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The Role of Lysyl Oxidase and Collagen Crosslinking during Sea Urchin Development

EDWARD BUTLER,* JEFF HARDIN,† and STEPHEN BENSON*,[1]

**Department of Biological Sciences, California State University, Hayward, California 94542, and †Department of Zoology, University of California, Berkeley, California 94720*

Lysyl oxidase, the only enzyme involved in collagen crosslinking, is shown to be present in embryos of the sea urchin *Strongylocentrotus purpuratus*. The enzyme specific activity increases over six-fold during development, showing the greatest rise during gastrulation and prism larva formation. The enzyme is inhibited by the specific inhibitor, β-aminopropionitrile (BAPN). Continuous BAPN treatment of *S. purpuratus* and *Lytechinus pictus* embryos from late cleavage stages onward increases the amount of noncrosslinked collagen present in prism larvae. When BAPN is added at the 128- or 256-cell stage it causes developmental arrest at the mesenchyme blastula stage. Embryos can be maintained in the arrested state for at least 96 h and will resume normal development and morphogenesis following BAPN removal. If BAPN is added after the mesenchyme blastula stage, it has little adverse effect on development; consequently nonspecific toxic effects of the drug are unlikely. The results suggest that lysyl oxidase and collagen crosslinking play a vital role in primary mesenchyme migration, gastrulation, and morphogenesis during sea urchin development and indicate that BAPN may be very useful in studying the extracellular matrix–cell interactions at the cellular and molecular level. © 1987 Academic Press, Inc.

The extracellular matrix (ECM), composed of collagen in association with proteoglycans and other extracellular glycoproteins, influences many developmental events. Cell shape, proliferation, migration, morphogenesis, and differentiation can all be affected by the surrounding ECM [5, 14, 20, 42].

Components of the ECM in sea urchin embryos have been described by morphological, biochemical, and immunological methods. Immunofluorescence studies using polyclonal antibodies to vertebrate collagens I, III, and IV indicate storage of these molecules in the unfertilized egg. Following fertilization these antigens redistribute and accumulate within the blastocoel and basal lamina [48]. Collagen synthesis can be detected at the mid-blastula stage and the rate of collagen accumulation increases through gastrulation to the prism stage, when typical striated collagen fibrils are visible within the blastocoel [8, 10, 11]. The presence of other ECM components such as proteoglycans, fibronectin, and laminin have all been detected in the ECM of sea urchin embryos [18, 35, 36, 48].

The role of the ECM and particularly collagen in sea urchin development is only beginning to be understood. Migration and differentiation of primary mesenchyme cells appear to require the presence of a collagen matrix [6, 47]. An earlier report [32] suggested collagen was the organic matrix of the calcareous spicule. Recent results do not support this idea and indicate that collagen is more likely a permissive substratum or spicule development [3, 4, 6].

[1] To whom reprint requests should be addressed.

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One method of examining the role of collagen in a developmental process is to inhibit collagen synthesis or deposition and observe the consequences. This approach is possible because a number of the co- and post-translational steps necessary for efficient collagen secretion and deposition may be inhibited. These modifications include hydroxylation of certain proline and lysine residues, glycosylation, cleavage of amino and carboxyl leader sequences, and inter- and intramolecular crosslinking of the mature collagen fibrils in the ECM [30, 31, 44].

Treatment of cells with proline analogs or inhibitors of the hydroxylation reaction results in disrupted collagen processing and decreased extracellular collagen accumulation [13, 33, 44, 46]. A potential problem with the use of proline analogs or prolyl and lysyl hydroxylase inhibitors is that they can also affect the synthesis of other proteins as well as general cell metabolism [13, 33, 44, 46]. A more specific inhibitor of collagen metabolism is the lathrytic agent β-aminopropionitrile (BAPN). BAPN is a specific inhibitor of lysyl oxidase [34, 37]. Lysyl oxidase plays a pivotal role in the crosslinking of collagen by oxidizing peptidyl lysine residues to peptidyl α-aminoadipic-semialdehydes, the reactive precursors to a series of covalent inter- and intramolecular polypeptide crosslinks [30, 31, 34]. These crosslinks are necessary for collagen fibril formation, tensile strength, and deposition [2, 12, 21, 24, 34, 38, 39].

Because of the important role of lysyl oxidase in collagen metabolism we have investigated the presence and potential role of lysyl oxidase and collagen crosslinking during sea urchin development. We report that lysyl oxidase activity is present in the embryo prior to gastrulation but undergoes a six- to seven-fold increase in specific activity during gastrulation. Embryos treated during late cleavage stages with BAPN do not show the postgastrula increase in lysyl oxidase activity, and morphogenesis is inhibited. The inhibited embryos remain viable for at least 96 h and will resume normal development following removal of BAPN. These observations suggest that collagen crosslinking and deposition play an important role in mesenchyme migration, gastrulation, and spicule formation.

MATERIALS AND METHODS

A. Embryo culture and enzyme extraction. Embryos of *S. purpuratus* and *L. pictus* were cultured in Millipore-filtered seawater (MFSW) at 2–6×10^3/ml with constant stirring (60 rpm) or without stirring in plastic petri dishes at 16°C. Cultures to be used for enzyme extraction were concentrated by centrifugation, washed with 1.5 *M* dextrose, and homogenized in 3 vol of 0.05 *M* Tris, 0.14 *M* NaCl, 6 *M* urea, pH 7.6. The homogenate was centrifuged at 28,000*g* for 10 min and the supernatant was dialyzed overnight against 0.05 *M* Tris, 0.14 *M* NaCl, pH 7.6. Extracts were assayed for lysyl oxidase activity immediately upon completion of dialysis. Protein concentration of the extract was determined by the method of Bradford [7].

B. Lysyl oxidase assay. Lysyl oxidase was assayed by the tritium-release method of Melet *et al.* [23] using a [^{3}H]lysine-labeled collagen substrate made from 15-day chick embryo calvaria. This assay is based on the quantitation of tritiated water produced by enzyme action on collagen labeled at the 6-carbon of lysine and hydroxylysine. The reaction mixture was incubated for 8 h at 22°C and stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 10%. After chilling on ice for 10 min the reaction mixture was centrifuged at 10,000*g* for 10 min. The TCA supernatants were passed through small Dowex 50 (H+) columns and the effluents were counted for radioactivity. Under these conditions enzymatic activity was linear with respect to protein concentration and time (data not shown). Controls included omitting the enzyme from reaction mixture and heating the enzyme extract

to 100°C for 3 min prior to addition to the reaction mixture. Activity values for these controls were usually equivalent and were subtracted as background.

C. Estimation of collagen crosslinking. The extent of collagen crosslinking can be estimated by the solubility of collagen in neutral and acidic solvents containing pepsin [12, 21, 22, 24]. Embryos (2×10^3/ml) cultured from late cleavage or early blastula stage in the presence and absence of 0.5 m*M* BAPN were collected by centrifugation, washed with dextrose, and homogenized in 0.05 *M* Tris, 1.0 *M* NaCl, pH 7.5. An aliquot of the homogenate was removed for total hydroxyproline content and the remainder was stirred at 4°C for 120 min and centrifuged at 28,000*g* for 10 min. The supernatant was saved and the pellet was extracted as above with 0.5 *M* acetic acid containing 0.5 mg/ml pepsin. The total homogenate, neutral salt, and pepsin solubilized supernatants were dialyzed against water and lyophilized, and the hydroxyproline content was determined as a measure of collagen content [49].

D. The effect of BAPN on embryonic development. Embryos of *S. purpuratus* and *L. pictus* were cultured in MFSW containing the indicated BAPN concentrations from late cleavage (256-cell stage) until controls reached the pluteus stage. Some cultures were removed from the BAPN by centrifuging at 500*g* for 60 s. The embryo pellet was washed twice in 50 vol of MFSW, resuspended in MFSW, and cultured exactly as controls.

RESULTS

A. Lysyl Oxidase Activity

Lysyl oxidase activity was determined at different times in control and BAPN treated cultures. A representative ontogeny is shown in Fig. 1. Embryos were cultured from the 256-cell stage in the presence or absence of 0.5 m*M* BAPN. Lysyl oxidase specific activity shows a slight but reproducible rise at 20 h coincident with blastula formation followed by a more significant increase during gastrulation and prism larva formation. During this period (38–70 h) lysyl oxidase specific activity increases over six-fold, whereas activity in BAPN-treated embryos is essentially unchanged, remaining at the preblastula level. It is unclear whether the activity that is present in the unfertilized eggs and persists in BAPN-treated embryos represents legitimate lysyl oxidase enzyme activity which is resistant to BAPN inhibition or represents another oxidative enzyme activity which results in the oxidation of the tritiated lysine collagen substrate. Embryos washed free of BAPN at 70 h and cultured for an additional 20 h have demonstrable lysyl oxidase activity (Fig. 1). Since BAPN inhibition is considered essentially irreversible (41), this result suggests that the synthesis of lysyl oxidase is still significant in prism and pluteus larvae.

B. Collagen Crosslinking

The physiological role of lysyl oxidase is to catalyze the oxidation of certain lysine and hydroxylysine residues to the corresponding aldehyde as the first (and only enzymatic) step in collagen crosslinking. It follows that inhibition of lysyl oxidase should increase the amount of uncrosslinked collagen. An estimate of the degree of collagen crosslinking can be made by determining its solubility in neutral solvents and acidic solvents containing pepsin. Solubilization of crosslinked collagen often requires acidic conditions and pepsin digestion [22, 25], whereas collagen containing few crosslinks is usually soluble in neutral salt solutions [12, 21, 25]. Control prism embryos and embryos treated with BAPN from the blastula stage onward were collected and sequentially extracted with 1

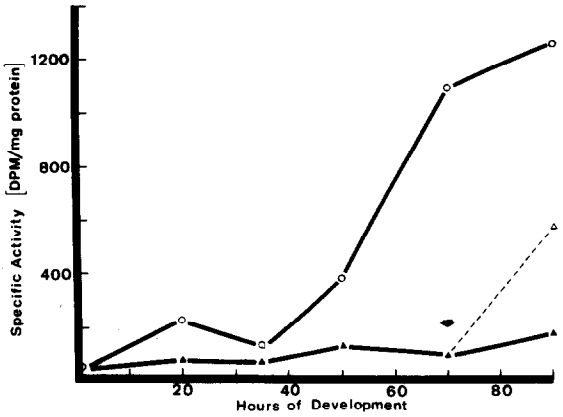

Fig. 1. Lysyl oxidase specific activity during normal development and the effect of BAPN. Embryos of *S. purpuratus* were cultured in the presence or absence of 0.5 m*M* BAPN. At the indicated times, embryos were collected and lysyl oxidase was assayed as described under Materials and Methods. At 70 h postfertilization an aliquot of BAPN-treated embryos was washed free of the inhibitor and cultured for an additional 20 h, after which embryos were assayed for lysyl oxidase activity. Each point represents the average of duplicate determinations and the ontogeny was repeated three times with different preparations of substrate. Since each substrate preparation had a different specific activity quantitative comparisons between developmental time courses cannot be made. Control (○), BAPN (▲), BAPN reversed at 70 h (△).

M salt and dilute acetic acid containing pepsin. The effect of BAPN on collagen content and solubility is shown in Table 1. Continuous treatment with 0.5 m*M* BAPN does not decrease the amount of collagen in the embryos but significantly elevates the amount of collagen soluble in neutral salt. This result is consistent with decreased collagen crosslinking due to BAPN inhibition of lysyl oxidase.

C. Effects on Morphogenesis

The effects of BAPN on morphogenesis of the sea urchin embryo are striking and very similar in the two species examined. Two effects may be observed,

TABLE 1

Effect of BAPN on collagen crosslinking

Experiment	Total collagen content (μg)	Percentage salt soluble	Percentage pepsin soluble	Percentage recovery
1 Control	68±8	48±9	41±5	89
BAPN	73±4	71±10*	22±6*	93
2 Control	79±6	51±8	28±7	79
BAPN	74±8	65±6	16±5	81

Note. S. purpuratus embryos (7.2×10^6) were cultured in the presence or absence of 0.5 m*M* BAPN from blastula until controls reached the pluteus stage (90 h), at which time hydroxyproline content was determined. Values for hydroxyproline content and solubility percentages were calculated by comparing the amount of hydroxyproline in each fraction to the total hydroxyproline content of the total homogenate. Values indicate the average ±SEM of four determinations.

* Significantly different from control values at $P\leq0.05$.

depending on the time of addition of the BAPN. Figure 2 illustrates the first effect, typical of *L. pictus* embryos when BAPN is added at the 256-cell stage. Embryos treated with BAPN are greatly retarded in their rate of development compared to controls. When control embryos are at the early prism stage (40 h postfertilization), BAPN-treated embryos have only just hatched (Figs. 2*A* and 2*B*). Embryos treated continuously with BAPN over several days are viable and swim normally, but are arrested at the mesenchyme blastula stage (Fig. 2*A*). The primary mesenchyme cells of such embryos ingress, but then fail to migrate away from the vegetal pole. More remarkable, however, is the complete reversibility of the effects of BAPN on morphogenesis. Embryos held in an arrested state of development by continuous BAPN treatment for 72 h (*L. pictus*) to 96 h (*S. purpuratus*) resume development when returned to normal seawater (Fig. 2*C*). Following such reversal, gastrulation occurs in essentially normal fashion, followed by the production of spicules at the prism stage (Fig. 2*E*).

When BAPN is added to *L. pictus* embryos at the hatched blastula stage, a somewhat different type of morphogenetic abnormality is observed (Fig. 3). At the gastrula stage, when the primary mesenchyme cells of control embryos aggregate into two clusters prior to overt spicule formation, the primary mesenchyme cells of BAPN-treated embryos display an impaired ability to migrate with little or no localization into aggregates. Instead, the primary mesenchyme cells tend to be rounded, have noticeably less protrusive activity, and appear to wander without direction through the blastocoel (Figs. 3*A* and 3*B*). Likewise, in BAPN-treated embryos the secondary mesenchyme cells at the tip of the archenteron are rounded, sending out fewer filopodial protrusions than those of controls (Fig. 3*A*). Often the archenteron seems flaccid in such embryos, although it still possesses the capacity to elongate, albeit normally. In embryos that gastrulate successfully, lateral buckling of the archenteron wall often occurs, giving the archenteron a "kinked" appearance (Fig. 3*B*).

DISCUSSION

The results of this study demonstrate that lysyl oxidase is present in sea urchin embryos and that its activity is important for gastrulation and subsequent development. Lysyl oxidase specific activity increases several-fold during the postgastrula period but our results suggest that a crucial period for lysyl oxidase is during the pregastrula phase of embryogenesis. Since lysyl oxidase is the only enzyme involved in collagen crosslinking, the results imply that crosslinked collagen is necessary for gastrulation and morphogenesis. This conclusion is supported by the observation that BAPN, a specific inhibitor of lysyl oxidase, causes developmental arrest if added before the mesenchyme blastula stage. BAPN-treated embryos progress to the mesenchyme blastula stage and stop further development. Likewise, although the total amount of collagen in BAPN-treated embryos is not reduced, the percentage of noncrosslinked collagen is increased in BAPN-treated embryos compared to controls. Crosslinked collagen may be necessary for gastrulation and morphogenesis and this is concordant with a number of

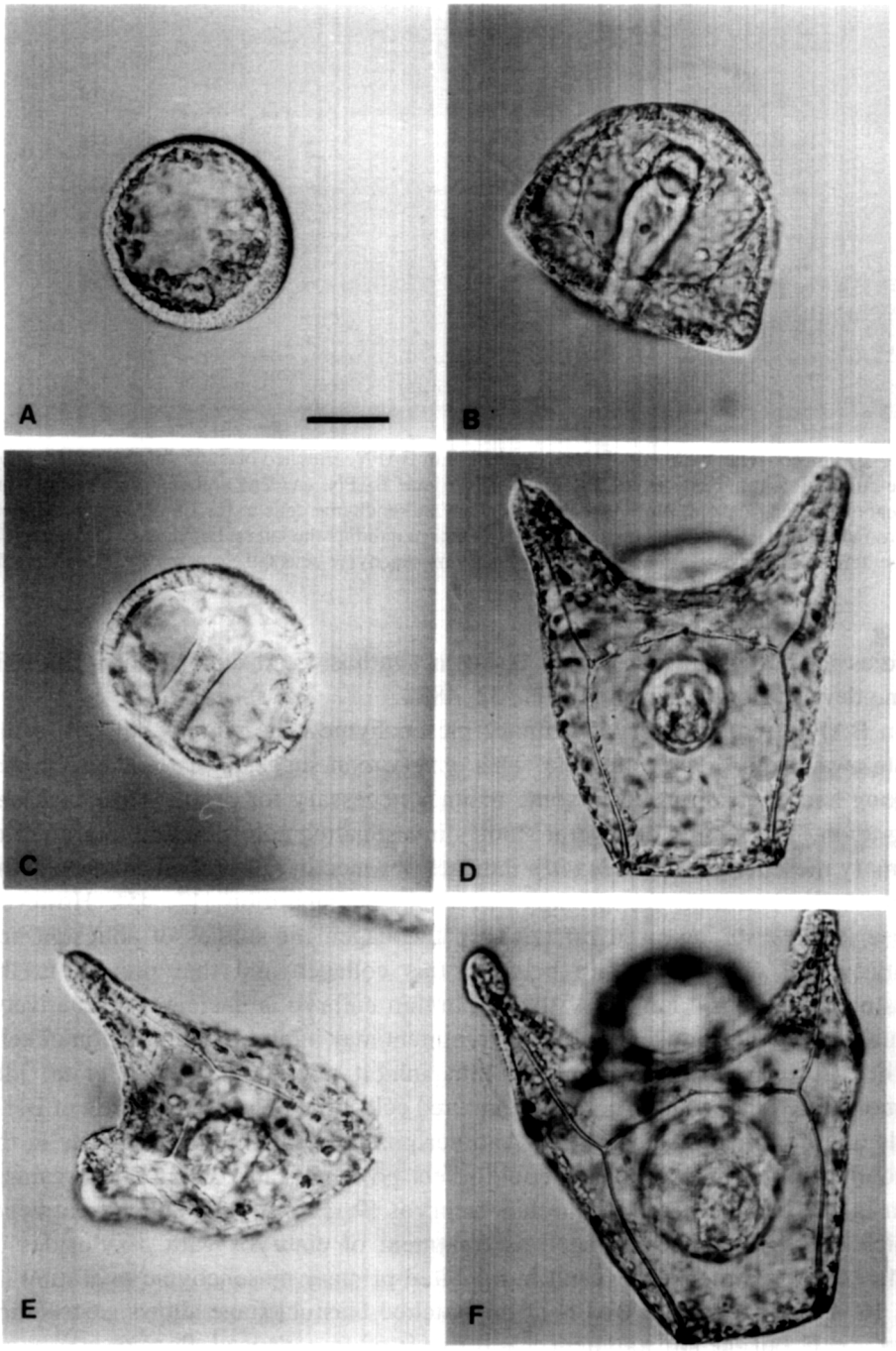

Fig. 2. Reversible inhibition of differentiation and morphogenesis by BAPN. Left-hand column shows *L. pictus* embryos treated with BAPN; right-hand column shows corresponding control embryos. (*A*) Embryos treated continuously with 0.75 m*M* BAPN from 256-cell stage at 40 h postfertilization. (*B*) Control embryos at 40 h. (*C*) Embryos treated for 40 h with BAPN, washed, and cultured until 72 h postfertilization in absence of BAPN. (*D*) Control embryos at 72 h. (*E*) BAPN-treated embryo reversed at 40 h and cultured until 96 h. (*F*) Control embryos at 96 h. Scale bar = 50 μm.

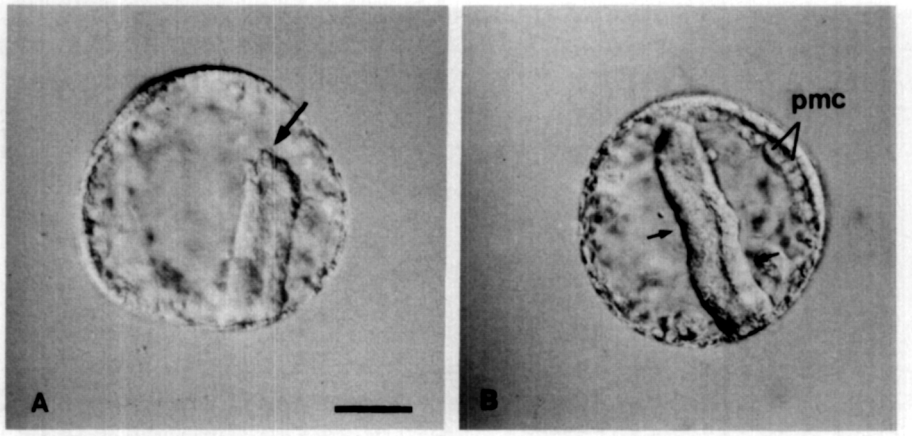

Fig. 3. Morphogenetic effects of late treatment with BAPN. Hatched blastula embryos of *L. pictus* were cultured continuously in the presence of 0.75 m*M* BAPN. (*A*) The archenteron elongates but appears somewhat flaccid. Note that the secondary mesenchyme cells at the tip of the gut rudiment are rounded and lack filopodia (arrow). (*B*) This embryo exhibits the lateral buckling or kinking of the archenteron wall (arrows). In addition, the primary mesenchyme cells fail to aggregate. Scale bar = 50 μm.

studies indicating an increase in collagen synthesis and deposition during this same developmental period [10, 11, 32, 48].

In BAPN-treated embryos primary mesenchyme cells ingress but fail to migrate away from the vegetal pole. This observation suggests that collagen crosslinking and deposition are in some manner necessary for primary mesenchyme migration. The results of several studies investigating adhesion and migration of primary mesenchyme cells *in vitro* indicate fibronectin rather than collagen is the preferred substratum for primary mesenchyme migration [17, 45]. However, those *in vitro* studies used rat tail type I collagen for studies of adhesion and motility and the relationship between this collagen and that present in the blastocoel and basal lamina of the sea urchin embryo is unclear. Alternatively, soluble collagen produced by BAPN treatment may inhibit the interaction of cells with a fibronectin substratum and thus inhibit migration. Nagata *et al.* [28] demonstrated that soluble collagen inhibited cell spreading and migration of BHK cells on a fibronectin substratum. Another possibility is that the decrease in the amount of crosslinked collagen could affect proteoglycan content and organization in the blastocoel of sea urchin embryos [9, 29, 40, 43]. Other treatments which alter proteoglycans, such as treatment of embryos with β-xylosides or sulfate-free seawater, also result in inhibited primary mesenchyme migration [1, 15, 16, 45]. Exposure to BAPN at the hatched blastula stage allows gastrulation to proceed, but the gut rudiment that forms is often collapsed, flaccid, or kinked. The simplest interpretation of this result is that crosslinking of collagen in the basal lamina and blastocoel provides structural support for the archenteron. The abundance of fibrillar collagen in the blastocoel [8] and the association of fibrillar material with the archenteron in normal embryos [1, 19] is consistent with this observation. Treatment of embryos with another inhibitor of collagen processing,

α,α'-dipyridyl, an inhibitor of proline hydroxylation, also causes archenteron collapse [26, 27], again indicating a role for collagen in stabilizing the archenteron.

An obvious concern in studies of this type is the potential toxic effect of the agent on the embryos. In the case of BAPN two observations argue against this possibility. First, postgastrula embryos treated with BAPN are apparently unaffected by the drug and develop normally. Second, embryos treated with BAPN at the early blastula stage can be removed from BAPN after 72–96 h of exposure and normal development resumes. The ability of BAPN-reversed embryos to complete normal development suggests that collagen synthesis continues normally in the presence of BAPN. We presume that when BAPN is removed, newly synthesized lysyl oxidase can then catalyze crosslinking and restore a suitable collagen substratum for continued morphogenesis. Our suggestion that newly synthesized lysyl oxidase catalyzes the renewed crosslinking stems from the observation that BAPN-reversed embryos show significant lysyl oxidase specific activity within hours after being washed free of the drug. Since BAPN inhibition of vertebrate lysyl oxidase is irreversible [41], demonstrable enzyme activity is likely due to *de novo* enzyme synthesis.

The evidence presented here confirms and extends previous reports of a role for collagen in sea urchin morphogenesis and differentiation [6, 11, 26, 27, 47, 48]. The mechanism by which collagen exerts its influence is not known. Given the myriad roles collagen can play in embryonic development, from providing structural support or a suitable substratum for adhesion and migration to serving as a permissive substratum for differentiation, any combination may prevail in this system. An interesting aspect of this study is the specificity and reversibility of BAPN as an inhibitor of collagen crosslinking. This may permit new approaches to investigate the cellular and molecular effects of the ECM on cell differentiation and embryonic development.

Note Added in Proof. Following the submission of this manuscript, Wessel and McClay published a paper where they reported the effects of BAPN on sea urchin collagen metabolism and morphogenesis. Our respective observations are similar. Their paper was published in *Developmental Biology* (1987) **121**, 149.

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