Involvement of Zinc in the Regulation of pH_i, 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, dipyridyl, 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 $^{65}Zn^{+2}$ from seawater, and EDTA removes 50% of the accumulated $^{65}Zn^{+2}$ by 5 min. Since both sperm motility and acrosome reactions are in part regulated by intracellular pH (pH_i), the effect of chelators on the sperm pH_i was examined by using the fluorescent pH sensitive probe, 9-aminoacridine. EDTA depresses sperm pH_i in both species, and 0.1 μM free Zn^{+2} reverses this pH_i depression. When sperm are diluted into media that contain chelators, both NH_4Cl and monensin (a Na^+/H^+ ionophore) increase the sperm pH_i 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 pH_i 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 (pH_i)[1] (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 pH_i and intracellular Ca^{+2} 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 Ca^{+2} or Mg^{+2} 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 pH_i. 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 pH_i, and Zn^{+2} also reversed this effect. This chelator inhibition of motility and acrosome reactions was reversed by reagents that increased the sperm pH_i. Since motility and

[1] *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 $CaCl_2$, 22.94 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$, and 10 mM Tris; EDTA-ASW, ASW to which 1 mM EDTA was added; OCaSW, ASW without $CaCl_2$; pH_i, intracellular pH.

acrosome reactions are known to be limited by pH_i, it appears that the effects of chelators on both motility and acrosome reactions are direct results of the chelator-produced pH_i depression.

MATERIALS AND METHODS

Animal Maintenance and Gamete Handling: *Lytechinus pictus* and *Strongylocentrotus purpuratus* were maintained in flow-through seawater holding tanks, and gametes were collected by intracoelomic injection with 0.2–0.4 or 3–5 ml, respectively, of 0.5 M KCl. The semen was collected undiluted ("dry") and stored on ice in microcentrifuge tubes. Eggs were spawned directly into seawater, diluted to a 1% suspension, and stored at 12–15°C until used. Eggs were used within 2 h of spawning and spermatozoa, within 6 h. Unless indicated otherwise, experiments with *L. pictus* were conducted at room temperature (21–23°C) and with *S. purpuratus*, at 12°C.

Seawater Formulations: Unless noted otherwise, the artificial seawater formulation (ASW) used consisted of 423 mM NaCl, 9 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 $NaHCO_3$, and 10 mM Tris. In OCaSW, the $CaCl_2$ was deleted from ASW. The pH was adjusted to 8.0 for all experiments with *S. purpuratus* and for the egg fertilization experiment with *L. pictus*. All other experiments with *L. pictus* were conducted at pH 7.8. For some experiments, chelators (with or without heavy metals) were added to ASW, and EDTA-ASW denotes ASW to which 1 mM EDTA was added.

Egg Jelly Preparation: Immediately after the eggs were spawned into Tris-free ASW (ASW that lacked Tris but was adjusted to pH 8.0), they were allowed to settle to the bottom of a beaker, and most of the seawater supernatant was decanted and discarded. Then the eggs were resuspended to a 10–20% egg suspension, the pH was adjusted with HCl to 4.5–5.0, and the suspension was gently swirled for 3 min. The eggs were then pelleted by hand centrifugation, the supernatant (egg jelly) was decanted, and the pH was adjusted to 7.8 (for *L. pictus*) or 8.0 (for *S. purpuratus*) with 0.1 N NaOH. The egg jelly was then centrifuged (12,000 *g*, 4°C, 30 min) to remove any particulate material, the supernatant was decanted, and the fucose content was determined according to the procedure of Spiro (19).

Sperm Acrosome Reaction Assays: Unless indicated otherwise, acrosome reactions were initiated by the dilution of 0.6 μl semen into 250 μl of ASW (± egg jelly, chelators, or other reagents). The egg jelly concentrations used were 11 μg and 8 μg fucose equivalents per milliliter, respectively, for *L. pictus* and *S. purpuratus*. The sperm were incubated 2 min (*L. pictus*) or 3 min (*S. purpuratus*) before being fixed with 17% formalin (250 μl sperm suspension plus 50 μl formalin). The sperm samples were then stored at 4°C for 16–28 h, placed on collodion- and carbon-coated grids for electron microscopy, washed with deionized water, air dried, and then scored (unstained) with transmission electron microscopy (× 4,000–7,500). 100 spermatozoa were counted for each sample, and only those with clearly formed acrosomal filaments were scored as acrosome reacted.

Fertilization Assays: To determine whether the presence of EDTA would alter the concentration of sperm required to fertilize *L. pictus* eggs, we added *L. pictus* spermatozoa (25 μl of a 40× stock suspension) to 1 ml of a 1% egg suspension. The sperm and eggs were both suspended in either ASW or EDTA-ASW, and the 40× stock suspension of sperm was made <30 s before addition of the sperm to the eggs. The final sperm concentrations (after addition to eggs) varied from 1 μl semen/10 ml to 1 μl semen/10^4 ml. 5 min after the sperm were added, the eggs were fixed by the addition of 100 μl formalin and scored (by phase contrast microscopy) for elevation of fertilization membranes. Experiments were done in triplicate, and 100 eggs were counted for each sample.

Elemental Analysis of Sea Urchin Spermatozoa: ASW, jelly, and sperm samples were dried in platinum crucibles at 100°C, and then ashed at 450°C. If a black deposit (carbon) remained, 20–50 μl of 30% H_2O_2 was added, and the sample was reheated to 450°C. If needed, the H_2O_2 addition and the reheating were repeated two more times, after which little carbon remained in any of the samples. 5 ml of ASW and 1–2 ml of each sperm sample were ashed. For the sperm washed in ASW, 1–2 ml of semen was diluted 50-fold into ASW, centrifuged (1,200 *g*, 20 min, 4°C), and the pellet was dried and ashed as indicated.

The ashed samples (25 mg each) were then analyzed for elemental composition by D.C. arc optical emission spectroscopy (20). The emission spectrum of each sample was examined for the presence of cations with atomic numbers 3–33, 38–42, 46–52, 56–58, 72–75, 79–83, and 90. The approximate concentration of each element was estimated from the intensity of its emission line(s). For a qualitative analysis, this method has an unequaled average sensitivity for these 60 elements, considering the small amount of sample available for analysis (20).

EDTA-buffered ASW Formulations: To adjust free metal ion concentrations, 1 mM EDTA was added to ASW (EDTA-ASW), and varying levels of metal ions were added. The MINEQL computer program (21), which simultaneously calculates the concentrations of all programed EDTA-metal complexes and inorganic ion complexes, was used to determine both the maximum free concentration of each ion possible in ASW, and the total amount of each ion that had to be added to EDTA-ASW to produce the desired free ion concentrations. The EDTA stability constants for Ca^{+2}, Cu^{+2}, Fe^{+3}, Mg^{+2}, Ni^{+2}, and Zn^{+2} were obtained from Anderegg (22), and constants for Al^{+3} and TiO^{+2} were from the MINEQL program (21). Where applicable, both metal-EDTA and metal hydroxide–EDTA constants were utilized. The constants for complexes of metal ions with other inorganic ions [e.g., $ZnCO_3$, $Fe(OH)_3$, etc.] are part of the basic MINEQL program. All constants were corrected to ionic strength 0.5 with MINEQL's activity correction subroutine. To compensate for pH, we used H^+-EDTA constants of 9.94 and 15.8 for complexes of 1 or 2 H^+ per EDTA, respectively.

The respective concentrations (μM) of Zn^{+2} and Ni^{+2} that were added (as $ZnCl_2$ or $NiCl_2$) to EDTA-ASW to produce free ion concentrations of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M were: Zn^{+2}, 20.9, 174, 665, and 949 μM; Ni^{+2}, 756, 965, 994, and 998 μM. Cu^{+2} was not soluble at 10^{-7} M; however, 10^{-9} and 10^{-8} M free Cu^{+2} were prepared by the addition of 884 and 986 μM, respectively, of $CuCl_2$ to EDTA-ASW. For some experiments, 1,010 μM $NiCl_2$ or $CuCl_2$ was added to EDTA-ASW, thus assuring that the maximum solubility was exceeded and therefore the maximum free ion concentration was obtained (1.2×10^{-8} M free Cu^{+2} and 2.1×10^{-6} M free Ni^{+2}). After the ASW, EDTA, and metals were mixed together, the pH was adjusted to 7.8 for *L. pictus* experiments and 8.0 for *S. purpuratus*.

Measurement of pH_i: The fluorescent amine, 9-aminoacidine (9AA), was used to monitor changes in the pH_i of sea urchin spermatozoa. As with other permeable amines, 9AA accumulates within cells or cellular compartments that are more acidic than the extracellular medium (in this case, ASW). This accumulation is proportional to the pH gradient, and 9AA has been used to monitor the pH_i of both *L. pictus* (3) and *S. purpuratus* (1, 23) spermatozoa.

The protocol used here was the same as that reported by Clapper and Epel (24) and Lee et al. (3). In summary, the fluorescence of a 2-ml solution of 9AA (2.5 μM) in ASW was measured in a fluorometer, then semen (5 μl) was added, and the change in fluorescence was monitored as 9AA equilibrated across the sperm membranes. The percent decrease in fluorescence (quenching) is proportional to the pH gradient between the spermatozoa and ASW (i.e., is proportional to the pH_i of the spermatozoa). At the end of each experiment, the pH gradient was discharged by the addition of monensin (a Na^+/H^+ ionophore) or monensin followed by Triton X-100. 9AA experiments with *L. pictus* and *S. purpuratus* were conducted at 19° and 12°C, respectively.

Although 9AA is convenient to use, it has the disadvantage of binding to the spermatozoa (probably to DNA) in addition to being concentrated via its distribution due to the pH gradient (1). In *S. purpuratus*, this binding is reversible and not saturated at the concentration of 9AA used in this study; therefore, the net effect of binding is to magnify the pH-dependent accumulation of 9AA within the spermatozoa. We used the calculation method of Christen et al. (1) to compensate for the 9AA binding and to allow the sperm pH_i in various seawater formulations to be determined. Although this method was verified only with *S. purpuratus* sperm, its application to *L. pictus* produced a similar pH_i in OCaSW (pH_i = 7.5; results of this study) to that measured by ^{31}P-NMR (pH_i = 7.6; reference 2).

Chemicals: Monensin was purchased from Calbiochem-Behring Corp. (La Jolla, CA). All other chemicals were of standard reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO), Baker, J. E., Chemical Co. (Phillipsburg, NJ), and Mallinkrodt, Inc. (St. Louis, MO).

RESULTS

Chelators Inhibit Jelly-initiated Acrosome Reactions and Fertilization

Results of this study confirm and extend the report of Johnson and Epel (12) and show that sea urchin acrosome reactions are inhibited by a variety of metal chelators. In triplicate experiments, dilution of *L. pictus* sperm into egg jelly that contained 0.1 mM EDTA, phenanthroline, dipyridyl, cysteine, or dithiothreitol produced only 3–7% acrosome reactions (compared to 71% in controls with egg jelly that lacked chelators, and a background of 2.3% acrosome reac-

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, phenanthroline, 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 $1:10^5$ was required in EDTA versus a $1:10^6$ dilution in the controls).

The chelators used are structurally dissimilar: EDTA coordinates metals with carboxyl groups; phenanthroline (1,10-phenanthroline) and dipyridyl (α,α'-dipyridyl) use N atoms embedded in otherwise uncharged aromatic rings; and cysteine and dithiothreitol use sulfhydryl 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 Ca^{+2} or Mg^{+2} 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^+, Ca^{+2}, and Mg^{+2} 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. Ba^{+2} and Sr^{+2} have lower affinities to the chelators than do Ca^{+2} and Mg^{+2} and would also not undergo a significant concentration change. The remaining elements have higher affinities to EDTA than do Ca^{+2} and Mg^{+2}, 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.

TABLE I

Semiquantitative Elemental Analysis of Sea Urchin Spermatozoa and Egg Jelly

	Cu	Ni	Zn
ASW	6	1	<15*
L. pictus			
Semen	20	<1*	20
Sperm washed in ASW	15	<1	<15
Jelly in ASW	5	<1	<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.

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

EDTA Removed ^{65}Zn from *L. pictus* Spermatozoa

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

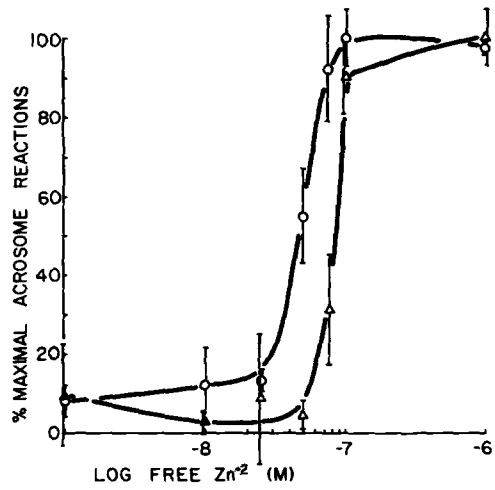

FIGURE 1 Zn^{+2} reversed EDTA inhibition of acrosome reactions in *L. pictus*. Semen (0.6 μl) was added to 250 μl of EDTA-ASW that contained sufficient Zn^{+2} to produce free Zn^{+2} concentrations of 10^{-9}–10^{-6} M. The Zn^{+2} solutions were prepared as indicated in Materials and Methods. Experiments with EDTA-ASW (that contained no added Zn^{+2}) produced results similar to those seen at 10^{-9} M. (The total contamination level of Zn^{+2} in ASW was determined by atomic absorption spectrometry to be 15 nM, which produced 7×10^{-13} M free Zn^{+2} in EDTA-ASW.) Experiments with solutions that contained (○) and lacked (△) egg jelly (11 μg fucose equivalents/ml) are plotted. Although Zn^{+2} initiated acrosome reactions in the absence of egg jelly, the Zn^{+2} threshold was lower in the presence of egg jelly. For a more accurate comparison of the Zn^{+2} thresholds, results are expressed as percentage of maximal acrosome reactions. Maximal acrosome reactions initiated in the presence and absence of egg jelly were 71 and 49%, respectively. Each point is the average of three determinations, and the bars indicate standard deviations. The difference in Zn^{+2} thresholds for these two experiments is not due to Zn^{+2} contributed by the egg jelly; the Zn^{+2} contributed by egg jelly (determined by atomic absorption spectrometry to be 65 nM total Zn) would produce an insignificant change (< 0.1%) in the level of free Zn^{+2} when added to EDTA-ASW that contained 8×10^{-8} M free Zn^{+2} (600 μM total Zn^{+2}).

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_i of Sea Urchin Spermatozoa

In sea urchin sperm, pH_i has previously been shown to increase upon sperm dilution into seawater (2), and this increased pH_i 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_i.

Using 9AA to monitor the pH_i, we found that *S. purpuratus* sperm diluted into ASW that contained EDTA had a lower pH_i than did spermatozoa diluted into media that lacked EDTA (Fig. 4*A*). The pH_i 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_i by 0.24 pH unit. This pH_i depression by EDTA was ~75% prevented by addition of Zn^{+2}.

In *S. purpuratus*, the effect of EDTA on pH_i (and its reversal by 10^{-7} M Zn^{+2}) is easily demonstrated in ASW (Fig. 4*A*).

TABLE II

Only Zn^{+2} Reversed Chelator Inhibition of Acrosome Reactions

	Log_{10} free metal ion concentration	Acrosome reactions	SD	*n*
	M	%		
I. *L. pictus*				
ASW control (without jelly or EDTA)	-	6.6	7.6	5
ASW + jelly (no EDTA)	-	72	12	5
ASW + jelly + EDTA (1 mM)	-	2.6	0.9	5
ASW + jelly + EDTA plus:				
Cu^{+2}	−9	1.3	0.6	3
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	3.2	3
Ni^{+2}	−5.68*	3.0	2.6	3
Zn^{+2}‡	−7	71	5	3
II. *S. purpuratus*				
ASW control (without jelly or EDTA)	-	0	0	2
ASW + jelly (no EDTA)	-	92	1.4	2
ASW + jelly + EDTA (1 mM)	-	28	6.4	2
ASW + jelly + EDTA plus:				
Zn^{+2}	−7	91	3	2
Zn^{+2}	−6	34	33	2
Cu^{+2}	−8	18	5	2
Ni^{+2}	−5.68*	23	11	2

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.

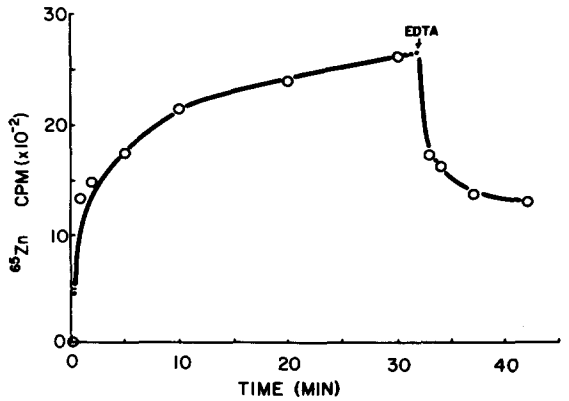

FIGURE 2 EDTA removes ^{65}Zn from *L. pictus* spermatozoa. ^{65}Zn (1 μCi/ml, 5 μM total Zn^{+2}, or 3.5 μM free Zn^{+2}) was added to a 1% sperm suspension in ASW (○). At the indicated times, triplicate 200-μl aliquots were removed and centrifuged (15,000 *g*, 45 s) through 300 μl diisobutylphthalate to remove the extracellular ^{65}Zn. With this procedure, the ASW (that contained the unbound ^{65}Zn) stayed on top of diisobutylphthalate, and the sperm passed through to form a pellet at the bottom of the centrifuge tubes).The ASW and diisobutylphthalate 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 ^3H_2O and [^{3}H]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 $^{65}Zn/\mu$l 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. 4*B*, 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_0 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. 4*B* as compared with Fig. 4*A*.]

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_4Cl 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_4Cl and monensin raise the sperm pH_i and reverse the effects of EDTA on both motility initiation and acrosome reactions. As shown in Fig. 4*A*, NH_4Cl (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_4Cl reversed ~50% of the

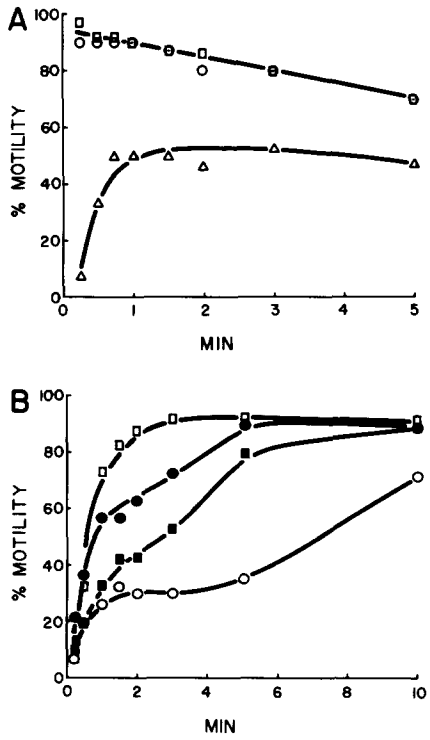

FIGURE 3 EDTA delays motility initiation in sea urchin spermatozoa. Semen (0.5 μl) was added to 500 μl of ASW or EDTA-ASW (± Zn^{+2}). Aliquots (15 μl) were removed at the indicated times and placed on a microscope slide (without a coverslip). The microscope was focused in the middle of the drop (to avoid surface effects) and viewed at 100× with a phase contrast microscope, and the percentage of motile sperm was estimated. Each point is the average of three determinations. (*A*) *L. pictus* 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} (□).

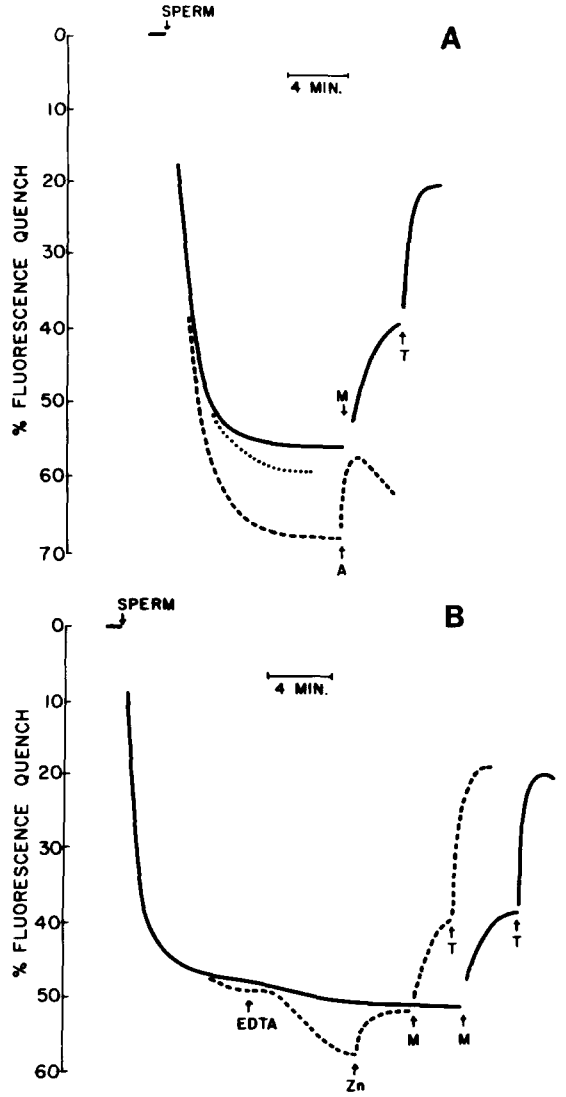

FIGURE 4 EDTA depresses the pH_i of *S. purpuratus* sperm, and Zn^{+2} reverses this depression. Each curve shows the initial fluorescence of 9AA in ASW (± EDTA or Zn^{+2}), then semen (5 μl) was added at the arrows marked *sperm*. Since the sperm pH_i is more acidic than ASW, the addition of spermatozoa caused 9AA to be concentrated within the spermatozoa, thus producing the observed fluorescence quenching for each curve. (*A*) The fluorescence quenching was greater in ASW that contained 1 mM EDTA (- - -) than in ASW that lacked EDTA (——), thus indicating a lower sperm pH_i in the presence of EDTA. Dilution of sperm into ASW that contained 1 mM EDTA plus Zn^{+2} (· · ·) reversed most of the EDTA suppression of pH_i; identical curves were produced by both 10^{-7} and 10^{-6} M free Zn^{+2}. At the end of each experiment, the pH gradient was discharged by the addition of 50 μM monensin (arrow marked *M*), then 0.005% Triton X-100 (arrow marked *T*). At the arrow marked *A*, 10 mM NH_4Cl was added. At 5 min after the addition of sperm, 0–5% acrosome reactions and 90–95% motility were observed in all three media. (*B*) Two experiments are shown: With the control (——), spermatozoa were added to ASW (that contained 9AA) and monitored for 21 min before addition of monensin (*M*) and Triton X-100 (*T*). In the experimental (- - -), spermatozoa were allowed to equilibrate in the same medium for 8 min before the addition of 100 μM Ca-EDTA (pH 8.0, arrow marked *EDTA*). This produced an intracellular acidification that was reversed by the subsequent addition of 65 μM $ZnCl_2$ (arrow marked *Zn*). This produced a free Zn^{+2} concentration of $\sim 10^{-7}$ M; addition of 95 μM Zn^{+2} (to produce $\sim 10^{-6}$ free Zn^{+2}) produced a nearly identical result. The additions of Ca/EDTA and $ZnCl_2$ produced extracellular pH changes of < 0.01 pH unit.

EDTA depression of pH_i (data not shown) which was followed by a more rapid re-acidification than was observed with *S. purpuratus*. Monensin also alkalinized spermatozoa of both species, but with slower kinetics (e.g., Fig. 4*A*).

Incubation in NH_4Cl 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 NH_4Cl and monensin reversed EDTA inhibition of sperm motility, with NH_4Cl 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 NH_4Cl 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 NH_4Cl (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_i (see, e.g., Fig. 4*A*).

High Zn^{+2} Also Raises pH_i in L. pictus But Not in S. purpuratus

As noted earlier, high Zn^{+2} (≥1 μM free Zn^{+2}) 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 Zn^{+2} to spermatozoa also produced an increase in pH_i in *L. pictus* (Fig. 5*A*), but not in *S. purpuratus* sperm (Fig. 5*B*). With *L. pictus*, Zn^{+2} alkalizes spermatozoa even in OCaSW (where no acrosome reactions occur because of the absence of Ca^{+2}); this indicates that Zn^{+2} initiates changes that precede the Ca^{+2}-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_i in sea urchin sperm, and via pH_i, 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

TABLE III

NH_4Cl Reversed Chelator Inhibition of Sea Urchin Acrosome Reactions

	L. pictus		*S. purpuratus*	
	%	*SD*	%	*SD*
ASW + egg jelly	52	26	92	1.4
ASW + mM EDTA	0.3	0.6	0	0
ASW + EDTA + jelly	4.5	2.1	28	6.4
ASW + EDTA + jelly plus:				
10 mM NH_4Cl	86	3	94	5
50 μM monensin	53	24	–	–
20 μM monensin	18	14	–	–
ASW + EDTA plus:				
10 mM NH_4Cl	3.3	4.2	1	1.4
50 μM monensin	22	8	–	–
20 μM monensin	9.7	10.5	–	–

The protocol was the same as that indicated in Table II. Experiments were done in duplicate, and percentage acrosome reactions and standard deviations are listed. –, Not determined.

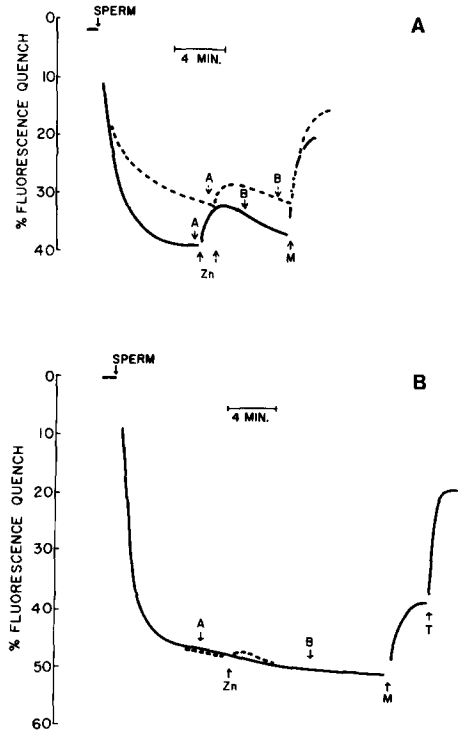

FIGURE 5 High levels of Zn^{+2} produced an increase in the pH_i and initiated acrosome reactions in *L. pictus* spermatozoa but not in *S. purpuratus* spermatozoa. 9AA was used to monitor pH_i as described in Materials and Methods and Fig. 4. (*A*) *L. pictus* spermatozoa. Spermatozoa were equilibrated in ASW (——) or OCaSW (- - -) before the addition of 20 μM $ZnCl_2$ (arrows marked *Zn*). Respective percentage acrosome reactions for ASW and OCaSW were 2% and 0% at *A* and 51% and 2% at *B*. Monensin (20 μM) was added at *M*. (*B*) *S. purpuratus* spermatozoa. In the control (——), spermatozoa were equilibrated in ASW for 21 min before addition of 50 μM monensin (*M*) and 0.005% Triton X-100 (*T*). In the experimental (- - -), 20 μM $ZnCl_2$ was added at the arrow marked *Zn*, and the pH_i monitored. No acrosome reactions were observed at *A* for either experiment or *B* for the Zn^{+2} experiment.

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 $^{65}Zn^{+2}$ 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 alkalinization is caused by the release of H^+ ions from sperm, the pH 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 μM in the absence of egg jelly, acrosome reactions were initiated in *L. pictus* but not in *S. purpuratus*. Indeed, 10–30 μM total Zn^{+2} inhibited jelly-initiated acrosome reactions in *S. purpuratus*, whereas

identical levels directly initiated acrosome reactions in *L. pictus*. In both species, the chelator and low Zn^{+2} effects are best explained by their effect on pH_i, and in *L. pictus* sperm, high Zn^{+2} may initiate acrosome reactions by its ability to increase pH_i above the normal resting level (Fig. 5). However, in *S. purpuratus* sperm, high Zn^{+2} produces no change in pH_i 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_i 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 would presumably act by altering the free Zn^{+2} concentration in the medium and thus remove Zn^{+2} from either external or internal sperm sites.

Zn^{+2} removal could depress the sperm pH_i 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 alkalinization mechanism that has been described in sea urchin sperm (1, 3, 27, 28) does not seem to be involved. When *S. purpuratus* spermatozoa were diluted into Na^+-free sea water and 10 mM NaCl was added to increase the pH_i and activate motility (references 1 and 3), the kinetics of the pH_i increase and the final equilibrium pH_i 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_i (e.g., Zn^{+2} removal might alter the membrane potential which could secondarily depress the pH_i); or (*c*) acting through a Zn^{+2}-dependent enzyme system that controls the sperm pH_i. [pH_i 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_i regulation of sperm and perhaps other cell types as well.

This work was supported by a grant from the National Science Foundation.

We thank Dr. George Knauer for measuring the levels of Zn in ASW and egg jelly, and Dr. Hon Cheung Lee for critical reading of the manuscript.

Received for publication 28 July 1983, and in revised form 2 January 1985.

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