THE ISOLATION OF A PUTATIVE COLLAGEN AND A STUDY OF ITS SYNTHESIS IN THE DEVELOPING SEA URCHIN EMBRYO

by

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"Often something works once with sea urchin eggs, then no more for twenty years."

Theodor Boveri

from p. 85, "Theodor Boveri: Life and Work of a Great Biologist"

—Fritz Baltzer
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Preface

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Introduction

Collagen is the major macromolecule of most connective tissues in vertebrates. Similarly, it has been identified in many invertebrate tissues (34). The major scope of this thesis is concerned with the evidence for the existence of an invertebrate collagen type in developing sea urchin embryos (*Strongylocentrotus purpuratus*). Before reviewing recent work on collagen in sea urchins, it is necessary to mention something about collagen polymorphism and biosynthesis in general.

I. Collagen Polymorphism and Biosynthesis

In recent years, it became apparent that the collagen fibers present in connective tissues of higher vertebrates were formed by the aggregation and precise alignment of individual collagen molecules. Subsequent studies indicated that the triple-stranded molecule comprised two identical chains (designated a1) and a distinct, but clearly homologous, chain (designated a2) (57). This collagen monomer is a long, thin rod-shaped molecule, approximately 1.5 μm in diameter and 300 μm in length, and the constituent alpha chains are coiled around each other in a right-handed triple helical conformation. The alpha chains contain about 1050 amino acids and have a molecular weight of 97,500 daltons. Glycine accounts for one-third of the total amino acids present in the polypeptide chain and is distributed such that every third residue is glycine. Thus, these polypeptide chains may be considered as having a simple triplet structure represented as \((-\text{Gly-}X-\text{Y-})_n\) in which the X and Y positions can be occupied by a variety of amino acids, but frequently the X position amino acid is proline.
and the Y- position is hydroxyproline. The ends of the three alpha chains are in at least approximate alignment; and while the triple helical structure extends throughout the body of the molecule, there are regions at the amino- and carboxy- terminii, approximately 15-20 amino acids in length, which are non-helical. These regions (telopeptides) are susceptible to proteolysis, whereas the triple-helical region of the molecule is resistant to most proteolytic enzymes and contain lysine or hydroxylysine residues involved in intermolecular cross-link formation.

The finding that such a molecule could be extracted from a variety of tissues, like skin, tendon, and bone, suggested that all vertebrate collagens fell into a simple class or type of molecule having the above chain composition. However, since 1970, this concept has been drastically changed, after the existence of several genetically distinct forms of collagen was demonstrated. The discovery of this polymorphism in the molecular species of collagen found in the extracellular matrix represents a major insight into the composition of this matrix. This has coincided with advances in our understanding of the mechanisms involved in the biosynthesis of collagen, particularly the elucidation of precursor forms (procollagens) for the currently defined collagen types. These findings have raised many important questions regarding the molecular and cellular biology of collagen synthesis and its regulation.
A. Interstitial Collagen Types

The first evidence for the existence of various types of collagen molecule was indicated in sternal cartilages from lathyritic chicks (43). These studies demonstrated a triple-stranded collagen molecule comprised only a single type of alpha chain exhibiting major differences in amino acid composition and sequence when compared to the a1 and a2 chains of the previously described form of collagen. This alpha chain derived from cartilage collagen (designated Type II collagen) more closely resembled a1 than a2 in composition and chromatographic behavior and was therefore termed the a1 (II) chain, with the previously described a1 chain now being referred to as a1 (I). Subsequent studies on the isolation and characterization of collagen molecules from mammalian cartilages, including human articular cartilage (58), have confirmed the existence of type II collagen in a variety of species.

Further evidence for the heterogeneity of interstitial collagen species came with the demonstration of a third type which has since been isolated and characterized from several tissues, such as skin, aorta, and external wall (44). These type III collagen molecules contain three identical a1 (III) chains, covalently linked by disulfide bonds in their carboxy-terminal regions (59).

These molecular species of collagen (types I-III) represent the major clearly defined interstitial collagens present in connective tissues. All the chains possess the characteristic features of collagenous polypeptides, namely, a high content of
glycine, proline, hydroxyproline, and alanine, together with the presence of hydroxylysine. The alpha chains of type III collagen are distinctive in containing cysteine residues which are involved in the interchain disulfide bonds in the molecule (45).

The fact that the different collagen alpha chains are distinct gene products is emphasized by studies on the primary structure of the chains. The complete sequence of amino acids in the α1 (I) chain is known (60), and about two-thirds of the sequence of amino acids in the α2 (I) chain is also established. In these studies, the chains were digested with cyanogen bromide. The order of the peptides along the chain was then deduced and the detailed sequence analysis of the various peptides was carried through to completion.

Analysis of CNBr-peptide distribution by ion-exchange chromatography and/or polyacrylamide gel electrophoresis followed by subsequent characterization of the peptides represents the major means of distinguishing between the known interstitial collagen types. It has also been used to identify potentially novel forms of collagen.

A type of alpha chain from one species is generally considered to be a single unique sequence of amino acids throughout its length. The complexity of collagen gene expression in cartilaginous tissues has been further extended, however, by the observations of Butler et al which have indicated that the α1 (I) present in bovine nasal cartilage collagen were the products of more than a single structural gene (61). Thus, it is concluded
that the polypeptide chains which result possess relatively few amino acid sequence differences. The evidence presented suggested that this microheterogeneity in the forms of α1 (II) arises from the expression of separate structural genes.

While the sequence data for the different types of interstitial collagen is incomplete, it is difficult to make any analyses to determine evolutionary relationships or any other quantitative measurement of species or tissue differences. A qualitative assessment which can be made is that where differences occurs, they usually arise from conservative substitutions. This suggests the maintenance of a molecular structure suited to a set of intermolecular interactions that produce a fibril structure and that this structure is similar for types I, II, and III collagens. Comparison of the amino acid sequence from various regions of the alpha chains of type I and type II collagens indicated that some regions are more subject to genetic variability than others (62) and these could be related to different requirements for specificity in different parts of the collagen molecule.

The existence of these genetically distinct interstitial collagens is of considerable interest since it suggests that collagen molecules and the fibers derived from them have other functions in addition to their structural role. In order for this question to be answered, the special functional requirements fulfilled by the presence in a particular tissue of a single type of collagen or a mixture of collagen types must be determined.
Type I collagen is the most abundant form of collagen and occurs in a larger number of tissues. Some of these tissues include bone, dentin and dermis.

Production of type II collagen has until recently been associated with cartilage and with tissues involved in the embryonic induction of cartilage. But it also has been found in the cornea, vitreous body, neural retinal tissues, and bovine cornea (51,63,64).

In addition to skin and the major blood vessels, type III collagen has been found in a wide variety of tissues including human lung and intestine and periodontal ligament (65,66,61). Thus, the tissue distribution of type III collagen appears to be almost as diverse as that of type I. It also has been found in recent evidence that type III always co-exists with type I. Thus, the existence of three distinct forms of collagen is now well established. Recent data has also suggested that further types of interstitial collagen may await characterization (5).

B. Basement Membrane Collagen Types

The range of diversity in the molecular forms of collagen in vertebrate tissues has been further extended by consideration of the data indicating the presence of collagenous components in basement membranes. Such membrane structures represent a class of cell surface materials lying at the interface of the extracellular matrix and the external plasma membrane of the cell. Early work on the the amino acid and carbohydrate composition of glomerular basement membrane (GBM) by Kefalides and co-workers
suggested the existence of a collagen-like protein in this structure together with other non-collagenous proteins (62). This structure contained a collagen comprised of three identical alpha markedly different in compositional features from the alpha chains of interstitial collagens (67). On the basis of this, the alpha chains of this basement membrane collagen have been designated \( \alpha_1(IV) \) chains. This collagen molecule, which has chain composition \( \alpha_1(IV)_3 \), was referred to as type IV collagen (68). The amino acid composition of the \( \alpha_1(IV) \) chain include high contents of 3-hydroxyproline and hydroxylysine, some cysteine, and a low content of alanine. Besides its presence in a variety of vertebrate tissues, type IV collagen has been shown to exist in various invertebrate tissues including the annelid Nereis, the nematode Ascaris, and the Hydra (69,70,71).

Thus, there would appear to be strong evidence for adding the type IV collagen found in basement membranes to the list of defined, genetically distinct collagen types, i.e., interstitial collagen types I-III. However, there is growing evidence that molecular heterogeneity of collagen type may exist within basement membrane structures as it does in the interstitial connective tissue matrix. Controversy has in fact existed for several years as to the molecular organization of basement membranes, including GBM, and this has extended to the nature and number of the collagenous components as well as to their interrelationship with other glycoprotein constituents. Based on accumulated evidence, basement membrane collagen would appear to cover a heterogeneous group of at least five collagen types.
comprised of six genetically distinct collagenous polypeptides, including \( \alpha_1(IV) \). The molecular origin of the "collagens" isolated by pepsin-solubilization techniques (see Materials and Methods) is also of importance since the methodology involves the removal of any noncollagenous regions. The possibility therefore exists that the collagens isolated may be only part of much larger proteins containing extensive nonhelical domains. These questions relating to the molecular and cellular origins of the various forms of basement membrane and, indeed, of interstitial collagens will require resolution not only by chemical techniques but also by the use of immunological methods and biosynthetic studies using cells in tissue culture.

C. Collagen Biosynthesis

In considering the transcriptional and translational mechanisms involved in collagen gene expression it is of critical importance to define the polypeptide chain products encoded in such genes. There is now a considerable body of evidence to indicate that the three most clearly defined collagen types (types I, II, and III) are synthesized in precursor forms called procollagens. These procollagens possess constituent polypeptides that are significantly larger than the respective alpha chains isolated. All the known procollagens contain peptide extensions at both ends of their three polypeptide chains (11, 72). The polypeptides of procollagen are called pro-\( \alpha \) chains. In the case of Type I procollagen, the two polypeptides are referred to as pro-\( \alpha_1(II) \) and pro-\( \alpha_2(II) \). The amino-terminal propeptide of the pro-\( \alpha_1(II) \)
chain has a molecular weight of about 20,000 daltons and contains three distinct structural domains: a globular amino-terminal domain, a central collagen-like domain, and another short globular domain (11). The pro-a2 (I) chain also contains an amino-terminal propeptide with a collagen-like domain that matches the collagen-like region of the pro-a1 amino-terminal propeptide. The carboxy-terminal propeptides of both the pro-a1 and pro-a2 chains have molecular weights of 30,000 to 35,000 daltons and globular conformations without any collagen-like domains.

Both the amino-terminal and the carboxy-terminal propeptides of type I procollagen contain cysteine, which is not found in type I collagen. In the amino-terminal propeptides of type I procollagen, the cysteine forms only intrachain disulfide bonds. In the carboxy-terminal propeptides, the cysteine is involved in both intrachain and interchain disulfide bonds. The propeptides also contain sugars not found in type I collagen.

The synthesis of collagen fibrils can be considered as occurring in two stages: (1) intracellular steps are required to assemble and secrete the procollagen molecule, and (2) extracellular steps convert the procollagen molecule to collagen and incorporate it into a stable, cross-linked collagen fibril.

Both the propeptide domain and the collagen domain of pro-a chains undergo extensive processing by post-translational reactions. As the amino-terminal ends of newly synthesized pro-a chains enter the cisternal of the rough endoplasmic reticulum, they encounter three hydroxylating enzymes located within this
\[ \text{NH}_2 - \text{TRIPLE HELIX} - \text{COOH} \]

**Procollagen**

\[ \rightarrow \]

**Collagen**

\[ \sim 300,000 \]

**Figure 1**
compartment (73). Two of these hydroxylases convert prolyl residues to 4-hydroxyproline or 3-hydroxyproline and the third converts lysyl residues to hydroxylysine. The reactions catalyzed by these three hydroxylases all involve the same cofactors: ferrous ions, molecular oxygen, α-ketoglutarate and ascorbic acid (74). As the substrates are hydroxylated, equimolar amounts of α-ketoglutarate are converted to succinate and carbon dioxide. This decarboxylation of α-ketoglutarate provides reducing equivalents for the hydroxylation of proline and lysine.

The three hydroxylases act only on non-helical substrates, and they do not act on collagen or collagen-like peptides that are triple-helical. This requirement for a non-helical conformation has an important role in the processing of the protein. The chains must have about 90 residues per chain of 4-hydroxyproline to form a triple-helix that is stable at 37°C. On the other hand, after the chains have acquired the 4-hydroxyproline and the inter-chain disulfide bonds necessary for helix formation, they fold into a triple-helical conformation that prevents further hydroxylation of prolyl or lysyl residues.

As lysyl residues in the newly synthesized chains are hydroxylated, sugar residues are attached to the resulting hydroxylysyl residues. These glycosylations are catalyzed by two specific enzymes: a galactosyltransferase and a glucosyltransferase. The first of these enzymes adds galactose to the hydroxylysyl residues and the second adds glucose to the galactosylhydroxylysyl residues. The addition of sugars may continue after the chains are released from ribosomes, but the effect of substrate
conformation on the enzymatic rxn assumes that glycosylation ceases when the collagen domains fold into a triple helix (75).

Another important step in the intracellular processing of the propeptides is the synthesis of both intrachain and interchain disulfide bonds (76, 8). It is not clear whether the synthesis of these linkages requires an enzyme or whether it occurs automatically during biosynthesis. Studies with isolated cells indicate that the sequence of events is hydroxylation of pro-α chains, synthesis of interchain disulfide bonds and then formation of the triple helix. If the hydroxylation of prplyl residues is prevented or the synthesis of interchain disulfide bonds inhibited, the molecule will not become triple helical and will remain as a nonfunctional protein (77).

The procollagen assembled in the rough endoplasmic reticulum passes through the Golgi complex before leaving the cell. The rate of secretion of procollagen depends on intracellular processing of the protein and, specifically, on folding of the collagen domain of pro-α chains into a triple-helical conformation.

The conversion of procollagen to collagen requires at least two enzymes, a procollagen aminoprotease that removes the aminopropeptides, and a procollagen carboxyprotease. Both the amino and carboxyproteases require a divalent metal, such as calcium (78). The procollagen aminoprotease cleaves the aminopropeptides from both the pro-α1 and pro-α2 chains of Type I. Collagen molecules, produced by cleavage of procollagen, spontaneously assemble into fibrils. The immature fibrils, however, do not have the necessary
tensile strength until they are cross-linked by a series of covalent bonds. Cross-linking of collagen in fibrils occurs in two or three states (79). The first step is the oxidative deamination of epsilon amino groups in certain lysyl and hydroxylysyl residues of collagen and this yields reactive aldehydes. The enzyme catalyzing the reaction, lysyl oxidase, is a copper-containing protein. The enzyme reacts poorly with isolated alpha chains, and it preferentially acts on native collagen fibers (80).

After aldehyde groups are generated from collagen by the action of lysyl oxidase, they can form two major kinds of cross-links (3). One kind is an intramolecular cross-link that joins the alpha chains of the same molecule and is formed by aldol condensation between an aldehyde derived from lysine, hydroxylysine, or glycosylated hydroxylysine and the epsilon amino group of a second lysine, hydroxylysine, or glycosylated hydroxylysine. The Schiff bases synthesized in this way are the source of the major intermolecular cross-links in collagen.

II. Collagen Synthesis in Sea Urchins

In view of collagen's important structural function in many tissues, the genetic diversity of the collagen forms found in higher vertebrates may not be too surprising in retrospect. There are, however, many facets of the polymorphism that remain to be resolved, including major questions as to the extent of the diversity and to the functional aspects of collagen structure which generated the need for it. There is strong evidence for
some ten distinct collagen types which are probably the products of at least 13 nonallelic structural genes. The number of the genes involved may well increase as more novel collagen types are discovered. Since there are close sequence homologies between the types, their gene linkage may be particularly interesting as may be the evolutionary relationship between these types and collagens of invertebrates like the sea urchin.

In order to explain the reasons for the choice of the sea urchin for this study and to provide the background for consideration of the data to be presented, it is necessary to review the embryogenesis of echinoderms.

A. Outline of Development

Sea urchin eggs are spawned after completion of both reduction divisions: the polar bodies are given off at one pole of the egg, defined as animal (designated An in figure 2). The opposite pole is called the vegetal pole (Veg in figure 2). After fertilization, the resulting zygote embarks on a series of cleavage divisions which occur very rapidly (¾ - 1 hour per cell cycle) and without intervening growth. Cleavage occurs within a rigid membrane, which forms at the time of fertilization. The pattern of the cleavages is shown in figure 2. The first two divisions are meridional and equal giving rise to a 4-cell embryo each of the cells of which is equivalent. The third cleavage is equatorial and equal giving an embryo of eight cells, all of the same size but qualitatively distinct. The fourth cleavage
Figure 2 - Diagrammatic outline of development of the sea urchin embryo.

All figures are orientated with the animal pole upwards except c, which is viewed from this pole.

Fertilization membrane has been omitted from a-f, nuclei from e-i, and cell boundaries from g-i, for the sake of clarity. Shading represents regions giving rise to, or arising from, micromeres (black), macromeres (cross-hatched) and mesomeres (white). An - animal pole. Veg - vegetal pole.

a. fertilized egg.  b. 2-cells.  c. 4-cells.  d. 8-cells.  e. 16-cells.
f. blastula.  g. mesenchymal blastula.  h. mid-gastrula.  i. plateus.
gives rise to the 16-cell stage embryo. This is the first unequal cleavage. In the animal half of the embryo the cleavage planes are meridional, producing a ring of eight cells of equal size, the mesomeres. In the vegetal half, the cleavage planes are approximately equatorial, but somewhat oblique, and the cleavages are very unequal, giving rise to two rings of cells: four large macromeres and four much smaller micromeres, the latter being derived from the most vegetal region of the egg. Hence, at this point in cleavage the embryo is composed of three different cell types, distinguishable on a size basis and, also as will be seen below, distinct as regards their developmental potentialities.

Subsequent cleavage divisions occur in all parts of the embryo eventually giving rise to a hollow ball of cells -- the blastula. The number of cells in the blastula depends on the species concerned. The reason for the development of the hole in the middle of the blastula, the blastocoel, appears to be that each cell retains a strong attachment to the hyaline layer, a gel-like, collagen containing, coating around the embryo (24). This causes the cells to be arranged with maximum contact with the hyaline layer and leads to a formation of the blastocoel. The period of cleavage lasts 8-18 hours depending on species and temperature. At the end of this time the embryo secretes an enzyme, hatching enzyme, which digests the fertilization membrane and the embryo emerges as a swimming blastula. It is covered with cilia, one per cell, and swims actively with the animal end forward.
The blastula becomes flattened at the vegetal pole and a ciliary tuft develops at the animal pole. This is composed of long, stiff, stereocilia. At the vegetal end cells free themselves from the blastula wall by rounding up, and by pulsatory and pseudopodial activity and enter the blastocoel. These cells comprise the primary mesenchyme and are derived from the micromeres, which can thus be seen to have undergone at least three divisions subsequent to the 16-cell stage. The primary mesenchyme cells move about on the wall of the blastocoel by the action of the pseudopodia. These extend up to 30 microns and are contractile. Thus, when a pseudopodium makes a stable contact, which can occur only at its tip, its subsequent contraction leads to translation of the mesenchymal cell from which it is derived. By such movements the mesenchymal cells become arranged in a ring around the vegetal end of the blastocoel and also in two ventrolateral branches extending towards the animal pole from the mesenchymal ring (Fig. 2). This arrangement appears to arise by random exploration by the pseudopodia and selective fixation in a pattern determined by the ectoderm which forms the blastocoel wall. This selection presumably occurs by variations in adhesiveness (81). The ectoderm in effect forms "a template" for the organization of the mesenchyme. The individual pseudopodia coalesce to form a mesenchymal ring, a small triradiate crystal of calcium carbonate forms within the matrix of the cable. The crystal is initially randomly orientated but becomes positioned so that its three axes point along the axes of the three arms of mesenchymal cable.
The crystal subsequently grows and the direction of growth is determined by the orientation of the cable. These in turn are brought about by exploration and attachment of individual pseudopodia to the ectoderm. Thus the eventual skeletal pattern results from an interaction of the primary mesenchyme with the ectoderm.

While the primary mesenchyme is undergoing the processes outlined above, the region of the embryo immediately animal to the micromere derivatives undergoes invagination to form the archenteron. The process has two phases. In the first, the ectoderm cells show some loss of adhesion for each other causing rounding up, and pulsatory activity. These processes are the same as those involved in formation of the primary mesenchyme but are less extreme, with the result that the cells remain in a sheet which folds inwards to form the archenteron. In the second phase, pseudopodia formed by the cells at the tip of the archenteron pull the latter fully across the blastocoel. The archenteron tip eventually makes contact with the ventral side of the blastocoel with which it fuses. Subsequently the mouth forms here. The factors which affect the pattern of invagination seem to be very similar to those discussed above for organization of primary mesenchyme. Subsequent development continues by elaboration of the same mechanisms to produce the pluteus larva.

The pluteus, thus is derived by a well described series of cellular movements from the blastula. Different parts of the
pluteus can be traced back to their ancestral cells in the early cleavage stages. The skeleton is formed via primary mesenchyme from the micromeres; the gut, via the archenteron from the vegetal part of the macromeres; and the external surface from the rest of the macromere derivatives and from the mesomeres. The morphogenetic processes by which the final larva is formed involve intimate interactions between the different cell types involved.

The role of collagen synthesis in this outline of development was the major question in this investigation. Differential gene expression would lead to divergence of the cells to produce different tissues in the embryo. Such differentiation would involve both the intrinsic programming of the different cell types and interaction between the latter. Biochemically, cellular differentiation is conveniently analyzed in terms of the spectrums of proteins synthesized by different cell types. The best available evidence indicates that the nuclei of multicellular organisms are equivalent in the informational content of their DNA. Thus the concept of differential gene activity is currently favored as an explanation of the different spectrums of protein synthesized by different cells of an organism. As an extension of the differential gene activity hypothesis to early development, it has been proposed that maternal localization determinants, laid down in the egg, are partitioned differentially into the cells of the early embryo. Subsequent interaction of these determinants with embryo nuclei elicits different patterns of
gene activity in the different cell types, leading to different patterns of protein synthesis and consequently progressive divergence of the cells to produce different tissues of the embryo. In this study, the question asked was: Is collagen synthesis turned on at a specific stage in development? The sea urchin embryo offers an excellent opportunity for attacking this problem. A very large body of evidence concerning echinoderm embryology, both biological and biochemical, already exists. Furthermore, the cells do show differential properties and the very existence of interaction between the different cell types involved is interesting in itself. In addition there are the practical aspects. Methods for working with sea urchin embryos have been developed over many years and reasonable quantities of dividing eggs can be obtained. But before a discussion of the role of collagen in development can be made, it is necessary to review the work showing that collagen exists in the sea urchin.

B. Sea Urchin Collagen

Collagen has been found in many invertebrate phyla, including echinodermata (15). The identity of segments of collagen from sea urchin, calf, and carp suggests that very little change of the quaternary and tertiary structure of the collagen molecule has taken place during evolution (32). Significant differences have been found, on the other hand, in the amino acid compositions of these collagens (34). This implies that their primary
structures have significant differences. Obviously, all the information necessary for collagen configuration and for fibril formation is contained in certain features of the collagen amino acid sequence, and it is these characteristics which are not subject to variation by mutation, insertion or deletion processes. Homology is thus found to be the limiting factor for evolutionary change in this structural protein. Unlike hemoglobin, cytochrome c or hypophyseal hormones, the collagen molecule exhibits pronounced constancy of its length during evolution. All these results point to the conclusion that the collagen molecule is involved in well-defined physiological functions.

There also appears to be a high content of glycine and hydroxyproline in sea urchin collagen which correlates well with that of most types of vertebrate collagen (13, 38). Travis also reported, via EM studies and X-Ray Diffraction, that collagen fibrils obtained from pure sea urchin spicules (a differentiated CaCO₃-containing product of mesenchyme cells) have axial repeating periods of 625 Å. In negative contrast, the dark-staining bands correspond to the "gaps" or "holes" described for mammalian collagen (13). Eyre and Glimcher have also reported the existence of similar cross-link structures in sea urchins and vertebrates (15). The insoluble collagenous matrix was recovered from the mineralised plates of fresh sea urchins which had been scraped free of spines and soft tissue, and demineralised in 3% acetic acid at 4°C. This collagen was analyzed after reduction with triated borohydride and revealed two peaks of tritium activity.
These peaks eluted in identical positions to the two major peaks of activity recovered by similar analyses of demineralized human bone and chicken bone. Bailey characterized the two tritiated peaks from chicken bone as syndesinol, which is the reduced aldo derived from the condensation of the aldehydes derived from hydroxylsine and lysine, and hydroxylsinorleucine, which is the reduced Schiff base between the aldehyde derived from lysine and the epsilon amino group of hydroxylsine (82).

Up until 1972, most of the research done was concerned with the characterization of collagen in adult sea urchins and not in the developing embryo. In 1972, Pucci-Minafra reported the existence of a hydroxyproline-containing protein in sea urchin embryos which underwent a multifold increase during gastrulation (29). This increase occurred jointly with the formation of spicules. This was the first evidence that supported the synthesis of a collagen in developing sea urchin embryos. Collagen synthesis was monitored by assaying for protein-bound $^3$H-hydroxyproline in $^3$H-proline-labeled embryos. It was found that $^3$H-HyPro incorporation increased during gastrulation. This increase was correlated with a concurrent increase in $^3$H-HyPro-incorporation in the spicule fraction. It was thus concluded that this hydroxyproline-containing protein was an important component of spicules. The only fault with this work was that the only criterion for the presence of collagen was a high hydroxyproline content. Characterization by other means like polyacrylamide gel electrophoresis, collagenase sensitivity, and pepsin-resistance was not utilized.
This result was confirmed with the same general methods by Golob et al (38). Hydroxyproline was again used as a marker for collagen. The ratio of radioactivity of hydroxyproline in collagen to that of proline in total protein was used to quantitatively measure the rate of collagen synthesis relative to that of total protein synthesis. The results showed that collagen synthesis began during gastrulation, increased rapidly between thirty and fifty hours, and then leveled off at about sixty to one hundred hours or late pluteus. This synthesis followed a sigmoidal curve, with a maximum rate increase at thirty hours. An increase in synthesis rate also coincided with an increase in spicule formation.

Gould and Benson also confirmed this result, and attempted to show that collagen synthesis was selectively inhibited by low actinomycin D concentrations (14). Embryos exposed continuously from hatching blastula to low actinomycin D concentrations failed to develop spicules, but were normal in all other aspects. The rate of collagen synthesis was reduced slightly in early and late gastrula and 75% in the pluteus stage (96 hour). On the other hand, total protein synthesis, which was determined by $^3$H-proline incorporation, was not inhibited. The authors go on to conclude that this selective inhibition of collagen synthesis by actinomycin D raises the possibility of further correlating the products of gene transcription and translation with cellular and morphological events during early development. The rate of collagen synthesis was determined from the total dpm of $^3$H
proline incorporated into collagen peptides during a six hour pulse from the following calculation:

\[
\% \text{ collagen} = 100 \times \frac{\text{dpm in collagenase digest}}{(\text{Total dpm incorp.} \times 4) + \text{dpm in collagenase digest}}
\]

Spiegel et al. have also found collagen in the hyaline layer of sea urchin embryos (24). Fibril-like structures that possessed a striated appearance and were sensitive to digestion by collagenase were identified in the hyaline layer, via EM, as early as ten minutes after fertilization to as late as the pluteus stage. The author conclude that these fibrils are "collagen" and that they are important in helping the hyaline layer maintain the shape and the "coherence" of the embryo.

In the most recent publication, Benson et al. observe the presence of collagen fibrils in the blastocoel of 72 hr. urchin embryos (12). This finding is the first ultrastructural EM evidence for non-spicule collagen fibrils in sea urchin embryos. The striated fibrils have a periodicity of 590-620 Å and measure approximately 200 Å in width which is similar to that reported in collagen of primary chick corneal stroma and adult chick tendon. They conclude that sea urchin collagen may have a role in cell attachment and orientation as well as spicule formation.

C. Experimental Objectives

In light of the established information on the presence of collagen in adult sea urchins, it is necessary to probe the synthesis of this structural protein in the developing sea urchin.
embryo. Recent evidence has focused on the identification of this protein only through assay by hydroxyproline content or visualization with EM.

The experiments to be described here are aimed at initiating more complete documentation of sea urchin collagen through investigation of the occurrence of collagen in the developing sea urchin embryo through new and more rigid criteria. In this work, collagen synthesis is monitored via $^3$H proline incorporation in developing embryos. The existence of collagen in these embryos is verified by co-migration on polyacrylamide gradient gels with $^{14}$C-pro labeled chick embryo collagen markers, sensitivity to collagenase digestion, and resistance to pepsin digestion. A new method for extraction and isolation of collagen from developing embryos is also described.
MATERIALS AND METHODS
MATERIALS AND METHODS

A. Collection of Gametes

The species of sea urchin used in these experiments was *Strongylocentrotus purpuratus*. This species was obtained from Pacific-Bio-Marine Company (Venice, California). The urchins were placed in an Aquarium Systems tank in sea water prepared from Instant Ocean Salts (Aquarium Systems, Inc., Eastlake, Ohio). The urchins were fed weekly with seaweed and/or lettuce. The temperature was kept constant at 12-14°C, and the specific gravity was maintained at 1.024-1.025.

Gametes were obtained by injecting 1-2 mls of potassium chloride (0.55 M) solution into the coelomic cavity of each fertile adult urchin to promote expulsion of gametes. The urchin was allowed to spawn into a beaker filled with Millipore-filtered sea water (MFSW). The MFSW used always contained 50 micrograms/ml of penicillin and 100 units/ml of streptomycin to inhibit bacterial growth. It was composed of: 446 mM NaCl, 22.5 mM MgCl₂, 18.2 mM MgSO₄, 9.0 mM KCl, 2.2 mM NaHCO₃, and 9.7 mM CaCl₂. The sex of the spawning sea urchin was easily determined by the color of the gametes. Eggs always appeared orange and were spawned in thin threads. The female urchin was allowed to spawn for 30 minutes or until all the eggs had settled to the bottom. This was then followed by aspiration of all but an equal volume of MFSW. Eggs (usually between 3 and 8 mls)
were then resuspended and then poured into another beaker through four layers of cheesecloth. The original beaker and cheesecloth were rinsed to recover remaining eggs. The eggs were then resuspended to 200 mls of MFSW. In order to determine the total packed volume of eggs, 2 mls of egg solution was pipetted into a 5 ml Hopkins vaccine tube and centrifuged on a table top centrifuge for forty-five seconds. The total volume of eggs was then calculated by multiplying this packed volume by 100. After the eggs had settled in the second beaker, the supernatant was aspirated and the eggs were suspended to 1% in MFSW.

Sperm was collected by either dissection of the entire gonad or by allowing the potassium chloride injected male to shed into a clean dry dish. Sea urchin sperm can be stored at 4°C and retains fertilization activity for up to five days.

B. Fertilization and Development

Fertilization was attained by addition of 1 ml of freshly diluted sperm per 100 ml of 1% egg suspension. The diluted sperm solution was prepared by addition of one drop of concentrated sperm to 10 mls of MFSW. Fertilization was indicated by the presence of a fertilization membrane. Experiments were continued only when there was 95% fertilization. Eggs and developing embryos were kept in suspension by incubation at 16°C in 250 ml bottles on a rotator. All operations were performed with MFSW.
C. ³H-Proline Labeling of Cultured Embryos in vivo

Eggs were fertilized and allowed to develop to desired stage with constant rotation at 16°C. One ml of a 1% embryo solution (0.1 mls embryos) is then pipetted into a Corex tube containing 100 µc of ³H-L-2,3 proline (NEN). The tube is then swirled to allow for complete incorporation of the label. The labeled embryos are then incubated for 60 minutes in the rotator at 16°C. When the incubation is complete, 2 mls of ice cold acid sea water is added to terminate incorporation. Embryos are then centrifuged on the International (2900 g x 25 sec), and the supernatant is aspirated off. This washing step is then repeated again, and the embryos are ready to be lysed either with 2% SDS or by douching.

D. Counting Procedures

Where necessary to insure complete precipitation, carrier BSA (100 µg) were added to samples. Samples were made 5-7.5% with cold TCA and collected on Whatman 3MM filter disks with 5 washes of cold 5% TCA (filters were dried by heating at 65°C). Dry filters were counted in toluene with 4.96 g/l PPO (2,5-diphenyleneoxazole, New England Nuclear) and 0.05 g/l POPOP (2-p-phenylene bis (5-phenyloxazole), New England Nuclear). A Beckman scintillation counter was used, and background radioactivity was generally 20 cpm for ³H.
E. Polyacrylamide Gel Electrophoresis

Sea urchin proteins (labeled or unlabeled) were analyzed by SDS-polyacrylamide gradient slab gel electrophoresis by a modification of the method of Laemmli (6).

The following were prepared or used:

1. Acrylamide-bisacrylamide (30:0.8) -- 30 grams of acrylamide and 0.8 grams of bisacrylamide were dissolved and diluted to 100 mls.

2. Sodium dodecyl sulfate (10%).

3. TEMED (N,N,N',N' tetramethylenediamine).

4. Ammonium persulfate (10%) -- 0.5 grams dissolved in 0.50 mls of water, made fresh.

5. Buffer 'L' -- 1.5 M Tris, pH 8.8.

6. Buffer 'M' -- 0.5 M Tris, pH 6.8.

7. Running Buffer -- 0.025 M Tris, 1.92 M glycine, 0.1% SDS, pH 8.3.

A 5--12.5% acrylamide gradient slab gel was poured, 0.1% in SDS and 1.5 M Tris, pH 8.8. Polymerization was effected by addition of TEMED (BioRad, Inc.) to a final concentration of 0.05% (V/V) and ammonium persulfate to 0.075% (W/V) immediately prior to pouring the gels. A 3% acrylamide stacking gel, 0.1% SDS and 0.5 M Tris-HCl-pH 6.8, 2% SDS, 6% sucrose, 5% 2-mercaptoethanol, and 0.001% bromphenol blue marker dye. Prior to layering on the gel, samples were heated for 15 minutes at 90°C. Electrophoresis at 120 volts was performed until marker dye reached the bottom of the gel.
Gels with unlabeled proteins were stained 75 minutes in 0.2% Coomassie Brilliant Blue - 0.1% Amido Black - 0.5% acetic acid - 5% methanol and were destained in 7.5% acetic acid - 5% methanol.

Gels containing ³H-proline labeled proteins were destained for two hours in order to "fix" the proteins. This was followed by three 20 minute DMSO washes. The gel was then soaked in 100 mls of a 22.5% PPO in DMSO solution for three hours. This was followed by three gd H₂O washes in order to remove excess PPO. The gel was then dried on vacuum at approximately 35°C for two hours. The gel was then exposed to X-ray film in the darkroom and stored at -70°C.

Approximate molecular weights of the polypeptides run on the SDS polyacrylamide gels was determined on the basis of the migration of the following standard proteins of known molecular weight: ovalbumin (M.W. 43,000), bovine albumin (M.W. 67,000) and B-galactosidase (M.W. 130,000).

F. Scanning of Gels

Gels were scanned at 600 um in an Ortec Visible and UV Densitometer outfitted with a gel transporter. Gels were placed in a 10 cm long cuvette with a 1 cm path length and were scanned. A 0.10 mm microslit was used for all experiments. In all cases, the point of lowest absorbance on each gel was defined as zero A₆₀₀.
G. Isolation and Extraction of Collagen from S. purp. Embryos

A modification of the schemes of Burgeson et al (83) and Madri and Furthmayr (84) was used. Labeled or unlabeled embryos in MFSW were centrifuged on the International (2,000 g x 25 sec) and the supernatant was aspirated. A volume of 1M NaCl-50mM Tris-pH 7.4 that was twenty times the volume of the embryos was added to the pelleted embryos. This suspension was then dounced on ice until complete lysis had occurred (approximately 25 strokes). Extraction of the salt-soluble material with stirring was carried out for forty-eight hours at 4°C. Insoluble material was removed from the extraction suspension by centrifugation at 10,000 g for 15 minutes. The suspermatant, containing neutral salt-soluble proteins was dialyzed against five liters of a buffer containing 1.7M NaCl-50mM Tris-pH 7.4 at 4°C for 48 hours.

This buffer was changed daily three additional times. Neutral salt insoluble proteins were resuspended in 0.5M acetic acid and extracted overnight at 4°C. This suspension was centrifuged as above and the supernatant made 1M NaCl by addition of solid salt with stirring. The precipitate which formed upon standing overnight at 4°C was acid-soluble collagen and was stored at -20°C. The pellet, which had been obtained via centrifugation of the acetic acid extraction suspension, was re-extracted with 0.5M acetic acid overnight at 4°C. This suspension was centrifuged as above and the supernatant made 1M NaCl by addition of solid salt with stirring. The precipitate which formed upon standing overnight at 4°C was another acid-soluble collagen fraction.
The neutral salt-soluble proteins present in the supernatant of the initial extraction suspension formed a precipitate upon dialysis with 1.7M NaCl. This material, which was salt-soluble collagen, was combined with the two acid-soluble collagen fractions into one erlenmeyer flask. The pH of this final mixture was measured to be 3.2. Consequently, the precipitate which then formed upon standing overnight at 4°C was, in effect, acid-soluble collagen and was collected by centrifugation as before. The pellet that was obtained was relatively pure acid-soluble collagen that could be collagenase treated and run on gels at a later time.

H. Collagenase Digestion of Collagens

Bacterial collagenase from Worthington, Inc. (CISPA 5275) was purified on a 1.6 x 130 cm Sephadex G-200 column equilibrated with 5mM CaCl₂-50mM Tris-HCl, pH 7.6, according to the procedure of Peterhofsky and Diegelmann (7).

Lysed embryos (in 2% SDS or MFSW) were divided into one ml fractions. To each tube containing 1 ml of lysed embryos, 0.1 ml of 20% NaAc was added followed by 2 ml of 95% cold ethanol. Proteins were allowed to precipitate overnight at -20°C and were collected by centrifugation (10,000 g x 10 min). This precipitate was lyophilized to dryness (3 hours) and was ready for collagenase treatment.

Lyophilized protein pellets or "extracted" collagen pellets were taken up in 0.5 ml of 0.1N NaOH and incubated for 5 minutes
PRO LABELING
3H OF EMBRYOS
INSOLUBLE PPT.
RE-EXTRACT 0.5M HAC
15 MIN. X 10,000 G
SUPT. MADE
1M NAACL
15 MIN. X 10,000 G
SUPT. MADE
3 FRACTIONS
COMBINE
ACID-SOLUBLE COLLAGEN=PPT.
SALT SOLUBLE COLLAGEN
1M NAACL
15 MIN. X 10,000 G
SUPT. DIALYSIS VS.
1.7M NAACL
15 MIN. X 10,000 G
SALT SOLUBLE
EXTRACTION
1M NAACL
EXTRACTION
15 MIN. X 10,000 G
INSOLUBLE PPT.
RE-EXTRACT 0.5M HAC
SUPT. MADE
1M NAACL
15 MIN. X 10,000 G
INSOLUBLE PPT.
RE-EXTRACT 0.5M HAC
SUPT. MADE
1M NAACL
15 MIN. X 10,000 G
SUPT. MADE
3 FRACTIONS
ACID-SOLUBLE COLLAGEN=PPT.
SALT SOLUBLE COLLAGEN
1M NAACL
15 MIN. X 10,000 G
SUPT. DIALYSIS VS.
1.7M NAACL
15 MIN. X 10,000 G
SALT SOLUBLE
EXTRACTION
1M NAACL
EXTRACTION
15 MIN. X 10,000 G
INSOLUBLE PPT.
RE-EXTRACT 0.5M HAC
SUPT. MADE
1M NAACL
15 MIN. X 10,000 G
INSOLUBLE PPT.
RE-EXTRACT 0.5M HAC
SUPT. MADE
1M NAACL
15 MIN. X 10,000 G
SUPT. MADE
3 FRACTIONS
ACID-SOLUBLE COLLAGEN=PPT.
SALT SOLUBLE COLLAGEN
1M NAACL
15 MIN. X 10,000 G
SUPT. DIALYSIS VS.
1.7M NAACL
15 MIN. X 10,000 G
SALT SOLUBLE
EXTRACTION
1M NAACL
EXTRACTION
15 MIN. X 10,000 G
INSOLUBLE PPT.
RE-EXTRACT 0.5M HAC
SUPT. MADE
1M NAACL
15 MIN. X 10,000 G
INSOLUBLE PPT.
RE-EXTRACT 0.5M HAC
SUPT. MADE
1M NAACL
15 MIN. X 10,000 G
SUPT. MADE
3 FRACTIONS
ACID-SOLUBLE COLLAGEN=PPT.
at 37°C. Then 0.5 mls of 0.08N HCl was added in order to neutralize the sample. 1 ml of 0.3M N-2-hydroxyethylpiperazine-N1-2-ethanesulfonic acid (Hepes; Sigma, Inc.) pH 7.2 was added to each sample. The samples that are to be collagenase-treated are distributed as 0.4 ml fractions. To each 0.4 ml fraction, 0.05 mls of 0.14 mg/ml collagenase in 5mM CaCl₂, 150mM Hepes, pH 7.2 is added along with 0.04 mls of N-ethylmaleimide (NEM; Sigma, Inc.) solution prepared at a concentration of 3.9 mg/ml. The samples that are not to be treated with collagenase are also distributed as 0.4 ml fraction, 0.05 mls of 5mM CaCl₂, 150mM Hepes, pH 7.2 is added along 0.04 mls of NEM. All samples are then incubated for one hour at 37°C. At the end of the incubation, 0.2 mls of 2X sample buffer is added to all fractions immediately prior to electrophoresis on gradient slab gels.

Pepsin-Solubilization of Sea Urchin Proteins

The procedure, which was utilized as a test for collagen, was a modification of the one reported by Benya et al (5). 0.6 mls of lysed embryos were incubated with 0.35 mls of 100 micrograms/ml Pepsin (Sigma, Inc.) in 0.1 M acetic acid for 12 hours at 4°C with constant stirring. The pepsin-treated sample was then lyophilized to dryness (6 hrs) and then titrated to neutrality by the addition of 1 M unbuffered Tris. 2X sample buffer was then added prior to electrophoresis on gradient slab gels.
J. Protein Determination

The method of Lowry et al was employed (85). The standard was normally BSA (Sigma, Inc.), and the concentrations of sample proteins were calculated from the optical density readings at 660 nm using a linear regression analysis.
RESULTS
A. Total Proteins of Developing Embryos

The protein content of a *Strongylocentrotus purpuratus* embryo is $40 \times 10^{-3}$ micrograms as measured by the Lowry procedure. These levels did not vary during the course of development (Figure 4), and thus there is no measurable net gain or loss of protein.

B. $^3$H-Proline Incorporation in Developing Embryos (in vivo)

My experimental approach began by determining the amount of $^3$H-L-2,3 praline that was incorporated into proteins of developing of *S. purp.* embryos. Since proline and hydroxyproline represent a large part of the amino acid residues contained in collagenous polypeptides, it was hypothesized that the amount of proline incorporated would be a suitable measure of the amount of collagen synthesized by the embryo during the course of development.

0.01 mls of embryos (packed volume) obtained at various stages of development were labelled for one hour in 100 $\mu$Ci of $^3$H-L-2,3 proline (Specific activity, 20 Ci/mMole) in vivo. 16 hr, 20 hr, 37 hr, 40 hr, 48 hr, 64 hr, and 72 hr embryos were used in this experiment. At the end of the incubation ($4^\circ$C), the labelled embryos were lysed in 2% SDS. After cooling on ice, triplicate one microliter samples were removed, precipitated by adding 10% TCA, and allowed to stand at least thirty minutes at $0^\circ$C. The precipitated fractions were collected onto Whatman glass fiber filters discs and subsequently washed with 5% TCA. The filters were dried overnight at $65^\circ$C. The filters were then counted to determine the amount of radioactivity in each sample.
Figure 4: Total protein content of *S. purp.* embryos during the course of development.
(See "Materials and Methods"). The results of this experiment can be seen in Figure 5 and Table I. There is a general increase in the amount of $^3$H proline incorporated during the course of development. The greatest rate of increase in amount of label that is incorporated occurs between 24 hours and 48 hours. At 48 hours, the amount of $^3$H proline incorporated begins to level off. But in order for this increased incorporation to be correlated with increased collagen synthesis, the $^3$H proline labeled proteins present at each stage of development had to be characterized by gel electrophoresis on gradient slab gels.

C. Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was conducted as described in "Materials and Methods" and was a most useful means of visualizing and analyzing sea urchin protein preparations.

Proteins precipitated from lysed, $^3$H-proline-labeled embryos with cold 95% ethanol were treated with collagenase to determine the presence of collagen. These proteins along with non-collagenase treated proteins were run on 5%-12.5% polyacrylamide gradient slab gels. The denaturing conditions here and in all electrophoretic experiments result in the breakdown of the collagen triple helical conformation to its constituent alpha chains.

To determine whether there were any contaminating collagenases in lysed embryo that could ultimately degrade any collagen that was present, $^{14}$C-proline labelled chick embryo procollagen (40,000 cpm) was added to the lysed embryos. If there were
Table 1: $^3$H proline incorporation into *S. purp.* embryo proteins at various stages in development.
<table>
<thead>
<tr>
<th>STAGE (hrs. after fert.)</th>
<th>$^3$H proline total cpm (1 hr. incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>3.5 x $10^6$</td>
</tr>
<tr>
<td>20</td>
<td>3.8 x $10^6$</td>
</tr>
<tr>
<td>37</td>
<td>9.6 x $10^6$</td>
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<tr>
<td>40</td>
<td>10.5 x $10^6$</td>
</tr>
<tr>
<td>48</td>
<td>12.5 x $10^6$</td>
</tr>
<tr>
<td>64</td>
<td>14.2 x $10^6$</td>
</tr>
<tr>
<td>72</td>
<td>15.0 x $10^6$</td>
</tr>
</tbody>
</table>
Figure 5: Graph of $^3$H proline incorporation into *S. purp.* embryo proteins at various stages during the course of development (1 hour incubation).
collagenases present, the chick collagen would degrade. Figure 6 shows the electrophoretic pattern of this labelled chick collagen (lane 2). In the adjacent lanes, this same chick collagen has been degraded with various concentrations of collagenase. In all cases, the collagen present has been degraded to lower molecular weight fragments, and there is no banding seen in collagenase-treated material in the regions which are occupied by alpha chains in the non-enzymatically treated sample. This result also indicates that bacterial collagenase is active in an environment containing sea urchin proteins.

Figures 7 and 8 are electrophoretic patterns of ethanol-precipitated proteins from lysed and \(^{3}H\)-proline labelled embryos that have been (+) and (-) collagenase-treated. In both gels, 2 distinct bands in the lanes containing labeled proteins from 46 hr embryos that were collagenase-treated can be seen. The alpha-one chain and the alpha-two chain slightly beneath it are prominent in the 95,000 to 100,000 dalton molecular weight range, and this is established via co-migration with chick collagen standard. In the adjacent collagenase-treated lanes, these 2 bands are witnessed again in the lane containing proteins from 72 hour embryos. These 2 bands again are degraded in the collagenase-treated lane confirming their authenticity as collagen.

In these gels, there are approximately 20,000 tritium counts/min per lane. A long four-week exposure was required in order to effectively visualize the two collagen bands. The fact that such
Figure 6: Autoradiogram of $^3$H proline labeled 16 hr. S. purp. embryo proteins and $^{14}$C proline labeled chick procollagen ((+) and (-) collagenase-treated) run on SDS-polyacrylamide gradient slab gels (See Materials and Methods). Lane 1) 16 hr. embryo proteins plus chick procollagen treated with 2.0 mg/ml collagenase, Lane 2) same as lane 1 but treated with 1.5 mg/ml collagenase, Lane 3) same as lane 1 but treated with 1.2 mg/ml collagenase, Lane 4) same as lane 1 but treated with 1.0 mg/ml collagenase, Lane 5) same as lane 1 but treated with .14 mg/ml collagenase, Lane 6) same as lane 1 but not treated with collagenase (less cpm in this lane), Lane 7) $^{14}$C protein standards. There were approximately 45,000 cpm per lane.
Figure 7: Autoradiogram of $^{3}$H proline labeled S. purpembryo proteins that have been (+) and (-) collagenase treated. 
Lane 1) 24 hr. embryos that have been treated with 0.14 mg/ml collagenase, Lane 2) 24 hr. embryo proteins (-) collagenase, Lane 3) 46 hr. embryo proteins treated with collagenase, Lane 4) 46 hr. embryo proteins (-) collagenase, Lane 5) collagen standards from chick Lane 6) $^{14}$C protein standards (30,000 cpm/lane).
Figure 8; Autoradiogram of $^3$H proline labeled S. purp. embryo proteins (in vivo) that have been treated with (+) and (-) collagenase. Lane 1) $^{14}$C protein standards, Lane 2) chick collagen standard (low $^{14}$C cpm in this lane), Lane 3) 48 hr. embryo proteins (-) collagenase, Lane 4) 48 hr. embryo proteins (+) collagenase, Lane 5) 24 hr. embryo proteins (-) collagenase, Lane 6) 24 hr embryo proteins (+) collagenase, Lane 7) 72 hr. embryo proteins (-) collagenase, Lane 8) 72 hr. embryo proteins (+) collagenase. (28,000 cpm/lane).
a low amount of radioactivity is present in these bonds established the need for an effective extraction procedure for collagen from S. purp. embryos.

D. Extraction of Collagen from Developing Strongylocentrotus purpuratus Embryos

The extraction procedure featuring salt precipitation of collagen at acid pH was performed as described in "Materials and Methods" and as outlined in Figure 3. From 1.5 mls of $^3$H-proline-labelled 40 hour S. purp. embryos, a precipitate containing acid-soluble collagen was obtained. This precipitate contained approximately 20,000 total tritium counts/min. The acid-soluble collagen was (+) and (-) collagenase-treated and run on 5%-12.5% polyacrylamide gradient slab gels (Figure 9). Each lane contains approximately 5,000 counts/min. Two distinct bands can be clearly visualized in the (-) collagenase-treated lanes. These bands comigrate in the same molecular weight range as $^{14}$C-proline labelled chick collagenase as evidenced in lanes five and nine.

The next experiment was designed to answer the question: is collagen synthesis developmentally regulated or is it constant throughout the course of development? If the two distinct bands characteristic of the 40 hour embryo stage can not be seen for other embryo stages, then it is safe to conclude that regulation of collagen synthesis is occurring during the course of development. 0.01 ml packed volumes of egg, 20 hour, 37 hour, 48 hour, and 72 hour S. purp. embryos were each labelled in vivo with 100 u Ci of $^3$H-L-2,3-proline as according to standard procedure.
Figure 2: Autoradiogram of extracted $^3$H proline-labeled 40 hr. S. purp. acid-soluble collagen (19,000 cpm/lane).
Lane 1) 40 hr. embryos plus chick collagen and treated with (-) collagenase, Lane 2) same as lane 1 but treated with (+) collagenase, Lane 3) extracted 40 hr. acid-soluble collagen treated with (-) collagenase, Lane 4) same as lane 3 but treated with (+) collagenase, Lane 5) same as lane 1, Lane 6) same as lane 2, Lane 7) same as lane 3, Lane 8) same as lane 4.
Figure 10: Scan of 40 hr. extracted *S. purp.* acid-soluble collagen from previous gel (lane 7). (scanned on Ortec densitometer)
RELATIVE ABS. 600 NM

1 CM GEL PER INCH
The egg was incubated with the $^3$H-proline for six hours, while the embryo stages were incubated in the label for one hour. An increase in the amount of $^3$H-proline incorporated was evidenced for the 48 hour and 72 hour embryos (Figure 11). The increase in $^3$H-proline incorporation after 37 hours of embryonic development also had been witnessed in previous $^3$H-proline *in vivo* incorporation studies. After the labeling, acid-soluble and neutral salt-soluble collagen were extracted from each embryonic stage. That is, salt-soluble material and acid-soluble material were not pooled together and then centrifuged as described in "Materials and Methods", but were collected as separate fractions and then centrifuged. This change in the procedure was committed in order to see if there was any difference in the electrophoretic patterns of acid-soluble and neutral salt-soluble sea urchin collagen. Although $^3$H-proline had incorporated well into the egg and the twenty hour embryonic stage, less than 1% of the original $^3$H cpm were recovered in the extracted acid-soluble fraction and less than 5% of the original $^3$H cpm was recovered in the extracted neutral salt-soluble fraction in both the egg and 20 hour embryo samples (Table 2). On the other hand, the extracted collagens from the other embryonic stages possessed a higher percentage of the original $^3$H cpm. Thus, it was concluded that gel electrophoresis of the extracted egg and 20 hour embryo samples would not be feasible since a very long exposure would be necessary to visualize the presence of any bands.
Figure 12: Autoradiogram of $^3$H proline-labeled extracted S. purp. acid-soluble collagen (14,000 cpm/lane).
Lane 1) 72 hr. extracted acid-soluble collagen treated with (+) collagenase, Lane 2) same as lane 1 but treated with (-) collagenase, Lane 3) 48 hr. extracted acid-soluble collagen treated with (+) collagenase, Lane 4) same as lane 3 but treated with (-) collagenase, Lane 5) 37 hr. extracted acid-soluble collagen treated with (+) collagenase, Lane 6) same as lane 5 but treated with (-) collagenase, Lane 7) $^{14}$C chick collagen standards, Lane 8) $^{14}$C protein standards.
Figure 14: Scan of 72 hr. *S. purp.* extracted acid-soluble collagen from gel in Figure 12(lane 2).
Consequently, (+) and (-) collagenase treated acid-soluble collagens from 37 hour, 48 hour, and 72 hour embryos were run on 5%-12.5% polyacrylamide gradient slab gels (Figure 12). There were approximately 15,000 cpm per lane (samples were counted in Tritosol) and a 6-week exposure was required for all bands to be visualized. Two distinct bands can be seen in the non-collagenase-treated lanes. The acid-soluble collagen extracted from 72 hour embryos appears to be much darker than the extracted acid-soluble collagens from the 48 hour and 37 hour embryos. This would indicate that much more collagen is synthesized at the 72 hour stage in embryonic development. This correlates well with the $^3$H proline incorporation results previously reported. The fact that these bands represent collagenous polypeptides in again indicated by their co-migration with chick-collagen standards and the susceptibility to collagenase digestion.

The neutral salt-soluble extracted collagen was also run on 5%-12.5% polyacrylamide gradient slab gels. The collagenase-treated samples did not possess sufficient $^3$H counts to make gel electrophoresis of them feasible. In Figure 15, 2 bands in addition to the two bands characteristic of acid-soluble extracted collagen can be seen. The collagenous nature of these bands could not be verified by this gel.

The next experiment attempted to determine the amount of collagen present in each embryo. Acid-soluble collagen was extracted from 37 hour, 48 hour, and 72 hour embryos, each with a packed volume of eighteen mls. Protein determinations by the method of
Figure 11: Amount of $^3$H proline present in extracted acid-soluble proteins. This graph shows the amount of radioactivity left after extraction of acid-soluble proteins from embryos labeled in vivo with $^3$H proline during the course of development (1 hr. incubation).
Table 2: Amount of $^3$H proline radioactivity (cpm x 10$^6$) present in S. purum embryo proteins before and after extraction of acid-soluble and salt-soluble collagen during the course of development.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Before Extraction</th>
<th>After Extraction</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>salt-soluble</td>
</tr>
<tr>
<td>Egg</td>
<td>1.2 x 10^6</td>
<td>7.2 x 10^3</td>
</tr>
<tr>
<td>20 hr.</td>
<td>2.1 x 10^6</td>
<td>12.5 x 10^3</td>
</tr>
<tr>
<td>37 hr.</td>
<td>2.3 x 10^6</td>
<td>14.5 x 10^4</td>
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<tr>
<td>48 hr.</td>
<td>3.8 x 10^6</td>
<td>14.4 x 10^4</td>
</tr>
<tr>
<td>72 hr.</td>
<td>3.9 x 10^6</td>
<td>10.3 x 10^4</td>
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</table>
Lowry were run on each extracted sample (Table 3). The value for the amount of collagen per embryo was calculated from the fact that there are $1.3 \times 10^6$ S. purp. eggs per ml of packed volume (86). The amount of collagen per embryo increases from $7.7 \times 10^{-7}$ ug at 37 hours to $1.17 \times 10^{-6}$ ug at 72 hours. Concomitantly, the percentage of total protein that is collagen increases 0.0019% in the 37 hour embryos to 0.0030% in the 72 hour embryos (pluteus).

The acid-soluble extracted collagen from each embryonic stage was then (+) and (-) collagenase-treated and run on a 5%-12.5% polyacrylamide gradient slab gel (Figure 16). After gel electrophoresis was completed, the gel was stained with Coomassie Blue, destained and then dried (See "Materials and Methods"). Two stained bands can be seen for each embryonic stage with the 72 hour stage possessing a more substantial enrichment of these bands. Again, these bands are susceptible to collagenase digestion as witnessed in the adjacent lanes. This result again confirms the collagenous nature of these extracted polypeptides.

E. Recovery of Chick Embryo Collagen by Collagen Extraction Method

The purpose of this experiment was to confirm that the extraction method utilized in this study was specific for collagen. Five microliters of $^{14}$C-proline labelled chick embryo collagen (obtained from Dr. Lukens' lab) possessing 40,000 total cpm was subjected to the collagen extraction in order to determine what percentage of these total cpm would be recovered. The labelled
Figure 15: Autoradiogram of salt-soluble collagen fraction from $^3$H proline-labeled S. purp.embryos. This fraction was collected after dialysis with 1.7 M NaCl was completed (See Materials and Methods). Lane 1) 37 hr. salt-soluble fraction, Lane 2) 48 hr. salt-soluble fraction, Lane 3) 72 hr. salt-soluble fraction (6,000 cpm/lane).
Table 3: Amount (micrograms) of acid-soluble collagen present in *S. purp.* embryos as determined by Lowry procedure. Amount of collagen per embryo is also indicated (See Results).
<table>
<thead>
<tr>
<th>Stage</th>
<th>Total Extracted Collagen</th>
<th>Amount of Collagen per Embryo</th>
<th>% of total protein of embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 hr.</td>
<td>25 ug</td>
<td>$7.7 \times 10^{-7}$</td>
<td>0.0019</td>
</tr>
<tr>
<td>48 hr.</td>
<td>28 ug</td>
<td>$8.6 \times 10^{-7}$</td>
<td>0.0021</td>
</tr>
<tr>
<td>72 hr.</td>
<td>38 ug</td>
<td>$1.17 \times 10^{-6}$</td>
<td>0.0030</td>
</tr>
</tbody>
</table>
Figure 16: SDS - polacrylamide gel electrophoresis on 5-12.5% acrylamide gradient slab gel as described in Materials and Methods. (Lanes counted from left to right). Lane 1) 72 hr. extracted acid-soluble collagen treated with (+) collagenase, Lane 2) same as lane 1 but treated with (-) collagenase, Lane 3), 48 hr. extracted acid-soluble collagen treated with (+) collagenase, Lane 4) same as lane 3 but treated with (-) collagenase, Lane 5) 37 hr. extracted acid-soluble collagen treated with (+) collagenase, Lane 6) same as lane 5 but treated with (-) collagenase. Gel was stained with Comassie Blue.
**Figure 17:** Summary of Results from chick collagen recovery experiment (See Results).
5 ul of ¹⁴C chick collagen
1M NaCl Extraction (40,000 cpm)
15 min. X 10,000 g

14,000 cpm
Extract w/ 0.5M HAc
15 min. X 10,000 g

10,000 cpm
Supt. made 1M NaCl
ppt re-extract w/ 0.5M HAc
15 min. X 10,000 g

ppt=1200 cpm

26,000 cpm
Dialysis vs. 1.7M NaCl
lost 12,000 cpm

14,000 cpm

10,000 cpm
Combine 3 fractions

26,800 cpm
15 min. X 10,000 g

Supt.=1800 cpm
25,000 cpm
62.5% recovery
14 of ¹⁴C cpm
chick collagen was brought up to 200 microliters in 1 M NaCl-50mM Tris pH 7.4 at the beginning of the extraction. The extraction was continued as according to "Materials and Methods".

At the end of each step in the extraction procedure, a 1 ul aliquot was removed, TCA-precipitated, and counted. In Figure 17, the counting results at each step in the extraction are summarized. Apparently, most of the collagen was lost during the dialysis step. This could have been attributed to counts adhering to the dialysis bag after the dialysis suspension was removed. In any event, 25,000 cpm were recovered at the end of the extraction or 62.5% of the total cpm which had been present at the beginning of the extraction. The final fraction was (+) and (-) collagenase treated and run on 5%-12.5% polyacrylamide gradient slab gels together with 14C-proline labelled chick collagen standard (Figure 18). There is essentially no change in the banding pattern of the "extracted" chick collagen and the non-extracted collagen standard. The fact that a high percentage of collagen was recovered indicated that this extraction method was a feasible one and that it was specific for collagen. The high salt condition used in this extraction also did not alter the banding pattern of the collagen.

F. Pepsin-Solubilization and Extraction of Sea Urchin Collagen

A final test for the collagenous nature of the polypeptides that migrated as two bands in the 95,000-100,000 molecular weight range on SDS-polyacrylamide gels in the results reported in this
Figure 18: Autoradiogram of $^{14}$C chick collagen which has been recovered by extraction method (See Materials and Methods). Lane 1) chick collagen which has been recovered by extraction, Lane 2) chick collagen (unextracted), Lane 3) $^{14}$C protein standards. (25,000 cpm/lane).
Figure 12: Autoradiogram of pepsin-solubilized $^3$H proline labeled 60 hr. S. purp. embryos (See Materials and Methods). Lane 1) pepsin-treated 60 hr. embryos. Lane 2) untreated 60 hr. embryos, Lane 3) 60 hr. embryos treated with (+) collagenase. (15,000 cpm/lane)
study was pepsin-solubilization (See "Materials and Methods"). A packed volume of 0.01 mls of sixty-hour S. purp. embryos were labeled in vivo with $^3$H-proline (100 u Ci). These were then lysed in 2% SDS and separated into three equal fractions. One fraction was treated with pepsin for 12 hrs. The other fraction was treated with collagenase as according to standard procedure. The third fraction was the control and remained untreated. These samples were then run on 5%-12.5% polyacrylamide gradient slab gels and then fluoroographed as according to standard procedure. In Figure 19, the pepsin-treated lane provides evidence for the existence of a pepsin-resistant polypeptide which can be safely labeled as collagen. Pepsin-resistance has been accepted as a criterion for the presence of collagen. This result confirms the existence of a collagen type in developing sea urchin embryos starting at 37 hours after fertilization.
Figure 20: Most recent data: Autoradiogram of $^3$H proline labeled S. purp. embryo proteins. Lane 1) 64 hr. embryos treated with (-) collagenase, Lane 2) 40 hr. embryos treated with (-) collagenase, Lane 3) 72 hr. embryos treated with (-) collagenase, Lane 4) 48 hr. embryos treated with (-) collagenase, Lane 5) 37 hr. embryos treated with (-) collagenase, Lane 6) same as lane 3 but treated with (+) collagenase, Lane 7) same as lane 4 but treated with (+) collagenase, Lane 8) same as Lane 5 but treated with (+) collagenase, Lane 9) $^{14}$C chick procollagen, Lane 10) $^{14}$C protein standards. (12,000 cpm/lane.).
DISCUSSION
DISCUSSION

Evidence has been presented here which demonstrates the existence of a collagen type in developing *Strongylocentrotus purpuratus* embryo. The results also indicate that this collagen first appears at 37 hours and continues up through 72 hours after fertilization. This conclusion which has been reported by other researchers was confirmed on the basis of the sequence of experimental findings outlined below.

The initial work with incorporation of $^3$H-proline into protein at various times after fertilization provided, to a limited extent, an index of the biosynthesis of the major type of collagen in the embryo. The results indicated that $^3$H-proline incorporation began to increase at gastrula (37 hours) and continued so until pluteus was reached. This increase correlates well with that reported by others for hydroxyproline: proline content at these stages in development (29,38). This result could not be equated with a concomitant increase in collagen synthesis without some kind of analysis of the proteins present at various stages in the course of embryonic development.

The results from the polyacrylamide gel electrophoresis of $^3$H-proline-labelled proteins at various stages in embryonic development show the existence of two distinct bands which co-migrate with $^{14}$C proline-labelled chick collagen standards at 37 hours, 40 hours, 48 hours, 64 hours, and 72 hours after fertilization. These same bands are also susceptible to collagenase digestion. No such bands can be seen at 24 hours after fertilization.
This evidence implies the existence of a protein that possesses collagenous polypeptides. The results also indicate a regulation of this collagenous protein during embryonic development. That is, there is no synthesis of this protein prior to gastrulation. Since these studies may have been complicated by the long exposure times needed to effectively visualize these collagenous polypeptides, it would have been interesting to look at these polypeptides without the presence of other labelled proteins. Subsequent work led to an extraction technique for acid-soluble collagenous proteins in *S. purp.* embryos.

The extraction method employed effectively isolated the 2 bands previously seen in concert on 5% -12.5% polyacrylamide slab gels. These 2 bands again co-migrated with chick-collagen standards and were also susceptible to collagenase digestion. The developmental study of these extracted collagenous proteins confirms the regulation of these proteins during the course of embryonic development. These two high molecular weight bands can be said to be two collagenous alpha-chains. They await further characterization, such as chromatographic separation of the two chains from each other, more information on amino acid composition, and cyanogen bromide peptide electrophoretic analysis. The fact that these polypeptide chains migrate in the 95,000 - 100,000 molecular weight region suggests that they are basement membrane collagen. The presence of contaminating polypeptides in the neutral salt-soluble extracted material on SDS-polyacrylamide gels underscores the incompleteness of the characterization of this collagen.
That the extraction method employed was a viable one was confirmed by the chick collagen recovery results. A 62.5% recovery of this chick collagen was a very good yield, and this result confirmed the collagenous nature of the material extracted from *S. purp.* embryos.

The pepsin-solubilization experiments provided further evidence for the presence of a collagen type in sea urchin embryos. Pepsin-solubilization of \(^{33}H\) proline-labelled proteins obtained from embryos resulted in the isolation of two collagenous polypeptides which migrated in the same molecular weight region as those polypeptides isolated from the extraction. Resistance to pepsin-digestion has been well-documented as a criterion for the collagenous nature of a polypeptide chain.

In summary, the evidence presented here establishes the presence of a developmentally regulated collagen type in sea urchin embryos. The collagenous nature of this protein confirmed on the basis of its co-migration with chick collagen standards on SDS-polyacrylamide gradient slab gels, its high content in proline, its susceptibility to collagenase digestion, and its resistance to pepsin digestion. This study marks the first time that SDS-polyacrylamide gel electrophoresis of labelled proteins has been utilized as a method of analysis to confirm the existence of a collagen type in sea urchin embryos. Isolation of this collagen by an acid-salt extraction procedure, is also a novel means of analysis in approaching this problem.
There arises the possibility of the existence of "informational" macromolecules which determine which cells in the embryo will ultimately synthesize collagen. Maternal determinants may be laid down in the egg, perhaps in the cortex, in such a way that they are partitioned differentially among the cells of early cleavage which therefore become distinct from each other. A second, related but separable, hypothesis is that these determinants interact with the nuclei of the cells causing them to express a pattern of gene activity specific for the particular part of the embryo. This differential gene expression would lead to divergence of the cells to produce different tissues in the embryo. Such differentiation would involve both the intrinsic programming of the different cell types and interaction between the latter.

As we have seen, collagen is mainly distributed in the hyalin and spicule tissues in the embryo. If the amounts of the collagens in a tissue are important for the maintenance of normal tissue architecture then this in turn must be critically dependent on the cells present maintaining their differentiated state with respect to the synthesis and degradation of those collagens. In this context the study of collagen synthesis by defined cell types in culture will be of much importance.

A necessary problem for further investigation is the mechanism of sea urchin collagen biosynthesis in localized tissues, like the spicules and the hyalin layer. This involves an assessment of the extent of post-translational modification of the collagen alpha chains in these particular cell types. A further area of
needed clarification is the functional significance of the collagens in these tissues. The needs of a given tissue can require highly individual modifications of collagen form and function.

An area of further study involves the establishment of a developmental profile of procollagen mRNA synthesis in the sea urchin embryo. Is the collagen which appears at 37 hours after fertilization translated from newly-synthesized procollagen mRNA or from procollagen mRNA which is constantly being synthesized throughout the course of development but which is somehow selected into a "translatable" (polysomal) class at 37 hours, instead of a "masked" (informosome) class during earlier stages in development. This question can be answered by picking out the procollagen mRNAs present in the embryo with a cloned collagen probe and then assessing the relative efficiency of translation of these mRNAs through the course of development. The presence of procollagen mRNAs sequences at stages earlier than 37 hours after fertilization would suggest that this message is selected into an inactive (informosome) class - via a possible "masking" of the message by specific binding proteins. A finding of this sort would essentially indicate that collagen synthesis is developmentally regulated at the post-translational level. The existence of regulation mechanisms of this complexity in the sea urchin embryo boggles the mind and certainly suggests that collagen synthesis is an interesting and unique developmental event that must be further characterized.
The possible existence of inactive procollagen mRNA in the form of free cytoplasmic mRNP particles in collagen-synthesizing cells remains to be established. But some of the alterations in the cellular expression of functionally-different collagens may be attributable to preferential inactivation of certain pro-a-chain mRNAs or some other form of control at the translational level.
V. REFERENCES


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