The Relationship between a Novel NAD(P)H Oxidase Activity of Ovoperoxidase and the CN\(^{-}\)-resistant Respiratory Burst That Follows Fertilization of Sea Urchin Eggs*

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The extracellular protein coat of the sea urchin egg is cross-linked after fertilization via dityrosyl linkages made by an exocytosed ovoperoxidase. The source of oxidant for this reaction is unknown, but eggs produce H\(_2\)O\(_2\) in amounts equivalent to the cyanide-insensitive O\(_2\) uptake “respiratory burst” that follows fertilization. Several possible H\(_2\)O\(_2\)-forming oxidase activities, including glucose, xanthine, fatty acyl, and fatty-acyl CoA oxidases, were absent from the egg cortex. However, an NAD(P)H oxidase activity oxidizing several substrates, including indoleacetic acid (Nakajima and Sugiyama, 1963), dihydroxyfumaric acid (Halliwell, 1977), and lactoperoxidase and thyroid peroxidase (Klebanoff, 1962) show no net production of H\(_2\)O\(_2\), but H\(_2\)O\(_2\) may be an essential intermediate (Akazawa and Conn, 1958), and lactoperoxidase and thyroid peroxidase (Klebanoff, 1962) show net production of H\(_2\)O\(_2\), but H\(_2\)O\(_2\) may be an essential intermediate (Akazawa and Conn, 1958; Yokota and Yamazaki, 1977). The oxidase activity of these peroxidases is greatly enhanced by the addition of Mn\(^{2+}\) and certain phenolic compounds, and when thus stimulated it is inhibited by low concentrations of CN\(^{-}\).

Since the respiratory burst of fertilization leads to H\(_2\)O\(_2\) production outside of the egg, we have examined isolated egg surface complexes (“egg cortices”) for oxidase activity. The egg cortex (Detering et al., 1977) includes the extracellular vitelline coat, plasma membrane, and secretory cortical granules which contain ovoperoxidase. Here we report the discovery of a Ca\(^{2+}\)-dependent NAD(P)H oxidoreductase activity from isolated sea urchin egg cortices and show that it can be accounted for by the oxidase activity of the recently isolated ovoperoxidase (Deits et al., 1984). The oxidase activity of ovoperoxidase, as that of other peroxidases, is sensitive to inhibition by CN\(^{-}\) when assayed in the presence of Mn\(^{2+}\) and phenols. However, when the NAD(P)H oxidase activity of ovoperoxidase is stimulated by Ca\(^{2+}\) and a small molecular weight egg cytoplasmic cofactor (factor ET), it becomes rela-
tively resistant to inhibition by CN-. The potential physiological significance of this novel oxidase reaction for the respiratory burst of fertilization is discussed.

EXPERIMENTAL PROCEDURES

Materials—Xanthine, GSH, GSSG, menadione, tyrosine, ATP, NAD, guanidino, NAHD, NADPH, fatty acids and their coenzyme A derivatives, N-acetyltyrosinamide, xanthine oxidase, lipoxidase, citrate synthetase, horseradish peroxidase, lactoperoxidase, catalase, superoxide dismutase, phosphorotransacetylase, and malate dehydrogenase were obtained from Sigma. 2,4-Dichlorophenol was obtained from Aldrich. Other chemicals were reagent grade or the highest grade commercially available.

S. purpuratus were gathered intertidally from the Strait of Juan de Fuca. Animals were spawned by intracelomic injection of 0.5 M KCl, and eggs were collected in 0.45-μm Millipore-filtered seawater. Manipulations of eggs and cortices were at 10 °C. Eggs were fertilized with freshly diluted sperm at an approximately 500:1 sperm to egg ratio. Fertilization membrane elevation occurred in >95% of eggs in all experiments.

Methods—Egg cortex preparation was by a modification of the method of Detcheff et al. (1977). Settled eggs were washed in 10 volumes of artificial seawater containing 0.5 M NaCl, 10 mM KCl, 2.5 mM NaHCO₃, and 25 mM EGTA, pH 8.0 (ASW-C), in which they spontaneously lose their jelly coats. The supernatant solution was decanted, and the eggs were resuspended in five times their original volume of ASW-C and homogenized in 10-ml lots with 10 strokes of a Teflon pestle tissue homogenizer (Thomas Scientific). The homogenate was then diluted to four times its original volume with artificial seawater containing 0.5 M NaCl, 10 mM KCl, 2.5 mM NaHCO₃, 25 mM EGTA, 10 mM Tris base, and 10 mM Hepes, pH 8.0 (ASW-B) and centrifuged in 12-ml conical centrifuge tubes at 9,000 rpm (1000 x g) in the clinical centrifuge. The flocculent white pellets were collected and resuspended in ASW-B.

Oxidoperoxidase was purified from the cortical granule exudate of artificially activated eggs by ammonium sulfate precipitation and ion-exchange chromatography as previously described (Deits et al., 1984). NAD(P)H oxidoreductase activity was assayed by the guaiacol method (Bergmeyer et al., 1974) in a 1-ml assay mixture containing 18 mM guaiacol, 0.3 mM EGTA, 0.4 mM NADH, but this activity was not measured.

Novel NAD(P)H Oxidase Activity of Ovoperoxidase

Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>O₂ uptake With 10 mM Ca²⁺</th>
<th>O₂ uptake Without Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact eggs (0.2 ml) (burst respiration)</td>
<td>23.4 nmol/min</td>
<td>-a</td>
</tr>
<tr>
<td>Egg homogenate (1 ml)</td>
<td>4.1 nmol/min</td>
<td>0.9 nmol/min</td>
</tr>
<tr>
<td>Egg cortices (0.1 ml) plus superenant (1.0 ml)</td>
<td>2.1 nmol/min</td>
<td>0.42 nmol/min</td>
</tr>
<tr>
<td>Egg cortices (0.1 ml) plus boiled superenant (1.0 ml)</td>
<td>17.0 nmol/min</td>
<td>3.3 nmol/min</td>
</tr>
<tr>
<td>Ovoperoxidase (13 units) plus boiled superenant (1.0 ml)</td>
<td>34.4 nmol/min</td>
<td>6.6 nmol/min</td>
</tr>
<tr>
<td>Cortices alone (0.1 ml)</td>
<td>0.45 nmol/min</td>
<td>-</td>
</tr>
<tr>
<td>Boiled superenant alone (1.0 ml)</td>
<td>0.85 nmol/min</td>
<td>-</td>
</tr>
<tr>
<td>Boiled cortices (0.1 ml) plus superenant (1.0 ml)</td>
<td>1.3 nmol/min</td>
<td>-</td>
</tr>
</tbody>
</table>

*—, not measured.

The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MESS, 2-(N-morpholino)ethanesulfonic acid.
Fig. 1. Co-purification of cortical oxidase and peroxidase. 425 mg of cortical protein, prepared from 30 ml of eggs as described under “Experimental Procedures,” was diluted 5-fold to 25 ml with H2O, causing cortical granule lysis (whole cortices, Table II), remaining particulate material was centrifuged out at 15,000 X g for 1 h. The soluble protein (lysed cortex supernatant, Table II) was dialyzed against a starting buffer of 10 mM Tris, 10 mM Hepes, 1 mM EGTA, 10% glycerol, pH 7.5, and applied to a DEAE-Sephacel ion-exchange column of 30-ml packed bed volume. The column was developed with a linear 0-400 mM NaCl gradient in the starting buffer, and fractions were assayed for NADH oxidase as described in the legend to Table I and for peroxidase (O) and protein (Δ) as described under “Experimental Procedures”.

Table II
Partial purification of the cortical granule oxidase activity

Cortical granule oxidase activity was purified as described in the legend to Fig. 1. Oxidase activity was measured spectrophotometrically; 1 unit of oxidase is the amount of enzyme required to consume 1.0 nmol of NADH/min under conditions standard for the factor ET assay (see “Experimental Procedures”) in the presence of 20 units of factor ET, 2 mM CN−, and 10 mM Ca2+.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Protein</th>
<th>Peroxidase</th>
<th>Oxidase</th>
<th>Oxidase/ Peroxidase</th>
<th>Oxidase/ Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml</td>
<td>mg</td>
<td>units/ml</td>
<td>units</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>600</td>
<td>3540</td>
<td>2220a</td>
<td>_b</td>
<td>3.0</td>
</tr>
<tr>
<td>Whole cortices</td>
<td>25</td>
<td>425</td>
<td>3220</td>
<td>7800</td>
<td>3.0</td>
</tr>
<tr>
<td>Lysed cortex supernatant</td>
<td>100</td>
<td>350</td>
<td>2800</td>
<td>7800</td>
<td>3.0</td>
</tr>
<tr>
<td>DEAE peak</td>
<td>14.4</td>
<td>5.0</td>
<td>1600</td>
<td>3900</td>
<td>2.4</td>
</tr>
<tr>
<td>Pure ovoperoxidase</td>
<td>1.0</td>
<td>1.0</td>
<td>1030</td>
<td>2440</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Assay of peroxidase in whole homogenates gives a significant lag which may affect the result.

b Assay not measured.

a Ovoperoxidase purified as described under “Experimental Procedures.” RZ > 1.4.

Assay mixture, which resulted in a rapid increase in O2 concentration.

Attempts to isolate the active heat-labile component of egg cortices led in every case to copurification of the NAD(P)H oxidoreductase and ovoperoxidase activity. Moreover, an amount of homogeneous ovoperoxidase (Deits et al., 1984), with peroxidase activity equal to the cortical preparation, was isolated from intact cortices maintained the same peroxidase/oxidase activity ratio throughout the purification.

Assay and Partial Purification of the Heat-stable Cytosolic Factor—The heat-stable factor (now called factor ET) of egg homogenates was prepared in quantity as described in the legend to Table III. Homogenization in EGTA-containing medium was not essential, but it simplified later steps by preventing cortical granule lysis and by preventing solubilization of the egg yolk lipid platelets.

Factor ET was assayed by measuring O2 consumption with the Clark electrode or NADH oxidation spectrophotometrically as described under “Experimental Procedures” with 10 mM Ca2+, 2.0 mM CN−, 0.4 mM NADH, and 117 nm ovoperoxidase added to the standard assay mixture (ASW-A). One unit of factor ET activity was defined as that amount needed for consumption of 1.0 nmol/min NADH (or 0.5 nmol/min NADP, see below). The factor ET assay showed hyperbolic kinetics with respect to the concentration of factor ET, as illustrated in Fig. 2. In order to conserve active factor ET, we routinely used 25 units/assay. Factor ET purified by the method above was heat-stable and Pronase- and trypsin-resistant. Factor ET passed a Mr >90% of the activity was lost over a few days at room temperature and over a few weeks at −5°C. Storage under nitrogen slowed the loss of activity, and long-term storage of active samples was possible under nitrogen at −80°C. Material purified and stored in this way was used in the experiments described below. Because residual EGTA in the preparation interfered with accurate manipulation of divalent cations at concentrations less than 1 mM, material for some experiments was further purified by ion-exchange chromatography on Dowex 1 OH−, eluted with a linear gradient of zero to 0.4 M ammonium formate, pH 7.0. Care was taken to minimize oxidation of the sample by purging solutions with N2 before use, and fractions were collected directly onto an ethanol/dry ice bath with subsequent lyophilization and storage under N2.
Novel NAD(P)H Oxidase Activity of Ovoperoxidase

Stoichiometry of Ovoperoxidase-Oxidase Reaction—Fig. 3 compares O₂ consumption to NADH consumption for the ovoperoxidase-factor ET system, measured simultaneously as described under “Experimental Procedures” in the absence of cyanide. The reaction rate fell to zero due to NADH exhaustion, as demonstrated by the additional O₂ consumption seen upon addition of a small quantity of NADH (data not shown). The stoichiometry of NADH:O₂ consumption when the reaction ceased was 1.6:1, suggesting that the overall reaction for most of the NADH equivalents is the complete (4e⁻) reduction of O₂ to water:

\[ 2 \text{NADH} + 2\text{H}^+ + \text{O}_2 \rightarrow 2 \text{NAD}^+ + 2\text{H}_2\text{O} \]  

but that some electrons are transferred to other acceptors. No increase in O₂ concentration was seen when catalase was added during the reaction, indicating that H₂O₂ was not formed quantitatively (data not shown). However, inhibition by catalase (shown below) indicated that hydrogen peroxide was an essential intermediate in the reaction, and in the presence of catalase or superoxide dismutase the final stoichiometry approached 2:1 (also below). Similar results were obtained in the presence of cyanide, with virtually identical stoichiometry, but there was an initial lag period and characteristically slower reaction rate.

The Phenol-Mn²⁺-dependent Oxidase Activity of Ovoperoxidase—In addition to the factor ET-dependent oxidase activity, ovoperoxidase was found to have an NAD(P)H-O₂ oxidoreductase activity requiring Mn²⁺ ion and certain phenols. This activity was similar to that found for horseradish peroxidase (Akazawa and Conn, 1958), lactoperoxidase (Klebanoff, 1962), and myeloperoxidase (Takanaka and O’Brien, 1975). Like horseradish peroxidase, ovoperoxidase utilized several phenols as cofactors including 2,4-dichlorophenol, N-acetyltirosinamide, and tyrosine. The apparent \( K_m \) for 2,4-dichlorophenol was 0.14 mM, with optimal activity near 0.5 mM and declining activity at higher concentrations leading to half-maximal activity again at 7 mM, and 1.17 mM ovoperoxidase. Oxygen consumption was corrected for a slow rate of background uptake by the electrode chamber and never actually fell below 40 μM.

Fig. 3. Stoichiometry of O₂ and NADH consumption measured in the oxygen electrode equipped for continuous-flow spectrophotometric monitoring at 360 nm. Assay mixture was 5 ml of ASW-A, pH 8.0, at 21°C with added 10 mM Ca²⁺, 0.375 mM NADH, 220 μM O₂, 75 units/μl factor ET, and 1.17 mM ovoperoxidase. Oxygen consumption was corrected for a slow rate of background uptake by the electrode chamber and never actually fell below 40 μM.

### Table III
Partial purification of factor ET

<table>
<thead>
<tr>
<th>Volume</th>
<th>Protein</th>
<th>Factor ET activity</th>
<th>( A_{360} )</th>
<th>Activity/ ( A_{360} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg homogenate</td>
<td>100</td>
<td>3,390</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Homogenate supernatant</td>
<td>85</td>
<td>1,420</td>
<td>38,500</td>
<td>420</td>
</tr>
<tr>
<td>Boiled supernatant</td>
<td>67</td>
<td>248</td>
<td>36,500</td>
<td>5,250</td>
</tr>
<tr>
<td>EtOH SN-1</td>
<td>3.5</td>
<td>-</td>
<td>38,300</td>
<td>420</td>
</tr>
<tr>
<td>EtOH SN-2</td>
<td>3.0</td>
<td>-</td>
<td>49,500</td>
<td>360</td>
</tr>
</tbody>
</table>

*The yield at each step approached or exceeded 100%, although discarded fractions had some activity; this is probably because of removal of substances which inhibited the assay.

* One \( A_{360} \) unit equals an \( A_{360} \) of 1.0 in a volume of 1 ml.

* - not measured.

* None detected.

### FIG. 2
Concentration of factor ET versus oxidase activity. NADH consumption was assayed under standard conditions as described in “Experimental Procedures” with (C) and without (O) 2.0 mM CN⁻. OPO, ovoperoxidase.
activity would be negligible. The optimum calcium concentration in the factor ET-Ca2+ system was 10 mM. Other ions tested at 1.0, 10.0, and 50 mM, including Mg2+, Sr2+, Ba2+, Ca2+, and Cu2+, did not appreciably stimulate NADH oxidation.

Fig. 5 shows the relative CN- insensitivity of the factor ET-Ca2+-dependent NADH oxidase activity of ovoperoxidase (as in Fig. 4) compared to its phenol-Mn2+-dependent NADH oxidase activity and peroxidase activity (see "Experimental Procedures"). Only the factor ET-dependent oxidase activity was relatively cyanide-resistant. In fact, the factor ET-dependent NADH oxidase activity was slightly stimulated at 10–50 μM CN−, concentrations that half-maximally inhibited the other activities.

**Inhibitors of the Factor ET-dependent Oxidase Activity of Ovoperoxidase**—Fig. 6 shows the effect of several inhibitors of the ovoperoxidase and of fertilization membrane hardening (Deits et al., 1984; Foerder and Shapiro, 1977) on the factor ET-Ca2+-dependent NADH oxidase activity of ovoperoxidase.

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**Fig. 4.** Cyanide inhibition of NADH oxidase activities. Cyanide inhibition of the factor ET-Ca2+-dependent oxidase activity of ovoperoxidase (O), horseradish peroxidase (□), and lactoperoxidase (△) is shown. Spectrophotometric assays were in ASW-A, pH 8.0, with 10 mM Ca2+, 25 units of factor ET, 0.4 mM NADH, 234 μM O2, and 130 nM ovoperoxidase or 300 nM horseradish peroxidase or lactoperoxidase, and the stated concentration of cyanide. Inset, cyanide inhibition of the phenol-Mn2+-dependent oxidase activity of ovoperoxidase (O), horseradish peroxidase (□), and lactoperoxidase (△). Assays were in ASW-A, pH 8.0, with 0.1 mM Mn2+, 0.5 mM 2,4-dichlorophenol, 0.4 mM NADH, 230 μM O2, and 50 nM ovoperoxidase, 50 nM horseradish peroxidase, or 24 nM lactoperoxidase, and the stated CN− concentration. 100% values were 17.2, 18.6, and 107.5 μmol/min/nmol of enzyme for ovoperoxidase, horseradish peroxidase, and lactoperoxidase, respectively.

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**Fig. 5.** CN− sensitivity of oxidase versus peroxidase activities of ovoperoxidase. Relative cyanide sensitivity of the factor ET-Ca2+-dependent (O) and phenol-Mn2+-dependent (●) oxidase activities of ovoperoxidase and of its peroxidase activity (□) is shown. Oxidase activity was measured by NADH consumption under standard conditions as described under "Experimental Procedures"; peroxidase activity was measured by guaiacol oxidation (see "Experimental Procedures").

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**Fig. 6.** Other inhibitors of the oxidase activity of ovoperoxidase. Inhibition of the factor ET-Ca2+-dependent oxidase activity of ovoperoxidase (OPO) by CN−, aminotriazole (ATA), N5−, and glycine ethyl ester (GEE) is shown. Assay mixture contained 1 ml of ASW-A at 20 °C with Ca2+, 0.4 mM NADH, 25 units of factor ET, 1.17 mM ovoperoxidase, and the stated inhibitor concentration.
Aminotriazole, azide, and glycine ethyl ester inhibited the oxidase at similar concentrations as for the other processes, but CN⁻ was markedly less effective as an inhibitor of the oxidase activity than of the ovoperoxidase activity (Fig. 6) or fertilization membrane hardening (Foeder and Shapiro, 1977; Hall, 1978).

**pH Dependence and Substrate Saturation**—Fig. 7 shows the pH dependence of the Ca²⁺-factor ET-ovoperoxidase system. Maximal activity was seen at pH 8.0, which corresponds to the pH optimum of its peroxidase activity (Deits et al., 1984) and to the pH of seawater.

The Ca²⁺-factor ET-ovoperoxidase system used NADH and NADPH indiscriminately; this has been observed with the phenol-dependent oxidase activity of horseradish peroxidase as well (Akazawa and Conn, 1958). The apparent $K_m$ for NADH was 50 μM and for NADPH 70 μM, with maximal rates at approximately 0.4 mM for both substrates, and a slight decline in rate at higher concentrations, under standard assay conditions with 25 units/ml factor ET. The apparent $K_m$ for the reduced pyridine nucleotides was unchanged by 2 mM CN⁻.

**Catalase and H₂O₂ Effects**—Fig. 8a illustrates the effect of catalase on the consumption of NADH by the Ca²⁺-factor.

**Discussion**

Hydrogen peroxide and other species it can generate, such as hydroxyl radical and superoxide, have a significant capacity for cellular damage. Virtually ubiquitous enzyme systems, catalase, glutathione peroxidase, and superoxide dismutase, exist to control this toxicity (Chance et al., 1979). It would seem reasonable in any case where H₂O₂ or superoxide was intentionally generated for an extracellular reaction that it should be produced outside the cell or near its surface to minimize damage to internal structures. For example, even in
the phagocytosing polymorphonuclear leukocyte, a cell with a much more limited future than the fertilized egg, the enzyme system forming H$_2$O$_2$ is located in the plasma membrane, which becomes the phagosomal membrane when the system is activated (Dewald et al., 1979). This system appears to be vectorial, so that the superoxide formed is delivered to the interior of the phagosome (Green et al., 1980). Furthermore, like virtually all aerobic cells (Chance et al., 1979), the sea urchin egg contains a substantial amount of catalase, the purpose of which is to destroy H$_2$O$_2$ produced as a by-product of aerobic metabolism; it is thus unlikely that H$_2$O$_2$ produced deep within the cell would ever reach the perivitelline space to interact with the ovoperoxidase. Finally, eggs treated with local anesthetics to prevent cortical granule exocytosis will fertilize and divide (Hylander and Summers, 1981), but do not have a respiratory burst (Foerder et al., 1978).

For the above reasons, our search for the respiratory burst oxidase has centered on the egg cortex, consisting of extracellular vitelline layer, plasma membrane, and cortical granules (Detering et al., 1977), supplemented by studies with whole egg homogenates. Although many oxidases are known, we focused on those which consume electron donors that might exist or be produced in sufficient quantity in the egg to account quantitatively for the oxygen consumed. A 2% (volume) suspension of dejellied eggs may cause a 30 μM drop in O$_2$ concentration via "burst" respiration in the 20 min following fertilization (Foerder et al., 1978, and this paper); thus, an appropriate two-electron donor must be present or produced at a cumulative concentration of >1.5 mM in the volume of the cells. This limits likely candidates for the oxidase substrate to major energy stores and intermediates, such as fatty acids, glucose, and reduced pyridine nucleotides, all of which are substrates for known H$_2$O$_2$-producing oxidases (Keevil and Mason, 1978). The initial screening for oxidase activities was done with the oxygen electrode, in the presence of cyanide, where we examined the effect of several possible oxidase substrates on egg cortices, with and without Ca$^{2+}$. Based on the possibility that a lipoxidase system or fatty acyl-CoA oxidase system like that of the peroxisome (Lazarow and de Duve, 1976) might be activated at fertilization, we tested several lipids, including 100 μM palmitic acid, 50 μM linoleic, oleic, and arachidonic acids, and similar concentrations of their CoA derivatives without success. Other ineffective substrates tested included xanthine, GSH, GSSG, menadione, and glucose. None of the compounds tested significantly increased CN$^{-}$-insensitive oxygen consumption (respiratory burst) in intact embryos. Although this is by no means an exhaustive search, it covers some of the more quantitatively likely possibilities. In this regard, Perry and Epel (1981) have shown a calcium-dependent oxygen consumption in Arbacia egg homogenates which is nonenzymatic and dependent on the lipid pigment echinochrome. However, echinochrome is not present in the eggs of S. purpuratus. They have also demonstrated a stimulation of lipid peroxidation after fertilization in S. purpuratus, but did not quantify it with respect to the respiratory burst or H$_2$O$_2$ production (Perry and Epel, 1985a, 1985b). At this point, there is little evidence that lipid

### TABLE IV

<table>
<thead>
<tr>
<th>Enzymes added</th>
<th>NADH:O$_2$ consumed</th>
<th>Ratio of NADH:O$_2$ consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase</td>
<td>Catalase</td>
<td>µg/ml</td>
</tr>
<tr>
<td>0, 40 (boiled)</td>
<td>0, 200 (boiled)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>2</td>
</tr>
<tr>
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<td>20</td>
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</tr>
<tr>
<td></td>
<td>100</td>
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</tr>
<tr>
<td></td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 9.** Effect of hydrogen peroxide on the NADH-oxidase activity of ovoperoxidase without (a) and with (b) cyanide and the effect of aging of the NADH stock solution (20 mM, pH 8.0) for 2 days prior to the assay (c). Assays were in 1 ml of ASW-A, pH 8.0, at 20 °C with 10 mM Ca$^{2+}$, 50 units of factor ET, 0.4 mM NADH, 234 μM O$_2$, 117 nM ovoperoxidase with (b and c) and without (a) 2.0 mM CN$^{-}$, and the stated concentration of hydrogen peroxide (micromolar).
peroxidation can account for these phenomena.

The only significant CN−-resistant oxidoreductase activity found in the egg cortex utilized reduced pyridine nucleotides, did not distinguish between NADH and NADPH, and was greatly enhanced by calcium ions and by a heat-stable factor from the cytosol (factor ET). The cortical enzymatic activity was shown to be due to the ovoperoxidase itself by isolating the enzyme directly from egg cortices and demonstrating the activity in ovoperoxidase purified by a standard procedure (Deits et al., 1984). A constant ratio of oxidase to peroxidase activity was seen throughout the purification, while the specific activity of both increased 100-fold relative to protein content (Table II). The cytoplasmic cofactor is of low molecular weight (<1000 from its behavior on Sephadex G-10) and is stable to 100°C but not ashing in a flame and is resistant to digestion with Pronase. Factor ET spontaneously oxidizes in air to an inactive form. It differs considerably from the phenolic compounds known to confer NAD(P)H oxidase activity on peroxidase, such as 2,4-dichlorophenol, resorcinol, and tyrosine, in that the oxidase activity with factor ET is significantly stimulated by Ca2+ (Table I) and is relatively cyanide-resistant (Figs. 4 and 5). It also could not be replaced by non-phenolic compounds known to affect peroxidase metabolism, including methimazole, ergothioneine, thiouracil, thiourrea, GSH, or GSSG (data not shown). The stoichiometry of O2 and NADH consumption in the ovoperoxidase-factor ET-Ca2+ system strongly suggests that factor ET is used catalytically, as an approximately 2:1 NADH:O2 stoichiometry is maintained throughout the reaction and H2O2 is not quantitatively produced. Isolation and identification of this cofactor are underway.

Although our data are compatible with the oxidase activity of ovoperoxidase being responsible for the respiratory burst of fertilization, a possibility previously considered by Hall (1978), several major problems exist with such an hypothesis. First, H2O2 is not produced in stoichiometric amounts by the ovoperoxidase oxidase reaction. However, fertilization membrane cross-linking does not require accumulation of H2O2, but only that oxidizing equivalents be available for peroxidative reactions. It is conceivable that under appropriate conditions some of the H2O2 produced in the oxidation reaction could be converted to cross-linking of the fertilization membrane in a tightly coupled sequence as shown in Equations 2 and 3.

\[
\text{NADH} + H^+ + O_2 \rightarrow \text{NAD}^+ + H_2O_2 \\
H_2O_2 + 2 \text{tyrosine} \rightarrow \text{dityrosine} + 2 H_2O
\]

We have shown that ovoperoxidase is capable of catalyzing both these oxidase (this paper) and peroxidase (Deits et al., 1984) reactions independently. The conditions under which they may be coupled have yet to be defined. It is interesting that there is a shift in the ratio of NADH to O2 from 1.6 in the absence of catalase to 2.0 in its presence (Table IV), suggesting that factor ET or another substrate in the reaction mixture may react with the low H2O2 concentration generated in a step similar to Equation 3, producing H2O. Whether factor ET is quantitatively oxidized in a step analogous to Equation 2 will be determined when homogeneous factor ET is available.

Considering ovoperoxidase as the respiratory burst enzyme also presents a topographical problem, in that it resides outside the egg during the burst, whereas NADH and NADPH are within the egg. We have not detected the net release of NADH or NADPH from the egg during the respiratory burst.2

Although it has long been known that an NAD+ kinase is activated at fertilization, no decrease in total pyridine nucleotide content indicative of NAD(P)H release has been observed (Epel, 1964), and indeed levels may actually increase (Whitaker and Steinhardt, 1981). One possibility is that NAD(P)H is released into the perivitelline space and resequestered after oxidation by ovoperoxidase. A more interesting possibility is that factor ET acts as a shuttle for transporting reducing equivalents across the membrane. Perhaps it is released inside the cell and released into the perivitelline space to transfer electrons to O2 via the ovoperoxidase, forming H2O2 which can be immediately used to cross-link tyrosyl residues. Factor ET activity has not been found in seawater surrounding eggs after the respiratory burst, but it may be resequestered by the egg or irreversibly oxidized. Virtually all of the activity of factor ET preparations is destroyed in minutes by incubation with millimolar concentrations of H2O2 (data not shown) or over days by exposure to air, indicating that factor ET can be irreversibly oxidized to a form from which activity is not recovered under our routine assay conditions. The reaction of factor ET with H2O2 in vitro also suggests an additional potential role: it may be part of a system that protects the cell from peroxidative damage, in a fashion similar to the glutathione-glutathione peroxidase system.

In broad perspective, the basic problem of the respiratory burst and dityrosine cross-linking that follow fertilization is this: to carry the reducing equivalents needed to reduce O2 to H2O2, and thus activate oxygen for peroxidative chemistry, across the cell membrane from the reducing milieu within the egg to the oxidizing one without. This transfer may be accomplished as H2O2 itself, with intracellular reduction of O2, risking peroxidative damage to cell structures or by some other transmembrane electron carrier, with external generation of activated oxygen species, perhaps in a way that leaves most of the activated oxygen enzyme bound. The results in this study suggest that the factor ET-ovoperoxidase system has some of the features characteristic of the system responsible for the respiratory burst. However, the physiological importance of this system will be better defined when the structure and biochemical reactivity of factor ET are known.

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