Journal of Cellular Biochemistry 30:181–193 (1986)
Biochemical and Molecular Epidemiology of Cancer 53–65

Copper Ions and Hydrogen Peroxide Form Hypochlorite From NaCl Thereby Mimicking Myeloperoxidase

Krystyna Frenkel, Fredric Blum, and Walter Troll

Department of Environmental Medicine, New York University Medical Center, New York, New York 10016

Sea urchins have elaborated multiple defenses to assure monospermic fertilization. In this work, we have concentrated on a study of the mechanism(s) by which hydrogen peroxide (H_2O_2) prevents polyspermy in *Arbacia punctulata*. We found that it is not H_2O_2 but probably hypochlorous acid/hypochlorite ($HOCl/OCl^-$) derived from H_2O_2 that is toxic to the supernumerary sperm. The spermicidal activity of H_2O_2 is potentiated by at least one order of magnitude by cupric ions (Cu^{2+}). This increased toxicity is not due to the formation of hydroxyl radicals ($\cdot OH$) because $\cdot OH$ scavengers did not counteract the activity of Cu^{2+}. Moreover, substitution of Cu^{2+} by ferrous ions (Fe^{2+}), which are known to cause formation of $\cdot OH$ from H_2O_2, had no effect on fertilization even at 10^2–10^3 times higher concentrations. In contrast, 3-amino-1,2,4-triazole (AT), an $HOCl/OCl^-$ scavenger, totally reversed the toxic effects of Cu^{2+}. Furthermore, we found that $HOCl/OCl^-$ is generated in solutions of H_2O_2 and Cu^{2+} in the presence of 0.5 M NaCl and that its accumulation is abolished by AT. Thus it is possible that the antifertility properties of copper are due to its ability to mediate formation of $HOCl/OCl^-$. $HOCl/OCl^-$ generated by Cu^{2+} from H_2O_2 and Cl^-, a low concentration of exogenously added $HOCl/OCl^-$, or increased concentrations of H_2O_2 has similar inhibitory effects on the fertilization process in sea urchins. Therefore, we suggest that polyspermy is prevented by the action of a myeloperoxidase that affects the formation of $HOCl/OCl^-$ from the Cl^- present in sea water through reaction with H_2O_2 generated by the newly fertilized egg.

Key words: sea urchins, copper ions, hydrogen peroxide, hypochlorous acid/hypochlorite, myeloperoxidase, spermicidal activity

The prevention of polyspermy is a complicated process [1]. Multiple defenses are necessary because fertilization by more than one sperm leads to an asymmetrical cleavage, which inevitably ends in the death of the embryo. In sea urchins, different mechanisms have developed to prevent polyspermic fertilization. The permanent block consists of raising a fertilization envelope (FE) that is totally impenetrable to

Received April 17, 1985; revised and accepted October 22, 1985.

the sperm. However, it takes up to 1 min to complete its formation. To protect eggs from supernumerary sperm during the time needed for completion of FEs, other, faster responses exist. These include a rapid, sodium-dependent electrical depolarization of the egg plasma membrane [2,3], various secretory products released by the egg's cortical granules [1,4], production of arachidonic acid oxidation products [5], and release of H_2O_2 [6–8].

In this work, we have concentrated on a study of the mechanism(s) by which H_2O_2 prevents polyspermy. It already has been shown that removal of H_2O_2 by incubation with catalase causes 100% polyspermy in *Arbacia punctulata* [6]. Similarly, soybean trypsin inhibitor, which prevents the release of H_2O_2 from eggs [6,9], also causes polyspermy [10]. These findings confirm the importance of H_2O_2 release to assure a monospermic fertilization. We have recently observed that Cu^{2+} interfered with the fertilization process by potentiating the effects of H_2O_2 [11]. H_2O_2 is generated by sea urchin eggs in response to a successful entry of the first sperm through the egg's membrane [6,7]. Transition metal ions are known to interact with H_2O_2. These interactions often lead to formation of more reactive activated oxygen species, such as $\cdot OH$ [12–16]. We thought it would be important to determine which (if any) active oxygen species is formed during the interaction of Cu^{2+} with H_2O_2 and, if possible, to determine its effect on fertilization.

The first hypothesis was that Cu^{2+} generates spermicidal $\cdot OH$ from H_2O_2. However, $\cdot OH$ scavengers did not counteract the activity of Cu^{2+}. Moreover, when Cu^{2+} was substituted by Fe^{2+}, which is known to cause formation of $\cdot OH$ from H_2O_2, Fe^{2+} had no effect on fertilization even at concentrations 10^2–10^3 times higher than that of Cu^{2+}. We then postulated that $HOCl/OCl^-$ was generated from H_2O_2 by Cu^{2+}. Exogenously added buffered $HOCl/OCl^-$ inhibited fertilization, whereas both the scavengers of $HOCl/OCl^-$ and catalase counteracted the activity of Cu^{2+} by increasing fertilization and polyspermy. We also found that $HOCl/OCl^-$ is indeed produced by the interaction of Cu^{2+} with H_2O_2 and Cl^- by using the formation of taurine chloramine as a measure of generated $HOCl/OCl^-$.

Based on these results, we concluded that the toxicity of Cu^{2+} ions to the fertilization process is due to the formation of $HOCl/OCl^-$ from Cl^- ions, which are present in high concentration in sea water and can be oxidized by H_2O_2 produced by the fertilized egg. Myeloperoxidase (MPO), an enzyme known to generate $HOCl/OCl^-$ from Cl^- and H_2O_2 by activated neutrophils [17–20], may be among the peroxidases present in gametes. Therefore, we suggest that $HOCl/OCl^-$ is generated by gametes through an MPO-mediated process as one of several mechanisms developed by sea urchins to prevent polyspermy.

MATERIALS AND METHODS

Materials

Arbacia punctulata sea urchins were supplied by the Marine Biological Laboratory (MBL) and kept in large glass tanks with flowing fresh sea water. Sea water was centrally distributed into MBL laboratories and filtered through Whatman No. 1 paper prior to use. Catalase, taurine, 3-amino-1,2,4-triazole, hydrogen peroxide, and mannitol were obtained from Sigma (St. Louis, MO), sodium hypochlorite from Aldrich (Milwaukee, WI) and Chelex 100 from Bio-Rad Laboratories (Rockville Centre, NY).

Methods

Fertilization experiments. Eggs and sperm were obtained by injection of 0.5 M KCl into the coelomic cavity of *A punctulata* as described by Coburn et al [6]. Fertilization was monitored microscopically and assessed by timing, morphology, and symmetry of cleavage. Standard experiments consisted of approximately 1×10^5 eggs fertilized with 1×10^7 sperm in 2 ml of filtered sea water. This ratio of eggs to sperm usually resulted in $\geq 90\%$ fertilization. Gametes were treated with different reagents for 30 sec just prior to fertilization.

Determination of HOCl/OCl$^-$ formation. Although HOCl/OCl$^-$ has a characteristic absorption at 292 nm, and therefore can be directly measured spectrophotometrically, its molar extinction coefficient of 3.5×10^2 is too low to be of use when only small amounts are present. Moreover, since H_2O_2 is produced during fertilization of sea urchins, the small amounts of HOCl/OCl$^-$ (if formed) would be readily reduced by H_2O_2 back to Cl^- and thus would go undetected. To circumvent these potential shortcomings, the method used by Weiss et al [18] for determination of HOCl/OCl$^-$ generated by another complex cellular system, human neutrophils, was adopted for this work. In this method, taurine is present in the reaction mixture and efficiently reacts with HOCl/OCl$^-$ as soon as it is formed. The resultant taurine chloramine is neither reduced nor oxidized by H_2O_2. However, its extinction coefficient of 3.98×10^2 is also too low and would not afford the required sensitivity. Since 1 mol of chloramine retains 2 mol oxidizing equivalents, its concentration can be sensitively determined by its ability to oxidize 2 mol of either 5-thio-2-nitrobenzoic acid to 1 mol of the disulfide or 2 mol of I^- to I_3^- [17,18]. Both methods show increased extinction coefficients of the products with $\epsilon = 1.36 \times 10^4$ for the former and $\epsilon = 2.29 \times 10^4$ for the latter. The oxidation of I^- was chosen because it has an ϵ almost twice as high as the other method. Overall, by using formation of taurine chloramine (from taurine and HOCl/OCl$^-$), and by coupling it with the oxidation of I^- to I_3^-, the sensitivity of the spectrophotometric method is increased by almost two orders of magnitude.

All assays were carried out in polypropylene tubes at 4°C. Glass distilled water and buffers were passed through Chelex 100 to remove adventitious transition metal ions. A standard reaction mixture of 1 ml consisted of 0.01 M taurine dissolved in 0.01 M potassium phosphate and 0.5 M NaCl buffer, pH 7.4, and was incubated with various concentrations of H_2O_2 and $CuCl_2$ for 10 min, after which time the reaction was stopped by the addition of 50 μg catalase. Catalase was included to prevent reduction of HOCl/OCl$^-$ by excess H_2O_2 [17–19]. After 5 min, KI was added to the final concentration of 20 mM, and, after an additional 5 min, absorbance at 350 nm, or spectra of liberated I_2 were recorded (Beckman, Model DU7). In other experiments, AT was incubated together with H_2O_2 and $CuCl_2$, or varying concentrations of NaOCl were used instead of H_2O_2 and $CuCl_2$. Appropriate blanks were prepared that contained all components except the one whose concentration was varied.

Two molecules of taurine chloramine are needed to liberate one I_2. Therefore, the measured amount of I_2 was multiplied by two to give an estimate of taurine chloramine formation. The standard curve of taurine chloramine formation by the exogenously added HOCl/OCl$^-$ was constructed. The efficiency of this reaction at different concentrations was used to calculate the total amount of HOCl/OCl$^-$ formed by $Cu^{2+}/Cl^-/H_2O_2$ mixtures and the percent of Cu^{2+} or H_2O_2 conversion to taurine chloramine.

RESULTS

The effects of Cu^{2+} ions on fertilization of *A punctulata* sea urchins are shown in Figure 1. The most pronounced inhibition of fertilization occurred between 1 and 5×10^{-7} M Cu^{2+}. In different experiments, values for inhibition by 6×10^{-7} through 1×10^{-6} M Cu^{2+} varied between 70% and 100%. As can be seen in Figure 1, addition of catalase restored fertilization even when the concentration of Cu^{2+} ions was as high as 10^{-4} M. These results show that in order to exert its activity Cu^{2+} must interact with H_2O_2.

Table I shows the effects on fertilization of Cu^{2+} alone, H_2O_2 alone, and their combination as measured by percent initial cleavage (at 45 min) and percent FEs. During the normal fertilization process, these two measures have the same values. However, with exposure to toxic agents, either cleavage or both cleavage and formation of FEs can be inhibited. For this reason, the occurrence of both was evaluated. Cu^{2+} alone had the strongest effect on eggs. Both cleavage and FEs were virtually abolished. Pretreatment of eggs with H_2O_2 inhibited cleavage at a higher concentration (5×10^{-4} M) but did not interfere with the formation of FEs. Pretreatment of eggs with a mixture of Cu^{2+} and H_2O_2 abolished both cleavage and the formation of FEs. When sperm were incubated with either Cu^{2+} or H_2O_2 prior to mixing with eggs, there was practically no effect on fertilization except at higher H_2O_2 concentrations (5×10^{-4} M) when some inhibition of cleavage occurred. However, mixtures of Cu^{2+} and H_2O_2 at all H_2O_2 concentrations tested, greatly reduced cleavage and abolished formation of FEs. These results indicate that another damaging agent might be formed from H_2O_2 by the action of Cu^{2+}. Since sperm do not produce H_2O_2, Cu^{2+} alone does not impair their ability to fertilize eggs. However, eggs are known to generate H_2O_2 [6,7]; therefore, Cu^{2+}-induced toxicity to eggs is probably caused by interaction with the endogenously formed H_2O_2. This is corroborated by the finding that incubation with catalase counteracted the effects of Cu^{2+} (Fig. 1).

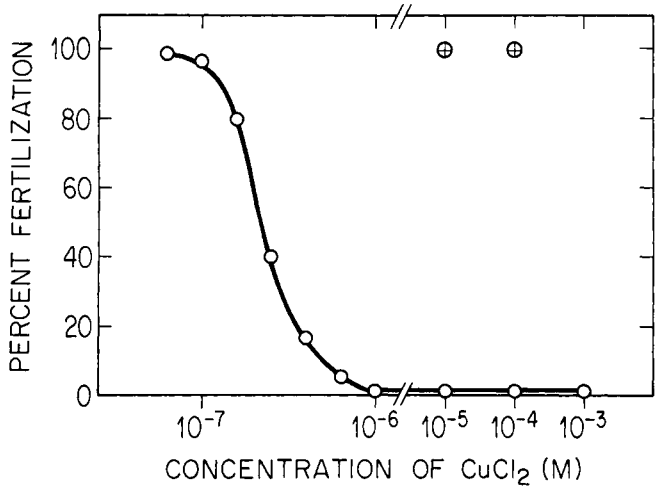

Fig. 1. Effect of Cu^{2+} on fertilization of *Arbacia punctulata* (○) and of Cu^{2+} in the presence of 1 mg catalase/ml (⊕).

TABLE I. Effects of Cu^{2+} Alone, H_2O_2 Alone, and Cu^{2+} in the Presence of H_2O_2 When Either Eggs (E) or Sperm (S) Were Pretreated Prior to Fertilization

Treatment	Pretreated gametes	Fertilization envelope (%)	Cleavage (%)
Control	—	100	100
Cu^{2+}	E	1–2	1–2
H_2O_2 (M)	S	>95	>95
5×10^{-5}	S	100	100
1×10^{-4}	E	100	>95
	S	100	100
5×10^{-4}	E	100	60
	S	100	40–80
1×10^{-3}	S	>95	0
H_2O_2/Cu^{2+}[a](M)			
5×10^{-5}	S	0	30
1×10^{-4}	S	0	6
5×10^{-4}	E	0	0
	S	0	0

[a]$Cu^{2+} = 1 \times 10^{-6}$ M.

TABLE II. Determination of Whether Spermicidal Activity of H_2O_2 Is Mediated by ·OH Radicals: Effects of Cu^{2+}, Fe^{2+}, and Cu^{2+} in the Presence of Mannitol, an ·OH Scavenger, on Fertilization

	Percent cleavage	
Treatment	−mannitol	+ 0.1 M mannitol
Control	96	87
Cu^{2+}(M)		
1×10^{-7}	95	88
2×10^{-7}	95	87
4×10^{-7}	85	20
8×10^{-7}	35	0
Control	80	
Fe^{2+}(M)		
1×10^{-7}	90	
1×10^{-6}	83	
1×10^{-5}	92	
1×10^{-4}	92	

Transition metal ions such as Fe^{2+} are known to produce ·OH from H_2O_2 in a Haber-Weiss reaction [12–14]. It has been assumed that Cu^{2+} also interacts with H_2O_2 in a similar manner. To test whether ·OH radicals were generated, eggs were fertilized in the presence of Cu^{2+} and mannitol, an ·OH scavenger [19,21]. Even at 0.1 M, mannitol did not reverse Cu^{2+}-induced inhibition of fertilization. On the contrary, it actually potentiated the toxic effects of Cu^{2+}, as can be seen in Table II. These results indicate that ·OH radicals are not formed by the action of Cu^{2+} and therefore could not be responsible for its toxic effects. To prove definitively whether ·OH are or are not involved in H_2O_2-induced effects on fertilization, eggs were fertilized in the presence of Fe^{2+} ions. As can be seen in Table II, Fe^{2+} ions did not

inhibit cleavage even at concentrations 10^2–10^3 times higher than that of Cu^{2+} ions, thus eliminating ·OH as a possible damaging agent.

It has been found that, like neutrophils, sea urchin eggs release arachidonic acid, oxidize it, and form prostaglandins and other cyclooxygenase-derived metabolites [5]. The inactivation of excess sperm by H_2O_2 released by a fertilized egg has been likened to the peroxidatic killing of bacteria by neutrophils [22,23]. It is known that neutrophils have several pathways that utilize H_2O_2 generated during the respiratory (oxidative) burst [20]. Among them, the MPO-halide-H_2O_2 system is prominent in inactivation of bacteria [20,23]. MPO catalyzes the oxidation of halide ions (mostly chloride since they are the most abundant) by H_2O_2 with the formation of the very powerful oxidant $HOCl/OCl^-$ [17,18,20,24]. Fertilization of sea urchin eggs occurs in sea water, which has a very high concentration of Cl^- ions (0.5–0.6 M). Therefore, we investigated the possibility that the MPO-halide-H_2O_2 system is responsible for the inactivation of the supernumerary sperm and that the interaction of Cu^{2+} with H_2O_2 in the presence of Cl^- mimics the MPO system. It is already known that the inactivation of a sperm by H_2O_2 is catalyzed by a peroxidase [6,7]. However, it is not certain whether this particular peroxidase is located in the egg or in the sperm, although the most recent evidence points to the latter [23,25].

To determine whether $HOCl/OCl^-$ could be formed by the interaction of Cu^{2+} with H_2O_2 released by the fertilized egg, fertilization was carried out in the presence of Cu^{2+} and AT, which is known to scavenge $HOCl/OCl^-$ [18]. Although AT also inhibits ovoperoxidase, this enzyme is thought not to participate in sperm inactivation [25]. As Table III shows, there is a dramatic reversal of Cu^{2+}-induced inhibition of fertilization as measured by both cleavage and FE formation. Furthermore, eggs fertilized in the presence of 10^{-5} or 10^{-6} M Cu^{2+} and 10 mM AT and left over night showed no apparent toxic effects.

To determine whether AT acted on the Cu^{2+}-induced intermediate or mediated H_2O_2-induced inhibition of fertilization, either eggs or sperm were preincubated with H_2O_2 in the presence or absence of 10 mM AT. In both cases, there were no significant

TABLE III. Effect of 3-amino,1,2,4-triazole on Cu^{2+}-Mediated Inhibition of Fertilization

Treatment	Fertilization envelope (at 5 min postfertilization, %)	Cleavage (at 45 min post-fertilization, %)
Control	99	99
AT (mM)		
10	100	96
100	99	92
Cu^{2+}(M)		
1×10^{-6}	0	0
5×10^{-6}	0	0
1×10^{-5}	0	0
Cu^{2+}(M) + AT (mM)		
$1 \times 10^{-6} + 1$	—	94
$1 \times 10^{-6} + 10$	99	99
$5 \times 10^{-6} + 10$	99	99
$1 \times 10^{-5} + 1$	—	94
$1 \times 10^{-5} + 10$	>95	99
$1 \times 10^{-6} + 100$	99	99

changes in H_2O_2 effects on fertilization, as measured by initial cleavage and FEs, proving that AT interacted with the Cu^{2+}-induced intermediate and scavenged it in situ before it could inactivate the sperm. These findings indicate that $HOCl/OCl^-$ might indeed have been formed through $Cu^{2+}/Cl^-/H_2O_2$ interaction. Next, we tested the influence of exogenously added $HOCl/OCl^-$ on fertilization. When sperm were pretreated with 7×10^{-4} M $HOCl/OCl^-$ buffered with sea water and then diluted to 1.7×10^{-5} M upon addition to the eggs there was no fertilization. When $HOCl/OCl^-$ was diluted to 7×10^{-6} M, fertilization, as measured by the formation of FEs, occurred at the level of 97% showing that there was no permanent effect on the sperm's ability to penetrate the egg's plasma membrane. However, there was no cleavage. Preexposure of eggs to $HOCl/OCl^-$ led to 60% fertilization, again with no apparent cleavage. The effects of the sea water-buffered $HOCl/OCl^-$ on cleavage were similar to those of Cu^{2+} and H_2O_2, thus strengthening the hypothesis that $HOCl/OCl^-$ is the active intermediate.

Hypochlorous acid/hypochlorite produced by the $MPO/Cl^-/H_2O_2$ system in stimulated neutrophils is known to interact with taurine, which is present in the cells, forming taurine chloramine [17,18]. If $HOCl/OCl^-$ is generated by $Cu^{2+}/Cl^-/H_2O_2$, then it also should interact with taurine. Taurine (1 and 10 mM), which by itself is relatively nontoxic, potentiated the toxic effects of Cu^{2+} and further inhibited fertilization from 66% (Cu^{2+} alone) to 28% and 3%, respectively. If taurine chloramine was formed, it would act as another powerful oxidizing agent. It has been shown that chloramines and chloramides are actually formed by the $MPO/Cl^-/H_2O_2$ system through $HOCl/OCl^-$ as an intermediate [17,18,26] and that they cause fragmentation of peptides and oxidation of bacterial components [17]. These destructive changes can be prevented by washing bacteria, which removes the chloramine derivatives [17]. Therefore, the results showing the potentiation of Cu^{2+} toxicity by taurine and the return of a capability to fertilize (as measured by FE formation) by diluting out $HOCl/OCl^-$ from pretreated sperm again point to the possibility that $HOCl/OCl^-$ is formed by the interaction of Cu^{2+} with H_2O_2.

To show unambiguously that $HOCl/OCl^-$ can be generated, H_2O_2 was incubated with Cu^{2+} and Cl^- ions in the presence of taurine. As soon as it is formed, $HOCl/OCl^-$ interacts with taurine producing taurine chloramine [17,18]. Chloramine has a characteristic absorption maximum at 250 nm; however, its molar extinction coefficient of about 400 is too low for quantitative analysis. Since it is a powerful oxidizing agent, it was allowed to oxidize KI to I_3^-, whose maximum is at 350 nm with a molar extinction coefficient of 2.29×10^4 [17,18]. Figure 2 shows that incubation of H_2O_2 with Cu^{2+} and Cl^- ions (——) generated the same product as commercially available $HOCl/OCl^-$ (- - - -). The formation of this product was abolished when any of the three substrates was omitted from the reaction mixture. When incubation was carried out in the presence of 10 mM AT, an $HOCl/OCl^-$ scavenger [18], there was no absorption at 350 nm either. To determine whether $\cdot OH$, a strong oxidizing agent, would also cause production of I_3^-, Cu^{2+} was substituted by Fe^{2+}, which is known to generate $\cdot OH$ from H_2O_2. Since there was no absorption maximum at 350 nm, it is concluded that in this system, $\cdot OH$ is not capable of generating I_3^-. All these results paralleled the findings obtained during fertilization of sea urchins. Therefore, it is concluded that Cu^{2+} indeed generated $HOCl/OCl^-$ from H_2O_2 in the presence of Cl^- ions and thereby mimics the action of myeloperoxidase.

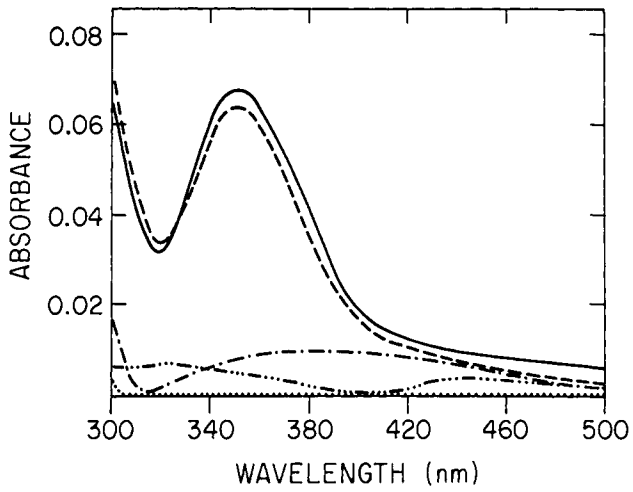

Fig. 2. Generation of I_3^- by taurine chloramine obtained by incubation of taurine dissolved in 0.01 M phosphate, 0.5 M NaCl buffer, pH 7.4, with: 1) synthetic 15 nmole $HOCl/OCl^-$/ml (- - - -), 2) 100 nmole Cu^{2+}/ml and 100 nmole H_2O_2/ml (——), 3) 100 nmole Cu^{2+}/ml in the absence of H_2O_2 (· · ·), 4) 100 nmole H_2O_2/ml in the absence of Cu^{2+} (-·-), 5) 100 nmole Cu^{2+} and 100 nmole H_2O_2 in the absence of Cl^- (-· · ·-).

Figure 3 shows that formation of $HOCl/OCl^-$ is linear between 10 and 100 nmole H_2O_2/ml reaction mixture when 100 nmole of Cu^{2+} is used and that it can be extrapolated to 0. Similarly, it is linear between 10 and 100 nmole Cu^{2+}/ml when 100 nmole H_2O_2 is used, but its slope is different. The lowering of Cu^{2+} concentration below 10 nmole/ml still results in measurable formation of $HOCl/OCl^-$. However, these values are located on a line with a different slope, which can be linearly extended to 0. These results suggest that there is no threshold for $HOCl/OCl^-$ formation even when minute amounts of H_2O_2 and Cu^{2+} are present. Figure 3 also shows that, when the synthetic $HOCl/OCl^-$ is used, formation of taurine chloramine coupled with oxidation of KI is also linear. However, under the conditions of this assay, the reaction did not go to completion. The yield of this reaction varied from 50% when 5 nmole $HOCl/OCl^-$/ml was used to 30% at higher concentrations (Table IV). The same reaction yields were assumed for $HOCl/OCl^-$ generated by a $Cu^{2+}/Cl^-/H_2O_2$ system. As Figure 4 shows, approximately 20% of H_2O_2 is converted into $HOCl/OCl^-$ by 100 nmole Cu^{2+}/ml regardless of H_2O_2 concentration. In contrast, when the concentration of H_2O_2 used for the reaction was held constant at 100 nmole/ml and that of Cu^{2+} was varied, the percent of H_2O_2 converted into $HOCl/OCl^-$ also varied from >50% for low concentrations of Cu^{2+} (2.5–10 nmole) down to 20% for 100 nmole Cu^{2+}. The comparison of the percent conversion into $HOCl/OCl^-$ by varying concentrations of H_2O_2 and Cu^{2+} shows that low concentrations of Cu^{2+} generate $HOCl/OCl^-$ more efficiently than low concentrations of H_2O_2. Furthermore, it seems that concentrations of Cu^{2+} higher than equimolar concentrations of H_2O_2 would suppress production of $HOCl/OCl^-$.

It is possible that the generation of $HOCl/OCl^-$ during interaction of Cu^{2+} with H_2O_2 and Cl^- involves reduction of Cu^{2+} to Cu^+. In such cases, $HOCl/OCl^-$, which is a strong oxidizing agent, might reoxidize some of the Cu^+ back to Cu^{2+}.

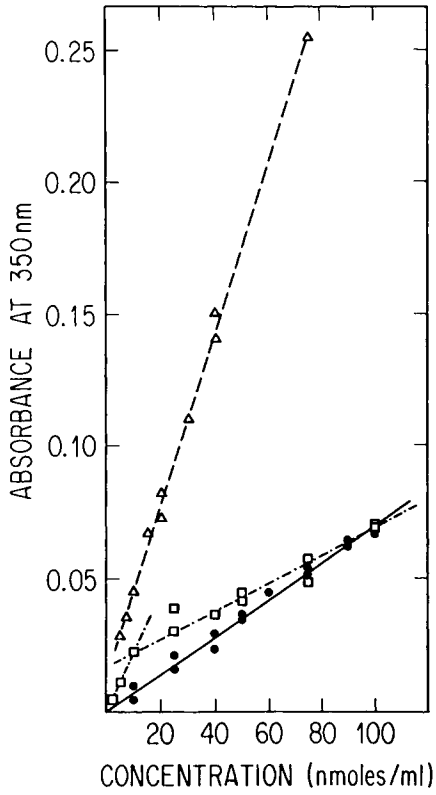

Fig. 3. Generation of I_3^- by taurine chloramine obtained by incubation of taurine dissolved in 0.01 M phosphate, 0.5 M NaCl buffer, pH 7.4, with varying concentrations of: 1) synthetic $HOCl/OCl^-$ (△), 2) H_2O_2 in the presence of 100 nmole Cu^{2+}/ml (●), and 3) Cu^{2+} in the presence of 100 nmole H_2O_2/ml (□).

Thus, in the presence of taurine, both production of taurine chloramine and reoxidation of Cu^+ may compete for $HOCl/OCl^-$. Therefore, the measured value would represent a net formation of $HOCl/OCl^-$. To determine the feasibility of this hypothesis, we had to prove that $HOCl/OCl^-$ is capable of oxidizing Cu^+ in the presence of taurine. Incubation of buffered $HOCl/OCl^-$ (15, 30, or 60 nmole/ml) with Cu^+ (100 nmole/ml) and taurine (10 μmole/ml) in 0.01 M phosphate, 0.5 M NaCl, pH 7.4 buffer resulted in about 20% inhibition of taurine chloramine formation, whereas the same amount of Cu^{2+} was without effect. Preincubation of the same concentrations of $HOCl/OCl^-$ and Cu^+ at 4°C for 10 min prior to addition of taurine resulted in 85%, 59%, and 53% reduction of taurine chloramine formation, respectively. These results show that $HOCl/OCl^-$ has a potential to oxidize Cu^+ in a dose-dependent manner. The amount of Cu^+ oxidized apparently depends on the ratio of $HOCl/OCl^-$ to Cu^+ in the absence of taurine. When taurine is present in the incubation mixture from the beginning of the incubation, the percent of Cu^+ that can be oxidized remains constant. When H_2O_2 (100 nmole/ml) was incubated with Cu^+, Fe^{2+}, or Fe^{3+} (100 nmole/ml) and taurine (10 μmole/ml) in the same buffer, there was no generation of taurine chloramine. All these results prove that it is Cu^{2+} that is needed to generate

TABLE IV. Efficiency of Taurine Chloramine Formation From Synthetic HOCl/OCl$^-$ and Taurine

Amount of HOCl/OCl$^-$ used (nmole)	$A_{350} \times 10^{-3}$[a]	HOCl/OCl$^-$ equivalents measured (nmole)[b]	Reaction efficiency[c]
5	29.0	2.53	0.51
7.5	37.5	3.28	0.44
10	47.0	4.10	0.41
15	62.5	5.46	0.36
20	78.5	6.86	0.34
25	95.0	8.30	0.33
30	111.0	9.70	0.32
40	145.0	12.66	0.31
50	176.0	15.37	0.31
75	257.0	22.45	0.30

[a]A_{350} values were obtained from Figure 3 (△).
[b]Iodine formation was calculated from molar extinction coefficient $\epsilon = 2.29 \times 10^4$ at 350 nm. To obtain HOCl/OCl$^-$ equivalents, that value was multiplied by 2 because two molecules of taurine chloramine derived from two molecules of HOCl/OCl$^-$ are needed to produce one molecule of iodine.
[c]Reaction efficiency was obtained by dividing HOCl/OCl$^-$ equivalents by the amount of HOCl/OCl$^-$ actually used.

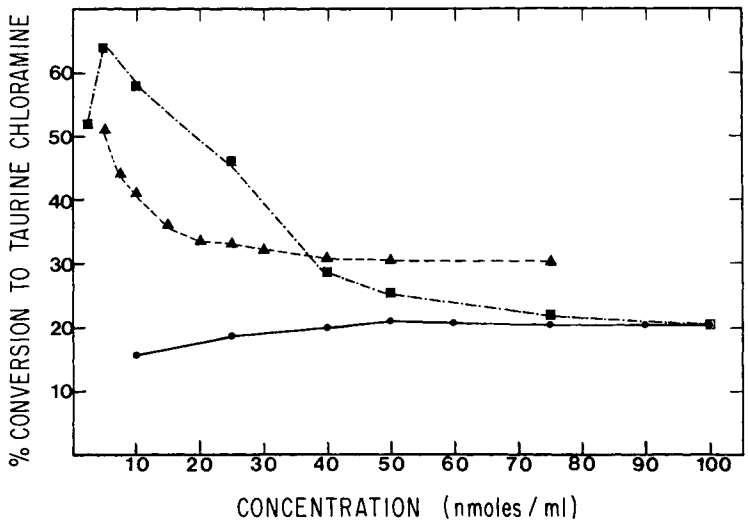

Fig. 4. Efficiency of conversion of taurine to taurine chloramine by HOCl/OCl$^-$. Reactions were carried out in 0.01 M phosphate, 0.5 M NaCl buffer, pH 7.4, with varying concentrations of: 1) synthetic HOCl/OCl$^-$ (▲), 2) H_2O_2 in the presence of 100 nmole Cu^{2+}/ml (●), and 3) Cu^{2+} in the presence of 100 nmole H_2O_2/ml (■).

HOCl/OCl$^-$ from H_2O_2 and Cl$^-$ and that during such a process Cu^{2+} is reduced to Cu^+. In turn, some of this Cu^+ is reoxidized to Cu^{2+} by HOCl/OCl$^-$, thus lowering the final yield of HOCl/OCl$^-$.

DISCUSSION

It has been known for a long time that copper is an essential element for normal metabolism [27] and that it also plays a role in inflammation [28,29]. It is thought

that some of its effects are due to interference with the biosynthesis of prostaglandins [30] and also with the ability to form complexes with a variety of ligands [28,29]. There are a number of postulated mechanisms that attempt to explain the various activities of copper. Among others, it is suggested that copper effectively scavenges the superoxide anion radical [31–33], which is associated with inflammation [34,35]. Depending on the valence, it is supposed to convert superoxide to either molecular oxygen (Cu^{2+}) or to H_2O_2 (Cu^{+} and Cu^{2+}) [36,37]. Therefore, we originally anticipated that the effect of Cu^{2+} on the fertilization of sea urchins was also due to its superoxide dismutase-mimetic capability. To our surprise, instead of generating H_2O_2, Cu^{2+} caused its disappearance. In this work, we proved that Cu^{2+} interacts with H_2O_2 in the presence of Cl^- and forms $HOCl/OCl^-$, a product known to be formed by MPO from H_2O_2 and Cl^-. Thus, we show that, in addition to mimicking superoxide dismutase, it also is capable of mimicking myeloperoxidase.

Sea urchins have developed multiple defenses to prevent polyspermic fertilization [1]. We and others have previously shown that rapid generation of H_2O_2 by the newly fertilized egg is a very effective defense mechanism [6,7]. The unresolved question left was how this H_2O_2 interferes with the ability of supernumerary sperm to refertilize the egg. It appears that the amount of H_2O_2 formed is very high—10 pmole H_2O_2 per egg [7]. Since 0.1 ml of packed eggs ($\sim 1 \times 10^5$) produces 1 μmole H_2O_2, then 10^{-5} μmole would be generated by one egg in a volume of 1×10^{-6} ml. This means that the initial concentration of H_2O_2 at the egg's membrane is as high as 10 mM. In our experiments, we have used an average of 1×10^5 eggs in 2 ml of sea water. Upon fertilization, these eggs would generate 1 μmole H_2O_2, a 5×10^{-4} M final concentration. When eggs were pretreated with H_2O_2 for 30 sec prior to addition of sperm, the inhibition of fertilization was observed starting with 5×10^{-4} M H_2O_2 (Table I), the same concentration as is generated by the eggs to prevent polyspermy [7]. Similar results were obtained upon preincubation of sperm. When gametes were treated with H_2O_2 in the presence of 1×10^{-6} M Cu^{2+}, the effects of H_2O_2 were potentiated and manifested one order of magnitude sooner.

In all experiments, we have found that Cu^{2+}/H_2O_2-induced effects on fertilization seemed to be qualitatively the same as those of H_2O_2 alone but occurred at lower concentrations of H_2O_2. These findings indicate that the actual mechanism of H_2O_2-mediated prevention of polyspermy is the same as that artificially created by the Cu^{2+}/H_2O_2 system.

Since the fertilization process occurs outside sea urchins in sea water [1], we propose that the Cl^- ions present in high concentrations (0.5–0.6 M) participate in a formation of the active intermediate, $HOCl/OCl^-$, upon reaction with H_2O_2. However, the formation of $HOCl/OCl^-$ from H_2O_2 and Cl^- requires a mediation by myeloperoxidase [17,18,20,26]. This enzyme has not been identified in sea urchins as yet, although there is evidence that the antipolyspermic activity of H_2O_2 is mediated by peroxidase [23,25]. Spermicidal activity present in mammalian systems has been shown to be mediated by peroxidase as well [38].

In our in vitro assays, we used concentrations of Cu^{2+}, H_2O_2, and Cl^- similar to those in the fertilization experiments. In the presence of an excess H_2O_2, 2.5×10^{-6} M Cu^{2+} caused formation of 1.3×10^{-6} M $HOCl/OCl^-$ from 1×10^{-4} M H_2O_2 (Table V). Assuming linearity at low concentrations, 1×10^{-6} M Cu^{2+} used in fertilization experiments might have caused formation of 0.5×10^{-6} M $HOCl/OCl^-$ from 5×10^{-4} M H_2O_2. Preincubation of sperm with exogenous $HOCl/OCl^-$

TABLE V. Rates of Conversion of $Cu^{2+}/Cl^-/H_2O_2$ Into $HOCl/OCl^-$ When Either H_2O_2 or Cu^{2+} Is In Limiting Amounts

Amount of H_2O_2 or Cu^{2+} used (nmole)	$A_{350} \times 10^{-3}$[a]	$HOCl/OCl^-$ equivalent measured (nmole)[b]	Reaction efficiency[c]	Total $HOCl/OCl^-$ formed (nmole)[d]	Percent conversion to $HOCl/OCl^-$[e]
H_2O_2/100 nmole Cu^{2+}					
10	7.5	0.66	0.41	1.6	16.0
25	18.0	1.57	0.33	4.8	19.2
40	28.5	2.49	0.31	8.0	20.0
50	36.0	3.14	0.30	10.5	21.0
60	42.5	3.71	0.30	12.4	20.7
75	53.0	4.63	0.30	15.4	20.5
90	63.5	5.55	0.30	18.5	20.6
100	70.0	6.11	0.30	20.4	20.4
Cu^{2+}/100 nmole H_2O_2					
2.5	4.5	0.39	0.3	1.3	52.0
5	11.0	0.96	0.3	3.2	64.0
10	20.0	1.75	0.3	5.8	58.0
25	31.0	2.71	0.3	9.0	36.0
40	39.0	3.41	0.3	11.4	28.5
50	44.0	3.84	0.3	12.8	25.6
75	57.0	4.98	0.3	16.6	22.0
100	69.5	6.07	0.3	20.2	20.2

[a]A_{350} values were obtained from Figure 3 for H_2O_2 (●) and for Cu^{2+} (□).
[b]The same as footnote b in Table IV.
[c]Values taken from the appropriate positions in Table IV.
[d]Values of "total $HOCl/OCl^-$ formed" were obtained by dividing "$HOCl/OCl^-$ equivalents measured" by the appropriate reaction efficiency.
[e]Percent conversion to $HOCl/OCl^-$ was calculated by dividing "total $HOCl/OCl^-$ formed" by the amount of the reagent (H_2O_2 or Cu^{2+}) used and multiplying by 100.

did not inhibit its ability to penetrate the egg's membrane as assessed by the presence of FEs when the concentration of $HOCl/OCl^-$ was 7×10^{-6} M. However, at that concentration, there was still no cleavage. It therefore appears that the exogenous $HOCl/OCl^-$ is as effective as inhibitory concentrations of H_2O_2 but at concentrations 10^2-10^3-fold lower than that of H_2O_2.

We have shown that Cu^{2+} mimics myeloperoxidase because it forms $HOCl/OCl^-$ from H_2O_2 and Cl^-. It is possible that this process is among those responsible for the known antifertility properties of copper [39,40]. Furthermore, $HOCl/OCl^-$ reoxidizes Cu^+, again similarly to MPO, which is oxidized by the same product. In the absence of scavenging agents, $HOCl/OCl^-$ is reduced by Cu^+. However, in the presence of scavengers such as primary amines and even NH_4^+ ions, it preferentially forms chloramines, which are also powerful oxidizing agents [17,18,24]. Since $HOCl/OCl^-$ generated by Cu^{2+} from H_2O_2 and Cl^-, exogenous $HOCl/OCl^-$, and higher concentrations of H_2O_2 all have similar inhibitory effects on the fertilization process in sea urchins, we suggest that it is myeloperoxidase that mediates formation of $HOCl/OCl^-$ from endogenous H_2O_2 to prevent polyspermy.

ACKNOWLEDGMENTS

These studies were supported by NIH grants CA-16060 (W.T.) and BRSG SO7 RR05399-23 (K.F.) and the New York University School of Medicine Honors Pro-

gram (F.B.). The authors wish to thank Catherine Bearce and Kazimierz Chrzan for excellent technical assistance. Part of this work was performed in the Marine Biological Laboratory, Woods Hole, MA, and was published as an abstract in Biol Bull 167:517, 1984.

REFERENCES

1. Schuel H: Biol Bull 167:271, 1984.
2. Jaffe LA: Nature 261:68, 1976.
3. Schuel H, Schuel R: Dev Biol 87:249, 1981.
4. Epel D: Curr Topics Dev Biol 12:186, 1978.
5. Schuel H, Traeger E, Schuel R, Boldt J: Gamete Res 10:9, 1984.
6. Coburn M, Schuel H, Troll W: Dev Biol 84:235, 1981.
7. Boldt J, Schuel H, Schuel R, Dandekar PV, Troll W: Gamete Res 4:365, 1981.
8. Aranov C, Eisen M, Zimmerman M, Troll W: Biol Bull 161:333, 1981.
9. Sinsheimer P, Coburn M, Troll W: Biol Bull 159:469, 1980.
10. Longo FJ, Schuel H: Dev Biol 34:187, 1973.
11. Blum F, Bearce C, Frenkel K, Troll W: Biol Bull 167:517, 1984.
12. Fridovich I: Annu Rev Pharmacol Toxicol 23:239, 1983.
13. Youngman RJ: TIBS 9:280, 1984.
14. Green MJ, Hill HAO: In Packer L (ed): "Methods in Enzymology, Vol 105." New York: Academic Press, 1984, pp 3–22.
15. Troll W, Frenkel K, Wiesner R: JNCI 73:1245, 1984.
16. Troll W, Frenkel K, Teebor G: In Fujiki H, et al (eds): "Cellular Interactions by Environmental Tumor Promoters." Tokyo: Japan Sci Press, Utrecht: VNU Sci Press, 1984, pp 207–218.
17. Thomas EL: Infect Immun 23:522, 1979.
18. Weiss SJ, Klein R, Slivka A, Wei M: J Clin Invest 70:598, 1982.
19. Rosen H, Klebanoff SJ: J Clin Invest 58:50, 1976.
20. Babior BM: N Engl J Med 298:659, 1978.
21. Levitz SM, Diamond RD: Infect Immun 43:1100, 1984.
22. Foerder CA, Klebanoff SJ, Shapiro BM: Proc Natl Acad Sci USA 75:3183, 1978.
23. Klebanoff SJ, Foerder CA, Eddy EM, Shapiro BM: J Exp Med 149:938, 1979.
24. Weiss SJ, Lampert MB, Test ST: Science 222:625, 1983.
25. Boldt J, Alliegro C, Schuel H: Gamete Res 10:267, 1984.
26. Harrison JE, Schultz J: J Biol Chem 251:1371, 1976.
27. Evans GW, Johnson WT: In Sorenson JRJ (ed): "Inflammatory Diseases and Copper." Clifton, New Jersey: Humana Press, 1982, pp 3–15.
28. Sorenson JRJ: In Sorenson JRJ (ed): "Inflammatory Diseases and Copper." Clifton, New Jersey: Humana Press, 1982, pp 289–301.
29. Lewis AJ, Smith WE, Brown DH: In Sorenson JRJ (ed): "Inflammatory Diseases and Copper." Clifton, New Jersey: Humana Press, 1982, pp 303–318.
30. Boyle E, Freeman PC, Goudie AC, Mangan FR, Thompson M: J Pharm Pharmacol 28:865, 1976.
31. Halliwell B: FEBS Lett 56:34, 1975.
32. Milanino R, Passarella E, Velo GP: Adv Inflam Res 1:281, 1979.
33. Kensler TW, Trush MA: Environ Mutagen 6:593, 1984.
34. Goldstein BD, Witz G, Amoruso M, Troll W: Biochem Biophys Res Commun 88:854, 1979.
35. Salin ML, McCord JM: J Clin Invest 56:1319, 1975.
36. Brigelius R, Spötl R, Bors W, Lengfelder E, Saran M, Weser U: FEBS Lett 47:72, 1974.
37. deAlvare LR, Goda K, Kimura T: Biochem Biophys Res Commun 69:687, 1976.
38. Smith DC, Klebanoff SJ: Biol Reprod 3:229, 1970.
39. Zipper J, Medel M, Prager R: Am J Obstet Gynecol 105:529, 1969.
40. Tatum HJ: Am J Obstet Gynecol 112:1000, 1972.