METABOLIC SIMILARITIES BETWEEN FERTILIZATION AND PHAGOCYTOSIS

Conservation of a Peroxidatic Mechanism

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Fertilization of eggs of the sea urchin, *Strongylocentrotus purpuratus*, or their parthenogenetic activation with the ionophore A23187, results in a burst of oxygen consumption, with much of the oxygen being converted to hydrogen peroxide (H_2O_2) (1). The H_2O_2 so formed can act as the substrate for a peroxidase (ovoperoxidase) which is released from the egg during the massive exocytosis of surface granules and associated alterations of the egg surface that follow fertilization (cortical reaction) (2). One function of the ovoperoxidase is to catalyze hardening of the glycoprotein coat of the egg; this vitelline layer is converted into a rigid structure, the fertilization membrane, by the formation of cross-links between tyrosyl residues. Fertilization also is associated with light emission (chemiluminescence) (1), increased hexose monophosphate shunt activity, as measured by glucose 1-^{14}C oxidation to $^{14}CO_2$ (3), and acid secretion from the egg (4).

These changes induced by fertilization in the sea urchin egg bear a striking resemblance to the changes induced by phagocytosis in polymorphonuclear leukocytes (PMNs).[1] A respiratory burst is initiated by phagocytosis (or more accurately by perturbation of the plasma membrane) (5), in which oxygen is converted to H_2O_2 (6). The PMN contains a peroxidase (myeloperoxidase, MPO) in cytoplasmic granules, and this enzyme is released into the phagosome after phagocytosis, where it interacts with H_2O_2 and a halide to form a potent antimicrobial system (7, 8). As with the fertilized egg, phagocytosis by the PMN is associated with the emission of light (9), increased hexose monophosphate shunt activity (5), and acid production (10, 11).

These findings prompted a search for other similarities between changes induced by fertilization of the sea urchin egg and phagocytosis in PMNs. Phagocytosis by PMNs is associated with the conversion of iodide to a trichloroacetic acid (TCA)-precipitable form (iodination) and this fixed iodine can be localized in part on the surface of the ingested organism (12). The thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), are deiodinated by phagocytosing PMNs with a portion of the

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[1] *Abbreviations used in this paper:* DMSO, dimethylsulfoxide; MPO, myeloperoxidase; MSW, Millipore-filtered sea water; PMN, polymorphonuclear leukocyte; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate; T_3, triiodothyronine; T_4, thyroxine; TCA, trichloroacetic acid.

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released iodine recovered as inorganic iodide and a portion as origin material, i.e., material which remains at the origin on paper chromatography and is presumably iodinated protein (13, 14). Estradiol is converted to an alcohol-precipitable form by PMNs during phagocytosis and the firm binding of the estrogen to cellular constituents can be visualized autoradiographically (15).

In this paper we confirm the presence of peroxidatic activity in the lamellae of the cortical granules of sea urchin eggs previously reported by Katsura and Tominaga (16), and show the release and partial incorporation of the responsible enzyme into the fertilization membrane after fertilization. Iodination, deiodination, T_4 degradation, and estradiol binding also occur and the fixed iodine and estradiol are found predominantly in the fertilization membrane. Thus a similar complex peroxidative mechanism which is activated in response to physiological stimuli has been conserved in two quite different cell types.

Materials and Methods

S. purpuratus eggs, sperm, and sea water were obtained as previously described (17). The sea water was filtered through a 0.45 μm Millipore filter (MSW) before use. Fertilization on exposure of eggs to sperm was greater than 90% in all experiments unless otherwise indicated. Ionophore A23187, kindly supplied by Dr. R. Hamill (Eli Lilly and Co., Indianapolis, Ind.) was dissolved at a concentration of 5×10^{-3} M in dimethylsulfoxide (DMSO) (J. T. Baker Chemical Co., Phillipsburg, N. J.). $Na^{125}I$ (carrier free in 0.1 M NaOH), L-thyroxine-^{125}I (85 Ci/mmol in 50% n-propanol), estradiol-17$^{\beta}$-4-^{14}C (59.0 mCi/mmol in benzene:ethanol 9:1) and estradiol [6,7-^{3}H(N)] (42.5 Ci/mmol in benzene:ethanol 9:1) were obtained from New England Nuclear, Boston, Mass. The estradiol and thyroxine were evaporated to dryness under nitrogen and the estradiol redissolved in ethanol and the thyroxine in DMSO. Methimazole (Tapazole) was kindly supplied by Eli Lilly and Co. Human albumin (fatty acid free fraction V), phenylmethyl sulfonyl fluoride (PMSF) and L-ergothioneine were obtained from Sigma Chemical Co., St. Louis, Mo., 3-amino-1,2,4-triazole was obtained from Schwarz/Mann, Orangeburg, N. Y., ascorbic acid from Merck Chemical Div., Merck & Co., Inc. Rahway, N. J., and sodium dodecylsulfate (SDS) from BDH Chemicals, Ltd., Poole, England. Catalase (beef liver, 9 mg/ml, 37325 U/mg), obtained from Worthington Biochemical Corp., Freehold, N. J., was dialyzed against water before use. All other reagents were of the highest quality available.

Preparation of Fertilization Product. A 10–20% suspension of eggs, treated with trypsin to prevent formation of the fertilization membrane (18), were fertilized and 5 min later the eggs were hand centrifuged. The supernatant solution was spun at 17,000 *g* for 5 min (2). The high-speed supernatant solution which contained the material released by fertilization was designated the fertilization product. The peroxidase activity of the fertilization product was determined by guaiacol oxidation (2).

Iodination. Iodination was determined by the conversion of iodide to a TCA-precipitable form by a previously described method (19) modified as follows. The components of the reaction mixture (see legends) were incubated at 13–15°C in 12 × 75-mm polystyrene test tubes for the periods indicated, in a water bath oscillating 70 times per minute. The reaction was stopped by the addition of 1.0 ml of cold 10% TCA. The precipitate was collected by centrifugation at 225 *g* for 5 min in an International PR-2 refrigerated centrifuge (International Equipment Co., Needham Hts, Mass.) and washed three times with 2.0 ml of 10% TCA. The test tube containing the washed precipitate was placed in a counting tube and the radioactivity of the sample determined in a gamma counter. A blank containing iodide-^{125}I in MSW was run with each experiment and the results subtracted from the experimental values. Less than 0.5% of the total added radioactivity was TCA-precipitable in the blank. A standard containing the total amount of ^{125}I in the reaction mixture was counted and the percent conversion to a TCA-precipitable form in the experimental tubes was determined as follows:

$$\text{cpm experimental} - \text{cpm blank}/\text{cpm standard} \times 100.$$

Each experimental value was determined in duplicate and the average employed as a single n for statistical analysis.

Release of ^{125}I from Autoiodinated Eggs during Development. Eggs at a final concentration of 10^5/ml were incubated for 10 min in 5.0 ml of MSW containing 1 μCi/ml carrier-free $Na^{125}I$ and 10^6 sperm/ml. The iodinated embryos were washed twice with 10 ml of MSW containing 0.1 mM KI, once with 250 ml of MSW and suspended in 50 ml of MSW. 5-ml aliquots were removed at intervals, the eggs washed three times by hand centrifugation in 5.0 ml of MSW, and suspended in 0.5 ml of a disaggregation buffer (0.05 M Tris HCl buffer pH 6.8, 1.1% SDS, 10^{-4} M PMSF, 20% glycerol, 1% β-mercaptoethanol). The suspension was heated at 100°C for 5 min and precipitated at 0°C for 1 h with 4.5 ml acetone. The precipitate was collected by centrifugation (12,000 *g*, 4°C, 3 min) and the pellet was resuspended in 1.0 ml of disaggregation buffer containing 0.1% SDS. Protein was determined in 10–25-μl aliquots with Coomassie Brilliant Blue (20). For radioactivity determinations, 50–100-μl aliquots were precipitated with 1.0 ml of ice-cold 10% TCA, with 50 μl porcine γ-globulin (10 mg/ml) as coprecipitant. The precipitates were collected on Whatman GF/C filters, washed twice with 5% TCA and twice with acetone, and counted in a gamma counter.

Deiodination of Exogenously-Labeled Eggs. The jelly coat of sea urchin eggs was removed by suspension of the eggs in MSW adjusted to pH 4.5 as previously described (21). The dejellied eggs were iodinated by incubation with $Na^{125}I$, myeloperoxidase and H_2O_2 as follows. To each 50,000 eggs was added 0.05 μCi $Na^{125}I$ (carrier free), 8 mU myeloperoxidase, and 5 nmol H_2O_2 (final concentration 10^{-5} M) in a total vol of 0.5 ml of MSW. After incubation for 20 min, the eggs were allowed to settle and were gently washed four times and suspended in MSW. Fertilization of the eggs was >90% after this procedure. For the measurement of deiodination, the iodinated eggs, incubated as described in the legend to Table V, were sedimented by centrifugation at 1,000 *g* and the supernatant fluid removed and counted. A TCA-precipitate was prepared and counted as described under Iodination and the non-TCA-precipitable iodine of the supernatant fluid was determined by subtraction. An aliquot of the supernatant fluid was chromatographed on paper as described under Thyroxine Degradation and autoradiograms prepared for the identification of released components. The TCA-precipitable iodine of the pellet (eggs) also was determined.

Thyroxine Degradation. Thyroid hormone degradation was performed as previously described (13). Briefly, the components of the reaction mixture (see legends) were incubated with ^{125}I-thyroxine in 12 × 75-mm plastic tubes (Falcon 2052) at 13–15°C in a water bath oscillating 70 times per minute. 0.05-ml aliquots were removed at intervals and added to 0.05 ml of 25% human serum albumin containing 0.01 M potassium iodide and 0.001 M propylthiouracil to stop the reaction. Duplicate 25-μl samples were applied to strips of Whatman No. 1 filter paper and the components separated by ascending chromatography for 16–18 h in butanol saturated with 2 N acetic acid. The strips were cut into three segments corresponding to the origin material (i.e. material which remained at the origin on chromatography), iodide and thyroxine spots and counted in a gamma counter.

Estradiol Binding. The conversion of estradiol to an alcohol-precipitable form was determined by a previously described method (15) modified as follows. Estradiol-^{14}C in ethanol (0.005 ml, 507 pmol, 0.03 μCi) was evaporated to dryness under nitrogen in polystyrene 12 × 75-mm test tubes. The components indicated in the legends were added and the mixture incubated at 13–15°C for the periods indicated, in a water bath oscillating 70 times per minute. The reaction was stopped by the addition of 1.0 ml of absolute ethanol and the tubes placed in an ice bath until filtration. The precipitate was collected on a Whatman 115 filter paper (2.5 cm diameter) in an ICN Precipitation Apparatus (ICN Chemical and Radioisotope Div., Irving, Calif.) and washed with 10 ml of absolute ethanol. The filter paper was placed in a liquid scintillation vial and 0.5 ml of protosol (New England Nuclear) was added. The vials were kept at room temperature overnight and counted in a liquid scintillation counter. A blank containing estradiol-^{14}C in MSW was run with each experiment and the results subtracted from the experimental values. Less than 0.5% of the total added radioactivity was alcohol-precipitable in the blank. A standard containing the total amount of estradiol-^{14}C in the reaction mixture was counted and the percent conversion of estradiol to an alcohol-precipitable form in the experimental tubes was determined as follows:

cpm experimental − cpm blank/cpm standard × 100.

Each experimental value was determined in duplicate and averaged.

Autoradiographic Localization. The reaction mixture was as employed in Table I except that 10 μCi of ^{125}I-iodide and 12.5 μCi of estradiol-^{3}H were employed for iodine and estradiol localization, respectively. After a 30-min incubation, an equal vol (0.5 ml) of fixative (4% glutaraldehyde, 0.2 M cacodylate buffer pH 8.0, 0.34 M NaCl) was added. After 30 min at 0°C, the eggs were gently washed four times with 1/2 strength fixative in sea water (containing 0.01 M potassium iodide in samples for iodine localization). The specimens were washed several times with 0.1 M cacodylate pH 8.0-buffered sea water and fixed at room temperature for 2–3 h in a solution containing 2% osmium tetroxide, 0.1 M cacodylate buffer pH 8.0, and 0.17 M NaCl. The eggs were then rinsed repeatedly in distilled water, dehydrated in 2,2-dimethoxypropane and embedded in Epon (Ladd Research, Burlington Vt.). 1-μm thick sections were mounted on microscope slides, coated at 35°C with Kodak NTB-2 emulsion diluted 1:1 with distilled water, allowed to dry, and stored at 4°C. After 1–4 wk of exposure, the slides were developed in half-strength Kodak Dektol for 2 min at 21°C, rinsed in distilled water, and fixed in half-strength Kodak Rapid-fix. The slides were allowed to dry, stained with 0.1% toluidine blue, and photographed with a Zeiss Universal microscope (Carl Zeiss, Inc., New York).

Peroxidase Localization. Unfertilized eggs or eggs 10 min after insemination with 100 sperm per egg were collected by hand centrifugation and suspended in 0.98 ml of buffer containing 5.6 mM 3,3′-diaminobenzidine, 0.45 M NaCl, and 0.1 M Tris-HCl buffer pH 8.0. Hydrogen peroxide (0.02 ml) was added to a final concentration of 6 mM and the samples were incubated for 10 min at 10°C. 2 vol of fixative consisting of 3% glutaraldehyde, 0.1 M cacodylate buffer pH 8.0, and 80% MSW were added and the specimens prepared for electron microscopy as described under autoradiographic localization. The sections were viewed and photographed with a Philips 300 electron microscope without further staining. In the aminotriazole-treated controls, unfertilized and fertilized eggs were preincubated for 10 min in 1.0 ml of 10 mM 3-amino-1,2,4-triazole, 0.45 M NaCl, and 0.1 M Tris-HCl buffer pH 8.0. The supernate was removed after hand centrifugation and the eggs were suspended in 2 mM 3-amino-1,2,4-triazole, 5.6 mM 3,3′-diaminobenzidine, 0.45 M NaCl, and 0.1 M Tris-HCl pH 8.0. H_2O_2 was added and specimens fixed and prepared for electron microscopy as described above.

In preliminary experiments, glutaraldehyde was found to strongly inhibit both the cytochemical reaction and the ovoperoxidase activity of the fertilization product as measured by guaiacol oxidation in the presence of H_2O_2. Over 99% inhibition was produced by 1.5% glutaraldehyde in 0.2 M cacodylate buffer at pH 8.0. Thus for cytochemical localization, it was necessary to incubate eggs with the peroxidase reagents before glutaraldehyde fixation.

Hardening Assay. Hardening of the fertilization membrane was determined as previously described (2). Briefly, eggs were mixed with sperm in scintillation vials and gently swirled every 30 s. At 5 min, the eggs were checked for fertilization by microscopic visualization of the fertilization membrane and at 20 min, 50 mM dithiothreitol was added. The eggs were allowed to settle for 10 min, vigorously mixed in a Vortex mixer and examined microscopically for the presence of the fertilization membrane. In the absence of hardening, dithiothreitol causes dissolution of the membrane and the percent of eggs with visible fertilization membranes falls.

Iodine Determination. Iodine determinations were performed by Reference Laboratory, Newbury Park, Calif.

Results

Cytochemical Localization of Ovoperoxidase. At fertilization, the ovoperoxidase is released from eggs and is found in the fertilization product (2). Treatment of eggs with trypsin to inhibit formation of the fertilization membrane increased ovoperoxidase content of the fertilization product (2), suggesting that the fertilization membrane acts as a barrier to exocytosed enzyme. This was supported by cytochemical studies (Fig. 1). In unfertilized eggs the peroxidase is located in the lamellae of the cortical granules (Fig. 1 A); specific staining is not seen in the presence of the ovoperoxidase

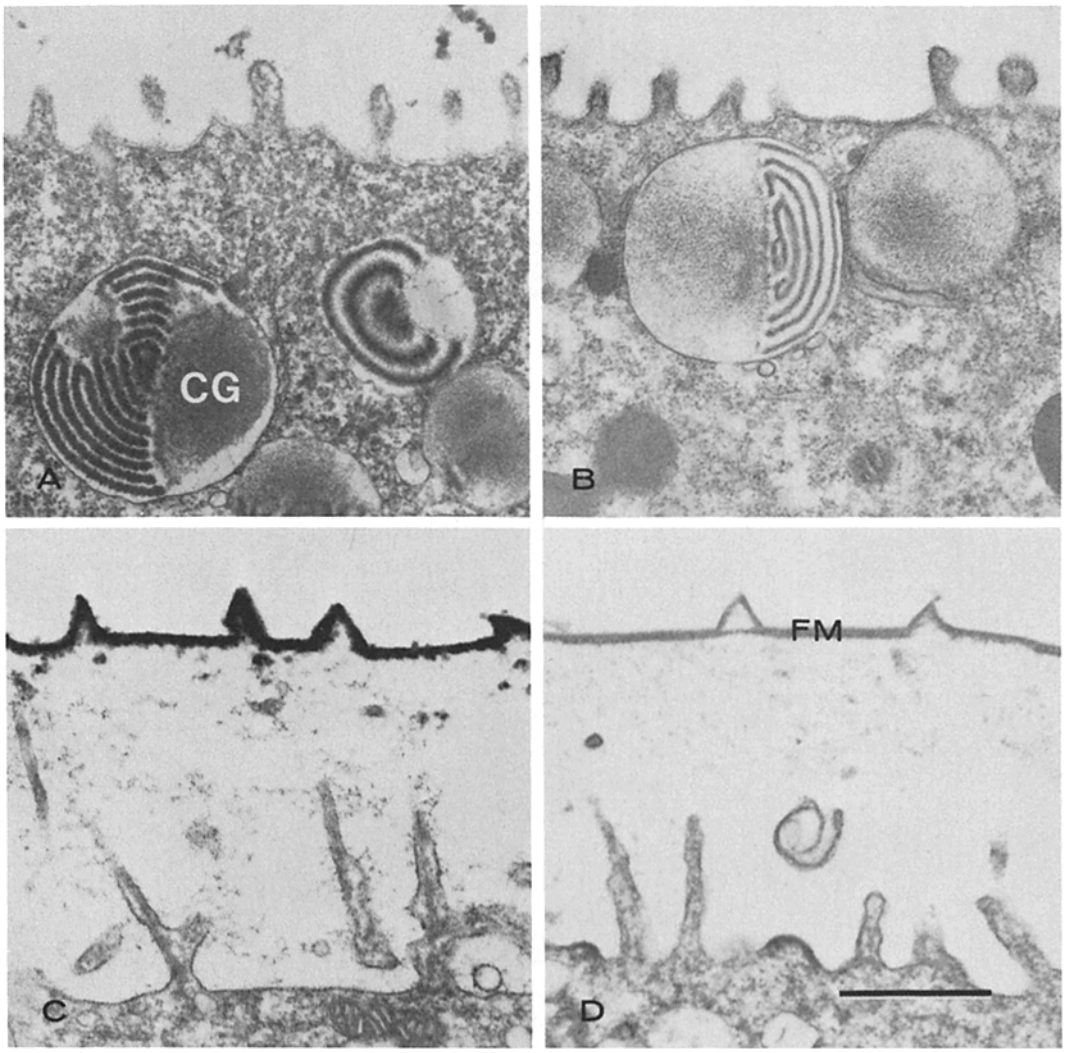

FIG. 1. Electron microscopic localization of peroxidase in unfertilized and fertilized eggs. Living eggs were exposed to diaminobenzidine and hydrogen peroxide and then fixed with osmium tetroxide. The reaction product indicative of peroxidase is present in the cortical granules (CG) of unfertilized eggs (A) and in the fertilization membrane (FM) of fertilized eggs (C). Addition of aminotriazole resulted in cortical granules (B) and fertilization membranes (D) similar in density to those of eggs not reacted for peroxidase (data not shown). Magnification, × 20,000. Bar = 1 μm

inhibitor, aminotriazole (Fig. 1 B). After fertilization the peroxidase activity is found in the elevated fertilization membrane (Fig. 1 C); this activity is likewise inhibited by aminotriazole (Fig. 1 D).

Iodination. Fertilization of sea urchin eggs, or parthenogenetic activation with the ionophore A23187, results in the conversion of iodide to a TCA-precipitable form (iodination) (Table I). Eggs, sperm, or ionophore alone were without effect. DMSO was employed as a solvent for A23187; it did not stimulate iodination by eggs at the concentration employed. Iodination increased in a linear fashion for the first 20 min

TABLE I

Iodination by Sea Urchin Eggs *

Components	Iodination
	%
Complete fertilization system	26.7 ± 2.2 (13)‡
Eggs only	0.1 ± 0.1 (3)
Sperm only	0.3 ± 0.01 (2)
Complete parthenogenetic system	20.1 ± 4.4 (5)
A23187 only	−0.1 ± 0.05 (4)§
Eggs + DMSO only	0.3 ± 0.2 (5)

* The complete fertilization system contained 50,000 eggs, 5 × 10^6 sperm, 0.05 μCi sodium iodide-^{125}I (carrier free), and MSW to a final 0.5 ml vol. In the complete parthenogenetic system 50 μM ionophore A23187 was substituted for sperm. DMSO employed as a solvent for A23187 was present at a final concentration of 0.13 M. Incubation period, 30 min.

‡ Mean ± SE of (*n*) experiments.

§ Negative numbers in this and other tables refer to values which are less than background.

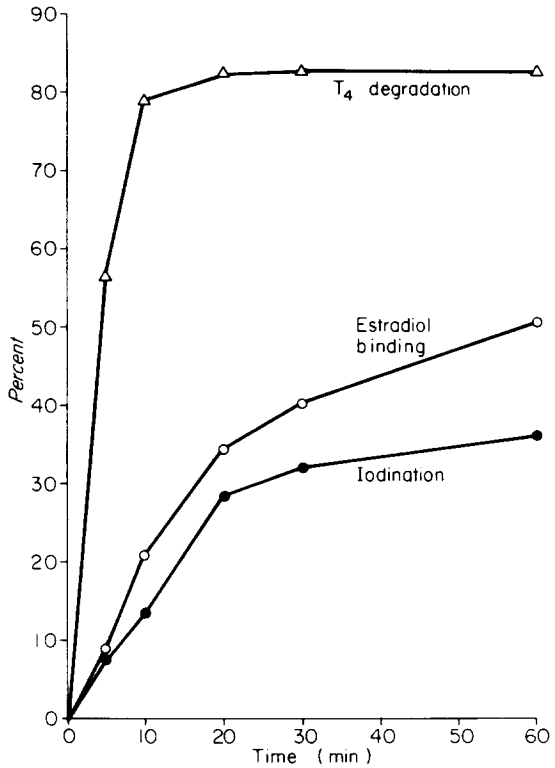

FIG. 2. Effect of incubation period on iodination, estradiol binding, and T_4 degradation. The reaction mixture was as described for the complete fertilization system in Table I (iodination), Table VI (T_4 degradation), or Table VII (estradiol binding) and determinations made at the periods indicated. Mean of three experiments.

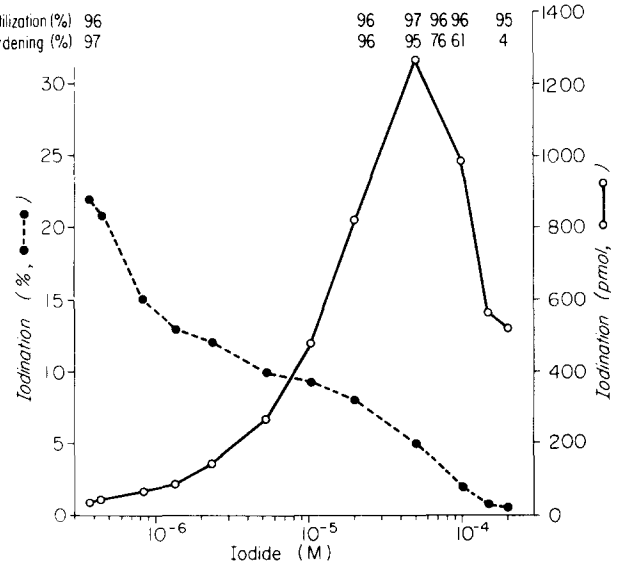

FIG. 3. Effect of iodide concentration on iodination, fertilization, and hardening of the fertilization membrane. The reaction mixture was as described for iodination by the complete fertilization system in Table I except that the amount of sodium iodide (unlabeled) was varied as indicated. The lowest concentration of iodide employed (3.7×10^{-7} M) was that of unsupplemented sea water. Percent fertilization and hardening were performed with identical concentrations of components; however, the volume of the reaction mixture was doubled. The results are the mean of three experiments.

after fertilization and then continued at a decreasing rate over a 60-min period (Fig. 2).

Carrier-free radioiodide was employed in the measurement of iodination in Table I and Fig. 2. The iodine concentration of the sea water employed was 4.5 μg/100 ml (3.5×10^{-7} M). Thus 175 pmol were present in the 0.5 ml of sea water employed in the reaction mixture of which 26.7% (Table I) or 47 pmol (0.94 fmol/embryo) were converted to a TCA-precipitable form in 30 min. The further addition of unlabeled iodide decreased the percent incorporation but total incorporation increased to reach a maximum of 1,259 pmol (25.18 fmol/embryo) at an iodide concentration of 5×10^{-5} M and then fell with a further increase in iodide concentration (Fig. 3). Percent fertilization remained high (≥95%) at all the iodide concentrations employed. However the percentage of eggs with hardened fertilization membranes as measured by their loss of solubility in dithiothreitol decreased at iodide concentrations greater than 5×10^{-5} M. The concentrations of iodide required for decreased hardening corresponded to those associated with a fall in total iodination.

The inhibitory effect of azide, cyanide, aminotriazole, methimazole, ascorbic acid, and ergothioneine on iodination by sea urchin eggs is shown in Table II. Cyanide was the most effective inhibitor with significant inhibition at 10^{-6} M. In contrast, iodination was not significantly affected by the addition of 90 μg/ml catalase (iodination 24.9 ± 3.8 [SE] without catalase; 22.3% ± 5.6 with catalase, $n = 8$).

The autoradiographic localization of the fixed iodine is shown in Fig. 4A–C. The silver grains predominate over the raised fertilization membrane (Fig. 4B), while

TABLE II

Effect of Inhibitors on Iodination *

Inhibitor	Inhibitor concentration				
	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Azide	94‡§	33§	11	0	0
Cyanide	100§	95§	74§	37§	0
Aminotriazole	91§	54§	0		
Methimazole	99§	99§	61§	15	
Ascorbic acid	99§	92§	35§	22	
Ergothioneine	99§	95§	46§	22	

* The reaction mixture was as described for the complete fertilization system in Table I except that the inhibitors were added in the concentrations indicated. The results are the mean of three to four experiments.

‡ Percent inhibition.

§ Difference from no inhibitor significant ($P < 0.05$).

there is no iodination of unfertilized eggs (Fig. 4A). Iodination of the fertilization membrane is completely inhibited by aminotriazole (Fig. 4C). The occasional unfertilized egg in the fertilized egg population was not iodinated (data not shown). This, and the fact that little iodination of plasma membrane components occurred (Fig. 4B), suggests that the reaction is localized, as is the enzyme (Fig. 1), to the fertilization membrane. As expected from these data, most of the radioactivity is lost from the embryo after hatching of the blastulae occurs (Table III). Although early development is associated with a slight loss of acid-precipitable ^{125}I, a dramatic loss occurs at the time of hatching, with release of 94% of the radioactivity.[2]

The fertilization product (the material containing ovoperoxidase that is released into the extracellular fluid during the cortical reaction) catalyzes the iodination of albumin in the presence of added H_2O_2. Table IV demonstrates the requirement for each component of the iodinating system (fertilization product, H_2O_2, albumin), the inactivation of the fertilization product by heat treatment and the inhibition of iodination by azide, cyanide, aminotriazole, methimazole, ascorbic acid, and ergothioneine. The pH optimum of this reaction was 7.0, as shown in Fig. 5.

Deiodination of Exogenously-Labeled Eggs. Eggs were prelabeled with ^{125}I by incubation with $Na^{125}I$, myeloperoxidase, and H_2O_2 and then extensively washed to remove unbound iodine and the exogenous iodinating system. A portion (22%) of the egg-associated radioiodine was released into the extracellular fluid after incubation of the labeled eggs in sea water for 30 min (Table V). When incubation was carried out in the presence of sperm, the proportion of released iodine increased to 42% ($P < 0.001$ vs. no sperm) and when incubation was with ionophore A23187, 40% of the radioiodine was present in the extracellular fluid ($P < 0.001$ vs. no A23187). 80% of the released iodine was non-TCA-precipitable and comigrated with inorganic iodide on paper chromatography.

Thyroxine Degradation. Table VI demonstrates the degradation of T_4 by sea urchin eggs during fertilization or parthenogenetic activation by A23187, as measured by the

[2] Since the completion of this manuscript, a paper describing iodination of the fertilization membrane has appeared (31).

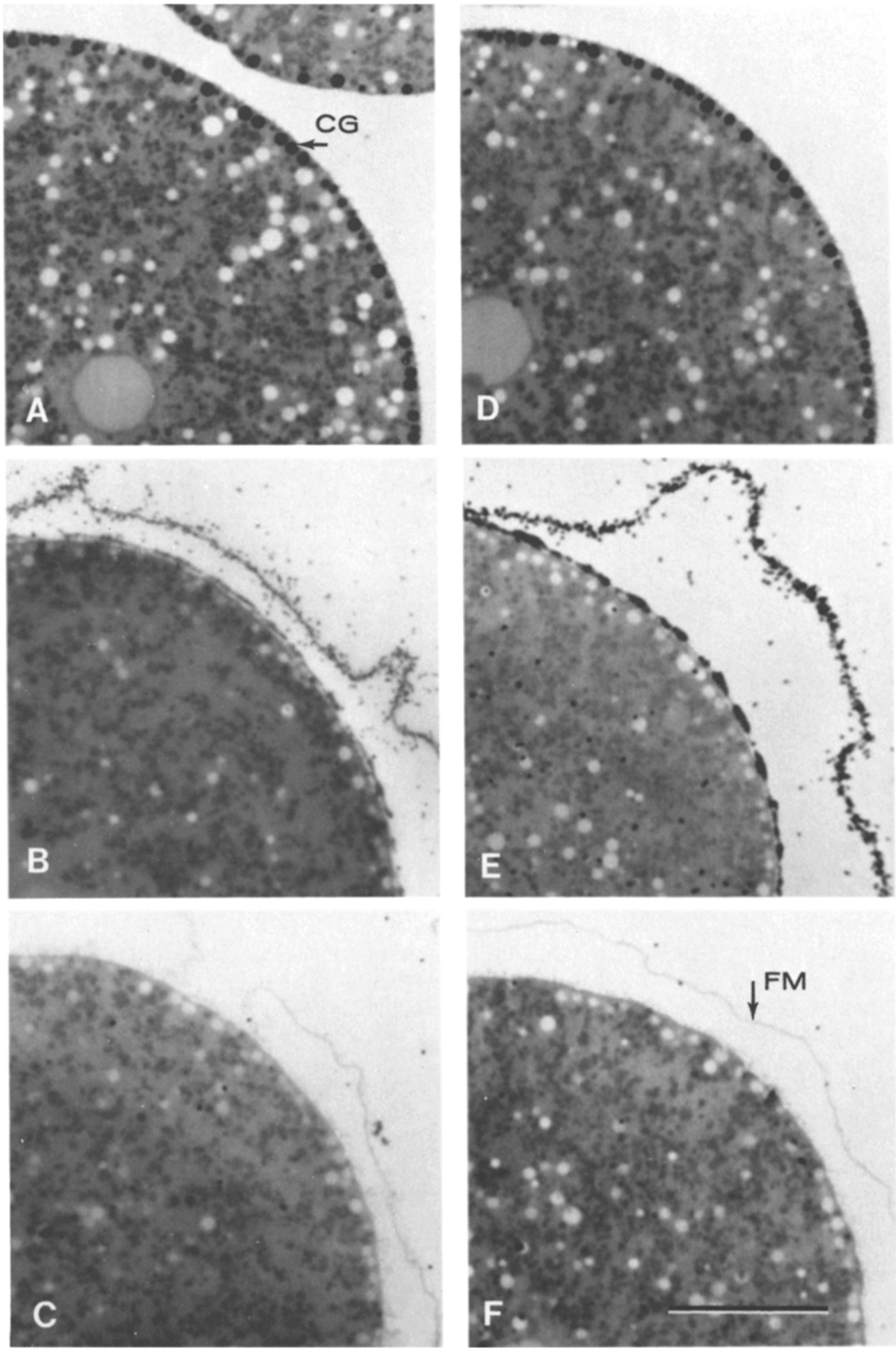
CG
A
D
B
E
C
FM
F

TABLE III

Release of ^{125}I from Autoiodinated Eggs during Development *

Developmental stage	Time after fertilization	Specific radioactivity
	h	*cpm/mg*
Single cell	1.2	8,600
Two cell	5.0	7,900
Early blastula (unhatched)	22.5	6,700
Hatched blastula	27.0	410

* Eggs were fertilized in the presence of $Na^{125}I$, washed free of unbound label, and monitored for the amount of bound label at various stages postfertilization as described in Materials and Methods.

TABLE IV

Iodination Catalyzed by the Fertilization Product *

Components	Iodination
	pmol
Complete system	3,375
Fertilization product omitted	85
H_2O_2 omitted	4
Albumin omitted	65
Fertilization product heated	95
Azide added	25
Cyanide added	120
Aminotriazole added	85
Methimazole added	−35
Ascorbic acid added	−10
Ergothioneine added	−55

* The complete system contained 0.06 M sodium phosphate buffer pH 7.0, 10^{-4} M sodium iodide (50 nmol; 0.05 μCi), 250 μg albumin, 10^{-4} M H_2O_2, and 0.05 ml fertilization product in a total 0.5 ml vol. Components were deleted, the fertilization product was heated at 100°C for 15 min, and inhibitors (10^{-3} M) were added where indicated. Incubation period, 60 min. Results are the mean of three experiments.

recovery of released radioiodine at the origin (labeled protein) or in the inorganic iodide spot on paper chromatography (13). DMSO employed as a solvent for both thyroxine and A23187 had no effect on T_4 degradation by eggs at the concentrations employed. Thyroxine degradation was essentially complete after 10 min of incubation with sperm (Fig. 2) and was inhibited by 1 mM azide, cyanide, aminotriazole, methimazole, ascorbic acid, and ergothioneine (Table VI).

Estrogen Binding. Estradiol was converted to an alcohol-precipitable form by sea urchin eggs during fertilization or parthenogenetic activation by A23187 (Table VII). Binding increased linearly for 10 min after fertilization and then at a decreasing rate for 60 min (Fig. 2). Estrogen binding during fertilization was inhibited by 10^{-3} M azide, cyanide, aminotriazole, methimazole, ascorbic acid, and ergothioneine (Table

FIG. 4. Autoradiographic localization of iodine and estradiol. Unfertilized eggs do not incorporate iodine (A) or estradiol (D), but they are present in the fertilization membrane of fertilized eggs (B and E). This incorporation is blocked by aminotriazole (C and F) which inhibits the egg peroxidase. Magnification, × 2500. Bar = 10 μm

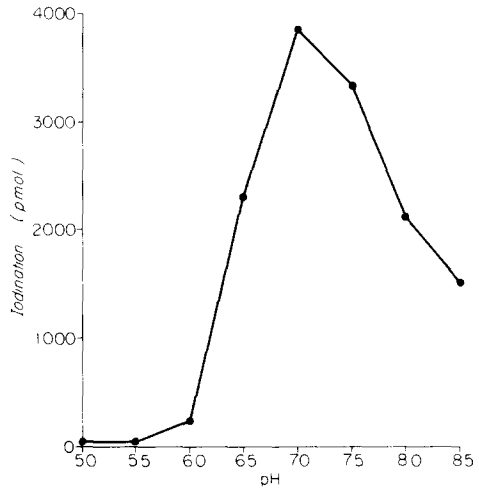

FIG. 5. Effect of pH on iodination by the fertilization product. The reaction mixture was as described for the complete system in Table IV except that the pH of the sodium phosphate buffer was varied as indicated. Incubation—60 min. Results are the mean of three experiments.

VII). As shown in Fig. 4 D–F, the bound estradiol was found predominantly in the fertilization membrane. Thus no estradiol was bound to unfertilized eggs (Fig. 4 D), whereas after fertilization there is a concentration of silver grains over the fertilization membrane (Fig. 4 E) that is inhibited by aminotriazole (Fig. 4 F). As was the case with iodination, estradiol is not bound to a substantial extent to the plasma membrane (Fig. 4 E).

Discussion

Fertilization of sea urchin eggs (or their parthenogenetic activation with the ionophore A23187) results in the conversion of iodide to a TCA-precipitable form and the bulk of the fixed iodine can be localized autoradiographically in the fertilization membrane. Iodination occurs at other sites in the mammalian organism and where investigated, it has been shown to require peroxidase and H_2O_2. For example, in the thyroid gland, T_4 synthesis is initiated by iodination of tyrosine residues of thyroglobulin by a thyroid peroxidase and H_2O_2 (22, 23). Iodination also occurs in neutrophils (12, 19), eosinophils (24, 25), and monocytes (26, 27) during phagocytosis and the evidence is strong for a peroxidative mechanism. Iodination by PMNs is inhibited by the peroxidase inhibitors azide and cyanide and is very much decreased or absent when PMNs which lack H_2O_2 (i.e., from patients with chronic granulomatous disease) or MPO (i.e., from patients with hereditary MPO deficiency) are employed (19). Isolated peroxidases are potent catalysts of the iodination reaction when combined with iodide and H_2O_2 (23).

Is a similar mechanism responsible for the iodination induced by fertilization in the sea urchin egg? The supporting evidence is as follows.

(*a*) A peroxidase is released from the cortical granules of the egg after fertilization (2) and can be demonstrated on the fertilization membrane where iodination occurs (Fig. 1).

TABLE V

Deiodination of Exogenously-Labeled Eggs *

	Extracellular iodine	Iodine bound to eggs	Total
	cpm	*cpm*	*cpm*
Eggs (4)	3,015	10,585	13,600
Eggs + sperm (4)	5,316	7,227	12,542
Eggs + A23187 (2)	4,934	7,468	12,402

* The reaction mixture was as described in Table I except that preiodinated eggs were employed and $Na^{125}I$ was not added. The radioiodine released into the extracellular fluid and that which remained bound to the eggs was determined as described in Materials and Methods. Incubation period, 30 min. The number of experiments is shown in parentheses.

TABLE VI

Thyroxine Degradation by Sea Urchin Eggs *

Components	T_4 Degradation		
	Origin	Iodide	Total
		%	
Complete fertilization system	50.4	32.3	82.7
Eggs only	1.9	1.3	3.6
Sperm only	−0.4	2.3	2.0
Azide added	12.3	6.4	18.7
Cyanide added	22.3	14.5	36.8
Aminotriazole added	5.5	6.7	12.2
Methimazole added	6.2	5.4	11.6
Ascorbic acid added	6.1	6.4	12.5
Ergothioneine added	9.4	12.3	21.7
Complete parthenogenetic system	57.8	21.0	78.8
A23187 only	0.1	−1.1	−1.0

* The reaction mixture was as described in Table I except 2 μCi L-thyroxine-^{125}I (≈30 pmol) in DMSO (10 μl) was employed instead of sodium iodide-^{125}I and the inhibitors (10^{-3} M) were added where indicated. Incubation period, 30 min. Results are the mean of two to three experiments.

(*b*) H_2O_2 is generated by the egg after fertilization (1).

(*c*) Iodination during fertilization is inhibited by agents which inhibit peroxidase-catalyzed reactions (Table II). Iodination however was unaffected by catalase under the conditions employed. This suggests either that H_2O_2 is not required for iodination or, more likely, that it is formed and utilized for iodination at a site unavailable to added catalase. The hardened fertilization membrane is impermeable to concanavalin A and other macromolecules (17) and catalase thus may be unable to penetrate this membrane to reach the site of interaction of ovoperoxidase and H_2O_2.

(*d*) The fertilization product which contains ovoperoxidase catalyzes the iodination of albumin when supplemented with H_2O_2 (Table IV). As with the fertilized egg, iodination is inhibited by azide, cyanide, aminotriazole, methimazole, ascorbic acid, and ergothioneine.

Because iodide is present in sea water (3.5×10^{-7} M for the sea water used in this study), iodination of egg components would be expected to occur during natural fertilization. What are the consequences of this iodination, if any? Tyrosine residues of protein are particularly prone to iodination by peroxidase and H_2O_2 with the

TABLE VII
Estradiol Binding by Sea Urchin Eggs *

Components	Estradiol binding	
	%	
Complete fertilization system	40.0	
Eggs only	0.2	<0.001‡
Sperm only	0.1	<0.001
Azide added	2.8	<0.01
Cyanide added	13.9	<0.05
Aminotriazole added	0.3	<0.01
Methimazole added	0.2	<0.01
Ascorbic acid added	0.04	<0.001
Ergothioneine added	0.1	<0.001
Complete parthenogenetic system	34.4	
A23187 only	0.1	<0.001
Eggs + DMSO only	0.2	<0.001

* The reaction mixture was as described in Table I except that 0.03 μCi estradiol-^{14}C (≈570 pmol) was added instead of sodium iodide-^{125}I and the inhibitors were added to a final concentration of 10^{-3} M where indicated. Incubation period, 30 min. Mean of three to four experiments.
‡ Significance of difference from complete system.

formation first of monoiodotyrosine and then diiodotyrosine (23). Iodohistidine and sulfenyl iodides also can be formed and it is possible that unsaturated fatty acids are iodinated. Fertilization is followed quickly by hardening of the fertilization membrane due to the formation of di- and trityrosine cross-links by ovoperoxidase and H_2O_2 (2). Approximately 15% of the tyrosine residues of the fertilization membrane participate in the cross-link reaction (2). It is presumably the carbon ortho to the phenolic hydroxyl group which is involved in the cross-link; the same carbon forms a covalent bond with iodine in the iodination reaction. Hardening of the fertilization membrane is inhibited by iodide only at very high concentrations. This inhibition may be due to (*a*) direct interference with the formation of cross-links by iodine incorporation; (*b*) competition for the available H_2O_2 since both iodination and cross-link formation require ovoperoxidase and H_2O_2; or (*c*) inhibition of peroxidase activity since hardening was decreased only at concentrations of iodide at which total iodination was declining (Fig. 3). The concentrations of iodide required for inhibiting the hardening reaction are in considerable excess of any which might be expected in sea water, suggesting that inhibition of hardening is not a physiological consequence of iodination of sea urchin eggs. An intriguing possibility is that substitution of the bulky iodine atom for hydrogen on the fertilization membrane may contribute to the loss of permeability that occurs with the hardening reaction.

Studies with eggs exogenously iodinated by the peroxidase system (Table V) and with T_4 radiolabeled with ^{125}I (Table VI) indicate that sea urchin eggs also can catalyze deiodination and transiodination reactions during fertilization or activation by A23187. A portion of the iodine released from T_4 is recovered as inorganic iodide and a portion as origin material, presumably largely iodinated protein. The latter may be formed by sequential deiodination and iodination or by transiodination in which iodine is transferred from one compound to another without an apparent iodide intermediate (28). The deiodination reaction, like the iodination reaction, may

be catalyzed by ovoperoxidase and require H_2O_2 because T_4 can be degraded by peroxidase and H_2O_2 (13) and degradation by eggs is inhibited by peroxidase inhibitors (Table VI). On the basis of these findings, we speculate that deiodination and transiodination occur in the fertilization membrane with the movement of the iodine among acceptor groups. In addition, limited proteolytic cleavage of iodinated surface components may occur. In a study with sea urchin eggs iodinated with lactoperoxidase (29), about 15% of the TCA-precipitable iodinated surface compounds were lost upon fertilization. This loss was blocked by soybean trypsin inhibitor suggesting a proteolytic mechanism.

Sea urchin eggs activated by fertilization or the ionophore A23187 also convert estradiol to an alcohol-precipitable form and the estrogen can be seen by autoradiography to be firmly bound to the fertilization membrane. The binding appears to be by covalent linkage, as the estradiol is not solubilized by ethanol or by the solvents employed in the autoradiographic studies. The inhibition of estradiol binding by the peroxidase inhibitors (Table VII), the localization of the bound estrogen in association with ovoperoxidase in the fertilization membrane (Fig. 4), and the conversion of estradiol to an alcohol-precipitable form by peroxidase and H_2O_2 in the presence of an appropriate acceptor (15) are suggestive of a peroxidative mechanism in the egg. The estrogen is presumably oxidized by peroxidase and H_2O_2 and the oxidized form binds to acceptor groups in the fertilization membrane. The physiological role of this reaction, if any, is unknown.

A kinetic study of iodination, T_4 degradation, and estradiol binding, indicates that activity is high during the first 20–30 min after fertilization (Fig. 2), i.e., for a substantial period of time after the hardening reaction has been completed (17). This is in agreement with the duration of H_2O_2 synthesis (1) as well as of the respiratory burst. A major function of the peroxidative system of sea urchin eggs appears to be in the hardening of the fertilization membrane (2) which serves as a block to polyspermy. It is not known whether additional functions are served by the continued activity of this system. One possibility, in view of the spermicidal activity of peroxidase, H_2O_2 and a halide (30), is that the peroxidase and H_2O_2 released by the egg contribute to the block to polyspermy by killing adjacent sperm. The microbicidal effect of an ovoperoxidase-H_2O_2-halide system also may be protective. Ovoperoxidase-dependent reactions do not seem to be involved in the regulation of development, because cell division occurs on schedule even when the peroxidase is completely inhibited by aminotriazole (R. Showman and C. A. Foerder, unpublished data).

The findings reported here emphasize the striking similarity between the metabolic response of sea urchin eggs to fertilization and the response of phagocytic cells to particle ingestion. In both, peroxidase, present in cytoplasmic granules, is released and this degranulation process is associated with a series of strikingly similar metabolic changes. These similarities raise the possibility that peroxidase release and H_2O_2 generation may be phenomena common to a number of diverse cell types with differing consequences; for example, in the thyroid gland, synthesis of the thyroid hormones; in the neutrophil, microbicidal activity; in the sea urchin egg, hardening of the fertilization membrane and possibly spermicidal activity. This conservation of a multienzyme peroxidative mechanism in diverse cells, which is dormant when the cell is at rest but which can be activated and channeled to specific and localized functions when the need arises, is striking.

Summary

At the time of fertilization, sea urchin eggs release a peroxidase which, together with H_2O_2 generated by a respiratory burst, is responsible for hardening of the fertilization membrane. We demonstrate here that the ovoperoxidase of unfertilized eggs is located in cortical granules and, after fertilization, is concentrated in the fertilization membrane. Fertilization of sea urchin eggs or their parthenogenetic activation with the ionophore A23187 also results in (*a*) the conversion of iodide to a trichloroacetic acid-precipitable form (iodination), (*b*) the deiodination of eggs exogenously labeled with myeloperoxidase and H_2O_2, (*c*) the degradation of thyroxine as measured by the recovery of the released radioiodine at the origin and in the inorganic iodide spot on paper chromatography, and (*d*) the conversion of estradiol to an alcohol-precipitable form (estrogen binding). The iodination reaction and the binding of estradiol occurs predominantly in the fertilization membrane where the ovoperoxidase is concentrated. From the estimation of the kinetics of incorporation of iodine, we determine that the peroxidative system is active for 30 min after fertilization, long after hardening of the fertilization membrane is complete. Most of the bound iodine is lost during the hatching process. Iodination of albumin is catalyzed by the material released from the egg during fertilization, when combined with H_2O_2 and iodide. Iodination, thyroxine degradation, and estradiol binding are inhibited by azide, cyanide, aminotriazole, methimazole, ascorbic acid, and ergothioneine, all of which can inhibit peroxidase-catalyzed reactions. These responses of the sea urchin egg to fertilization are strikingly similar to the changes induced in polymorphonuclear leukocytes by phagocytosis and, in both instances, a peroxidative mechanism may be involved.

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