effective concentration of Zn\(^{2+}\) (\(\sim 10^{-7}\) M free Zn\(^{2+}\)) is reasonable for a physiologic action. We cannot, of course, rule out the possibility that an additional element is involved which was present at too low a concentration to be detected by the qualitative analysis method used. Also, the absolute effective free Zn\(^{2+}\) concentration of \(10^{-7}\) M is approximate, since this determined concentration (a) depends on the accuracy of the stability constants used, and (b) could be decreased somewhat by Zn\(^{2+}\) sequestration by the sperm.

The observation that EDTA inhibits the pH\(_{i}\) rise of sperm diluted into seawater provides an explanation for the observed delay in motility initiation, since motility requires an elevated pH\(_{i}\). As regards the prevention of the acrosome reaction by chelators, these results suggest the sperm pH\(_{i}\) must rise above a critical level before or near the time of egg jelly binding for acrosome reactions to occur. It is known that jelly induces enzymatic changes within seconds (e.g., a protein dephosphorylation occurs by 5 s, reference 27); and if the critical pH\(_{i}\) hypothesis is correct, this pH\(_{i}\) level must be attained shortly after jelly binding for the acrosome reaction to occur. When sperm are diluted into ASW that contains chelators plus egg jelly, however, the pH\(_{i}\) increase is delayed and would reach this critical level later so that the acrosome reaction would then not occur. If, however, sperm are diluted first into the chelator and then egg jelly is added 5 min later, the sperm pH\(_{i}\) has passed the critical level and acrosome reactions can occur now.

The equilibration time of 9AA (~8 min) is too slow to determine whether the rate of pH\(_{i}\) increase in sperm is slowed by EDTA during the first 5 min after dilution into seawater. However, since this dilution-associated alkalization is caused by the release of H\(^{+}\) ions from sperm, the pH\(_{i}\) of the extracellular medium can be monitored as an indicator of changes in pH\(_{i}\) (2, 28, 29). Indeed, experiments in which the extracellular pH was monitored as S. purpuratus sperm were diluted into seawater showed a 35% lower rate of H\(^{+}\) release in the first 5 min after dilution in the presence of EDTA (unpublished results), therefore supporting the hypothesis that chelator inhibition of both sperm motility and acrosome reactions is due to a slowing of the dilution-associated pH\(_{i}\) increase.

The results of this study are compatible with previously reported chelator effects on sea urchin spermatozoa; however, this is the first study to identify Zn\(^{2+}\) as the metal being removed and to identify pH\(_{i}\) depression as the probable mechanism by which chelators inhibit sperm motility and acrosome reactions. The chelator-produced depression of sperm pH\(_{i}\) would explain the depression of sperm respiration reported by others (e.g., 11, 14) and also the inhibition of sperm motility (16) and of acrosome reactions (12). In a study contemporary with ours (Christen, R., R. W. Schackmann, and B. M. Shapiro, manuscript in preparation), it was also found that EGTA and dithiothreitol depress the sperm pH\(_{i}\), depress respiration, and prolong the fertilizable life of S. purpuratus sperm. However, they assayed for fertilizability of sperm and did not monitor either motility or acrosome reactions, and did not correlate the EGTA and dithiothreitol effects with the chelation of a trace metal. Several previous workers have correlated both Zn\(^{2+}\) and Cu\(^{2+}\) with the chelator effects on sperm (e.g., 14, 15, 16, 18); however, our use of EDTA-metal buffers to individually control the level of each metal ion (at submicromolar levels) indicates that Zn\(^{2+}\) alone reverses the chelator effects. [The observation by Morrisawa (18) that Zn\(^{2+}\) alone alters the axoneme structure also implies that Zn\(^{2+}\) may have an additional structural role in sperm motility.]

This study also reveals species differences in the responses of L. pictus and S. purpuratus spermatozoa to low and high levels of Zn\(^{2+}\). In both species, chelators inhibited motility and acrosome reactions, and \(10^{-7}\) M Zn\(^{2+}\) reversed these effects, but when Zn\(^{2+}\) was increased to 1 \(\mu\)M in the absence of egg jelly, acrosome reactions were initiated in L. pictus but not in S. purpuratus. Indeed, 10–30 \(\mu\)M total Zn\(^{2+}\) inhibited jelly-initiated acrosome reactions in S. purpuratus, whereas
identical levels directly initiated acrosome reactions in \textit{L. pictus}. In both species, the chelator and low Zn$^{2+}$ effects are best explained by their effect on pH, and in \textit{L. pictus} sperm, high Zn$^{2+}$ may initiate acrosome reactions by its ability to increase pH above the normal resting level (Fig. 5). However, in \textit{S. purpuratus} sperm, high Zn$^{2+}$ produces no change in pH, and inhibits acrosome reactions, therefore showing that high Zn$^{2+}$ is either toxic to those sperm or inhibits an essential step required for acrosome reactions in this species. Species differences in the effects of Zn$^{2+}$ on sperm motility and acrosome reactions have also been seen in mammals and other invertebrates (30–37).

The most interesting aspect of the current study is the finding that pH regulation in sperm involves Zn$^{2+}$. The Zn$^{2+}$ is apparently not limiting in vivo, but its essential participation is revealed by the chelator studies. Since a highly charged molecule such as EDTA is unlikely to enter the sperm cell, it is apparently not limiting in vivo, but its essential participation is revealed by the chelator studies. Since a highly charged molecule such as EDTA is unlikely to enter the sperm cell, it is likely that Zn$^{2+}$ removal could depress the sperm pH by any of several mechanisms, which include: (a) slowing the movement of H$^+$ through a membrane channel or a H$^+$-exchange mechanism [The Na$^+$-dependent alkalization mechanism that has been described in sea urchin sperm (1, 3, 27, 28) does not seem to be involved. When \textit{S. purpuratus} spermatozoa were diluted into Na$^+$-free sea water and 10 mM NaCl was added to increase the pH, and activate motility (references 1 and 3), the kinetics of the pH increase and the final equilibrium pH reached were the same in the presence and absence of 1 mM EDTA]; (b) affecting the movement of other ions which, in turn, could alter the pH (e.g., Zn$^{2+}$ removal might alter the membrane potential which could secondarily depress the pH); or (c) acting through a Zn$^{2+}$-dependent enzyme system that controls the sperm pH. [pH may be regulated by the level of phosphorylation of an essential ion pump, and phosphoprotein phosphatases in sea urchin sperm and elsewhere are inhibited by Zn$^{2+}$ (38, 39).] Whatever the mechanism, the results of this study indicate that a previously unsuspected role of a trace metal, most likely Zn$^{2+}$, is that of pH regulation of sperm and perhaps other cell types as well.

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