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9. These microfaunal horizons, described by M. L. Natland [thesis, University of California at Los Angeles (1952)], are based on benthonic foraminifera which are controlled by water depths rather than evolutionary changes in fauna. The terms Repettian, Venturian, Wheelerian, and Hallian, as used in Fig. 1, are microfaunal stages erected by Natland. However, within a single restricted environment, the abyssal plain, they may approach time lines and are so used in Fig. 1. In this environment in the Ventura Basin, the Repettian stage is early Pliocene, the Venturian late Pliocene, the Wheelerian Plio-Pleistocene, and the Hallian early Pleistocene in age. The water depth shoals from about 2000 m in the Repettian to 400 m near microfaunal horizon 5 where it is within the deep-water sandstone sequence; the water depth is unrelated to the sedimentation rate within the region covered by Fig. 1.
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Hydrogen Peroxide Induces Spawning in Mollusks, with Activation of Prostaglandin Endoperoxide Synthetase

Abstract. *Addition of hydrogen peroxide to seawater causes synchronous spawning in gravid male and female abalones, and certain other mollusks as well. This effect is blocked by exposure of the animals to aspirin, an inhibitor of the enzyme catalyzing oxidative synthesis of prostaglandin endoperoxide. Hydrogen peroxide activates this enzymatic reaction in cell-free extracts prepared from abalone eggs (a very rich source of the prostaglandin endoperoxide synthetase); this effect appears to reveal a fundamental property of prostaglandin endoperoxide synthesis. Applicability of these findings to both mariculture and medical purposes is suggested.*

Although the marine invertebrate animals constitute a vast and protein-rich food resource, control of their reproduction remains one of the principal barriers to their economical cultivation for human consumption (*1*). Accordingly, we have begun to investigate chemical means for controlling the reproduction and early development of the abalone, a large herbivorous marine snail (mollusc an gastropod) valued as a highly palatable source of meat protein in parts of the United States, Mexico, Australia, Africa, China, and Japan. We have found that the addition of hydrogen peroxide to seawater causes gravid male and female abalones (as well as certain other mollusks) to spawn, and that this effect may result from a direct activation of the enzyme-catalyzed synthesis of prostaglandin endoperoxide.

Experiments described here were performed with *Haliotis rufescens*, the red abalone, as gravid specimens may be found throughout the year (*2*); this is also the species of principal importance to the commercial fishery in the United States. These bottom-dwelling animals reproduce by broadcast spawning, with synchronous and copious liberation of gametes from animals of both sexes resulting in external fertilization and the subsequent development of free-swimming planktonic larvae (*3, 4*).

Spawn liberated from abalones of either sex can trigger responsive spawning in animals of the opposite sex (*3, 5*). We have found that this response can be detected with sensitivity and specificity by testing animals singly at 13° to 14°C, at which the spontaneous spawning of gravid animals is effectively suppressed (Table 1, section 1).

Under these conditions, we have tested compounds known to affect reproduction in other species (Table 1, section 2). Prostaglandin E and prostaglandin F [both known to mediate various physiological reactions, which in turn regulate ovulation, fertilization, pregnancy, and birth in humans and other species (*6*)] are occasionally effective in inducing spawning in both male and female abalones, under conditions in which comparably gravid animals, receiving control treatment in parallel, fail to spawn. Specificity of this effect is indicated by the failure of both mammalian gonadotropin and thyroxin [the latter capable of inducing spawning in male oysters (*7*)] to elicit any detectable response under these conditions.

The biosynthesis of prostaglandins is known to depend on the prior synthesis of prostaglandin endoperoxides; these unique, short-lived intermediates contain both unusual endoperoxide and hydroperoxy moieties, sequentially introduced from molecular oxygen by the catalytic activity of the enzyme fatty acid cyclooxygenase (prostaglandin endoperoxide synthetase) (*8*). Prostaglandin endoperoxides are the direct precursors of both the prostaglandins and the physiologically potent thromboxanes; the endoperoxides themselves may also be physiologically active (*8–10*). We were thus interested in investigating possible effects resulting from direct addition of the uniquely reactive oxidant hydrogen peroxide (H_2O_2) to seawater containing gravid abalones.

Addition of H_2O_2 (to a concentration of ~ 5 m*M* in alkaline seawater, *p*H ~ 9.1) reproducibly causes spawning in both male and female abalones within $3 \pm \frac{1}{2}$ hours after the first addition (Fig. 1 and Table 1, section 3). This induction can be blocked by aspirin, added to the seawater 15 minutes before the H_2O_2. Aspirin (acetylsalicylic acid) is a well-characterized inhibitor of the fatty acid cyclooxygenase-catalyzed first step in the conversion of arachidonic acid to prostaglandin endoperoxide (*10, 11*). The inhibition of the H_2O_2 induction of spawning by aspirin suggests, then, that cyclooxygenase activity and prostaglandin endoperoxide synthesis may be required in the animal during its exposure to H_2O_2 for the induction of spawning.

Rapid and specific extracellular destruction of H_2O_2 by catalase, added to seawater before the peroxide, also effectively blocks the induction of spawning (Table 1, section 3), which proves that it is the H_2O_2 molecule itself (rather than some contaminant) that is responsible for this induction. Blockade by the generalized reducing agent mercaptoethanol is consistent with the hypothesis that the action of H_2O_2 responsible for the induction of spawning is essentially an oxidative one. In fact, we have found that oxygen gas, bubbled through seawater containing gravid abalones, is marginally

effective in inducing spawning (*12*); the percentage of animals induced, and the speed of the induction, however, are both much lower than those reported here with H_2O_2. Kikuchi and Uki (*13*) recently demonstrated prompt and efficient induction of spawning in abalones and other mollusks exposed to seawater that had been irradiated with ultraviolet (UV) light. We have found that this effect is mediated by a mechanism similar to that characterized here, depending on oxidants generated by the energetic decomposition (UV photolysis) of the parent water molecule (*12*). A high degree of specificity in the physiological requirement for this oxidative activity is indicated by the failure of a variety of other common oxidants to induce spawning (*12*). A requirement for some specific, electronically activated form of oxygen is thus indicated.

The results presented above suggest that H_2O_2, or some product derived from this molecule, may act in concert with (or on) the prostaglandin endoperoxide–forming cyclooxygenase (or on some substrate formed as a consequence of the activity of this enzyme), for induction of spawning to occur. In Table 2, we demonstrate that H_2O_2 does, in fact, directly increase the rate of the reaction catalyzed by the prostaglandin endoperoxide–forming cyclooxygenase from reproductive cells of the abalone.

We find that abalone eggs, and the gonads from gravid animals of both sexes, are particularly rich in this cyclooxygenase; these tissues contain approximately 100 times higher specific activities of the enzyme than are found in rabbit prostate (*14*). [Mature sperm released by the abalone appear to contain only traces of this enzyme, however (*14*).] Stimulation of the reaction catalyzed by the enzyme from abalone eggs is proportional to the concentration of H_2O_2 up to a maximum at about 0.3 m*M*; increasing concentrations above this become progressively inhibitory. The "unstimulated" reaction, in the absence of exogenous H_2O_2, is completely inhibited by a small amount of purified catalase (Table 2); this finding has been verified in assays performed at higher resolution as well (*14*). This suggests that H_2O_2 must be produced and utilized as an obligatory substrate during the reaction. Under the conditions described in Table 2, it is this production of H_2O_2 that is rate-limiting for overall catalysis. [This finding also suggests that catalase may be one of the cytoplasmic "inhibitors" occasionally found to mask the activity of the cyclooxygenase in crude extracts of mammalian tissues (*6*).]

Specificity of the H_2O_2-stimulated, enzyme-catalyzed reaction is verified (Table 2) by the dependence on a heat-labile factor provided by the extracts, dependence on substrate (arachidonic acid), and sensitivity to inhibition by aspirin. The aspirin inhibition of the H_2O_2-stimulated enzymatic reaction in vitro parallels the aspirin inhibition of H_2O_2-induced spawning observed in vivo (Table 1). In contrast, indomethacin, a potent inhibitor of the cyclooxygenase from mammalian tissues (*10, 11, 15*), fails to inhibit the enzyme from abalone eggs (*14*); similarly, indomethacin fails to block induction of spawning by H_2O_2 in vivo (*12*). Diethyldithiocarbamate, a chelator with high specificity for copper, inhibits the enzyme from abalone eggs (Table 2); similar inhibition has been observed with the fatty acid cyclooxygenase from mammalian tissue (*16*) and is common to many of the true oxygenases. EDTA, a chelator specific for other heavy metals and quite inefficient in complexing copper, has no effect at similar concentrations.

Activation by H_2O_2 appears to be a general property of the prostaglandin endoperoxide–forming cyclooxygenases; we have observed this effect with crude enzyme from rabbit kidney and prostate, and from the Pacific gorgonian *Lophogorgia chilensis* (a soft coral) as well. In contrast, however, we find that oxidation of arachidonic acid by *lipoxygenase* is neither enhanced by H_2O_2 nor inhibited by catalase; thus the effect appears somewhat specific for the cyclooxygenase reaction. Organic peroxides, including those formed by the mammalian cyclooxygenase (and the related lipoxygenase), are capable of autocatalytic stimulation of their respective oxygenase-catalyzed syntheses (*17*). We observe that addition of H_2O_2 eliminates the lag prior to autocatalytic activation of the molluscan fatty acid cyclooxygenase; this suggests that the endoperoxide product may be stimulating this reaction simply by acting as a donor of active oxygen.

We consider it likely that H_2O_2, and the organic peroxides derived from it, serve as donors of some electronically

Table 1. Induction of spawning in *Haliotis rufescens*. Comparably gravid, sexually mature animals (maximum length, 10 to 25 cm), which had been well fed on the giant kelp *Macrocystis pyrifera*, were incubated individually in 12-liter tubs of well-aerated fresh running seawater (100 ml/min, 50 percent replacement per hour) and thermostatically maintained at 13° to 14°C. Groups of 6 to 12 animals, including those receiving control and experimental treatments, were tested in parallel for 5 to 24 hours after a 1-hour equilibration. Spawnings in response to prostaglandin F occurred within 3 to 16 hours after the first addition; all other spawnings reported here occurred within 3 ± ½ hour after onset of the experimental treatment. Spawning females (F) generally released ~ 10^5 to 3 × 10^6 eggs; males (M) released ~ 10^{11} to 10^{12} sperm. In section 1, control treatments included hourly additions (50 ml) of either fresh water, seawater, or seawater from tubs containing unspawned gravid abalones; eggs (~ 10^5 in 50 ml) or sperm (~ 10^9 in 50 ml) were obtained from spontaneously spawned animals and added promptly in single additions. In section 2, animals were tested with hourly additions of dimethylsulfoxide (DMSO, 1 ml) containing other substances as indicated. In section 3, tris(hydroxymethyl)-methylamine (tris, 6 ± 2 m*M*) was used to maintain *p*H 9.1 (± 0.1); without this buffer the seawater was *p*H 7.8 (± 0.1). Mean effective concentrations (maintained by fresh hourly additions to within ± 33 percent) were: prostaglandins E and F (unfractionated isomeric mixtures), 3 × 10^{-12}*M*; gonadotropin (pregnant mare), 50 IU/liter; thyroxin, 10^{-5}*M*; H_2O_2 (prepared by fresh dilution from 30 percent solution), 5 m*M*; aspirin, 5 × 10^{-4}*M*; catalase (bovine liver, 90 percent pure), 12 μg/ml; β-mercaptoethanol, 7.5 m*M*. Virtually all nonspawning animals, with the exception of those treated with aspirin, were subsequently found capable of spawning in response to H_2O_2 or some other characterized inducer.

Treatment	Total number of animals	
	Tested	Spawned
	Section 1	
Controls	77 (47 F, 30 M)	3 (2 F, 1 M)
Eggs	3 M	3
Sperm	4 F	2
	Section 2	
DMSO	30 (17 F, 13 M)	0
+Prostaglandin E	13 (9 F, 4 M)	5 (3 F, 2 M)
+ Prostaglandin F	15 (10 F, 5 M)	7 (5 F, 2 M)
+ Gonadotropin	10 (7 F, 3 M)	0
+Thyroxin	10 (7 F, 3 M)	0
	Section 3	
H_2O	18 (13 F, 5 M)	0
H_2O_2	31 (17 F, 14 M)	30 (16 F, 14 M)
Aspirin, then H_2O_2	26 (12 F, 14 M)	2 M
Catalase, then H_2O_2	5 M	0
Mercaptoethanol, then H_2O_2	5 M	0

Table 2. Hydrogen peroxide stimulation of the fatty acid cyclooxygenase-catalyzed reaction in extracts of abalone eggs. Aliquots of a cell-free extract prepared from abalone eggs (spontaneously spawned; 1 mg of protein per milliliter in 10 m*M* tris-HCl buffer, *p*H 7.85) were incubated for 10 minutes at either 0° or 98°C, and then diluted 200-fold into the same buffer containing 1 m*M* arachidonic acid and epinephrine for spectrophotometric assay (at 20°C) by a modification of the method of Takeguchi and Sih (*15*). The complete assay mixture also contained hydrogen peroxide (0.6 m*M*); the rate of the reaction catalyzed by this complete system corresponds to 0.19 μmole of arachidonic acid consumed per minute. Results shown are the averages of duplicate determinations. Catalase (bovine liver, twice crystalized) was tested at a concentration of 0.1 μg/ml in the absence of exogenously provided H_2O_2. Inhibition by aspirin (freshly neutralized sodium acetylsalicylic acid, 1 m*M*), sodium diethyldithiocarbamate (1 m*M*), or ethylene-diaminetetraacetic acid (EDTA, tetrasodium salt, 1 m*M*) was determined by preincubation and assay in the presence of these inhibitors. Details of the methods of extract preparation and assay will be reported elsewhere (*12, 14*).

Condition	Relative reaction rate (%)
Complete system	100 ± 10.9
Omit extract	< 1.3 ± 0
Heated extract	< 1.3 ± 0
Omit arachidonic acid	< 1.3 ± 0
Omit H_2O_2	8.7 ± 1.7
Omit H_2O_2, add catalase	< 1.3 ± 0
Plus aspirin	18.0 ± 5.1
Plus diethyldithiocarbamate	4.8 ± 1.2
Plus EDTA	102 ± 4.8

activated species of oxygen (such as the hydroperoxy free radical, HOO·, or the peroxy diradical, ·OO·) that is uniquely reactive and suitable as substrate for the cyclooxygenase-catalyzed addition of oxygen. In support of this suggestion, we note that the induction of molluscan spawning by H_2O_2 in vivo and the stimulation of the fatty acid cyclooxygenase reaction by H_2O_2 in vitro both require an alkaline environment, indicating that both phenomena may depend on the obligatory participation of some product derived from the hydroxide radical–catalyzed decomposition of H_2O_2 (*12*). Further support for this suggestion comes from our observation that the cyclooxygenase-catalyzed reaction is also markedly stimulated in vitro by addition of either sodium peroxide (Na_2O_2) or potassium superoxide (KO_2), both of which dissociate in water to yield electronically excited oxygen. From the sensitivity to glutathione peroxidase which they observed, Smith and Lands (*18*) concluded that a hydroperoxy radical was required for at least one of the reactions catalyzed by the cyclooxygenase from mammalian tissues; a similar suggestion was made by Flower *et al.* (*19*).

We suggest that H_2O_2 activation of prostaglandin endoperoxide synthesis may account for the aspirin-sensitive, H_2O_2 dependent induction of spawning that we observe (*20*). If the fatty acid cyclooxygenase reaction is indeed rate-limiting in the physiological sequence of reactions leading to spawning in the gravid abalone, we might expect that it normally be controlled, and physiologically triggered, by neural or hormonal factors, or both; there is considerable evidence for such control of cyclooxygenase activity in various mammalian tissues (*6*). Our preliminary data also suggest that synthesis of prostaglandin endoperoxide within the eggs may play an essential role in controlling fertilization and early embryonic development (*12, 14*).

Under optimal conditions, >98 percent of the abalone eggs and sperm shed in response to H_2O_2 induction were fully competent for normal fertilization and embryonic development, yielding hatched, free-swimming planktonic larvae 28 hours after fertilization (*12*). Hydrogen peroxide also induces synchronous spawning in male and female mussels (*Mytilus californianus*), and may be widely applicable for the control of reproduction in a number of different species (*12*). These or similar inexpensive and easily controlled chemical methods may thus offer unique advantages for synchronous control of reproduction in large numbers of animals, for both cultivation and genetic-breeding purposes.

These findings also open the possibility, particularly through the use of less reactive, less caustic analogs of H_2O_2, for the localized control of prostaglandin endoperoxide synthesis in humans and other mammals. Numerous therapeutic applications can be suggested for such control, as the prostaglandin endoperoxides (and their derivative prostaglandins and thromboxanes) are known to control fever, pain, the inflammation response, the clotting of blood, and cardiovascular, renal, and gastric function, as well as ovulation, fertilization, pregnancy, and birth (*6*).

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