ECHINODERM CALCIFICATION-REGENERATION OF SEA URCHIN TEST

ABSTRACT

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Calcification in Echinoderms was investigated by drilling the test of sea urchins and allowing them to regenerate. Regenerating mineral components were studied by scanning electron microscopy and regenerating non-mineral components by histology, histochemistry and electron microscopy. Calcite replacement involved coelomocytes of the perivisceral fluid. Regeneration was divisible into three distinct stages: 1) Initial filling of the wound by leucocytes; 2) Elongation of leucocytes and differentiation into connective tissue elements; 3) Interaction between spherule (and possibly vibratile) cells and leucocytes resulting in deposition of new platelets. Spherule cells appear to contribute protein pigment (echinochrome) and acid sulfated mucopolysaccharide to formation of calcite matrix. Leucocytes are intracellularly calcified by mineral deposition in or on this matrix. Neither collagen nor the enzyme carbonic anhydrase appear to be directly involved as limiting local factors in echinoid skeletogenisis.

CALCIFICATION IN ECHINODERMS: REGENERATION OF THE TEST OF THE SEA URCHIN STRONGYLOCENTROTUS DROEBACHIENSIS

by

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I think I could turn and live with the animals, they are
so placid and self-contained,
I stand and look at them long and long.

They do not sweat and whine about their condition,
They do not lie awake in the dark and weep for their sins,
They do not make me sick discussing their duty to God,
Not one is dissatisfied, not one is demented with the
mania of owning things,
Not one kneels to another, nor to his kind that lived
thousands of years ago,
Not one is respectable or unhappy over the whole earth.

- Walt Whitman

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Chapter I

HISTORICAL BACKGROUND

1. Introduction

The work on echinoderm calcification can be divided into three main headings: (1) the organic constituents including experiments and histological studies on both larval and adult forms; (2) the inorganic constituents dealing with the calcite endoskeleton, its unique structure and crystallography, and certain geological facts directly applicable to its mode of deposition; and (3) physiological studies on echinoderm calcification including studies on larval and adult forms.

2. Organic Constituents of the Test

2.1 Developmental Studies:

Much of what is known about the calcification of echinoderms has resulted from studies on their larval forms. Many of these have been descriptive studies of gross morphological changes accompanying growth and development of the larvae, but few have detailed the cellular activity which accompanies skeletal deposition. I shall limit this description to (i) primarily members of the class Echinoidea, and (ii) those studies which dealt with the cytological details of larval skeletal deposition.

Calcareous skeletons are usually two-phase systems. By this is meant that in most animals a mineral, usually a calcium salt, is deposited on and in an extracellular

organic matrix. This has been found to be the case in vertebrates, molluscs, arthropods, coral and even unicellular foraminifera. The organic matrix is usually a high molecular weight protein such as collagen, conchiolin or chitin. The close association of extracellular protein and mineral salt has become an accepted fact in most animal phyla (see Moss 1964). In the case of echinoderms, however, the relationship between the organic phase and the mineral calcitic phase is by no means a resolved issue. In the larvae of echinoids and, indeed, all echinoderms, the skeleton first appears as a series of tiny calcareous rods or spicules which occur inside the blastular cavity within forty hours after fertilization (Hyman 1955).

The spicules are laid down by the cells of the primary mesenchyme. These cells are derived from the ectoderm (Wolpert and Gustafson 1961a). Thirty to forty hours after fertilization the primary mesenchyme cells aggregate into small clumps of from three to ten cells on either side of the blastula. In 1879 Selenka, studying five different echinoderm larvae, observed a minute calcareous body which was deposited inside two of these mesenchymal cells. This small granule grew into a triangular spicule. As it did so, the cell from which it arose moved to one of the apices of the spicule and two other mesenchyme cells moved to the other two apices. These three cells then added to the growth of the spicule. Selenka observed that this growth occurred in three directions, thus the name triradiate spicule. It is not clear, however, whether Selenka thought that the

calcareous granule remained enclosed by cellular material or whether it was extruded by the cell and additional deposition occurred extracellularly.

Semon (1887) also observed the intracellular granule reported by Selenka, in larvae of Strongylocentrotus lividus. He found that as the granule grew into the triradiate spicule, it was enveloped by a thin membrane which originated from the primary mesenchymal cell. Thus the calcareous granule and resulting spicule were essentially intracellular. He thought that the membrane originated from the spicule-producing primary mesenchyme cells which then lost their nuclei, leaving only the cell membrane surrounding the spicule.

Théel (1892), studying spicule deposition in the developing larvae of Echinocyamus pusillus, found that the calcareous spicules originate within the ectoplasm of the pseudopods of the spicule-producing cells. This ectoplasm is displaced from the main portion of the cell. Further growth of the spicule took place by the gradual deposition of calcareous material within a pseudopodal clump formed from the fusion of the pseudopodia of several calciferous cells. He disputes the conclusions of Selenka and Semon which seemed to indicate that the granules originated from one cell. He saw the granule as originating from and growing inside of the fused pseudopodia of several calciferous cells. Théel then postulated that the ectoplasm of the pseudopods of the calciferous cells were the calcifying matrix and that these cells determined the shape of the larval spicules. He believed that the cells,

after elaborating the membrane-matrix and depositing the skeletal rod within in, moved away to a new center of calcification. It is significant that Théel drew a parallel between the formation of echinoid triradiate spicules and vertebrate calcification. He further stated "...it seems to me very credible that the ossifying ground substance is not formed in an intracellular substance but is originated by direct conversion of the plasma of the osteoblasts." (p. 39). Théel (1894) extended his observations to include spicule absorption. He found that the spicules were first surrounded by the pseudopodia of the "wandering cells." They were gradually eroded until pieces of spicule broke off and became engulfed by the cell to be dissolved, for use at a later time. He assumed the presence of a dissolving fluid extruded by the cells.

Chun (1892) observed the development of the skeletal wheels of the Holothurian auricularia larvae. In these structures he noted a departure from the triradiate and tetraradiate form of most larval echinoderm spicules. The auricularia wheels are formed from a series of jelly-like cells which separate from the mesodermal tissue cells. A wheel-shaped structure was formed from their fused pseudopods. This wheel contained many nuclei in a syncytium of fused cell membranes. Calcification then occurred within the fused pseudopods, very closely following the shape of the membranes of the cells. Thus the auricularia wheels are, according to Chun, formed intracellularly in much the same way as the triradiate spicules of normal larval echinoderms.

MacBride (1903) studied the development of Echinus esculentus. He describes two calcareous stars, both of which impinge on the ectoderm, but states only that they are derived from primary mesenchyme cells, and does not detail the cytological phenomena. Woodland (1906) extended MacBride's work on E. esculentus. He found that the primary mesenchyme cells became protoplasmically continuous at the point in time just before the appearance of the first so-called spherical granule. The spherical granule appears inside one of the more centrally located cells of the primary mesenchyme. More than one granule may originate in a given cellular clump but only one spicule continues to grow. As growth of the granule proceeded into the triradiate shape, he saw "strands of protoplasm" streaming from the tips of the growing spicule. He was unable to determine whether the protoplasmic connections between the mesenchymal cells served as a mold for the developing spicule or whether the growing spicule was responsible for the orientation of the mesenchymal cells which then contributed the lime for extension of the spicule.

Prenant (1926a & b) described what he termed "anastamosing tracts" between the cells of the primary mesenchyme. These tracts joined the exoplasm of the mesenchyme cells. He found that calcification of the spicules occurred along these tracts and that they therefore functioned as the organic matrix of the spicule in the larva.

Bouxin (1926b) confirmed the presence of "une gaine protoplasmique étroitement accolée au squellette" (p. 451),

seen by Prenant (1926b). He also placed early gastrula larvae in acidic seawater and observed material which stained vitally as the calcareous part of the spicule was dissolved.

Von Ubisch (1937) studied the echinoids Echinocyamus pusillus and Psammechinus miliaris. He observed the same calcareous granule formed inside the fused pseudopods of several primary mesenchyme cells. He found that triradiate spicules had their tips continuous with a sort of syncytium of fused pseudopods and that their elongation followed along the course determined by the position of the syncytial mass.

Bevelander and Nakahara (1960) studied skeletal development in the larval sand dollar Echinarachnius parma. They found that primary mesenchyme cells first become vacuolate and thenpproduce a small triangular crystal. This stage then proceeded intracellularly. Subsequent growth occurs when the young triradiate spicule "appears" outside its cell of origin and on a "delicate fibrous matrix." The authors believed the process of skeleton formation in the mineral phase to be analagous to crystal growth in inanimate systems with the initial seed crystal being elaborated by the first primary mesenchymal cell. Their photographs show the "delicate fibrous matrix" which bears very close resemblance to the skeletal envelope of Okazaki (see below).

Okazaki (1960) described in detail the formation of skeletal rods in several sea urchins. Her results are as follows: after the mesenchymal cells migrate to either side

of the blastocoel, they can be seen to send out fine pseudopodia in all directions. These pseudopodia fuse into large hyaloplasmic masses which concentrate at the center of the mesenchymal aggregate and grow even further as more cells contribute their pseudopodia. The net result is an organic "envelope" which is composed of many fused pseudopods and is triangular in appearance. Meanwhile, lagging somewhat behind this envelope formation, more mesenchymal cells fuse their pseudopods, forming a chain which extends up the walls of the blastocoel. These cell chains extend to the three corners of the mesenchymal envelope and gradually fuse with it. The calcareous spicule noted by previous workers arises within this envelope and elongation of the spicules occurs within the pseudopods which have fused with the envelope's apices. Okazaki dissolved away the calcareous elements of isolated spicules and found a "cord of the same shape as the spicule" left behind. This cord stained with Nile blue. She states: "In short, if the matrix is considered to be a part of the mesenchyme cells the spicule is intracellular, but if independent of these cells, it will become extracellular." (p. 317).

Gustafson and Wolpert (1961a)used time lapse cinematography in their study of the development of the skeletal arms of the sea urchin Psammechinus miliaris. They saw the pseudopods of the skeletogenic mesenchyme cells surrounding and converging on the tips of the forming arms. Turning to the earlier stages in a later paper (Gustafson and Wolpert 1961b), they extended these observations further. They confirm the

formation of a cable-like string of connected pseudopods in which granules first appear. Growth of the spicule begins in a "triangle" of connected pseudopods (again resembling Okazaki's "envelope"). The spicule starts as a triangular crystal which gradually assumes the typical triradiate form. The triradiate spicule becomes re-oriented within the blastocoel and growth of the arms of the spicule follows the main pseudopodal cable. Mesenchymal skeletogenic cells always enclose the skeletal spicules in a sheath. Sometimes however, they found the pseudopods to be very fine and not distinctly organized into a cable. They attribute the growth of the triradiate spicule as a crystalline-controlled process rather than one completely biogenic in origin. They stated that changes in direction of crystal growth, however, were always mediated by the presence of pseudopods at points where the direction changed. The authors attribute the change in position of the mesenchyme cells as changes in these cells' adhesiveness to the ectoderm. Final control of the skeletal shape is ultimately dependent on the ectodermal influences on the skeletogenic mesenchymal cells.

Gibbons et al (1969) conducted a series of studies which dealt with the control of cell shape by microtubules. They used sea urchin blastulae of Arbacia punctulata for this study. Electron microscopy of primary mesenchyme cells confirmed the presence of pseudopods attached to these cells. These pseudopods were connected to the main body of the cells by narrow stalks. The pseudopods formed a true syncytium (called the cable) with one another and with the main cell

bodies of the primary mesenchyme cells. Pseudopodal cables contained mitochondria, ribosomes, vesicles and microtubules, but no Golgi. In addition, the connecting stalks were seen to contain large numbers of microtubules. They observed changes in the orientation of the microtubules which correlated with changes in the shape of the cable. Thus they proposed that the control over the shape of the pseudopods (and hence the skeleton) rested in the microtubules. This is the opposite view from that of Gustafson and Wolpert who believed that control over pseudopodal shape was determined by ectodermal influences. Within the cable cytoplasm, Gibbons and his colleagues observed the formation of a vacuole in which was deposited the calcite spicule. They were unable to section these calcite granules. They found that the skeletal material which was formed inside the cable was never continuous with the cable nor did it ever occur outside it. They observed coated and double vesicles which they believed may have been involved with the process of larval skeletogenesis. In addition, they observed a matrix prior to the appearance of the spicule. They were unable to detect any fibrous elements within this matrix and so concluded that it was probably a protein or mucoprotein produced locally in the cable cytoplasm. They observed dark electron opaque granular material which surrounded "holes" in the embedding medium where the spicules had fallen out. They propose that vacuoles containing organic and inorganic skeletal components fuse inside the cytoplasm of the pseudopods to form the skeleton.

Thus, from the foregoing studies it appears that the skeletons of echinoderm larvae are intracellular in origin and surrounded by a close fitting membrane derived from the fusion of pseudopods of the cells of the primary mesenchyme.

2.2 Adult Test Structure:

2.2.1 Development and Morphology:

We shall now consider the development and structure of the soft parts of the post larval and adult echinoid skeleton. This section will deal only with those studies which are directly relevant to possible physiological mechanisms of skeletal deposition.

The development of the triradiate spicule, described in Section 2.1, and its mechanism of deposition is central to the understanding of skeletogenesis in the echinoderms, particularly the Echinoidea. All the calcareous elements of sea urchins including spines, pedicellariae, sphaeridia and the lantern of Aristotle are formed by the elaboration of the triradiate spicule (Hyman 1955).

Woodland (1907) describes the development of the test in a post-metamorphosed larva. He starts with the triradiate spicule along the arms of which are situated the scleroblastic cells. The nuclei of these cells then divide into two daughter cells which contain a part of the spicule between them. The arms of the spicule continue to bifurcate again and again under the influence of the scleroblasts along their length. From gastrula, the young urchin develops further into the more

complex pluteus. During this period, an elaborate spicular skeleton develops which grows into a network of fine calcite rods. Towards the latter part of pluteal stage, the actual plates of the adult urchin begin to form (Hyman 1955). Gordon (1926) found that each plate begins as a small spherical granule inside the scleroblast (mesenchyme cell). This granule then grows into a small triradiate or tetraradiate spicule. By repeated dichotomous branching, the spicule forms a meshwork extending in three planes to form the "beams and rafters" stereom which is the basic organizational unit of each adult echinoid plate.

It appears that the calcite stereom remains internal to the dividing mass of protoplasm and that the protoplasm becomes pushed aside as skeletogenesis occurs (Nichols 1962). The new spicules which form the plates arise independently of the larval spicules. Spines and sphaeridia are also formed by repeated branchings of tetraradiate and triradiate spicules. The end of the pluteus stage is marked by a remarkable metamorphosis after which the larvae acquire the characteristic ovoid shape of the adult. Gordon (1926) described the newly metamorphosed larva as covered by a series of interconnected plates. Each plate is an open meshwork of calcite rods formed by the repeated branching of triradiate spicules. Plates at the aboral end of the animal are known to undergo resorption (Raup 1966). Sea urchins add to their test size by increasing individual plate size and increasing the number of plates in any given row (Deutler 1926; Raup 1966). Plate growth occurs

in the directions described by Kobayashi and Taki (1969) (see Section 4.2). Plates are added on at the aboral end of the animal so that the oldest plates are the oral ones.

Hoffman (1871) described the structure of the adult echinoid test. On the external surface he saw a ciliated epithelium which covered all of the test except for the tips of spines and sphaeridia. He described a loose connective tissue layer below this epithelium which is rich in pigmentation. Pigments were either loose as granules or contained within amoeboid pigment cells. Below the connective tissue layer are situated the actual plates of the echinoid test. The test consists of calcareous spicular elements between which run what Hoffman termed a granular interstitial region. The outer peripheral regions of the plates were seen by Hoffman to be heavily pigmented, while the inner regions were usually colorless. In addition, these inner regions contain thicker rounded calcite spicules from 18-26 μ in cross section, as opposed to peripheral regions which are usually oval and 8-12 μ in cross section. Plates situated in ambulacral regions are thinner than plates in interambulacral regions. Between the calcite spicules Hoffman saw the net-like organic matrix. This "matrix" consisted of a network of fine interconnected fibrillar cells with nuclei scattered throughout their cytoplasms. He found little variation between the five echinoids he looked at except for variations in the structure of madreporite plates. The inner layer of the echinoid plate is covered by a fragile skin of connective tissue overlaid by a narrow band of ciliated epithelium.

Prouho (1887) described the test of Dorocidaris papillata. He saw an anastamosing network of "conjunctive canalicules" with very thin walls. The walls, he said, abutted against the calcitic phase of the skeleton. Inside the canalicules he saw many nuclei as well as other coelomocyte types. This is the central portion of the test. The conjunctive tissues of this central area then formed a basement membrane, supporting what Prouho believed to be an internal ciliated peritoneum. On the outside, covering the whole of the test, including all its appendages, was an external epidermis. Prouho describes spherical brown globules and "muriform corpuscles" within the conjunctive canaliculi. Between each plate he saw the sutural fibers which anchor the plates to one another. He observed an accumulation of nuclei where new calcite was appearing, but did not pursue these observations.

Hamman (1887) also described the test of echinoids. He saw a series of "reticular" fibers which were derived from lengthening of the connective tissue (dermal) cells. These, he states, function as supporting fibers for the calcite plates. Between the plates Hamman describes the "interstices". These are probably the same structures described by Prouho as the "conjunctive canalicules" and by Hoffman as the matrix. Within the interstices were a series of nodular points containing cell nuclei and surrounded by a granular substance. The interstices for Hamman consisted of a syncytium of connected star-shaped cells, in which individual cells could

not be differentiated. Areas between these cells and their fibrous extensions are completely calcified. Each plate was penetrated by interconnecting fibrous elements which were packed into thick bundles and served to connect the plates. Where the growing calcite had pushed the reticular fibers away from the center of the plates and towards the coelomic cavity, they were seen to collect in fibrous bundles between the calcite spicules and the internal peritoneal lining. He saw several amoebocytes and wandering cells which he stated to be derived from the cells of the perivisceral fluid. He did not (along with Hoffman and Prouho) describe these cells in detail.

Cuénot (1891) confirmed the results of Prouho, Hoffman and Hamman. He further extended his observations on the wandering cells found in theoorganic part of the test. He believed that these amoebocytes functioned as nutritive cells to the scleroblasts as well as carriers for excretion products. He further stated that the spicules of the test were covered by a membrane but did not possess a demonstrable organic matrix. He believed that the spicules developed outside the dermal cells and hence were extracellular. Cuénot described a cuticle directly continuous with the cells of the dermis, but did not show or describe a separate epithelial epidermis between the cuticle and the dermal cells.

Kindred (1924) described the test of Strongylocentrotus droebachiensis. His work is detailed in Part II, Section 2.1.

Moss and Murchison (1966) studied the anal and pharyngeal teeth of an adult holothurian Actinopygia mauretania. The anal and pharyngeal teeth are homologous to sea urchin test plates and, as we shall see below, are very similar in structure. These authors describe an epithelium (epidermis) consisting of pseudostratified columnar cells resting on a base of connective tissue fibers which join the epidermis to the underlying tooth. After decalcification, they observed a residual basophilic organic stroma, penetrated by connective tissue fibers. Within the stroma they found two types of cells: a small stellate basophilic cell and a large vesicular, deeply basophilic cell. The granules of the organic stroma showed varying degrees of basophilia, as evidenced by methylene blue extinction test. Argyrophilic fibers were also seen within the stroma. The authors believed the anal and pharyngeal teeth to be formed locally as spicules secreted by scleroblasts. They postulate an organic matrix as the organizer of spicular shape but do not state which of the cells they believe to be the scleroblasts nor do they present any evidence for the presence of the organizing organic matrix. They base their assumptions on the polycrystalline nature of the spicule.

Moss and Meehan (1967) studied the sutural connective tissues of the plates of an echinoid Arbacia punctulata. These sutural tissues join the test plates to each other. The authors present histochemical data which suggest to them that the sutural fibers are uncalcified collagen embedded in an acid mucopolysaccharide ground substance. They describe five cells

within the mesoderm of the plates: three large cells, 10 μ in diameter, and two small cells of 3 μ and 6 μ each. One of the large cells contained reddish spherules when stained with Mallory's triple stain, the other contained orange "dots" and the third had a brown cytoplasm. The 3 μ cell had a clear cytoplasm and the 6 μ cell was green. Just what cells these were and how they relate to the cells of the perivisceral fluid, as described by many authors (see Johnson 1969a), is not clear, nor do the authors make any attempt at classifying them. They suggest that the larger cells may be involved in acid mucopolysaccharide production.

Pilkington (1969) made a detailed study of the organic component of the spines of Echinus esculentus. He also studied the ultra-structure of spine sclerocytes. He found three types of cells besides the sclerocytes: those with large but not intensely stained granules, those with intensely stained granules and those without granules but containing unstained vesicles. He also described first and second order spherical bodies outside the sclerocytes in the stereomal spaces. These were frequently connected to each other by thin strands of protoplasm and, in turn, to the membrane which surrounds the calcite of the skeleton. He suggests that the spherical bodies may be involved in the maintenance of this membrane. The inorganic phase of the spines he found to be topographically intracellular. In the extracellular spaces Pilkington found collagen fibers similar to those described by Travis et al (1967) (see below). Associated with the growing tip of the spine he

found intense acid phosphatase activity and less intense aminopeptidase and esterase activities. These three hydrolytic enzymes were also found in sclerocytes associated with the connective tissue layer in the spine base. Pilkington rejected Travis' suggestion that the collagen present in the extracellular spaces is calcified collagen, since it could easily be connective tissue collagen associated with the spine bases. He treated the skeletal calcite with sodium hydroxide and protease and found a nitrogenous organic fraction (as analyzed by the Kjeldahl method), resistant to these two substances. He postulated that this material represented the remains of a tightly bound organic fraction which disappears during maturation. He suggests that the organic phase can exist in a single crystal as an "isomorphous" phase of the crystal lattice. He supports this statement with electron micrographs of replicated calcite surfaces which show a rough appearance.

2.2.2 Role of Collagen:

Collagen plays a major role in vertebrate skeletal systems (Eastoe 1968). It provides the flexible phase of a two-phase system and hence serves a structural function. It has been suggested (Glimcher 1960) that collagen in bone may function as a matrix which actively organizes the crystals of hydroxy-apatite, thus acting as a template for this, the inorganic phase of the skeleton. According to this theory then, active sites on the collagen bind with corresponding groups on the crystals and act as nucleation sites on which

seed crystals form. Mineralization proceeds by growth from these seed crystals. In the light of this suggestion, many investigators have focused on collagen as physiologically significant in the calcification process.

Invertebrate collagens are far more variable from species to species than are vertebrate collagens. In general they contain a uniformly large amount of glycine, have a smaller proportion of amino acids, more total dicarbonylic and hydroxy amino acids, and fewer non-polar amino acids when compared to vertebrate collagens (Piez and Gross 1959).

Marks et al (1949) performed wide and small angle X-ray diffraction on fibers isolated from the body wall of the holothurian Thyone sp6 They also examined fibers isolated from decalcified body wall of Asterias sp. and the peristome of Arbacia sp. Wide angle diffraction revealed collagen which was essentially similar to vertebrate collagen in all cases. Small angle diffraction studies showed fibers with axial repeat patterns of 635 Å - 670 Å, again in all three animals. They did find that echinoderm collagens showed variations in distribution of diffraction orders when compared to vertebrate diffraction patterns. This indicated to the authors the probability that these echinoderm collagens contained proportions of certain amino acids which were different from those found in vertebrates.

Roche et al (1951) determined the amino acid content for the echinoid Arbacia aequituberculata as well as several molluscs. They found the values of glycine in the decalcified test to be similar to those of mollusc collagen (and generally

higher than the vertebrates). They were unable to find correlations between the type of connective tissue protein present in molluscs and echinoderms and the form of mineral (calcite or organite) present in their shells.

Randall et al (1952) found fibers exhibiting regular periodicity in the body wall of Asterias rubens and Psammechinus miliaris. These fibers were thinner and fewer in number in Psammechinus.

Gross et al (1958) examined the collagen of Thyone to determine its amino acid composition. They found the amount of proline to be equal to or slightly greater than hydroxyproline, and that sugar moeities (Hexase) were low (less than 5%). There appeared to be a close association (although not conclusively proven) between fucose and the protein fraction in Thyone collagen.

Piez and Gross (1959) examined the collagen present in the body wall of Thyone sp. using electron microscopy and amino acid analysis techniques. They found that the structure of this collagen was similar to vertebrate collagen and showed typical axial repeat patterns of 640 Å - 670 Å. They also found that Thyone collagen contained one third to one fifth as much lysine and hydroxyproline as vertebrate collagen.

Watson and Silvester (1959) examined the amino acid and carbohydrate fractions of the cuvérian fibers of the holothurian Holuthuria forskali. They found that the sugar moeities were glucosamine and galactosamine. They found similar amino acid residues to Gross et al and, in addition, found very high values for glycine.

Katzman et al (1969) repeated earlier work on Thyone briareus. By purification of the collagen extracts and subsequent gelatinization they were able to demonstrate two collagen fractions which contained only glucose and galactose and one carbohydrate fraction which contained fucose mannose, arabinose, glucosamine and galactosamine. They found no carbohydrate associated with hydroxylysine. They explained that reports of many different carbohydrates being associated closely with echinoderm collagen were due to impure sample preparations.

Travis, François, Bonar and Glimcher (1967) studied the tests of Strongylocentrotus and Lytechinus. They present X-ray diffraction and amino acid analyses of a collagen which the authors state to be the organic matrix of the echinoid calcite. They also show electron micrographs of collagen banded at about 640 Å.

It must be emphasized that all of these studies were probably performed on connective tissue collagen, which is not equivalent to calcified collagen. This is certainly the case for all studies on holothurian collagen. In those cases reported on echinoid and asteroid material no mention of efforts to separate calcified from connective tissue collagen are mentioned, in fact in three cases (Marks et al 1949; Roche et al 1951; Randall et al 1952) mention is made of the thinness of fibers obtained from echinoid tests. It is likely that these fibers were either the sutural connective tissue fibers (which are very thin) or fibers which connect the

musculature of spines and pedicellariae to the dermal cells. Travis et al (1967) also made no attempt to separate collagen from sutural fibers, from "calcified" collagen.

Klein and Currey (1970) used purified extracts of the highly specific enzyme collagenase to determine whether or not collagen was indeed functioning as an organic matrix in echinoid tests. They conducted their investigations on Strongylocentrotus droebachiensis. Presence of hydroxyproline or proline is considered to be diagnostic of collagen. First they analyzed distal spines, base of the spines, tests with sutures, and Aristotle's lantern by chromatography. They found very small amounts of hydroxyproline. Dentin and bone are resistant to collagenase unless they have been decalcified at neutral pH (Klein and Currey 1970). Collagenase then may be used as a means of distinguishing calcified collagen from soft tissue collagen. The authors applied these principles to the study of sea urchin tests. They incubated the spines, bases of spines and Aristotle's lantern in collagenase for ten days until no more hydroxyproline was released. The amounts so released were comparable to those released from mammalian soft tissue. By comparing post-enzyme treated tissue weights with pre-enzyme treated tissue weights, they were able to determine that only trace amounts of hydroxyproline are present in calcified echinoid tissue. They did find .1 to .3 per cent noncollagenous protein which they relate to Pilkington's (1969) findings of a tightly bound organic fraction present in the spines. They assume collagen reported previously to be derived from soft connective tissues.

The preceding discussion serves to point out the many controversial aspects surrounding investigations of the organic components of echinoid skeletal tissues in general and the test in particular. However, based on the most recent investigations, collagen does not seem to function as an organizing or structural matrix in the echinoid skeletal tissue. It also appears that the echinoid skeleton is topographically intracellular, In this respect, it is very different from mollusc and vertebrate skeletal material.

3. Inorganic Constituents of the Test

3.1 Chemical Components:

We turn now to consideration of the non-organic constituent of the echinoid skeleton. This presentation will be limited to the echinoid test (and spines) and will not deal with tooth structure. A considerable amount of data is available on the crystallography and functional morphology of the echinoid test.

The endoskeleton of echinoids (and all other echinoderms) consists of a fenestrated network of calcite arranged in a porous lattice of interconnected rods (Nichols and Currey 1968). Between the rods are spaces which are filled with the organic component (or stroma) dealt with in the preceding section. The organic material accounts for as much as sixty per cent of the volume of a single skeletal element, e.g., plate or spine (West 1937); the rest is composed of calcium carbonate in the crystal form of calcite (Raup 1966). Clarke and Wheeler (1922) made the first comprehensive analysis

of the inorganic constituents of echinoid skeletons. They found that $CaCO_3$ in solid solution with $MgCO_3$ made up from eighty-five to ninety-four per cent of the test. They surveyed a large number of species from around the world. In addition, they found that magnesium carbonate comprised some six to fifteen per cent of the test. Urchins from colder regions were generally lower in their magnesium content than those species from the tropics.

Calcite($CaCO_3$) and dolomite $CaMg(CO_3)$ are present in solid solution in many marine organisms, particularly echinoderms (Raup 1966; Chave 1952). Chave (1952) showed that the relatively high magnesium content of echinoid endoskeletons cannot be produced by physical precipitation alone and that some biological intervention is necessary to account for this high value. He further showed that fossilized echinoderms have low magnesium. This is the result of reversion to low magnesium in the absence of organic material to maintain the normal condition of high proportions of that metal. In 1954 Chave extended the work of Clarke and Wheeler. He found that for echinoids there is a considerable variation among individuals of a population with respect to their magnesium content. He confirmed the correlation between high magnesium and high water temperature. Within individuals of a population he found differences in the magnesium content between spines and plates. This, Chave thought, was due to resorption of the inside of the shell as the animals grow larger. However, resorption only occurs in the anal plates of newly metamorphosed larvae (Gordon 1926). Growth occurs by increases

in the number of plates and by addition to the outside edges of each plate (Kobayashi and Taki 1969).

Weber and Raup (1966a) analyzed the isotopic content of echinoid teeth, plates and spines. They found differences in $^{C13}/_{C12}$ and $^{O18}/_{O12}$ in different parts of the lantern and between interambulacral and ambulacral columns of different species. Since isotope fractionation takes place during decarboxylation, the variations in these fractions indicate differences in relative amounts of carbon and oxygen that are contained in $CaCO_3$. This in turn means that the carbon and oxygen atoms of the calcite are derived from both seawater bicarbonate and metabolic carbon dioxide and that they are derived in different proportions in different species. In a subsequent paper, Weber and Raup (1966b) state that variations in isotopic fractionation between various species are principally due to phylogenetic and genetic factors rather than local physiological and environmental factors. In addition, although they found that the calcite of spines was isotopically similar to inorganically precipitated marine carbonates, the test varied greatly from species to species in respect to its isotopic composition.

Weber (1969) again studied the magnesium content of echinoid skeletons. He found that within individual echinoids there were large differences in magnesium content between spines, tooth parts and plates. He also found that magnesium was lower in spines, but was well correlated with the temperature of the water in which the animal lived, i.e., confirmation of Clarke and Wheeler (1922) and Chave (1954).

3.2 Crystallography of the Test:

Raup has summarized the available crystallographic data (Raup 1960, 1966). Two principle methods of study have been used in determining the crystallographic properties of echinoid calcite: optical and X-ray methods.

West (1937) found that each spine, when studied by X-ray diffraction, behaved as a single crystal of calcite rather than being an accretion of many tiny micro- or macro-crystals.

Garrido and Blanco (1947) determined the crystal structure of spines of the sea urchin Strongylocentrotus lividus using X-rays. They concluded that each spine behaved as if it were a single crystal or made up of perfectly oriented microcrystals. The axis of this "monocrystal" was parallel to the length of the spine.

Lucas (1953) examined the apical plates of several recent and extinct urchins by polarization microscopy. He found that each plate behaved as if it were a single crystal and that the crystallographic axis of these plates defined bilateral axis of symmetry across the test. He correlated shifts in crystallographic symmetry with those shifts in morphological symmetry that occurred during echinoderm evolution.

Donnay (1956) indicated that the skeleton of plutei of Strongylocentrotus franciscanus are single crystals of calcite, their length coinciding with the C-axis of the crystal. She also quotes from work which indicates that

each of the plates of the adult skeleton is a single crystal of calcite. These results were based on optical methods. Raup (1959) studied the crystallography of a large number of recent and extinct echinoids. By optical means he established that new crystals of calcite are always added in optical continuity with those already present. He found that he could divide up the echinoids into two groups based on the crystallographic orientation of their plates. One group showed the C-axis of ambulacral and interambulacral plates nearly tangent to the surface of the test and the other group had their C-axis perpendicular to the surface of the test. He found some variation from plate to plate within a given individual. In a later study, Raup (1960) related these two variations in C-axis orientation to adaptations to light sensitivity. Those echinoids which are light negative have tangential C-axes, those which are light positive have perpendicular C-axes. When the C-axis is perpendicular to the plate, light is scattered at the crystal-tissue interface. This has not been subsequently confirmed, although work by Millott (1956) on Diadema may be supportive.

Nissen (1963) gives X-ray data on the spines of echinoids suggesting that each skeletal element was made up of tiny oriented crystals. Each crystal, he stated, was oriented parallel to the C-axis as a whole.

Raup (1965) found that the crystallographic axes of individual plates of the apical system define an axis of bilateral symmetry corresponding to that of the larvae.

With the introduction of the scanning electron microscope, studies on echinoid calcite have been renewed. Currey and Nichols (1967) showed that fracture planes of echinoid spines were similar to fracture planes of a single crystal of mineral calcite. The authors thereby conclude that echinoid calcite was monocrystalline. However, working with platinum-carbon replicas on the standard electron microscope, Towe (1967) found that while the inner surface of the plates showed smooth fracture planes, the outer surface showed needles of calcite, 2 μ wide, which were oriented in a preferred direction. He suggested that growth of the echinoderm skeletal element consisted of a process of oriented polycrystalline growth on an organic matrix followed by maturation involving recrystallization by continued fusion and coalescence of the micro-elements.

Donnay and Pawson (1969), using both optical and X-ray techniques, studied echinoderm skeletal calcite. By using X-rays, they demonstrated that each skeletal unit of Strongylocentrotus droebachiensis was indeed a single crystal and that cleavage of these crystals yielded no fracture lines. Optically, they found the calcite plates to be imperfectly anisotropic, although the variation was small. Only echinoid teeth proved to be polycrystalline. They explain Towe's results as being due to mechanical abrasion on the outer surfaces of the plates and tubercles. This caused pulverization of these surfaces, causing them to appear "powdery" in replica.

Nissen (1969) extended this work by use of the scanning electron microscope. By X-ray methods he found the calcite of

spines and plates of echinoids to be monocrystalline. He also showed, however, that although two plates show identical C-axis orientation, they may differ in their A-axis orientation. He also presents evidence from cleavage planes and patterns of calcite layering that indicate different secretion conditions during growth of the plate. He concludes by stating that there are large variations in crystal lattice orientations between different species of echinoids and that phylogenetic relationships are difficult to establish without further data.

Donnay and Heatfield (1970) found that portions of spines of the sea urchin Arbacia punctulata, which were fractured and allowed to regenerate, did so in perfect crystallographic continuity and that X-ray diffraction patterns revealed no evidence of the past fractures.

3.3 Functional Morphology:

Several studies have been concerned with the functional morphology of the echinoid skeleton. Currey and Nichols (1967) showed that the calcite trabeculae of echinoids serves as a stress breaker in the skeleton. Stress will be transferred from one trabecula to the other, rather than through the organic phase as in vertebrate bone. Furthermore, cracks forming in the meshwork will rapidly run out of solid phase and meet the organic amorphous stage. This will prevent further propagation of the crack, much as holes in the pockets of rubber clothing prevent tears from occurring at a weak point in the material.

Cockbain (1966) correlated the pentamerous symmetry of echinoderms to the shape of a calcite crystal. Each plate

is a single crystal of calcite, and calcite has a cleavage angle of 75°. The cleavage direction is parallel to the plate surface. If the calcite crystal is placed in a pentamerous arrangement only two cleavage directions are possible across each plate, reducing the possibility of breakage. This he gives as an adaptive function of the pentamerous symmetry so characteristic of the echinoderms.

Weber et al (1969) showed that the crushing strengths for echinoid tests were as high as mollusc shells of comparable dimensions and showed an effective strength-to-weight ratio higher than mollusc shells and comparable to bulk limestone.

Donnay (1970) showed that the echinoid stereom was grown with a minimal surface as interface between the calcite and organic stroma. This gives maximum possible calcite surface exposed to the organic stroma. This also gives an increase in the rationof crystalline to amorphous volume with increasing age. This was confirmed by measuring densities of older plates and newer plates. It was found that densities of the plates increase with age.

The inorganic endoskeleton of echinoids is an apparently single phase monocrystalline solid. Crystallographic data correlate well with studies on the functional morphology. The outstanding problem, therefore, is how this skeleton, so different from that of other calcified tissues, is elaborated and what processes physiologically are involved in its deposition. The next section will summarize the information available in this area.

4. Physiology of Test Formation

4.1 Studies on Larvae:

Most of the studies which have been made on the physiology of test formation have dealt, for the most part, with the larval skeleton.

Pouchet and Chabry (1899) studied the effects of lowering the calcium content of seawater on larvel skeleton deposition. They reduced the calcium level and replaced it with potassium. They found that as more and more calcium was removed (by precipitation with sodium oxalate), there was more and more retardation of skeleton formation. A one-tenth reduction of Ca^{++} was sufficient to cause abnormal skeleton deposition. At this concentration they found that although the larvae developed to plutei before dying, no skeleton ever appeared.

Herbst (1904) found that sulphate in addition to calcium plays an important role in skeleton formation. He felt that the sulphate content of the skeleton indicated some active role on the part of these ions in skeletogenesis. He also reported abnormal skeleton formation in low magnesium seawater.

In 1925, Rapkine and Prenant studied spicule deposition in Paracentrotus lividus. They employed microinjection techniques to inject indicator dyes into the blastular cavity. They found a sudden rise in blastular pH from 7.3 to 8.5 just at gastrulation. When the pH reached 8.0 spicules began appearing. It remained at 8.5 for about six hours and then dropped back to 7.3 within twenty-four hours. They correlated

the appearance of primary mesenchyme cells to the initial pH rise. The subsequent drop in pH they attributed to precipitation of calcium carbonate which acts as a buffer against the hydrogen ions produced by an increase in carbon dioxide intake. They cite further work to show that 8.0 is the optimum pH for calcite deposition.

Vlés and Gex (1925) repeated the work of Rapkine and Prenant but, in addition, followed the changes in pH in the seawater immediately surrounding the larvae. They reasoned that internal pH changes and spicule deposition should cause changes in the external medium due to entry of Ca^{++} ions and evolution of metabolic CO_2. They observed an initial drop in pH due to accumulation of respiratory CO_2 from closely packed embryos. At the point in time when spicules began to appear in the larvae, the external pH rose to nearly 8.5. This maximum occurred before the internal maximum. A final stage in which the pH dropped again to 7.9 was observed when 100% of the larvae had spicules. The final drop in pH they attributed to accumulation of metabolic CO_2 in the holding vessel. The rise in pH during spicule deposition was due to loss of CO_2 from the area immediately surrounding the larva. This CO_2 then becomes utilized as carbonate in the spicule. The authors also suggest that bicarbonate functions as a CO_3 source in spicule formation.

Bouxin (1926a) studied the effects of several acids on the skeleton of the larvae of Paracentrotus lividus. Included were acetic, sulphuric and hydrochloric acids.

He found all acids to be alike with respect to the way in which they affected the skeleton and so concluded that the effects he observed were due to H^+ ions only and not to the effects of other ions. At a pH of 8.1-7.2 there was no effect. From pH 7.2-6.6 development was slowed down; 6.6-6.4 caused arrest of development and from 6.4 to 5.4 the skeleton began to regress.

Rapkine and Bouxine(1926) studied pH again to determine how closely internal changes were affected by changes in the external medium. Working with Paracentrotus lividus and using injected dyes, they found that internal pH changes followed external pH changes down to about 6.2, at which point the larvae were able to maintain their internal pH despite further acidification of the surrounding medium. Skeletal regression occurred from 6.4 downwards until death occurred around pH 5.4. The authors visualized skeletal regression acting as a kind of buffer against the extremes of acid pH.

Chambers and Pollack (1927) repeated these experiments with the sea urchin Arbacia sp. In all cases they found that the pH of the blastocoel corresponded to that of the surrounding seawater. They attribute the variations observed by the French workers as due to acid reactions produced when the dyes were injected.

Hirabayashi (1937) again repeated this work on Toxopneustes pileolus. He found a rise in pH from 8.3 to 8.5 which occurred at the seventeenth hour after fertilization

and continued to the twenty-fifth hour. These are the times which delimit the appearance of the primary mesenchyme cells and the formation of triradiate spicules. When he lowered the pH of the surrounding seawater, he found that the blastocoel remained slightly more acid than the external medium down to pH 6.0. At more acid pH's, they were equal. He also found that embryos treated with lithium or magnesium were unable to regulate pH. His results seem to support and extend the findings of the French workers and contradict those of Chambers and Pollack. In summary then, alkalinity increases appear to accompany the appearance of spicules in echinoid larvae, although none of the above investigators were able to locate precisely where in the blastocoel the changes occurred. This is perhaps due to obscuring of the mesenchymal aggregates by overall color changes in the blastocoel which surrounds them.

Lowndes (1944) found that zygotes and blastulae had a density of 1.07. Seawater was 1.03. On the basis of other considerations, the density of the embryos would be expected to be 1.05. He attributesthe increase in density to the formation of tiny calcitic granules (not Ca^{++} ions). He supports this finding by quoting the ash content of blastulae, which is much higher (by a factor of three) than newly fertilized eggs.

Okazaki (1956) studied the effects of lowered calcium concentration on skeleton formation in larvae of Pseudocentrotus depressus. She found that the time for skeletal formation was

delayed by one-half for calcium concentrations of .1N and was slightly increased by concentrations up to ten times normal. In addition, the proportion of the thickness of the spicule to its length was inversely proportional to the calcium concentration of the medium. This last observation indicated to Okazaki that growth of the spicules was related to the shape of the mesenchymal "envelope". Support for this statement was drawn from the following observations. The higher the concentration of calcium ions, the earlier the formation of the spicules occurred and generally the more developed the spicule. However, past the initial stages of spicule deposition, growth was inhibited by high calcium. In calcium low media, the mesenchyme cells were farther apart while in calcium high media, they were closer together. This is due to the smaller blastocoel size in hyperosmotic media. Thus, in high calcium media, growth beyond the initial spicule stages was inhibited because the cells laying down the spicules were too close together.

Bevelander and Nakahara (1960) performed similar experiments on Echinarachnius parma. They found that "vacuole" formation in primary mesenchyme cells was inhibited in low calcium (7.2). This, in turn, produced inhibition of spicule formation. They found that high calcium triggered spicule formation in several cells instead of just the two that normally are spicule producing, and that skeletons so produced were abnormally large. Rearing in acid seawater (pH 6.7-6.9) produced the same abnormal large skeletons as in Okazaki's

study, except that they were more delicate. Labelling with Ca^{45} showed that Ca^{++} was mobilized from seawater and then concentrated by primary mesenchyme cells to be added to the growing skeleton. They did not, however, show the relationships of cell to spicule.

Okazaki (1960) further investigated the relationships between the mesenchyme cells and the growing spicules. She placed developing larvae in acidified seawater pH 5.6 and found that the organic envelope (see Section 2.2) was deformed and contracted, and the spicules dissolved. While the spicules were dissolving, she describes envelopes from amoeboid-like cells which contained calcareous fragments inside. She again confirmed and extended her earlier work on the effects of low Ca. Low calcium produced independent strands and chains of mesenchyme cells, each of which in turn provided spicules. Since these chains never fused, many separate skeletal units were formed throughout the blastocoel. She also made observations which suggested to her that although the primary mesenchyme cells seem to contribute to the formation of a "matrix", it is not proven that they all do, which leaves open the possibility that only some of the primary mesenchyme cells are specialized as matrix formers.

Magnesium is an important constituent of the larval spicule (see Section 3.1). Okazaki (1961) found that an excess of magnesium x 2, 2.5, 3 and 4 produced effects identical to .4, .2, .1 and .05 respectively of calcium. Moreover, she found that the absolute values of the concentrations of these

ions were more important than the ratio of their concentrations. This suggests that magnesium may compete with calcium in the formation of spicules.

From the above it can be seen that the effects of various ions have been quite thoroughly investigated in the case of the larvae of sea urchins. It seems likely that many of the skeletal aberrations produced by varying the ionic content of the seawater are probably due to effects on the primary mesenchyme cells responsible for their deposition. None of these studies has suggested the underlying mechanism of spicule deposition, the only indication being a possible involvement of pH changes at the time of the appearance of the skeleton.

4.2 Studies on Adults:

Nichols and Currey (1968) and Raup (1966) both state that calcification in adult echinoderms is probably similar to that of the larvae. There is, however, no definitive proof of this statement and much needs to be done with regard to physiological studies especially on adults of echinoderms.

It is relevant here to mention the study of Holland (1965) on tooth renewal in Strongylocentrotus purpuratus. By treating the animals with tritiated thymidine, he followed DNA production in the tooth cells. He found that the aboral end of the tooth contained the chief source of new cells for tooth renewal. As time went on, the labelled zone of cells moved in an aboral direction down towards the tip of the tooth. The time for tooth renewal was about seventy-five days.

Kobayashi and Taki (1969) studied growth of sea urchin plates by means of tetracycline labelling. It is known that tetracycline exhibits the phenomenon of in vivo fixation in tissues which are undergoing mineralization. They found differences between specimens taken in winter and those taken in summer. Summer specimens showed much less growth than winter specimens and the sutural fibers between their plates were more easily seen. Based on tetracycline binding, the authors present the following gradient with respect to growth of the parts of the individual plates: longitudinal suture >> inner surface = mammelon and part of boss in tubercle > latitudinal suture > outer surface of base of spine shaft > outer surface of plate itself. Thus the growth of sea urchin plates occurs by outward and inward expansion, and not by addition to the outside, as has been frequently suggested (Raup 1966). Increases in test size as a whole occur by addition of plates to the apical region. This is confirmed by embryological data (see Section 2.2.1). The authors further suggest that the sutural fibers play a role in calcification of the plates since most growth occurs on the periphery where the fibers are located. This is difficult to imagine, however, since growth occurs in spines where there are no sutural fibers. It could be explained if the mechanism of skeleton deposition is different in the two areas.

Travis (1970) has examined the plates of Strongylocentrotus droebachiensis using electron microscopy and X-ray diffraction techniques. On the basis of surface replication

of test plates she ascribes a polycrystalline nature to the calcite.. She found that these crystals show a preferred orientation with their crystallographic C-axes paralleling that of the fiber axis of the sheets in which they are deposited. She finds two types of collagen. One normal fibril with 600 to 700 Å repeats and one thin 150 Å wide fibril with faint axial repeat bands. Travis divides echinoderm calcification into primary and secondary stages. During primary calcification young calcite crystals are deposited randomly within the collagen fibrils. They occur in regions of non-overlap of the constituent protofibrils of tropocollagen which make up the collagen fibrils. This primary stage crystallite deposition is followed by the secondary stage. During this stage there is a further increase in the amount of crystallization and recrystallization of crystals of calcite until axial repeat of the collagen fibrils becomes obliterated. Thus crystals deposited during the primary stage of calcification are seeded by the collagen present in the amorphous ground substance. From these initial "seed" crystals the rest of the calcite is deposited by epitaxial growth or "recrystallization".

Travis does not outline the mechanism in which calcite deposits come to lie within "holes" in the collagen. Presumably it is through banding with "active sites" on constituent amino acids of the collagen (Glimcher 1960). Thus, deposition of calcite in echinoderms and hydroxyapatite in vertebrates are essentially similar processes according to Travis' view.

4.3 Role of Enzymes in Echinoid Calcification:

Enzymes have been implicated in calcification of vertebrate bone (Maclean and Urist 1955), mollusc shell (Bevelander and Benzer 1948) and in coral formation (Goreau 1959). In the case of molluscs and coral, the enzyme carbonic anhydrase is thought to aid calcification by catalyzing the the reaction $CO_2+H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^-+H^+$. Carbonate could be obtained via the bicarbonate ion and combine with ionic calcium to form calcium carbonate.

Stolkowski (1948) studied the effect of a carbonic anhydrase inhibitor benza-sulfamide on the development of the larval skeletons of Paracentrotus lividus and Arbacia aequituberculata. He determined that a concentration of N/400 of the inhibitor produced two small round calcified spots in either corner of the gastrula. Lower concentrations interfered with normal spicular development at different stages between early gastrula and the formation of normal triradiate spicules. He also found various abnormalities of later larval development which he attributed to the absence of proper skeletal rods.

Heatfield (1970) performed experiments using Ca^{45} on regenerating spines of the sea urchin Strongylocentrotus purpuratus. He found increases in Ca^{45} uptake in isolated spines following their fracture. Based on Ca^{45} uptake, the fractured spines showed a Q10 of 2.7-4.7, from 5°C to 20°C. A carbonic anhydrase inhibitor, Diamox, was found to reduce Ca^{45} uptake. He concluded that carbonic anhydrase was involved in deposition of calcium carbonate. He made no attempt at

localizing the enzyme, and further suggests that hydroxylation of CO_2, while involved, is probably not the rate limiting factor in mineralization of echinoid spines.

From this chapter then we see that there are few studies which have been concerned with the actual mode of calcite deposition in echinoids. The vast majority have been concerned with the effects of ionic concentrations on larval skeleton formation. While these are valuable, they do not indicate the relationship between the organic and inorganic phase, with respect to their interaction on a physiological level. In addition, studies on adult echinoids have been very few. Since sea urchins show a high capacity to replace broken or lost parts (the test included), this seemed to be an ideal calcifying system in which to study mineralization in echinoids. The next chapter will detail a study of regeneration of the test of sea urchins from the point of view of obtaining information relevant to physiological mechanisms of calcification in these animals.

Chapter II

RESULTS:

REGENERATION OF THE TEST OF STRONGYLOCENTROTUS DROEBACHIENSIS

1. SCANNING ELECTRON MICROSCOPY AND OBSERVATIONS AND EXPERIMENTS ON LIVING MATERIAL.

1.1 Introduction

Members of the phylum Echinodermata are noted for their capacity to regenerate body appendages and organs following autotomy or removal. Many investigators have focused on regeneration of the spines in echinoids. The first incidence of regeneration of spines of sea urchins was reported by Carpenter (1870) after Quekett (1854). Since then, Mackintosh (1875, 1878), Poso (1909), Krizenecky (1916), Deutler (1926), Chadwick (1929), Hobson (1930), Borig (1933), Cuénot (1948), Swan (1952), Cutress (1965), Ebert (1967), Weber (1969), Heatfield (1970), Donnay and Heatfield (1970) and Heatfield (1971) have investigated regeneration of spines by sea urchins. Investigations have been based on observations of autotomy or experimental removal of spines. Only Heatfield (1970) has dealt with quantitative aspects of spine regeneration. Few studies have dealt with regeneration of the test in echinoids. Prouho (1887) reported that Dorocidaris papillata was able to repair wounds to the epidermis of its test by covering the region with a "membrane". He found that replacement of the test occurred underneath this "membrane" and concluded that the cellular

components contained therein were skeletogenic. Prizbram (1904) reported regeneration of larval skeletons after they had been dissolved away in acidic seawater. Nusbaum-Hilarowicz and Oxner (1917) found several specimens of Echinus esculentus, Acartus sphaerechinus and Sphaerechinus granularis, with numerous smaller and more heavily pigmented plates, in regions normally occupied by single plates. They also describe specimens which had piled two regenerated plates on top of one another (supra-regeneration). However, these descriptions were based on tests found in nature, and the investigators did not differentiate between plate anomalies due to teratological effects and those due to regeneration. Koehler (1922) reviewed a large number of echinoid plate anomalies due to both deformities (teratological) and trauma. He found that plates which had been removed were replaced by many smaller hexagonally shaped platelets. These smaller plates had tubercles and pores which were displaced or rearranged. Crozier (1919) reported reformation of lunules in Mellita (sand dollar) by inward regrowth of the test edges so as to enclose and reform new lunules. Okada (1926) removed plates of the ambulacral and interambulacral region of Heliocidaris purpuratus and Pseudocentrotus depressus. He observed that the wounds were closed by "dark brown material" and plates were replaced by many hexagonal platelets within two months' time. He found that ambulacral plates were replaced in a more irregular manner than interambulacral plates. He did not report the "supraregeneration" (piling of plates on top of one another)

seen by Nusbaum-Hilarowicz and Oxner. Kindred (1924) removed pieces of test from the aboral surface of Strongylocentrotus droebachiensis. After two weeks the hole was covered by a "reddish membrane". During the third week the membrane toughened and some skeletal material appeared around the edges of the wound. Skeletal deposition continued during the fourth week, at which time some of the animals showed portions of regenerated tests. He examined the "membrane" histologically and concluded that deposition of calcite was due to "phagocytic leucocytes" of the perivisceral fluid. Schinke (1950) also mentions a "membrane" which formed after removal of test plates to prevent entry of seawater into the body cavity of Psammechinus miliaris.

None of the above studies was concerned with quantitative aspects of test regeneration or calcification. Since the test is calcium carbonate in the crystallographic form of calcite (Hyman 1955; Raup 1966), it is reasonable to suppose that carbonate could be derived from hydration of carbon dioxide and used by the animal to build its test. Since the enzyme carbonic anhydrase is known to catalyze this reaction (Meldrum and Roughton 1933), several investigators (see Wilbur 1964) have implicated it in formation of mollusc shell. Although inhibition of Ca^{45} uptake in echinoid spines has been shown to occur in the presence of the carbonic anhydrase inhibitor Diamox (Heatfield 1970), no quantitative data relating to its presence in these or any other calcified echinoderm tissue is available. If implication of carbonic

anhydrase in echinoid calcification is supported, it is relevant to consider whether greater amounts of it are present in calcifying (or calcified) tissue than in non-calcified tissue. The regenerating test can reasonably be supposed to calcify by mechanisms similar to that of the normal test. For this reason, both hard and soft tissues of the echinoid Strongylocentrotus droebachiensis were assayed for the presence of carbonic anhydrase.

High concentrations of carbonic anhydrase have been reported in many molluscs (Freeman and Wilbur 1948; Florkin and de Marchin 1941; Wilbur 1960; Ferguson, Lewis and Smith 1937), and in crustacea and coelenterates (Brinkman 1933; Sobotka and Kann 1941). Reports of carbonic anhydrase occurrence in the echinoderms have been made by Brinkman (1933), who found small amounts in the gonads (ovary and spermatazoa) of Asterias and a sea urchin of unspecified type. Van Goor (1937) reported the presence of carbonic anhydrase in the gonad and intestine (probably pyloric caeca) of the asteroid Asterias glacialis, in the gonad of the echinoid Arbacia pustulosa, and in the gonad, respiratory tree and musculature of Holothuria tubulosa. Sobotka and Kann (1941) also reported the enzyme in the gut tentacles, respiratory and digestive gland of Stichopus moebii. None of these investigators assayed calcified tissues.

By using the modified micromethod for determination of carbonic anhydrase of Maren (1960), a survey was made of both calcified and calcifying tissue and soft tissues of an echinoid Strongylocentrotus droebachiensis.

1.2 Methods and Materials

1.2.1 Scanning Electron Microscopy:

Three species of sea urchin were examined in the present investigation: Strongylocentrotus droebachiensis, Strongylocentrotus palladis and Strongylocentrotus purpuratus. The first two were collected intertidally, the third subtidally, during the late spring. During the winter, specimens of St. droebachiensis were obtained commercially and kept in Instant Ocean salt water aquaria at 4°C.

Newly collected animals were allowed to acclimate and feed for several days, after which 3.5 mm diameter holes were drilled in the interambulacral area AB (Hyman 1955 after Carpenter), approximately 2.5 cm from the anal opening. The wounds were cleaned of broken test and spine fragments and the animals returned to the aquaria.

Regenerating wounds forty days old were carefully dissected out of the test in which they were regenerating, placed in large depression glass slides, bleached over night, washed in distilled water and then affixed to aluminum grids. Older, 150-day old wounds were removed by cutting out a piece of test containing the regenerated plates. These were treated in the same manner. All samples were coated with palladium and examined in a Cambridge Stereoscan Mk2A scanning electron microscope.

1.2.2 In Vivo Observations:

Animals were collected and drilled as described above. St. palladis was particularly suited to observations of events

occurring in its dermis during regeneration because of small amounts of obscuring pigments in its epidermis. Besides 3.5 mm holes, some animals had 1 cm square pieces of test removed. These wounds were covered by coverslips held on with elastic bands. After 3-5 days when extensive clotting had occurred, the coverslips with clotted coelomocytes attached were removed and the animals allowed to continue regenerating. The coverslips were fixed in formal seawater and processed for histological examination (see 2.2.1). Thin slits, 2 mm x 20 mm, were cut in the tests of other animals, using a high speed jeweller's saw. These animals were also observed during regeneration.

Experimental animals were immobilized by placing them in plasticene-lined dishes with their spines pushed into the plasticene. They were observed under high power (up to 80x) binocular dissecting microscopes at regular short intervals for periods up to 180 days. At periods of twenty, thirty and fifty days sections of test containing the regenerating area were removed, fixed in 10% neutral formal-seawater and bleached over night (when necessary) in 6% Hydrogen Peroxide. These pieces were then dehydrated in a graded series of alcohols, cleared in Xylene and mounted in thick-depression slides for examination.

1.2.3 Determination of Carbonic Anhydrase:

The procedure used was a modification of the technique of Maren et al (1960) for determination of carbonic anhydrase and its inhibitors. This technique allows rapid determination

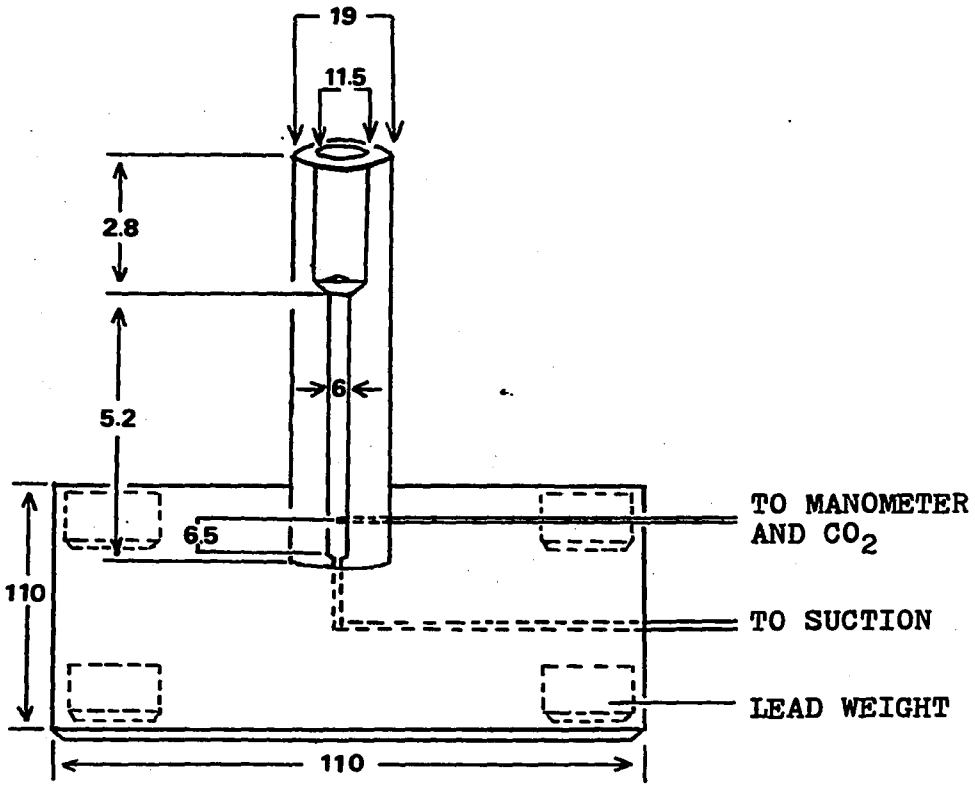


FIG. 1.1 DIAGRAM OF PLEXIGLASS REACTION VESSEL USED TO DETERMINE CARBONIC ANHYDRASE ACTIVITY. ALL DIMENSIONS IN MILLIMETERS.

of the presence of small amounts of carbonic anhydrase in tissue extracts.

The vessel shown (Fig.1.1) gives a reaction volume of 1.3 ml. The pieces of 1 mm O.D. tubing on the bottom and middle are connected to suction (for draining reaction vessel) and CO_2 respectively. Tubing inserted into the middle of the reaction vessel produces a non-tubulent but vigorous flow of CO_2. Both tubes connect to three-way valves. One arm of the CO_2 tubing connects to a manometer to regulate the flow of CO_2 into the reaction vessel. The whole of the reaction vessel was immersed in an ice bath which was continuously stirred.

Reagents used were as follows:

Indicator: 12.5 mg phenol red was dissolved in 1 liter of .0026 M $NaHCO_3$.

Buffer: 300 ml of 1 M Na_2CO_3 was added to 206 ml of 1 M $NaHCO_3$ and the whole made up to 1 liter

Enzyme: Urchins of the species Strongylocentrotus droebachiensis were used. Pieces of body wall, regenerating test (3-4 weeks), gonad, peristomium and regenerating spines (3-4 weeks) were dissected, carefully weighed, and diluted with fifty times their weight of distilled water. They were homogenized and extracted for eighteen hours at 4°C (Ferguson et al 1937). Samples of perivisceral fluid were diluted in ten times their volume and extracted in a similar manner. This gave final dilutions of 1:150 for tissue extracts and 1:30 for extracted perivisceral fluid. As positive controls,

dogs' blood diluted 1:100 (final concentration 1:400) and mantle tissue of the freshwater bivalve Lampsilis radiata (Gmelin) diluted in fifty times its weight in distilled water were also used.

In carrying out assays the reaction vessel was immersed in ice and filled with ice cold distilled water. Gas flow was regulated to 14 mm of mercury as registered on the manometer. Gas flow was adjusted for each reading. All reagents were kept immersed in ice. One-half ml glass syringes were used to deliver the reagents to the reaction vessel. Distilled water was substituted for enzyme extract to obtain an uncatalyzed reaction. Phenol red (.5 ml) was added to the reaction vessel, followed by .4 ml of distilled water. The solution immediately turned yellow, indicating carbon dioxide saturation. Buffer (.3 ml) was added and the solutions in the reaction vessel immediately turned red. The time for the reagents to revert to their original yellow color was taken as the uncatalyzed (baseline) reaction time. Five consecutive runs were performed to establish a baseline. Next the sample of dog's blood was tested and its end point determined. Another uncatalyzed run was performed and then an experimental sample was tested. Between each sample the uncatalyzed time was checked. Times for each run were recorded. Half of each sample was tested in this manner. The other half of the sample was immersed in a boiling water bath for ten minutes, to inactivate any enzyme present. The whole procedure was then repeated using the boiled samples. Activities of carbonic anhydrase are computed on the basis of the ratio of catalyzed to uncatalyzed reaction time.

Fig. 1.5. Scanning electron micrograph of newly regenerated platelet. Note thinness of trabeculae. Newly bifurcating spicules indicated by arrows. Regeneration time = 50 days.

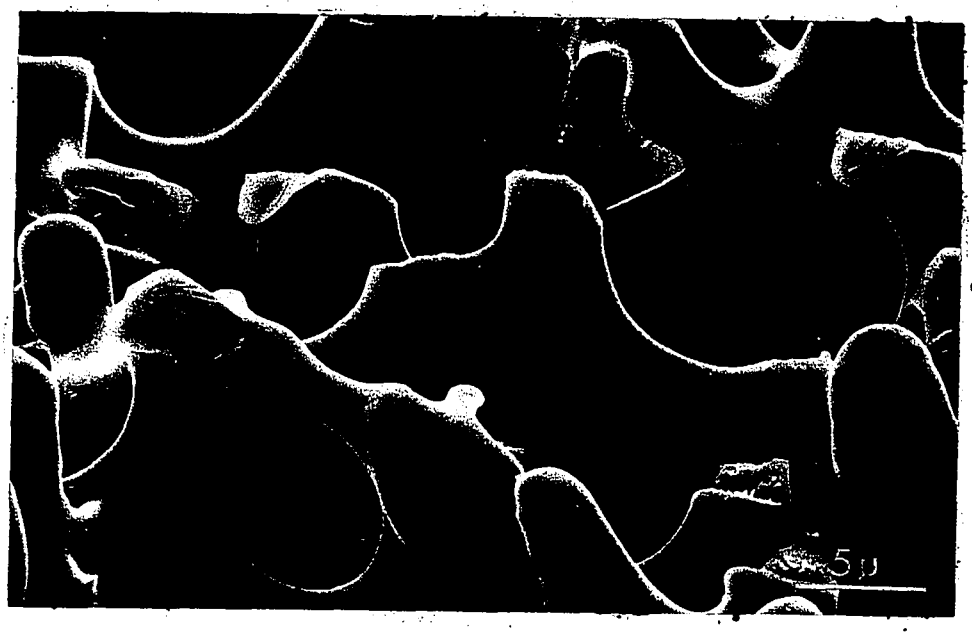
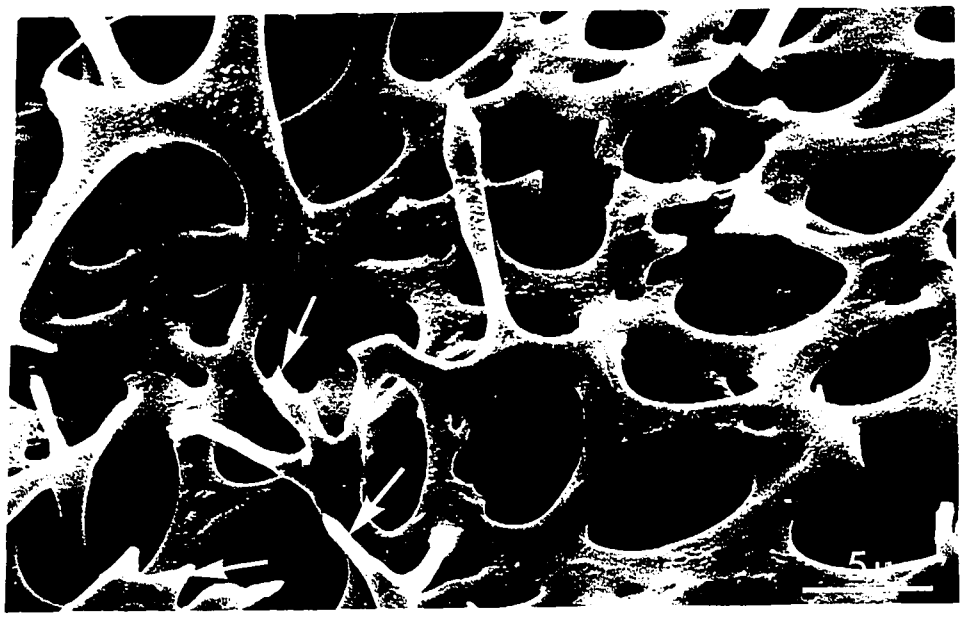


Fig. 1.6. Scanning electron micrograph of edge of normal plate. Compare size of trabeculae to those of Fig. 1.5.



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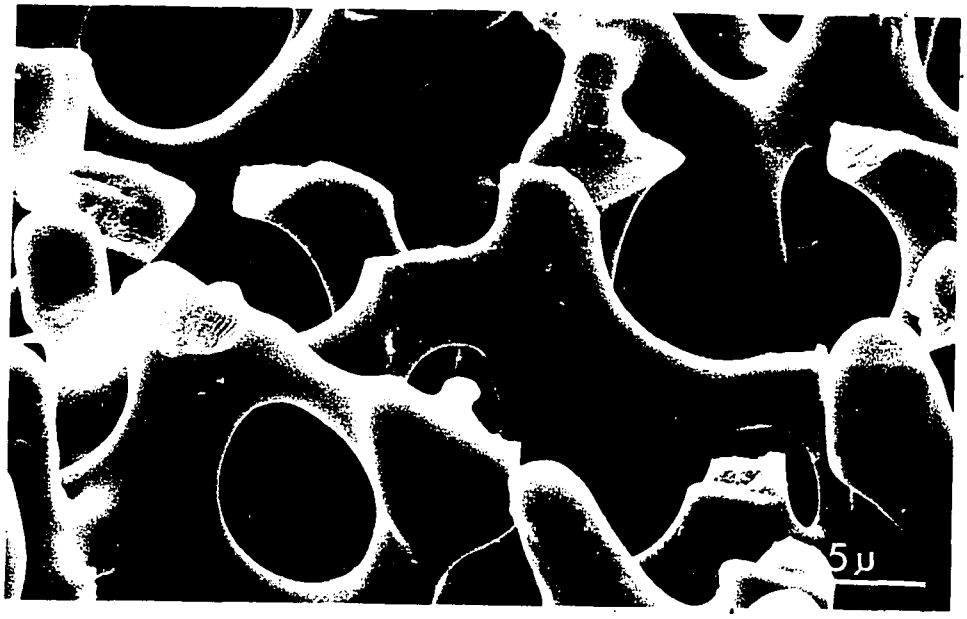


Fig. 1.6. Scanning electron micrograph of edge of normal plate. Compare size of trabeculae to those of Fig. 1.5.

Fig. 1.4. Photomacrograph of similar region as in Fig.1.3. Original hole is delimited by white area. Besides new platelets parts of original plates have grown in to partially fill the hole(dotted line).Regeneration time 180 days. S=spine tubercles; P=platelets.

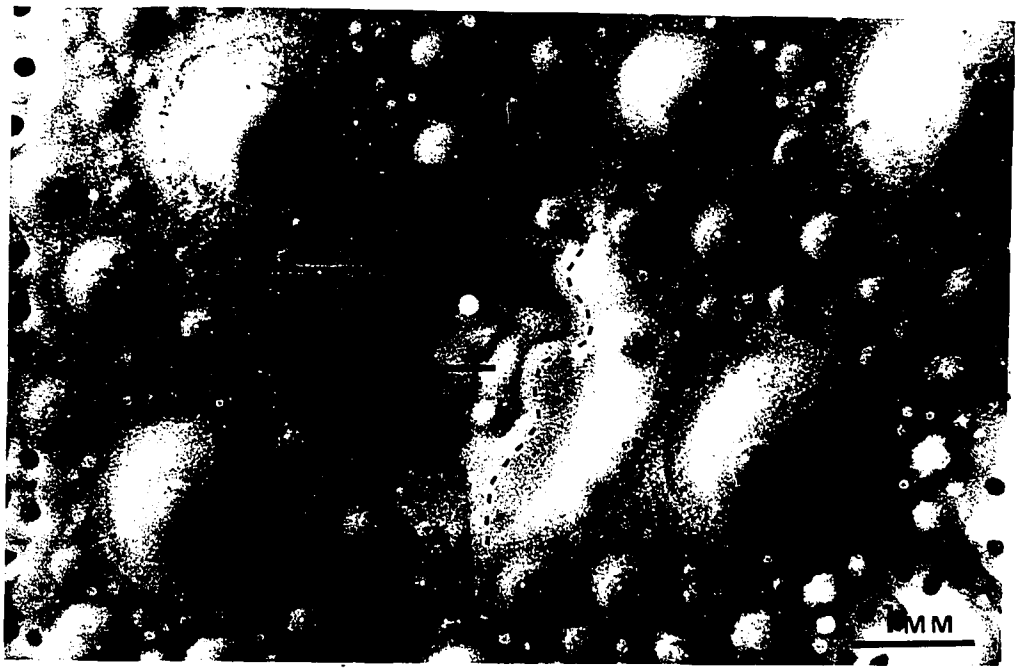


Fig. 1.4. Photomacrograph of similar region as in Fig.1.3. Original hole is delimited by white area. Besides new platelets parts of original plates have grown in to partially fill the hole(dotted line).Regeneration time 180 days. S=spine tubercles; P=platelets.

Fig. 1.2. Photomacrograph of normal plates of interambulacral region of St. droebachiensis (St. dr.). S=spine tubercle

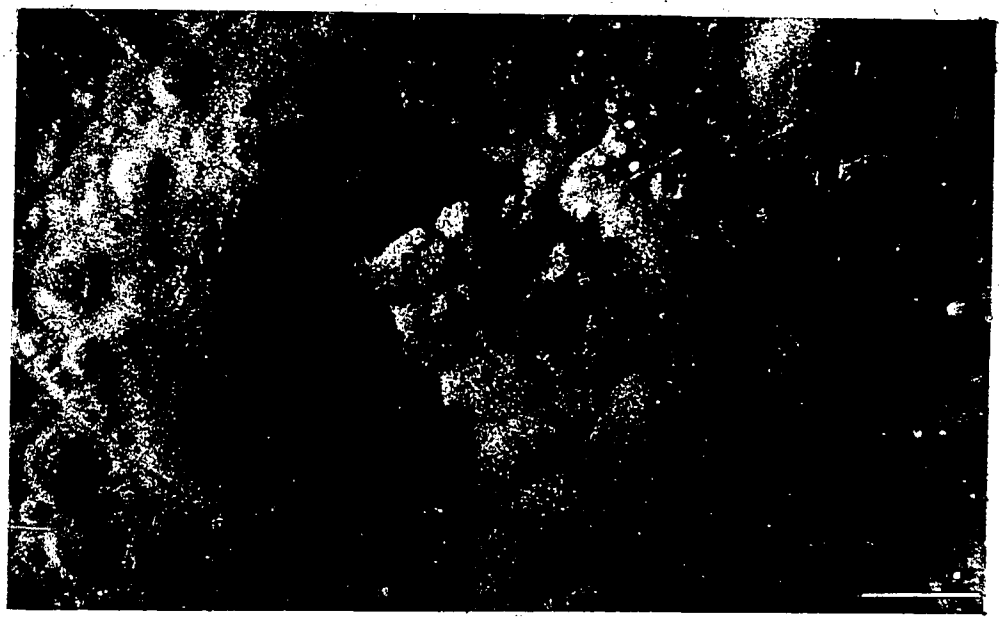


Fig. 1.3. Photomacrograph of similar region as in Fig.1.2 containing regenerated platelets(P). Regeneration time 60 days.

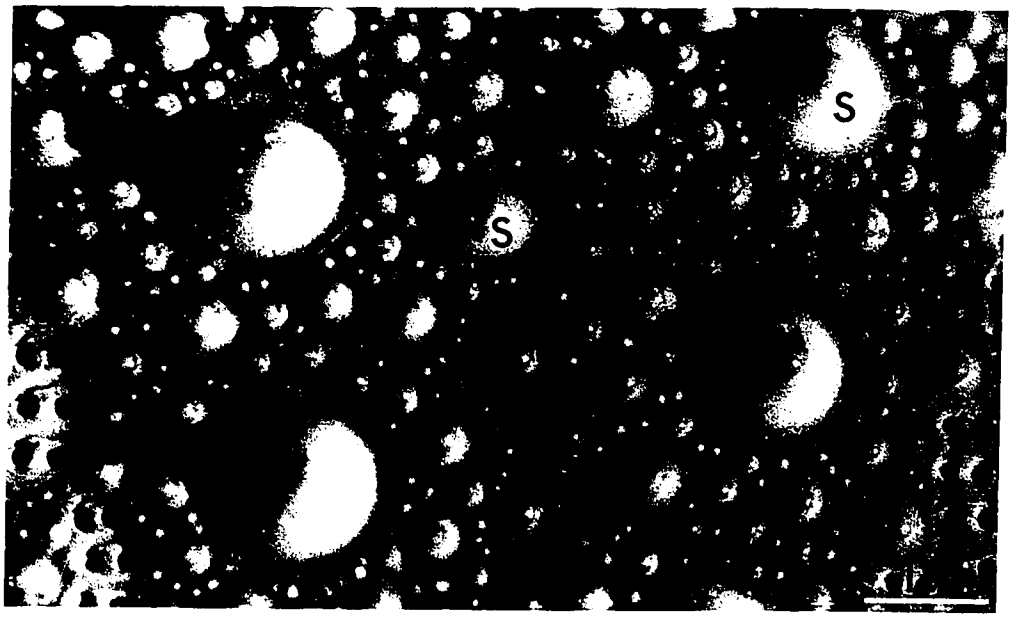


Fig. 1.2. Photomacrograph of normal plates of interambulacral region of St. droebachiensis (St. dr.). S=spine tubercle

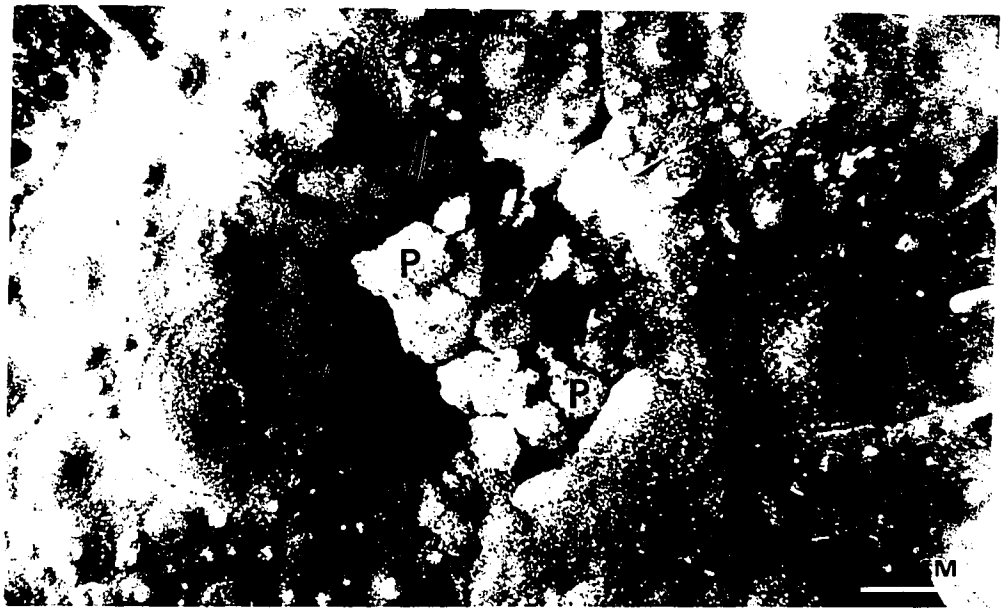


Fig. 1.3. Photomacrograph of similar region as in Fig.1.2 containing regenerated platelets(P). Regeneration time 60 days.

1.3 Results

1.3.1 Scanning Electron Microscopy of Normal and Regenerated Plates:

Figure 1.2 shows the configuration of the inter-ambulacral plates of a normal animal. Regions which have regenerated fill the holes drilled in the tests (Fig. 1.3, 1.4) and were seen to consist of more numerous, smaller, often hexagonal platelets. Shape, size and number of platelets was seen to vary from animal to animal. These variations were not investigated.

The appearance of regenerated plates in the scanning electron microscope (Fig. 1.5) is similar to normal plates (Fig. 1.6) except that the trabeculae are thinner in regenerating plates. These trabeculae thickened as the plates became older so that those which had been regenerating for six months had trabeculae nearly as thick as normal plates (Fig. 1.9).

Figure 1.5 shows a plate from a forty-day old regenerated blastema. The spicules have grown together to form the characteristic sponge-like structure. At several places new arms of the spicules have begun to bud. It appears as if growth is taking place by continual bifurcation of growing spicules. The trabeculae or cross branches are much thinner than in the normal plate (compare to Fig. 1.6). This also shows that the spaces, which are filled in the live animal with organic material, are larger in newly formed plates. The average diameter of the holes is approximately 15 μ.

Fully regenerated plates (Figs. 1.7 - 1.12) are closely associated with surrounding plates and often the two are

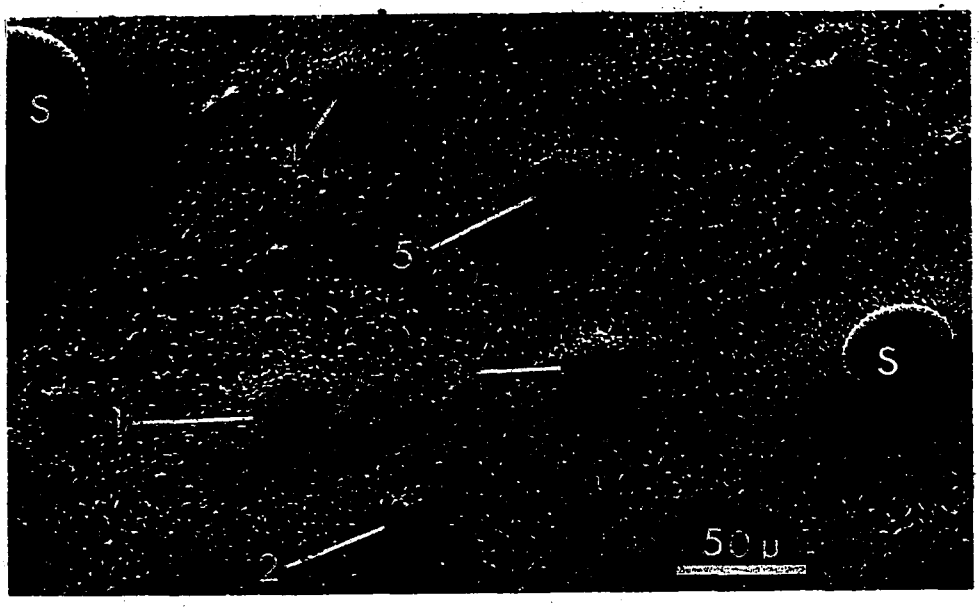


Fig. 1.11 Low power scanning electron micrograph of 180 day regenerated test. Numbers indicate stages in regrowth of tubercles. Movement of spines has smoothed surfaces of older tubercles (S).

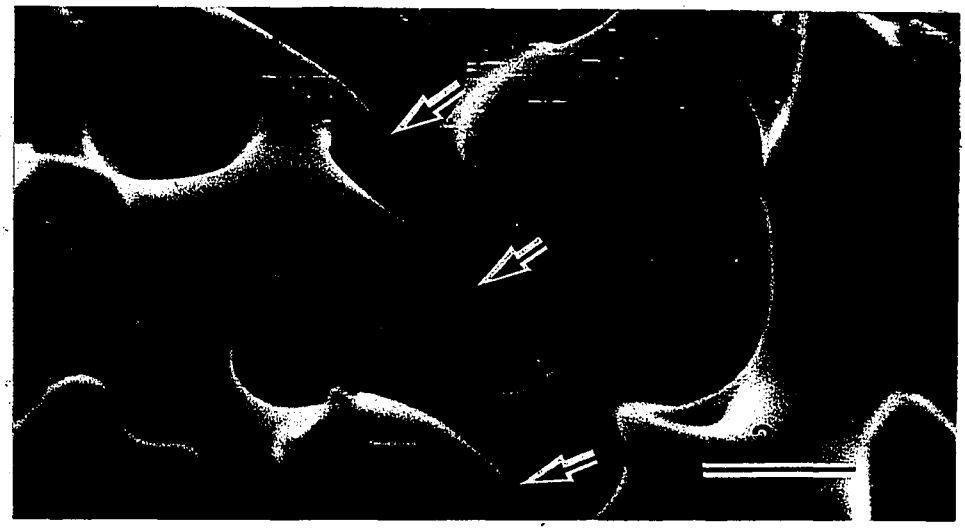
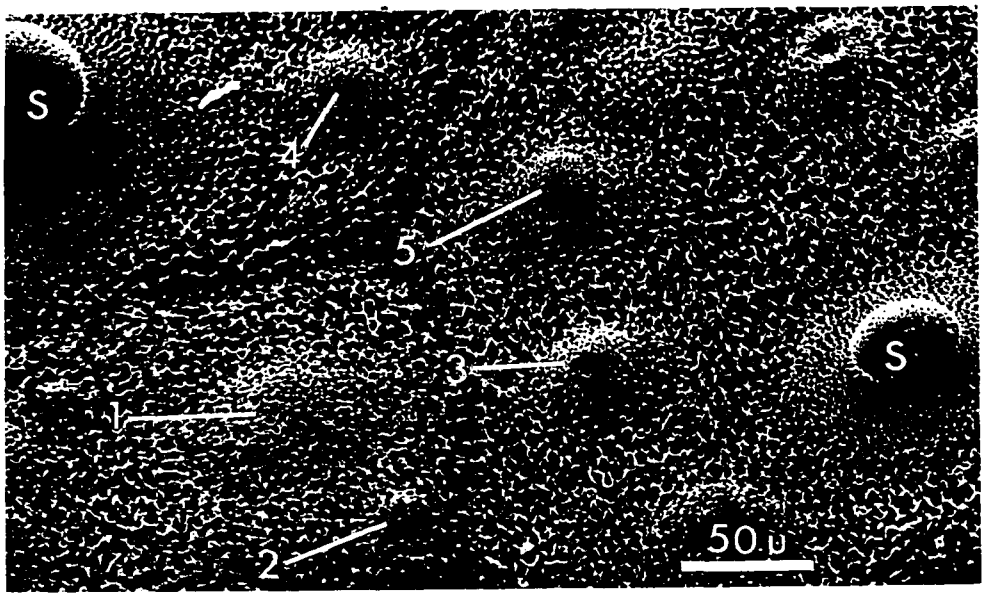


Fig. 1.12 High power scanning electron micrograph of hook-like appendages which normally anchor platelets to one another (arrows).



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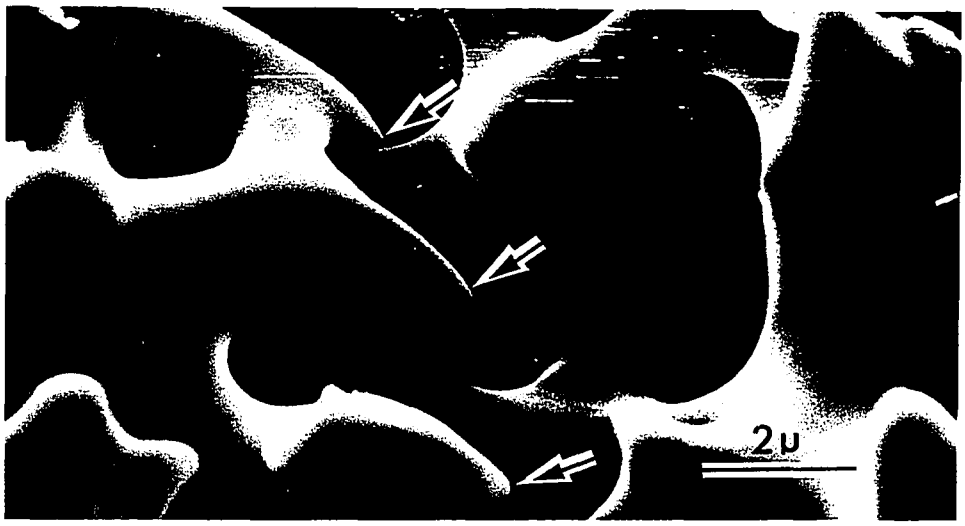


Fig. 1.12 High power scanning electron micrograph of hook-like appendages which normally anchor platelets to one another (arrows).

Fig. 1.9 Detail of boundary as seen in Fig. 1.8. White flecks are organic material not removed by bleaching. Note connections between platelets (arrows) and newly forming trabeculae (T).

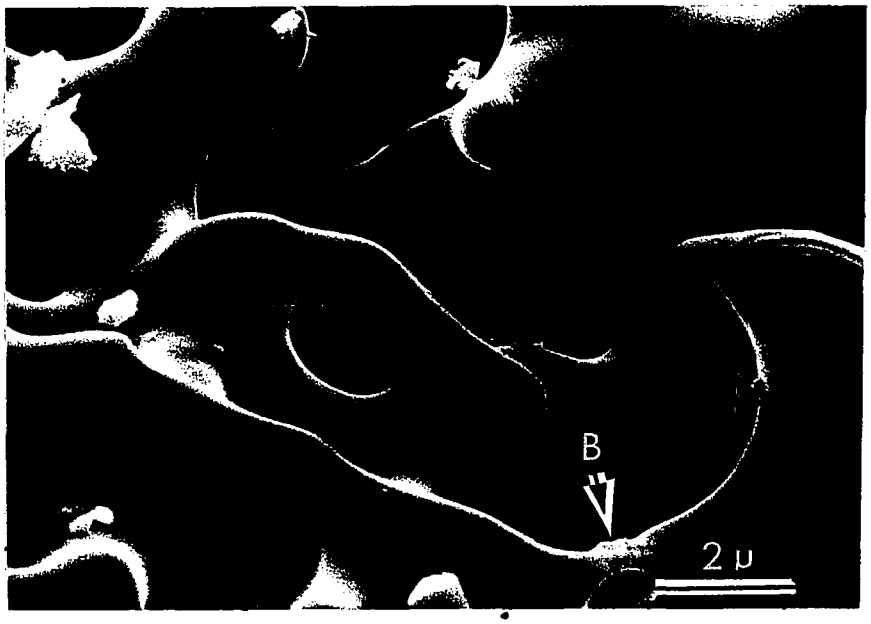
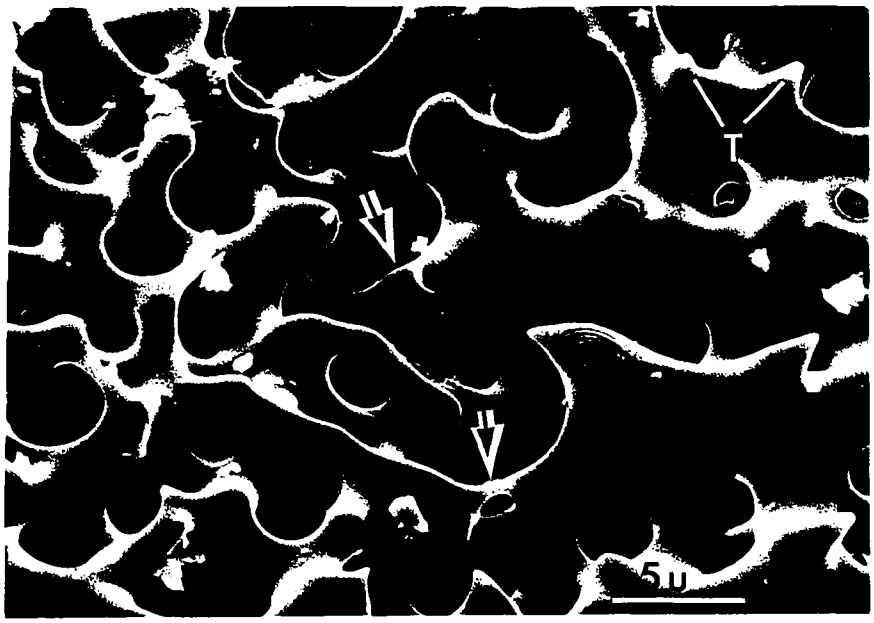


Fig. 1.10 Detail of connections as seen in Fig. 1.9 between "old" and "new" platelets. Note rough appearance of connection at 'B'.



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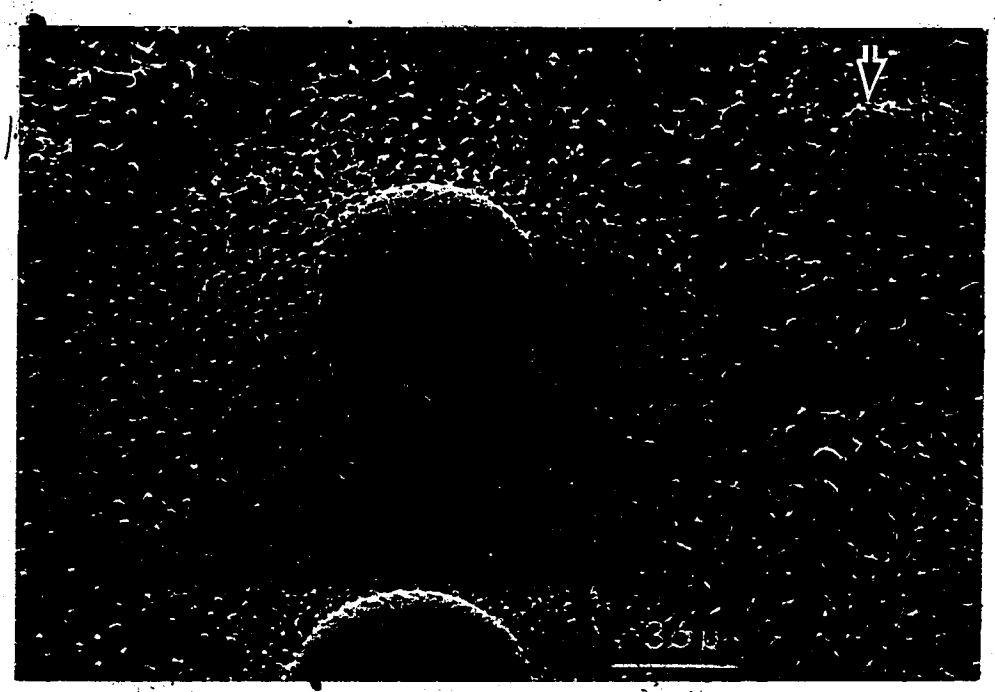

Fig. 1.7 Scanning electron micrograph of 180 day regenerated test. Note jagged boundary separting central platelet from older surrounding ones. Arrow indicates newly forming tubercle.

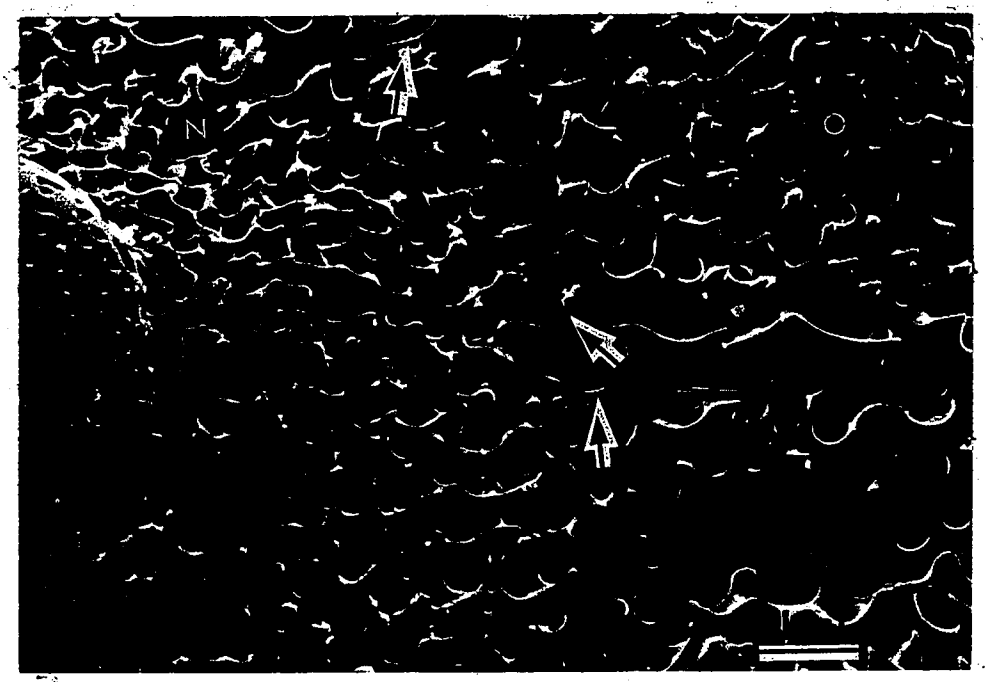

Fig. 1.8 Detail of boundary between older (O) and newer (N) platelet seen in Fig. 1.7. Note thicker trabeculae in older platelet and calcite connections across boundary (arrows).

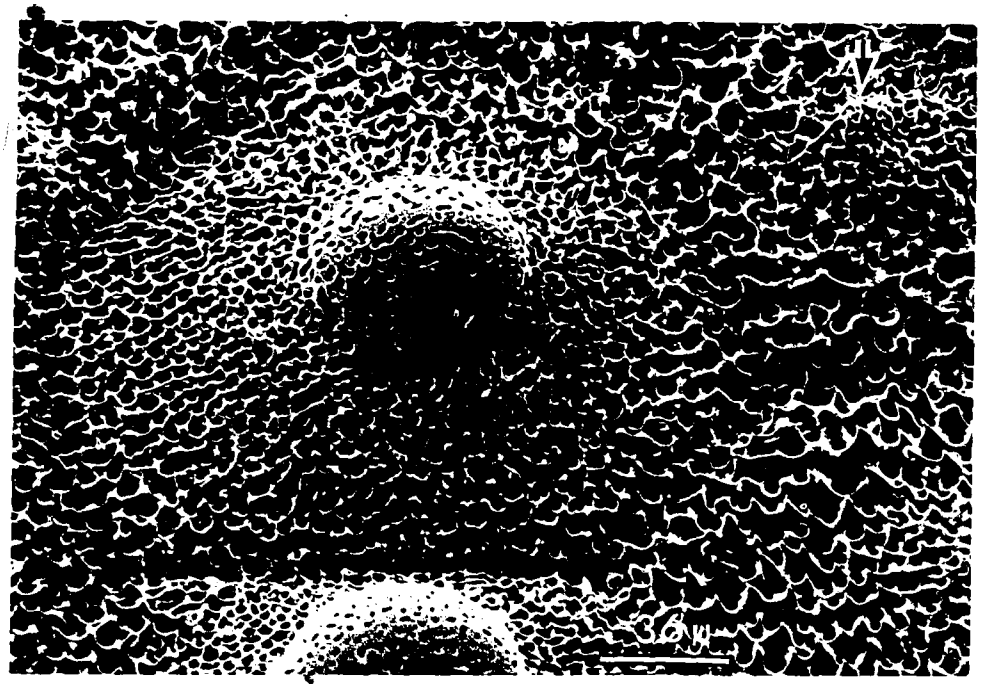

Fig. 1.7 Scanning electron micrograph of 180 day regenerated test. Note jagged boundary separting central platelet from older surrounding ones. Arrow indicates newly forming tubercle.

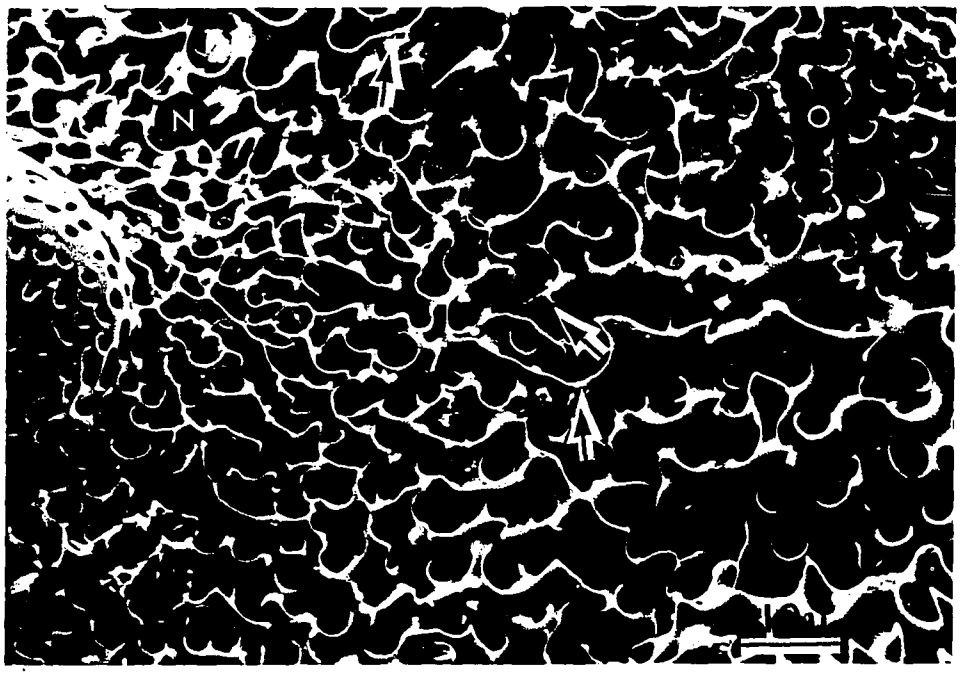

Fig. 1.8 Detail of boundary between older (O) and newer (N) platelet seen in Fig. 1.7. Note thicker trabeculae in older platelet and calcite connections across boundary (arrows).

attached to each other at several points (Figs. 1.9, 1.10). Normally however, there is a distinct boundary visible between plates (Fig. 1.8). Curved pointed hooks interlock between them but are not continuous across the boundary with calcite from the other plate (Fig. 1.12). At points that do not connect, the boundary between plates is rough in appearance. Newly forming tubercles first appear as raised portions of the trabeculae (see Figs. 1.7 and 1.11). These are later smoothed off by the rubbing action of regenerated spines (see Fig. 1.7).

Older plates (from six-month regenerated blastema) have thicker trabeculae than new plates, but thinner than normal plates (compare Fig. 1.5 to 1.9). Holes in the plates of six-month regenerated blastema average about 7 μ in diameter, whereas holes in normal plates average about 5 μ in diameter.

Since the normal and regenerated plates resemble one another so closely, it is here assumed that conclusions drawn from observations on regenerating tests are relevant to normal growth processes.

1.3.2 Macroscopic Description and Stages of Regeneration:

In the description of regeneration to follow, the process has been divided into three arbitrary stages. These stages were not described solely in terms of their temporal sequence for the following two reasons: (1) division of regeneration processes on the basis of temporal sequences alone is often misleading due to wide variations between individuals (Needham 1952), and (2) in the present investigation

several factors were seen to influence the temporal sequence of plate replacement; animal size appears to be a factor since larger animals take a shorter period of time to fill in holes in the test. As might be expected, hole size affects the length of time for regeneration. In general, wider wounds take more time to repair. Long, thin wounds take approximately the same time as round wounds of the same width. Often gonadal material becomes trapped within the regenerating wound. This was seen to affect regeneration times. These factors were not investigated in the present experiments. Results presented here are based on holes of a uniform size (3.5 mm) which were drilled in the same area of each animal.

What follows is a generalized description of plate repair.

1.3.2.1 Stage I

As soon as a hole was drilled in the test, all intact spines in the immediate area of the wound were seen to bend inwards, interlocking with each other over the hole. Debris was washed from the periphery of the hole and subsequent events were observed under a dissecting microscope. Coelomic fluid immediately began to ooze from the wound but before more than a few millilitres could escape, it was drawn back into the coelomic cavity by suction produced by the coelomic membrane. As the coelomic membrane is drawn upwards, the perivisceral fluid again oozes out into the seawater and the cycle repeats itself. The perivisceral fluid of echinoids contains at least four types of coelomocytes (see Johnson 1969a).

One of these, the bladder amoebocytes, are known to fuse into gel-like clots when exposed to seawater. As the perivisceral fluid is sucked back and forth past the edges of the wound, some of these bladder amoebocytes were seen to clot, sticking to the sides of the hole and to each other; others were drawn back into the coelomic cavity. As the cycle continues, clotted bladder amoebocytes build up along the edges of the wound and plug up the hole. The compression-suction cycle, then, prevents the loss of large amounts of perivisceral fluid while at the same time exposes more cells to seawater. Seawater is known to increase the clotting time of coelomic fluid (Boolootian and Giese 1958). This mass of clotted cells will hence be referred to as the blastema. Trapped inside the blastema is a second type of cell, the red spherule cell (Type I) or eleocyte (see Johnson 1969a). These are large red cells which impart to the blastema a deep crimson color. Holes of 3.5 mm were completely filled in twenty-four to thirty-six hours. On the outside of the test the blastema is expanded laterally over the surrounding epidermis, and held by bent over spines.

Internally the blastema is also expanded laterally and often pressed against the plates by the gonad, which is attached to the peritoneum. The peritoneum is normally removed from the immediate area of the wound by drilling of the hole. Often the wound will be temporarily stopped up and healing retarded by the gonad or a loop of intestine.

If left undisturbed, the blastema eventually becomes firmly anchored in place. This anchoring occurs by fusion of

the clotted amoebocytes and dermal sclerocytes into a permanent syncytial mass (see Section 2.3.1.3) which may then only be removed by cutting. Frequently animals were observed to cover the wounded region with bits of shell or plant material from the aquarium. In the present investigations, anchoring occurred in about ten days. The blastema also decreases in thickness and normally by the end of Stage I, is nearly level with the surrounding epidermis, so that the wound is filled by a neat plug of reddish-brown tissue.

1.3.2.2 Stage II

Stage II is that period during which there is marked reduction in the size and thickness of the blastema, as well as a toughening of the regenerating tissue. Beginning at the outer periphery of the blastema, a rim of thinned tissue becomes wider and wider until the whole wound is thinned down. This thinned area is visible as a crimson band about 1mm in width around the perimeter of the blastema, fifteen to eighteen days after drilling of the hole.

At about the same time that wound thinning became discernible, a thin white membrane was seen around the outside edge of this thinned area. This membrane was seen to be continuous with the surrounding epidermis when lifted off the underlying tissue mass. The membrane is almost transparent except for the large number of red spherule cells which have penetrated it. This is the regenerating epidermis. It continues to grow inwards, preceded slightly by the front of thinning blastemal tissue. The wound is not completely covered

by new epidermis before the first calcite crystals appear. If the epidermis was lifted off the blastema and removed, regeneration was seen to be retarded. A new epidermis was partially regrown before regeneration proceeded further.

Regions of the blastema covered by regenerating epidermis were very thin, usually 1-1.5 mm. Beneath the epidermis, thin white fibers arranged in flattened bundles were seen to criss-cross the whole of the regenerating area. Some of these could be followed to their origin on the tubercles of spines and pedicellariae which surround the blastema and which were often damaged or removed during drilling of the test. It seems likely then that some of the fibers are the connective tissue elements of the appendages. The fibers appear to keep the wound firmly in place by anchoring it to surrounding appendages and dermis.

Regeneration of the peritoneal membrane was observed by removing a piece of test containing the wound. This was done at various intervals during regeneration. By Stage II there is no differentiation of the peritoneal membrane under the blastema. As healing proceeds, a thin transparent sheath becomes differentiated at the extreme edge of the wound. It appears a week or more later than the regenerating epidermis and is discernible only when the first calcite crystals become visible. The rest of the peritoneum (that in the center of the blastema) was continuous with and seemed to be a part of the fibrous elements described above. Differentiation of the peritoneum proceeds from the outer edge towards the center of the wound.

Late in Stage II the blastema appears as a depression in the test. Those areas covered by epidermis are deep crimson and are more depressed than areas more central and not covered by epidermis. These central regions are usually dark brownish-red. Calcite granules were seen to appear around the periphery of the wound about eighteen to twenty-five days after drilling of the hole. Thus, within any one blastema just before calcite first appears, there is a central region in Stage I, a concentric depressed area surrounding this in Stage II, and a peripheral band around the blastema which is thinned down and will contain the first calcite granules.

1.3.2.3 Stage III

During this final stage the actual porous plates characteristic of the echinoid endoskeleton are laid down (see Fig. 1.3. and 1.7).

The first indication of incipient plate formation is the appearance of small crystalline granules around the periphery of the blastema. Granules were often seen at the same time in more central regions, provided these areas were covered by an epidermis. Holes, 3.5 mm in diameter, normally required about twenty-five days before the first tiny crystallites were visible.

The epidermis was carefully removed and the underlying dermis observed under a dissecting microscope at 80x. In regions that are forming granules the blastema is very thin, about 1 mm. The granules can clearly be seen spread randomly throughout the dermal tissue.

The fibers mentioned in Stage II are easily seen at this time. As regeneration proceeds, this initial stage of granule deposition develops into the next phase, that of plate formation. Regions of the blastema which are forming new plates appeared at the beginning to consist of a white opaque mass. On closer observation, this region was seen to consist of elongated granules (spicules) closely associated with, and often on top of long flat bundles of white fibers. This opalescent mass of crystalline-with-fibroid material resembled the "calcification front" often referred to in vertebrate studies (Hancox and Boothroyd 1964). Not all areas of calcification showed fibers present. Centers of spicule formation continue to grow and increase their density until they resolved into delicate porous plates rarely more than 1.5 mm in diameter (Fig. 1.3). The increase in plate density appeared to be due to an increase in the amount of crystalline spicular material. These plates are very delicate at first (Fig. 1.5). The spicules which make them up are longer and branch less frequently than those of the fully matured plates (compare Figs. 1.5 and 1.6). As regeneration proceeds, other plates become visible within the blastema. Usually four to twelve plates were visible inside the blastemas of 3.5 mm diameter. As plate formation spreads to central areas of the blastema, it becomes thinner and more depressed. Plates do not appear to be laid down in any distinct pattern and usually four or five forming plates were visible at any one time. Plates were often formed in layers piled two deep. The crystallography of a few of these plates was examined and preliminary

data from X-ray diffraction seem to indicate that each behaves as a single crystal, although they are closely oriented (sub-parallel).

The longest period of time any experimental series was left to regenerate in the present study was six months. At this time the regenerated area could be seen as a depression in the test. On the inside surface were 8-10 plates, .5-1 mm in diameter. On the outside surface were 5-8 somewhat larger plates. This suggests that plates were added in layers, starting from the middle with subsequent plates having been added on to the epidermal side of the blastema.

1.3.3 The Presence of Carbonic Anhydrase in Hard and Soft Tissue

The results of end point determinations for tissues assayed for carbonic anhydrase are shown in Table 1.1. The highest level of carbonic anhydrase measured by the present methods was in dog's blood. Values for echinoid body organs are all very close to each other. Significant differences between treated and untreated extracts were found in perivisceral fluid, body wall, gonad and regenerating test. No significant difference was found for peristomium, and possibly regenerating spines (see Discussion).

Table 1.2 is a comparison of the ratio of heated extracts to unheated extracts, to the ratio of distilled water to unheated extracts. Where these ratios are not significantly different, they are expressed as a single ratio. For regenerating spines and mollusc mantle the ratios differ slightly and both are given.

Table 1.1

Endpoint Determinations for Presence of Carbonic Anhydrase

Sample	Time (sec) Mean	Number Determ.	S.D.	Confid. d=.90±	t test	° of Freedom	Signific. at .95
No Extract	110.28	42	3.55	.931	-	-	
Heated Ext. Dog's Blood	109.75	4	.83	1.13	57.46	10	Yes .001
Extract	30.13	8	1.69	1.21			
Heated Ext. Mollusc Mantle	135.67	3	18.11	37.56	8.80	6	Yes .001
Extract	71.40	5	6.09	6.49			
Heated Ext. Perivisceral Fl.	113.60	5	2.65	2.82	4.20	8	Yes .001
Extract	101.60	5	4.84	5.15			
Heated Ext. Body Wall	111.00	5	.89	.947	3.05	10	Yes .01
Extract	102.86	7	5.17	4.09			
Heated Ext. Gonad	114.00	5	5.33	5.68	2.98	12	Yes .02
Extract	100.11	9	11.07	7.28			
Heated Ext. Regen. Test	111.00	2	2.00	12.72	2.86	5	Yes .05
Extract	104.20	5	3.12	3.32			
Heated Ext. Regen. Spines	105.33	3	7.54	15.57	.89	6	No .10
Extract	101.40	5	5.99	6.38			
Heated Ext. Peristomium	113.33	3	3.40	7.02	1.33	7	No .10
Extract	110.33	6	3.20	2.89			

Table 1.2

Comparison of Heated Extract to No Extract

Sample Tested	Heated Extract	Distilled Water Extract	t value of Heated vs Distilled	Ratio(s) Used
Dog's Blood	3.64	3.66	.273	3.6
Mollusc Mantle	1.90	1.55	9.742*	1.9 1.6
Perivisceral Fluid	1.12	1.10	1.692	1.1
Body Wall	1.08	1.07	.373	1.1
Gonad	1.14	1.10	1.811	1.1
Regenerating Test	1.06	1.06	.365	1.1
Regen. Spines	1.04	1.09	2.35	1.0 1.1
Peristomium	1.03	1.00	1.538	1.0

* $p > .02$

In order to determine whether or not carbonic anhydrase is concentrated in calcifying tissues, a comparison between hard and soft tissues was made. Table 1.3 shows the results of this comparison.

Table 1.3

Comparison of Hard and Soft Tissue

Samples Tested	Number Tested	Mean	S.D.	t test
Hard: Body Wall, Regenerating Wound Spines	17	102.82	5.06	.011*
Soft: Gonad, Peristomium, Perivisceral fluid	19	103.16	9.25	

* Not significant.

Based on the present experimental method then, there appears to be no difference at any level of significance between calcified and non-calcified tissues of Strongylocentrotus droebachiensis with respect to the level of carbonic anhydrase in their tissues.

1.4 Discussion

The observations by scanning electron microscopy, revealing that newly deposited plates had much thinner trabeculae than normal or older plates, confirms the work of Donnay and Pawson (1969) who found that older plates showed higher densities, as measured on a Berman balance, than new plates. They made their measurements on animals which had been growing normally. These observations also provide evidence that regeneration of test parts and normal growth probably occur by similar physiological mechanisms.

As noted, there are certain differences between regenerated and normal plates in that the former are more numerous than the original. Preliminary data (unpublished X-ray diffraction) indicate that each of the newly regenerated plates behaves as if it were a single crystal. This would mean that many separate "seeding centers" of calcite crystals occur throughout the blastema. If crystal growth were being initiated and oriented by the "fibrous elements", then for any one given plate these fibers would have to be oriented in one direction (Travis 1970). Bundles of fibers were observed to criss-cross the blastema in all directions as well as occur in layers. Based on macroscopic observations, test regeneration has been divided into three stages. The first stage consists of plugging of the wound by clotting amoebocytes, the second stage is characterized by reduction in blastemal thickness. This thinner clot is probably the "membrane" reported by Kindred (1924). The "membrane" was seen to

consist of a very thin outer epithelium (epidermis) and an internal dermal tissue layer, and it is within this tissue layer that deposition of calcite takes place. As new plates are formed within the dermis, the third or peritoneal layer becomes differentiated. The presence of fibers within the dermis is significant in view of the importance placed on their appearance in calcifying tissue (Glimcher 1960; Travis 1970). Examination of blastema *in vivo* did not reveal the extent to which the calcite and these fibers were associated. It is probable that these fibrous elements are formed from differentiating cellular elements of the dermis. Differentiation of these cells into fibrous material probably forms the sutural connective tissue which extends between plates to anchor them. These sutural connective fibers have been described in the normal echinoid by Moss and Meehan (1967). It may be that where the dermal leucocytes have not differentiated into sutural fibers they have deposited calcite. This would account for the observed bridges across plate boundaries (Fig.1.9 and 1.10).

Examination of newly regenerated plates showed the presence of newly bifurcating spicules. This phenomenon was observed by Gordon (1926) during test formation in newly metamorphosed larvae. Recently, Heatfield (1971) has observed similar growth patterns in regenerating spines of sea urchins. However, unlike regenerating spines, plate regeneration is not confined to extensions of pre-existing calcite material. New plates may form at points in regenerating blastema that are not connected by calcite. If such separate seeding centers

for calcite deposition occur, as the observations would suggest, then it seems plausible that plate size may be controlled by the presence or absence of connective tissue sutures. Another difference between regenerating spines and regenerating test is that regenerating spines appear to deposit new spicules which are the same thickness as old ones (Pilkington 1969; Heatfield 1971), whereas newly regenerated plates have much thinner trabeculae when first deposited.

Carbonic anhydrase may be considered relevant to calcite deposition because it catalyzes the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$ (Meldrum and Roughton 1933). It has been held to be an important factor in calcification of mollusc shell (Wilbur 1964). In living systems carbonate $CO_3^=$ could be obtained from the bicarbonate ion of this reaction. In combination with Ca^{++} present in seawater, $CaCO_3$ could then be precipitated *in vivo*.

Quantitative measurements of carbonic anhydrase levels in the hard and soft tissues of *Strongylocentrotus droebachiensis* revealed that although carbonic anhydrase appears to be present in most tissues, it does not seem to be more concentrated in hard calcified tissue or in tissues actively secreting calcite than in soft tissues. This being the case then, one would expect that inhibitors of carbonic anhydrase would affect the physiology of both hard and soft tissues alike. It is instructive to compare the results obtained in the present investigation with values of carbonic anhydrase for mollusc tissue. Freeman and Wilbur (1948) found carbonic anhydrase to be present

in the mantle and other tissues of twelve species of pelecypods and eight species of gastropods. The extracts they examined were diluted by 500 times (as compared to 300 in the present experiment). They found heated to unheated extract ratios of from .6-5.1, with an average value of 3.0. In the present experiment, the mantle of Lampsilis radiata (Gmelin) gave a ratio of 1.9 for heated to unheated extracts. From this it may be stated that if carbonic anhydrase were present in large amounts in the tissues of the sea urchin investigated, it would have in all likelihood been detected, since the separate values for mollusc tissue are close in both experiments. Results obtained with dog's blood are comparable to Maren's original values (Maren 1960). The results with regenerating spines indicate very low levels of carbonic anhydrase in these tissues. Heatfield (1970) recorded fifty to sixty-one per cent inhibition of Ca^{45} uptake over a concentration range of 10^{-3} to 10^{-6} M of the carbonic anhydrase inhibitor Diamox. Samples of regenerating spines were the smallest samples assayed, since care was taken to include only the regenerating tips. In view of this and the fact that distilled water and boiled extracts differed substantially (see Table 1.2), levels of the enzyme may be similar in regenerating spines as in other echinoid tissues surveyed. Although the results obtained do not preclude the involvement of carbonic anhydrase in calcification of sea urchin tests, it is postulated that carbonic anhydrase is not the major local factor, since it is not present in significantly greater amounts in calcified

tissues than in soft tissues. The only other determinations of carbonic anhydrase activity in sea urchins were reported by van Goor (1937) and by Brinkman (1933) and Sabotka and Kann (1941). These investigators recorded slight carbonic anhydrase activity in the gonads of Arbacia pustulosa and another unspecified sea urchin respectively. All found no activity in the perivisceral fluid, contrary to results obtained in the present investigation. They used different assay methods than those used in the present investigation.

Davis (1961) mentions that inhibition of carbonic anhydrase due to the presence of carbonate ion occurs with indicator methods of the enzyme assay. Maren (1960), however, encountered no carbonate ion inhibition with the same system as used in the present investigation. However, enzyme inactivation at high pH and some diffusion limitations (although minimal due to vigorous bubbling) are known to occur in all indicator methods (Davis 1961). For this reason, no attempt was made to determine absolute levels of enzyme concentration. Relative levels of enzyme concentration in various tissues were determined in order that the possibility of high concentrations of carbonic anhydrase in those tissues which are calcifying or have calcified would be obvious. Since no difference between these tissues and soft tissues was detected, it may be concluded that carbonic anhydrase probably does not play a major role in deposition of calcite in the tissues of Strongylocentrotus droebachiensis.

In summary then, echinoids show a capacity for regenerating pieces of test which have been experimentally removed. The regeneration process has been divided into three stages. Cells of the dermal layer are responsible for calcite deposition and perhaps also for control of plate size by their differentiation (or absence of differentiation) into sutural connective tissues. When examined in the scanning electron microscope, regenerated plates resemble normal plates except for the thickness of their trabeculae. The thickness of these trabeculae increases as the plates age. Carbonic anhydrase is probably not the major local factor in precipitation of echinoid calcite.

2. HISTOLOGICAL OBSERVATIONS: LIGHT AND ELECTRON MICROSCOPY

2.1 Introduction

Studies dealing with the formation of the echinoderm skeleton have been mainly concerned with skeletogenesis in larval forms. Selenka (1879), Cuénot (1891), and Bevelander and Nakahara (1960) observed a granule which first formed inside the primary mesenchyme cells of the blastula. The rest of the skeleton they felt was formed extracellularly by growth of this granule into a spicule and from there into the complicated system of calcite rods which make up the larval echinoderm skeleton. Semon (1883), Théel (1892, 1894), Chun (1892), MacBride (1903), Woodland (1906), Prenant (1926a & b), Bouxin (1926), von Ubisch (1937), Okazaki (1960), Gustafson and Wolpert (1961a & b), Gibbons et al. (1969) observed the same intracellular granule, but present data to show that the granule remains intracellular and that growth of the larval skeleton takes place inside the cytoplasm of a cable formed from elongated primary mesenchymal cells. The deposition of calcite, according to these investigators, is intracellular following the boundary of the cable. Okazaki (1960) postulated that the cable membrane functioned as the organic matrix of the spicule, but Gibbons et al.(1969), using electron microscopy, found that the envelope was discontinuous with the spicule and that skeletal material was probably deposited intracellularly in a non-fibrous organic matrix.

Studies on adult echinoderms have mostly been concerned with skeletogenesis of echinoids. There is considerable debate in the literature as to whether the collagen which is

present in the test of sea urchins acts as a calcifiable matrix or functions solely as connective tissue. Travis et al (1967) describe collagen fibrils, each with a 640A axial repeat unit, which they claim to be the calcite matrix. Travis (1970) describes this same collagen with tiny embedded calcite crystallites which the author claims to be the seed crystals of the calcite spicules of the test. Kobayashi and Taki (1969) found that tetracycline was absorbed onto both spine "muscles" and sutural connective tissue collagen, during test growth. Crystallographic data (see Raup 1966 for a review; Donnay and Pawson, 1969), indicates that the calcite is monocrystalline or perhaps recrystallized polycrystalline aggregates (Towe 1967), a fact difficult to reconcile with collagen-seeded calcification theories (Glimcher 1960). Pilkington (1969) found that the calcite of the spines of Echinus esculentus is intracellular and is associated to a closely bound, non-collagenous nitrogen containing organic fraction. Klein and Currey (1970) present biochemical data which indicates the absence of a collagenous matrix in the spines of Strongylocentrotus droebachiensis and Strongylocentrotus lividus.

Echinoderms are able to regenerate and repair body organs, appendages and, in the case of the echinoids, test wounds (Prouho 1887; Kindred 1924). For this reason, test regeneration was found to be a good calcifying system for studying calcite deposition in vivo in these animals (see Section 1.0). There has been one published account of the histology of test regeneration. Kindred (1924) examined the membrane which formed to cover the holes in regenerating tests. He distinguished

three regions: (1) a syncytium of closely packed "leucocyte" cells which had amoebocytes with spherules wandering through them; (2) a lacunar region adjacent to this area which contained fewer wandering amoebocytes with spherules; (3) a highly lacunar region which contained the regenerated calcite and no amoebocytes with spherules. He concluded that the phagocytic leucocytes which originated in the perivisceral fluid were responsible for deposition of new calcite.

Many other investigators have focused on these perivisceral fluid coelomic cells in attempts to determine their origin and functions. The following investigations have been concerned with the coelomic elements of the perivisceral fluid of echinoids: Hoffman (1871), Geddes (1880), Théel (1891, 1896, 1921), Cuénot (1891, 1906), Kindred (1921, 1924, 1926), Ohuye (1934, 1936), Donnellon (1938), Bookhout and Greenberg (1940), Liebman (1946, 1950), Schinke (1950), Boolootian and Giese (1958), Boolootian (1959), Abraham (1963, 1964), Holland et al.(1965), Burton (1966), Pequignat (1966) and Johnson (1969a & b).

There are four types of cells in the coelomic fluid of echinoids, listed here in order of their abundance: (1) Bladder amoebodytes (also known as leucocytes, phagocytes, filiform amoebocytes, fusiform corpuscles, hyaline hemocytes). These are 20-30 μ in diameter with large petaloid extensions and very granular cytoplasms. They are involved in clot formation (Geddes 1880) and have also been reported to be scleroblastic and phagocytic (see Johnson 1969a for review).

(2) Vibratile cells, spherical 7-14 u in diameter, containing a granular cytoplasm and having a long flagellum attached. They have no known function, but Cuénot (1891) believed they kept the perivisceral fluid in motion, and Johnson (1969a) indicated that they may serve a defensive function by forming a gel to limit the spread of escaping coelomic fluid, or to entrap invading organisms.

(3) Red spherule cells (also known as eleocytes, trephocytes, colored morula cells, Type I spherule cells). These are usually described as basophilic cells 10-15 u in diameter containing 1-1.5 u red granules known to contain a naphthoquinone pigment called echinochrome. They have been assigned a respiratory function by McMunn (1885), nutritive function by Awerinzew (1911), Cuénot (1891), Pequignat (1966), and were claimed to be involved in mucopolysaccharide synthesis by Rollefson (1967). Vevers (1960) and Johnson and Chapman (1970) indicated they may perform bacteriocidal or algistatic functions.

(4) Colorless spherule cells (Type II spherule cells, colorless morula cells, white amoebocytes). These are 10-15 u spherical acidophilic cells containing yellow or yellow-green granules. They have short lobular pseudopodia, and have been assigned fat storage functions by Cuénot (1891) and nutritive functions by Kindred (1924). Nothing is known about the relationship of these cells to calcite deposition, although Schinke (1950) presented evidence to indicate that colorless spherule cells were derived from the connective tissue elements surrounding the calcite and that bladder amoebocytes were derived from the

colorless spherule cells. Vibratile cells appear to derive from the peritoneal lining of the coelomic cavity (Holland 1965).

The following investigations on test repair in the echinoid Strongylocentrotus droebachiensis were undertaken for four reasons:

(1) to determine if calcite replacement in adult echinoids occurs intra- or extracellularly

(2) to determine the role of the elements of the perivisceral fluid in the replacement of calcite in regenerating tests

(3) to provide further information on the nature of the organic matrix of echinoderm endoskeletons, and

(4) insofar as it is reasonable to suppose that test regeneration occurs by mechanisms similar to the normal process of calcification, the regenerating test may be used as a system for investigation of the physiological basis for these mechanisms.

2.2 Methods and Materials

2.2.1 Light Microscopy

Animals were collected, kept and examined live as described in Section 1.2.2. For histological examination, pieces of normal test were used, as well as tests which had regenerated for the following number of days: 4, 7, 9, 10, 11, 12, 13, 18, 20, 25, 26, 33, 41, 42, 47, 60, 76, 78, 80 and 82. These animals were processed in the following manner: Pieces of test containing the regenerated area were cut out, using a high speed cutting tool. They were fixed in cold 10% neutral buffered formol seawater for a minimum of twenty-four hours. The samples were then washed in tap water for thirty minutes and placed in a decalcifying solution. The decalcifying solution was made up of 40 gms of Ethylenediaminetetracetic acid (EDTA, versene), 4.4 gms of sodium hydroxide and 12 gms of sodium chloride dissolved in 850 ml of distilled water. This gave a near neutral (pH 7.3) solution of 930 mOsm as a decalcifying reagent. Pieces of tissue were decalcified for 2-4 days until completely soft. They were then washed in running tap water for three hours, rinsed in distilled water and placed in 70% alcohol for twenty-four hours. They were dehydrated quickly through a graded series of alcohols and embedded in paraffin in the usual manner. In some cases only the regenerating area was dissected out and processed: those in the early stages of calcite deposition were embedded without decalcification; the others were decalcified and embedded as described above. Paraffin embedded sections were cross-sectioned at 5-10 μ on an AO

rotary microtome. In addition, tissues which had regenerated for 8, 10, 15, 25 and 40 days were sectioned longitudinally - i.e., parallel to the epidermis. All sections were affixed to albuminized glass slides and stained. General stains used were: Harris' Hematoxylin-eosin, Mallory's Aniline blue-orange G, Van Kossa's stain for calcium. Mallory's Aniline blue-orange G was found to be the best general stain. (Appendix 8.0)

In addition, whole mounts as described in Section 1.2.2 were examined.

2.2.2 Electron Microscopy

A 3% solution of glutaraldehyde in .1 M Sorenson buffer (pH 7.6 osmolarity 450 mOsm) was saturated with calcium chloride and tissues fixed in it for two hours. They were then placed in a decalcifying solution and decalcified in the same manner as light microscope sections. After decalcification the tissues were rinsed in the Sorenson buffer and then postfixed in 1% O_sO_4. They were rinsed in distilled water, dehydrated quickly through a graded series of alcohols, embedded in Spurr medium (Spurr 1969) or Epon 812, and sectioned on a Porter Blum ultramicrotome. Sections were picked up on uncoated or formvar coated grids.

In addition to normal tests, tests which had regenerated for 20-30 days (in which calcite granules had begun to appear - see Section 1.3.2.3) were fixed and embedded in Epon 812 and sectioned without prior decalcification. Thin sectioning of this material was difficult due to the presence of calcite granules which split many of the sections.

Both normal and regenerated tests were stained for two minutes in a .5% solution of Uranyl acetate in 50% alcohol. They were counter-stained with lead citrate prepared in the following manner (Venable and Coggeshull ,1965): .03 gms of lead citrate were added to 10 ml of glass distilled water, .1 ml of 1N NaOH was added, and the mixture shaken until the lead citrate had dissolved. The whole staining solution was then centrifuged for five minutes. Counterstaining was for two minutes. All sections were examined on a Zeiss EM 9A electron microscope. In addition, 1 μ thick Epon sections were stained with .5% Toluidine blue and mounted in permount for examination by light microscopy.

Fig. 2.1A Normal test. Cross section of test of St. dr. showing spine base and dermis. E = epidermis; D = dermis; P = peritoneum. Note that connective tissue to spine is continuous with cells of the dermis (arrows). H+E.

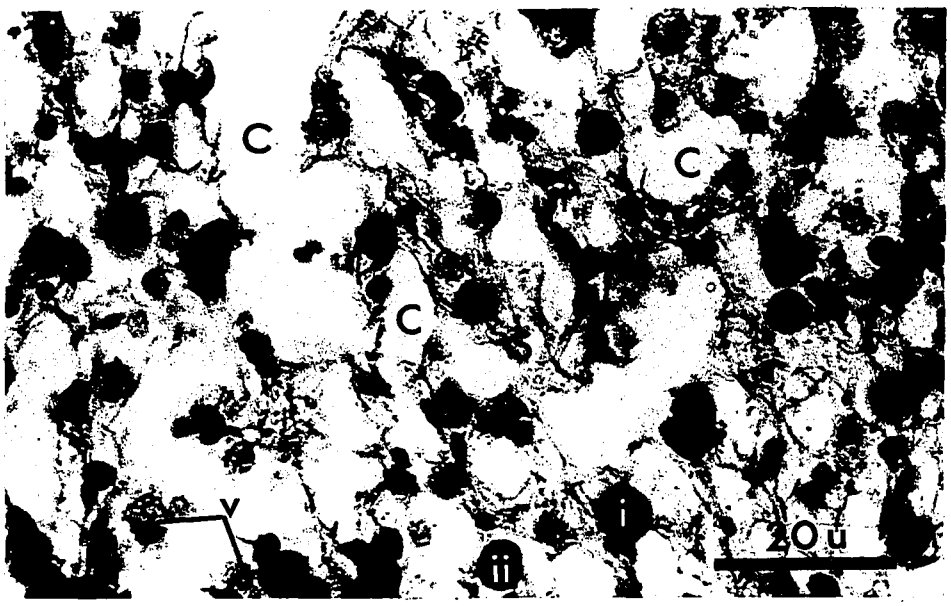

Fig. 2.1B Normal test. Detail of dermis showing syncytial leucocytes (S) and several other cell types - i = Type I; ii = Type II; v = vibratile cell; C = areas formerly occupied by calcite. Compare to Fig. 1.6. H+E.

Fig. 2.1A Normal test. Cross section of test of St. dr. showing spine base and dermis. E = epidermis; D = dermis; P = peritoneum. Note that connective tissue to spine is continuous with cells of the dermis (arrows). H+E.

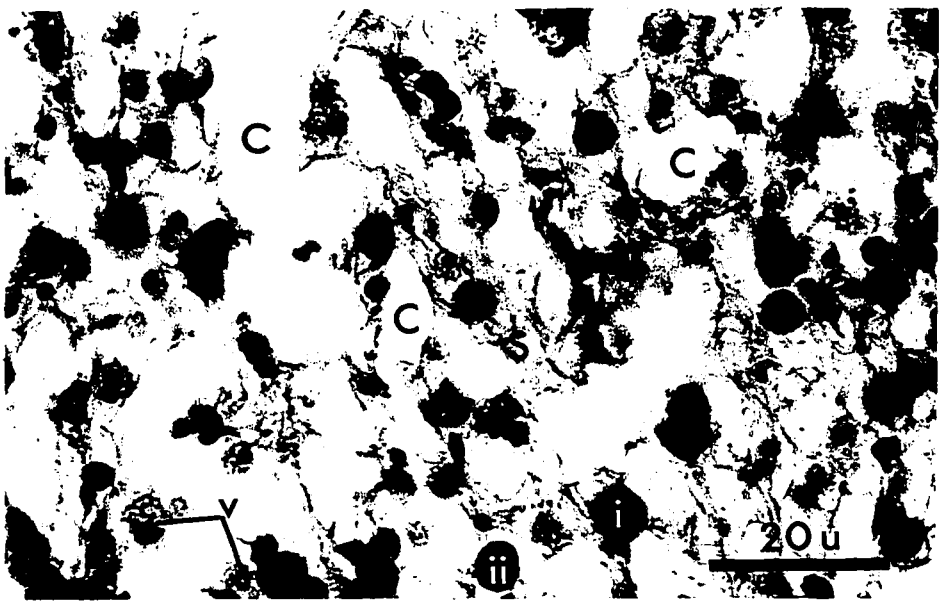

Fig. 2.1B Normal test. Detail of dermis showing syncytial leucocytes (S) and several other cell types - i = Type I; ii = Type II; v = vibratile cell; C = areas formerly occupied by calcite. Compare to Fig. 1.6. H+E.

2.3 Results

2.3.1. Light Microscopy

2.3.1.1 The Normal Test of St. droebachiensis

Several authors have described the test of sea urchins as seen in the light microscope: Valentin (1841), Prouho (1887), Hoffman (1871), Hamman (1887), Kindred (1924) described briefly the test of St. droebachiensis. It will be described here for comparison with the regenerating test.

The external surface of the test is a 40 μ thick pseudostratified epithelium called the epidermis. This epidermis covers all plates and appendages. The cells of the epidermis are irregularly arranged and have eccentrically placed nuclei. Over the top of these cells is a thin (.5 μ) layer that is usually termed as a"cuticle" (Hyman 1955) because of its appearance in the light microscope. The epidermis is bordered internally by a thin layer of connective tissue through which run the subepidermal nerve fibres.(Fig. 2.1A).

Beneath the epidermis occurs a middle tissue layer, the dermis, which is about 2-2.5 mm. thick and contains both the organic and inorganic elements of echinoid endoskeleton. (Compare Fig. 2.1 B to Fig. 1.6)

The organic component of the dermis consists of a syncytium of cells (here called syncytial leucocytes) which have no visible plasma membrane between them. This is the so-called stroma. Nuclei of syncytial leucocytes appear regularly throughout the syncytium. (Fig. 2.1B) In the cross section the cells appear to be

perforated by large membrane-bound vacuoles 10-20 u in diameter. (Fig. 2.1B). These vacuoles are occupied, in the living animal, by the calcite spicules of the endoskeleton. The membrane of the"vacuole" is very closely applied to these calcite spicules. Each echinoid plate with its dermis has a spongy structure, the organic stroma, which may be represented by the soft elements of a sponge, and the calcite corresponding to the holes of the sponge. The spicules of the echinoid endoskeleton are always surrounded by organic stroma. The stroma consists of syncytial cells surrounding the calcite together with several other cell types which appear in spaces between the stroma and the calcite. Whether they occur within the syncytium (intracellularily) is not clear from the light microscope picture. These cell types included all four types of cells seen in the perivisceral fluid of echinoids (see Introduction).

Most abundant in sections examined in the present investigation were spherule cells. Three types were distinguishable on the basis of their staining with Mallory's Aniline blue-orange G. The Type I basophilic cells stained blue with brown granules, Type II eosinophilic cells contained red granules, and a third spherule cell, intermediate between the other two, contained blue, brown, and yellow granules. Many of the syncytial leucocytes contained red, brown and blue granules which originated from disintegrated spherule cells. Also numerous were the vibratile cells which were light blue with Mallory's. The flagella of vibratile cells were never seen in section, probably due to the plane of section passing below or above this fine appendage.

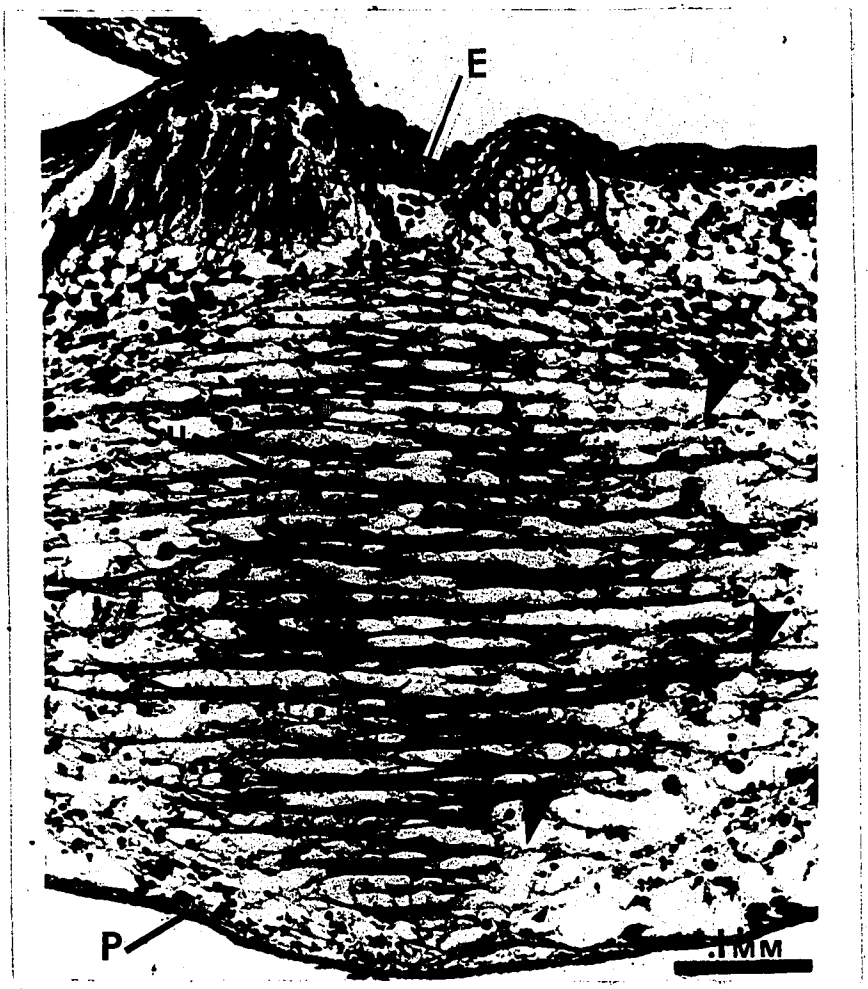

Fig. 2.1C Normal test. Detail of sutural connective fibres which join test plates. Note their continuity with dermal leucocytes (arrows). Su = sutural connective fibres; E = epidermis; P = peritoneum. H+E.

Fig. 2.1C Normal test. Detail of sutural connective fibres which join test plates. Note their continuity with dermal leucocytes (arrows). Su = sutural connective fibres; E = epidermis; P = peritoneum. H+E.

The third layer seen in a cross section of the test is the thin peritoneal lining (Fig.2.1C). The peritoneum consists of a single layer of cells with very prominent nuclei. These nuclei occur very close together and are similar in appearance to a string of beads along the inner layer of the peritoneum. On the coelomic side, the cells are ciliated, and on the dermal side they surround a bundle of banded fibres. The peritoneum is usually 15-25 μ thick with the fibrous region occupying approximately 5-10 μ of this. The cytoplasm of the cells which make up the peritoneum is continuous with those of the dermis. The peritoneum, then, appears to be a layer differentiated from the dermis.

Throughout the test connective tissue fibres exhibiting varying degrees of basophilia can be seen. These were seen to consist of two basic types. The first were connective tissue elements and musculature of the test appendages, such as spines, pedicellariae and sphaeridia. These connective tissue elements appear to be continuous with the cells of the dermis. The second type of connective tissue element seen was the sutural connectives which join the plates of the test to one another. (Fig. 2.1C). The structure and histochemistry of sutural fibres of Arbacia punctulata was described by Moss and Meehan (1967). They found the fibres to be continuous with the cells of the dermis and show histo-chemical reactions similar to those of collagen.

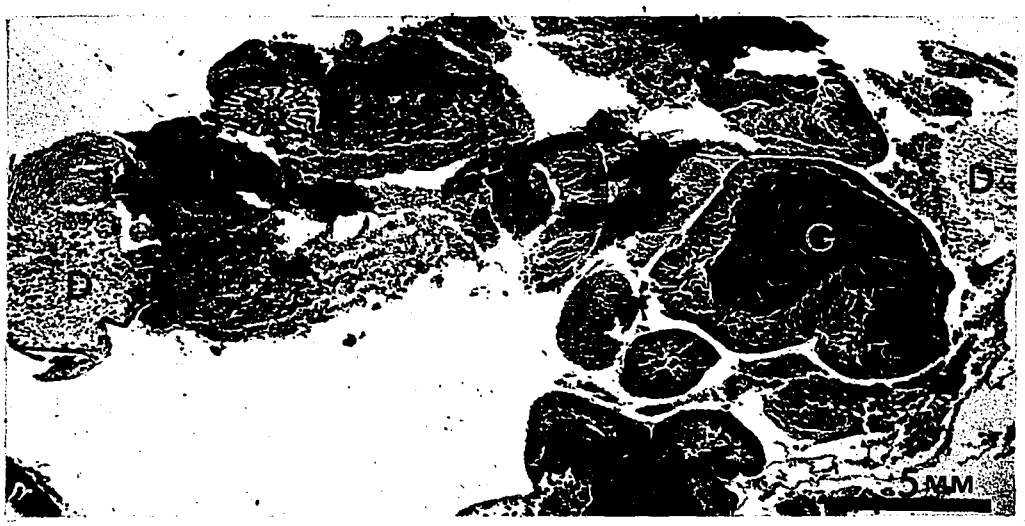

Fig. 2.2A Stage I: regenerated 6 days. Clotted mass of leucocytes containing a trapped spine (Sp) and gonadal material (G). D = normal dermis. H+E.

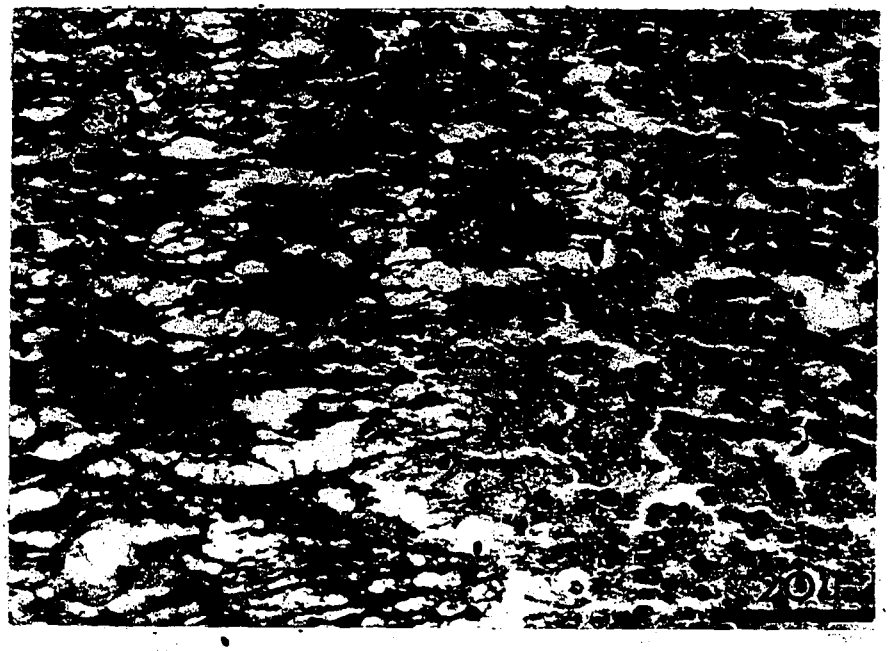

Fig. 2.2B Stage I: regenerated 12 days. Loosely (L) and densely (D) organized leucocytes of blastema. Note packed nuclei of dense region. Alcian Blue-Nuclear Fast Red (AB-NFR).

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2.3.1.2 Regeneration: Stage I

Based on in vivo observations of regenerating blastema (Section 2.0), the histology of regeneration may be considered divisible into three stages. They are described below. The first stage begins immediately holes are made in the test (Section 1.3.2.1)

Clots (blastema) which formed as a result of drilling 3.5 mm holes in the test were examined histologically in both fresh and formol-seawater fixed condition. Newly formed clots were seen to consist of an aggregated mass of coagulated cells. When removed from the animal and examined in the fresh condition, cells with large petaloid extensions could be seen moving within the clot mass. When they stopped moving, they were seen to loose their petaloid extensions and fuse to the clot mass. These cells, 20-30 μ in diameter, appeared to be similar to the bladder amoebocytes described by many authors (Section 2.1). These will be referred to as leucocytes in this paper.

Trapped within this mass of leucocytes were seen the other three types of cells known to oocur in clotted coelomic fluid (Johnson 1969a). They were in descending order of frequency of occurrence; the red (Type I) spherule cells, flagellated vibratile cells and colorless Type II spherule cells.

When the clotted cell mass was fixed in neutral buffered formol-seawater and stained with Harris' H+E or Mallory's Aniline blue-orange G, the following details were seen:(Fig.2.2A) leucocytes appeared to be aggregated into two distinct regions.

In the first, the cells were packed closely together with little visible cytoplasm and distinct, closely packed nuclei, that were often arranged in a row. This region will be referred to as the dense region of the blastema. In newly formed clots, most of the coagulated cell mass is densely packed. Spread out between these dense regions were areas of decreased cell density. In these areas the cells were arranged into a loose syncytium with long cytoplasmic extensions and few nuclei (Fig. 2.2B). Mallory's stain showed many red spherules presumably from the colorless spherule cells, free in the clotted cell mass. In other respects, formol-fixed material resembled the fresh material.

As the blastema ages, there is a decrease in the number of cell types other than leucocytes. Halfway through Stage I, very few red spherule cells remain except for the external border of the blastema which is exposed to seawater. There were few intact vibratile cells, most of them being broken and lysed. A few free acid fuschin positive spherules could be seen randomly distributed throughout the blastema. These are probably derived from Type II cells. The contents of many of the broken and lysed cells appeared to be phagocytized by the leucocytes. In order to determine the phagocytic capacity of the leucocytes, a seawater suspension of carmine particles was injected into the coelomic cavity of several animals. Twenty-four hours after the carmine was injected, a hole was drilled in the test and the animals allowed to regenerate for various periods of time. Many of the cells which participated in filling the wound contained

Fig. 2.4A Stage II: regenerated 24 days. Late Stage II blastema covered by regenerated epidermis. New peripheral platelets are continuous with adjoining normal plates. P = normal plates; dotted line = boundary of adjoining normal plates; arrows = direction of regrowth. H+E.

Fig. 2.4B Stage II: regenerated 24 days. Detail of Fig. 2.4A centre. Note leucocytes are more spread out and nuclei very prominent. E = epidermis. H+E.

Fig. 2.4A Stage II: regenerated 24 days. Late Stage II blastema covered by regenerated epidermis. New peripheral platelets are continuous with adjoining normal plates. P = normal plates; dotted line = boundary of adjoining normal plates; arrows = direction of regrowth. H+E.

Fig. 2.4B Stage II: regenerated 24 days. Detail of Fig. 2.4A centre. Note leucocytes are more spread out and nuclei very prominent. E = epidermis. H+E.

Fig. 2.3A Stage I: regenerated 12 days. A late Stage I blastema which has become firmly anchored in place. There has also been a reduction in the mass of the clot. Note degenerated trapped gonadal material (G) and compare to Fig. 2.2A. Sp = trapped spine; D = normal dermis. Mallory's Aniline.

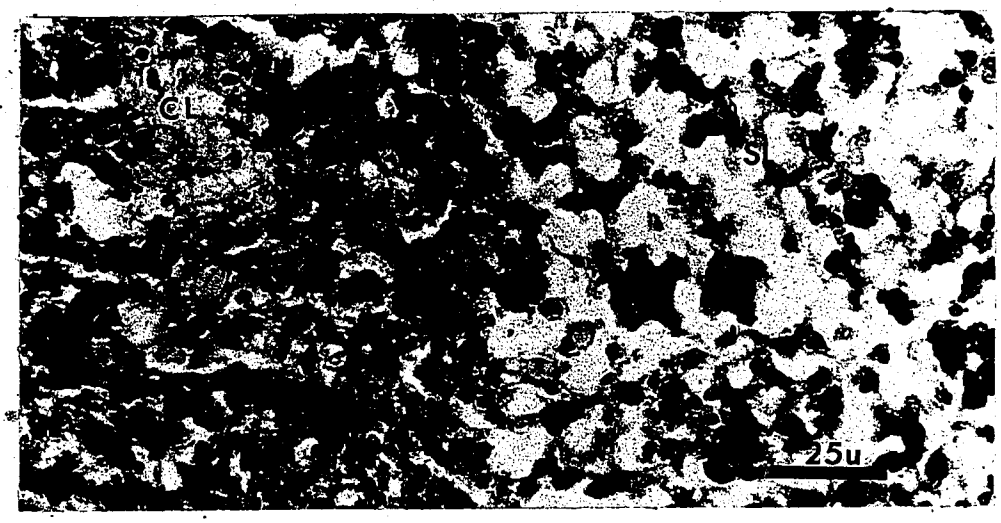


Fig. 2.3B Stage I: regenerated 12 days. Detail of Fig. 2.3A showing fusion of clotted leucocytes (CL) to syncytial leucocytes (SL) of adjacent dermis. H+E.

Fig. 2.3A Stage I: regenerated 12 days. A late Stage I blastema which has become firmly anchored in place. There has also been a reduction in the mass of the clot. Note degenerated trapped gonadal material (G) and compare to Fig. 2.2A. Sp = trapped spine; D = normal dermis. Mallory's Aniline.

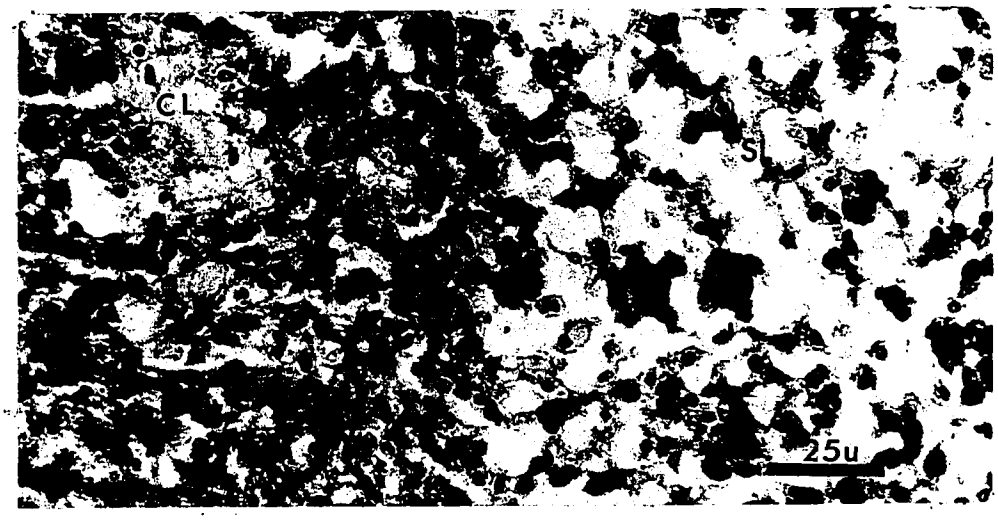


Fig. 2.3B Stage I: regenerated 12 days. Detail of Fig. 2.3A showing fusion of clotted leucocytes (CL) to syncytial leucocytes (SL) of adjacent dermis. H+E.

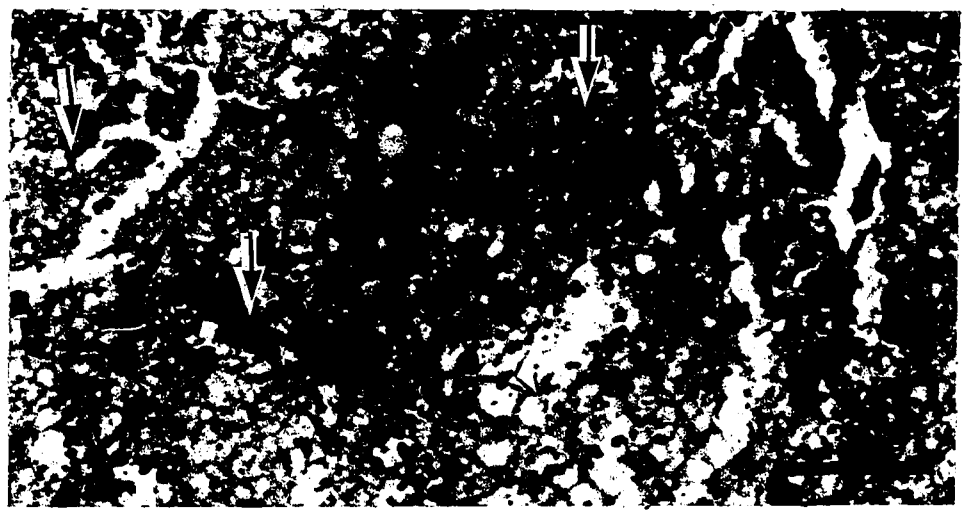


Fig. 2.2C Stage I; regenerated 12 days. Note carmine particles (arrows) phagocytized by clotted leucocytes of blastema. H+E

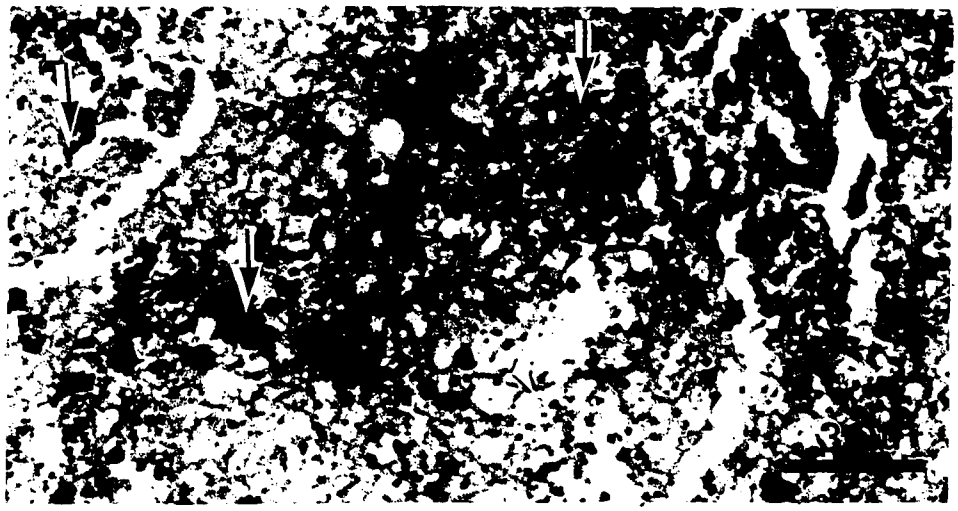


Fig. 2.2C Stage I: regenerated 12 days. Note carmine particles (arrows) phagocytized by clotted leucocytes of blastema. H+E

carmine particles. In animals allowed to regenerate for ten to fifteen days, carmine particles were still visible trapped within the aggregations of leucocytes (see Fig.2.2C).

The end of Stage I is marked by the firm anchoring of the clot, which occurs by the actual joining of the leucocytes to the syncytial cells of the dermis adjacent to the blastema (Fig.2.3A). When examined in section, the leucocytes which have joined up with the dermal cells are not distinguishable from them except by virtue of their more closely packed configuration and the absence of spherule cells within the blastema as compared to the dermis. (Fig.2.3B). The other histological event evident at the end of Stage I was an increase in the amount of densely aggregated leucocytes over those more loosely packed. Stage I usually lasted from thirteen to eighteen days in tests drilled with 3.5mm holes.

2.3.1.3 Stage II

During Stage II the blastema becomes thinner and more resilient (Fig.2.4A). Dense regions of leucocytes continue to spread toward the center of the blastema. However, during this stage their cytoplasms become more elongated so that they appear as if they have been stretched across the wound. Nuclei are less packed and more prominent. The cytoplasms of leucocytes are stretched into thin fibrous bands, most of which run lengthwise in an ambital direction. There are slightly more fibrous elements concentrated in the peritoneal region. When fully developed, the fibres stain a deep blue with aniline blue. The sutural connective fibres between echinoid plates are derived from cytoplasmic extensions

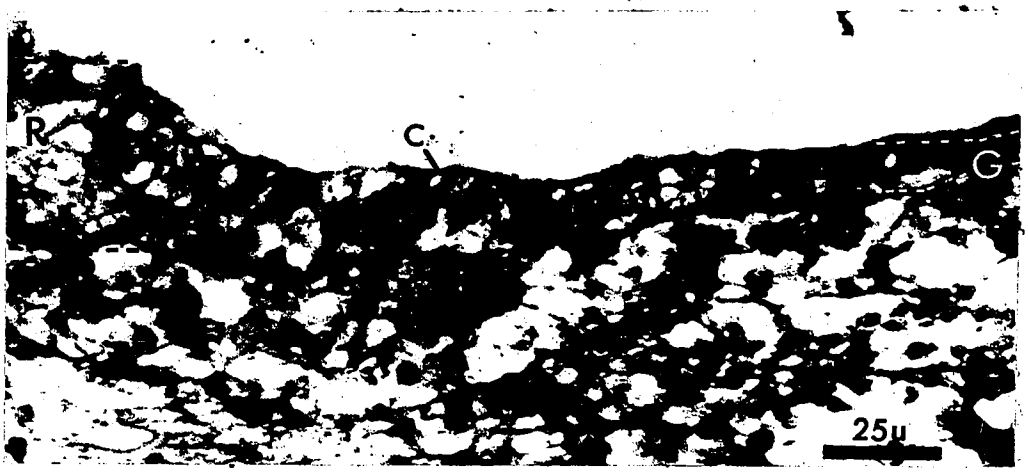


Fig. 2.6 Stage II: regenerated 25 days. Detail of pattern of growth of epidermis. Thick, fully regenerated part (R) near periphery of blastema tapers into thinner advancing layer (G) near centre of blastema. Mallory's Aniline. C = cuticle

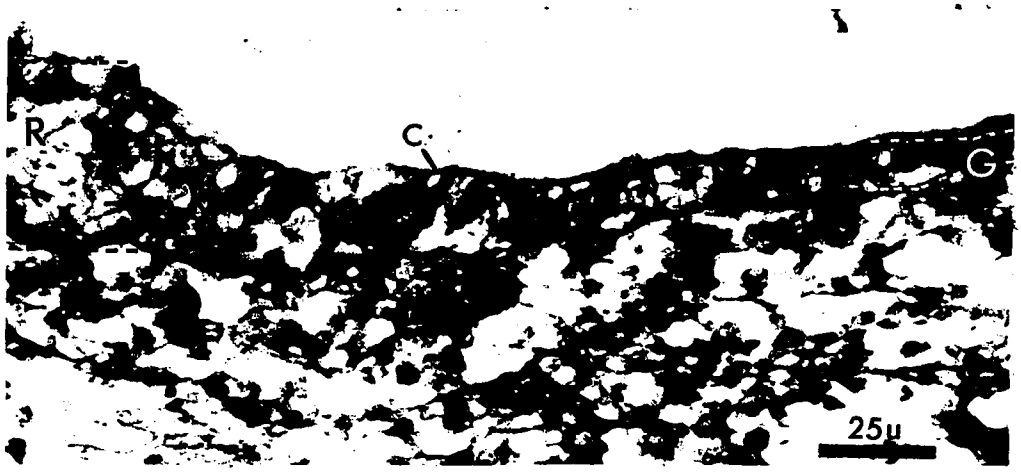


Fig. 2.6 Stage II: regenerated 25 days. Detail of pattern of growth of epidermis. Thick, fully regenerated part (R) near periphery of blastema tapers into thinner advancing layer (G) near centre of blastema. Mallory's Aniline. C = cuticle

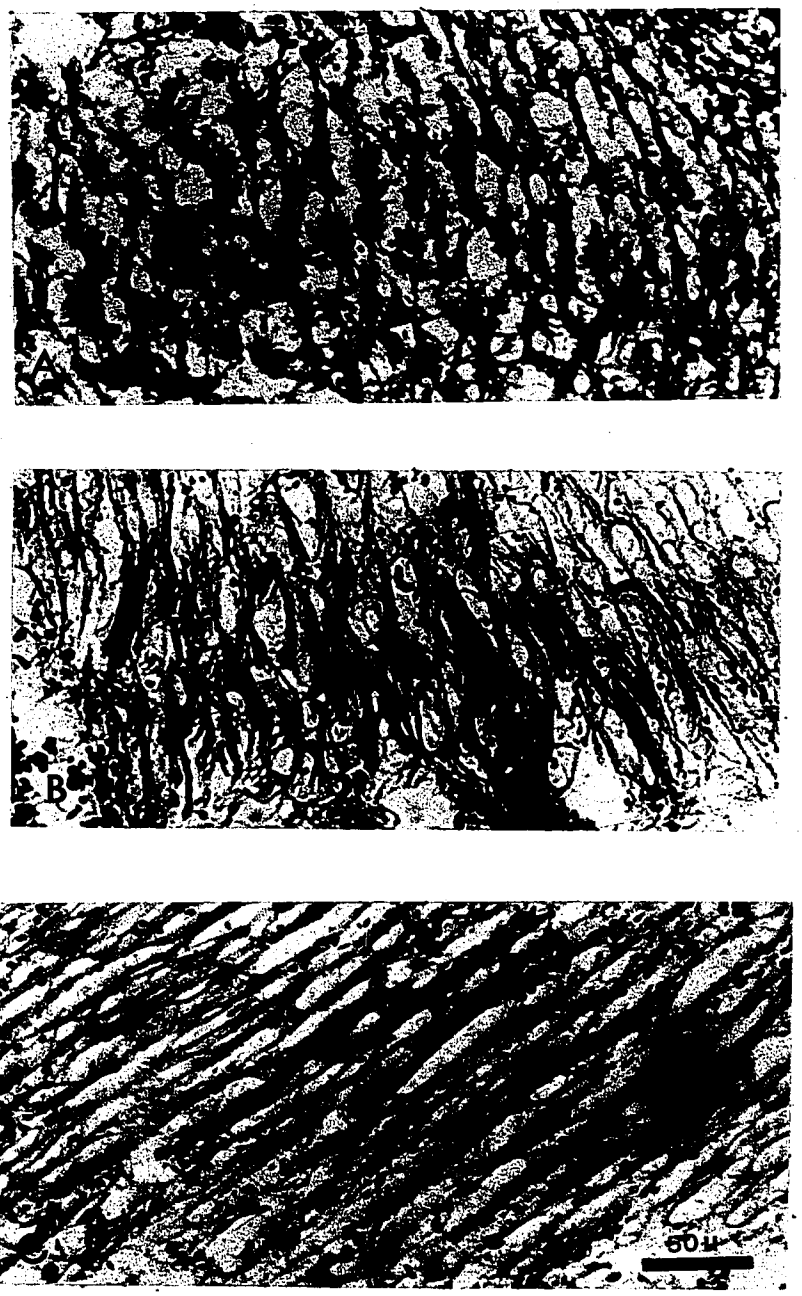


Fig. 2.5 A,B,C Stage II-III. Sequence of changes undergone by leucocytes in forming new sutural connective tissue between regenerating platelets. A and B: Mallory's Aniline; C: H+E. Compare to Fig. 2.1C. All at same magnification.

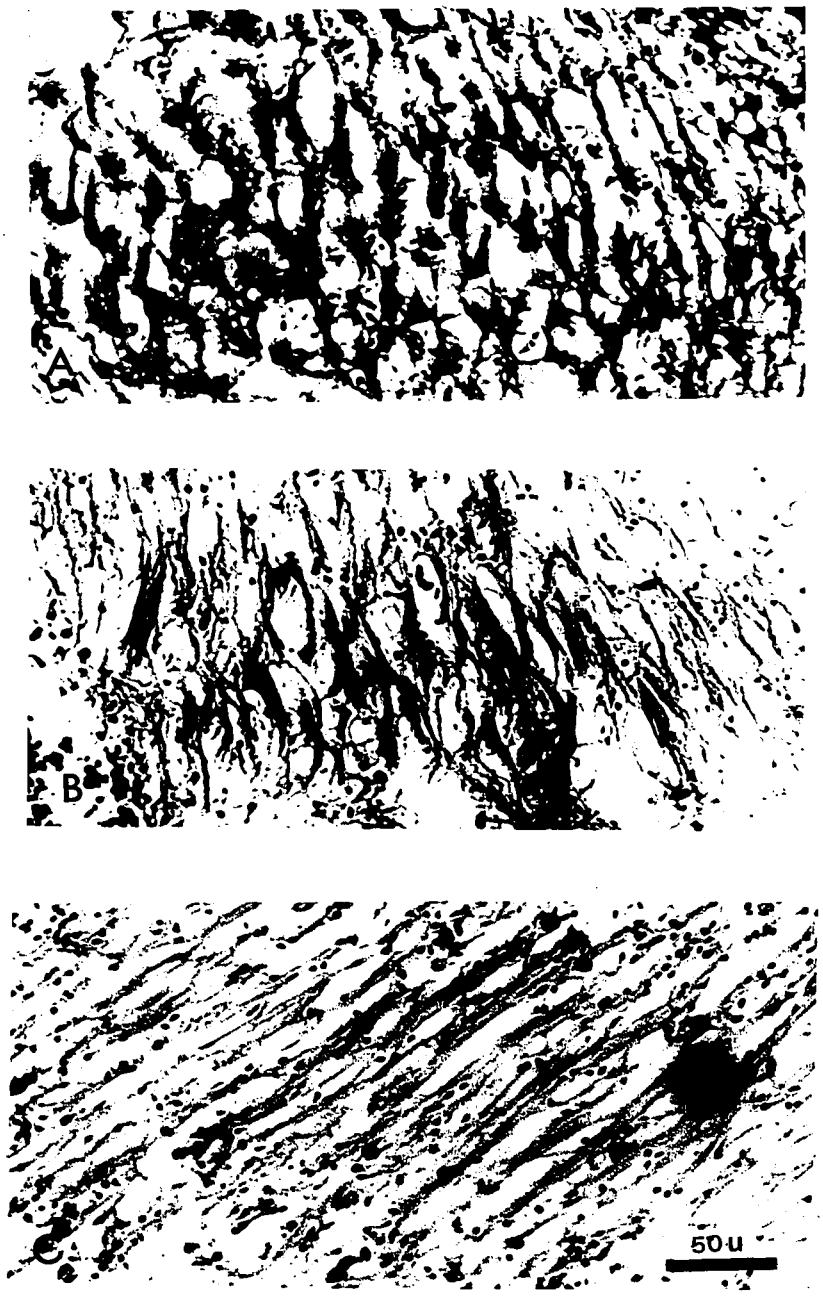


Fig. 2.5 A,B,C Stage II-III. Sequence of changes undergone by leucocytes in forming new sutural connective tissue between regenerating platelets. A and B: Mallory's Aniline; C: H+E. Compare to Fig. 2.1C. All at same magnification.

of dermal cells (Fig. 2.1). It seems possible then that the fibrous elements which appear (in vivo) towards the middle of Stage II (see Section 1.3.2.3) are derived from the elongated filiform leucocytes that are forming new sutures between the irregular plates (compare Fig. 2.5 A,B,C, to Fig. 2.1 C).

Afew of these dark blue staining fibrous elements may be followed to their origin on spines and tubercles which surround the blastema. However, only about one quarter of the fibres are related to the test appendages in this way; the rest originate from the bladder amoebocytes and criss-cross only the blastema. At this point the cells begin to resemble the syncytium of the dermis, and spaces between them are common, although it is difficult to ascertain whether or not they form a true syncytium.

The other important event which occurs during Stage II is the growth of the epidermis. Very early in this stage it is distinguishable in cross section as an advancing layer of very thin cells at either end of the blastema. The edge of the epidermis closest to the periphery of the blastema is about 40 μ thick, tapering down to 20 u near the centre (Fig. 2.6). There is a very prominant .5 μ "cuticle" on top of the pseudostratified epithelium. Areas of the bladder amoebocytes covered by epidermis have prominant nuclei (Fig. 2.4 B). The epidermis continues to grow over the top of the blastema from all directions, much as a diaphragm lens decreases around a central point. The first calcite crystals appear before the blastema has been completely covered by epidermis.

Fig. 2.7 Stage III: regenerated 26 days. Note accumulation of spherule cells (S) among dense leucocytes of blastema. E = epidermis; L = leucocytes. Alcian Blue- Nuclear Fast Red.(AB-NFR).

Fig. 2.8 Stage III: regenerated 25 days. Detail showing relationship between spherule cells (S) and surrounding leucocytes (L). Note strands of cytoplasm (arrows) between spherule cells and leucocytes. Epon- Toluidine Blue.

Fig. 2.7 Stage III: regenerated 26 days. Note accumulation of spherule cells (S) among dense leucocytes of blastema. E = epidermis; L = leucocytes. Alcian Blue- Nuclear Fast Red (AB-NFR).

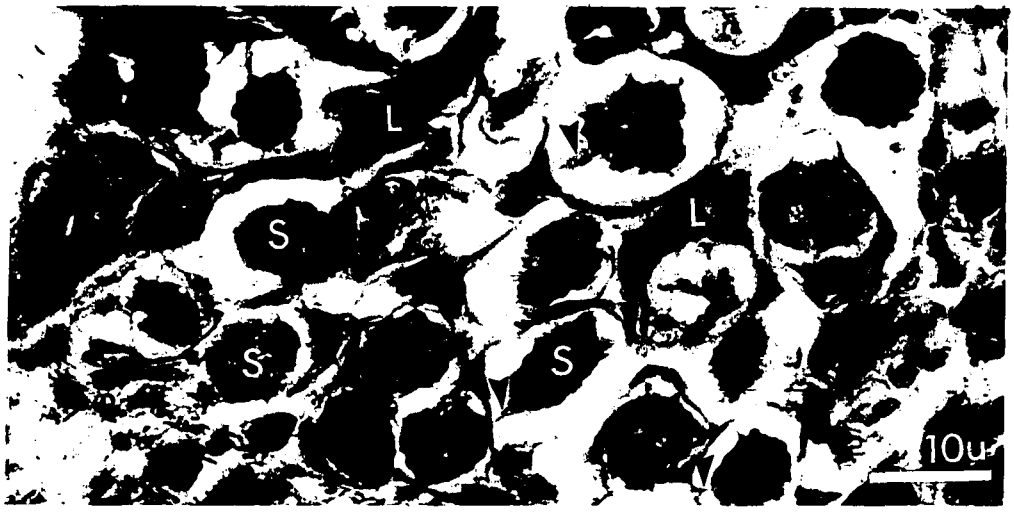


Fig. 2.8 Stage III: regenerated 25 days. Detail showing relationship between spherule cells (S) and surrounding leucocytes (L). Note strands of cytoplasm (arrows) between spherule cells and leucocytes. Epon- Toluidine Blue.

The peritoneum appears much later than the epidermis. The only evidence of differentiating peritoneum is an obvious concentration of fibrous bundles in the lower region of the blastema and the appearance of strings of nuclei at the extreme periphery of the wound. Cell bodies which may be associated with these nuclei are not distinguishable from the bladder amoebocytes at this time. Calcite crystals first appear randomly throughout the epidermis-covered blastema after twenty-five to thirty days' regeneration.

2.3.1.4 Stage III

The blastema was seen to undergo several changes prior to the appearance of the small crystalline granules. The first change is a marked increase in tissue basophilia of the dermal part of the blastema, especially near its periphery and epidermis. These centers of increased basophilia were seen to occur in the following manner: intact, deeply basophilic spherule cells are concentrated in an area extending from the middle of the dermis to just below the epidermis, (Fig. 2.7). These aggregations appeared to be more heavily concentrated towards the peripheral regions of the blastema. In many cases, vibratile cells were also seen, but were less conspicuous due to their light blue staining. As more and more cells appear, older spherule cells were seen to lyse and release their contents. Spherules, both free and in small clumps were spread around, on and between the dense leucocytes of the blastema. Where many spherules had amassed, the tissue appeared more granular and deeply basophilic. In many cases,

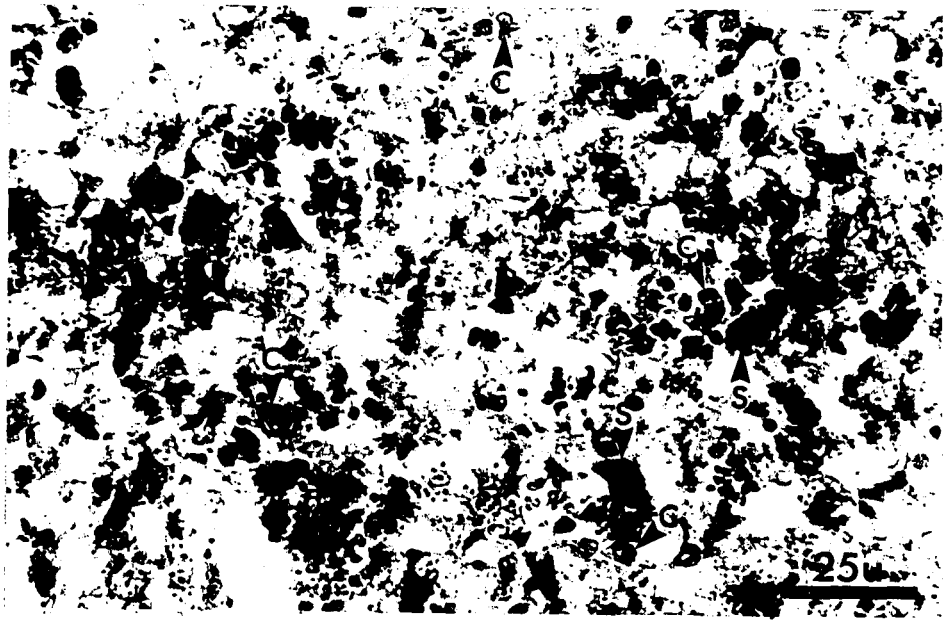


Fig. 2.9A Stage III: regenerated 26 days. Deposition of initial calcite granules (C). Note that many are associated with spherule cells or parts of spherule cells (S). Mallory's Aniline. Phase contrast.

Fig. 2.9B Stage III: regenerated 30 days, periphery of blastema. Newly formed spicules (Sp) are beginning to fill the blastema. Epon- Toluidine Blue. Phase contrast.

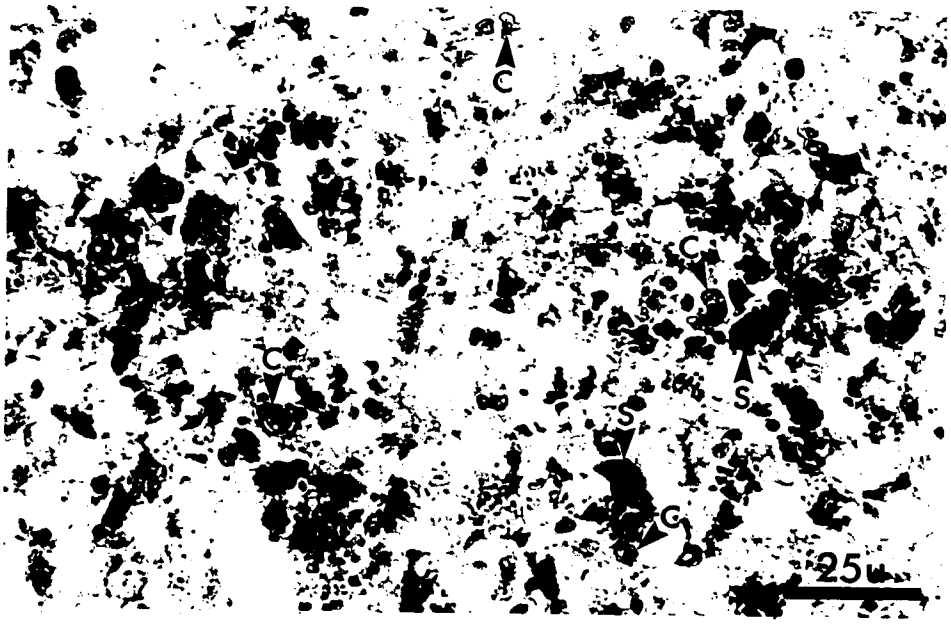


Fig. 2.9A Stage III: regenerated 26 days. Deposition of initial calcite granules (C). Note that many are associated with spherule cells or parts of spherule cells (S). Mallory's Aniline. Phase contrast.

Fig. 2.9B Stage III: regenerated 30 days, periphery of blastema. Newly formed spicules (Sp) are beginning to fill the blastema. Epon- Toluidine Blue. Phase contrast.

spherules, spherule cells and vibratile cells were entirely surrounded by leucocytes. This gave a granular, basophilic appearance to the leucocytes (Fig. 2.8). The regions of the blastema surrounding this middle region are less basophilic. Internally the peritoneal region is much lighter and fibrous with few nuclei. On the epidermal side, the leucocytes are little changed from Stage II.

Blastemae which contained small crystalline granules (see Section 1.3.2.3) were cut out and embedded in paraffin or Epon without prior decalcification. Sections 1 μ to 8 μ thick were examined under polarized light to determine the location of the granules. When this was done, areas which contained granules which rotated the plane of polarized light were examined with oil immersion lenses. Close examination showed vacuoles" (or round open areas) 5-10 μ in diameter among the leucocytes (Fig. 2.9 A). Within these "vacuoles" were seen whole spherule cells or individual spherules, depending on the plane of section. These spherules were red, brown or dark blue when stained with Mallory's Aniline blue-orange G. With Harris' H+E they were light brown or black. Spherules of different colors were seen to occur within any one vacuole. Vacuoles which were only partially filled were also observed. The outer edge of many vacuoles was outlined by the edge of a spherule cell, much as if the vacuole had been formed by removing a piece from the middle of the spherule cell, leaving a basophilic concentric ring which then acts as the vacuole wall.

It is within these vacuoles with their colored

Fig. 2.10 Stage III: blastema regenerated 40 days. New platelets have been deposited in the stereomal region (St.). Per. = peritoneal region; Pr. = prestereomal region; SL = stereomal leucocytes C = areas calcified; E = epidermis. H+E.

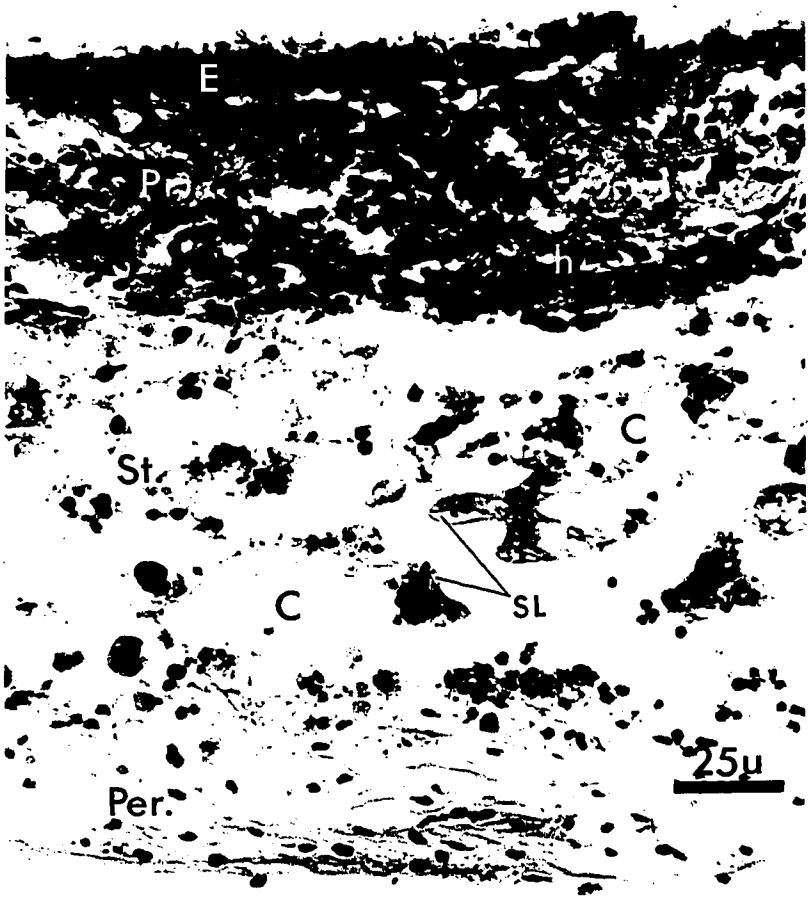


Fig. 2.10 Stage III: blastema regenerated 40 days. New platelets have been deposited in the stereomal region (St.). Per. = peritoneal region; Pr. = prestereomal region; SL = stereomal leucocytes C = areas calcified; E = epidermis. H+E.

granules, that the first crystalline granules become visible. They range in size from 3-5 μ and are nearly always seen in section to be associated with whole spherule cells, spherules alone or small clumps of spherules (Fig. 2.9 A). Those sections which showed crystalline granules alone appeared to be due to the plane of the section passing through the the softer tissue but breaking off the granules, leaving them apparently without any associated soft tissue. The crystallites appeared as single units or as associated subunits which could be distinguished by a fine boundary between them. This first part of Stage III was seen to occur twenty-two to twenty-eight days after drilling of a hole of 3.5 mm in the test.

At this point in Stage III, the dermis of the blastema may be divided into three rather distinct regions which extend from the epidermis to peritoneum (Fig. 2.10). It must also be borne in mind that the regeneration still proceeds more or less from the periphery inwards, so that these three regions become distinct only in those areas which are covered with epidermis and have begun to deposit calcite granules. These regions have been called the prestereomal region, the stereomal region and the peritoneal region. They are usually but not invariably found in the following relationship: the prestereomal region extends from just below the epidermis to the center of the dermis and contains newly deposited calcite granules. It is distinguishable by deep basophilia due to the accumulation of spherule and vibratile cells. The leucocytes of this region appear to be cytoplasmically continuous with those of the stereomal

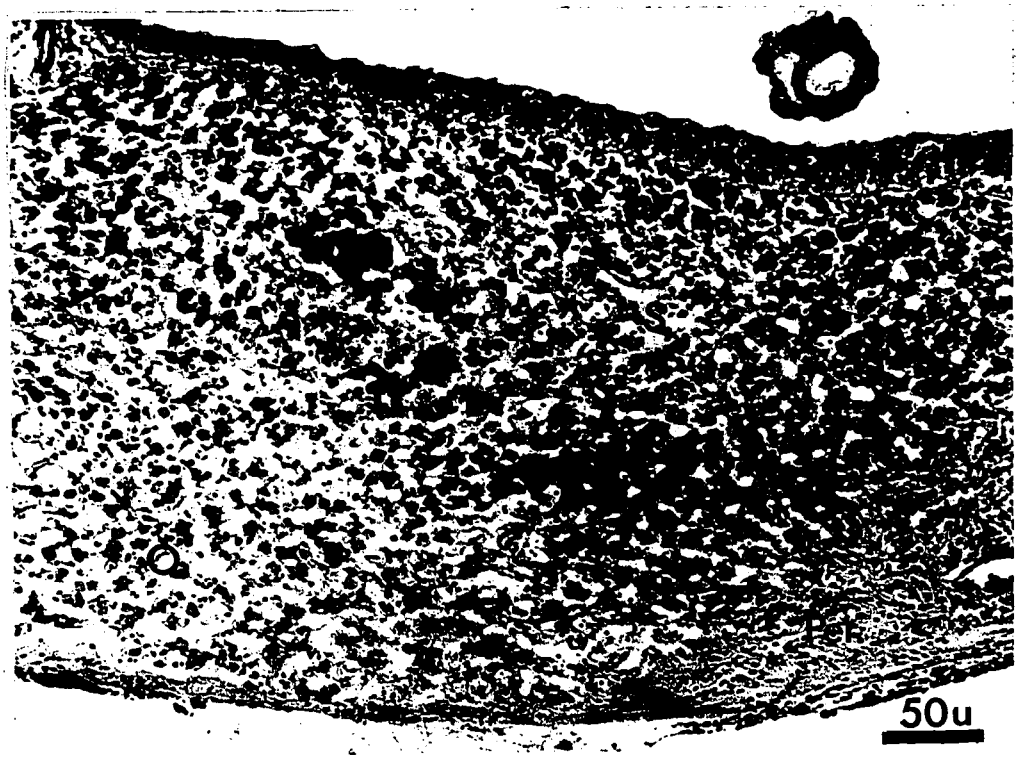


Fig. 2.11 Stage III: regenerated blastema 45 days. Only stereomal (S) and differentiating peritoneal (Per.) regions remain. The oldest stereomal region is on the left (O). H+E.

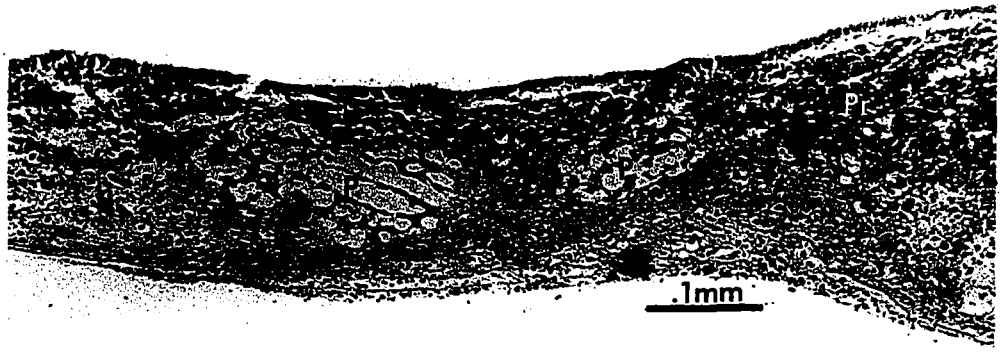


Fig. 2.12 Stage III: regenerated blastema 40 days. Isolated new platelets have formed in two regions (P). Per. = peritoneal region; Pr. = presterecomal region. AB-NFR.

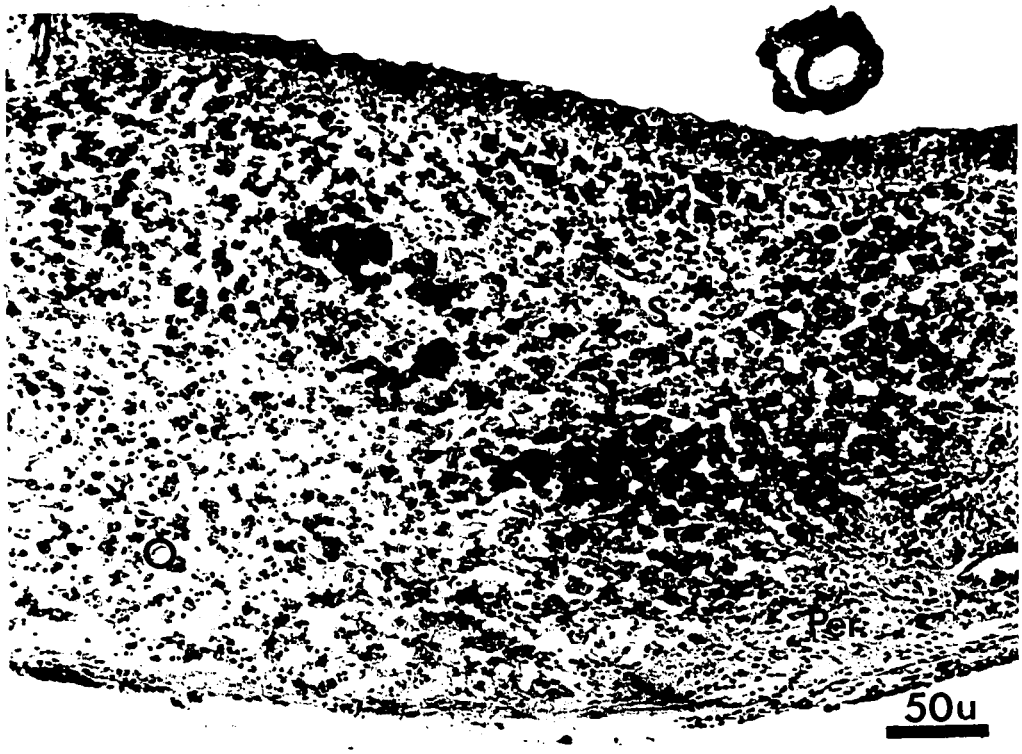


Fig. 2.11 Stage III: regenerated blastema 45 days. Only stereomal (S) and differentiating peritoneal (Per.) regions remain. The oldest stereomal region is on the left (O). H+E.

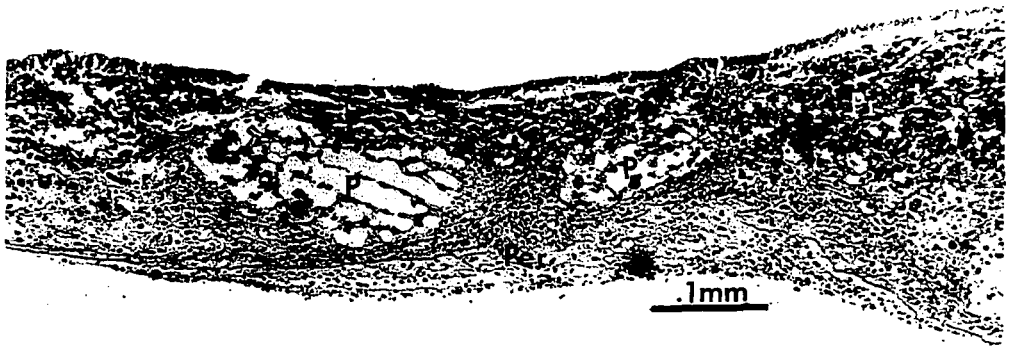


Fig. 2.12 Stage III: regenerated blastema 40 days. Isolated new platelets have formed in two regions (P). Per. = peritoneal region; Pr. = prestereomal region. AB-NFR.

region. This central, or stereomal region is the region in which the calcite granules are growing larger and displacing the leucocytes. The peritoneal region is very light staining and consists of elongated cells with many fibrous extensions. There are few nuclei present. Many of the nuclei are surrounded by fibrous whorls, as if the cytoplasm had been stretched tightly around them. Much of the cytoplasm of these leucocytes appears to have been stretched into fibrous elements (Fig. 2.10).

The crystalline granules described above continue to increase in lenght and diameter during Stage III (Fig. 2.9 B). As the granule size increases to larger than 5 μ, it becomes necessary to decalcify sections for examination in the microscope. Decalcified sections show that the vacuoles in the leucocytes of the stereomal region continue to increase their size (Fig. 2.11). Many have irregular shapes, reflecting the three-dimensional growth characteristics of the granules (compare Fig. 1.5 to Fig. 2.11). When growth of the granules has reached this stage, they are referred to as spicules. Vacuoles which contain spicules are often surrounded by a halo of basophilic cytoplasm. Growth of the spicules occurs at the expense of the surrounding leucocytes, which decrease their cell volume as the blastema becomes more and more filled with calcite spicules. The size of the vacuoles ranged from 5-15 μ in diameter. At this point, the stereomal region begins to resemble the normal test seen after decalcification. Meanwhile, the prestereomal region is also going through the same sequence of events - basophilia, followed by small granule deposition and then growth into spicules,

Fig. 2.13A Stage III blastema: regenerated 60 days. Nearly the entire blastema is calcified except for the prestereomal area in the centre (Pr). The peritoneal region can be seen differentiating on the right (arrows). H+E.

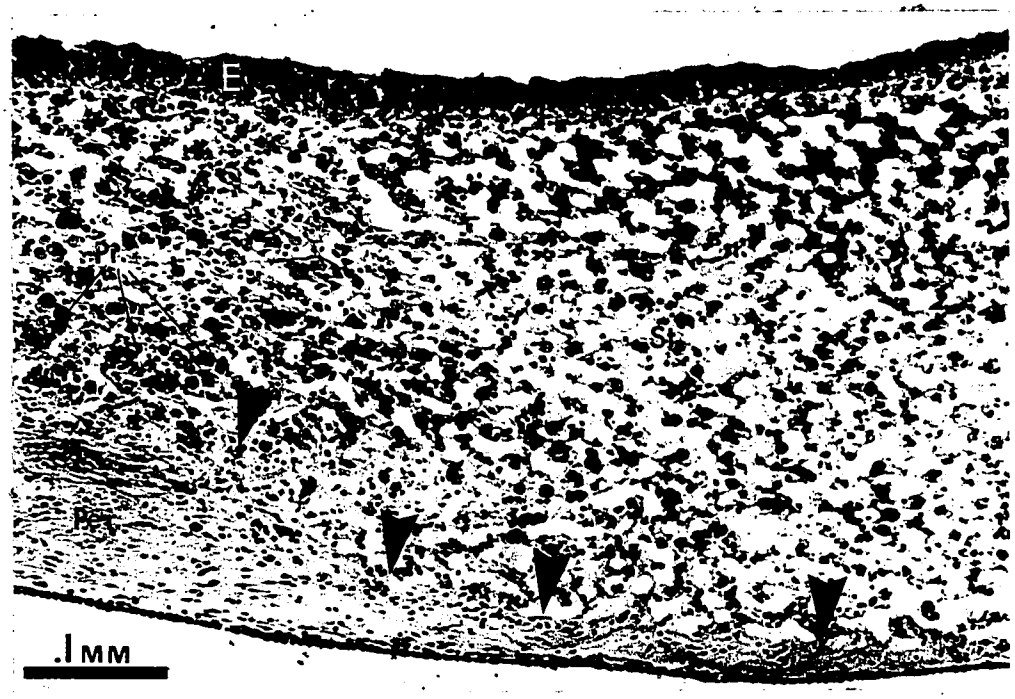


Fig. 2.13B Stage III blastema. Close-up of Fig 2.13A showing peritoneal differentiation (arrows). Pr = prestereomal area; Per. = peritoneum; St = stereomal region; E = epidermis. H+E.

Fig. 2.13A Stage III blastema: regenerated 60 days. Nearly the entire blastema is calcified except for the prestereomal area in the centre (Pr). The peritoneal region can be seen differentiating on the right (arrows). H+E.

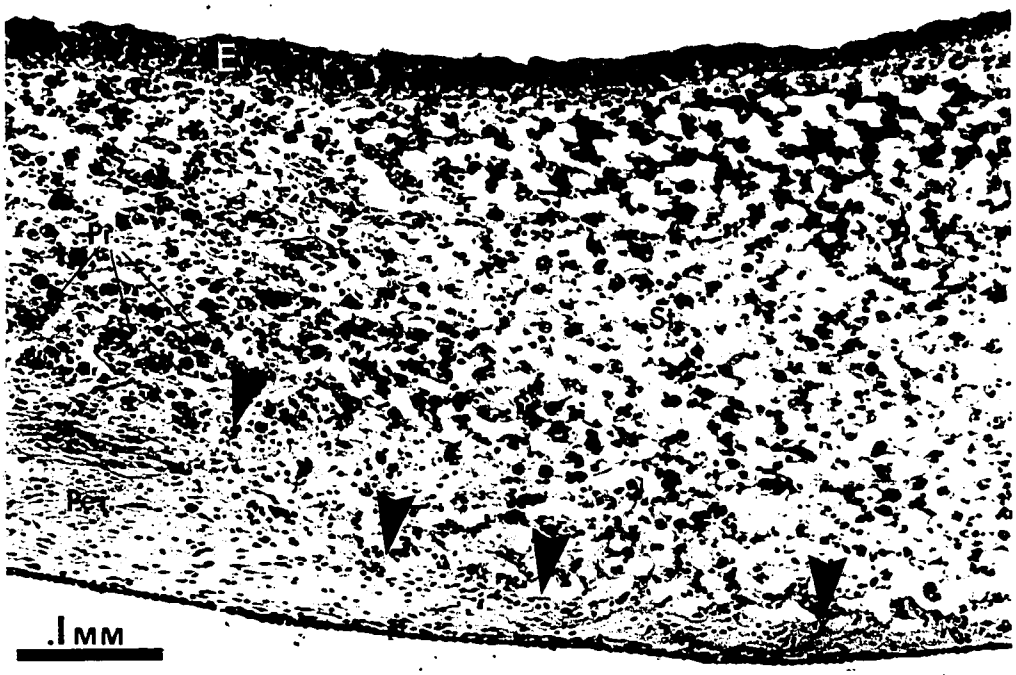


Fig. 2.13B Stage III blastema. Close-up of Fig 2.13A showing peritoneal differentiation (arrows). Pr = prestereomal area; Per. = peritoneum; St = stereomal region; E = epidermis. H+E.

at which point it too becomes a stereomal region. Vacuoles may be separated expanses of leucocytes. This appearance in paraffin sections is due to the plates which form in scattered regions of the blastema, so that many plates develop to fill the hole (Fig. 2.12, compare to Fig. 1.4).

The peritoneal region was seen to undergo changes, quite different from those of the stereomal and prestereomal regions. It is delimited on the inside by the plates forming in the stereomal region, and consists of many fibrous elements organized into a tract, 15 - 25 u wide, which runs across the blastema in an ambital direction (Fig. 2.13 A). As the plates thicken, this region becomes more clearly differentiated and much thinner. Eventually it appears as a 7-20 u wide band of fibrous elements with a few scattered nuclei (Fig. 2.13 B). On the coelomic side of the peritoneal region, the nuclei which are present are concentrated in a narrow band. On the dermal side, the fibrous elements are continuous with the cells of the stereomal region. This explains the <u>in vivo</u> observation (see Section 1.3.2.3.) of thin filamentous fibres continuous with the cellular elements of newly formed plates which were directly connected to the peritoneum. As the plates grow larger, they displace more and more of the peritoneal fibres. The regenerated peritoneum consists of a thin layer of cells surrounding the fibrous connective tissue. The formation of the peritoneum appears to be a result of the differentiation of the dermal tissue. This contrasts with epidermal regrowth which is an extension of the preexisting epidermis over the top of the blastema.

Fig. 2.14A Stage III: regenerated 70 days. Blastema almost completely regenerated containing carmine particles (arrows) in new stereom (St) and in presteream (Pr). Mercury Bromphenol Blue (MBPB).

Fig. 2.14B Stage III blastema: regenerated 40 days. Close-up of unstained new stereomal region containing new spicules (Sp) and carmine particles (black granules, see arrows). Phase contrast.

Fig. 2.14A Stage III: regenerated 70 days. Blastema almost completely regenerated containing carmine particles (arrows) in new stereom (St) and in prestereom (Pr). Mercury Bromphenol Blue (MBPB).

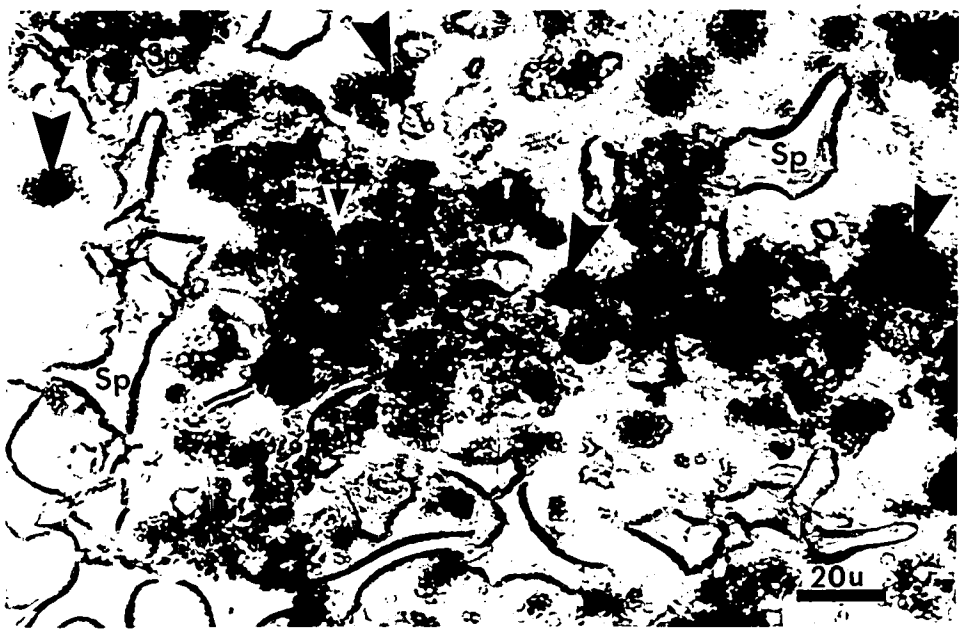


Fig. 2.14B Stage III blastema: regenerated 40 days. Close-up of unstained new stereomal region containing new spicules (Sp) and carmine particles (black granules, see arrows). Phase contrast.

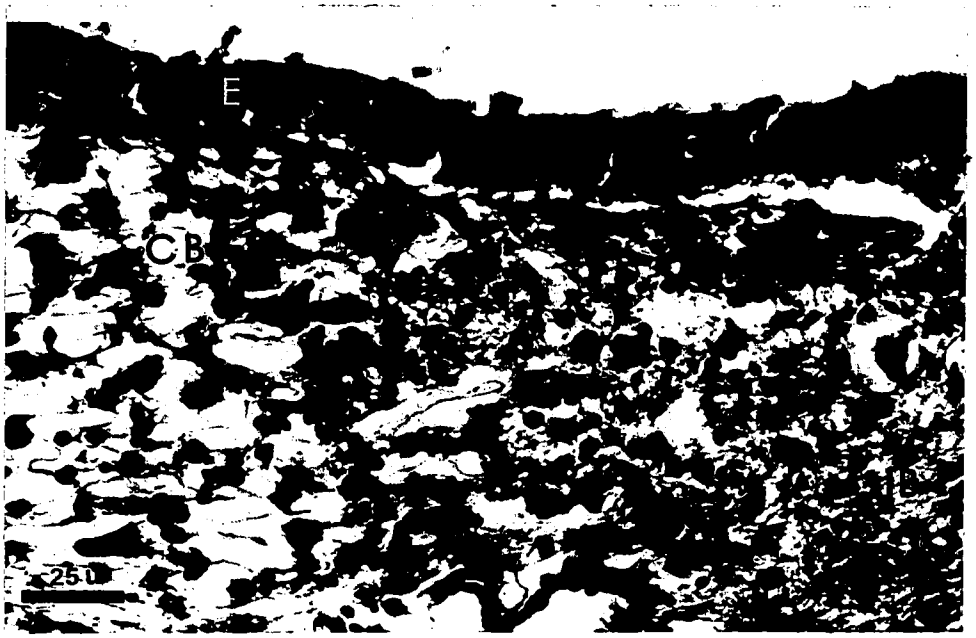
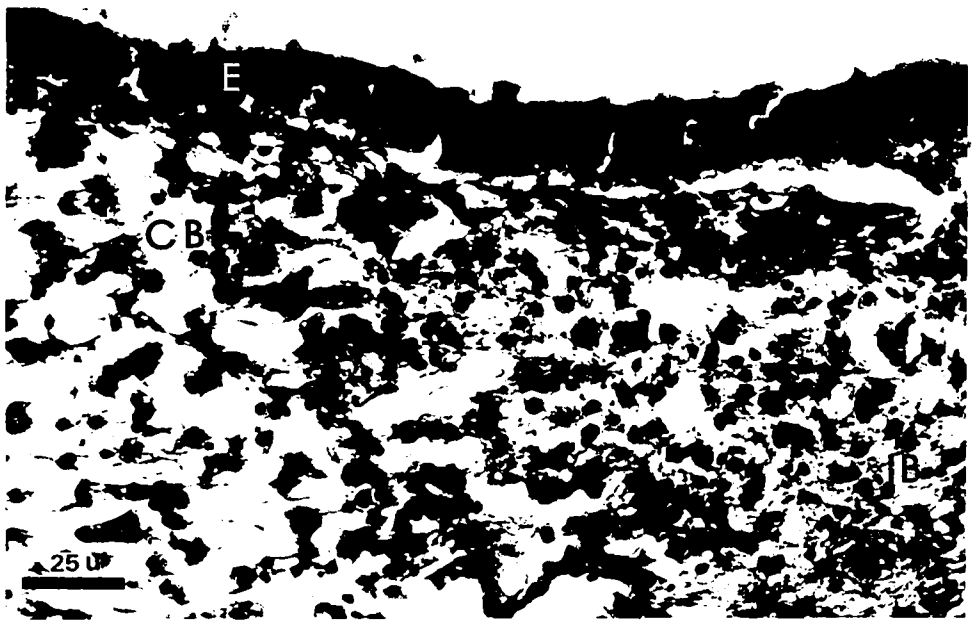


Fig. 2.15 Stage III: regenerated 80 day blastema near centre. Plate deposition has resulted in reduction in granularity in region CB; whereas region IB, containing fewer spicules, is more basophilic and granular. E = epidermis; CB = calcified blastema; IB = incompletely calcified blastema. H+E.



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It seems likely that the filamentous peritoneal fibres help to anchor new plates together so that they are kept in place as the hole fills in. Whether these filaments are involved in actual calcite deposition directly is not clear from histological observations. Calcite deposition appears to occur from the middle of the dermis outwards and no vacuoles were ever found in peritoneal regions. The displacement of fibrous elements by the expanding plates does seem to occur, but the plates appear to be undergoing compression as well, so that the extent to which calcite directly "replaces" (or is deposited on) fibre is not clear.

Animals which were injected with carmine and allowed to regenerate to Stage III contained carmine particles massed inside the cytoplasm of leucocytes which were depositing or had deposited calcite (Fig. 2.14 A,B). This suggests that these cells are the same as originally clotted in the wound and that they are the cells responsible for the deposition of new plates. However, it is not possible at the resolution of the light microscope to determine wether or not the spaces occupied by the carmine particles are intra- or extracellular. This is due to the unique structure of syncytial leucocytes discussed in the following section.

As the stereomal regions continue to spread to the rest of the blastema, the cells become both less granular and smaller. This is accompanied by an increase in the amount and size of calcite spicules (Fig. 2.15). The syncytial leucocytes appear more "empty" as more calcite is deposited (Fig. 2.15). The spicules of a regenerated plate are surrounded by a syncytium of leucocytes

which have mostly non-granular cytoplasms containing distinct nuclei and a few engulfed spherule or vibratile cells. The number of engulfed cells decreases as the stereom ages.

In summary then, the deposition of calcite in regenerating blastema occurs by the action of the leucocytes which may be the same cells as those present in the perivisceral fluid. The spherule cells also appear to be involved with skeleto-genesis since calcite granules are nearly always associated with these cells or parts of these cells.

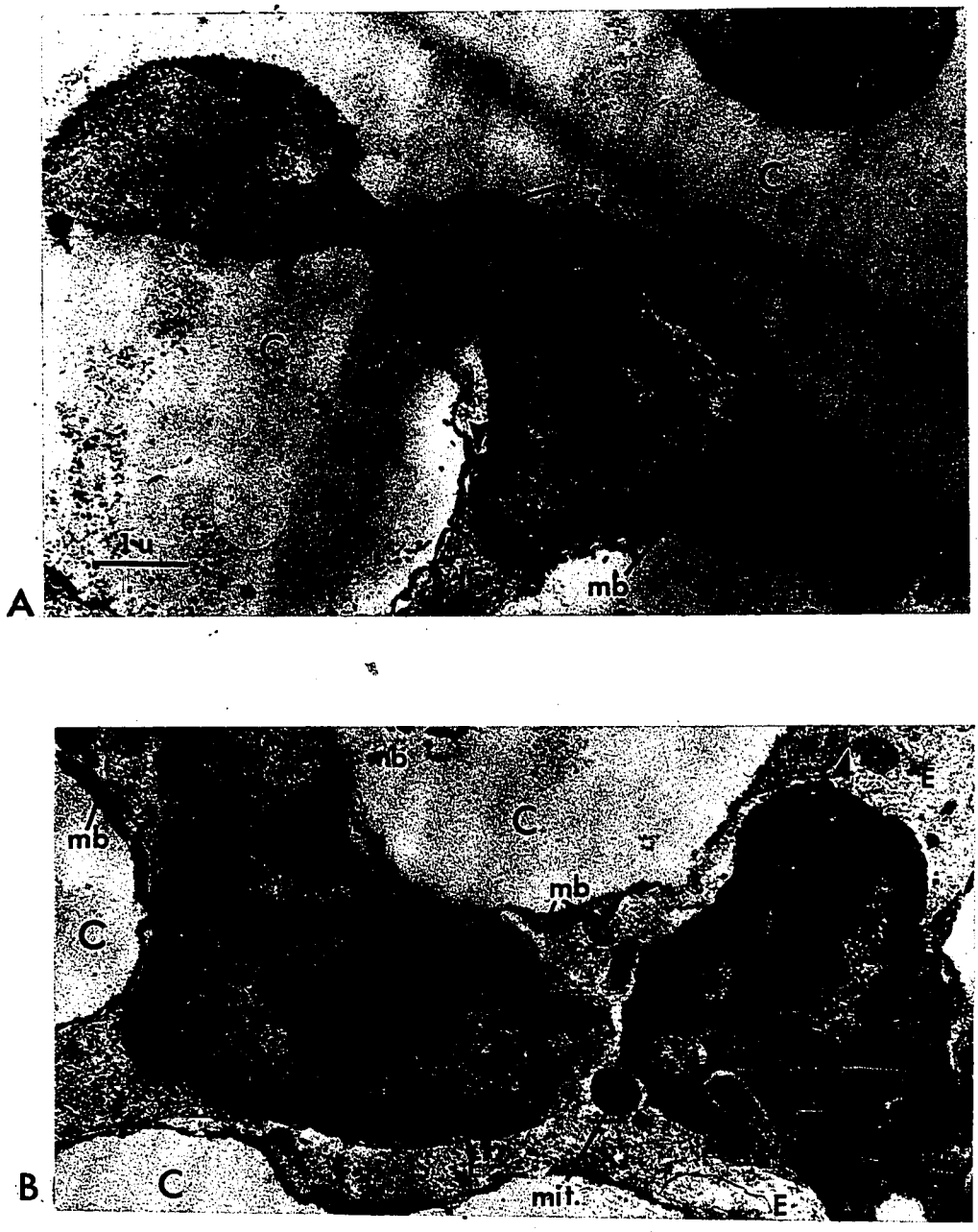
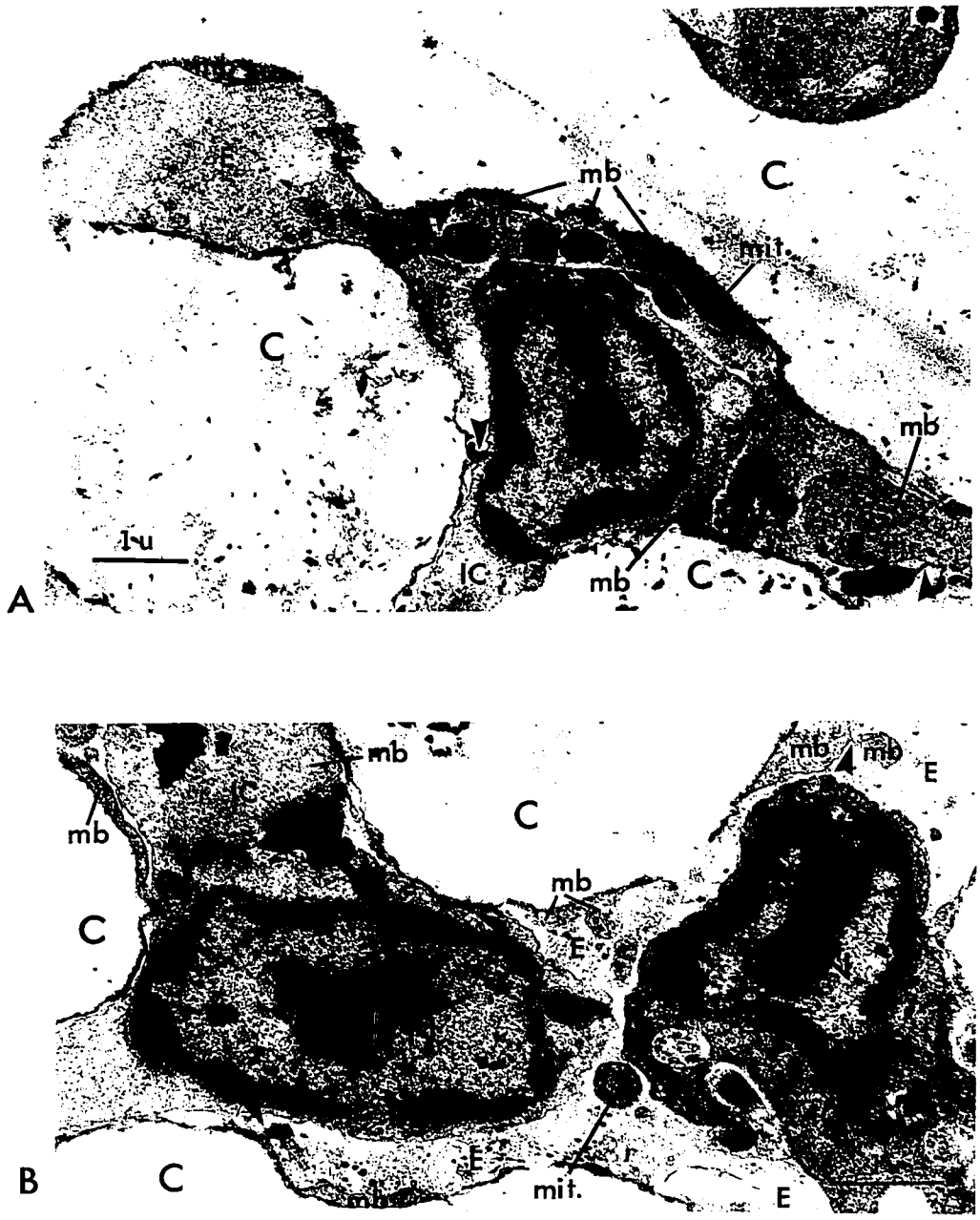


Fig. 2.17 A,B Normal test. Electron micrographs of x sections of syncytial leucocytes. Note membrane-bound bodies (mb) which interconnect (arrows) and contain many organelles. Note that membrane-bound bodies are continuous with calcite (C) - also intracellular. Note double membrane between extracellular fluid (E) and calcite. Compare to Fig. 2.26C. N = nucleus; mit. = mitochondria; IC = intracellular fluid. A- x15500 B- x16500



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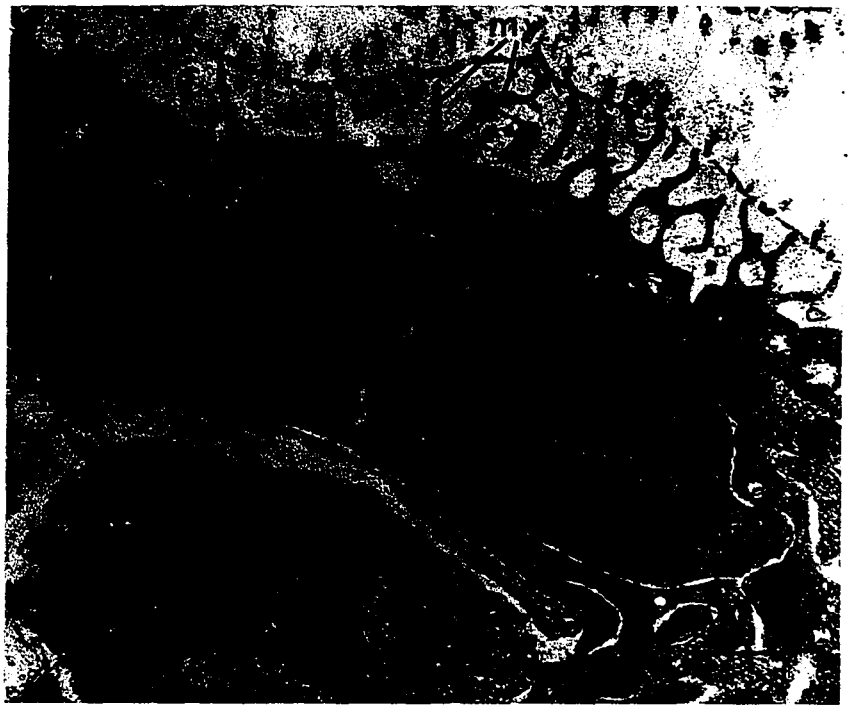


Fig. 2.16 Normal test. Electron micrograph of x section of top of epidermis showing cuticle (C) which consists of amorphous material between microvilli (mv). N = nucleus. x 13,800

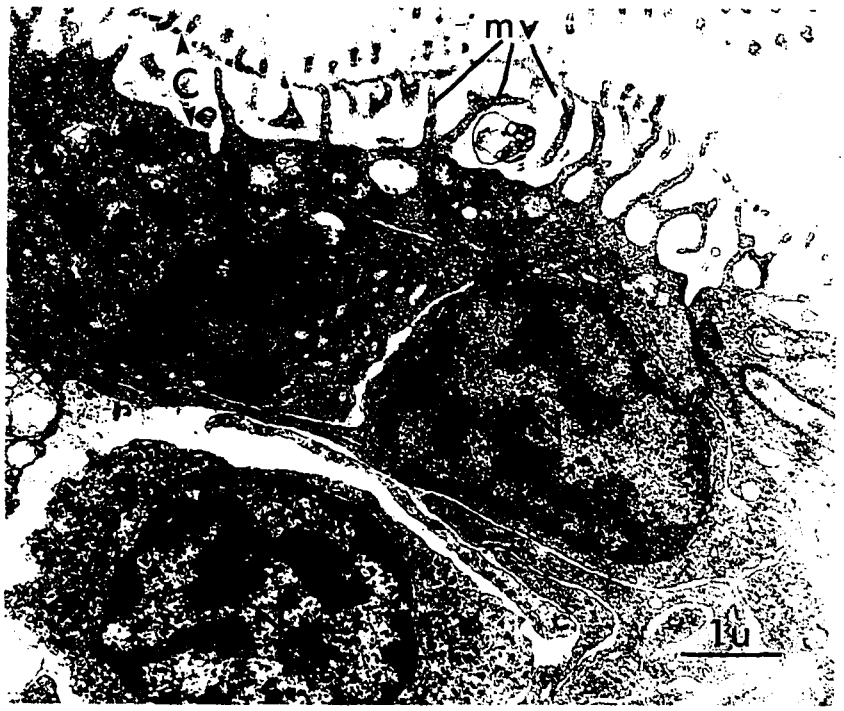


Fig. 2.16 Normal test. Electron micrograph of x section of top of epidermis showing cuticle (C) which consists of amorphous material between microvilli (mv). N = nucleus. x 15,800

2.3.2 Electron Microscopy

2.3.2.1 Normal Test

Electron microscopy of the epidermal region confirmed the pseudostratified nature of this epithelium. Nuclei appear scattered throughout this tissue layer and cell boundaries are indistinct. The outer edge of the epidermis is extended into fine microvilli .6 u x .06 u (Fig. 2.16). Between these microvilli an amorphous substance extending nearly to the tip of each microvillus is visible. This substance is mostly removed by fixation. The presence of the microvilli with amorphous material between them probably constitutes the "cuticle" described by many authors. Beneath the epidermis is the beginning of the dermis, in which the syncytial leucocytes (sclerocytes) which form the organic stroma can be seen (Fig. 2.17 A). The cytoplasm of the sclerocytes appears to be filled with many membrane-bound bodies. These membrane-bound structures were seen to contain the organelles of the cells, such as nuclei and mitochondria, and many of them are visibly joined (Fig. 2.17 B). Also visible along this membrane are dark spherical, expanded regions. These may be other membrane-bound components which have fused or are fusing with the membrane. Where the membrane is discontinuous there is often a membrane-bound body which opens directly into the cavity formerly occupied by calcite. Thus the calcite appears to be intracellular. Connections visible between some membrane-bound structures may indicate that all these structures are interconnected. Regions not contained within these membranous (spherical) structures are

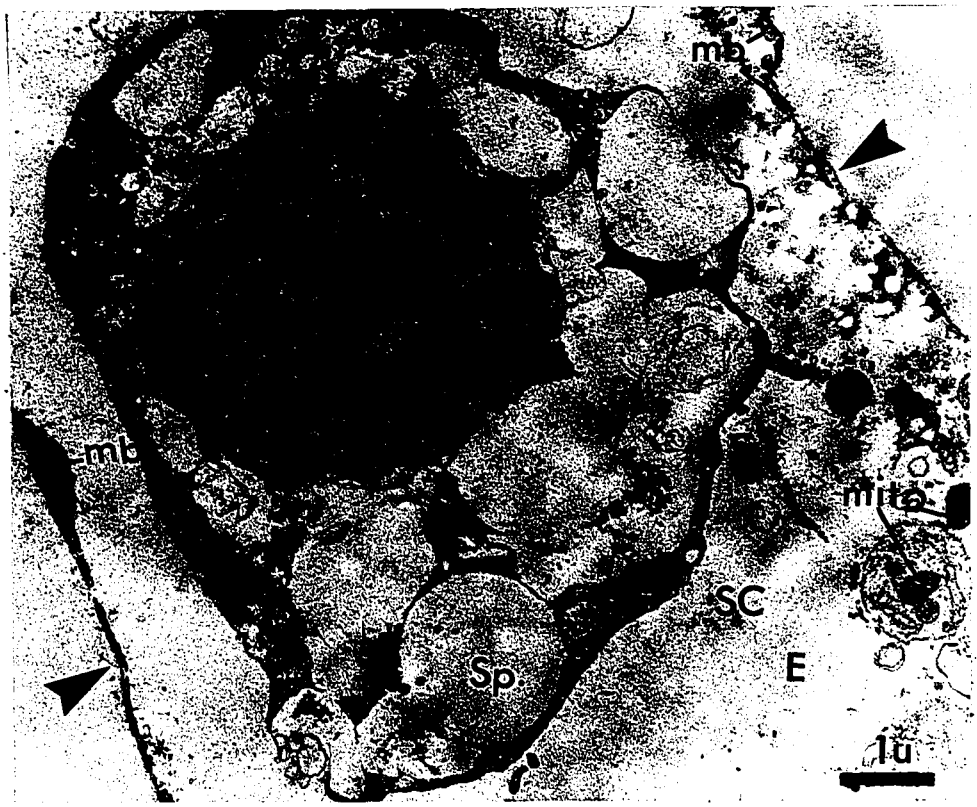
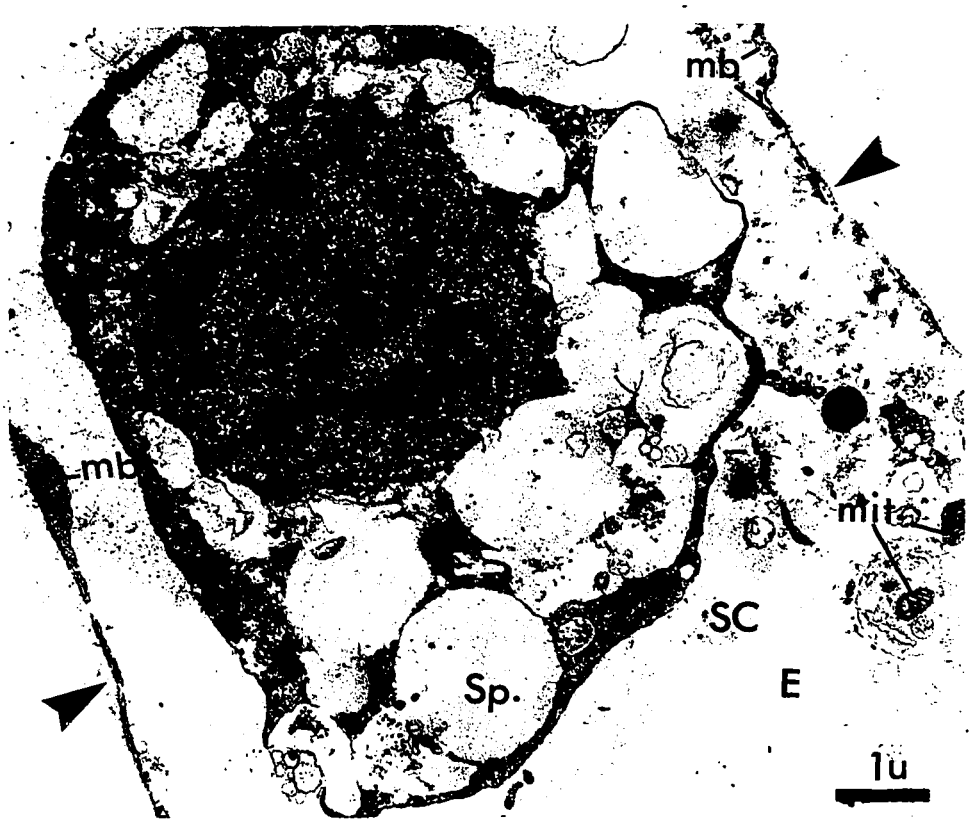


Fig. 2.19 Normal test. electron micrograph of spherule cell (Sc) contained within extracellular space (E) of syncytium. Boundary of syncytium is indicated by arrows. mit. = mitochondria; mb = membrane bound body; N = nucleus; Sp = spherule. x13,500

Fig. 2.20 Normal test. Detail of x section of peritoneum. Note that fibrous elements (F) run in several directions. D = dermal side; C = coelomic side; mb = membrane-bound body. x13,500



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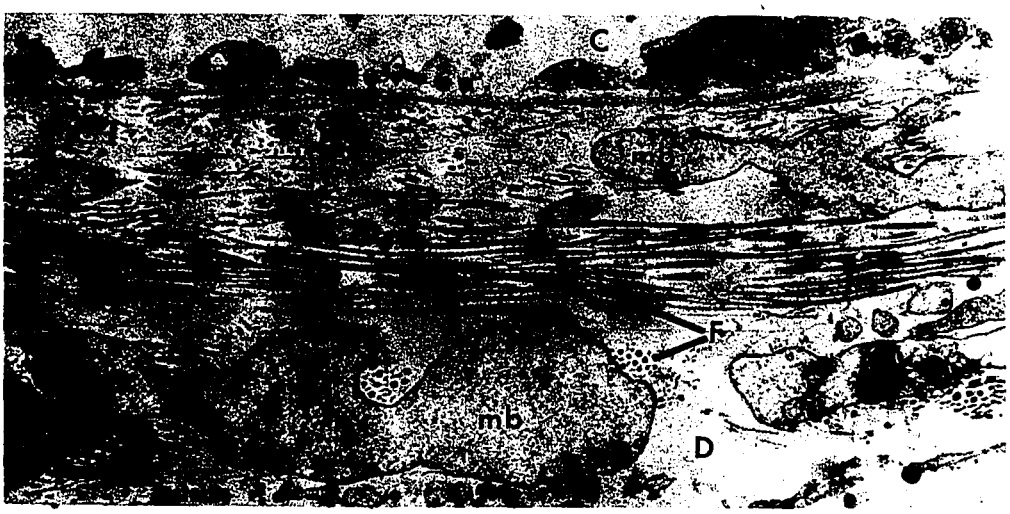

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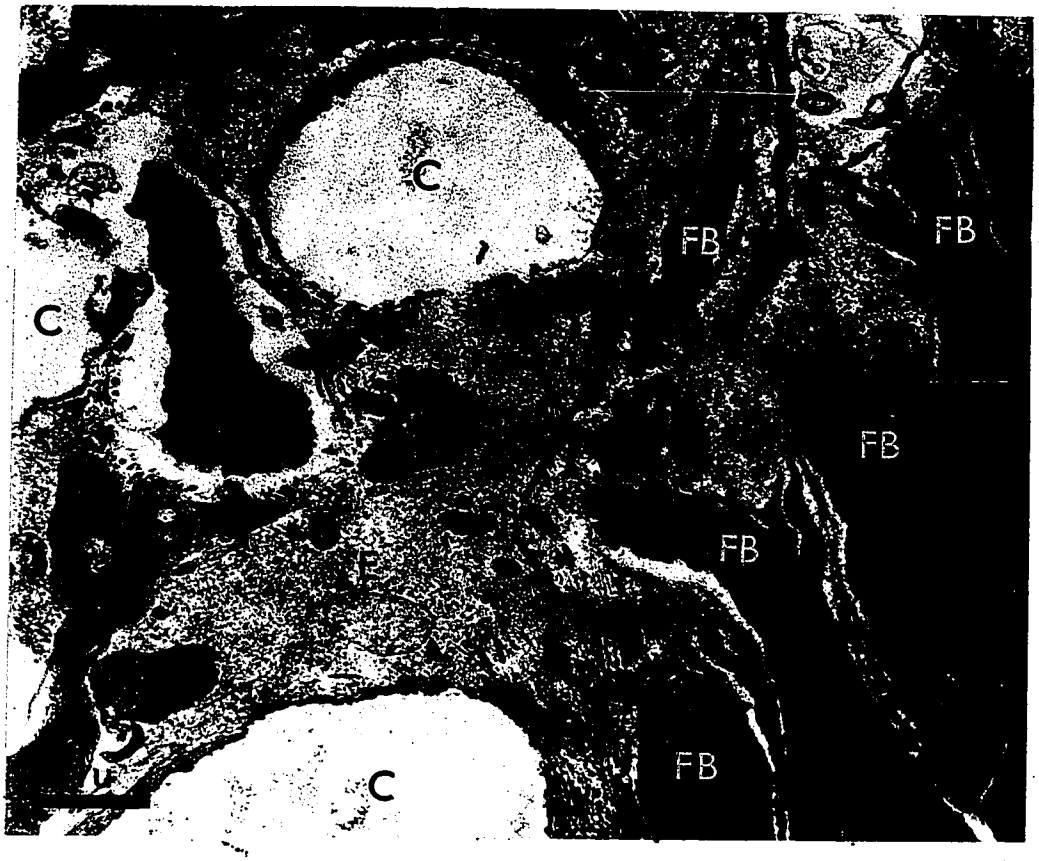


Fig. 2.18 Normal test. Electron micrograph of region immediately below epidermis. Note connective tissue elements (F) which are outside membrane-bound bodies (mb). Dark fibrous material (FB) is connective tissue bundles which fuse with leucocytes. C = calcite; E = extracellular fluid. x16500

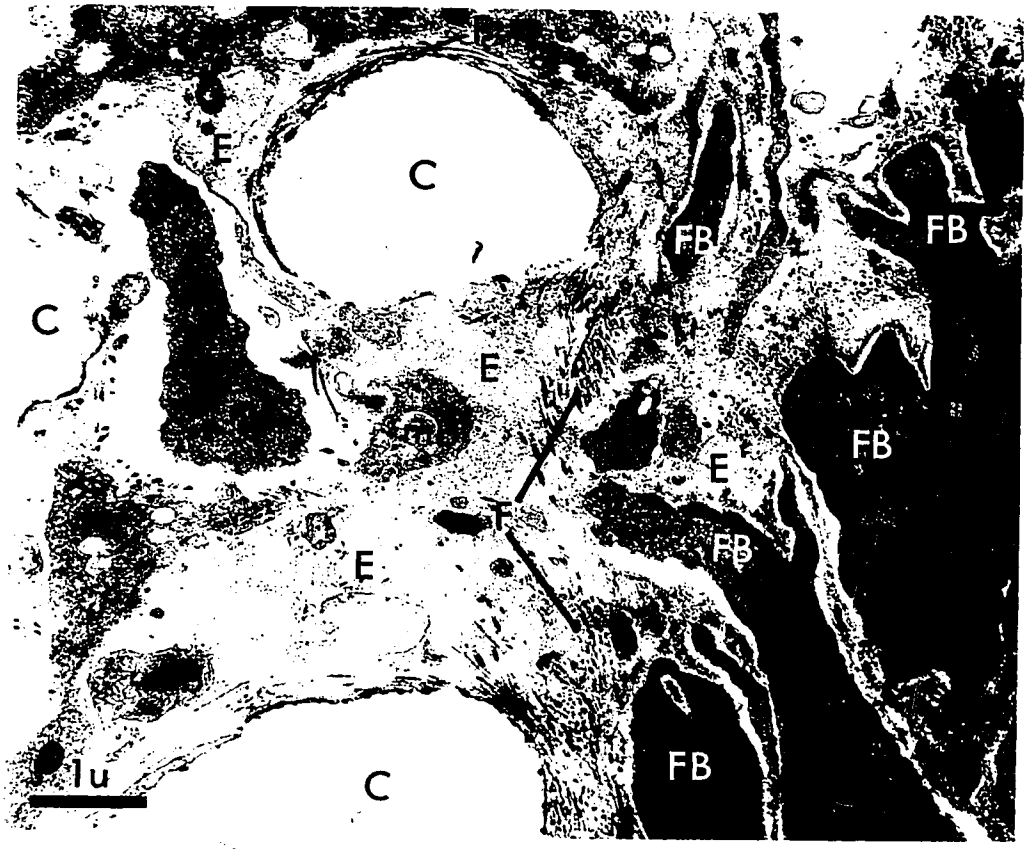


Fig. 2.18 Normal test. Electron micrograph of region immediately below epidermis. Note connective tissue elements (F) which are outside membrane-bound bodies (mb). Dark fibrous material (FB) is connective tissue bundles which fuse with leucocytes. C = calcite; E = extracellular fluid. x16500

thus extracellular. What appears in the light microscope to be a membrane-bound sclerocyte (i.e. Fig. 2.1 B) is actually a combination of extracellular fluid (regions outside connected spherical regions) and a highly convoluted membrane which encloses the cellular components (see Fig. 2.2C).

Regions immediately below the epidermis showed an abundance of fibrous material. These fibres were not contained within membrane-bound bodies and hence are extracellular. Sections from areas which contain very thick fibrils show that they are concentrated in bundles. Where these fibrils have been cross-sectioned they appear as solid rods viewed end on or obliquely (Fig. 2.18). In the light microscope many of the connective tissue elements were seen to "fuse" into the syncytial leucocytes. From their appearance in the electron microscope this close association with the syncytial sclerocytes is confirmed.

When the syncytial sclerocytes of the mid-dermal region are examined, there is a conspicuous absence of fibrillar material. These cells contain the same membrane-bound bodies seen in the other regions of the dermis.

Many regions of the syncytium were seen to contain other cells within their extrace lular fluid (Fig. 2.19). These cells appeared to be spherule cells or possibly vibratile cells. They contained light, empty vacuoles. It is possible that tissue processing removed the contents of the vacuoles, although in sections of regenerating material spherule cells containing both dark and light

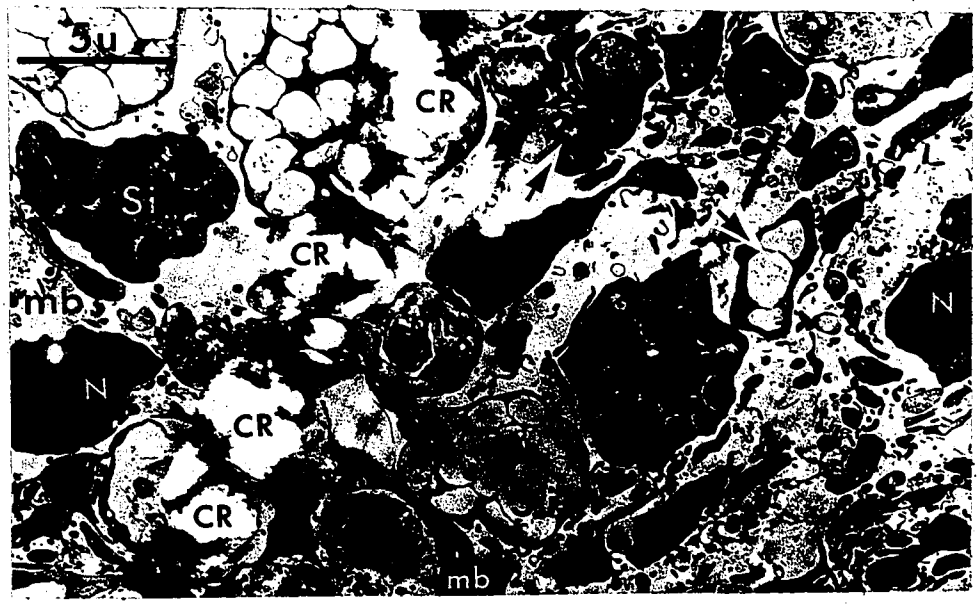
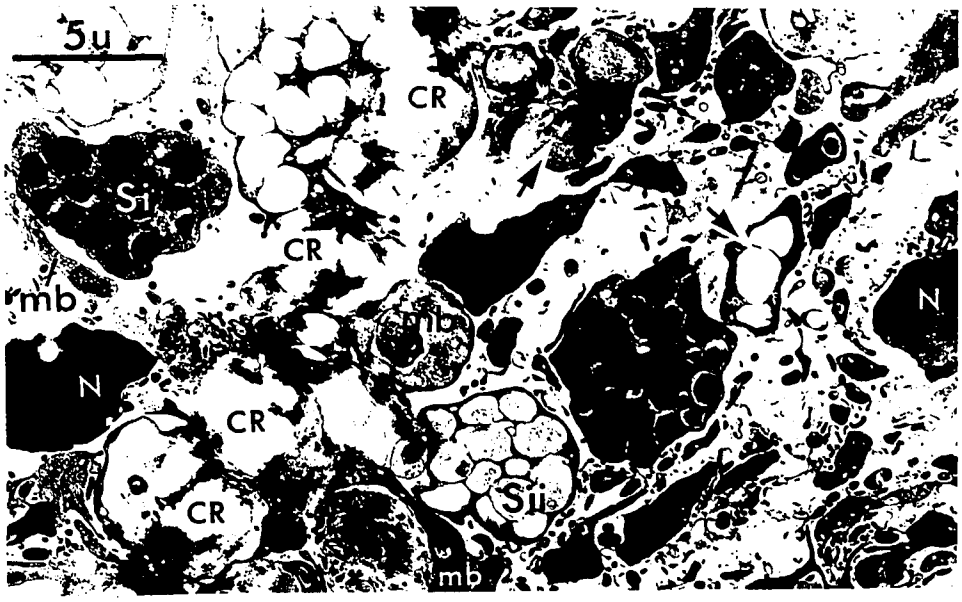


Fig. 2.21 Regenerating test 30 days. Two types of spherule cells are visible within the blastema. Some appear to have contained calcite which has been ripped from the section (CR). Parts from disintegrating spherule cells may be seen at arrows. Note scattered membrane-bound bodies (mb) belonging to blastemal leucocytes. N = nuclei of blastemal leucocytes; S i = dark granular spherule cell; S ii = agranular spherule cell. x4250



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granules were seen together (Fig. 2.2d).

The peritoneal region contained fibrils surrounded by the same membrane-bound connected spherules seen in other parts of the mesoderm. These fibrils were seen to run in two directions and appeared to be banded (Fig. 2.2e). The connected membrane-bound spherules of the peritoneal region were frequently seen to be continuous with those of the dermal region. Nuclei (again enclosed by spherules) were seen to line the coelomic side of the peritoneum.

2.3.2.2 Electron Microscopy of Stage III Regenerating Tests

Results obtained from studies with the electron microscope on regions depositing calcite granules must be considered preliminary. They were mainly performed with the view of extending the "effective resolution" of those results obtained with the light microscope. They were not intended to resolve the ultrastructural relationships of subcellular components of the regenerating blastema.

When regions of the blastema which contain calcite granules were sectioned, the calcite granules usually were ripped out of the section because of jamming on the knife. However, a few sections were obtained where some granules were left intact. In regions actively engaged in calcite depostion, a large number of spherule cells were seen (Fig. 2.21). Three types are distinguishable on the basis of their morphological appearance: cells containing only dark spherules, cells containing only light (empty?) vacuoles and a third cell type which appeared to contain fragments or whole dark spherules

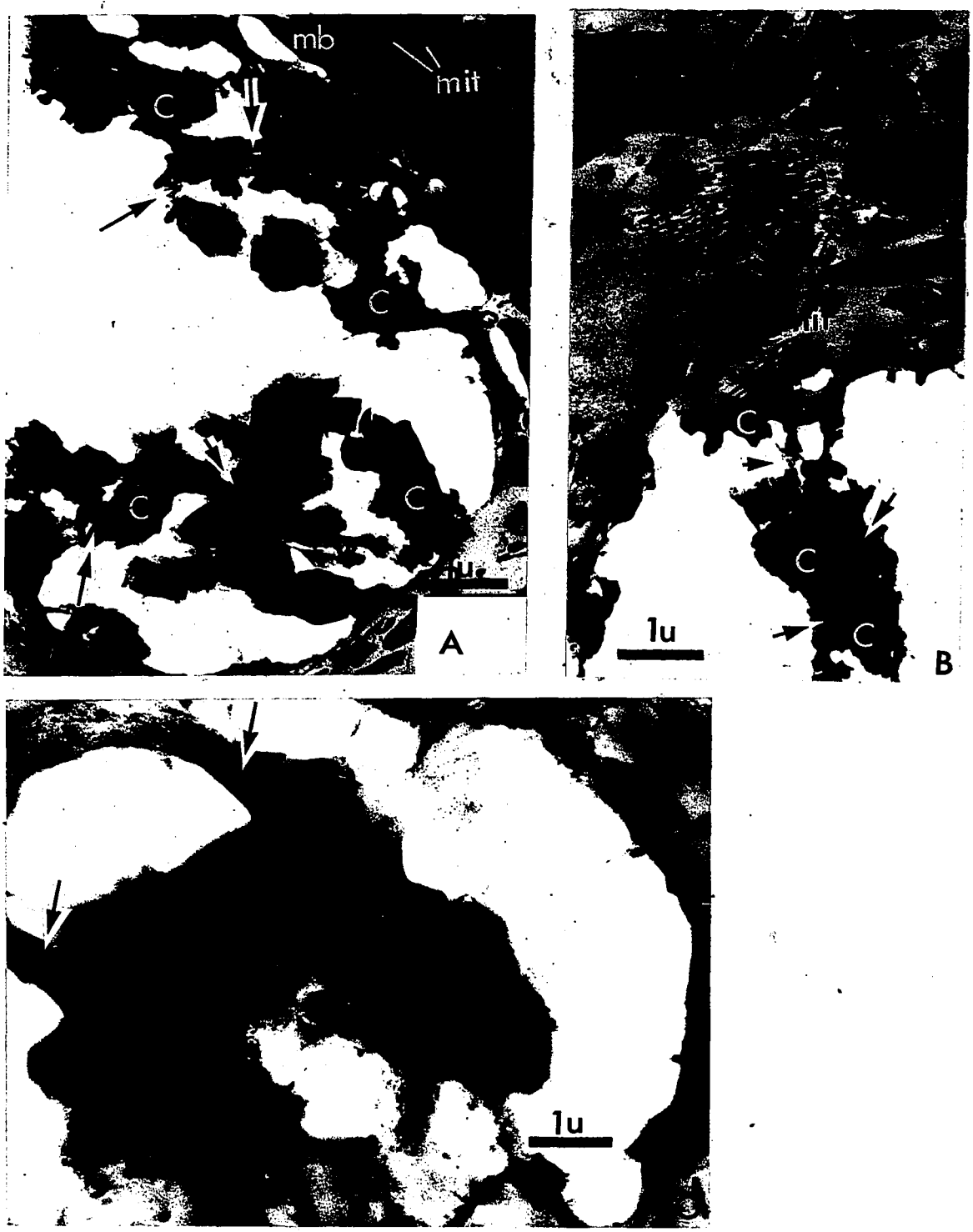
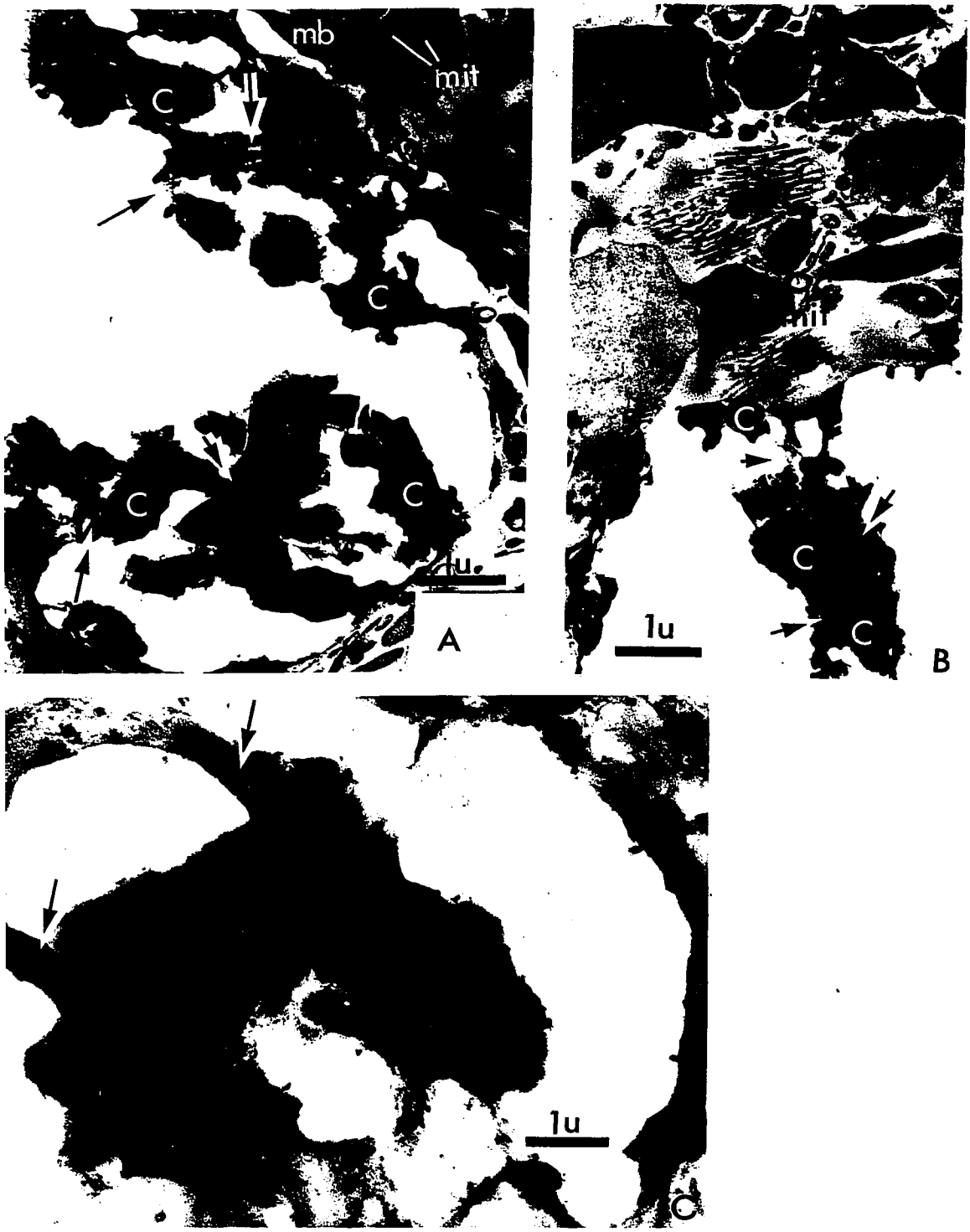


Fig. 2.23 A,B,C Regenerating test 33 days. A and B- details of 'vacuoles' which form within the calcifying blastema. C- detail of calcite granule. Note amorphous material joining scattered calcite fragments (arrows). Dark membrane bound (mb) intracellular regions in A and B contain mitochondria (mit.). C.= calcite granules. A- x13200; B,C- x14400.



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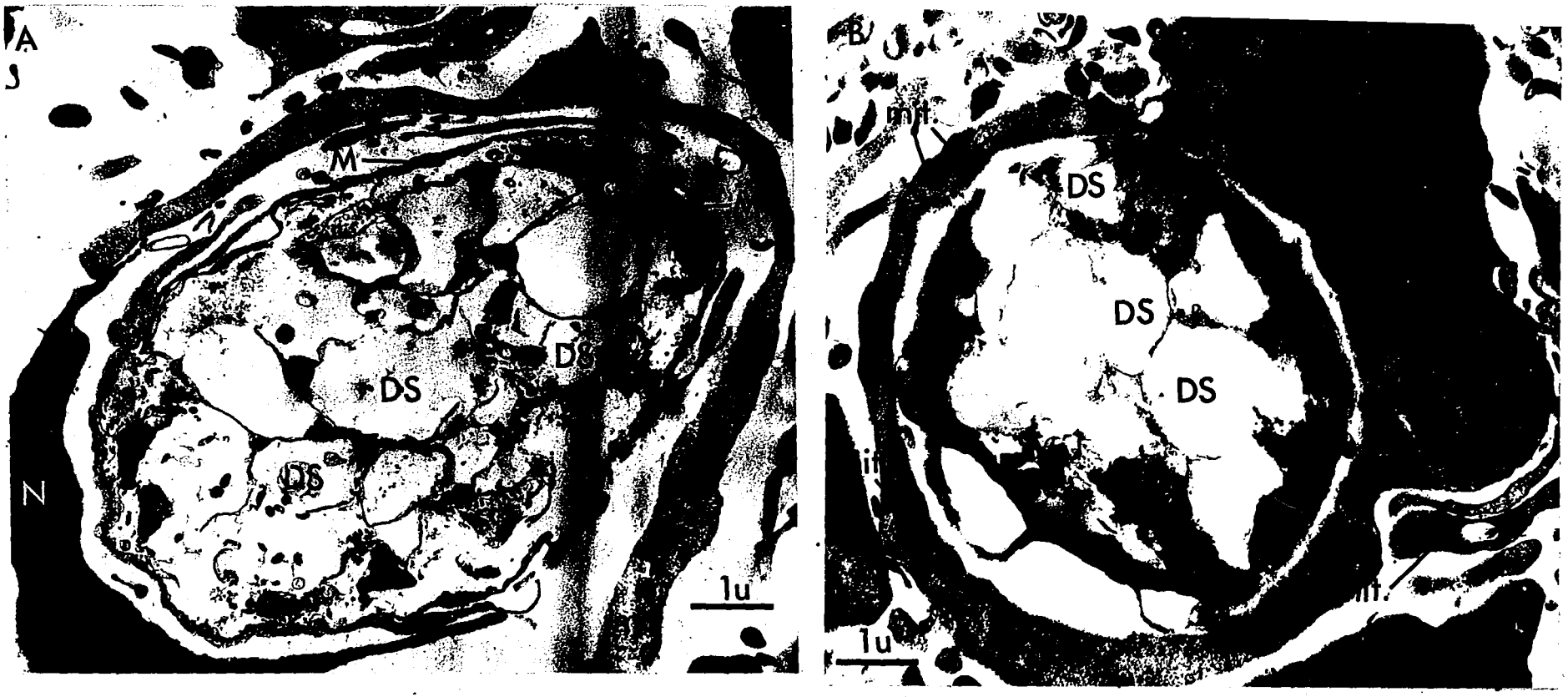


Fig. 2.22 A,B Regenerating test 33 days. Disintegrating spherule cells contained within a calcifying blastema. A and B are progressive stages in disintegration. In A, the membrane-bound body (mb) is partially surrounding the spherule cell which is still contained by a semi-intact membrane (M). In B, the cell has lost its membrane and fused with surrounding membrane-bound body. Nucleus (N) of blastemal leucocytes is closely associated with both spherule cells. Note in B the more granular membrane-bound body, a large number of mitochondria (mit.) and an active Golgi (G). DS = disintegrating spherule. A- x13,400 & B- x13,800

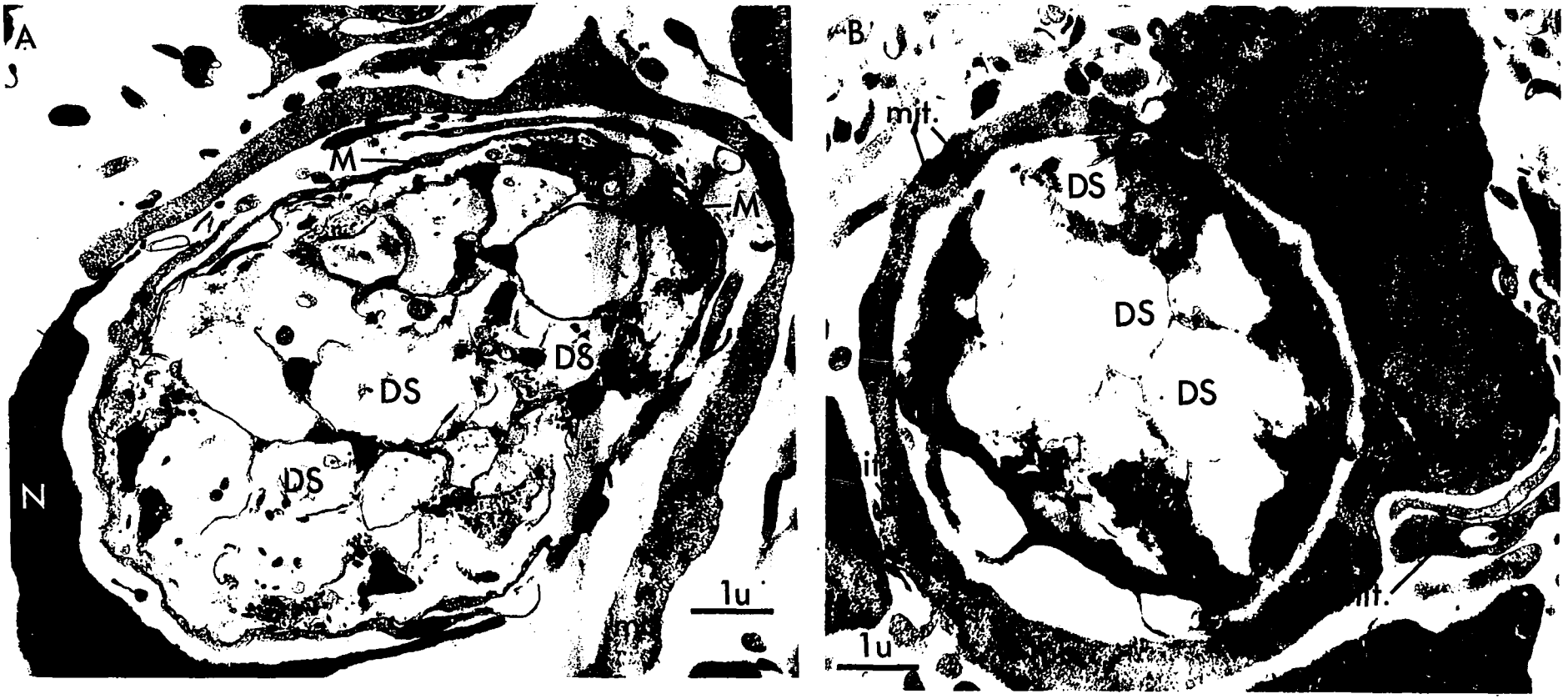


Fig. 2.22 A,B Regenerating test 33 days. Disintegrating spherule cells contained within a calcifying blastema. A and B are progressive stages in disintegration. In A, the membrane-bound body (mb) is partially surrounding the spherule cell which is still contained by a semi-intact membrane (M). In B, the cell has lost its membrane and fused with surrounding membrane-bound body. Nucleus (N) of blastemal leucocytes is closely associated with both spherule cells. Note in B the more granular membrane-bound body, a large number of mitochondria (mit.) and an active Golgi (G). DS = disintegrating spherule. A- x13,400 & B- x13,800

in addition to white empty vacuoles. Many of the spherule cells with empty vacuoles were also seen in various states of disintegration. Clumps of two or three isolated spherules were scattered throughout the extracellular spaces. Some of these disintegrating spherule cells had large pieces of their cytoplasm removed by sectioning. This may be due to the presence of calcite granules inside the cells which "jammed" during sectioning. (Fig.2.21).

Many of the disintegrating spherule cells were surrounded by dark membrane-bound regions which presumably derive from the syncytial leucocytes (Fig.2.22A). In many cases fusion of the plasma membrane of the spherule cell with that of the surrounding membrane-bound leucocyte eytoplasm had occurred (Fig.2.22B).

The leucocyte components of the blastema are present as granular and dark staining membrane-bound regions. These regions are not as spherical as in the case of the normal syncytial sclerocytes.

Examination of Stage II paraffin and Epon embedded sections in the light microscope revealed the presence of "vacuoles" in the leucocytes of the blastema. In many of these vacuoles the first calcite granules were deposited. When examined in the electron microscope, very few of these vacuoles contained intact material. Most consisted of broken and scattered electron dense granules, connected by amorphous material stretched between them (Fig. 23A,B,C). These broken granules exhibited the same shape as broken calcite trabeculae

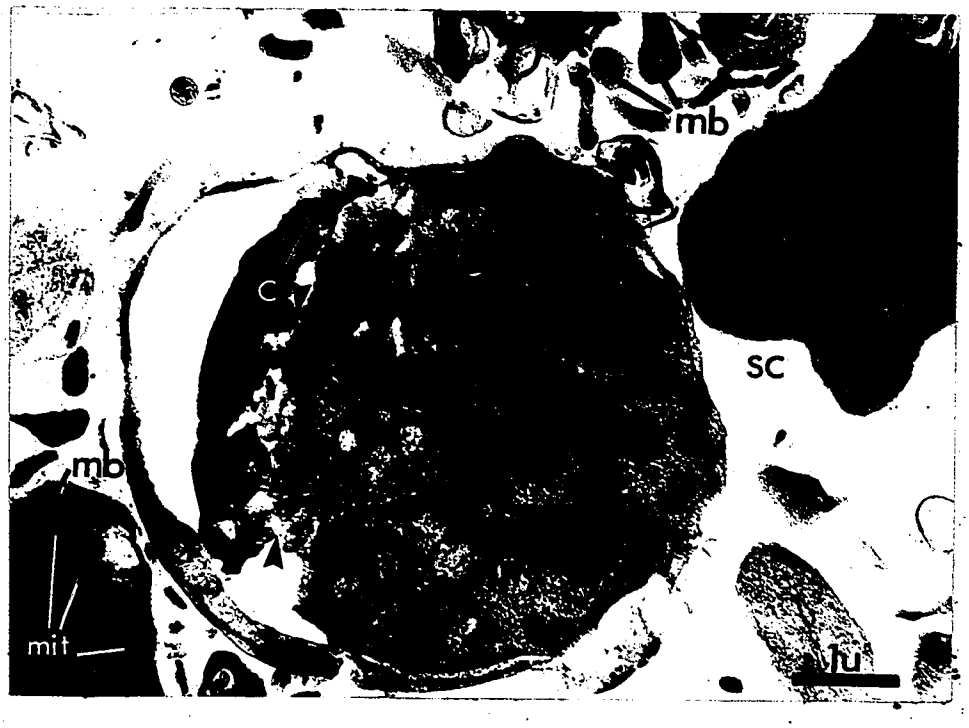
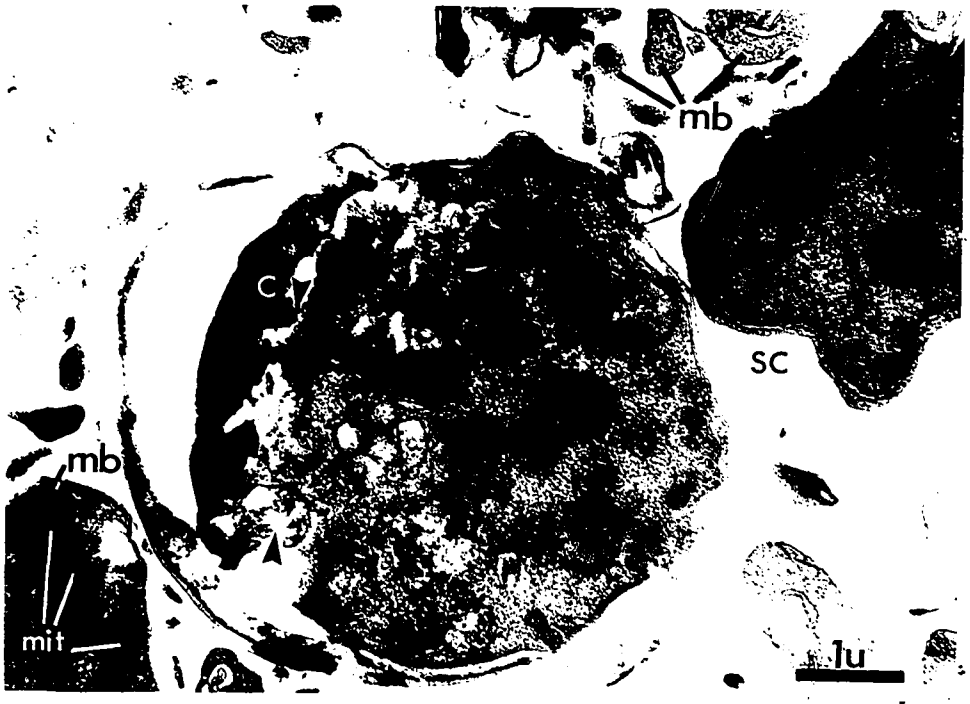


Fig. 2.24 Regenerating test 30 days. Vacuole containing calcite crescent (C) closely applied to spherule containing cell (SC). Spherule (arrow) appears to be fusing to calcite. N = nucleus of blastemal leucocyte; mb = membrane-bound body; mit. = mitochondria. x1500



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seen in undecalcified light microscope sections. In a few sections, both hard and soft components of the "vacuoles" were preserved intact (Fig. 2.24). In these cases the vacuole was seen to be surrounded by a membrane which appears to have derived from the spherule cell contained within it or by fusion of the spherule cell membrane with the cell membrane of the leucocytes. In Fig. 2.24 the contents of the vacuole have been displaced slightly by the knife. The top of the vacuole contains a crescent of very dark electron opaque material. Below it is a spherule cell containing dark spherules. If the electron opaque material is assumed to be calcite (a reasonable assumption in view of its appearance), then the close association of spherule cell and calcite observed in paraffin sections is confirmed in the electron microscope. It is not known whether all such vacuoles contain spherule cells and calcite associated in the same way. Most sections show either calcite granules but no cellular components or only cellular components without calcite. No fibrous material of any sort was seen in any sections of regenerating blastema.

2.4 Discussion

2.4.1 Structure of the Test

Since it is on the same side of the plasma membrane as other subcellular components such as the nucleus and mitochondria, the endoskeleton of the test of the echinoid Strongylocentrorus droebachiensis (and probably other urchins as well) is here considered to be intracellular. This fact is not readily apparent when sections of the test are examined in the light microscope, although the sheath which covers the calcite is very thick and easily stained by conventional methods. The electron microscope has revealed a large number of membrane-bound structures which are probably continuous from cell to cell and thus form a syncytium. Extracellular fluid is contained between the calcite and these membranous structures. Unequivocal evidence for the syncytial nature of the stroma must await examination of serial sections of the test. A similar organization of the sclerocytes of the spines of Echinus esculentus has been postulated by Pilkington (1969). The present investigation provides confirmation of the structural homology between spines and test in sea urchins. Slight differences were noted as follows. Pilkington called the membrane-bound structures containing nuclei, mitochondria, etc., first order spherical bodies, and the smaller, more dense structures near the membrane, second order spherical bodies. In the present material there are fewer of the second order spherical bodies. This is reflected in the less basophilic appearance

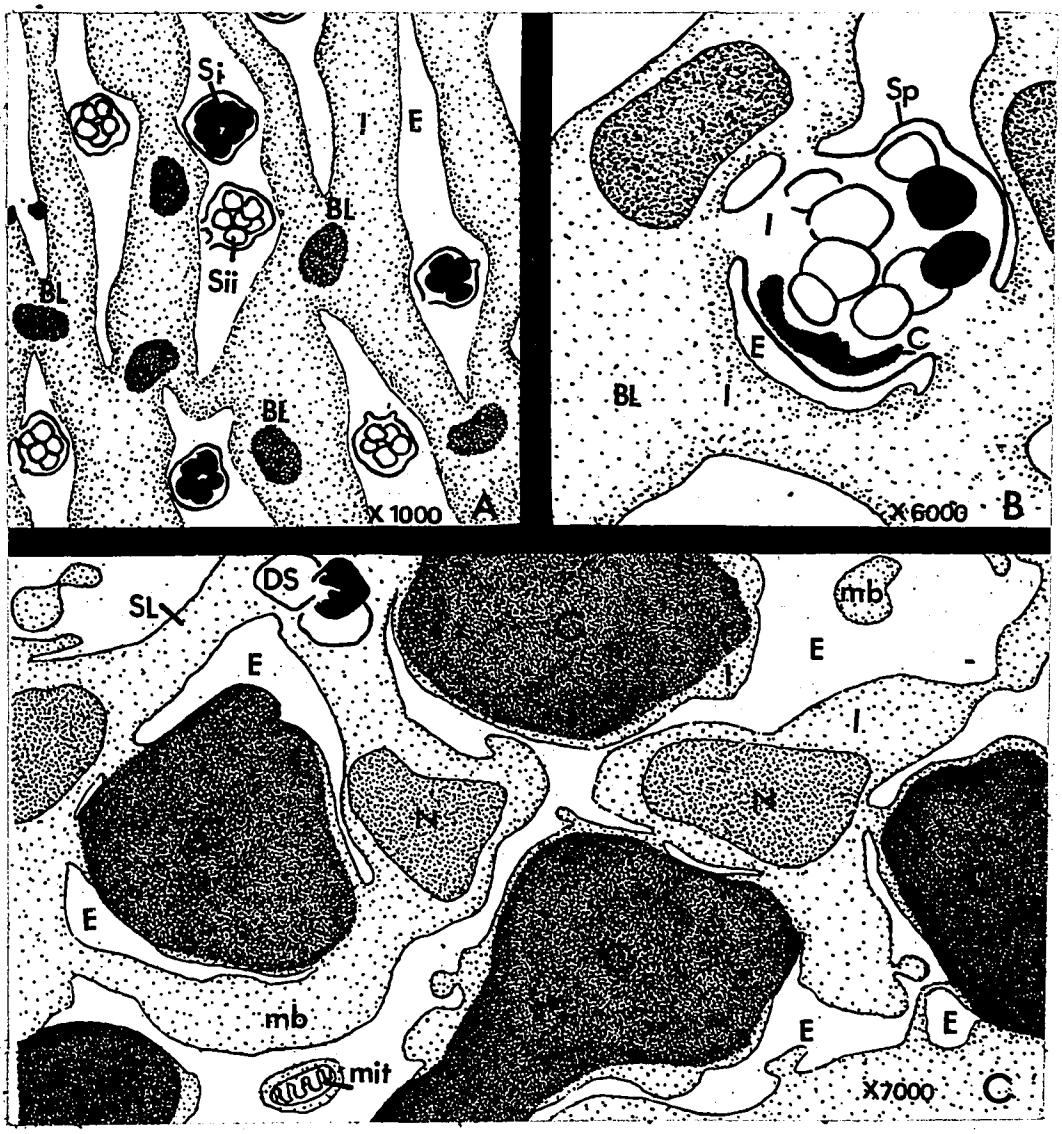
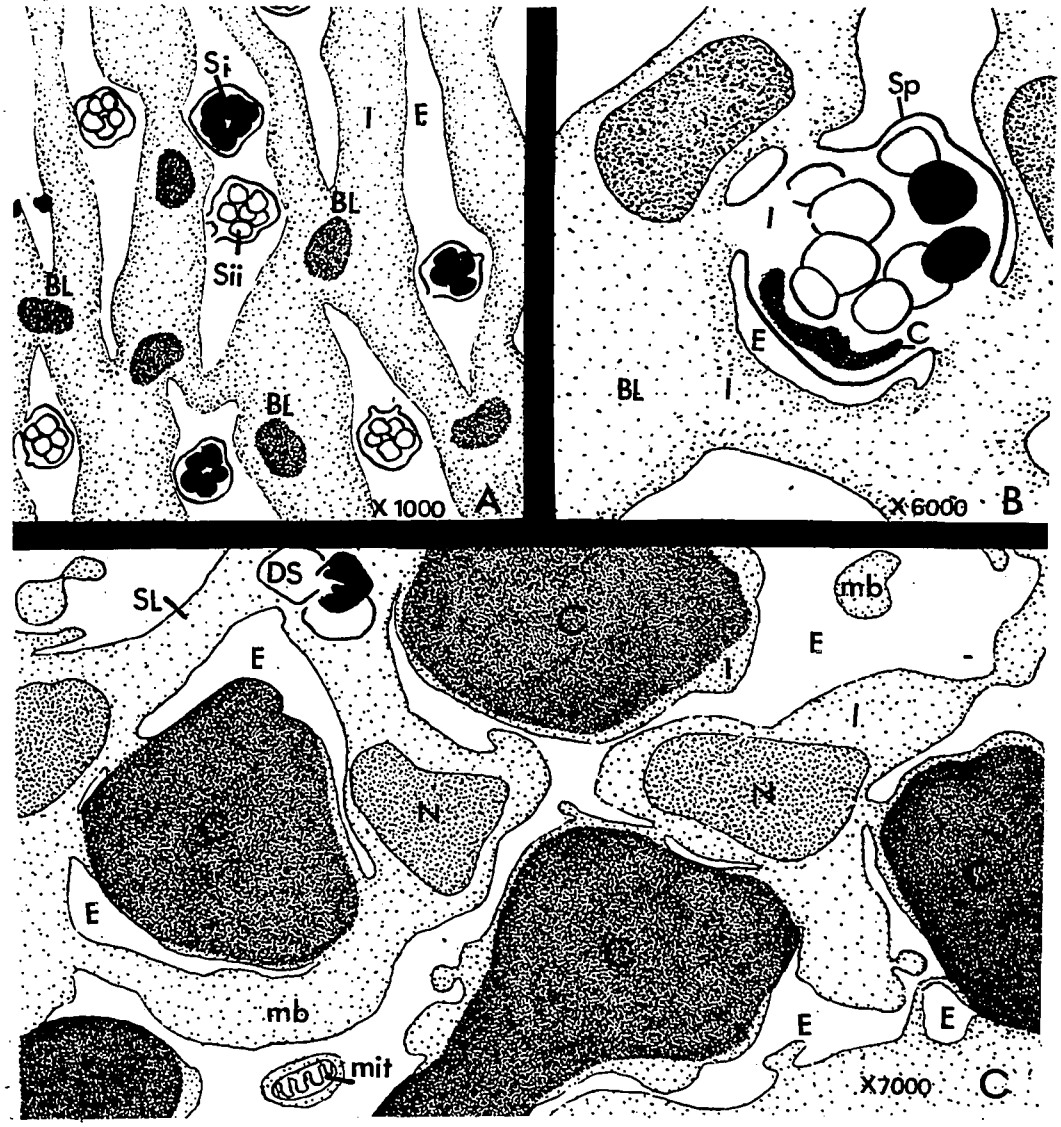


Fig. 2.25 A,B,C Stage III regeneration. Diagram to illustrate possible changes undergone by leucocytes and spherule cells during regeneration and calcification. Note changes undergone by intra (I) and extracellular (E) spaces as calcite (C) grows and displaces various membranes. BL = blastemal leucocytes; SL = syncytial leucocytes; SP = spherule; Si = dark granular spherule cell; Sii = agranular spherule cell; N = nucleus; mit. = mitochondria; mb = membrane bound body; DS = disintegrating spherule cell. Magnification approx.



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of test sclerocytes as compared to spine sclerocytes when these are examined in the light microscope. The decrease in basophilic granularity that was seen as the stereomal regions mature into a normal stroma may reflect decreases in the number of basophilic spherical bodies. Electron microscopy of cells in the later stages of regeneration would be needed to provide confirmation of this.

The spicular skeleton of echinoid larvae is also intracellular, as shown conclusively by Okazaki (1960) and confirmed by Gustafson and Wolpert (1961 a and b), and Gibbons et al. (1969). The larvae were examined in the electron microscope by Gibbons et al. (1969) and their skeletal parts shown to be contained within and surrounded by a membranous sheath or envelope. This envelope, however, was found not to be continuous with the calcite of the spicule, as light microscope examination had previously indicated (Okazaki I960). I have also seen this same sheath surrounding isolated spicules in the scanning electron microscope (unpublished observation). Results presented in Section 2.3 indicate that a similar membrabous sheath probably surrounds the calcite of mature tests.

2.4.2 Role of Coelomocytes

The perivisceral fluid coelomocytes were seen to be involved in all stages of regeneration and calcite deposition. Although specific functions to each cell type cannot be stated with certainty, a discussion of their possible roles in the events outlined seems warranted. A tentative proposal for the relationship between coelomocytes and calcite during later stages of regeneration is diagrammed in Fig. 2.25.

2.4.2.1 Leucocytes

The leucocytes (bladder amoebocytes) were seen to form extensive clots to plug up holes made in the test. They were also observed to phagocytize carmine particles injected into the coelomic fluid. On the basis of the observation that carmine particles are trapped within the leucocyte syncytium from Stage I to the end of Stage III, it has been postulated that these leucocytes may be scleroblastic. The difficulty in determining where in the syncytium carmine ends up, i.e., intra- or extracellularly, is acknowledged, although it is probable that if the entire cell population of the blastema were renewed, much less (or no) carmine would remain. Non-regenerating areas of injected animals showed little or no carmine present in their dermis, thus the possibility that carmine was being excreted from regenerating areas (as is often the case, Hyman 1955) seems unlikely. Furthermore, the amount of carmine present during Stage I and II and beginning of III is much greater than would be expected if only excretion were occurring. The fusion of the clot leucocytes with the mesenchymal cells of the dermis seen around the middle of Stage I suggests that the leucocytes can and do remain active for extended periods of time. The thromboblastic and phagocytic ability of these cells has been reported by numerous investigators (Johnson 1969a). Kindred (1924) and Théel (1892, 1921) also assigned a scleroblastic function to the leucocytes based on their observations. Schinke (1950) presented evidence to suggest that leucocytes of the perivisceral fluid were replaced by dermal amoebocytes,

(leucocytes). Pilkington (1969) also concluded that the phagocytic leucocytes were able to undergo transformation into scleroblasts. Johnson and Chapman (1970) came to a similar conclusion from observations on regenerating spines.

Results of the present investigation also show a decrease in the amount of cellular material as regeneration proceeds. No mitotic figures were ever seen. Johnson (1969a) suggested that nuclear migration through cytoplasmic channels demarcated by microtubules may occur in leucocyte syncytia, especially if the cells are arranged in a row. I saw leucocytes of dense regions arranged in rows during Stage I and part of Stage II, suggesting that exchange of nuclear material may occur in the way envisaged by Johnson. Holland et al (1965) concluded, on the basis of tritium labelling of urchin coelomocytes, that these cells resembled expanding rather than renewing cell populations. Cowden (1968) studied cutaneous wound healing in the connective tissue of a holothurian. He noted that no mitotic figures were seen in the "fibroblasts" that affected wound closure by migrating to the wound site. He concluded that cell proliferation had no role in affecting wound closure.

It appears then from the foregoing that the cells which form the clot in Stage I of regenerating blastema are the same cells responsible for calcite deposition in later stages.

2.4.2.2 Spherule and Vibratile Cells

Observations with the light and electron microscope

on regenerating blastema showed the presence of cell types which seem to be intermediate between Type I and Type II spherule cells. This may mean that either each in turn goes through a cycle of morphological and presumably physiological changes, or that interconversion between the two cell types occurs. The role of vibratile cells in this scheme is obscure since they were not always distinguishable from spherule cells in tissue sections. Also, great variability in appearance and staining with different fixation techniques is known to occur in all these cells (Liebman 1950; Johnson 1969b), making comparison with living material difficult. The origin and possible interconversion of cell types which occur in the perivisceral fluid has been mentioned by several investigators. Frenzel (1892) suggested that Type I spherule cells differentiated into bladder amoebocytes. Kindred (1926) thought that bladder amoebocytes differentiated into Type II spherule cells which then formed Type I spherule cells. Liebman (1950) suggested that vibratile cells originated from the peritoneal lining and then differentiated into bladder amoebocytes. He also believed that Type I spherule cells transformed into Type II spherule cells. Schinke (1950) reported that Type II spherule cells arise from connective tissue cells of the dermis and possibly differentiate into bladder amoebocytes. Hetzel (1965) suggested that lymphocytes (bladder amoebocytes) originate from mesenchymal cells in hemal vessels of holothurians and later differentiate into other cell types (note that vibratile cells are absent from holothurians). From autoradiographic

observations of perivisceral fluid cells and nearly all organs of sea urchins implicated in coelomocyte production, Holland et al.(1965) came to no conclusions except for the observation that bladder amoebocytes, Type I and Type II spherule cells take up the label and divide in the circulation, whereas vibratile cells do not. This and other observations suggested that vibratile cells originate in the parietal peritoneum. Johnson (1969b) was also unable to determine any definite relationship between the cells, except to suggest a constantly changing biological state as characteristic of the coelomocytes. My observations of a large number of spherule cells (and possibly vibratile cells) in calcifying blastema would seem to support suggestions of a mesenchymal origin for these cells, since they were observed in mature stromae and in Stage III blastemae. However, the cells occurred in the greatest concentration in the prestereomal regions, were present to some extent in maturing stereoms, and then declined to very low concentrations in fully matured stereoms. Also the spherule cells appeared to be undergoing changes in the blastemae which ended in their disintegration. If they were originating in the newly formed stereom, then it is reasonable to suppose that they would leave the dermis intact to complete whatever cycle they may undergo in the perivisceral fluid. Definite conclusions concerning the relationship of Type I and Type II cells are difficult to make as they are known to variably lose or retain their spherules in many fixatives and stains (Johnson, 1969b).

Vibratile cells were also seen in prestereomal regions. Since there is some evidence to suggest that these

cells originate in the parietal peritoneum, their presence in the blastema suggests that they may have migrated there with the other spherule cells. Finally, none of these observations precludes the possibility that coelomocytes were formed in regions of the test which were not regenerating, and that they then migrated to the regenerating blastema.

Spherule cells (and possibly vibratile cells) were seen to be concentrated in large numbers in the prestereomal and, to a lesser extent, the stereomal regions of regenerating blastema. They were most concentrated during early Stage III, just prior to the appearance of the first calcite granules. In addition, a very close association was often seen between calcite granules and isolated spherules or whole spherule cells in both the light and electron microscope. Some electron micrographs appeared to show spherule cells with remnants of very dark electron opaque granules inside them. From this it may be suggested that spherule (vibratile?) cells are involved in formation of new plates in the test. Prouho (1887) and Kindred (1924) also noted the large number of Type I spherule cells in regenerating tests. More recently, Johnson and Chapman (1970) reported a large number of amassed eleocytes (Type I spherule cells) in spines that were regenerating new calcite. They suggested they were acting as a defense against invading microorganisms. Vevers (1963, 1966) suggested an algistatic function for these cells which contain a napthoquinone pigment echinochrome A. Gustafson and Wolpert (1961a) noted aggregations of pigment cells around the tips of growing arms of larval spicules.

Rulon (1941) noted the absence of a skeleton when pigment cells were experimentally excluded from the larvae. Besides a possible algistatic (bacteriological) function mentioned above, Type I spherule cells which contain the pigment Echinochrome A have been said to be involved in respiration (McMunn 1885; Griffiths 1892; Cannan 1927), nutrition and nutrient distribution (Kindred 1924; Boolootian and Lasker 1964; Burton 1966), secretion (Frenzel 1892) and digestion (Pequignat 1966). Type II spherule cells have principally been implicated in nutrition and nutrient dispersal (Geddes 1880; Kindred 1924, 1926; Boolootian and Lasker 1964; Pequignat 1966; Burton 1966). They have been shown to contain protein possibly of 3 indolyl derivative, and/or phospholipid and fatty acid. In short then, most recent evidence suggests that Type I spherule cells are involved in defense of the echinoid from invading microorganisms and Type II spherule cells are involved in nutrition. My results do not negate either of these possible functions. In regenerating blastemae, Type I spherule cells are present at the very beginning of regeneration, particularly in the center of the wound, but are absent during Stage II. They again appear during calcification in Stage III. They are present in the growing epidermis. If defense against infection was the sole function of the Type I spherule cell, then they would be expected to occur throughout the regeneration cycle rather than at specific stages, as they apparently do. Furthermore, outside of references to Vevers' (1966) suggestion, there has been no experimental evidence to indicate unequivocal algistatic properties , although many quinone-type molecules

exhibit fungicidal properties (Fieser and Fieser, 1961). The findings of Boolootian and Lasker (1964) suggest a nutritive function for the Type I and possibly Type II spherule cells. My results are consistent with the idea that the spherule cells are distributing nutrient materials to the actively regenerating blastema. Their conspicuous absence during Stage II of regeneration may indicate that the leucocytes are actively differentiating (i.e. lag period) before beginning skeletogenesis. Just how the naphthoquinone pigment fits into a nutritive function is unclear. It is not unreasonable to suppose in view of the diversity of function of the leucocytes, that spherule cells could also be capable, under different conditions, of assuming different functions.

Reports that Type II spherule cells contain protein is intriguing, particularly since one report (Gibbons et al, 1969) has indicated that the larval skeleton appears to be secreted in a non-fibrillar protein or glyco-protein matrix. This will be discussed further in the next section.

Vibratile cells have been seen to form a protective gel to prevent escape of body fluids or invasion of foreign material (Johnson 1969a). I have also observed gel-like material surrounding newly formed clots, and large numbers of vibratile cells appear around the periphery of these clots. In addition, vibratile cells were difficult to see in clots older than five or six days, thus suggesting they are rapidly phagocytized by the leucocytes.

2.4.3 Role of the Epidermis

Growth of the epidermis over the blastema appears to be a prerequisite for the appearance of calcite in tissue sections. In addition, spherule cells amass in early Stage III blastema after regrowth of the epidermis. Regeneration was seen to be retarded if the epidermis was removed. These observations may suggest an inductive role of this tissue, similar to that observed in other regenerating systems (Goss 1969). The radial nerve has been shown to be involved directly in regeneration of starfish arm (Huet, 1967); the same may be true for the ectoneural nerve trunks associated with the echinoid epidermis.

2.4.4 The Organic Matrix

Studies using polarized optics, X-ray crystallography and scanning electron microscopy have shown that each echinoid skeletal unit (plate or spine) behaves as a single crystal of calcite (Raup 1966). Studies which have indicated a polycrystalline nature have been based on observations on sectioned or polished material, a technique which does not preserve the crystallographic integrity of materials so treated (Towe and Hamilton 1968). A single crystal arrangement could arise by appositional growth from a seed crystal or by epitaxial growth from highly oriented subunits. It is likely that both configurations arise by deposition in some sort of organic substratum, and it is the nature of this so-called organic matrix that remains controversial. Travis et al.(1967) and Travis (1970) claimed to have isolated and analyzed a native type collagen

from the tissues of two echinoids. While the material appears to be collagen, there is some question as to whether it is the organic matrix of the calcite. Moss and Meehan (1967) found dense collagenous bundles in echinoid tests, but suggested that it was not calcified. Klein and Currey (1970) showed that there was no collagen bound to the calcified tissues of Strongylocentrotus drobachiensis. Instead they found .1-.3% noncollagenous protein associated with these tissues. Similarlly, Pilkington (1969) concluded that the material seen by Travis et al. (1967) was probably connective tissue collagen. He makes the point that "the more accurately aligned and closely packed the crystallites are, the smaller the conceptual and physical differences between a polycrystalline aggregate and a unit structure." Pilkington (1969) postulated an organic phase isomorphous with the calcite crystal lattice to explain his results which were based on total nitrogen analysis of the coronal plates of Echinus esculentus which had been treated with sodium hydroxide or bacterial protease. He found that the material analyzed contained about .1 mg N/gm calcite. Gibbons et al. (1969) describe a non-fibrillar material, probably protein or glycoprotein, which they say may form the matrix of larval spicules in the echinoid Arbacia.

Results of the present investigation do not support a collagenous matrix for echinoid calcite. Results of electron microscopy show fibrous material, presumably collagen, concentrated only near the epidermal and peritoneal regions. Furthermore, this collagen was seen to be extracellular. Although none was found in regenerating blastema, it may have been removed by "jamming". Since echinoid calcite appears to be intracellular,

it is difficult to reconcile a collagen matrix.

Spherule cells either Type I of Type II appear to congregate in calcifying regions of the regenerating blastema (Fig. 2.26). There is a decrease in their numbers as more calcite becomes deposited. Some possibility exists then, that they may be involved in matrix elaboration. Kollmann (1908), Ohuye (1936), Burton (1966) and Johnson (1969b) have all found that Type II spherule cells contain a protein, probably "albuminous". The present investigations of regenerating blastema using the electron microscope have revealed vacuoles containing granules (probably calcite) which appear to be connected by amorphous material. This amorphous material extends to the walls of the vacuoles. There is some indication that these vacuoles derived from or are closely associated with one or the other spherule cell. The amorphous material may be a glycoprotein or protein postulated by Gibbons et al. (1969).

The spherule cells have been compared to vertebrate mast cells and basophil leucocytes (Cowden 1968). Doyle and McNeill (1964) postulated, on the basis of electron microscope observations of the holothurian respiratory tree, that spherule cells of some type (probably Type II) were involved in elaboration of connective tissue ground substance. Cowden (1968), suggests that the contents of the spherules of these cells is an acid mucopolysaccharide, which may be involved in cutaneous wound repair in holothurians. There is some indication that these cells are involed in ground substance production in echinoids from the results of Moss and Murchison (1966).

Type I spherule cells are known to contain the pigment echinochrome A (McMunn 1885). This pigment is a pentahydroxy-quinone derivative of the naphthoquinone, B ethylnaphthalene (Fieser and Fieser 1961). Its function was thought to be respiratory (McMunn 1885; Griffiths 1892). However, Cannan (1927) found that echinochrome formed no dissociable compound with oxygen. He also found that because of its very low redox potential, echinochrome entered into oxidation reduction reactions very easily. In the form in which it is found in the spherule cells it appears to be in the oxidant form. Cannan postulated on this basis that it was an oxygen activator. Mention has been made of the possible role of echinochrome as an algistatic substance (Vevers 1963; Johnson and Chapman 1970). Echinochrome A and a closely related ∝ naphthoquinone spinochrome A (hydroxyechinochrome) are known to occur in the spines and test of many echinoids (Nishibori 1961; Fox and Hopkins 1966). Furthermore, the color of the spines and test (purple to olive green) of these echinoids (Strongylocentrotus droebachiensis among them) is due to the formation of calcium salts between the pigments and calcium carbonate of the skeletal structures (Goodwin and Srisukh 1950). Ball and Cooper (1949) showed that the pigment when extracted from soft and hard tissues of sea urchins (in this case Arbacia) always has a protein moeity associated with it. They note that the ionizable hydroxyl group of the pigment does not seem to be involved in the linkage to protein carrier. Kuhn and Wallenfels (1940) showed that naphthaquinone pigments do not occur in the free state in Arbacia eggs, but are conjugated with a high molecular weight

protein in the form of a binary or tertiary complex involving the acidic B -hydroxyl groups of the quinone and basic amino groups of the protein. This protein binding is significant in view of the possible role of echinochrome-protein complexes as a possible organic matrix of echinoid calcite.

Millott (1957), also noted the ease with which these types of pigment formed calcium salts and thought that they existed in the cells in a buffered colloidal state bound to a protein. Stern (1938) and Suto (1938) also noted this close association of echinochrome and protein. Values for the concentration of echinochrome in the test of Arbacia are .19 mg gm of tissue (Ball and Cooper 1949). Pilkington (1969) found .1 mg gm as the concentration of proteinaceous material in Echinus esculentus. Although such comparisons may be misleading, it is interesting that the two substances occur in concentrations of similar orders of magnitude. They are present in the eggs of many urchins and in some appear at gastrulation just prior to the appearance of spicules. Although not all families of echinoids possess naphthoquinone pigments, quinone pigments of some type occur in representatives of all classes surveyed (Fox and Hopkins 1966). Quinones are also known to easily form bonds with the sulfhydryl groups of proteins (Fieser and Fieser 1961).

Thus there appears to be some evidence from the present investigation and some support from previous work that a calcium-pigment-protein complex may exist in an isomorphous state in the skeletal parts of sea urchins.

The possibility also exists that there is no organic matrix in the echinoid test skeleton. Those determinations that have been made indicate very low concentration of nitrogenous material which may have become trapped in the calcite during its precipitation. Since the calcite is intracellular, active uptake by the cells could result in conditions of supersaturation in which calcite would precipitate spontaneously. This seems unlikely however, in view of the pecularities of crystal form exhibited by these animals.

Large numbers of mitochondria and active golgi were observed within the membrane-bound bodies of calcifying blastema. Although ultrastructural morphology is beyond the scope of the present investigation, this observation is important in view of recent suggestions (Eanes and Posner 1970) that these components may be involved in the organization of matrix or mineral of calcifying tissues.

3. Histochemical Observations

3.1 Introduction

The investigations reported in this section are concerned with the histochemistry of the regenerating test of Strongylocentrotus droebachiensis, particularly as it relates to skeletogenesis. The studies of Moss and Meehan (1967), and Pilkington (1969) have dealt with the histochemistry of the normal test of echinoids. In general, cellular material responsible for test deposition termed the stroma, consists of mesenchymally derived cells called sclerocytes which, in addition to being slightly metachromatic and positive to Alcian Blue, contain substantial amounts of basophilic granular material. The granular material may be PAS positive and usually stains below pH 3.5. Often argyrophilic fibres are associated with some of the stroma cells, particularly in regions near the sutural connective tissues. Granular material in the stroma is thought by Pilkington (1969) to contain lipid and/or carbohydrate, part of which may be an acid mucopolysaccharide.

Coelomic elements of the perivisceral fluid are normally present in mature tests and have been implicated in the physiology of the stroma cells. Aspects of the histochemistry of these cells have been studied by Kollmann (1908), Ohuye (1936), Boolootian and Lasker (1964), Burton (1966) and Johnson (1969b). Johnson (1969a) has reviewed the literature concerned with the nomenclature and appearance of these cells. The cytoplasm of the bladder amoebocytes (leucocytes) is weakly

positive to protein stains and is metachromatic, containing granular material which may be acid mucopolysaccharide with a protein core. Red spherule cells (Type I) contain a red pigment echinochrome A (McMunn 1885) and possibly glycogen (Boolootian and Lasker 1964). The Type II or colorless spherule cells have often been confused histochemically with Type I spherule cells (Johnson 1969a). However, in general they have been reported to contain a protein possibly of the indolyl type (Burton 1966). Vibratile cells appear to contain a carbohydrate fraction, neutral or acidic, associated with a proteinaceous core.

The mechanism of calcification of echinoids is unknown. Studies on the physiology of skeleton formation have been mostly concerned with larval forms: Pouchet and Chabry (1899), Herbst (1904), Rapkine and Prenant (1925), Vlès and Gex (1925), Bouxin (1926 a & b), Rapkine and Bouxin (1926), Chambers and Pollack (1927), Hirabayashi (1937), Lowndes (1944), Okazaki (1956), Bevelander and Nakahara (1960), Okazaki (1960, 1961). These studies have shown that the pH of the blastular cavity rises during spicule deposition. This is possibly due to incorporation of $CO_3^{=}$ into the skeleton, HCO_3^{-} ions being absorbed to supplement those derived from respiratory CO_2 (Nicol 1967). The primary mesenchyme cells responsible for spicule deposition are able to concentrate Ca (Ca^{45}). As might be expected, variations in calcium and hydrogen ion concentration (and many other ions such as sulphate and magnesium) have pronounced effects on the final shape and form of the larval

skeleton. It should be emphasized that most of these changes in the spicules appear to be due to adverse effects on form (and presumably function) of the primary mesenchyme cells. Most recent evidence (Gibbons et al. 1969; Pilkington 1969), suggests that echinoid larval skeletons may be deposited intracellularly in a non-fibrillar proteinaceous or glycoproteinaceous matrix, but this has not been shown conclusively.

It has been stated on several occasions that calcification in adult echinoids probably occurs by mechanisms similar to those of the larvae (Nichols and Currey 1968; Raup 1966). There are certain similarities between the two, but the mechanizm of skeletogenis is unknown. Particularly lacking are studies on cellular aspects of echinoid calcification. Travis (1970) has proposed that test deposition occurs by epitaxial growth from crystals seeded by specific active groups on a collagen matrix, in a manner similar to that proposed for vertebrate calcification (Glimcher 1960). Kobayashi and Taki (1969) have suggested that the fibrous elements (sutural connective tissues) are directly involved in test deposition and that there may be two more or less distinct modes of calcification of echinoid tests - one responsible for increases in plate size, another responsible for increases in the number of plates. Several studies have indicated that no collagenous calcified matrix is present in the echinoids (Currey and Nichols 1967; Nichols and Currey 1968; Pilkington 1969; Klein and Currey 1970). Motohiro (1970) found the concentration of protein and a chondroitin sulphate-like substance in the perivisceral fluid to be much lower in summer specimens of Strongylocentrotus

intermedius than in winter specimens. Since summer specimens are known to cease test growth, Motohiro has postulated that these substances may be involved in increases in plate size and number.

Inhibitors of the enzyme carbonic anhydrase have been shown to interfere with normal skeleton formation in larvae (Stolkowski 1948) and reduce the uptake of Ca^{45} by regenerating spines of adults (Heatfield 1970). The implication is that this enzyme may be involved in supplying $CO_3^{=}$ by catalyzing the hydration of carbon dioxide.

The present investigation was undertaken for three reasons: first, to obtain further information by histochemical means concerning the "organic matrix" of the echinoid endoskeleton; second, to provide a basis for comparison between the histochemical reactions of the regenerating echinoid test with those of other calcifying systems; and third, to examine features of the histochemistry of test regeneration which may provide evidence concerning the mechanism of calcification in echinoderms.

3.2 Methods and Materials

Pieces of test of the sea urchin Strongylocentrotus droebachiensis were perforated as described previously (Section 2.2) and allowed to regenerate for varying periods of time, ranging from two to eighty-two days. Pieces of test containing the regenerating region, henceforth referred to as the blastema, were removed, fixed, decalcified, embedded and sectioned, as described previously. A variety of histochemical tests were performed on this material. Details of the methods used are contained in the Appendix. Each stain will be outlined in the appropriate section of Results below.

3.3 Results

3.3.1 Introduction

The process of regeneration of the test elements of echinoids may be considered divisible into three stages. Stage I; initial clotting by leucocytes (bladder amoebocytes) into a syncytium consisting of a region of loosely organized cells and a region of densely organized cells. Within this clotted syncytium, or blastema, spherule cells and vibratile cells may also become trapped. Stage II; thinning and strengthening of the blastema during which its constituent leucocytes become more elongated, some forming fibrous elements criss-crossing the wound. There are few spherule cells and vibratile cells present in the syncytium. Regrowth of the epidermis occurs so that the wound may be almost completely covered by this tissue by the end of Stage II. During Stage III, the blastema becomes filled with numerous regenerated platelets. This stage is divided into two parts. Stage IIIA; the initial deposition of calcite granules occurs within the blastema. The appearance of calcite is accompanied by a significant increase in the number of spherule and vibratile cells in calcifying regions. These regions have been called prestereomal regions. Stage IIIB; is the stage during which the test plates are replaced by many smaller hexagonally shaped platelets. The granular blastema now consists of three regions: the prestereomal region which is depositing new calcite, the stereomal region, which contains the first large trabeculae

of the new plates; and the peritoneal region which is undergoing first differentiation into fibrous elements, and then thinning down to form the normal peritoneal fibres of the fully regenerated test. As the plates mature, there is a decrease in overall staining of the organic material of the stroma as well as a decrease in the ratio of organic to inorganic components.

Calcite is deposited intracellularly within the blastema. As the calcite granules elongate they displace the cells of the syncytium compressing their membranes into many complex convolutions. The extracellular fluid is between these convolutions, but is not easily resolved in the light microscope (see Fig. 2.25C).

Results of histochemical procedures for detection of protein, carbohydrate, pigment and reducing material, and connective tissue reticular elements, are given in Tables 3.1 - 3.7. Table 3.1 shows the histochemical reactions of the normal test elements which are the same as those of the fully regenerated test. In some cases the spherule cells were not distinguishable from each other or from vibratile cells on the basis of color reaction and/or morphological appearance. In these cases they are treated as if they were a single cell.

In the description below components of the blastema have been divided into (1) the cellular elements, including leucocytes, spherule cells and vibratile cells (2) connective tissue elements including the sutural connective tissue elements and the peritoneum.

3.3.2 Protein Stains

Two stains for protein were used: the mercury bromphenol blue (MBPB) stain of Mazia et al.(1953), which is a general stain for protein, and Millon's reaction (Pearse, 1968) which is specific for tyrosyl groups of protein. Since there is some question as to the specificity of the MBPB reaction (Baker, 1958; Kanwar, 1960) it was used with a deaminated control which increases the specificity of the staining reaction by completely destroying amino groups (Lillie, 1965).

3.3.2.1 Cellular elements

Leucocytes: Leucocytes in the dense configuration when stained with MBPB had dark blue cytoplasms throughout the three stages of regeneration (Table 3.2). As the blastema became more mature there was a decrease in cytoplasmic staining. There appears to be a decrease in proteinaceous material within the leucocytes during latter stages of regeneration which was also related to decreases in the overall size of the syncytial leucocytes. Millon's reaction did not stain the cytoplasm of cells in regenerating blastema, indicating the absence of tyrosyl containing proteins in these cells.

Granular inclusions which appear in the prestereom during early Stage III contained granules which stained red and dark blue with MBPB. Some stained yellow with Millon's. These granules probably contain protein and some may contain a tyrosyl or similar group.

Loose regions of leucocytes were stained much less

strongly by MBPB and not at all by Millon's, suggesting little protein and no tyrosine containing material.

Deamination eliminated staining in most leucocytes except for some granular inclusions. Since a few muscle fibres were also still stained lightly following deamination it may be that the treatment employed was not long enough to produce complete deamination (16 hours at 20°C).

Spherule and Vibratile cells: Spherule cells of two types were seen; those containing dark red granules which obscured the cytoplasm were taken to be Type I cells and those with dark blue granular or amorphous cytoplasms were taken to be Type II cells or vibratile cells. These cells appeared similar in all stages of regeneration when stained with MBPB. Deamination left cells with blue granules (Type I) and cells with refractile granules (Type II or vibratile cells). There was a larger number of cells with refractile granules in Stage IIIB following MBPB, indicating a possible turnover of granular material in these cells. Millon's reagent colored one of the spherule cells very lightly, but it was not clear which of the two it was. Vibratile cells were clearly distinguishable only in fully regenerated tests. It is likely then that the spherule cells contain a protein (and some tyrosine) associated with their granules and possibly also their cytoplasms. Vibratile cells may contain protein in their cytoplasms.

It is clear from the foregoing that the granular contents of Stage III leucocytes are similar in their staining properties to the granules of the spherule cells (and also

perhaps vibratile cells). Since these sections were decalcified prior to embedding and sectioning, it is unlikely that the granular material is a calcium salt. Evidence from the MBPB stain for proteins indicates that the granules in both Stage III leucocytes and spherule cells have a protein component associated with them, and at least some of the granules contain tyrosyl groups. It is suggested that spherule cells may be contributing material to the leucocytes. Since the granularity of the leucocytes decreases as regeneration proceeds, it is possible that this material is being incorporated into newly forming calcite. There also appears to be some turnover of protein material within the spherule cells themselves.

3.3.2.2 Connective Tissue

The peritoneal region showed a steady increase in staining, by the MBPB method, from its first appearance during Stage IIIB to the normal condition in fully regenerated plates. Both the mature peritoneum and the sutural connective fibres were dark blue, indicating the presence of a protein fraction in these structures. Deamination reduced staining to a light wash of blue or to a refractile state. Connective elements were negative to Millon's, indicating the absence of tyrosyl groups in these structures.

3.3.3 Carbohydrate Stains

In animal tissue carbohydrate is present as neutral, weakly or strongly acid, sulfated or unsulfated, mucopolysaccharide. The periodic acid Schiff (PAS) technique was here used

to detect all but the more acidic polysaccharides (Pearse 1968). Neutral polysaccharides (glycogen) were visualized by combining the PAS technique with the enzyme diastase, which hydrolyzes glycogen only. Tissue basophilia (and hence its acidic or basophilic quality) was detected by the Methylene blue extinction series (MBE). Metachromasia was detected by the basophilic dyes Azure A and Toluidine blue. Acidic groups (either sulfate or carboxyl) were detected by the Alcian Blue stain. In addition, the above stains were used with the technique of sulfation to increase specificity of PAS and metachromatic methods, and methylation followed by saponification to distinguish sulfated from non sulfated acid mucosubstances.

3.3.3.1 Cellular Elements

Leucocytes: In general leucocytes showed a pattern of staining which closely followed the course of regeneration (Table 3.3). There was an increase in PAS reactivity from Stages I to III of the cytoplasm of these cells. This was followed by a decrease in staining intensity to a weakly positive reaction in mature fully regenerated tests. No significant difference between treated and untreated sections was seen following treatment with diastase, indicating that glycogen is probably not present in these structures, or not preserved by the methods used. Sulfation abolished staining of leucocytes except in Stage III where a wash of red often remained. This may have been due to retained echinochrome from engulfed pigment cells.

Cytoplasmic metachromasia (Table 3.3) was seen to

increase from weakly beta (B) to ortho (o) metachromasia in Stage I, to deeply beta to gamma (γ) metachromasia during Stage III. The dermal region of the blastema also showed a gradient of increasing metachromasia from prestereomal to peritoneal regions. This increase in metachromasia was accompanied by an increase in the Methylene blue extinction point (MBEP) from 3.9 in Stage I to 5.3 in Stage III. Fully regenerated plates contained sclerocytes (leucocytes) whose cytoplasms were unstained with either of these stains. Cytoplasmic metachromasia was enhanced by sulfation. Leucocytes were well-stained by Alcian Blue being more deeply stained in the more alkaline media. A positive reaction to Alcian Blue was also seen to increase during regeneration. Mature leuco-sclerocytes were slightly stained by Alcian Blue. Mild methylation abolished Alcian Blue staining of fully mature, Stage I and Stage II leucocytes, but appeared to have less effect on the staining of Stage III leucocytes. Methylation was reversed by saponification, although loss of material from the sections affected the interpretation of this procedure. It seems likely that the decrease in intensity of staining following saponification of some sections may have been due to the presence before methylation of sulfate containing moieties. When stained by the Alcian Blue PAS (AB-PAS) technique, the blastema was dark blue in some regions, purple in others. The fully regenerated test contained cells which stained dark blue. These staining reactions probably are due to the presence of acidic sulfated mucopolysaccharides (sulfomucins) (Spicer 1963). There is also the

possibility that some components of the leucocyte cytoplasms show increased capacity for protein binding as regeneration proceeds (see discussion, Section 3.4).

Leucocytes in the loose configuration were different in their staining characteristics from the densely organized cells. They were weakly periodate reactive and diastase resistant. They showed metachromasia which was enhanced by sulfation, and appeared unaffected by methylation, although no loose regions were seen in saponified sections, possibly due to removal of material in the highly alkaline medium. The cells were stained light blue in AB-PAS stain and showed little change during regeneration. Thus loosely organized leucocytes may contain an acidic sulfated mucopolysaccharide (Spicer, 1963). Their low MBEP indicates more sulfate groups than dense leucocytes.

The granular material which appears in the prestereomal leucocytes at the beginning of Stage III is periodate reactive, and consists of two types of granules; the first was observed to be derived from the Type I spherule cells and it is probably the red pigment echinochrome. This material is the same color in tissue section as PAS positive material. It seems likely then that some masking of the color reaction by the pigment is taking place. Another type of granule less deeply stained was probably derived from the Type II spherule cells or the vibratile cells. The amount of periodate reactive granular material decreases as more calcite is deposited. In mature tests there are a few scattered granules and some intact spherule cells which stain with PAS. The granules appear to be diastase

resistant, indicating absence of glycogen. Some of the granules were sulfation resistant probably due to retained echinochrome. The granules were orthochromatic or deeply B metachromatic, and stained below pH 3.9. When stained with Alcian Blue, they were brown, green and black, indicating that they were probably not positive to Alcian Blue, although some stained blue and purple with AB-PAS. Results of methylation are difficult to interpret owing to wide variability in the response of the granules. In one case, however, green granules were left unstained following methylation and then stained blue after saponification.

Since the granular contents of prestereomal leucocytes are PAS reactive, and orthochromatic or B metachromatic at low pH (below 3.9), it is likely that these granules have a weakly acidic sulfated mucopolysaccharide core (Spicer 1960). However, masking of the color reactions by the granules makes interpretation of histochemical tests difficult. Some granules remained blue with AB-PAS. These are probably more sulfated than the other granules. It appears from the foregoing that accumulation of acidic sulfated mucosubstances is occurring within the leucocytes just prior to and during calcification.

Spherule Cells: In many cases the staining of the contents of spherule cells obscured the color reactions of their cytoplasms. They show variability in staining reactions and are often not distinguishable from one another. Type I spherule cells contained deeply PAS positive granules which obscured the cytoplasm. This material was probably sulfation (Table 3.3) and diastase resistant. The granules were deeply orthochromatic

with an MBEP below pH 2.6. They did not seem to react with Alcian Blue, although they were purple and black with AB-PAS. Some Alcian Blue staining of the cytoplasms of these cells was evident. This cytoplasmic staining with Alcian Blue was abolished by methylation and restored by saponification, indicating the presence of an acidic mucopolysaccharide. Staining of these granules was very similar to some of the Stage III A leucocyte granules. It is likely then, that the echinochrome of Type I spherule cells contains an acid mucopolysaccharide associated with it.

Type II spherule cells displayed far more variability in morphology and staining than Type I cells. In general their cytoplasm were periodate unreactive although they appeared to contain some PAS positive granules. The cytoplasm was B or γ metachromatic with an MBEP of 3.9. This metachromasia was enhanced by sulfation, indicating perhaps the presence of sulfate esters (Pearse 1949). The cells stained with Alcian Blue and were very slightly affected by mild methylation, further indicating sulfate groups. The granules were usually orthochromatic, but less deeply stained than those of Type I cells. They were also periodate reactive and green or brown with Alcian Blue. These granules resemble the second type of granule contained in prestereomal leucocytes and probably have associated with them a slightly acidic weakly sulfated mucopolysaccharide.

Spherule cells appear to undergo changes during regeneration because far mor despherulated cells were seen in Stage III than during earlier stages.

Vibratile cells were not always distinguishable. In general they were periodate reactive γ or B metachromatic and had MBEPs of 3.9 to 4.9. They were seen to be positive to Alcian Blue, although only slightly affected by mild methylation. In general they contained inclusions which were periodate reactive. This was confirmed by AB-PAS which also showed periodate reactive but Alcian Blue unreactive granules. Slight decreases of staining intensity following incubation with malt diastase may mean that these cells contain glycogen, although results were not consistent, perhaps due to diffusion of glycogen out of the section (Pearse 1968). The role of vibratile cells in regeneration is also unclear. Certainly they became engulfed in the syncytial blastema and are visible after regeneration. It could not be determined, however, if these cells went through the same changes during regeneration as seen in the spherule cells.

3.3.3.2 Connective Tissue

The peritoneal region which differentiates during early Stage III was seen to increase in periodate reactivity during regeneration. The fibrous elements of this region showed deep sulfation enhanced γ metachromasia with an MBEP of 3.9. In addition they stained blue with Alcian Blue and were not affected by mild methylation. When fully regenerated this Alcian Blue staining was distinct especially following AB-PAS where they remained blue. These histochemical tests are indicative of acidic sulfated mucopolysaccharides (Spicer 1963;

Pearse 1968).

The sutural connective fibres were remarkably similar to the peritoneum with respect to metachromasia, periodate reactivity and affinity for Alcian Blue. They did, however, exhibit a purple tinge with AB-PAS, indicating stronger periodate reactivity and perhaps a more closely bound or unmasked sugar moeity than that seen in the peritoneal fibres. Since both these fibres stain above pH 3.9 and are PAS reactive, they may contain a considerable amount of bound protein associated with the carbohydrate fraction, indicating the presence of glycoprotein (Pearse 1968).

3.3.4 Pigment Stains

The presence of reducing substances was tested by the Argentaffin reaction. To increase the specificity of this reaction the Ferrous iron uptake (FIU) of Lillie (1957), said to be specific for melanin; the dehydrated Nile blue (DNB) reaction, Lillie (1956a), said to distinguish between melanin (blue-green) and lipofuscin (yellow); and the Prussian blue reaction of Thompson (1966) were also used.

3.3.4.1 Cellular Elements

Leucocytes ; No elements of regenerating tests were stained by the Prussian blue reaction for iron. The cytoplasm of leucocytes in Stage III blastema was lightly stained by the Argentaffin reaction, but was negative to FIU and DNB. As is common for basophilic cellular constituents, these cells were

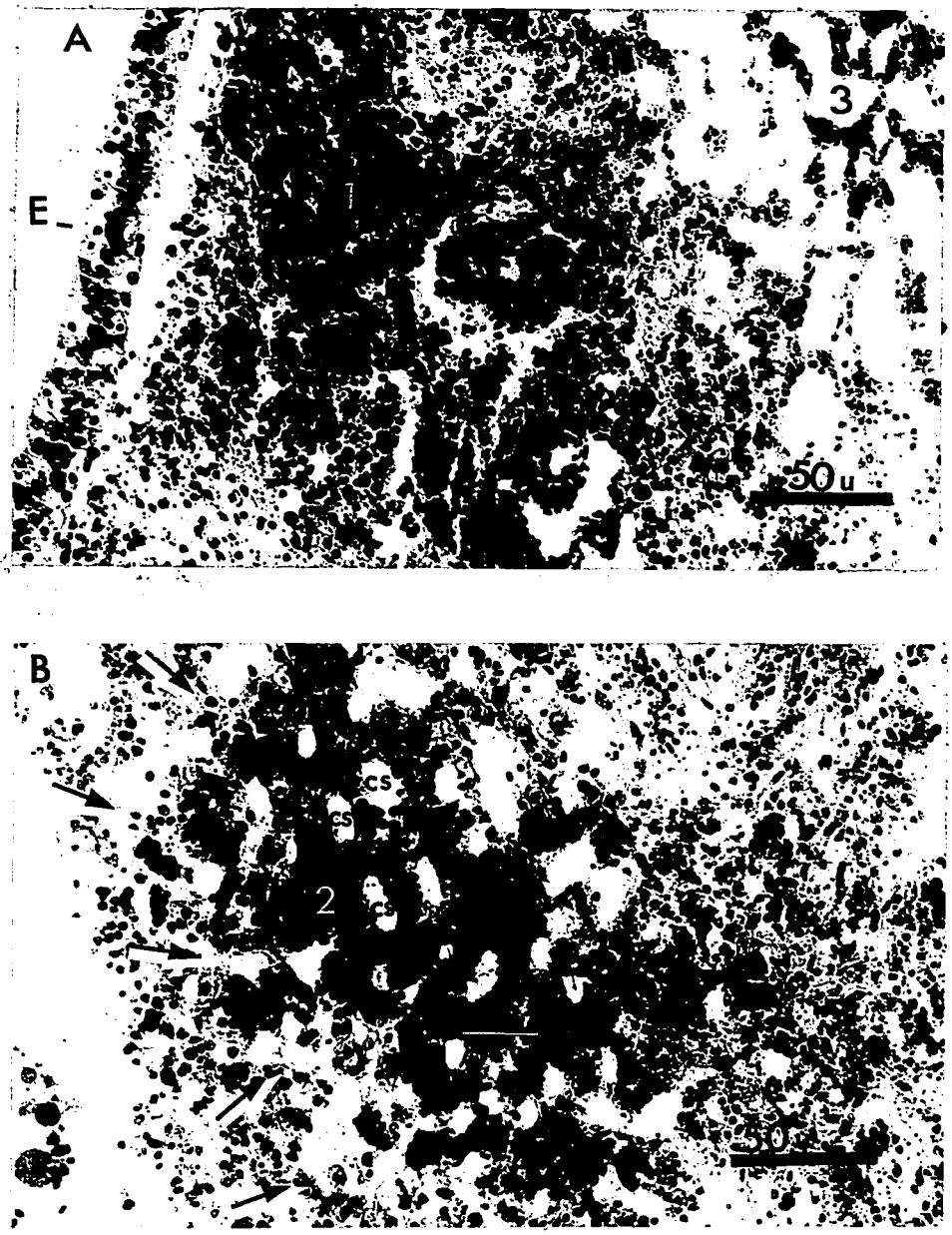
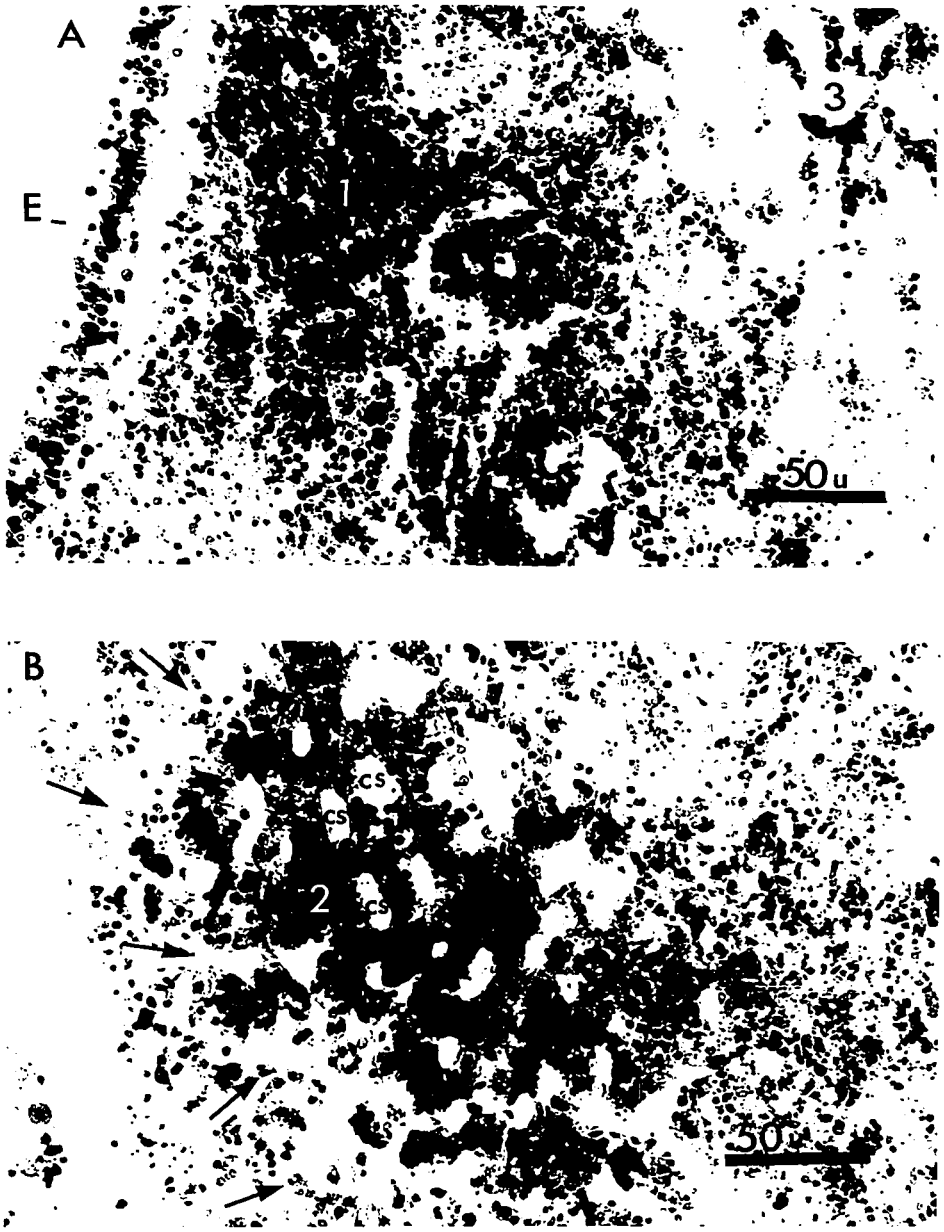


Fig. 3.1 Regeneration 25 days. Sequence showing progressive changes in granule configuration. In A (1) the prestereom is very granular and unorganized. In B (2) the first calcite spicules (CS) have been deposited and the characteristic stereom is forming. The edges of B (arrows) show decreases in granularity. At A (3) the stereom is nearly complete and contains few dark granules. E = epidermis. Argentaffin-NFR.



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stained blue in gelatin mounted Nile blue-stained slides.

Loosely organized leucocytes were negative to all stains except the aqueous and dehydrated Nile blue stain. The reason for the staining of these cells following dehydration is not evident. They contrasted sharply with the dense regions which were refractile following dehydration. Vibratile cells exhibited similar staining properties as the loose region, with respect to DNB (see below).

The granular material which appears in Stage III leucocytes stains black with the Argentaffin reaction, (Fig. 3.1 A B) and yellow with FIU, and blue and yellow with DNB. This indicates the presence of melanin and lipofuscin in the granular inclusions of Stage III leucocytes. The mechanism of staining of FIU occurs by chelation of the reactive iron by the oquinhydrone complex of melanin (Lillie 1957). Since this type of structure occurs in echinochrome, and this pigment is known to occur in Type I spherule cells, and since these cells are closely associated to the regenerating blastema during Stage III it may be that the color seen is due to the presence of this pigment and not melanin. As the calcite trabeculae thicken, there is a decrease in granular contents of the leucocytes. (Fig 3.1 A). In the normal fully regenerated plates a few yellow DNB reactive granules remained, although these were few in number and scattered.

From these results then there is further evidence of a cyclic change in the granular contents of leucocytes as the blastema calcifies. They appear just prior to calcite deposition and show a gradual decrease in concentration as the plate spicules increase in length and width.

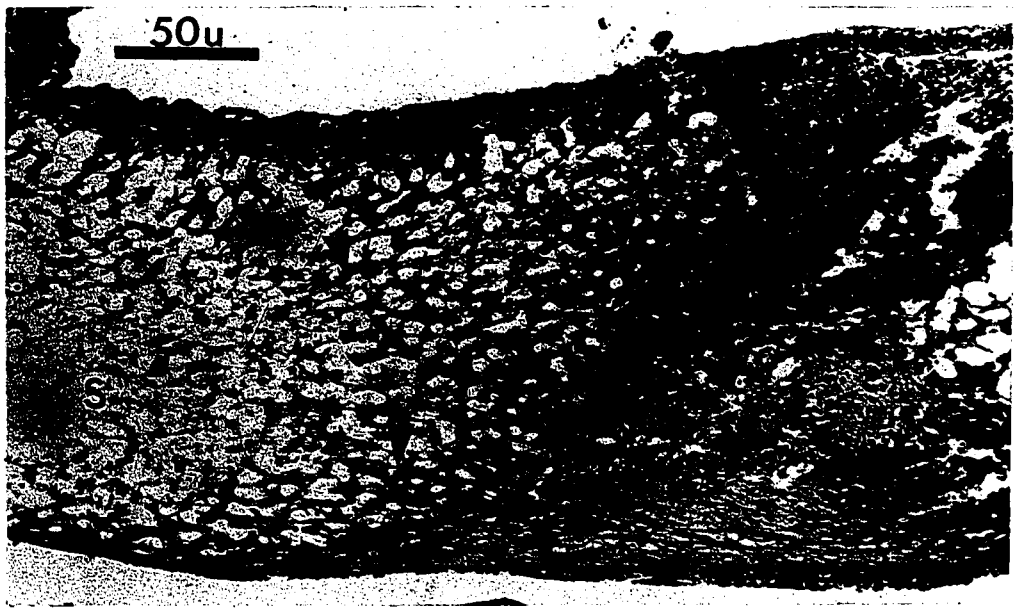

Fig. 3.2 Regenerated 45 day blastema. Note presence of dark reticulin fibres (arrows). There are fewer fibres in the fully regenerated stereom (S) than in the differentiating peritoneum (P). Gridley's Reticulin NFR.

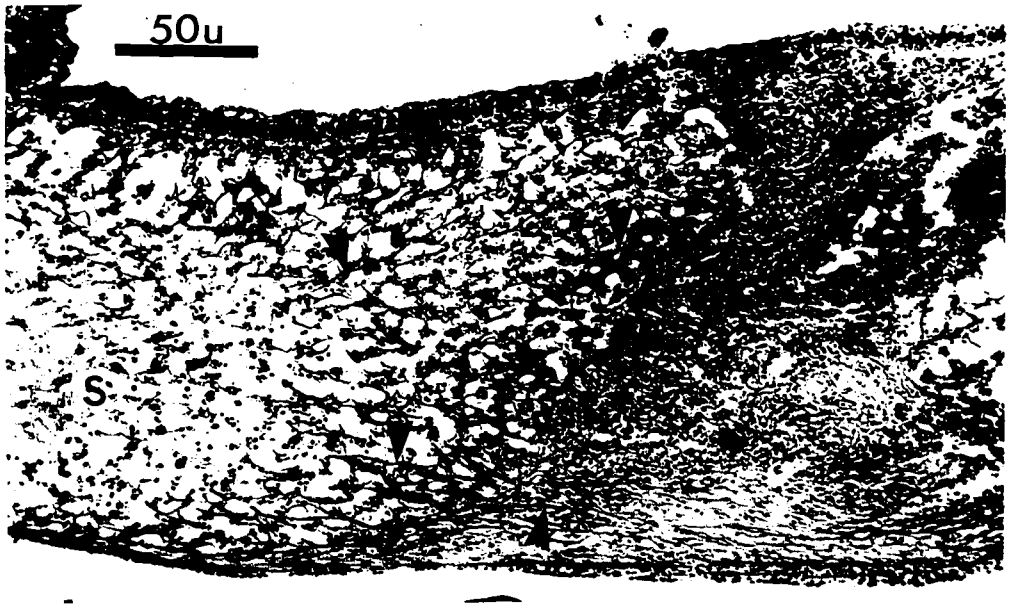

Fig. 3.2 Regenerated 45 day blastema. Note presence of dark reticulin fibres (arrows). There are fewer fibres in the fully regenerated stereom (S) than in the differentiating peritoneum (P). Gridley's Reticulin NFR.

It was also observed that undecalcified blastemae contained spicules which took up ferrous iron, indicating the presence of melanin or echinochrome-like material in these structures.

Spherule Cells: TypeI cells contain granules which stain black with the Argentaffin, green with FIU and dark blue-green with the DNB reaction. The possibility that the green FIU reaction is due to quinhydrone moeities of echinochrome has been mentioned above. In the late Stage III some Type I cells showed deep blue granules when stained by the DNB reaction. Since they were not seen in Stage I cells, it is possible that these spherules represent some melanin-like material left behind during the turnover that appears to accompany calcite deposition.

Type II spherule cells showed a light brown color when stained by the Argentaffin reaction, greenish-yellow with FIU and yellow with DNB. No cytoplasm staining was evident. The staining reactions indicate the presence of a lipofuscin. Since there were few stainable granules in Stage III and fully regenerated blastema, it is likely these cells are undergoing a cyclic change with regard to granule turnover (Fig. 3.2). The cytoplasm of vibratile cells was sometimes stained lightly by the Argentaffin raction. It was blue following FIU and remained blue in aqueous and DNB. In this latter respect it resembled the loosely organized leucocytes.

3.3.4.2 Connective Tissue

Both the sutural connective tissue and the developing

peritoneum were Argentaffin and aqueous Nile blue positive. With DNB some color remained in sutural fibres only.

3.3.5 Reticulin Stain

Reticulin positive material first appeared in some late Stage II blastemae. Two types of fibres were seen; the first were short, 50 μ in length, randomly oriented and continuous with the cytoplasmic extensions of the blastemal leucocytes; the second were also continuous with the leucocytes but longer, extending from them to connective tissue elements outside the regenerating area. (Fig. 3.2).

In general, some reticulin positive material appeared within prestereomal regions, less in stereomal regions and in fully regenerated tests was confined to areas bordering between plates. Since both peritoneal and sutural connective tissue elements are reticulin positive it is likely that the fibres which are seen in prestereomal and stereomal regions represent either of these two connective tissue elements in the early stages of their formation. Since regenerated plates are smaller than normal plates (Section 1.0) there are many more sutural regions in any one blastema than in the equivalent area of normal plates. It is possible that differentiation of the blastemal leucocytes into connective tissue elements serves to limit the size of newly regenerated plates. Certainly once the plate size has become established there are no reticular fibres except where two platelets meet.

3.4 Discussion

3.4.1 Histochemical Results

Because of the complex nature of the syncytium of fused leucocytes which form the regenerating blastema and the organic stroma in the fully regenerated test, it is difficult to determine at the level of the light microscope, which regions are intracellular and which are extracellular. Since calcite is being deposited intracellularly, histochemical changes which have been recorded are here considered to be occurring within the confines of the cell membrane.

In general protein, carbohydrate and pigment (reducing substances) were all seen to undergo similar cyclic variations in their concentration within the leucocytes of regenerating blastema. Changes observed were closely linked in time with deposition, growth and expansion of the inorganic, spicular, calcite skeleton.

From both histological and histochemical observations the primary organic constituents responsible for calcite deposition appear to be the densely organized leucocytes. The leucocytes do not, however, function alone, but appear to be closely dependent on the activity of three other cells which originate in the perivisceral fluid, and are found in calcifying regions. These are the Type I and II spherule cells and the vibratile cells. The role of the latter is much less clear due to difficulty in distinguishing it from the other two in paraffin sections.

Leucocytes show slight increases in the protein content of their cytoplasms as regeneration proceeds up to the point where calcification begins. At this time there is a decrease in MBPB positive material as more calcite is deposited. This is probably closely linked to reduction in the size of leucocytes as they are displaced by growing calcite spicules. These same leucocytes show increasing metachromasia (primarily B) and PAS reactivity as well as distinct increases in the pH at which their cytoplasms will take up the basic dyes, Methylene blue and Alcian Blue. This decreasing basophilia indicates an increased dissociation of side groups (polyanions) attached to the carbohydrate backbone (Quintarelli et al 1964a & b). Since carboxyls do not stain at low pH, in contrast to sulfates, which do exhibit this property (Quintarelli et al 1964a & b ; Dempsey and Singer 1946) it is likely that the acidity of the present material is due to the presence of carboxyl groups. Methylation abolished staining in Stage I and II leucocytes (dense) but had less effect on cells in later stages. Methylation times used were 15 hours at 20°C. Recommended times for methylation vary from 15-72 hours at 20°C (Drury and Wallington 1967) to 8-96 hours at 37°C (Pearse 1968). Since the time and temperatures employed in the present study were in the lower range of these values, it is possible that tissue sections were incompletely methylated. Those stages which exhibited decreased staining with Alcian Blue following methylation had staining restored by saponification. This is indicative of acidic carboxyl groups (Spicer 1963). The decrease in effect of methylation during later stages of regeneration may be due to incorporation of more methylation resistant groups at this time. Sulfate groups are more methylation resistant than carboxyl groups (Dempsey etaal 1947), hence

it is possible that sulfates bound as full esters may be incorporated into the cytoplasm of regenerating leucocytes. This is supported by the effects of sulfation on metachromatic staining of the tissue as well as staining with metachromatic dyes alone. In this capacity Holland and Nimitz (1964) found that incorporation of S^{35} occurred wherever staining with basic dyes appeared in gut mucopolysaccharides of sea urchins stained with Azure A. They concluded that detectable amounts of S^{35} sulfate were incorporated as sulfate esters without creating enough free electronegative surface charges to cause metachromasia. This is in distinct contrast to mammalian gut mucopolysaccharide where increasing concentrations of sulfate esters produce proportionate increases in metachromasia. Thus the metachromasia observed in the present investigations is probably due to the presence of carboxyl groups, although there is some evidence to suggest the presence of sulfate esters. Sialic acid, hyaluronic acid and chondroitin sulfate exhibit the aforementioned properties. Gross et al. (1958) performed amino acid analyses on connective tissues of holothurians and found very low levels of sialic acid. Motohiro (1970) found decreases in a chondroitin sulfate-like substance, a hyaluronate-like substance, other acid mucopolysaccharides, and protein which correspond closely to cessation of test growth of mature specimens of the urchins Strongylocentrotus intermedius and St. nudus. This suggests an association of these substances with plate growth. Johnson (1969b) also noted the presence of hyaluronidase labile material in the leucocytes of St. purpuratus. Sialidase and hyaluronidase extractions were not performed in the present investigation.

It has been suggested by Springall (1954) that increases in MBEP such as occurred in the present material may indicate increases in the capacity of the material to bind protein due to the presence of more dissociated COOH groups.

The leucocytes in dense configurations were seen to take up (contain) dense granular material. This accumulation began in early Stage III and continued as calcification proceeded. Subsequent decreases in the amount of granular material present accompanied increases in the size of the calcite spicules. Closely associated with the leucocytes at this stage were the spherule and vibratile cells of the perivisceral fluid. These cells contained granules which were identical in histochemical properties to the granules contained within the leucocytes. These granules all showed the capacity to reduce silver in solution. They appeared to consist of two basic types, one of which bore very close resemblance to granules of Type I spherule cells known to contain echinochrome (Johnson 1969a). The staining of these granules was similar to that shown by melanin. The staining of melanin by Ferrous iron uptake and perhaps also by dehydrated Nile Blue is due to the presence of o-quinhydrone groups in this compound (Lillie 1957). Since echinochrome also contains such groups, it has tentatively been suggested by the present writer that staining of this pigment may occur by a mechanism similar to that of melanin. Granules which reacted positively to stains for lipofuscin were also seen in both spherule cells and Stage III dense leucocytes. One of these leucocyte granules was also slightly stained with Millon's

reagent, as was a spherule cell of undetermined type. Since Burton (1966) found indolyl positive reactions to occur in Type II spherule cells, the positive reaction to Millon's which is specific for tyrosine, is tentatively assigned to these Type II cells. Furthermore, the presence of Millon's positive granules in dense Stage III leucocytes suggests that they were derived from Type II spherule cells. These cells contained granules which were strongly stained by MBPB. The granules appeared to loose this staining in Stage IIIB blastema, further indicating the possibility that the cells were contributing proteinaceous granules to the dense leucocytes.

The granular contents of Stage III leucocytes as well as spherule and vibratile cells appear to be associated with a carbohydrate fraction. This statement is subject to the reservation that masking of histochemical reactions by pigments present in these granules is known to occur (Burton 1966; Johnson 1969b). This appeared to be the case for the PAS reaction in which the color given by this stain is nearly identical to the natural color of echinochrome. Type I cells and some leucocyte granules contained PAS positive (?), orthochromatic, and slightly Alcian Blue positive material associated with them. They exhibited an MBEP below 2.6 and were bluish purple with AB-PAS. This may indicate quite strongly sulfated mucopolysaccharides (Spicer 1963). Type II spherule cells and the second class of leucocyte granules showed more γ to B metachromasia as well as higher and more variable MBEPs (pH 2.6 - 4.9). This may be indicative of less sulfated polysaccharide and/or more bound protein,

(Springall 1954). Moss and Meehan (1967) noted that spherule cells appeared to be associated with mucopolysaccharide. They described one cell (unspecified) which showed an MBEP of 5.4. This was probably a Type II spherule cell. Johnson (1969b) reported that many Type II spherule cells did not stain until pH 6.2 - 7.5. Vibratile cells sometimes appeared red with AB-PAS indicating the possible presence of a neutral polysaccharide. However, they also exhibited γ metachromasia and very high MBEPs. The difficulty in distinguishing between spherule cells and vibratile cells in the present material prevents definite conclusions from being drawn regarding the nature of these cells.

In addition to contributing granular material to the leucocytes it is likely that the cytoplasmic contents of spherule cells also become incorporated into those cells engulfed by Stage III leucocytes. Since Type II spherule cells may contain sulfated mucopolysaccharides, it is possible that apparent increases in sulfate containing groups of the cytoplasms of Stage III leucocytes is due to incorporation of the contents of the spherule cells. This staining of phagocytized contents of leucocytes has also been noted by Johnson (1969b) in hanging drop preparations of perivisceral fluid cell elements of sea urchins.

From the foregoing it seems very likely that the dense leucocytes acquire protein, pigment and possibly sulfated mucopolysaccharide from the spherule cells. The fate of this material appears to be incorporation into the forming calcite endoskeleton of the test. In a previous section (2.0) it has been suggested

that in calcite, echinochrome and perhaps a glycoprotein may exist as an "isomorphous" phase in the test of echinoids. Undecalcified blastemae contain spicules which stained positively for melanin, and electron micrographs of undecalcified blastema revealed an amorphous material between the forming calcite granules which may have been proteinaceous. Decreases in protein, carbohydrate and pigment within the leucocytes during regeneration may indicate incorporation of these substances into the calcite endoskeleton since they were never observed in other regions of the blastema.

The spherule cells themselves also appear to be undergoing changes. From the present results it is not possible to determine whether these cells are interconverting between one another or whether each is undergoing a cycle of its own. Certainly the histochemical results suggest physiological changes which may be associated with calcification of regenerating tests. The determination of the exact role of these spherule cells must await more exact methods of mapping of the cells than those employed here.

Loosely organized leucocytes seen in Stage I and II were weakly periodate reactive or totally unreactive. They exhibited deep sulfation enhanced γ metachromasia at low pHs, positive reactions to Alcian Blue and were stained blue by the AB-PAS reaction. This may indicate the presence of highly sulfated acidic mucopolysaccharides (Spicer 1963; Pearse 1968). Results with methylation and methylation followed by saponification were equivocal due probably to insufficient length of treatment and low temperature at which it was carried out. The lack

of reactivity of this tissue component to methylation may support the results obtained with metachromatic dyes and MBEP which indicate the presence of sulfate esters.

The peritoneal region was strongly stained for protein as well as sulfated acid mucopolysaccharide and in this latter characteristic resembled the loosely organized leucocytes. The sutural connective fibres also resembled the peritoneal region.

Results of reticulin staining show the presence of these connective fibres in late Stage II and Stage III blastema. They were never seen to occur in vacuoles containing calcite. It was not possible in the light microscope to determine whether they occurred intracellularly or extracellularly, but their close association with filiform extensions of the leucocytes suggests that these fibres probably constitute the forming sutural connective tissue seen between newly regenerated plates, and are possibly differentiated from these cells directly.

3.4.2 Significance of Histochemical Findings

The association of quininoid pigment, protein and acid mucopolysaccharide (sulfated as well as carboxyl) with the leucocytes of calcifying regions of regenerating blastema is significant in view of results obtained by other investigators of calcification. Abolins-Krogis (1958, 1963a,b,c & 1968) found that regeneration of mollusc shell was accomplished by deposition of small crystallites within an organic sheet that formed over the wound. These crystallites are preceded by so-called "organic crystals", which appear to consist of hyaluronidase-

labile acid mucopolysaccharide which had the same shape as the mineral crystals. This same author observed that these "organic crystals" contained proteinaceous granules (b-granules) which are derived intracellularly from the hepatopancreas and which may act as calcification centres of the "organic crystals". Granules which are eosinophilic and metachromatic and which precede or initiate calcification have been observed by other investigators of shell regeneration in molluscs (Bevelander and Benzer, 1948). Mollusc shell is covered by a layer of quinone-tanned protein (Beedham 1954). This same author found that in addition to quinone-tanning, calcifying mollusc shell contained protein, lipid and PAS positive material. Durning (1957) noted the presence of pigment cells in the epithelium of regenerating mollusc as well as a non-fibrous glycoprotein matrix.

The Crustacea contain quinone-tanned protein in their epicuticle (Dennell 1960). Travis (1957, 1960) found that increases in the amount of glycoprotein occurred during mineralization of the spiny lobster. In the calcifying gastrolith of the hepatopancreas, this same author noted that the concentration of acid mucopolysaccharides and proteins decreased as this structure calcified.

In general the invertebrates appear to contain matrices with higher polysaccharide to protein ratios than do the vertebrates (Moss 1964). Nevertheless, some of the histochemical properties of calcifying tissues of echinoids resemble those of the vertebrates. Carbohydrate-protein complexes have been seen in the cytoplasm of odontoblasts and the ground substance of

bone (Sognnaes 1955). These reactions decrease as calcification proceeds. Follis (1960) found a protein-polysaccharide complex in calcifying cartilage. This same author observed destruction of these cells at fronts of calcification. Bélanger (1954) showed that S^{35} (sulfate esters) appears first within cells and then in the ground substance of cartilage, bone, and dentin of young rats. This incorporation of sulfate esters was accompanied by increases in the degree of metachromasia. Thus the appearance of sulfate esters is associated with calcification in vertebrates. Wislocki et al (1948) found metachromasia and low MBEP in calcifying ground substance of enamel. Bevelander and Johnson (1950) also found protein and mucopolysaccharide in fibroblasts and bone. These substances are present in regenerating echinoid tests, but it is likely that there are fewer sulfate groups associated with the mucopolysaccharide. Quinone-tanning and acid mucopolysaccharide-protein complexes appear in other invertebrate phyla where calcification occurs.

Histochemical results presented in this study indicate certain basic similarities in the chemical nature of calcified tissues of echinoids and the calcified tissues of other vertebrate and invertebrate phyla. For the most part, histochemical findings have shown the presence of acidic groups

in calcifying tests of echinoids. In addition there is some evidence from the present histochemical results that increases in sulfated mucopolysaccharides are associated with calcification in echinoids. This being the case, then it is possible that they are associated to ground substance production in this tissue. Sobel (1955) has shown that in calcifying cartilage some local activating factor is responsible for calcification. He has postulated that the first step in calcification may involve the combination of Ca^{++} ions with the "local activating factor". Sobel further postulated that this activating factor is, in the case of calcifying cartilage, chondroitin sulfate. Since sulfate ester polysaccharides appear to accumulate in leucocytes of regenerating blastema, it may be that a mechanism similar to that in calcifying cartilage is operating in skeletogenesis of echinoid endoskeleton.

The histochemical findings of the present investigation are mainly relevant in providing background information about the kinds of chemical constituents associated with echinoid skeletogenesis. The significance of what was known previously and has been revealed by the present study with respect to mechanisms of calcification will be considered below.

3.4.3 Towards a Theory of Calcification in Echinoids

Much work is necessary to resolve the details of the basic general mechanism of calcification. In fact, it is quite unlikely that all phyla calcify via identical pathways. In the case of the echinoids there is lack of detailed information on cellular events accompanying calcification. This study has attempted to explore some aspects of this problem.. While no-one theory suggests itself as the underlying explanation of the events described it is here considered relevant to review some of the theories currently being suggested as possible mechanisms of calcification and to examine which aspects of which theories may be applicable to the present study. Theories of calcification may be divided into four basic categories:

1) Enzymatic - suggested first by Robison et al.(1930), this theory proposes that in bone an enzyme alkaline phosphatase raises the local concentration of Ca x PO_4 to the point where apatite (bone mineral) is spontaneously precipitated. This theory has been extended to include the molluscs by Wilbur (1960) and others who have proposed that the enzyme carbonic anhydrase may be involved in increasing local concentrations of calcium carbonate. Heatfield (1970) also presented evidence to suggest a role for this enzyme in echinoid calcification. The enzyme concept has also been applied to suggest that enzymes (alkaline phosphatase) may remove crystal poisons (phosphate ions) present in calcifying media, thus allowing crystallization in supersaturated solutions to occur freely. This idea has also been applied to calcite deposition (Simkiss 1964). The principal objection

to enzyme theories in vertebrate material has been the finding that alkaline phosphatase is present in areas that do not normally calcify (Maclean and Urist 1955; Eanes and Posner 1970). In the present investigation, carbonic anhydrase was found in the same concentration in echinoids in both calcifying and non-calcifying tissue (see Section 1.0). Sea water is known to be from 150% to 300% supersaturated with respect to calcium carbonate, depending on what part of the ocean is assayed (Wattenberg 1933). The idea that crystal poisons which would have to be present in seawater, are being removed by enzymes would require remarkable uniformity of biochemical pathways among the many diverse phyla in which calcification occurs. Furthermore, difficulties arise in the case of the higher vertebrates where the concentration of Ca x (PO_4) is considerably lower than the concentration of calcium carbonate in sea water. Moreover, it seems from more recent evidence (Sognnaes 1955), that alkaline phosphatase is probably involved in organic matrix production rather than its mineralization.

2) The second theory proposed to explain calcification has focused on the role of collagen as an initiator of mineralization, particularly in the vertebrates. First proposed by Neuman and Neuman (1958) and later extended by Glimcher (1960), this theory proposes that sites on or in the collagen fibres act as active groups on which inception of crystallization occurs. Further mineralization then occurs by epitactic growth from these initial seed crystals. Solomons and Irving (1958) obtained evidence to suggest that ε-amino groups of lysine and hydroxylysine may be the active initiation sites of mineralization.

This theory has recently been extended by Travis (1970) to include echinoid mineralization. The experimental evidence for collagen nucleation is based largely on in vitro precipitation of apatite crystals from solutions which often contain much more than the normal Ca x (PO_4) concentration in serum (Taves 1965). Furthermore, Fleisch (1964) has pointed out that non-mineralizing collagen will calcify <u>in vitro</u> under conditions where many bone collagens will not. Sobel (1955) has shown that chondroitin sulfate may also initiate calcification in cartilage. Collagen seeding is highly dependent on the interaction between specific amino acid groups and the crystals of forming bone or calcite. Invertebrate collagen contains different sugar moeities and different proportions of amino acids than vertebrate collagen (Gross and Piez 1960). Furthermore, there is also much variability between invertebrate groups with respect to amino acid composition of their collagens (Gross & Piez 1959). There is no proof that the collagen seen by Travis et al.(1967) and Travis (1970) is not connective tissue collagen (Pilkington 1969), and indeed Klein and Currey (1970) present evidence to indicate that echinoid collagen is not calcified. Evidence from other sources, especially scanning electron microscopy and X-ray diffraction indicates that echinoid calcite does not contain a fibrous organic matrix (see Section 2.3). Finally, to explain the single crystal (or oriented polycrystal) nature of echinoid calcite, some mechanism different from that of mammalian bone and teeth, which are unoriented and polycrystalline in nature, must be proposed. In the present investigation reticulin

positive material was seen to be associated with the subepidermal and sutural connective tissue elements. No evidence was obtained to suggest that these fibres are involved in calcite initiation and growth. Furthermore, these fibres were never found in intracellular regions where calcite deposition was seen to be occurring. Finally the shape of the inorganic stroma of regenerating echinoid tests reflects the shape of the leucocyte syncytium in which the calcite has become intracellularly deposited. This does not exclude the fibrous elements as being possible factors in controlling plate size of regenerated tests, and in fact differentiation or lack thereof of densely organized leucocytes into the sutural connective tissues may be responsible for limiting plate size.

3) The third category of calcification theories have proposed that electrochemical factors may be involved in deposition of bone, (Bassett 1962, 1964, 1968 ; Digby 1966), mollusc shell, (Digby 1968) and crustacean cuticle (Digby 1967a & b). Bassett indicated that mechanical stress acting on bone mineral may produce charge separation by piezoelectric effects causing bone to be deposited in regions of cathodic activity. Bassett et al.(1964) found mineral deposition at cathodes of batteries which had been implanted in dog's femurs. This has not been confirmed by Hambury et al. (1971). Digby has proposed an electrochemical theory based upon short circuiting through gradients of electric potential by semi-conducting complexes. The charge separation producing the potential gradient could be produced by diffusion as occurs through crustacean cuticle,

streaming effects such as that caused by blood flow through capillary loops in bone, or by suction of sea water through mollusc periostracum. The essential idea of the theory is that small differences in pH, which are produced by electrode action caused by short circuiting of low d.c. potentials cause precipitation of mineral from a more or less saturated solution of the ions concerned. The semi-conducting substances may be quininoid-like material in molluscs and crustacea or ubiquinones or vitamin-like substances in bone and teeth. Implication of electrochemical theories involves detection of electrical potentials (and/or pH differences) across the boundaries between calcified and non-calcified tissues and the presence of semi-conducting materials at the interface. Detection of potential or pH differences is not possible in all calcified material, and is especially difficult in the echinoid endoskeleton where calcified and uncalcified material are separated by a cell plasma membrane. The inorganic structure of different types of mineral in calcified tissues appears to vary from one phylum to another, but is quite specific for a given species. Thus some molluscs contain only the aragonitic form of calcium carbonate, others contain only calcite, others vaterite and some mixtures of the three. Simple precipitation without the influence of any other factors would not be expected to produce the highly ordered and specific arrangement of mineral seen in many calcified tissues. Thus it seems likely that the organic matrix functions in some capacity as an ordering influence on the inorganic mineral. Whether it functions as the initiator of calcification is at present uncertain

and more relevant experimentation is needed in this area. In the case of the echinoids the presence of quinone-like material in calcifying regenerating tests is suggestive of a possible role for semi-conducting materials in calcification initiation of these animals, although it is likely that ordering of the mineral phase is dependent on matrix-mineral interaction. The intracellular origin and locus of calcite presents technical difficulties in direct measurement of pH. Perhaps the problem may be overcome by the use of substances such as radioactive DMO* in which ionization is proportional to the pH of the media.

All the foregoing theories have been concerned principally with calcification as an extracellular phenomenon. This is because most systems which calcify do so extracellularly. Recently, however, attention has been focused on the possibility of intracellular origins and control of calcifying material, (Taves 1965; Eanes and Posner 1970). According to these workers, apatite does not precipitate directly from serum. Instead, initial calcification loci may be cellularly-derived globules originating from subcellular components such as the Golgi. It has been proposed that calcium and phosphate ions may be accumulated extracellularly or intracellularly by ion "pumping" (Eanes and Posner 1970). When the ions become sufficiently concentrated, precipitation of amorphous calcium phosphate would occur. This amorphous calcium phosphate being more soluble than bone mineral (apatite) would provide the serum levels of calcium phosphate needed for extracellular formation of apatite. A matrix may provide for preferential sites for

*5,5-Dimethyl-2,4-Oxazdidinedione.

nucleation, but is not directly involved in control over bone deposition. Several problems are evident from this theory, the primary one being that calcification is known to occur in the absence of cellular intervention in calcifying cartilage (Follis 1960; Dixon and Perkins 1965). This fourth theory of calcification is a reconciliation between extracellular factors of bone deposition and intracellular ones. Echinoid calcification appears to be intracellular (Section 2.0; Pilkington 1969; Gibbons 1969). This fact and the unique phylogenetic position of the echinoderms suggests that both intracellular and matrix-dependent factors may be operating in echinoid calcification. The formation of calcite within a cellular envelope in which occurs growth of mineral, possibly initiated epitaxially, continues by apposition throughout areas which are continuous with one another. This would explain the unique single crystal structure of echinoid plates. It was observed in the present investigation that many small platelets replace the large plates during test regeneration. As long as the blastemal leucocytes form a continuous syncytium, appositional continuous growth of calcite could be envisaged to occur. Where this syncytium is interrupted, calcite growth ceases. In this way, many centres of calcification could occur in separate regions of the blastema. The boundaries of these centres are defined by the presence of fibrous elements which have differentiated from the leucocytes.

In regenerated plates examined in the scanning electron microscope (Section 1.3.1) boundaries between plates were often bridged by thin calcitic connections (see Fig. 1.10). These

connections may represent places where intracellular calcification occurred before the leucocytes differentiated into sutural connective fibres. In this respect it is worthy to note that Towe and Hamilton (1968) and Bevelander and Nakahara (1969) have shown that mineralization of mollusc shell occurs within a .5 μ porous envelope which forms within the pallial fluid. Each envelope contains a crystal of calcite or aragonite. Crystal formation within this membrane-bound compartment is induced epitaxially by other crystals which may be in contact with the fluid within the envelope through the porous membrane. In this way, crystal orientation is dependent on where induction occurs. If this "envelope" concept is extended to include a cellular syncytium, then some analogy between the calcification of molluscs and echinoids is evident. In the case of echinoids, crystallization induction would be intracellular in origin. Subsequent growth of calcite would be in crystallographic continuity with this first crystal. This is supported by preliminary unpublished data which indicates that each newly regenerated plate behaves, when examined by X-ray diffraction, as a separate crystal. The question remaining of course is the mechanism whereby the first crystal is "precipitated". In this regard, it should be noted that precipitation initiation in supersaturated solutions can be accomplished by the presence of any foreign material, which then acts as a seeding crystal. Epitaxial induction and further growth by apposition may occur in non-living systems without the intervention of "activated" molecules. This does not obviate the role of these molecules in determination

of crystalline form after they have precipitated. From studies on echinoid larval forms, it has been shown that (see Section 3.1) ionic calcium, as well as bicarbonate ion, are mobilized during initiation of spicule development. Thus initiation of calcification may be by purely physical precipitation in a supersaturated intracellular environment. However, Pilkington (1969) Gibbons (1969) and results of the present investigations seem to indicate that a pigment-glycoprotein material is involved in some way with calcification in these animals. Although nucleation by fibrous material such as collagen is probably not occurring in echinoids, there are several other compounds present which contain groups known to be active in fixing ionic calcium. The very acidic nature of the polysaccharides contained in regenerating blastema suggests they may be involved. Dixon and Perkins (1956) have shown that highly acidic groups in cartilage matrix are able to fix calcium ions. These investigators suggested the sulfates of chondroitin sulfate as the group involved. In the regenerating test of echinoids there are almost certainly carboxyl groups. Some evidence suggests there may also be sulfates although more definite proof would be provided by S^{35} uptake experiments. Fixation of protein by carboxyl groups may also be occurring, although this is highly speculative at the present time. The formation of isomorphous salts between calcite and napthoquinone pigments has been suggested (Section 2.4) as a possible explanation of the non-fibrillar nature of the echinoid matrix. Taves (1965) has suggested the intracellular formation of calcium phosphoprotein complexes may initiate

calcification in bone. In this capacity it should be noted that mitochondria and active golgi were observed to be concentrated within granular membrane-bound spherules of the leucocytes of regenerating blastema (Fig.2.22B, 2.23). Further study is necessary however, to determine whether these subcellular components are directly involved in calcification.

The presence of semi-conducting substances (naphthoquinones) in calcifying tests of both adult and larval echinoids (Wolpert and Gustafson 1961) may indicate calcite precipitation by alkanity production through an electrochemical reaction (Digby 1967). Larval forms are known to increase pH of the blastocoel prior to calcite deposition. However, it cannot be determined if this pH rise is occurring intracellularly or extracellularly within the blastocoel.

The similarities between calcification of cartilage and echinoid skeletogenesis from both cytological, histochemical and perhaps biochemical standpoint presents interesting avenues for future research. This is particularily true because of the unique phylogenetic position of the echinoderms and the possibility that the regenerating blastema may be cultured in-vitro.

LIST OF ABBREVIATIONS FOR TABLES 3.1-3.7

0-4 = intensity of staining estimated by eye on a 4 point scale.

o = ortho: dark blue } Metachromasia

B = beta: violet purple } Metachromasia

γ = gamma: red } Metachromasia

* = granular material: if preceded by a number, granules are same color as stain: if preceded by a color abbreviation, granules are color indicated (e.g. gr.* = green granules).

> = very few

/ = number after this sign refers to lowest pH at which color appears (e.g. B/3.9 = beta metachromasia at pH 3.9)

BL. = blue

bl. = black

br. = brown

G = grey

gr. = green

R = red

P = purple

yel. = yellow

ABBREVIATIONS CONTINUED

ALC. BL.	=	Alcian Blue
ARGENT.	=	Argentaffin
CONN. TISSUE	=	Connective Tissue
D	=	Dehydrated
DEAM.	=	Deaminated
DIAS.	=	Diastase
FERROUS I.	=	Ferrous Iron
GRIDLEY'S RET.	=	Gridley's Reticulin
M	=	Methylation
M.B.E.	=	Methylene Blue Extinction
M.B.P.B.	=	Methylene Bromphenol Blue
METACHROM	=	Metachromasia - Aqueous Mounted
METACHROM -D	=	Metachromasia - Dehydrated
METACHROM -S	=	Metachromasia - Sulfation
METACHROM -SC	=	Metachromasia - Sulfation Control
NILE BL.	=	Nile Blue
PAS	=	Periodic acid - Schiff
PERIT.	=	Peritoneum
PRESTER.	=	Prestereomal
S.	=	Sulfation
SAP	=	Saponification
STEREOML.	=	Stereomal
VIB.	=	Vibratile Cells

Table 3.1 HISTOCHEMICAL OBSERVATIONS ON NORMAL TESTS

STAIN	REACTION	LEUCO-SCLEROCYTES	SPHERULE CELLS TYPE I	TYPE II	VIB.	CONN. TISSUE SUTURE	PERIT.
PAS	RED	0-1,3*	4*	0,1*-3*	2,1*-3*	1	2
PAS-S.	RED	0,>2*	------3*------			0	0
PAS-DIAS.	RED	0-1,3*	4*	0,1*-3*	2,1*-3*	1	2
M.B.E.	oBγ	0,o2*-4*/2.6	o3*/3.9	B/3.9 o4*/2-6	0- 1/4.9	γ2 /3.9	γ2 /3.9
METACHROM	oBγ	0,o2*	o4*	B3-o1	γ1	B1-γ2	γ2
METACHROM-D	oBγ	o3*	o4*	----- B3 -----		B2	B3
METACHROM-S	oBγ	0,0*	o4*	B3-o1	γ2	γ2	γ3
METACHROM-SC	oBγ	0,0*	o3*	---- B2-o1 ----		0	0
ALC.BL. pH1.0	BL	0-1	BL2 gr*,br*	BL2	BL2,0*	0-1	BL-1
ALC.BL. pH2.5	BL	1-2	BL3 br*,bl*	BL3	BL3,0*	2-3	BL1-2
ALC.BL.-PAS	BL,R,P	BL,P2-3*	BL2,P3* bl*	BL3,P2* br*	P2	P1-2	BL3
ALC.BL.-M	BL	0	gr*,br*	BL2 gr*	BL2,0*	BL2-3	BL2
ALC.BL.-SAP	BL	BL1-2	BL3 gr*,BL*	BL3	BL3,0*	BL1	BL1-2
ARGENT.	br,bl	0>bl*3	0,bl4*	0,br1-3*	br0-1,0*	br1-2	br1-2
FERROUS I.	gr,BL	0	0,gr4*	0,gr1-3* yel*	BL1,0*	0	0
NILE BL.	BL	BL1,BL3*	0,4*	2,2*	BL2	BL2	BL1
NILE BL.-D	BL,yel,gr	0,yel*	0,gr3*	0,yel*	0	0-BL1	0
GRIDLEY'S RET.	G fibers, bl	0	0,bl4*	0,bl1-3*	bl1-2,0*	br1-3	br3
MBPB	BL,gr,RED	BL2	BL3,RED4*	BL3,BL3*	BL2,0*	BL3	BL3
MBPB-DEAM.	BL,gr,RED	0	----- BL3* -----		0	BL0-1	BL0-1
MILLON'S	yel	0	---------- 0,yel2* ----------			0	0

Table 3.2 HISTOCHEMICAL OBSERVATIONS ON REGENERATING TESTS: CONNECTIVE TISSUE PROTEINS

STAGE I

Stain	Reaction	Leucocytes Loose	Leucocytes Dense	Spherule Cells Type I	Spherule Cells Type II	Vib.
GRIDLEY'S RET.	G(fibers), bl	0	0	---- 0,bl2-3* ---		N.S.
MBPB	BL,gr,RED	BL2	BL3,gr1	?,RED*	------- BL2,0* -------	
MBPB-DEAM.	BL,gr,RED	0	gr1	---------- BL3* ----------		
MILLON'S	yel	0	0	---------- yel2* ----------		

STAGE II

Stain	Reaction	Leucocytes Loose	Leucocytes Dense
GRIDLEY'S RET.	G(fibers), bl	0	G3 var
MBPB	BL,gr,RED	BL2	BL3, gr1
MBPB-DEAM.	BL,gr,RED	N.S.	0,gr1
MILLON'S	yel	0	0

STAGE III A

Stain	Reaction	Leucocytes Prestereom	Spherule Cells Type I	Spherule Cells Type II
GRIDLEY'S RET.	G(fibers), bl	G2 var	----- 0,bl4* -----	
MBPB	BL,gr,RED	BL3	0.RED3*	bl3*
MBPB-DEAM.	BL,gr,RED	0-gr1	------ bl3* ------	
MILLON'S	yel	0,yel0-1	---- yel1-2* -----	

STAGE III B

Stain	Reaction	Leucocytes Prester.	Leucocytes Stereoml.	Leucocytes Perit.	Spherule Cells Type I	Spherule Cells Type II	Vib.
GRIDLEY'S RET.	G(fibers), bl	G3,bl4*	G3,bl2*	G2	0,bl4*	--- 0,bl3* ---	
MBPB	BL,gr,RED	BL3,RED3*	BL2,RED3* BL3*	BL1-2	RED3*	--- BL2,0* ---	
MBPB-DEAM.	BL,gr,RED	0,BL1*	0,gr1	0,gr1	BL3*	---- 0,0* ----	
MILLON'S	yel	0,yel1-2*	0	0	------ 0,yel1-2* -------		

Table 3.3 HISTOCHEMICAL OBSERVATIONS ON REGENERATING TESTS
Periodic acid-Schiff; Methylene Blue Extinction

STAGE I

Stain	Reaction	Leucocytes Loose	Leucocytes Dense	Spherule Cells Type I	Spherule Cells Type II	Vib.
PAS	RED	0-1	1-3	4*	0-1,1*-3*	1,1*-3*
PAS-S.	RED	0	0	----- 2* -----		?
PAS-DIAS.	RED	0-1	1-2	4*	0,1*-3*	2,1*
M.B.E.	oBγ	γ1/2.6	B1.o3/3.9	o4*/2.6	o4*/2.6 B1/3.9	0-γ1/4.9 o2*

STAGE II

Stain	Reaction	Leucocytes Loose	Leucocytes Dense
PAS	RED	0	3
PAS-S.	RED	>2	>2
PAS-DIAS.	RED	0	3
M.B.E.	oBγ	γ2/2.6	B3-o2/4.9

STAGE III A

Stain	Leucocytes Prestereom	Spherule Cells Type I	Spherule Cells Type II
PAS	2-3,4*	---- 2,3*-4*	----
PAS-S.	0,3*	---- 0,2*-3*	----
PAS-DIAS.	2-3,4*	---- 2,3*-4*	----
M.B.E.	o3*/2.6-3.9 γ2/4.9	o4*/2.6	o3*,B2-1/2.6

STAGE III B

Stain	Reaction	Leucocytes Prester.	Leucocytes Stereoml.	Leucocytes Perit.	Spherule Cells Type I	Spherule Cells Type II	Vib.
PAS	RED	2-3,4*	3,4*	0-1	4*	---- 2,0*	----
PAS-S.	RED	0,3*	0,3*	0	3*	---- 0	-----
PAS-DIAS	RED	2-3,4*	3,4*	0-1	4*	---- 2,0*	----
M.B.E.	oBγ	o3*/2.6 γ1/5.3	o4*/2.6 γ1/5.3	γ/3.9	o4*/2.6	o4*B2/2.6	γ2/3.9

Table 3.4 HISTOCHEMICAL OBSERVATIONS ON REGENERATING TESTS: METACHROMASIA

STAGE I

Stain	Reaction	Leucocytes Loose	Leucocytes Dense	Spherule Cells Type I	Spherule Cells Type II	Vib.
METACHROM	oBγ	γ3-4	o2-B2	o4*	γ3-B1,o3*	B3
METACHROM-D	oBγ	B3-4	0-o1	o4*	B3,o3*	B2
METACHROM-S	oBγ	γ4	0-o1	o4*	------ γ3-01 ------	
METACHROM-SC	oBγ	γ1	0	o3*	------- B3 --------	

STAGE II

Stain	Reaction	Leucocytes Loose	Leucocytes Dense
METACHROM	oBγ	>γ3	B3-o1
METACHROM-D	oBγ	N.S.	B1-0
METACHROM-S	oBγ	γ3	B4-o1
METACHROM-SC	oBγ	>γ2	o3-0

STAGE III A

Stain	Leucocytes Prestereom	Spherule Cells Type I	Spherule Cells Type II
METACHROM	B3-γ1,o4*	o4*	B3
METACHROM-D	B2,o2*	o4*	B2
METACHROM-S	B3-4,o3*	o4*	B3
METACHROM-SC	0-o3,o3*	o3*	0-B1

STAGE III B

Stain	Reaction	Leucocytes Prester.	Leucocytes Stereoml.	Leucocytes Perit.	Spherule Cells Type I	Spherule Cells Type II	Vib.
METACHROM	oBγ	γ2,B4*	γ2-3,B3*	γ2-3	o4*	B3,o2*	B2
METACHROM-D	oBγ	o2*	o2-B2,B3*	B2	o4*	>B4,o1*	B2
METACHROM-S	oBγ	γ2,B3*	γ1-B2,B2*	γ3	o3*	---- B3,o1 ----	
METACHROM-SC	oBγ	0*-o3*	0*-o3*	0	o3*	------ 0 ------	

Table 3.5 HISTOCHEMICAL OBSERVATIONS ON REGENERATING TESTS: METHYLATION SAPONIFICATION

STAGE I

Stain	Reaction	Leucocytes Loose	Leucocytes Dense	Spherule Cells Type I	Spherule Cells Type II	Vib.
ALC.BL.-C	BL	BL1-2	BL2-3	0,br*	BL3,br*	0
ALC.BL.-M	BL	BL3	BL0-1	0,gr*	N.S.	0
ALC.BL.-SAP	BL	N.S.	BL2-3	0,br*	N.S.	N.S.

STAGE II

Stain	Reaction	Leucocytes Loose	Leucocytes Dense
ALC.BL.-C	BL	BL2	BL2
ALC.BL.-M	BL	BL3	BL0-1
ALC.BL.-SAP	BL	N.S.	BL2

STAGE III A

Leucocytes Prestereom	Spherule Cells Type I	Spherule Cells Type II
BL2-3 br*,gr*	BL2-3 br*,gr*	BL2-3
BL1-2 br1*	BL2, br*	BL2
BL3,br1*	------ BL3 ------	

STAGE III B

Stain	Reaction	Leucocytes Prester.	Leucocytes Stereoml.	Leucocytes Perit.	Spherule Cells Type I	Spherule Cells Type II	Vib.
ALC.BL.-C	BL	BL2-3	BL2-3, gr*	BL1-2	BL2-3, gr*	BL2-3,0*	BL3,0*
ALC.BL.-M	BL	BL2	BL1-2, >gr*	BL2-3	BL3, gr*	BL2,0*	BL2,0*
ALC.BL.-SAP	BL	BL3	BL3,BL3*	BL2-3	BL3	N.S.	BL3

Table 3.6 HISTOCHEMICAL OBSERVATIONS ON REGENERATING TESTS: ALCIAN BLUE
ALCIAN BLUE-PAS

STAGE I

Stain	Reaction	Leucocytes Loose	Leucocytes Dense	Spherule Cells Type I	Spherule Cells Type II	Vib.
ALC.BL. pH 1.0	BL	BL1-2	BL2-3	0,br*	BL3,br*	0
ALC.BL. pH 2.5	BL	BL-2	BL3	0,br*	0,gr*	0
ALC.BL.-PAS	BL,R,P	BL2	P3	P4,BL2*	P4,BL2*	>R1-2

STAGE II

Stain	Reaction	Leucocytes Loose	Leucocytes Dense
ALC.BL. pH 1.0	BL	BL2	BL2
ALC.BL. pH 2.5	BL	>BL2	BL2-3
ALC.BL.-PAS	BL,R,P	>BL2	P3

STAGE III A

Leucocytes Prestereom	Spherule Cells Type I	Spherule Cells Type II
BL2-3 br*-gr*	BL2-3, gr*-br*	BL2-3
B3 bl*	BL3,bl*	BL3
P4	P4,bl*	P3

STAGE III B

Stain	Reaction	Leucocytes Prester.	Leucocytes Stereoml.	Leucocytes Perit.	Spherule Cells Type I	Spherule Cells Type II	Vib.
ALC.BL. pH 1.0	BL	BL2-3, gr*	BL2-3, gr*	BL1-2	BL2-3, gr*	BL2-3	BL3,0*
ALC.BL. pH 2.5	BL	BL3-4, br*,gr*	BL3, br*	BL2-3	BL4, br*	BL3,0*	BL3,0*
ALC.BL.-PAS	BL,R,P	P4	BL3,P3*, BL3*,R3*	BL1-2	BL3, bl*,P3*	R3-P3	P2,R2*

Table 3.7 HISTOCHEMICAL OBSERVATIONS ON REGENERATING TESTS: REDUCING REACTIONS PIGMENT STAINS

STAGE I

Stain	Reaction	Leucocytes Loose	Leucocytes Dense	Spherule Cells Type I	Spherule Cells Type II	Vib.
ARGENT.	br,bl	0	0	0,bl4*	0,br1-3*	0
FERROUS I.	gr,BL,yel	BL1-2	0	0,gr4*	0,gr1-3*yel*	BL1,0*
NILE BL.	BL	BL2-3	BL3	0,BL4*	0,BL2*gr*	BL3,BL3*
NILE BL.-D	BL,yel,gr	BL2	0	0,gr3*	yel2*,gr*	BL3,0*

STAGE II

Stain	Reaction	Leucocytes Loose	Leucocytes Dense
ARGENT.	br,bl	0	0
FERROUS I.	gr,BL	BL1-2	0
NILE BL.	BL	BL1-2	BL3
NILE BL.-D	BL,yel,gr	N.S.	0

STAGE III A

Leucocytes Prestereom	Spherule Cells Type I	Spherule Cells Type II
br1-2,bl3-4*	0,bl4*	0,br3*
0,gr3-4*,yel3*	0,gr4*	0,gr2*
BL2-3,BL4*	0,BL4*	BL2,BL4*
0,BL2*,yel2*,0*	0,gr3-4*	0,BL3*

STAGE III B

Stain	Reaction	Leucocytes Prester,	Leucocytes Stereoml.	Leucocytes Perit.	Spherule Cells Type I	Spherule Cells Type II	Vib.
ARGENT.	br,bl	br1-2 bl2-4*	br1-2, bl4*	br1-2	bl4*	0,br2-3*	br0-1,0*
FERROUS I.	gr,BL	0,gr2-4*	0,gr4*	0	0,gr4*	0,gr2-3*	BL,0*
NILE BL.	BL	BL3,4*	BL1-2, BL3*	0-1	0,BL3*	BL3,gr3*	BL2,BL3*
NILE BL.-D	BL,yel,gr	0,yel3*	0,yel3*	0	0,gr3*, BL3*	BL3,gr3*	BL1,0*

APPENDIX

Forward:

The following section is an appendix to the histochemical stains and histochemical methods reported in Section 3.0.

It is included here to provide the reader with a brief description of the chemical mechanisms responsible for each of the stains, the procedure used in each case and the results to be expected from them.

The histochemical results detailed below are based on observations on some one hundred sea urchins. About fifty blocks were prepared from selected specimens and a total of about 1800 slides subjected to the histological and histochemical procedures outlined both below and in section 2.2.

APPENDIX NO. I

1.1 Periodic Acid Schiff

Mechanism of Reaction: Periodic acid cleaves carbon-carbon bonds when these are present as 1:2 glycol groups (CHOH-CHOH). These groups are then converted to dialdehydes of the type CHO-CHO. Carbonyl groups of the type CHOH-CHOH are characteristic of many carbohydrates but not all. Following conversion to dialdehydes no further oxidation occurs in these groups. A magenta-red color is provided by the union of fuschin-sulphurous acid with the dialdehydes; the color being proportionate to the number of groups present (Pearse 1968).

Expected Results: Substances known to be PAS reactive after aqueous fixation are polysaccharides including neutral mucosubstances and mucins, hyaluronic acid and mucoproteins (Pearse 1968). It is worthy to note that strongly acidic mucopolysaccharides are PAS negative.

Technique Used: Method used was according to McManus after Pearse (1968) with some modifications as noted below.

Solutions: (1) Schiff's Reagent (De Tomasi 1936)after Pearse (1968) - reagent prepared as described in Pearse(1968).

(2) Periodic Acid - .5% periodic acid in aqueous solution.

(3) Metanil Yellow - .25% solution in .25% acetic acid.

(4) Blocking Agent - 2,4 dinitrophenylhydrazine (2,4,DNP) saturated in 15% acetic acid.

Procedure: Slides for PAS (a, b, c, d, e, f, g):

a,b,g: triplicate sections from all tissue blocks embedded - see Methods.

c,d: representative sections from Stage I, II, III, beginning IIIa, IIIb.

e,f: duplicate sections of frogskin to serve as positive controls.

a,b,c,d,e,f,g:	(1) Bring sections to distilled water.	
a,b,c, e,f,g:	(2) Treat with 2,4,DNP	10 mins.
a,b,c, e,f,g:	(3) Wash in tap water	30 mins.
c :	(4) Treat with 1 N HCl at 60°C	30 mins.
g:	(5) Treat with Sulphuric Acid-Ether (See Appendix No. 5)	5 mins.
a,b,c,d,e, g:	(6) Rinse with distilled water	15 mins.
a, e, g:	(7) Treat with periodic acid .5%	8 mins.
a,b, d,e,f,g:	(8) Rinse in distilled water	Rinse
a,b,c,d,e,f,g:	(9) Treat with Schiff's reagent	15 mins.

a,b,c,d,e,f,g,:	(10) Wash in tap water	15 mins.
a,b,c,d,e,f,g:	(11) Counterstain with Metanil Yellow	1 min.
a,b,c,d,e,f,g:	(12) Distilled water	Rinse
a,b,c,d,e,f,g:	(13) Dehydrate and mount.	

Expected Results:

a) PAS+ due to presence of oxidized carbonyl groups.

b) PAS- not treated with periodic acid.

c) PAS+ slight reaction due to reversal of aldehyde blockade.

d) PAS+ slightly positive due to blockade of aldehydes present.

e) PAS+ due to oxidized carbonyl groups.

f) PAS- due to non-treatment with periodic acid.

g) PAS- due to acidification by sulfate addition.

1.2 PAS + Diastase

Diastase is an enzyme which specifically hydrolyzes glycogen. Sections incubated in diastase and then treated with the PAS reaction are colorless in those areas which formerly contained glycogen.

Method according to Drury and Wallington (1967).

Solutions: (1) .1% malt diastase.

Procedure:

	(1) Bring duplicate sections a,b to water.	
a	(2) Treat with .1% malt diastase at 40°C	50 mins.
a,b	(3) Wash in running tap water	5 mins.
a,b	(4) Treat with PAS technique (see Section 1.1)	

Expected Results:

a) PAS- in areas which contained glycogen.

b) PAS+ in areas which are unstained in (a).

Appendix No. 2

Metachromatic Staining and Sulfation Techniques

Mechanism of Staining: Metachromasia is defined as staining in which the absorption spectrum of the tissue dye complex is different from the ordinary dye color or tissue-dye color, thus giving a contrasting color (Pearse 1968). Color appears to be due to the formation of dye-polymers. For a substance such as Toluidine blue, the monomeric (α) form is blue, the dimeric form (B) is violet, and the polymeric form (γ) is red. The prerequisite for the appearance of metachromasia in a tissue is a minimum density of negatively charged groups such as SO_3H^- and $COOH^-$ spaced at 4-6Å intervals. It is generally believed (Pearse 1968; Thompson 1966) that substances which exhibit metachromasia are those which contain free sulphate, phosphoryl or carboxyl anionic groups at a minimum distance of 4-6Å. Shifts in color when the dye forms aggregates are presumed to be the consequence of electronic interaction between adjacent stacked dye molecules. Thompson (1966) states that practically all tissue components in aqueous mounting exhibit metachromasia. For this reason several authors (Lillie 1965; Thompson 1966; Pearse 1968) recommend that comparison be made between dehydrated mounted sections and water-mounted sections. Substances which are metachromatic after alcoholic dehydration are considered to be high molecular weight polysaccharide ester-sulfates.(Thompson 1966; Pearse 1968). It is not possible to determine which specific groups stain metachromatically,

the only certain statement that can be made is that metachromatic substances exhibit a certain density of free electronegative surface charges.

Kramer and Windrum (1954) developed sulphation as a histochemical technique when it was found that treatment of tissue sections with sulphuric acid increased basophilia of basement membranes. It is thought to be due to the sulfation of neutral or partially substituted anionic polysaccharides which results in the esterification of the primary hydroxyl groups of the carbohydrate. 1,2 glycols and 1,2 aminohydroxy groups are not esterified, particularly by the Gomori technique used in the present investigation (Thompson 1966). Treated sections are compared to controls to determine if metachromasia has been induced by the sulfation technique.

Technique Used:

Methods: Azure A staining technique after Kramer and Windrum modified after Thompson (1966).

Toluidine Blue staining technique after Pearse (1968).

Sulfation technique after Gomori from Thompson (1966).

Solutions:

(1) Azure A .1%: Azure A .1 gm
Dist. water 100 ml

(2) Toluidine Blue .5%:
Toluidine Blue .5 gm
Dist. water 100 ml

(3) Acetic Toluidine Blue .01%

I Toluidine Blue 1 gm
Distilled Water 100 ml

II	Solution I	1 ml
	1% Acetic acid	9.9 ml

(4) Sulphuric Acid-Ether

Sulphuric acid	25 ml
Ethyl ether	25 ml

Procedure:

a) quadruplicate series of sections a,b,c,d was brought to distilled water then treated as follows:

1)	a)	stained .1% Azure A	10 mins.
	b)	stained .5% Toluidine blue	3 hours
	c)	treated Sulphuric Acid-Ether	5 mins.
	d)	control left in distilled water	5 mins.
	c,d)	stained .01% Acetic Toluidine blue	20 mins.

2) a,b,c,d: all sections examined in distilled water.

3) a : dehydrated through alcohols and mounted in permount.

b,c,d: mounted in glycerine jelly.

Expected Results:

a) determines presence of alcohol - stable metachromasia.

b) determines presence of metachromasia in aqueous solutions.

c) enhanced metachromasia due to sulfation of anionic polysaccharides.

d) control slide for sulfation.

Appendix No. 3

Methylene Blue Extinction

Mechanism of Staining: The Methylene Blue Extinction method was extended by Dempsey et al (1947) to characterizing mucopolysaccharides according to the pH at which their affinity for that dye was extinguished. It is generally agreed (Thompson 1966) that the pH of the staining reaction can shift the degree of ionization of a tissue component so that it may function as an acidic (anionic) or basic (cationic) substance and hence display increased or decreased affinity for basic dyes such as Methylene Blue. Carbohydrates contain anionic groups such as sulphates, hydroxyls and carboxyls. When the pH of the staining solution is on the acid side of the isoelectric point of a particular tissue component, the dissociation of the anionic (+) groups is suppressed. Staining of a tissue component with a basic dye at various pH's will show a point at which the tissue component no longer stains. This is the Methylene Blue extinction point (MBEP) and corresponds to the dissociation characteristics of the anionic groups of the basophilic components of the tissues.

Dempsey et al (1947) found that components which stain at very low pH have acid radicals so strong that dissociation is not suppressed except in very acidic solutions. They found this to be true of sulfated mucopolysaccharides. He found that glycoprotein and mucoprotein do not bind Methylene Blue below about pH 6.0.

Pearse (1968) cautions that specific distinction and identification of carboxyl and sulphate groups is not possible using only the Methylene Blue extinction method.

Technique Used: Method according to Pearse (1968) after Dempsey and Singer (1946).

Solutions:

1) Veronal Acetate Buffer (Pearse 1968, after Michaelis 1931)

   a) .1 N HCl

   b) Veronal Acetate

Sodium Acetate Crystals	19.4 gm
Sodium Diethyl Barbiturate	29.4 gm
Distilled water to make	1 litre

2) Methylene Blue .0005 M

Methylene blue (.1869 gms) was added to 1 litre of .1 N HCl, 1 litre of Veronal acetate solution, and 1 litre of distilled water, respectively.

These three solutions were then mixed together in the proportions shown below. The pH of the solutions was checked on a pH meter before slides were immersed in the solution.

pH	Veronal Acetate ml	.1 N HCL ml	Distilled Water ml
2.6	5	15	5
3.9	5	13	7
4.4	5	11	9
5.3	5	8	12
6.1	5	7	13
7.4	5	5	15
8.2	5	2	18
8.5	5	1	19

Method:

1) Sections brought to water.
2) Representative sections from each of the four stages were stained in a graded buffer series containing Methylene Blue for 24 hours.
3) Sections examined under water.
4) Sections mounted in glycerine.

Appendix No. 4

Alcian Blue: PAS, Methylation, Saponification

Mechanism of Staining: The use of Alcian Blue as a stain for mucins was introduced by Steedman in 1950 (Pearse 1968). It is claimed to stain acid mucopolysaccharides but not glycoproteins. Alcian blue carries 2-4 positive charges. In the presence of added electrolytes NaCl, KCL, Quintarelli et al(1964a,b.)found that staining with Alcian blue was enhanced. They assumed that blocking of extra anions by these electrolytes increased the amount of dye bound by tissues. This indicated that Alcian blue staining was by salt linkage formation. Pearse (1968) following work by Scott et al states that in solutions containing less than .3 M of electrolytes, carboxyl groups of both sulphated mucins and acid mucopolysaccharides stain. Above .8 M electrolyte concentration only sulphated mucosubstances stain. With sulphated acid mucopolysaccharides the sulphate groups may be removed by methylation resulting in substitution of a hydroxyl group. Restoration of alcianephilia is not possible because the sulphate groups are converted to free methyl sulfate esters. In carboxylated acid mucopolysaccharides methylation results in formation of esters of the carboxyl groups. By using potassium hydroxide these esterified carboxyls may be reconverted to normal carboxyls after hydrolysis which occurs in the solution.

Spicer (1960) found that prolonged methylation of tissue sections eliminated Alcian Blue staining of acidic mucopolysaccharides. He also found that strongly acidic

sulfated mucins were unstained with Alcian blue. If tissue sections treated in acid methanol for four hours have Alcian blue staining first blocked and then restored by mild saponification, it is likely that they are acid mucopolysaccharides of the non-sulfated type and that the Alcian blue affinity is due to strongly acidic carboxyl groups. Sulphate-free mucins are known to be particularly labile with respect to their alcianophilia after mild methylation.

Spicer found a third category of mucopolysaccharides which are increasingly Alcian blue reactive from pH 1.5-3.0. These substances were found to contain sulfate by radioautography. In addition, they are very resistant to methylation.

In combination with the PAS technique, Alcian blue staining reveals simultaneous demonstration of acid mucopolysaccharides and 1,2 glycols. According to Thompson (1966) after Mowry (1956), exclusively acid mucopolysaccharides are stained blue, neutral polysaccharides are red-magenta. In tissue components which react to both PAS and Alcian blue, the color is purple.

Technique Used:

Methods: Alcian blue: 1.0, 2.5 and Alcian blue PAS after Pearse (1968).

Alcian blue methylation and saponification after Drury and Wallington (1967).

Solutions:

I Alcian blue pH = 2.5

Alcian blue 8 Gx	1 gm
3% Acetic Acid	100 ml

II Alcian blue pH = 1.0

Alcian blue 8 Gx	1 gm
.1 N HCl	100 ml

III Methylation Solution:

Concentrated HCl	1 ml
Methyl Alcohol	100 ml

IV Saponification Solution

N/10 Potassium Hydroxide

V Nuclear Fast Red

See Appendix No. 5.

Technique:

1) quintuplet series of sections (a,b,c,d,e) brought to distilled water.

2) a) stain in Alcian blue pH 1.0 — 30 mins.
   b) stain in Alcian blue pH 2.5 — 30 mins.
   c) methylate 1% HCl in methanol — 15 hours
   d) saponify N/10 Potassium Hydroxide — 30 mins.
   e) stain in Alcian blue pH 2.5 — 2 hours

3) a,b,d,e: rinse in water — 2 mins.
   c: rinse in absolute ethyl alcohol — 2 mins.

4) a,b: counterstain with Nuclear Fast Red 5 mins.

c,d: stain in Alcian blue 2.5 30 mins.

e: rinse in 3% acetic acid and then immerse in distilled water

5) a,b,c,d: wash in tap water 5 mins.

e: perform PAS without counter-stain (Appendix No. 1).

6) Dehydrate clear and mount in permount.

Expected Results:

a) only sulphated mucosubstances stain; acid mucins usually negative or weak.

b) weakly acidic sulphated mucosubstances

hyaluronic acid sialomucins dark blue

strongly acidic sulphated mucopolysaccharides are stained weakly.

c) strongly acidic sulphated mucopolysaccharides staining is blocked.

d) sulphated acid mucopolysaccharides do not have staining restored.

acidic mucopolysaccharides which are sulphate free have staining restored, very strongly sulphated are refractile.

e) exclusively acid mucopolysaccharides, neutral polysaccharides, lipochromes: red-magenta.

hyaluronic acid sialomucins weakly

acidic mucosubstances blue-blue purple.

Appendix No. 5

Reticulin Stain

Mechanism of Staining: Demonstration of reticulin fibers in tissue section is achieved by oxidation of the tissue to produce an aldehyde. The aldehyde so formed is capable of reducing solutions of silver nitrate to the metallic silver state. Demonstration of sites of silver impregnation are accomplished by "developing" or reducing the silver with formaldehyde. Gold chloride reacts with the silver to form silver and gold chloride (Thompson 1966). Location of reticulin appears black in sections.

Technique Used: Method used was Gridley's (1951) Reticulin stain after Thompson (1966).

Solutions:

I .5% aqueous periodic acid.

II 5% aqueous silver nitrate.

III 10% aqueous sodium hydroxide.

IV Ammoniacal silver nitrate

5% aqueous silver nitrate	20 ml
10% aqueous sodium hydroxide	20 drops
ammonium hydroxide 28%	(see below)

5% silver nitrate was placed in a 100 ml graduated glass cylinder. 10% sodium hydroxide was added. Ammonium hydroxide was then added drop-wise until a few granules of the precipitate remained on the bottom of the cylinder. Volume was made up to 60 ml.

VI 30% aqueous formalin.

VII .5% aqueous gold chloride.

VIII 5% sodium thiosulphate.

IX Nuclear fast red

aluminum sulphate	5 gm
distilled water	100 ml
nuclear fast red	.1 gm

Procedure:

1)	Bring sections to distilled water.	
2)	Periodic acid .5%	15 mins.
3)	Distilled water	Rinse
4)	Silver nitrate 2%	30 mins.
5)	Distilled water (2 changes)	Rinse
6)	Ammoniacal silver nitrate	15 mins.
7)	Distilled water	Rapid Rinse
8)	Formalin 30% (agitate)	3 mins.
9)	Distilled water (3 changes)	Rinse
10)	Gold chloride	*5 mins.

*sections were toned until examination showed them to be a uniform lavender grey.

11)	Distilled water	Wash
12)	Sodium thiosulphate 5%	5 mins.
13)	Running tap water	5 mins.
14)	Nuclear fast red	10 mins.
15)	Tap water	Wash
16)	Dehydrate and mount.	

Expected Results:

Sites of reduced silver: black.

Appendix No. 6

Reduction Reactions: Argentaffin, Ferric Ferricyanide.

6.1 Argentaffin Reaction

Mechanism of Staining: The argentaffin reaction is based on the intrinsic ability of some tissue components to act as reducing substances and thereby to reduce silver present in ammoniacal solutions.(Thompson 1966). In argyrophilic reactions the tissue must first be impregnated with silver salts and then be reduced. Many substances are known to be reducing in nature and hence give a positive reaction. The argentaffin reaction must be interpreted with caution. Pigments such as melanin and lipofuschin are very strongly reducing.

Technique Used: Method according to Thompson (1966) after Lillie (1954).

Solutions:

I Weigert's Iodine

I_2	1 gm
KI	2 gm
Dist.water	100 ml

II Diamine silver

To 2 ml of 28% ammonia water 5% silver nitrate was added until faint turbidity was reached.

Procedure:

1) Sections brought to distilled water.	
2) Treated with Weigert's iodine	10 mins.
3) Sodium thiosulphate	2 mins.
4) Tap water	10 mins.
5) Distilled water	Rinse

6)	Diamine silver at 60°C approximately until dark particles were visible.	10 mins.
7)	Distilled water	Rinse
8)	Sodium thiosulphate	1-2 mins.
9)	Tap water	5 mins.
10)	Nuclear fast red .1%	2 mins.
11)	Tap water	Rinse
12)	Dehydrate and mount.	

Expected Results:

Substances reduced by silver stain dark brown to black.

6.2 Ferrous Iron Uptake

Lillie (1957) introduced the ferrous iron reaction which he claimed to be specific for melanin. He suggested that components possessing an o-quinhydrone configuration were capable of chel ting ferrous iron. The Fe^{++} ion so bound may then be demonstrated by treatment with potassium ferricyanide which produces ferrous ferricyanide (Turnbull blue) (Thompson 1966).

Technique Used: Method after Lillie (1957).

Solutions:

I Ferrous sulphate

Ferrous sulphate $FeSO_4 .7 H_2O$	2.5 gm
Distilled water	100 ml

II Potassium Ferricyanide

Potassium ferricyanide $K_3Fe(CN)6$	1 gm
Distilled water	99 ml
Glacial acetic acid	1 ml

Procedure:

1) Bring to distilled water.	
2) Ferrous sulphate	1 hour
3) Distilled water - 4 changes	20 mins.
4) Potassium ferricyanide	30 mins.
5) 1% acetic acid	1-2 mins.
6) Dehydrate, clear and mount.	

Expected Results:

Melanin: green

Background: faint green

6.3 Acid Nile Blue

Mechanism of Staining: When tissues are treated with Nile blue sulphate dissolved in acid solutions, two mechanisms of staining occur (Lillie 1956). The first is an acetone or alcohol labile fat solubility mechanism which is determined by the indicator properties of the dye and operates at low pH levels (under 1.0). Thus the dye becomes soluble in lipid containing material. The second method of staining is acetone resistant and involves formation of salt linkages between basophilic tissue components and the dye. Although the mechanism of Nile blue sulphate staining of lipids is controversial and perhaps non-specific, it is useful for distinguishing between melanin and lipofuschin (Thompson 1966). These two substances may be distinguished by their acetone lability. Following acid extraction and acetone dehydration, lipofuschins are stained light yellow to brown, whereas melanin remains dark green or blue.

Technique Used: Method from Humason (1962) after Lillie (1956a).

Solutions:

Nile Blue A	.05 g
1% Sulfuric Acid	100 ml

Procedure:

	1) Bring duplicate sections a,b to distilled water.	
a, b	2) Stain with Nile Blue A solution	20 mins.
a	3) Wash in running water	10-20 mins.
b	4) Rinse quickly in 1% sulfuric acid	
b	5) Acetone 4 changes	15 secs.
a	6) Mount in glycerine jelly	
b	7) Clear and mount in permount.	

Expected Results:

a)	lipofuscins	dark blue or blue green.
	melanin	pale green.
b)	melanin	dark green.
	lipofuscins	yellow-brownish.

6.4 Prussian Blue Method

Potassium ferrocyanide forms Prussian blue $Fe_4(Fe(CN)_6)_3$ with reactive ferric salts in acid solutions (Thompson 1966). This iron will react even when bound to protein, although in some cases special "unmasking" treatments must be used to free it.

Technique Used: Thompson (1966) after Gomori (1936).

Solutions:

I Potassium Ferrocyanide 10%

Potassium ferrocyanide	10 gm
Distilled water to make	100 ml

II Acidified Ferrocyanide

Dilute HCl	20 ml
10% Potassium ferrocyanide	20 ml

made up immediately before use.

III Nuclear Fast Red

See Appendix No. 5.

Procedure:

1) Bring sections to distilled water.	
2) Acidified ferrocyanide	20 mins.
3) Distilled water	Rinse
4) Nuclear fast red	5 mins.
5) Dehydrate, clear and mount.	

Expected Results:

Sites of active iron - deep blue.

Appendix No. 7

Protein Stains

Two stains for protein were used in the present investigation: Millon's stain for tyrosine and the Mercury Bromphenol Blue stain for proteins.

7.1 Millon's Reaction

The reaction appears to occur by two steps (Pearse 1968): The first is production of nitrosophenol by substitution of NO on the hydroxyl of the phenol of tyrosine. Secondly Hg^{+2} is incorporated into a new ring by chelation. Thus a positive reaction with Millon's reagent indicates only a phenolic compound which is unsubstituted in the position meta to the hydroxyl group. These compounds do not occur free in tissues (usually) and hence a positive reaction is indicative of the presence of a protein containing the amino acid tyrosine.

Technique Used: After Pearse (1968) using commercially obtained Millon's Reagent.

Solution:

Millon's Reagent- Mercuric Nitrate Solution (Hartman-Leddon Co., Philadelphia, U.S.A)

Nitric Acid (conc.)	400 ml
Mercuric Nitrate	Saturated
Distilled Water	600 ml

Procedure:

Positive control used was paraffin sections of mollusc periostracum.

1) Bring sections to xylene.

2) Rinse in acetone and air dry.

3) Place in Millon's reagent at 60°C until full color develops 1-3 hrs.

4) 2% nitric acid Rinse

5) Dehydrate, clear and mount.

Expected Results:

Proteins containing tyrosine - orange to rose red.

7.2 Mercury Bromphenol Blue

The Mercury Bromphenol Blue of Mazia et al (1953) is adapted from chromatographic spot test technique for electrophoresis of proteins. It does not appear to stain any specific amino acid but rather reacts with the NH_2 groups of basic proteins by direct salt formation. Mazia et al (1953) also speculated that SH groups, aromatic groups and free COOH may contribute to the overall reaction. The function of mercury appears to be as a linkage which permanizes the binding of the dye to the reactive groups of the protein. (Baker (1958) and Kanwar (1960) have criticized the technique on the grounds that it was not specific to protein. It was used in the present investigation combined with a deamination procedure of Johnson (1969b).

Technique Used: Method after Pearse (1968).

Solutions:

I Deamination solution

60% Sodium nitrite	20 ml
1% Acetic acid	60 ml

II Mercury Bromphenol Blue

Mercuric Chloride $HgCl_2$	1 gm
Bromphenol Blue	.05 gm
2% acetic acid	100 ml

Procedure:

1) Duplicate sections a,b are brought to distilled water.

a	2) Deamination solution	16 hrs.
a, b	3) Stain in Mercury Bromphenol Blue	2 hrs.
a, b	4) .5% acetic acid	5 mins.
a, b	5) Distilled water	Rinse
a, b	6) Dehydrate, clear and mount.	

Expected Results:

a) Lightly or unstained due to removal of protein.

b) Protein stained clear blue color.

Appendix No. 8

General Stains

General methods used in the present study were Harris' Hematoxylin-Eosin, Mallory's-Aniline Blue-Orange G.

For visualizing Ca ions in section, Von Kossa's stain for calcium was used.

8.1 Harris' Hematoxylin - Eosin

Technique Used: After Thompson (1966).

Solutions:

I Harris' Hematoxylin

Hematoxylin	5.0 gm
Alcohol 100%	50 ml
Aluminum Ammonium Sulphate	100 gm
Distilled water	1,000 ml
Mercuric Oxide	2.5 gm

II Eosin

Eosin Y	1.0 gm
Distilled water	20 ml
Alcohol 95%	80 ml

Working solution: 3 parts alcohol 80%, 1 part Eosin; just before use add .5 ml acetic acid per 100 ml eosin solution.

Procedure:

1)	Deparaffinize and hydrate to water.	
2)	Harris' hematoxylin	15 mins.
3)	Tap water	4 dips
4)	Acid alcohol	5 dips

5) Tap water	Wash
6) Ammonia water	5 dips
7) Tap water	15 mins.
8) Eosin	30 secs.
9) 95% alcohol	Rinse

10) Dehydrate, clear and mount.

8.2 Mallory's Aniline Blue-Orange G

Method Used: Mallory (1938) after Thompson (1966).

Solutions:

I Aqueous acid fuschin .5%

II Aniline Blue-Orange G (make up fresh)

Aniline blue (water soluble)	.5 gm
Orange G	2.0 gm
Phosphotungistic Acid	1.0 gm
Distilled water to make	100 ml

Procedure:

1) Bring sections to distilled water.	
2) Place formalin fixed tissue in saturated mercuric chloride at 60°C	3 hours
3) 1% alcoholic iodine	5 mins.
4) Running tap water	Wash
5) 5% sodium thiosulphate	5 mins.
6) Running tap water	15 mins.
7) Distilled water	Wash
8) .5% acid fuschin	5 mins.
9) Aniline Blue-Orange G	55 mins.
10) 95% alcohol	2 mins.

11) Dehydrate, clear and mount.

Results obtained with echinoid test of Strongylocentrotus droebachiensis, Strongylocentrotus purpuratus:

Epidermal cuticle:	dark blue.
Epidermal cells:	dark blue around edges; nuclei orange and red; red and yellow granules.
Basement lamina:	dark blue.
Bladder amoebocytes, stereomal cells including cells around peritoneum:	grey cytoplasm that is often outlined in dark blue; orange (yellow) nuclei.
Vibratile cells:	cytoplasm light blue-grey, refractile; nucleus orange.
Type I spherule cells:	light blue - grey; dark blue; yellow nuclei.
Type II spherule cells:	deep red, granular - yellow orange granular.
Sutural connective fibers:	dark blue.
Peritoneal fibers:	dark blue.
Muscle sheets:	deep red - orange red.
Connective tissue:	light - dark blue.

8.3 Von Kossa Technique for Calcium

Mechanism of Staining: The technique is based on metal substitution; calcium carbonate, phosphate, oxalate, etc. exchange anions with silver nitrate. The resultant silver salt undergoes photic reduction to metallic silver in the presence of bright light. It should be borne in mind that the technique is not specific for calcium per se or any particular anion of

calcium - that is, any of the anions listed above will react even if they are not combined with Calcium (Thompson 1966).

Technique Used: Method after Thompson (1966) after Mallory (1942).

Solutions:

I 5% silver nitrate.

II .1% nuclear fast red.

Procedure:

1)	Bring sections to water.	
2)	Silver nitrate expose to bulb	30 mins.
3)	Distilled water	Rinse
4)	5% sodium thiosulphate	3 mins.
5)	Distilled water	Rinse
6)	Nuclear fast red	3 mins.
7)	Distilled water	Rinse
8)	Dehydrate, clear and mount.	

Expected Results:

Calcium anions: black.

Note: Although Von Kossa's stain for calcium was used, it was generally found that examination under crossed Nicol's was a much more effective way of localizing calcite deposits.

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