RECONSTRUCTION OF SINGLE COMB FROM DISSOCIATED CELLS ON THE CHORIOALLANTOIC MEMBRANE

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RECONSTRUCTION OF SINGLE COMB FROM DISSOCIATED CELLS ON THE CHORIOALLANTOIC MEMBRANE

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ABSTRACT

Reaggregation of White Leghorn single comb cells was studied in culture. Entire comb organs from 8 and 10 day incubated chicks were excised and dissociated in a calcium-magnesium-free trypsin solution. The dissociated cells were washed in Tyrode's solution, centrifuged, transferred by pipette to the chorioallantois of host chicks, and developed for approximately 10 days.

Some comb structure was reconstructed in the grafts. Two organ patterns formed; elongate ridge and irregular points. Several tissues developed. Typical comb epidermis formed in many grafts. Dense regular dermis and a comb-like vascular pattern formed in all grafts. Loose irregular tissue was frequent. Several grafts formed non-comb tissues of cartilage and bone.

Tissue interactions between epidermis and dermis and between cartilage and dermis were apparent. Where thin epidermis formed on the free margin of the comb ridge, underlying dermis was usually organized in typical comb pattern. Cartilage formed an elongate rod, and when present, comb ridge was prominent and the vascular and dermal patterns were most typical. There was evidence that mass of tissue was significant: cartilage formed in all whole comb cellular suspensions, but was missing in most of the partial combs.

Comb mesenchymal cells possess some pleuripotency; a capacity expressed in formation of cartilage and bone. Pleuripotency did not include feather papillae. Since comb is an integumentary derivative, the lack of feather formation in any of the grafts is considered significant.

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INTRODUCTION

The purpose of this study was to find out if the phenomenon of aggregation of embryonic chick comb would occur and to what extent the geometric pattern of comb organogenesis could be reconstructed.

I used a modification of Moscona's experimental technique to study aggregation of single comb cell suspension on the chorioallantoic membrane (Moscona, 1952). A specific objective was to determine whether dissociated and isolated comb cells from 9 and 10 day embryos would aggregate to form comb.

The form of the single comb is basically a dorsoventrally symmetrical ridge. The highest part of the ridge is found in the posterior blade. From this peak, the ridge decreases in size in a series of points anteriorly. The lowest section of the ridge is an anterior unserrated division. Histologically, typical comb epidermis is thin and without feathers and can be distinguished from the surrounding epidermis. The dermis is chiefly fibrous and consists of three strata. The fibers are most compact in the peripheral layer and are loose and more random in the center (Lawrence, 1963). These morphological patterns are easily recognized, and results from cellular aggregation experiments should lend themselves to reasonable interpretation.

Organogenesis is brought about by a series of genetically determined interactions which control characteristic tissue arrangements of a given organ. Organ-specific regional environments develop which determine, in part, the nature of the developing cells. The cells become determined as specific types at certain stages in development;

and, following this, environmental influences may become less pronounced. If these cells are transplanted to another area on the embryo at an older stage, they will develop into a "determined" type and assume organ specific tissue patterns. Whole organs may self-differentiate in uncharacteristic areas.

Several related experiments have been performed to determine whether a group of cells transplanted as an intact tissue contained an extracellular determinant which may cause characteristic organ formation, or whether each individual cell contained an intracellular determinant which may cause characteristic organ formation. For example, H. V. Wilson (1908) dissociated adult sponge cells by passing them through silk bolting. In culture, these sponge cells clump together and aggregate as small sponges with epidermis, collar cell chambers, and archeocytes, in the same relative positions as in the original sponge.

Later dissociation studies have included vertebrate embryos. Dissociation of tissue cells can be brought about in various ways: 1) mechanical, by straining through silk or nylon or by forcing the cells apart with a mortar and pestle; 2) chemical, by raising the pH of the surrounding medium which results in the breaking of the connections between the cells; and 3) enzymatical, by means of a digestive enzyme which destroys intercellular bonds (Weiss and Taylor, 1960).

If the cells of an amphibian embryo in the late blastula or early gastrula stage are dissociated and isolated in culture, the presumptive ectoderm, mesoderm, and endoderm, will assume characteristic positions.

A. A. Moscona (1952) was a pioneer in enzymatical dissociation of embryonic cells. He successfully used trypsin. He found that briefly

exposed cells will not be damaged even in relatively large concentrations of the enzyme. At first he placed the cells in in vitro culture where aggregation occurred without obvious outward stimulation, and found that the cell numbers must be of a critical mass before they would aggregate. This was also observed by Weiss and Taylor (1960). Rotating flasks were used to speed clumping of the cells to the critical mass for aggregation. This method improves reproducibility and consistency of results (Moscona, 1961a). The cellular mass in in vitro cultures would reach a certain size and then cease to grow. Cessation of growth was attributed to a lack of vascularity in the tissues (Weiss and Taylor, 1960; and Moscona, 1961b) so in vivo culture was tried. The chorioallantoic membrane of host chick embryos can be used to provide a vascular system sufficient for further growth on a relatively indifferent medium (Hamburger, 1966). In this case dissociated cells are centrifuged to form a pellet of cells before they are grafted to the membrane. This technique has been used in studying several vertebrate organs.

REVIEW OF LITERATURE

The capacity of cells to aggregate after partial or complete dissociation has been investigated extensively. It is well established that the organs of certain invertebrates can be broken down and the separated cells will associate into characteristic structures (Weiss and Taylor, 1960). Wilson (1908) pioneered such work with his studies of sponges. The capacity to aggregate is found in vertebrates also. Holtfreter (1948) observed that early amphibian embryonic cells would aggregate in the same relative positions as in whole embryos. Furthermore, it was found that cellular suspensions of chick and mannal tissue would form characteristic patterns (Moscona, 1952; and Weiss and Andres, 1952).

Invertebrate and amphibian cellular aggregates are able to maintain themselves in isolation for some length of time as self-contained units (invertebrates) or with stored yolk providing nutrients (amphibians) (A. Moscona and H. Moscona, 1952). The Mosconas found that work that is done with chick and mammal embryos requires more exacting technique as the nutritional and thermal conditions have to be supplied by the experimenter. Weiss and Andres (1952) met the requirements for dissociated chick embryos by injecting mechanically separated cells into the vascular route of host embryos. These cells settled in various places along the route and continued to differentiate. Some of the aggregates formed were located by the color difference in feathers. In these early studies, whole embryos were used for their cellular suspensions and various types of tissues were obtained in the aggregates. Enzymatical dissociation of cells from homiothermic animals was employed by Moscona (1952). He

tested hyaluronidase for dissociation of the cells, but it brought about irreversible abnormalities in a large number of the cells. Trypsin did not attack the cells even in relatively large concentrations so this enzyme was used in his later studies. With enzyme dissociation, the number of viable cells in a cellular suspension was increased. He used a calcium-and magnesium-free Tyrode's solution with 1% salt-free trypsin and found that the absence of calcium and magnesium salts decreases the stability of the intercellular materials and the mutual adherence of cells. The digestive enzyme, trypsin, in a calcium-and magnesium-free saline solution, attacks selectively the peptide linkage adjacent to lysil and arginyl residues (Moscona, 1963). The procedure of dissociation is effective in most cases for chick embryos up to about 10 days. After this time, the basement membrane becomes resistant to the trypsin and another enzyme is needed (Grover, 1960). Collagenase may be used for basement membrane breakdown.

In his initial studies, Moscona cultured the tissues in a hanging drop (Moscona, 1952; and A. Moscona and H. Moscona, 1952) and the cells formed self-aggregates. The technique was later improved by the use of rotating flasks so that the cells could be concentrated (Moscona, 1961a). Still later the membrane of host chick embryos was used. The chorioallantois provided a vascular supply which supported grafts for longer periods on a relatively indifferent medium (Hamburger, 1966; Weiss and Taylor, 1960; and Garber and Moscona, 1964).

The first studies Moscona performed were on limb bud rudiments in four-day chicks. He later worked with mesonephros and skin. From the studies, he found that the dissociated cells would aggregate to form

characteristic tissues (Moscona, 1952; and A. Moscona and H. Moscona, 1952).

The principle of reassociation was carried over to older chick embryos. It is now apparent that dissociated tissue from 7-14 day chicks will reassociate by cell migration and selective adhesion to form characteristic patterns (Weiss and James, 1955; and Steinberg, 1963). Steinberg states the cells regroup each with others allied to it and reconstruct the various original tissues.

In an effort to find out whether a particular group of cells will combine with cells from other tissues, Moscona used tissues from Swiss albino mice as markers. The mouse cells have a larger nucleus and stain differently from chick cells (Moscona, 1957). If cellular suspensions of like organ rudiments of chick and mouse embryos are intermixed, the cultures will aggregate and combine to form chimeric tissues. Moscona finds that aggregates skin from chick and mouse embryos will form feathers and hair respectively. In co-aggregates of mouse and chick cells, however, the feathers are suppressed but hair is not (Garber and Moscona, 1964; and H. Moscona and A. Moscona, 1965). Recent studies by Moscona (1967) indicate that age is critical for feather formation in the chimeric aggregates. The structure developing from cellular suspensions form recognizable morphology rather than a chaotic assemblage of cells (Balinsky, 1964). Furthermore, Weiss and Taylor (1960) find evidence of aggregated liver and kidney tissues being functional. Such studies indicate controlled aggregation takes place which results in a characteristic pattern.

METHOD AND MATERIALS

The White Leghorn breed was used in this study. Eggs were obtained directly from the Castlebury Poultry Farm, Apex, North Carolina. Many of the eggs were stored for short intervals at 17°C before incubating. A 600 egg capacity cabinet incubator was used. Average dry bulb temperature was 37.7°C while wet bulb readings ranged from 30.0 - 30.5°C. These are optimal value and were recommended by the manufacturer of the incubator.

The technique for dissociation followed the standard procedure described by Moscona (1952) with some modifications. A one percent trypsin solution was made, using crystalline 2x salt-free trypsin (obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio) in calcium-and magnesium-free (CMF) Tyrode's solution. This solution was warmed to incubation temperature before using.

Eggs were incubated for nine or ten days, which allows the embryos to develop to stage 36 or 37 (Hamburger and Hamilton, 1951). At these stages, the comb ridge is conspicuous and the points are visible. Before operation, the eggs were candled and were divided into donor and host groups. Initially the grouping was arranged to insure a host embryo for every donor. Later one donor was used for every four or six hosts. The donor eggs were prepared by opening them into a medium size finger bowl and removing the extraembryonic membranes from around the embryo. The exposed embryos were than placed in a watch glass containing warm, sterile 0.86% NaCL solution and staged. Watchmaker's forceps were used to hold the embryo in position during the operation. The comb

organ was removed with iridectomy scissors by cutting through the skin posteriorly and anteriorly to the ridge, and then laterally along each side. The entire organ was severed from adjacent tissues by making a fifth and last incision at right angles to and underneath the comb ridge. The excised organ was removed to a dish, minced with the scissors and placed in a small sterilized test tube which contained approximately 2 ml of 1% trypsin in CMF Tyrode's solution. The cells were then incubated at 37.7°C in the trypsin for 30-45 minutes or until the cells were dissociated. The tubes were shaken at intervals and the medium agitated by rapidly pipetting it a number of times. Occasionally a portion of the cellular mass was examined under the microscope to confirm cellular dissociation.

The tubes containing dissociated comb cells were lightly centrifuged using an International Clinical Centrifuge to concentrate the cells. The trypsin solution was decanted and the cells washed with complete Tyrode's solution (Davenport, 1960) to remove any remaining enzyme. The cells were washed in repeated changes of Tyrode's ten times, centrifuging and decanting the solution after each wash. Following the washing, the tubes containing the dissociated cells were placed in the incubator no longer than 15-30 minutes while the hosts were prepared.

The host chicks were exposed by cutting a circular opening in the shell above the embryo using a drill provided with a circular abrasive dental disc. The shell membranes over the air sac were punctured to cause the embryo to settle to a lower place inside the shell. This allowed more room beneath the opening for the donor cells to develop (Wenger, 1951). The dissociated clump of donor cells was then transferred

from the tube to the chorioallantoic membrane by means of the standard pipette. The opening through the host membranes was then covered with a sterile, circular cover glass and sealed with melted paraffin. The host egg was identified by a number and returned to the incubator for eight or nine more days. Chorioallantoic grafts were removed at around nineteen days incubation before the host membranes started to dry prior to hatching. Some of the comb grafts were transferred to a second ten day host for another nine day period of development. These cellular masses were permitted to grow for a more extended length of time in order to develop larger grafts.

The grafts were excised at the termination of development and placed in Bouin's fixative. Following fixation, the grafts were stained with iron hematoxylin in toto (Wenger, 1951), embedded in paraffin, sectioned at 10 microns, and mounted with Permount for histological observation. Graphic reconstructions were made by projecting the histological sections with a bioscope and marking the vertical and horizontal dimensions for every fifth section. Finally, photomicrographs were taken of representative tissues.

RESULTS

Table 1 presents a summary of the experimental operations performed during the investigation. Grafts were made of whole comb and partial comb cellular aggregations. Host and graft survival was greater in the cases where only a portion of the tissue was used. A whole comb was apparently too large for some host embryos to support. Table 1 shows that of 39 sectioned grafts, 25 developed comb-like tissue or comb organ. Some comb organ structure was recognizable in all grafts and usually consisted of an elongate ridge with some irregular surface projections. Five other grafts were not sectioned. The remaining 29 survivors did not yield successful grafts.

Table 2 presents a tissue analysis of the 25 sectioned grafts of aggregated comb. Vascularity and dense regular dermis are characteristic of comb and were used as criteria in establishing the presence or absence of comb. In all cases, dermis with dense regular organization and a vascular pattern were seen. Thin comb-like epidermis developed in 17 of the grafts. Generally, where characteristic epidermis formed, the dermal pattern was more organized and was similar to the dense regular tissue of the peripheral layer in the normal comb (Plate III, Figure 10). Some non-comb tissues differentiated; these were two grafts with bone, six with cartilage, twelve with reticular, and ten with loose irregular connective tissue. However, the loose irregular tissue resembled, in pattern, that tissue forming the central layer of normal comb. No feather papillae were noted in any of the comb skin grafts.

Table 1. Summary of Experimental Operations on Dissociated Comb

Amount of Comb	Number of grafts	Host survival number	%	Number of grafts recovered	Sectioned number sectioned	grafts number with comb
Whole comb	88	16	18.2	10	5	2
Portions of comb	103	57	55.3	34	34	23
Totals	191	73	38.2	44	39	25

Table 2. Tissue Analysis of Sectioned Grafts of Aggregated Comb

Code Number	Differentiated Tissue						
	Bone	Carti-lage	Dermis	Dermal organization			Epidermis
				comb-like	dense irr.	loose	
Complete comb							
27	-	+	+	+	+	+	+
38	+	+	+	+	-	+	+
Partial comb							
73	-	+	+	+	-	+	+
76	-	-	+	+	-	-	-
79	-	-	+	+	-	-	+
80	-	-	+	+	-	-	+
81	-	-	+	+	-	-	+
84	-	-	+	+	+	+	+
85	-	-	+	+	-	-	+
90	-	-	+	+	+	+	+
93	-	-	+	+	-	+	+
94	-	-	+	+	+	-	+
96	-	-	+	+	+	-	-
100	-	-	+	+	+	-	+
104	-	-	+	+	-	+	+
110	-	-	+	+	-	-	-
114	-	-	+	+	+	-	-
132	-	-	+	+	-	-	-
133	-	-	+	+	+	-	-
140	-	+	+	+	+	-	-
142	+	+	+	+	-	-	+
143	-	+	+	+	+	-	+
191	-	-	+	+	+	+	-
192	-	-	+	+	-	+	+
196	-	-	+	+	+	+	+
Totals	2	6	25	25	12	10	17

Plate I presents graphic reconstructions of three of the grafts. Figures 1-a, b, and c show the lateral views and Figures 2-a, b, and c are diagrams of partial comb grafts. A comb ridge was seen in all three cases. The ridge is irregular and contains projections which may be comparable to points found on normal comb.

Plate II contains photomicrographs of graft Number 38. The levels of the photographs on the tissue are indicated in Figures 1a and 2a. Unlike normal comb, the dense dermis is central in position with loose material on the periphery. Figures 4 and 6 show different levels taken of the same tissue section from graft Number 38. A cartilage rod is under the middle portion of the graft but does not extend the entire length of the graft. Bone is near the center of the section (note Figure 5 also). Bone and cartilage were found in close proximity in the two cases where bone occurred. Figures 3, 5, 6, and 7 show the projecting free surface of the graft and clearly show dermis as a core in the center of the graft. A very distinct epidermis is present. In spite of the fact that the dermal layers are reversed, there is no lack of organization even in these layers. Figure 5 is of a complete tissue section from the middle of the graft and the presence of the cartilaginous rod is conspicuous. On the other hand, Figure 7 is from a section near one end of the graft and the cartilage rod has almost disappeared. There was no cartilage beyond this point.

Plate III and Figures 8 and 9 are photomicrographs of graft Number 93. The levels of these photographs are given in Figures 1c and 2c. There is a distinct comb-like pattern of the tissues in this case with the dense regular dermis being found outside the loose irregular and

vascular tissue in the center. Though typical epidermis is not present, the aggregate is similar to normal comb in the pattern of dermal tissues. Plate III and Figure 12 was taken of graft Number 92. This specimen also shows organization of tissue that is characteristic of comb. In graft Number 92, typical comb epidermis is present. Organization of dermis is more regular in the comb-like process of graft Number 85 (Plate III, Figures 10 and 11). In this specimen, typical comb-like epidermis covers the free surface.

Plate III and Figure 13 shows the cartilage rod that formed in graft Number 73. As noted above, this rod does not extend the entire length of the graft. Graft Number 143 is shown in Figure 14 (Plate III). A definite ridge with a dermal pattern typical of comb formed. Cartilage was present in a basal position throughout most of the graft.

DISCUSSION

The material presented in this study indicates that dissociated single chick comb cells cultured in vivo will aggregate to form comb-like tissue and some comb organ structure.

Comb-like dense regular dermis and vascular pattern were recognized in all of the sectioned grafts. The consistency in the formation of these tissues can be correlated with development of definitive structure. In typical comb, the most obvious histological feature is seen in the organization of dense dermis in a superficial layer. The vascular network of the central layer is also pronounced. Less apparent is the loose irregular tissue in the intermediate layer. In the grafts, the underlying dermis was most organized where a thin comb-like epidermis was present. The regular alignment of these dermal cells and fibers is so striking as to suggest the force of some important interaction between the tissue layers. Even without epidermis, the aggregates reveal the strength of some regulative capacity among comb cells, especially in the establishment of dense regular dermis.

The dermal pattern of normal comb with dense regular tissue in the periphery and loose irregular in the center was seen in most of the grafts. In graft Number 38 (Plate II, Figures 3, 5, 6, and 7), the exact opposite arrangement was present. Even here, however, there was some formative interaction for comb organization. Several comb-like patterns were noted in formation of an elongate ridge, a cartilaginous rod, a dense dermis, and in vascularity. All of these tissues formed in close association as if in an organizing center with controlled interaction.

Only comb tissue was excised in these experimental proceedures, and that a variety of tissues formed is interesting. Certainly, the presence of bone, cartilage, and reticulum indicates that comb dermal cells are pleuripotent and can differentiate into tissues that are different from comb and skin. The comb dermis is derived from mesenchyme. And, in general, mesenchyme is considered to be on a low level of differentiation and is found in adult formative areas of bone, cartilage, and reticular tissue. Perhaps this explains, in part, the occurrence of some diverse tissues in the grafts.

The fact that aggregated comb tissues are orderly and identifiable indicates a certain amount of selectivity among the cells. In the grafts that contained cartilage, only a single cartilage rod developed. If some selectivity or organizing influence had not been operating, several patches of cartilage might have been formed throughout the grafts. Furthermore, aggregation into an elongate structure that conformed to comb ridge rather than into an oval or spherical shape shows complex control in origin of geometric pattern. The latter control may be one of axiation or polarity.

It is to be noted that cartilage developed only in the middle region of the combs and not under the anterior-and posterior-most portions. Furthermore, the two whole comb grafts had cartilage, but cartilage was missing in all of the partial combs except four. In both situations, the results may be due to the number of cells used in the grafts: the cells may not have been sufficiently populous to initiate cartilage differentiation. The cartilaginous rod formed in these grafts is similar to the one noted by Lawrence (1963) in undissociated primordial grafts of

comb cultured on the chorioallantois. In the primordial grafts the cartilage was thought to be derived from the frontonasal system, perhaps ethmoidal in origin. Certainly this is not the case in these aggregate grafts, though it may be assumed that low differentiated mesenchymal cells along the basal surface of the comb could have been included in the excisions.

There is some correlation between prominence of comb ridge and the development of the underlying cartilage rod. Though this rod is not necessary for support of the comb as seen with development in most of the grafts, it appears to have some organizing influence in comb ridge formation. Characteristically, the comb organ is a surface protrusion that describes an elongate ridge and it is important that many of the aggregates formed a recognizable ridge. In association with epidermis and cartilage, development of ridges was most distinct. As noted above, dermal differentiation was also more typical in these combs. The interactions that apparently occur between development of cartilage and dermis and between development of epidermis and dermis may be very important in formation of definitive pattern under both dissociated and undissociated cellular states.

Some irregularities are seen along the comb ridge in the graphic reconstructions. These may be points. If so, they are not arranged in a regional pattern of distribution. Lawrence (1963) found that comb grafts which grew on the chorioallantois were compressed and points were atypical. In more recent studies, he found that comb would not elongate without beak (Lawrence, 1964). Combs that do not elongate have thick and fewer points. It is not surprising, then, that comb aggregates from

atypical points in shape and number. The interest here is that comb cells do possess some ability to regulate point formation.

There was little apparent anterior-posterior differential in pattern of components seen in the grafts. Thus, graphic reconstructions show no clear regional divisions that are characteristic of normal comb. It would seem, then, that dissociated comb cells have limited ability to regulate a complete anterior-posterior gradient.

The comb is a featherless integumentary derivative. That none of the reconstructed grafts developed feathers, while some other non-comb tissues formed, has important implications. Furthermore, these other non-comb tissues were non-integumentary. Lawrence (1963) found in tissue recombination grafts of feather determined ectoderm with presumptive comb mesoderm an inhibition in the development of previously formed feather placodes. Amprino and Camasso (1959) also observed feather suppression with comb mesoderm. In chimeric aggregates of chick and mouse skin, feather germs are usually suppressed, though hair follicles will develop (Levak-Svajger and Moscona, 1964; and Moscona, 1967). In this case an age dependent shift is seen to operate where chimeric feathers are formed. These data suggest dermal specificity such that comb dermis is not compatible to feather development.

These cumulative results show that dissociated comb cells possess some capacity to aggregate comb structure. Ridge and point are organ patterns that are regulated. Since comb will not regenerate in later ontogeny (Hardesty, 1931), regulative ability is apparently lost at some point during histogenesis. Dense regular dermis and an organized vascular tissue are patterns that form generally through comb cell interactions. The cells respond weakly in aggregating regional distribution

of parts along an anterior-posterior axis. Finally, comb dermal cells are pleuripotent, but this capacity does not extend to feather papilla formation.

SUMMARY

Comb from nine and ten day embryos was dissociated and the cells were cultured as chorioallantoic grafts to determine whether aggregation will occur. Based on the experiments, these conclusions were drawn:

1. Dissociated comb cells possess some ability to reconstruct comb structure. An aggregate of such cells can form elongate ridge and irregular points.

2. Characteristic dense regular dermis and vascular pattern form as a result of tissue interactions. Some correlation is seen between organized tissue structure and presence of thin epidermis and cartilage.

3. Dissociated comb dermal cells have a capacity to differentiate into a variety of non comb-like tissues, i.e., bone, cartilage, and reticular.

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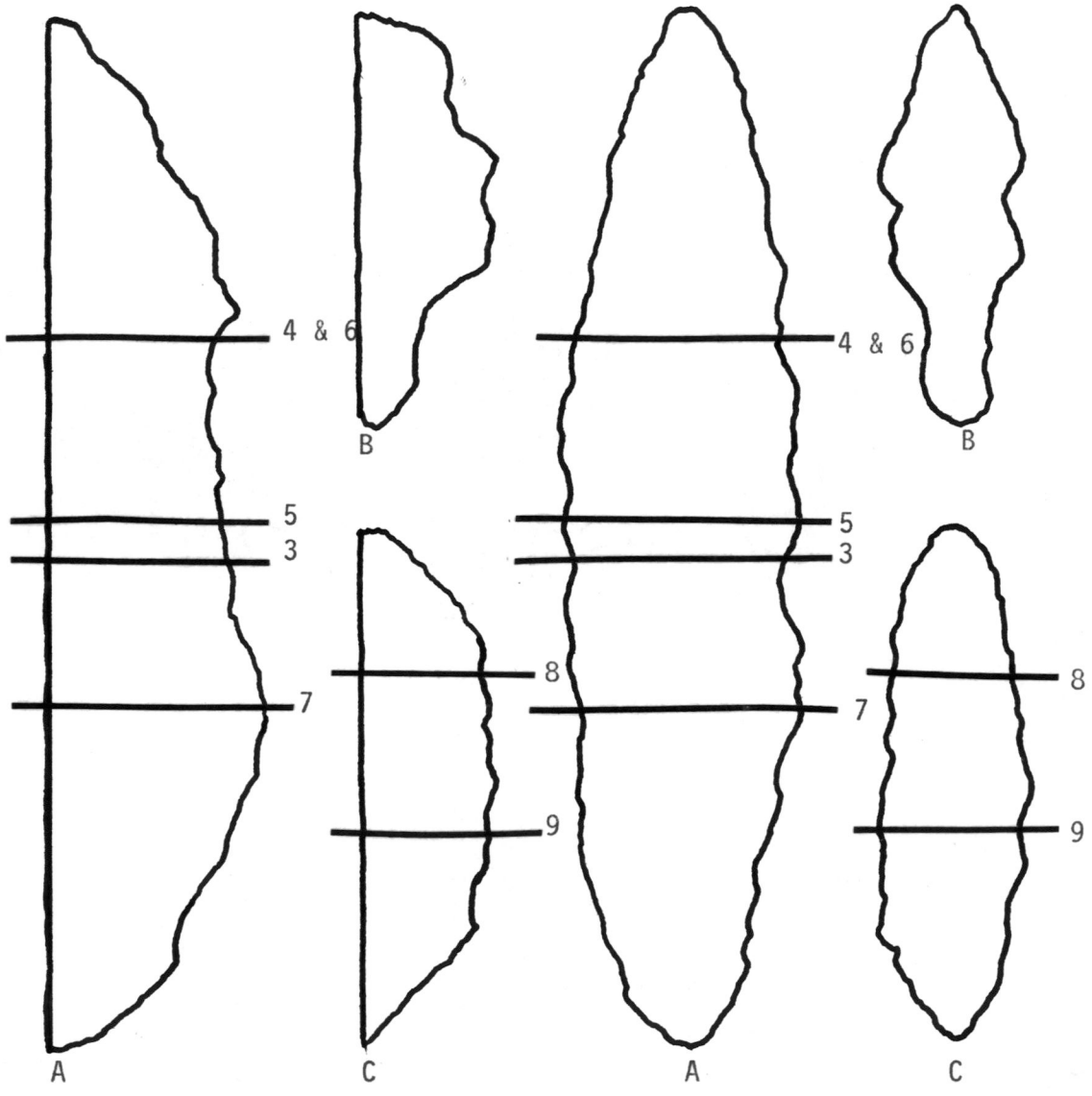


Plate I.

1mm=10μ

Figure 1. Graphic reconstruction
Lateral view

Figure 2. Graphic Reconstruction
Vertical view.

Legend: a graft Number 38, b graft Number 192, c graft Number 93, 3-9 are levels of photographs in Plates II and III.

Plate II.

All Figures in Plate II are of graft Number 38 and show tissue sections from different levels. These levels are indicated in Plate I, Figures 1a and 2a.

Figure 3. The free margin near the middle of the grafts. Dense dermis occurs centrally and loose vascular tissue peripheeally. A thin comb-like epidermis is also present.

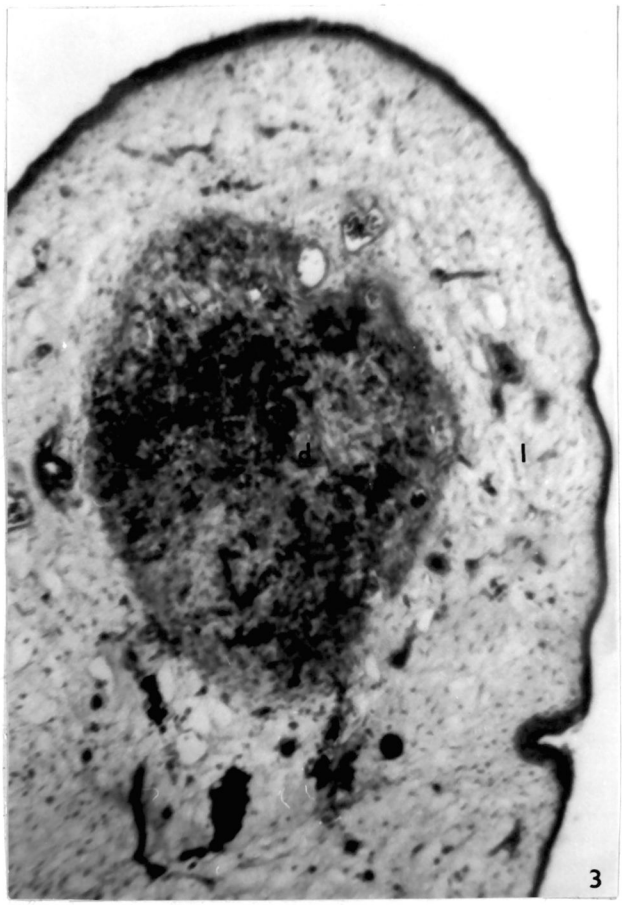
Figure 4. The basal area near one end of the graft. Cartilage and bone are differentiated.

Figure 5. An entire section near one end of the graft. Cartilage, bone, and irregular dermis appear. The basal position of cartilage in relation to graft attachment on the chorioallantois is apparent.

Figure 6. The free margin of the same section as Figure 4.

Figure 7. An entire section near the end of the graft and opposite to Figure 5. The cartilagenous rod terminated four sections behind this level.

Legend. c- cartilage, b- bone, l- loose irregular tissue, d- dense regular dermis, and e- epidermis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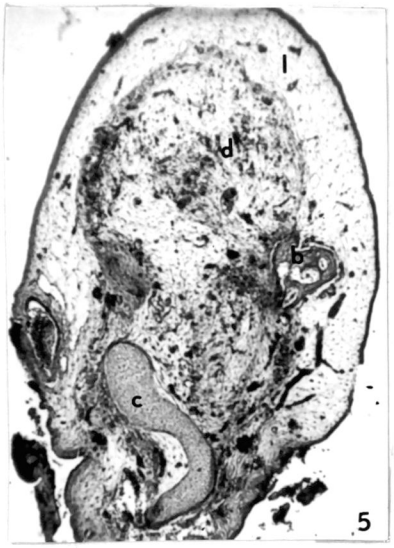
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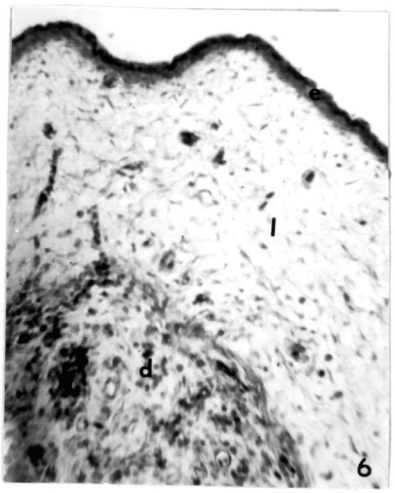
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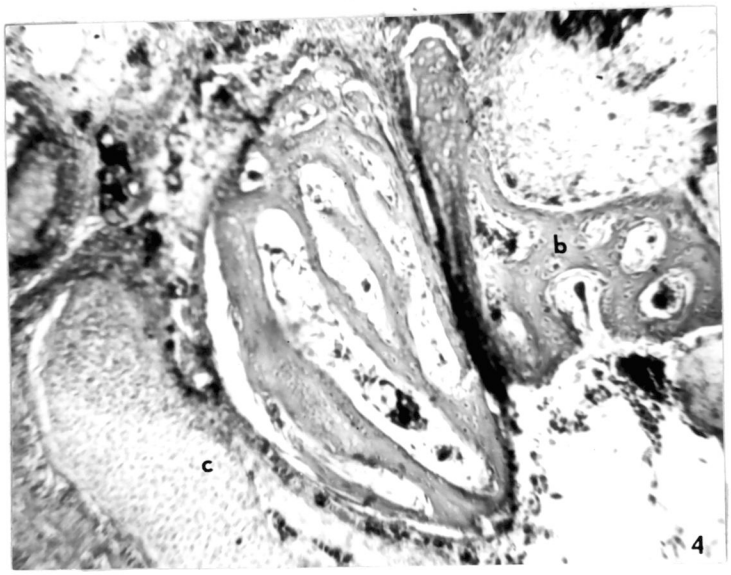
b
c
4

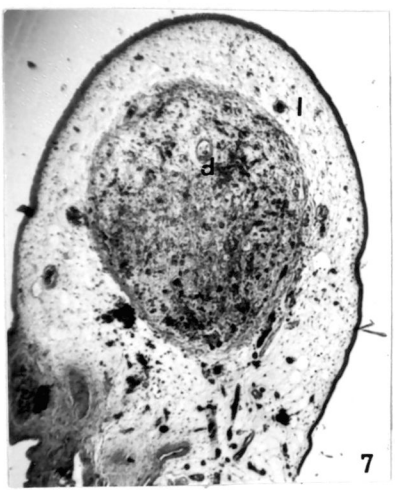
l
d
7

Plate III.

Figure 8. A cross-section taken from one end of graft Number 93. Dense dermal organization that is comb-like is seen peripherally. The vascular core is apparent. See Figure 1c for level.

Figure 9. A cross-section from another level of graft Number 93. A tissue pattern similar to that of Figure 8 occurrs.

Figure 10. A cross-section of graft Number 85 taken from near the center. The free process projecting superiorly shows a transverse arrangement of dermal tissue components. Comb-like epidermis is clearly seen along the margin.

Figure 11. An over all cross-section of graft Number 85. Showing attachment to the chorioallantoic membrane.

Figure 12. A cross-section of graft Number 192 taken from the center. The separate structure is typical comb.

Figure 13. A cross-section of a typical condition of the cartilage rod. Graft Number 73 is shown.

Figure 14. Prominent ridge is formed in graft Number 143 and is shown in cross-section.