Chapter 9

DISRUPTION OF THE EPITHELIAL-MESENCHYMAL INTERFACE OF THE REGENERATING NEWT LIMB WITH SALT AND STUDIES ON SALT-SEPARATED TISSUES

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Introduction

ONE OF THE CLASSIC PROBLEMS in the study of growth control is the phenomenon of inductive interaction between developing tissues. Experimental studies on developing chick and amphibian limbs have shown that an inductive interaction occurs between the epithelium at the distal tip of the limb and the underlying mesenchyme. By still unknown mechanisms, the epithelium controls the outgrowth and differentiation of the underlying mesenchyme, and the mesenchyme maintains the epithelium in its inductive capacity. In the regenerating limb a similar epithelial-mesenchymal interaction occurs, but after seventy years of investigation, the mechanism of this interaction is still unknown.

Interdependence of the Epithelium and Mesenchyme in the Regenerating Newt Limb

If the limb of the newt is amputated, a new limb regenerates. During the first thirteen hours after amputation, epithelial cells of the stump skin migrate over the wound surface on a fibrin-like clot (1). During the first fourteen days after amputation, mesenchymal blastema cells accumulate beneath the epithelium. Ultimately, the limb tip grows from a budlike protruberance to a new limb with digits virtually indistinguishable from the original.

What is known about the nature of the interaction between the epithelium that covers the amputation surface and the underlying mesenchymal tissue (Fig. 9-1)? It was shown by Goss (2) that regeneration cannot proceed unless an epithelium covers the stump surface. Amputated limbs inserted into the coelomic cavity were never covered by an epithelium and did not regenerate. Indirect experimental evidence (3, 4) indicated that, once the amputation surface was covered by epithelium, the mesenchymal stump tissue induced the thickening of the most distal epithelium into a structure called the apical cap. Carefully executed experiments of Thornton (5) showed that regeneration did not ensue if apical cap formation was inhib-

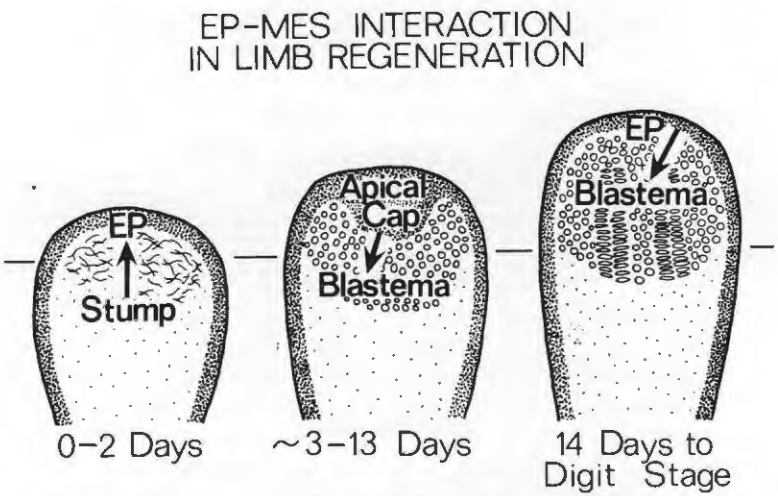

Figure 9-1. During the first several days after the epithelium closes over the open amputation surface, indirect experimental evidence suggests that the tissues of the stump mesenchyme induce the thickening of the apical epithelium (EP). Between approximately three and thirteen days after amputation, the apical epithelial cap influences the accumulation of mesenchymal-like blastema cells beneath itself and directs the outgrowth of the regenerate. Even though the apical cap disappears at approximately fourteen days, the epithelium is required for continued distal outgrowth of the regenerate until the formation of digits is observed.

ited by ultraviolet light irradiation. In addition, Thornton (6) and Thornton and Steen (7) showed that by nicking the epithelium adjacent to the apical cap, the apical cap became positioned eccentrically as the skin wound healed. The ultimate outgrowth of regenerating limbs with eccentric apical caps was at an angle to the normal axis of growth. This indicated that the apical cap influenced the accumulation of blastema cells beneath itself. Finally, experiments of Stocum and Dearlove (8) have shown that, until the formation of digits, the epithelium is required for continued distal outgrowth of the regenerate. (See 9, 10 and 11 for reviews.)

A Technique for Separating the Epithelium and Mesenchyme

The molecular nature of the epithelial-mesenchymal interdependence is not known. Perhaps more than anything, a technical stumbling block has inhibited the investigation of this problem. This stumbling block has been the lack of a technique for cleanly and reliably removing the epithelium intact from its underlying mesenchyme so that these tissues can be experimentally manipulated. Such a technique, which allows the easy removal of the epithelium from the underlying mesenchyme like a mitten or glove, has been developed (12). In the following discussion this technique

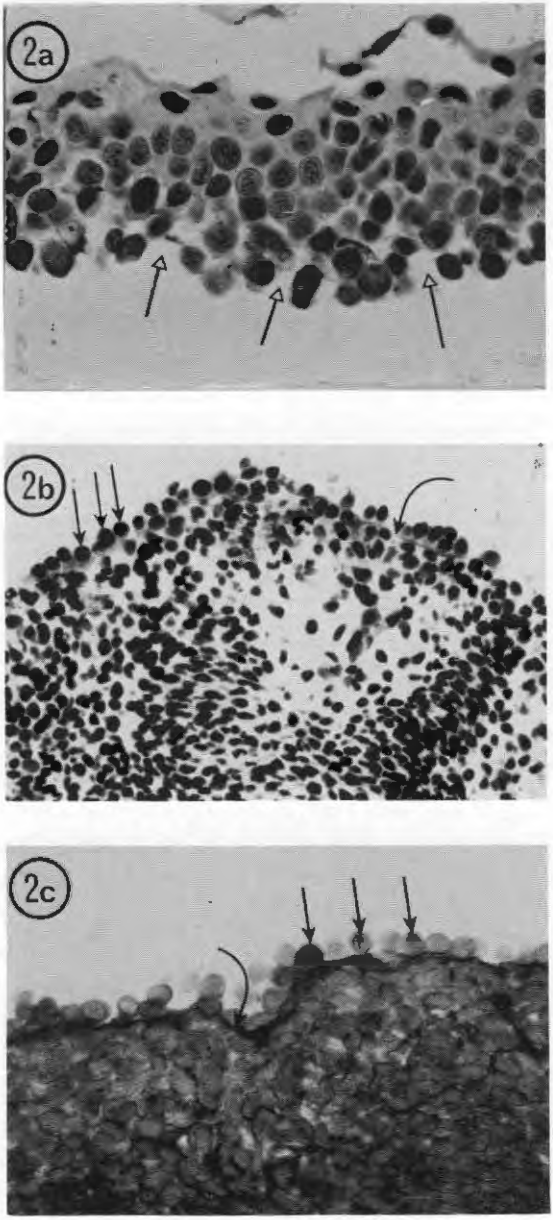

Figure 9-2. Sections of the epithelium and mesenchyme of the newt limb regenerate after an unsuccessful separation attempt. *A*. The basal epithelial surface contained gaps (open-headed arrows) that indicated cell loss. × 260. *B*. Basal epithelial cells remained adherent to the mesenchymal surface (straight arrows), but with hematoxylin and eosin staining the developing basement membrane (curved arrow) was seen only in fortuitous sections. × 130. *C*. Mallory's trichrome stain clearly accentuated the developing basement membrane (curved arrow) and allowed easy identification of the overlying basal epithelial cells (straight arrows). × 210.

will be described, and information gained by studying separated tissues will be presented.

In search for a separating medium, limb regenerates were isolated from the newt at thirteen to thirty-five days after amputation (24°C) and were incubated in solutions containing 1% or 3% Difco® 1 : 250 trypsin, 10 mM EDTA, or 150 units collagenase per ml medium. The common vehicle for each of these reagents was 100 mM sodium phosphate buffer at pH 7.5. Trypsin, EDTA, and collagenase have been used to separate the epithelium from the mesenchyme of embryonic tissues, as well as epidermis from dermis in a variety of skin preparations. Although these solutions allowed the removal of a major portion of the epithelium from the mesenchyme of the newt limb regenerate, histological examination of the epithelium showed that the basal surface was rough, indicating cell loss (Fig. 9-2A). Examination of the mesenchyme showed that these basal epithelial cells were adherent to the mesenchymal surface. Although not readily appreciated with hematoxylin and eosin staining (Fig. 9-2B), Mallory's trichrome stain accentuated the developing basement membrane in older regenerates and allowed easy identification of the overlying epithelial cells (Fig. 9-2C).

In these studies, it was noted that the common vehicle buffer chosen, 100 mM sodium phosphate, was by itself as effective a separating agent as the buffer containing trypsin, EDTA, or collagenase. After some trials, a separating medium was designed that contained 100 mM sodium phosphate buffer, 111 mM sodium chloride, and 5.6 mM potassium chloride (pH 7.5, 400 milliosmols/kg). Incubation of a limb regenerate isolated from the animal in this medium for sixty minutes at 37°C allowed the easy separation of the epithelial component of the regenerate from the mesenchyme. As seen in section, the epithelium removed from a regenerate after incubation in this saline-phosphate medium presented a smooth basal surface, suggesting no cell loss (Fig. 9-3A). The mesenchyme presented a smooth surface with no adherent epithelial cells. This was the case for both paraffin (Fig. 9-3B) and epon (Fig. 9-3C) embedded tissues.

The reliability of this technique was evaluated (Table 9-I). Twenty-five paraffin (wax) embedded mesenchymal components ranging in age from thirteen to twenty-five days (24°C) were serial sectioned, adherent epithelial cells were counted on one out of three sections, and the total epithelial cells per mesenchyme were calculated. Twenty-three mesenchymal components were rated *Clean* since each had less than twenty epithelial cells adherent to its surface. Sixteen mesenchymal components were embedded in epon and several sections from each specimen were evaluated on a more subjective basis. Fourteen mesenchymal components were rated *Clean* since no epithelial cells were seen on any section. It was concluded that this was a reliable separation procedure.

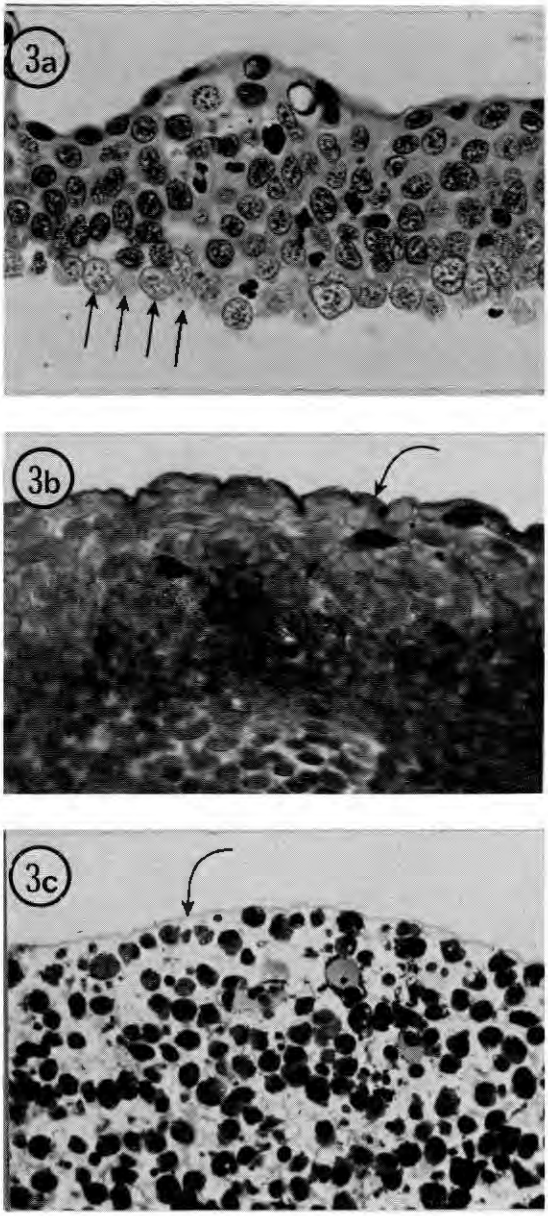

Figure 9-3. Sections of the epithelium and mesenchyme of the newt limb regenerate after a successful separation, for example using the saline-phosphate (salt) medium. *A*. The basal epithelial cell surface was lined by a continuous layer of basal epithelial cells (straight arrows). ×230. *B*. Mallory's trichrome stain of a paraffin section clearly accentuated the developing basement membrane (curved arrow) and showed that no epithelial cells were adherent. × 190. *C*. Tissues prepared for epon embedding (under conditions when limited shrinkage artifact occurs) were also free of epithelial cells at the surface of the developing basement membrane (curved arrow). ×200.

TABLE 9-I
EVALUATION OF MESENCHYME

	Wax	*Epon*
Nos. Trials	4	2
Nos. Limbs	25	16
Evaluation of mesenchyme:		
Poor (>50 Epith. Cells)	0	0
Good (20-50 Epith. Cells)	2	2
Clean (<20 Epith. Cells)	23	14

Studies on the Epithelial-Mesenchymal Interface Using Salt-Separated Tissues

The structure of the epithelial-mesenchymal interface of developing limbs has been studied extensively (13-20). These studies have demonstrated that, whatever the nature of the epithelial-mesenchymal inductive interaction in limb development, it takes place across the epithelial basal lamina. This, as well as other studies (21), favors the idea that an inductive substance diffuses from or is secreted by the epithelium into the underlying mesenchyme, and that direct contacts between epithelial and mesenchymal cells do not occur.

Few studies have been made of the epithelial-mesenchymal interface in regenerating limbs. Results of two early studies (22, 23) suggested that, unlike all developing limbs, an epithelial basal lamina is not found at the epithelial-mesenchymal interface of the young regenerating limb. What, if anything, provides a boundary between the epithelium and mesenchyme? Disruption of this interface with salt provided the opportunity to examine both the mesenchymal and epithelial sides of the interface with scanning electron microscopy. The information so obtained could be used to interpret the limited information obtained from transmission electron micrographs of the intact interface. It appeared that this was a valid approach to the study of the interface, especially the nature of the epithelial substratum. In experiments where the intact interface, as well as the mesenchymal and epithelial surfaces separated by the salt medium, were examined with transmission electron microscopy, dissolution or precipitation of materials at the interface was not demonstrable. It appeared that the separation occurred between the basal epithelial cell and its underlying substratum, the nature of which changed throughout the course of limb regeneration (24 and Jasch, preliminary results).

Wrist regenerates between three and twenty-five days after amputation (24°C) were removed from the animal and incubated in the saline-phosphate medium (Fig. 9-4). The epithelium was then removed from the mesenchyme. The mesenchymal components of these regenerates will be considered first. At three to fourteen days after amputation, the central

portion of the surface of the mesenchymal component was studied in detail using the scanning electron microscope. At the bulb, cone, and paddle stages, the central and distal tip of the now more elongated mesenchymal components were examined. At twenty-five days at the two and three digit stages, the tip of a digit of the mesenchymal component was examined. Mesenchymal components at all stages were also embedded and sections of the same areas described above were examined with transmission electron microscopy.

Correlation of data from transmission and scanning electron microscopy showed that the surfaces of all the mesenchymal components thus far

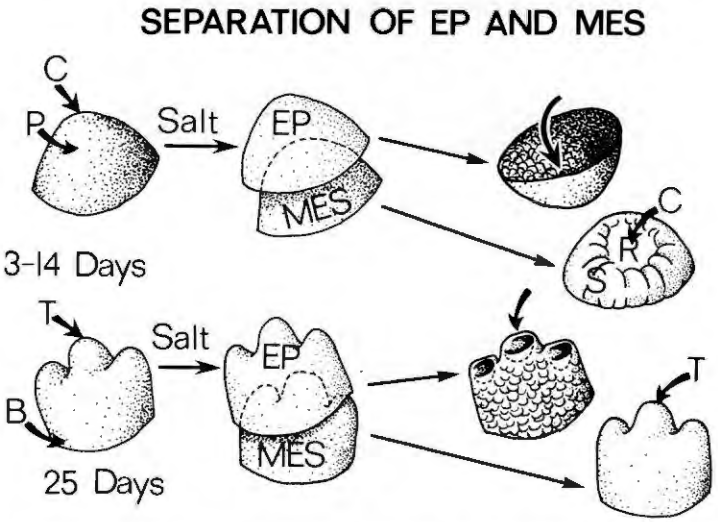

Figure 9-4. Intact regenerates between three and twenty-five days after amputation were removed from the newt limb with a razor blade cut. The isolated regenerates were incubated in a medium containing sodium chloride, potassium chloride, and sodium phosphate buffer (SALT). After one hour incubation at 37°C the epithelium (EP) was easily dissected away intact from the mesenchyme (MES). The *mesenchymal* portion of regenerates (R) between three and fourteen days after amputation were flattened and frequently were surrounded by a ring of stump tissues (S). Only the central portion (C) of the regenerate was examined with scanning and transmission electron microscopy. Regenerates at the bulb, cone, and paddle stages were more elongated, and only the distal tip of the newly exposed mesenchymal surfaces of these regenerates were examined. Finally, as digits developed, only the distal tip (T) of the mesenchymal component of the digit regenerate was examined. (See Figs. 9-5 — 9-8.) The *epithelial* components of three to fourteen day regenerates were fixed so that the tissues did not collapse. The newly exposed basal epithelial surface was examined at the area indicated (arrow). At twenty-five days the epithelial glove was too deep to examine; thus, it was everted (like turning a glove inside out) but the tips of the digits were left in their normal position. Only the newly exposed basal epithelial cells at the tips of the digits were examined (arrow). (See Figs. 9-15 — 9-16.) Arrows on the left side of the diagram indicate the regions of intact regenerates examined. Explanations are given with Figures 9-9 — 9-13.

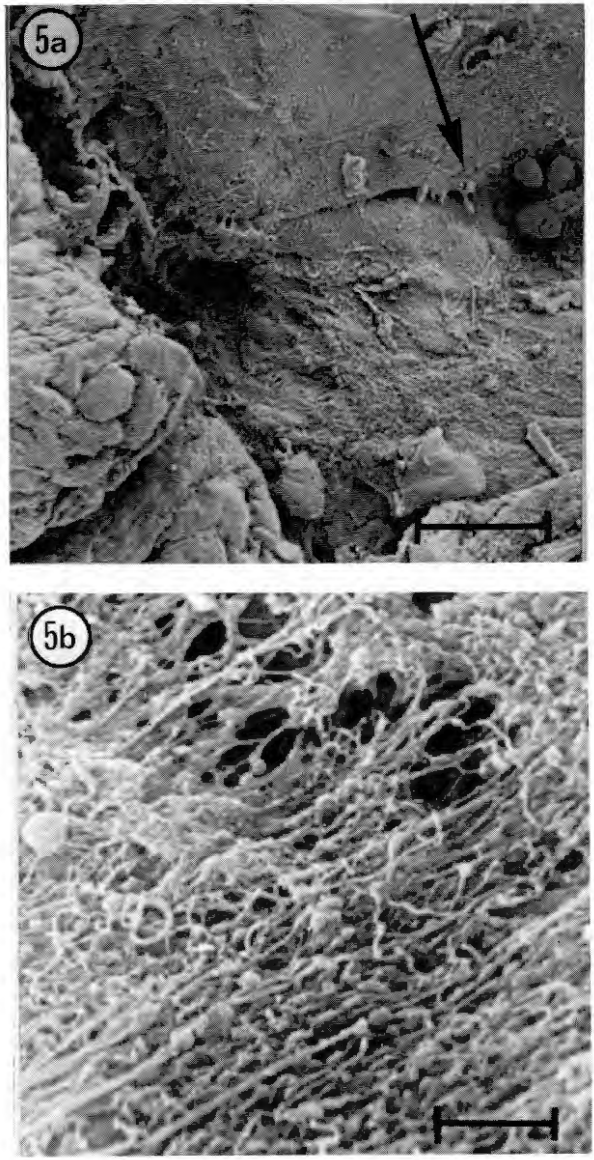

Figure 9-5. Scanning electron micrographs of mesenchymal component of five day regenerate. *A*. Low power view for orientation. Surface of regenerate at right, junction with stump tissue at left. Area at arrow is shown at higher magnification in B. ×400. Marker 40 μm. *B*. A fibrin-like mat consisting of fibers up to 140 nm in diameter covered most of the surface of the mesenchymal component of the regenerate. ×3,500. Marker 4 μm.

examined were coated by extracellular material. Considering here only the scanning electron microscopic data, preliminary observations of regenerates between three and fourteen days after amputation showed that at five days (Fig. 9-5) the mesenchymal component of the regenerate was coated

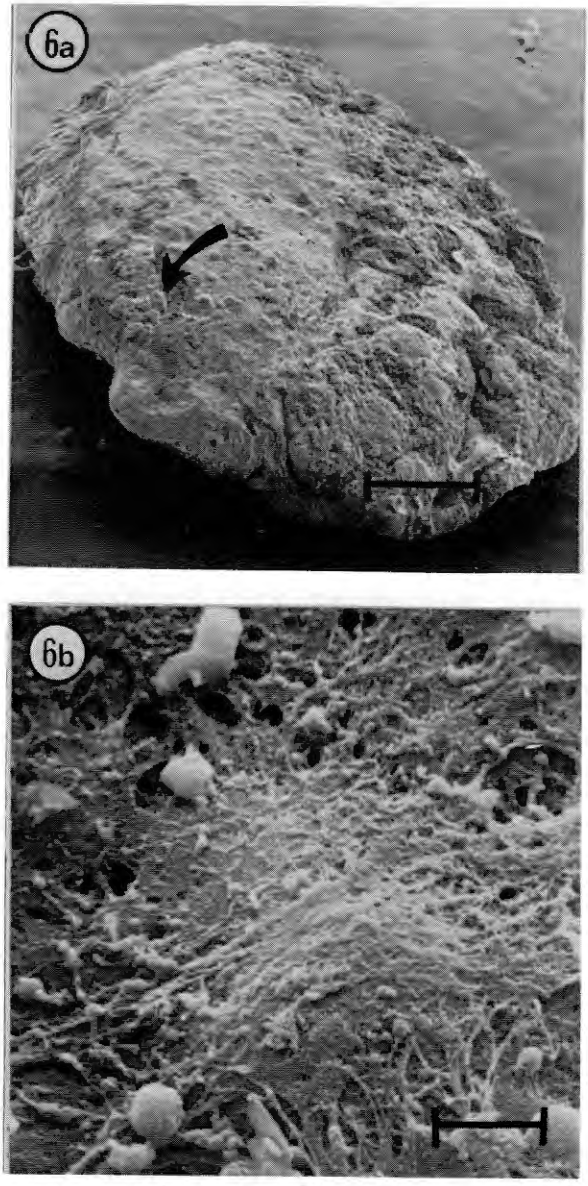

Figure 9-6. Scanning electron micrographs of mesenchymal component of a fourteen day (wound epithelium stage) regenerate. *A.* Low power view for orientation. Surface of regenerate towards left, stump tissue to right. Area at arrow shown at higher magnification in B. ×65. Marker 200 μm. *B.* A different type of material was found at the mesenchymal surface. This consisted of a meshwork of fine fibers up to 75 nm in diameter and an amorphous material filling the interstices between the fibers. ×3,250. Marker 4 μm.

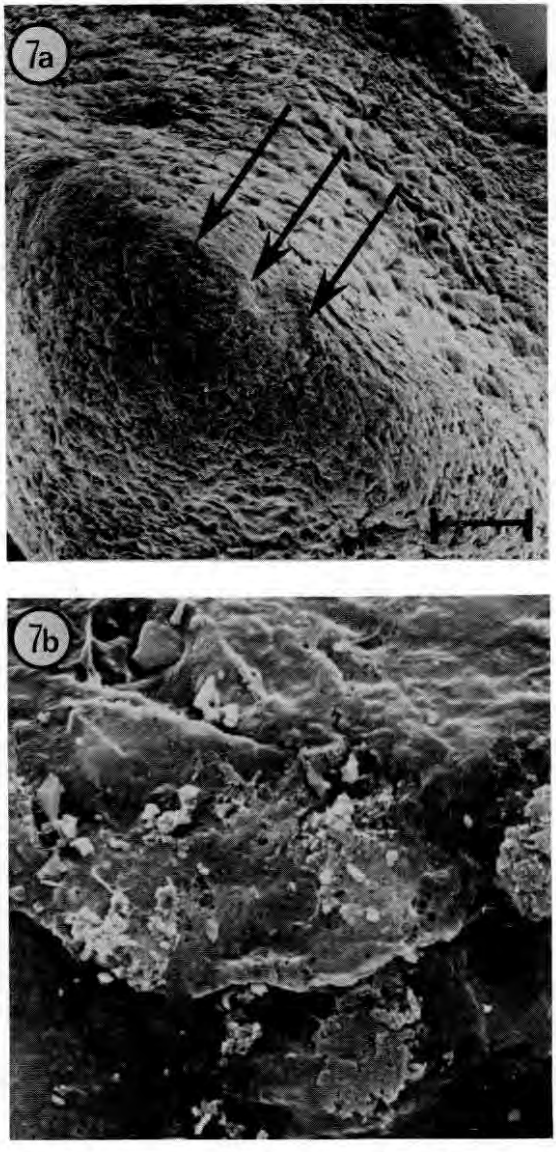

Figure 9-7. Scanning electron micrographs of mesenchymal component of a twenty-one day (paddle stage) regenerate. *A*. Low power view for orientation. Much outgrowth of the regenerate occurred and the tip of the regenerate was dorso-ventrally flattened. Area similar to that at arrows is shown at higher magnification in B. ×120. Marker 100 μm. *B*. Most of the surface of the mesenchymal component of the regenerate was coated by an amorphous material. Fragments of epithelial cell membrane adhered to the mesenchymal surface up to this stage. ×1,700. Marker 5 μm.

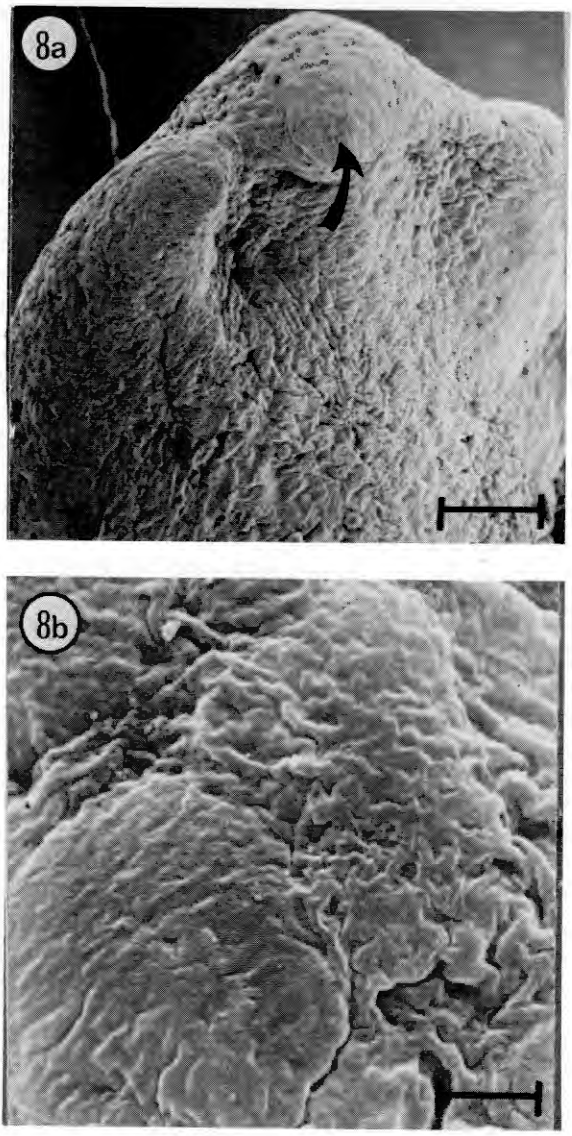

Figure 9-8. Scanning electron micrographs of mesenchymal component of a twenty-five day (three-digit stage) regenerate. *A*. Low power view for orientation. Digits have formed, and an area at the tip of a digit similar to the one shown at the arrow is shown at higher magnification in *B*. ×120. Marker 100 μm. *B*. The mesenchymal surface was coated by an amorphous material. ×3,000. Marker 4 μm.

by coarse fibers up to 140 nm in diameter. These fibers were perhaps persisting strands of fibrin, part of the blood clot formed at amputation that served as an epithelial substratum at wound closure (1). At fourteen days at the wound epithelium stage (Fig. 9-6) the surface of the mesenchymal component of the regenerate was coated by what seemed to be a different type of material. This consisted of a meshwork of fibrils and fibers up to 75 nm in diameter; the interstices between these fibrils were filled with an apparently amorphous extracellular material. At the bulb, cone paddle (Fig. 9-7), and digit (Fig. 9-8) stages, the surfaces of the mesenchymal components became more completely covered by the new extracellular coat.

Returning to transmission electron microscopy of intact regenerates and using the information obtained from scanning electron microscopy of the surfaces of the isolated mesenchymes to assist in interpreting the images, it appears that, up to about five days after amputation (Fig. 9-9), the basal epithelial cells sit on a substratum of fibrinlike material. In addition, some areas of the basal epithelial cell surface may be directly supported on cellular and extracellular debris at the amputation surface. Preliminary examination of regenerates at about eight days after amputation, during the period of the apical cap, suggests that the debris is removed and very little extracellular material is interposed between the epithelium and mesenchyme at this time.

By fourteen days, at the wound epithelium stage and as the apical cap disappears, a layer of extracellular material is found closely applied to the basal epithelial cells (Fig. 9-10). At the tip of developing digits a compact layer of extracellular material underlies the epithelium (Fig. 9-11). Mesenchymal cell processes abut against this layer. It is stressed that this material at the distal tip of the regenerates does not have the configuration of a basal lamina. A developing basal lamina is found at the periphery of the fourteen day regenerates (Fig. 9-12) or at the base of digit stage regenerates (Fig. 9-13) as previously described by Salpeter and Singer (22). The

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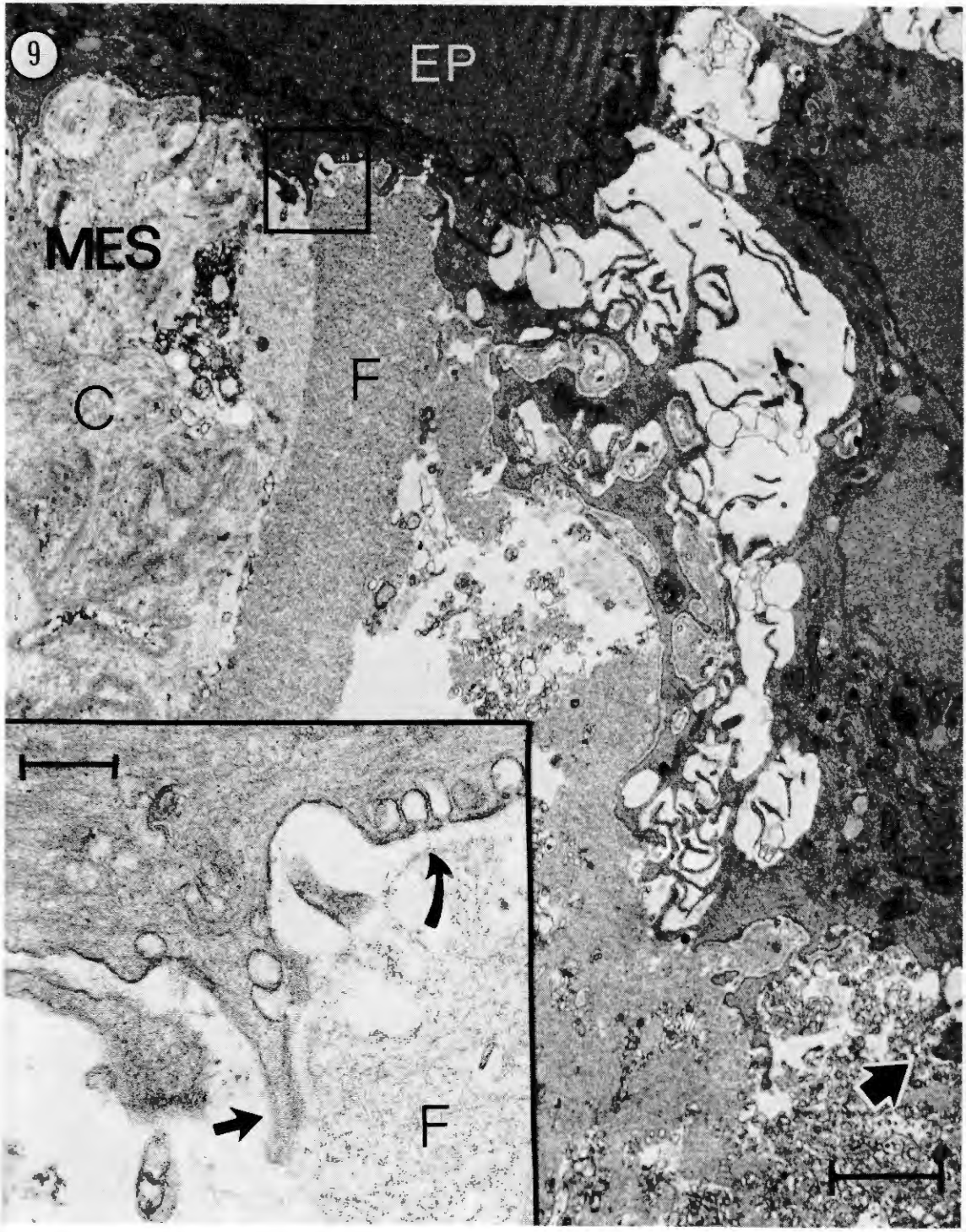
Figure 9-9. Epithelial-mesenchymal interface in the region of the apical cap of a five day regenerate. The section was taken from the area indicated by the arrow (C) on the left side of Figure 9-4. Epithelial cells (EP) were supported by different components of the mesenchyme (MES) including a fibrin-like substratum (F), collagen fibers (C) from tendons at the amputation surface, and membrane bound vesicles (arrow) possibly derived from dead cells or disintegrating myelin. Area in box shown at higher magnification in inset. ×8,350. Marker 2 μm.

INSET. It appeared that the epithelium rested directly on the fibrin substratum. It was not clear whether the material at the curved arrow was a basal lamina or a condensation of the fibrin substratum at a set distance from the epithelial cell membrane. Some areas of the epithelial membrane were devoid of any directly subjacent substratum (straight arrow). ×54,000. Marker 0.25μm.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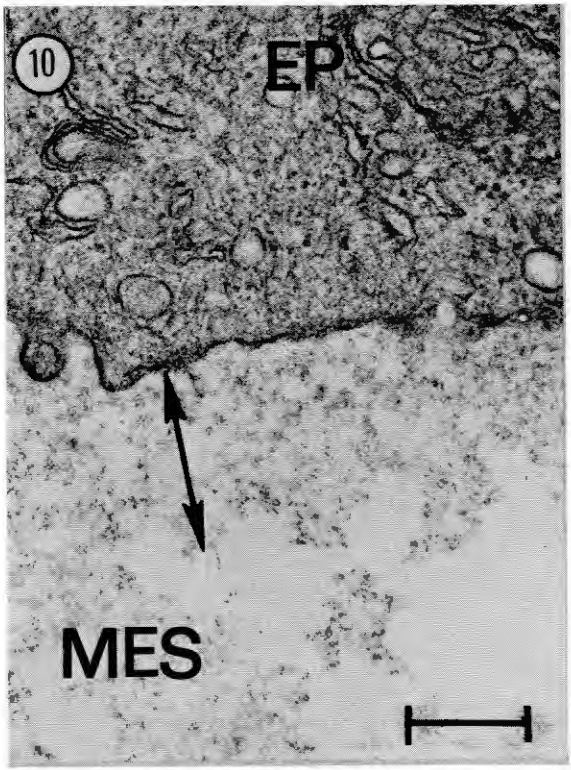


Figure 9-10. Epithelial-mesenchymal interface of an intact fourteen day (wound epithelium stage) regenerate. This section was taken near the central and distal-most area of the regenerate (C, left side of Fig. 9-4). There was no thickened apical cap in this specimen and the epithelium (EP) was only several cell layers thick. The epithelial substratum (arrow) was composed of an apparently nonfibrous extracellular material closely applied to the basal epithelial cell plasma membranes. ×59,600. Marker 0.25 μm.

basal lamina itself is approximately 50 nm wide and is separated from the basal epithelial surface by a 30 nm space.

In summary, correlation of data from transmission electron microscopy of intact regenerates and direct *en face* observation of the mesenchymal surface after removal of the epithelium (at the plane indicated by the arrows, Fig. 9-14) suggests that the nature of the epithelial substratum changes throughout the course of regeneration. At the time of apical cap formation, the epithelium rests on cellular and extracellular debris from the amputation surface and a fibrinlike material. This substratum is perhaps the subepithelial fibrocellular "scar" described by Salpeter and Singer (22). During the period of the apical cap, the fibrinlike material and debris are removed and a study of the nature of the epithelial-mesenchymal interface during this critical time period is still in progress. As the apical cap disappears, the epithelium rests directly on an extracellu-

lar material with no intervening space. It is within this layer of extracellular material that the basal lamina subsequently regenerates.

These observations lead to several suggestions that can be tested in future experiments. First, the apical cap may possess fibrinolytic as well as histiolytic properties for the removal of debris at the amputation surface. Second, the extracellular material appearing at the epithelial-mesenchymal interface as the apical cap disappears may be secreted by the epithelium, may be composed in part of proteoglycans, and may play a role in morphogenesis. It is known that epithelia secrete extracellular materials, including their own basal laminae (25). The basal laminae of many embryonic epithelia contain glycosaminoglycans, some in association with protein-forming proteoglycans. It has been suggested that these substances play a role in morphogenesis by stimulating responding mesenchymal cells (20, 26-29). It has been shown that the regenerating limb synthesizes the glycosaminoglycans hyaluronate and chondroitin sulfate,

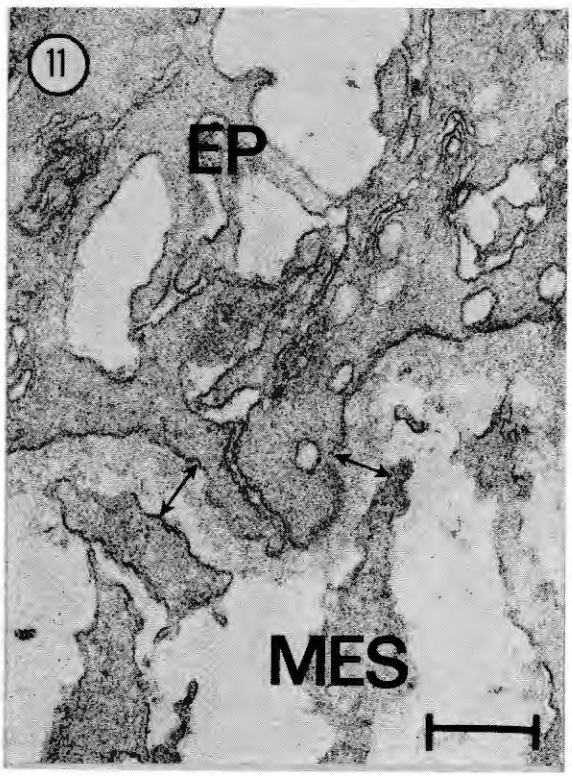
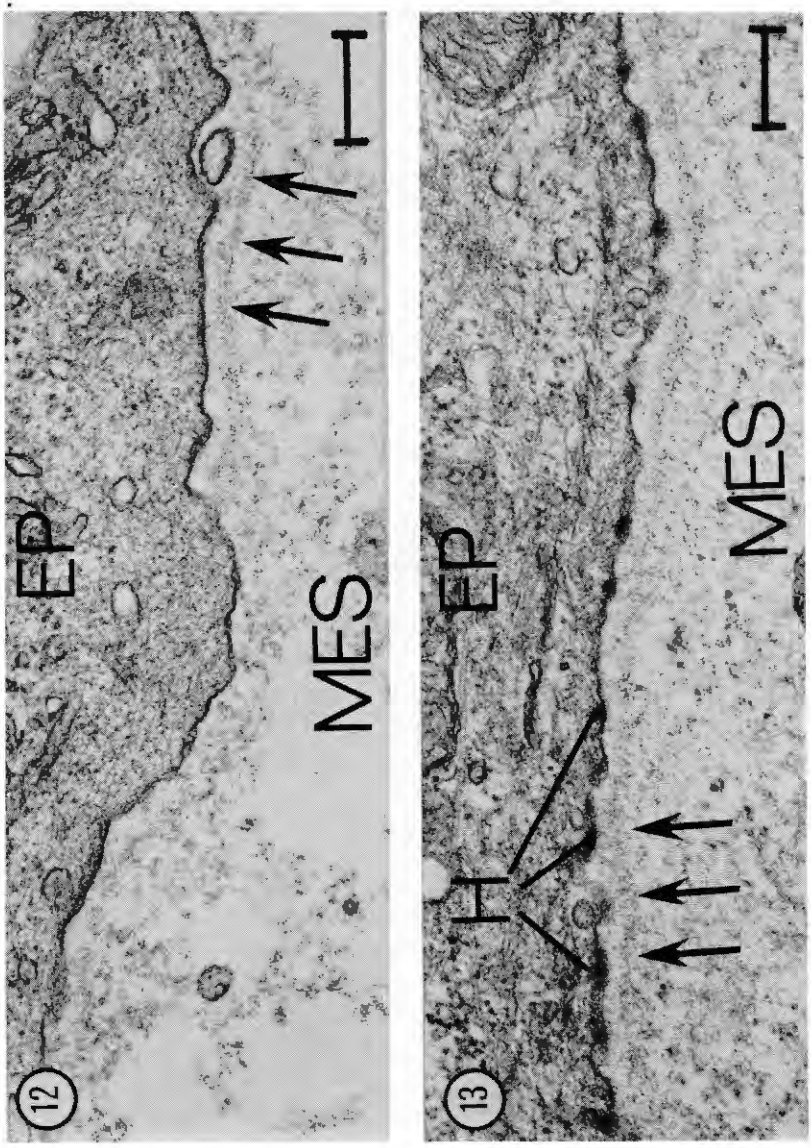


Figure 9-11. Epithelial-mesenchymal interface of an intact twenty-six day (three-digit stage) regenerate. This section was taken from the tip of a digit (T, left side of Fig. 9-4). The epithelial substratum (arrow) was a compact layer of apparently nonfibrous extracellular material closely applied to the basal epithelial cell (EP) plasma membranes. Mesenchymal cell processes (MES) abutted against this material. ×52,500. Marker 0.25 μm.


EP
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but the cells of origin are not known (30). It is also possible that the extracellular material at the epithelial-mesenchymal interface is in part glycoprotein. There is evidence that the epithelium of the regenerate can incorporate the glycoprotein component fucose and secrete a fucose-containing material into the underlying mesenchyme (31). The morphogenetic properties of at least one glycoprotein have been demonstrated (*see* Chapter 21).

What can be learned about the epithelial side of the interface by correlation of transmission and scanning electron microscopic observations? For transmission electron microscopy, epithelia were fixed immediately after removal from the regenerate after incubation in the salt medium. For scanning electron microscopy, once the epithelium was removed from the young regenerates at three to fourteen days (Fig. 9-4), the epithelial component was fixed in an open position so that the newly exposed (i.e. basal) surface could be examined. As the regenerate became larger, the epithelial glove was too deep to examine; thus, it was everted (like turning a glove inside-out), except for the tips of the digits, which remained in their normal positions. Only the area at the tips of the digits was observed.

In general, transmission electron microscopy showed that the newly exposed basal epithelial cells were free of extracellular material. Blebs containing cytoplasmic contents were frequently seen at the exposed cell surfaces and were interpreted as artifacts of the separation procedure. Scanning electron microscopy demonstrated that during the first fourteen days when an apical cap was present (Fig. 9-15A) there was a difference in the surface morphology of regions, thought to be nonapical cap epithelium (Fig. 9-15B) and apical cap epithelium (Fig. 9-15C). Cells of the nonapical cap regions appeared relatively smooth and well organized, whereas the cells of the apical cap appeared to react to the removal of their substrate by extensive blebbing. At the three-digit stage (Fig. 9-16A) the basal epithelial cells of the tip of a digit showed much less blebbing and the contours of individual cells were easily seen (Fig. 9-16B).

A final consideration with respect to the epithelium is that a problem

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Figure 9-12. Epithelial-mesenchyml interface of an intact fourteen day (wound epithelium stage) regenerate. This section was taken from the peripheral area of the regenerate (P, left side of Fig. 9-4) toward its junction with stump tissue. Segments of developing basal lamina (arrows) were found adjacent to the hasal epithelial cell (EP) membrane. ×52,900. Marker 0.25 μm.

Figure 9-13. Epithelial-mesenchymal interface of an intact twenty-six day (three-digit stage) regenerate. This section was taken from the hase of the regenerate (B, left side of Fig. 9-4) toward its junction with stump tissue. A complete basal lamina (arrows) lined the basal epithelial cell (EP) surface. Hemidesmosomes (H) punctuated the basal cell membranes. ×51,800. Marker 0.25 μm.

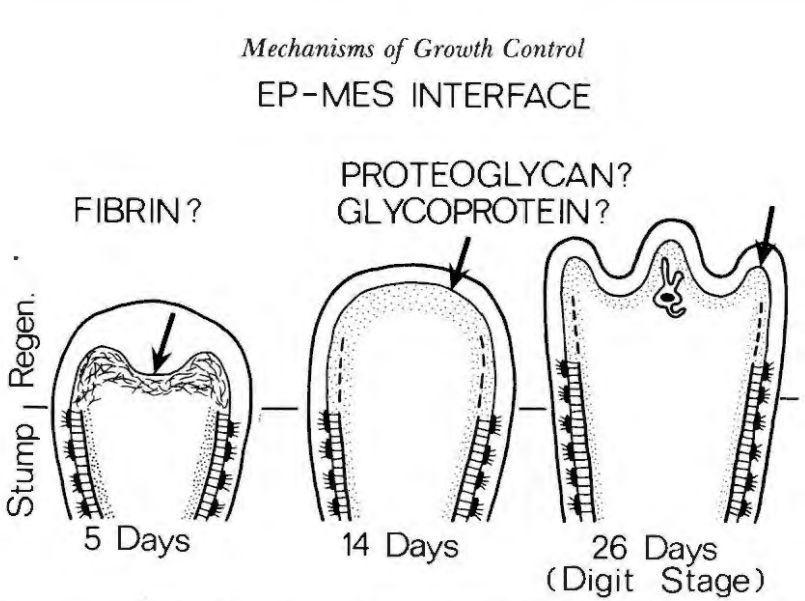

Figure 9-14. The nature of the epithelial-mesenchymal interface (arrows) changes throughout the course of limb regeneration. At five days after amputation the apical cap epithelium rests on a fibrin-like substratum, as well as cellular and extracellular debris at the amputation surface. By fourteen days (wound epithelium stage) the apical cap has disappeared, the debris at the amputation surface has been removed, and mesenchymal blastema cells have accumulated. At this time, and also at twenty-six days (three-digit stage) an extracellular material is found closely applied to the basal epithelial cells (stipples). Areas with developing basal lamina (dashed line) and mature basal lamina (solid line) are indicated.

with attempting to experimentally manipulate the apical cap of young newt limb regenerates is that the apical cap cannot be visualized externally because it is an internal thickening of the epithelium (Fig. 9-17A). Thus the question of whether branching of the apical cap precedes abnormal development of digits has never been answered. Some experimental conditions (32, 33) are known to induce the development of multiple digits. If the epithelium of regenerates of these experimentally manipulated limbs were removed during the first twelve days of regeneration, fixed, stained with uranyl acetate (and embedded in epon if sections were required), the apical cap could be observed directly and it could be determined if apical cap branching had occurred. In the normal regenerate, the cap appears as

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Figure 9-15. Scanning electron micrograph of the basal surface of the epithelial component of a ten day regenerate. *A.* Low power view for orientation. Areas at arrows shown at higher magnification in B and C. ×75. Marker 200 μm. *B.* Epithelial cells at a region thought to be nonapical cap. Individual cells were clearly seen. ×1,300. Marker 10 μm. *C.* Epithelial cells at a region thought to be the apical cap. Extensive blebbing (B) of the epithelial cells has occurred, perhaps as a reaction to the loss of substrate during the separation procedure. ×1,200. Marker 10 μm.


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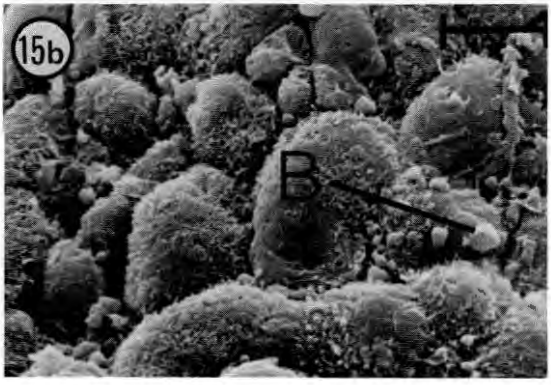
b
c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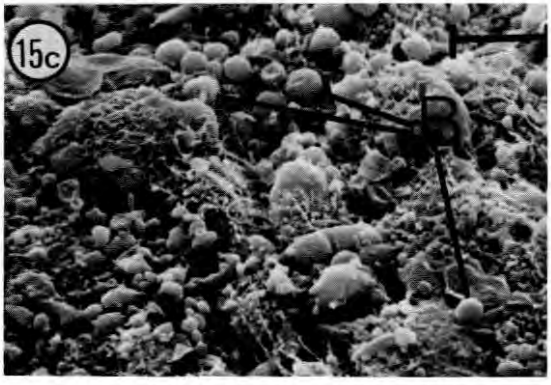
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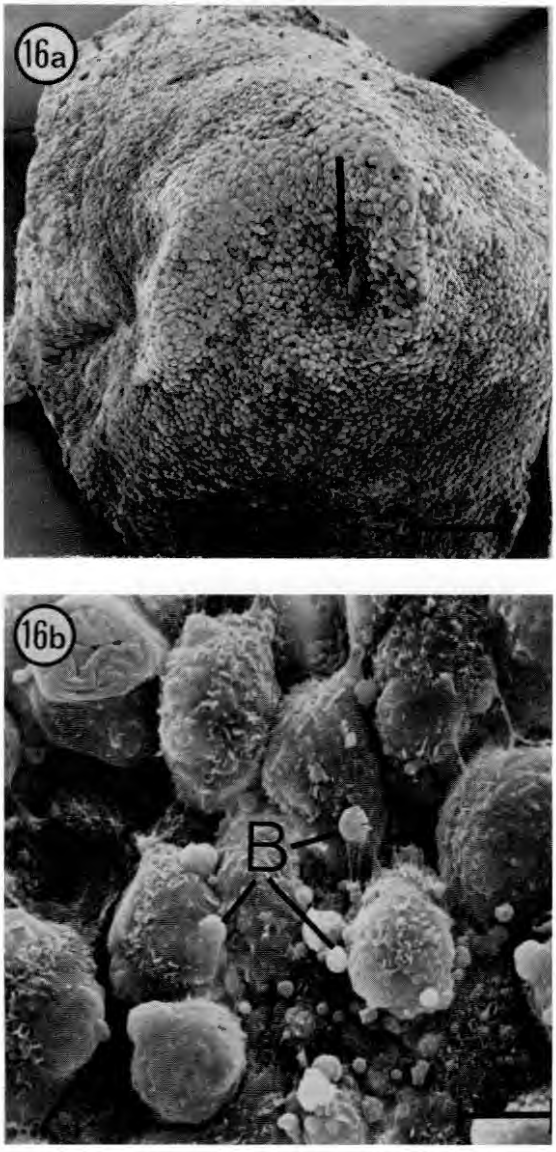


Figure 9-16. Scanning electron micrograph of the basal surface of the epithelial component of a twenty-six day (three-digit stage) regenerate. *A*. Low power view for orientation. Area at arrow shown at higher magnification in B. ×60. Marker 200 μm. *B*. Again individual cells were clearly seen. Some blebbing (B) has occurred but this was not as extensive as at the cap region of younger regenerates. ×1,400. marker 10 μm

an oval to round density at the junction of dorsal pigmented and ventral nonpigmented epithelium (Fig. 9-17B). A section through the dense area of this tissue demonstrated that the dense area was the region of the thickened apical cap (Fig. 9-17C).

The Mechanism of Action of the Salt Medium

After the nature of the epithelial-mesenchymal interface of the newt limb regenerate was examined, it was of interest to know why the saline-phosphate medium worked as a separating agent. Understanding the mechanism of action of the salt medium might provide clues as to the physi-chemical nature of the bond between the epithelium and its substratum. Although regenerates at all stages can be separated into their epithelial and mesenchymal components by the salt medium, only regenerates undergoing morphogenesis (late cone to four-digit stages) were used in these experiments. At this time the epithelium rests on a closely adherent extracellular material described above, a developing basal lamina, or a newly regenerated basal lamina.

Limb regenerates were isolated and incuhated in the saline-phosphate medium at 24°C or 37°C or in one of several other media designed to test the mechanism of action of the saline-phosphate medium. After incubation, an attempt was made to separate the epithelium from the mesenchyme. Mesenchymal components were embedded in paraffin and serial sectioned. Adherent epithelial cells were counted on one out of three sections and the total number of epithelial cells per mesenchyme were calculated.

First, it was noted that the saline-phosphate medium was an effective separating agent only at 37°C. Increasing the temperature of the tissues may increase the fluidity of some epithelial cell membrane components (34, 35) and thus facilitate the separation of the cells from the underlying substrate. Second, it was noted that, if the saline-phosphate medium was broken down into its component parts (physiologic saline and sodium phosphate buffer) and each of these media was tested as a separating agent, neither agent mimicked the effect of the combined medium (Table 9-II and Fig. 9-18). The saline-phosphate removed the epithelium cleanly from the mesenchyme so that, on the average, thirteen epithelial cells remained adherent to each mesenchyme (Experiment 1); physiologic saline alone did not allow the removal of any epithelium at all (Experiment 2); and sodium phosphate buffer allowed the removal of much of the epithelium, but on the average 300 epithelial cells remained adherent to each mesenchyme (Experiment 3). What feature of the combination of the two media allowed separation?

The saline-phosphate medium is hyperosmotic at 400 mOsm (whereas newt blood is 250 mOsm according to Conn et al.: 36) and hypertonic (about three times the ionic strength of physiologic saline). Therefore, separations were attempted with saline made hyperosmotic with sucrose and saline made hyperosmotic and hypertonic with sodium sulfate (Experiments 4 and 5). These solutions allowed the removal of most of the

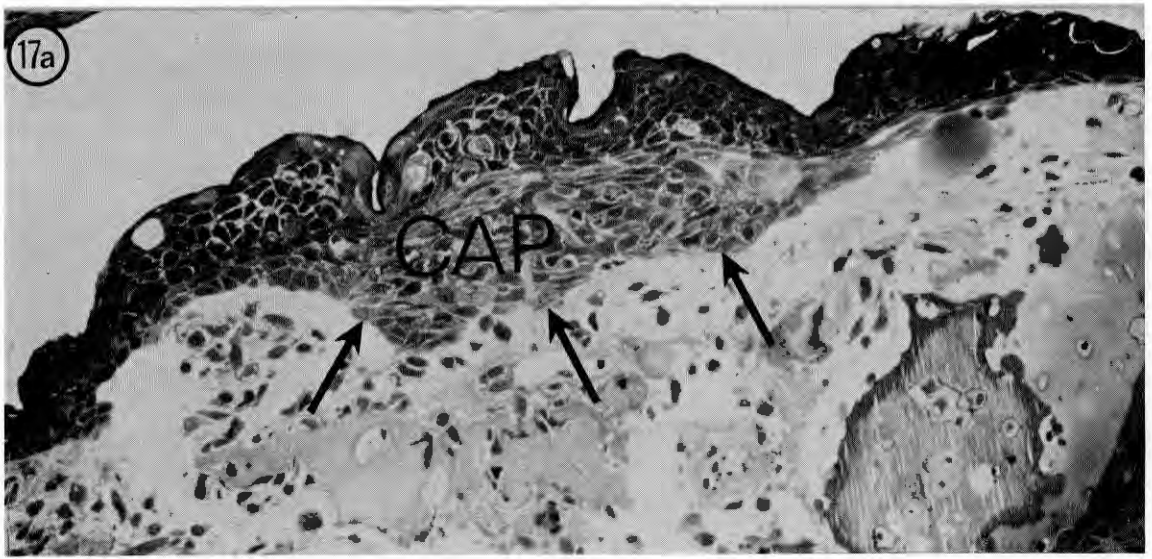
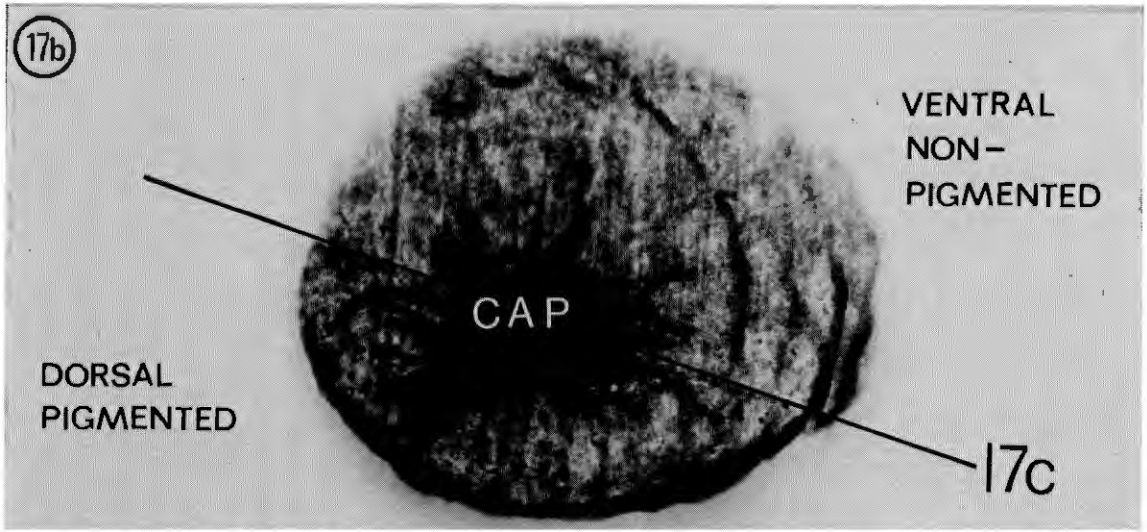
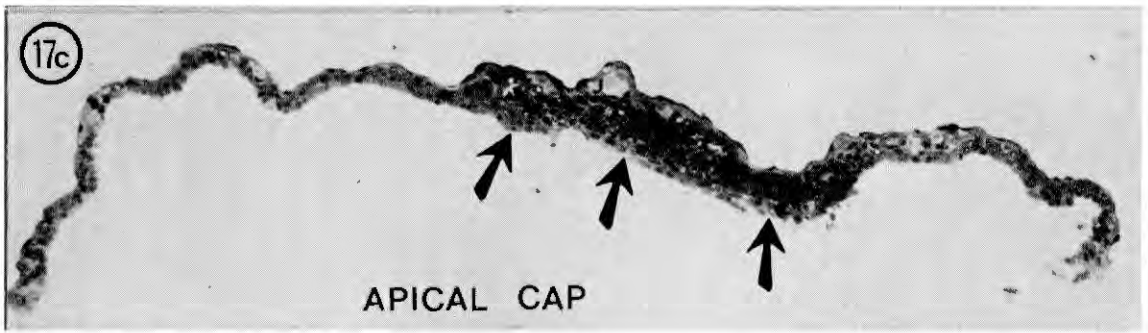


Figure 9-17. *A.* Intact regenerate at eleven days. The apical cap (CAP) was an internal thickening (internal limit at arrows) of the epithelium. × 133.



B. Gross specimen of epithelial component of a ten day regenerate after fixation, staining, and embedding in Epon. The apical cap (CAP) appeared as a density at the junction of the ventral non-pigmented and the dorsal pigmented epithelium. ×38.



C. A section of the specimen shown in B proved that the dense area of the gross specimen was the area of the thickened apical cap. ×76.

TABLE 9-II
MECHANISM OF SEPARATION

Experiment	*1*	*2*	*3*	*4*	*5*	*6*
*Nos. Limbs**	*31*	*4*	*10*	*13*	*22*	*13*
Contents of Medium†						
sodium chloride (mM)	111	111		111	111	111
potassium chloride (mM)	5.6	5.6		5.6	5.6	5.6
sodium phosphate (mM)	100.		100			
EDTA (mM)						10
sodium sulfate (mM)					89	
HEPES (mM)		10		10	10	10
sucrose		+		+		+
Osmolality (mOsmol/kg)	400	211	210	398	399	400
Ionic Strength (μ)	.39	.13	.26	.13	.39	.13
Time (min)	60	60	60	60	60	30
Eipth. Cells/Mes.	13	N/C	293	93	86	17
SEM	±2		±93	∓19	±19	±7

* Regenerating limbs between 21 and 39 days after amputation (advanced cone to four-digit stages).
† All media were at pH 7.5, 37°C.
Key:
EDTA ethylenediamine tetraacetate
HEPES = *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
\+ = sucrose was added to bring the osmolality to the value stated
N/C = not counted
SEM = standard error of tbe mean.

epithelium, leaving on the average ninety epithelial cells per mesenchyme. Thus it appeared that the hyperosmolarity alone contributed to the separation but was not sufficient.

Since the saline-phosphate medium readily precipitates calcium, it was possible that part of its activity as a separating agent was a result of this property. A solution was tested that contained the divalent cation chelator EDTA in saline with sucrose added to make the medium hyperosmotic (Experiment 6). This solution, as the saline-phosphate medium, removed the epithelium from the mesenchyme so that on the average only seventeen epithelial cells remained adherent to each mesenchyme. However, unless the incubation period is carefully monitored, the EDTA quickly attacks the desmosomes of the epithelium and the epithelium disintegrates as the separations are attempted. The saline-phosphate medium does not attack desmosomes. Although these experiments suggest that the mechanism of action of the salt medium may be to precipitate calcium from the epithelial-mesenchymal interface of the regenerate, the role of increased sodium, the possibility that small amounts of materials at the interface are solubilized, and the possibility that phosphate in some way inhibits the binding of anionic substances (Glycosaminoglycans?) to the basal epithelial plasma membrane have not been investigated.

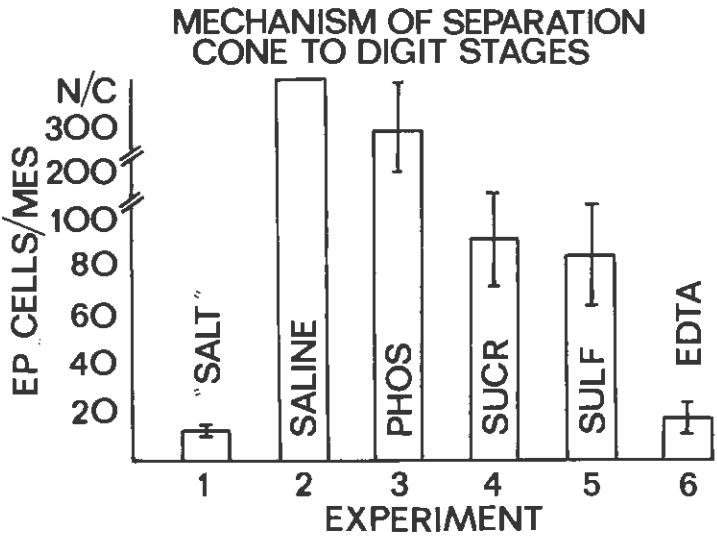


Figure 9-18. Data from Table 9-II. Bars indicate ± one standard error of the mean. Application of Student's T-test for unpaired samples showed that there was a significant difference between Experiment 1 and Experiments 3, 4 or 5 ($p < .001$) but that there was no significant difference between Experiments 1 and 6.

Conclusion

In conclusion, a saline-phosphate medium has been presented that for the first time allows the separation of the epithelium intact from the underlying mesenchyme of the newt limb regenerate. It has been suggested that this medium works by virtue of its elevated temperature, hyperosmolarity, and possibly the ability of the medium to precipitate calcium. Separated epithelial and mesenchymal components have been used to study the nature of the epithelial-mesenchymal interface throughout the course of limb regeneration. It is hoped that future studies using separated tissues will help elucidate the nature of the epithelial-mesenchymal interaction during the process of limb regeneration.

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