AMER. ZOOL., 13:1067-1083 (1973).

Glycosaminoglycans and Epithelial Organ Formation

MERTON R. BERNFIELD, RONALD H. COHN, AND SHIB D. BANERJEE

Division of Developmental Biology, Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305

SYNOPSIS. The concept that extracellular matrix materials are involved in the morphogenetic process is supported by substantial indirect evidence. Essential morphogenetically active materials are obscure with regard to their nature, their mode of action, and whether they are causally involved in tissue interactions.

Studies are presented indicating that glycosaminoglycans are components of embryonic epithelial basal laminae, and that materials within the basal lamina which are, at least in part, glycosaminoglycan are required for establishing and maintaining braching epithelial morphogenesis. The tissue of origin and molecular nature of basal laminar glycosaminoglycan are described and speculations are made regarding its possible mode of action in the context of a model for branching morphogenesis.

INTRODUCTION

Morphogenesis involves the organization of cell populations into specific arrangements ultimately resulting in organs with unique structures. The central question in understanding this process is "How do the cells come to be where they are?" Certain aspects of the developmental process are under direct genomic control, but substantial evidence suggests that cell and tissue interactions are the primary regulatory mechanisms for the assembly of cells into organs. The behavior of individual cells within a tissue involves recognition and communication among the participating cells. However, organogenesis encompasses not only the behavior of the component cells, but also their behavior as a group. Groups of cells (tissues) influence the behavior of dissimilar cell groups resulting in the development of discrete organ forms.

Although virtually every organ in the mature organism arises in the embryo as a result of tissue interactions, relatively little is known about how they occur. The development of organs composed of an epithelial and mesenchymal (or stromal) component are particularly convenient for study since the interaction involves only two tissues, and since it results in the formation of a unique structure. In epitheliomesenschymal organs, the attainment of final organ form is strictly dependent upon the interaction between the two components. This interaction is not a single event, but a continuous process, required during the formation of the organ primordia and usually persisting through the period of organogenesis.

Supported by National Institutes of Health research grants HD 06763 and HD 02147 and training grant HD 00049, and the National Foundation-March of Dimes. Prepared while M.R.B. was a John Simon Guggenheim Memorial Foundation fellow at the Biozentrum der Universität, Basel, Switzerland.

EXTRACELLULAR MATERIALS AND EPITHELIAL MORPHOGENESIS

Extracellular matrix materials at the epithelial-mesenchymal interface were implicated in organogenetic tissue interactions after studies suggested that direct tissue contact was not required for the interaction (Grobstein, 1967). The interface includes the epithelial basement membrane with its associated materials. Ultrastructurally, embryonic basement membranes resemble their adult counterparts, showing an electron lucent zone adjacent to the epithelial plasma membrane, an electron dense basal lamina and a superficial layer of varying structure traversed by collagen fibrils (Kallman et al., 1967; Rifkind et al., 1969). By analogy with basal laminae isolated from specialized adult tissues (Spiro, 1970; Kefalides, 1970) (e.g., renal glomeruli, lens capsule), collagen of a type dif-

ferent from fibrillar collagen as well as two distinct glycoproteins may be the major components of the basal lamina. Additionally, electron-dense granules of varying size and shape, sometimes reported as membrane-bound (Slavkin et al., 1969), have frequently been observed near the basal lamina on embryonic epithelia undergoing morphogenesis (Bernfield and Wessells, 1970; Cohen and Hay, 1971). These bodies have been categorized into types and some are reported to contain ribonucleoprotein (Slavkin, 1971).

The basal lamina appears to be continuous over embryonic epithelia, but the abundance of fibrillar collagen varies among specific sites. For example, on bronchial epithelia, the greatest amounts are on the trachea and between the bronchial buds (Wessells, 1970). On salivary epithelia, there is heavy accumulation along the stalk and in the clefts between the lobules (Grobstein and Cohen, 1965). Minimal amounts of collagen fibrils are near the morphogenetically most active sites: on the lung, at the tips of the buds, and on the salivary, at the surface of the lobules.

During the last several years, substantial data have accumulated which support, but do not prove, the hypothesis that extracellular materials direct differential changes in tissue behavior during embryogenesis (Grobstein, 1967). Studies have focused on the epithelial basement membrane and on collagen, however, the way in which these materials act, whether they are causally involved in the tissue interaction, as well as the precise nature of the essential or morphogenetically active material(s) are unknown. One striking result is that collagenase reversibly alters the morphology and interrupts the development of certain epithelia (lung, salivary, ureteric bud) (Grobstein and Cohen, 1965; Wessells and Cohen, 1968). Where studied, the surfaces of these rudiments show markedly fewer collagen fibrils, dissolution of the epithelial basal lamina, and loss of the matrix adjacent to the basal lamina. The presumption that materials susceptible to collagenase treatment (presumably collagen) are required for normal morphogenesis is consistent with other observations on developing skin, cornea, teeth, muscle, and other systems (Stuart and Moscona, 1967; Konigsberg 1970; Cohen and Hay, 1971). Collagen has been suggested to be an "inductive-factor," mediating the influence of one tissue upon another (Grobstein, 1967; Cohen and Hay, 1971).

Our search for the involvement of additional matrix materials was prompted by three major pieces of evidence. First, the amount of collagen deposited by prelabeled mesenchyme on epithelia undergoing collagenase-sensitive morphogenesis was similar in amount to that on epithelia either not undergoing morphogenesis or undergoing morphogenesis insensitive to collagenase (Bernfield, 1970). These results implied that the morphogenetic effect of mesenchyme is probably not mediated exclusively by collagen. Second, commercial preparations of purified collagenase, the enzymes used in the studies implicating collagenase-susceptible materials in morphogenesis, were shown to contain appreciable mucopolysaccharidase activity (Bernfield et al., 1972). This enzymatic contamination raised the question of the possible involvement of mucopolysaccharides (or glycosaminoglycans, as they are now known). Finally, Kallman and Grobstein (1966) showed that salivary epithelium deposits hyaluronidase-susceptible, ^{3}H-glucosamine-labeled material at its surface.

GLYCOSAMINOGLYCAN (GAG) AT EPITHELIO-MESENCHYMAL INTERFACES

Various histochemical procedures established that these molecules were associated with embryonic epithelial surfaces (Bernfield and Banerjee, 1972). Alcian Blue 8GX, a cationic dye that dissociates from GAG at characteristic counter-ion concentrations, stains the epithelio-mesenchymal interface of 13¼-day mouse embryo salivary glands at 0.2, 0.3 and 0.4 M Mg^{2+} concentrations at which authentic GAG binds the dye (Quintarelli and Dellovo, 1965) (Fig. 1). The stain is nearly uniformly distributed over the epithelial surface,

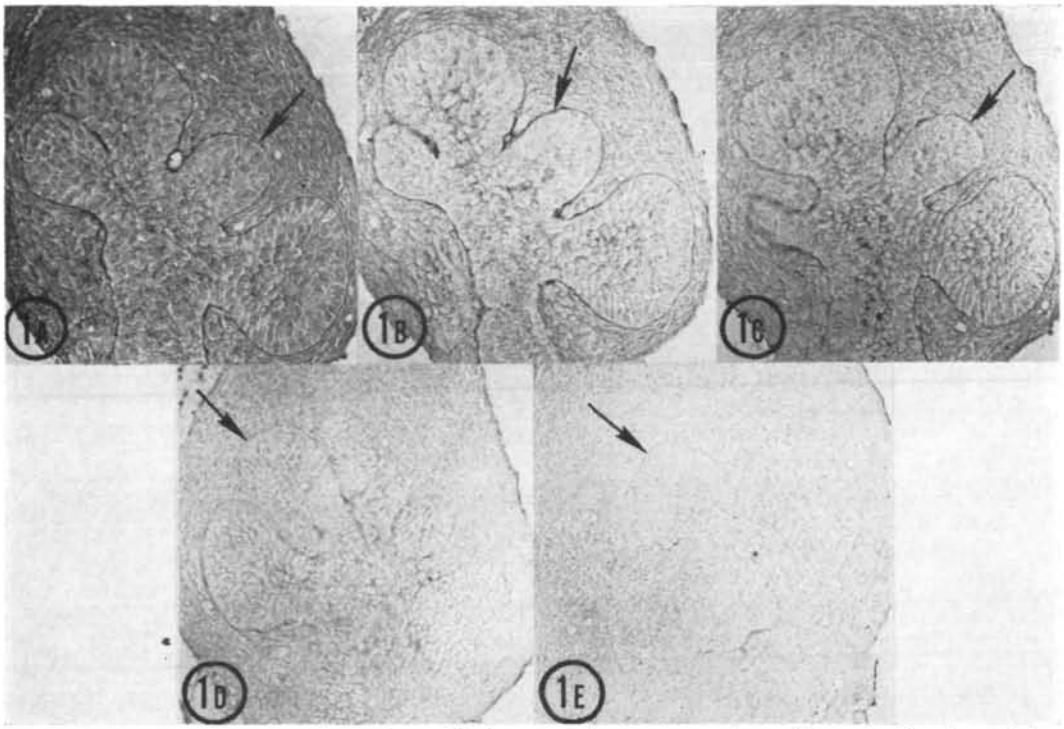


FIG. 1. Mouse embryo 13¼-day submandibular salivary gland stained with Alcian Blue 8 GX at various magnesium concentrations. ×166. Alcian Blue stains materials at the epithelial-mesenchymal interface (arrows) at 0.2 M $MgCl_2$ (*A*), 0.3 M $MgCl_2$ (*B*), and at 0.4 M $MgCl_2$ (*C*). The stain is nearly uniformly distributed over the epithelial surface, with a slightly more intense stain within the interlobular clefts. At higher magnesium ion concentrations (0.6 M in *D*), the staining of the interface (arrows) has markedly decreased and at 0.7 M (*E*) the stain at the interface has almost completely disappeared, indicating that the concentration of $MgCl_2$ at which most of the Alcian Blue is displaced from the material at the interface is between 0.4 and 0.6 M. (From Bernfield and Banerjee, 1972.)

with a slightly more intense stain within the interlobular clefts. At Mg^{2+} concentrations of 0.6 and 0.7 M, the stain markedly decreases, a result consistent with the presence of authentic GAG. Another characteristic of GAG is their non-reactivity in the usual 10 or 15 min periodic acid-Schiff (PAS) stain. Periodic acid attacks GAG by very slowly oxidizing the uronic acid residues (Scott and Dorling, 1969) and this difference in oxidation rate was used to identify the glycans which encircle the epithelium in a manner identical to that seen with Alcian Blue.

Ruthenium red, a polyvalent cationic dye similar in a number of ways to Alcian Blue, readily binds to authentic GAG, with the complex becoming highly electron dense in the presence of osmium tetroxide (Luft, 1971*a*). The basal epithelial surface reveals copious ruthenium red staining under the electron microscope. Collagen fibrils appear to be coated with ruthenium red positive material and the basal lamina which is continuous over the entire epithelium appears uniformly dense (Bernfield et al., 1972).

Further evidence for epithelial surface-associated GAG comes from autoradiographic studies (Bernfield and Banerjee, 1972). Salivary glands labeled with ^3H-glucosamine show most of the label within the lobules and at the epithelial surface. However, only the surface label is removed by the protease-free mucopolysaccharidases, testicular hyaluronidase, chondroitinase ABC or chondroitinase AC, confirming its

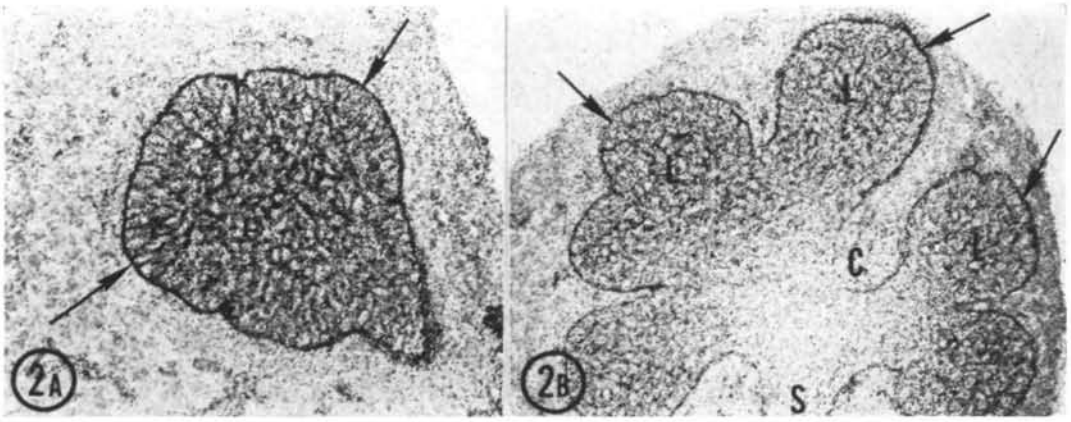


FIG. 2. Autoradiograms of 12¼- and 13¼-day salivary glands incubated immediately after explanation for 2 hr in the presence of ^{3}H-glucosamine. *A*, 12¼-day salivary glands demonstrate equivalent distribution of ^{3}H-glucosamine label over the epithelial surface (arrows). At this early stage of salivary development there is no distinct lobule formation. *B*, 13¼-day glands have distinct lobules (L) and the distal surfaces of the lobules show maximal amounts of glucosamine label (arrows)—much less is seen at the surfaces of the stalk (S) and interlobular clefts (C). ×145.

presence within authentic GAG (Bernfield and Wessells, 1970; Bernfield et al., 1972).

In contrast to the nearly uniform distribution of total surface GAG as revealed by the staining procedures, the distribution of newly synthesized GAG may vary. Lobules are not yet present in 12¼-day salivary glands and there is equivalent distribution of ^{3}H-glucosamine label over the epithelial bud after a 2 hr incorporation. However, with the formation of distinct lobules and interlobular surfaces in 13¼-day glands, the surfaces of the distal ends of the lobules show the maximal amounts of the glucosamine label—much less is seen at the surfaces of the stalk and the interlobular clefts (Fig. 2). This same distribution, with most rapid accumulation of GAG label at the surface of the lobules, is seen when using either a glucosamine or radiosulfate label and also on such glands after 24 hr in culture.

To get an idea of the general occurrence of epithelial surface-associated GAG, two other branching epithelia were studied: lung, which shows branching of the primary bronchi, and ureteric bud, which elongates to give rise to long, thin bifurcating branches. In addition, the embryonic pancreas was studied. The pancreas does *not* pass through a similar stage of branching as in the three other organs. Embryonic lung and metanephros (11 to 13 days) show Alcianophilic material on the epithelial surface at 0.3-M Mg^{2+}. In each instance, the Alcianophilic material encompasses the epithelium nearly uniformly, with the surface of the buds showing slightly less stain (Fig. 3). Minimal Alcian Blue stain (0.3-M Mg^{2+}) is seen on the surface of the 11-day pancreatic epithelium, and even less is seen on the older (12- and 13-day) pancreas (Fig. 3).

The presence of GAG on these epithelia was confirmed by autoradiography after a 2-hr ^{3}H-glucosamine incorporation and hyaluronidase treatment of the sections (Fig. 4). In the lung, label is maximal at the surface of the ends of the bronchial buds and similar distribution characterizes the ureteric bud (Fig. 5). In the pancreas at 10¼ to 12½ days, hyaluronidase-susceptible label is seen, but is small in amount when compared with the other epithelia. Older pancreatic epithelia show sparse surface label on their acini.

On epithelia which undergo branching morphogenesis, total and newly synthesized GAG are distributed distinctly. The epithelia are encompassed by a nearly uniform layer of GAG, but the rate of accumulation of newly synthesized GAG is maximal at the morphogenetically active sites—the

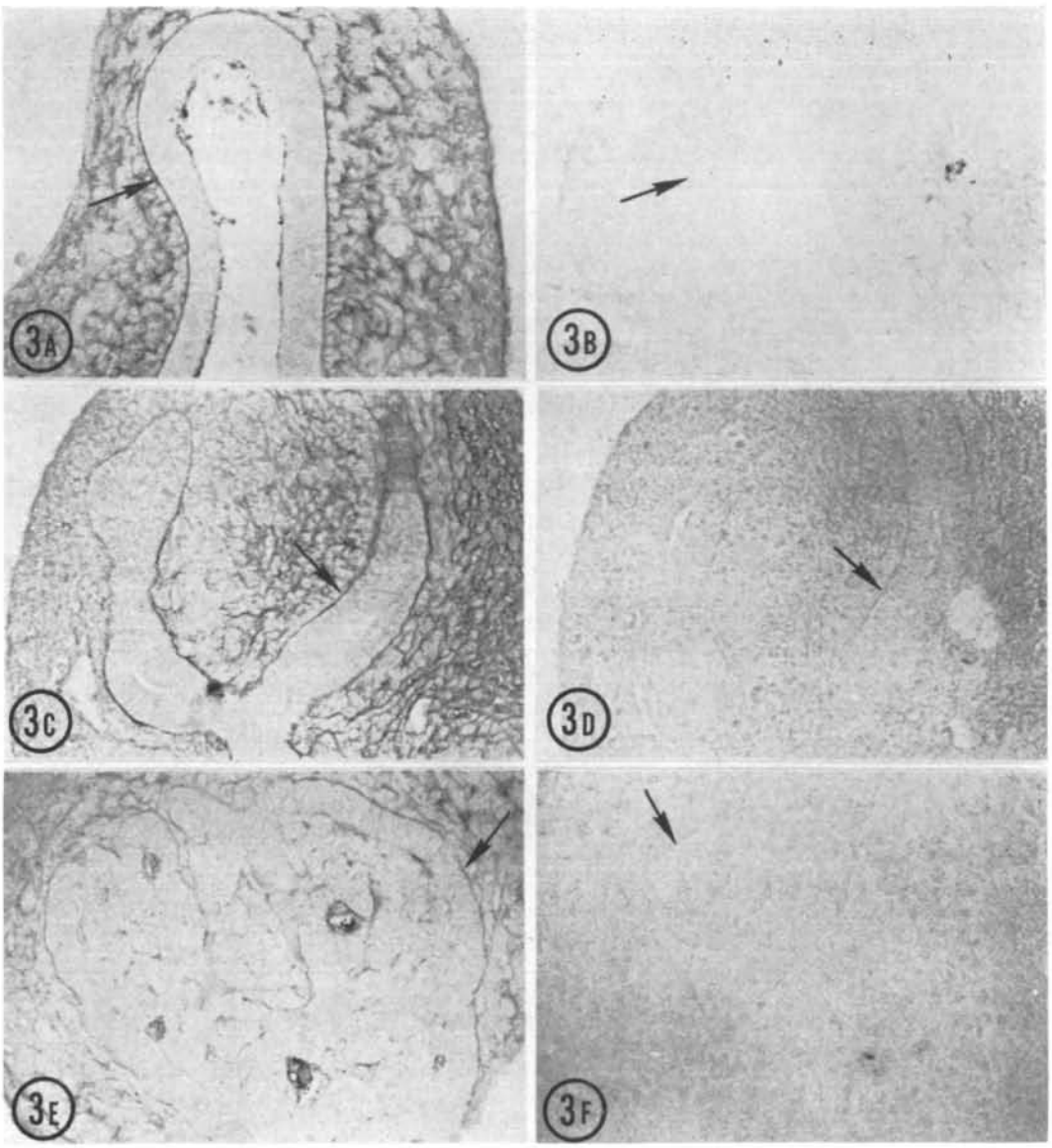


FIG. 3. Alcian Blue staining of mouse embryo lung, ureteric bud (metanephros) and pancreas at 0.3 M $MgCl_2$ (left) and 0.6 M $MgCl_2$ (right). *A*, 11-day lung at 0.3 M $MgCl_2$ shows a nearly uniform layer of alcianophilic material at the epithelial surface (arrow), however slightly less material is seen at the distal end of the epithelium (top). *B*, Minimal staining of the lung epithelial surface (arrow) is seen at 0.6 M $MgCl_2$. *C*, 11-day ureteric bud at 0.3 M $MgCl_2$ shows intense alcianophilic material at the epithelial surface (arrow) with less stain at the distal bud (top left). *D*, Minimal staining of metanephros occurs at 0.6 M $MgCl_2$ (arrow). *E*, 11-day pancreas shows minimal staining of the epithelial surface at 0.3 M $MgCl_2$ (arrow). *F*, No stain is seen on the pancreatic epithelial surface at 0.6 M $MgCl_2$ (arrow). ×227.

buds and lobules which undergo cleft formation and branching—and minimal at the quiescent sites—the surfaces of the clefts between the branches and the stalk. The nature of the relationship between morphogenetic behavior and the differing rates of new GAG accumulation needs closer study. The data might be accounted for on the basis of differences in the rate of surface GAG turnover, a greater rate

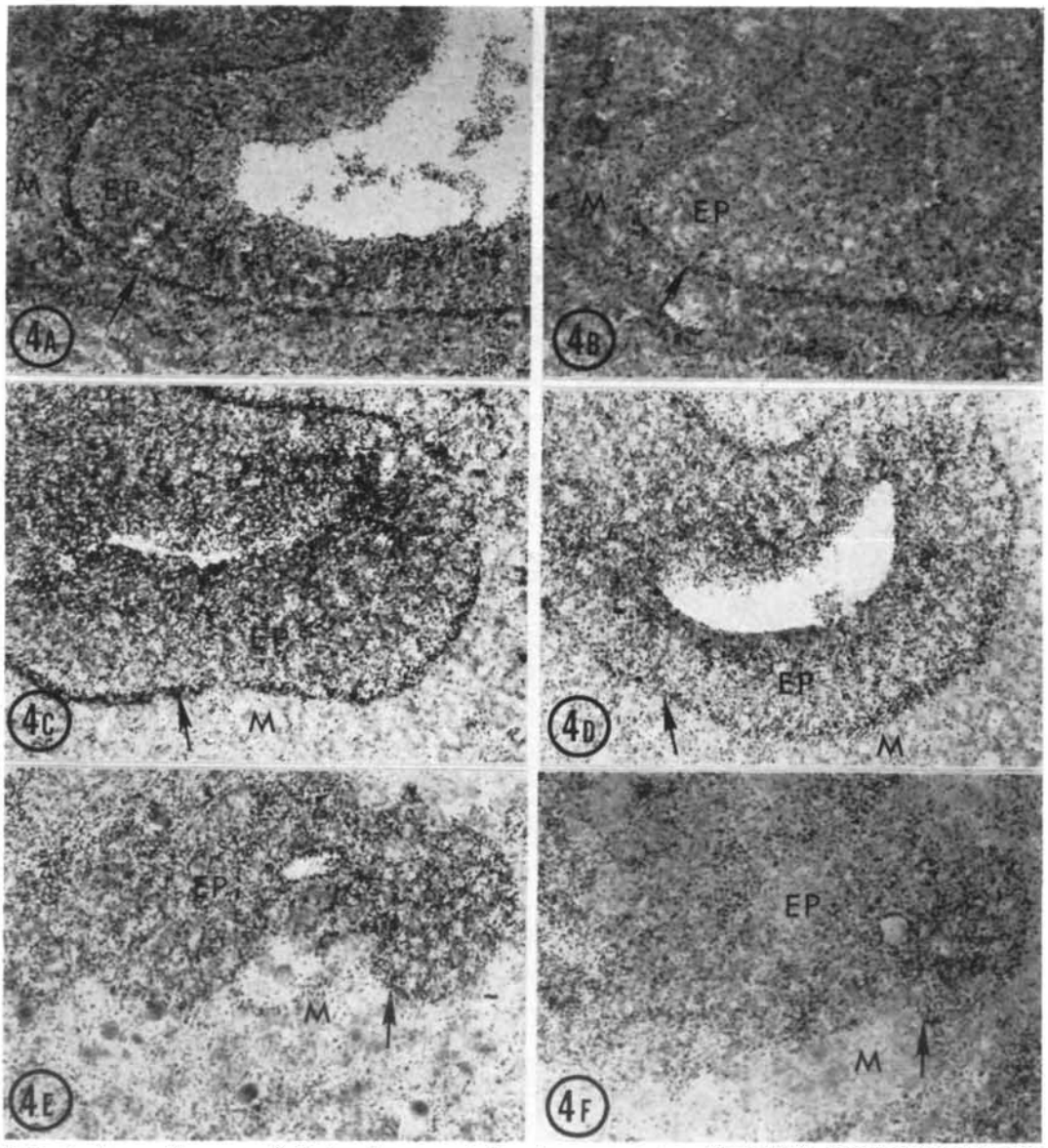


FIG. 4. Autoradiograms of 11-day lung, ureteric bud, and pancreas incubated after explantation for 2 hr in the presence of ^{3}H-glucosamine. Localized ^{3}H-glucosamine label is seen on the epithelial surfaces of (*A*) lung and (*C*) metanephros (arrows), however comparatively little localized label is seen on the (*E*) pancreas (arrow). *B*, *D*, and *F*, The sister sections in the right column were treated with testicular hyaluronidase and most of the epithelial surface label is removed (arrows). ×480.

of new GAG accumulation implying a greater rate of GAG degradation. The sites of heaviest collagen fibril localization show less new GAG deposition and slightly greater amounts of stainable GAG, suggesting that GAG associated with fibrillar collagen may turn over at a slower rate than basal laminar GAG.

The presence of GAG within embryonic epithelial basal laminae is unexpected, since basal laminae of adult epithelial tissues are ruthenium red negative (Luft, 1971*b*) and, on the basis of analyses of isolated basement membranes, are not thought to contain GAG (Spiro, 1970; Kefalides, 1970). An exception may be the

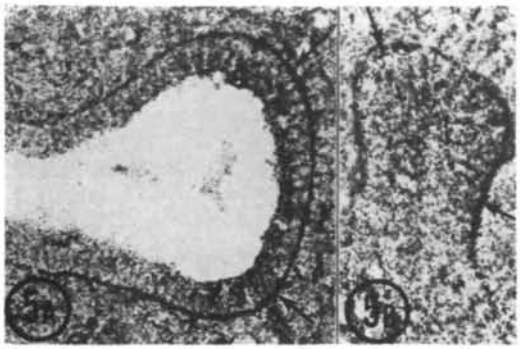


FIG. 5. Autoradiograms of 12-day lung and metanephros labeled for 2 hr in ^{3}H-glucosamine after explantation. *A*, Lung shows an intense accumulation of ^{3}H-glucosamine label at the epithelial surface on the distal growing end of the bronchial bud (arrows). Much less surface label is seen on the quiescent stalk of the bud (left). ×230. *B*, Metanephros similarly shows intense ^{3}H-glucosamine epithelial surface label on the growing bud (arrows) with less label on the stalk (lower half). ×380.

bovine lens capsule which, although composed principally of collagen with a high density of hydroxylysine-linked carbohydrate, contains GAG in young animals (Fukushi and Spiro, 1969; Spiro and Fukushi, 1969). Possibly, GAG may be present only in the basal laminae of epithelia from embryonic or immature animals.

Glycosaminoglycans in epithelial morphogenesis

To evaluate the developmental role of the surface-associated GAG, the morphogenesis of epithelia containing surface glycan was compared with those from which surface glycan had been removed (Bernfield et al., 1972). In culture, mouse 13¼-day sub-mandibular salivary glands undergo progressive budding and branching within the mesenchymal tissue (Fig. 6). To selectively remove the surface GAG, procedures were needed to strip off the mesenchyme and leave the epithelium and surface materials intact. Removal of the mesenchyme with crude trypsin-pancreatin, the time-honored procedure for isolating epithelia, also removed the surface GAG. When such trypsin-pancreatin isolated epithelia were placed in culture in contact with fresh mesenchyme, the epithelia lost their lobules, rounded up and formed a "ball-like" mass of tissue. With continued culture, outgrowths formed from the "ball-like" mass and branching morphogenesis resumed (Fig. 7). Treatment of the glands with low concentrations of purified collagenase along with gentle microdissection yielded epithelia free of mesenchyme *without* removing all of the surface glycan. When these epithelia were recombined with mesenchyme, they did not lose their shape and morphogenesis continued normally without interruption (See Fig. 11). This result showed that transient removal of mesenchyme *per se* does not alter morphogenesis and provided a means of obtaining epithelia retaining surface GAG.

Epithelia isolated in low collagenase concentrations were treated for 10 min with various enzymes in an attempt to selectively remove the surface GAG. The epithelia were recombined with fresh mesenchyme and subsequent morphogenesis was observed. Treatment of low collagenase-isolated epithelia with testicular hyaluronidase, crystalline trypsin, or either of two bacterial mucopolysaccharidases, chondroitinase ABC and chondroitinase AC, caused the epithelia to lose their lobular shape and form a "ball-like" mass in a fashion similar to the trypsin-pancreatin treated epithelia (Figs. 8-10). With time, outgrowths formed from which branching morphogenesis resumed. Similar treatment with sialidase did not alter the behavior of the low collagenase-isolated epithelia; these epithelia maintained their lobular shape and developed normally (Fig. 11).

To assess surface GAG, epithelia were isolated from glands pre-labeled with ^{3}H-glucosamine and treated by the identical procedures prior to autoradiography (Figs. 12-15). The autoradiograms revealed that surface GAG is removed by those enzyme treatments that interrupt morphogenesis and is retained on those epithelia which maintain their shape and continue morphogenesis unabated. These data demonstrate that loss of surface glycan correlates with loss of normal morphology and interruption of morphogenesis, suggesting that

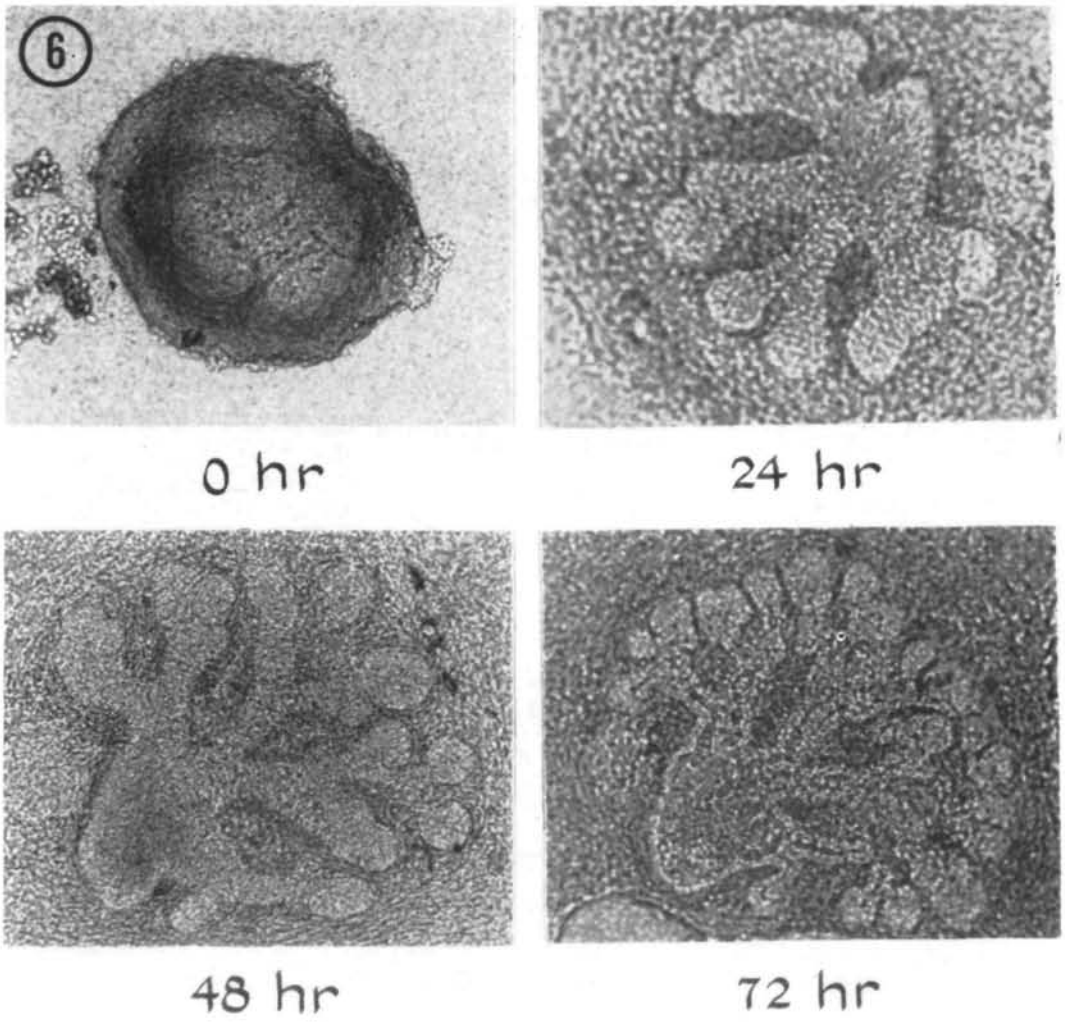


FIG. 6. Living whole 13¼-day submandibular salivary gland, explanted and immediately cultured (0 hr). The epithelium continues to undergo progressive budding and branching unabated within the mesenchyme (24, 48, and 72 hr). ×50.

maintenance of normal morphology and progressive branching morphogenesis require the continued presence of GAG at the epithelial surface.

Ultrastructural studies show that surface GAG resides within the basal lamina and not to a significant degree within the associated fibrillar or amorphous materials. Intact glands show fibrillar collagen and amorphous materials external to the basal lamina. Low collagenase-isolated epithelia retain a distinct basal lamina, but the fibrils and amorphous materials are absent by either ruthenium red or usual staining procedures (Figs. 20*A*, *B*). After exposure to hyaluronidase, the epithelial surface is essentially devoid of any lamina-like materials (Figs. 20*C*, *D*), but remnants of these materials frequently remain in clefts. This result suggests that (i) enzymatic digestion of GAG causes the collagenous and glycoprotein components of the basal lamina to be released from the tissue surface, and (ii) the loss of surface glycan observed autoradiographically represents removal of the entire basal lamina, possibly implicating any part or all of the basal lamina in the maintenance of lobular

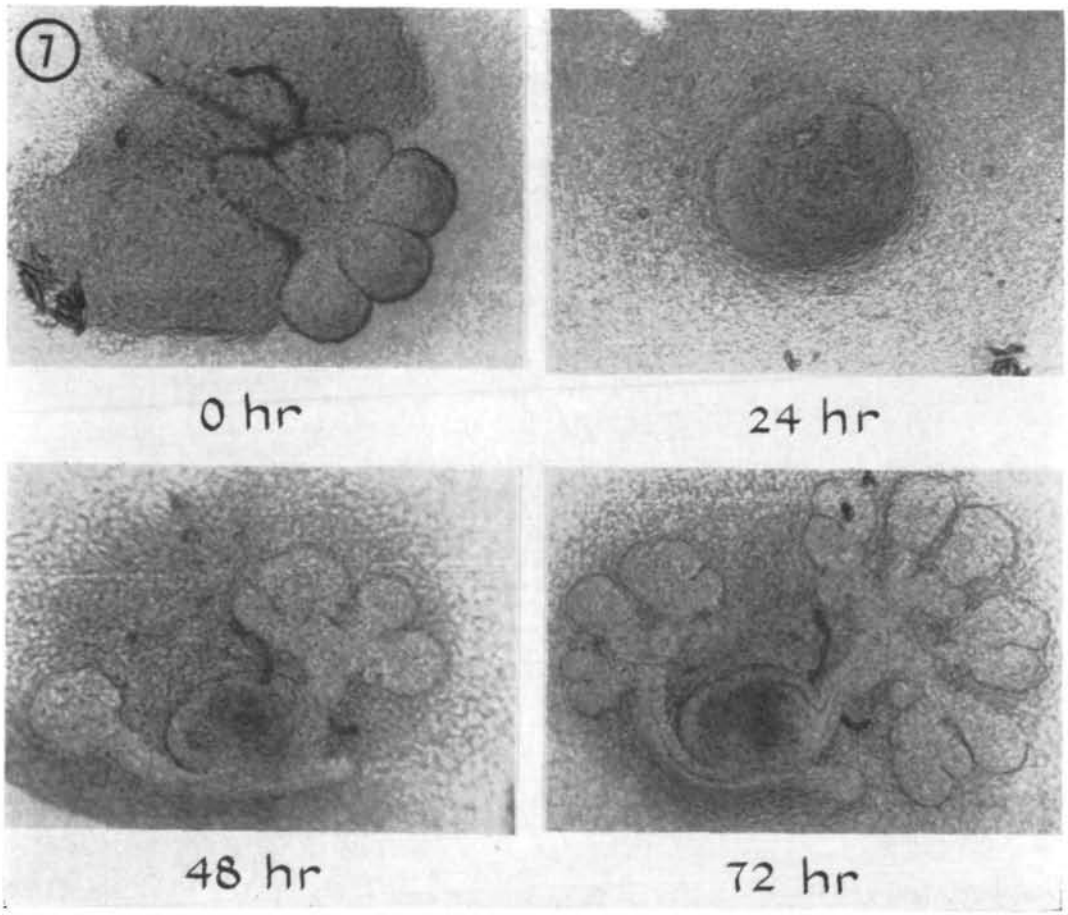
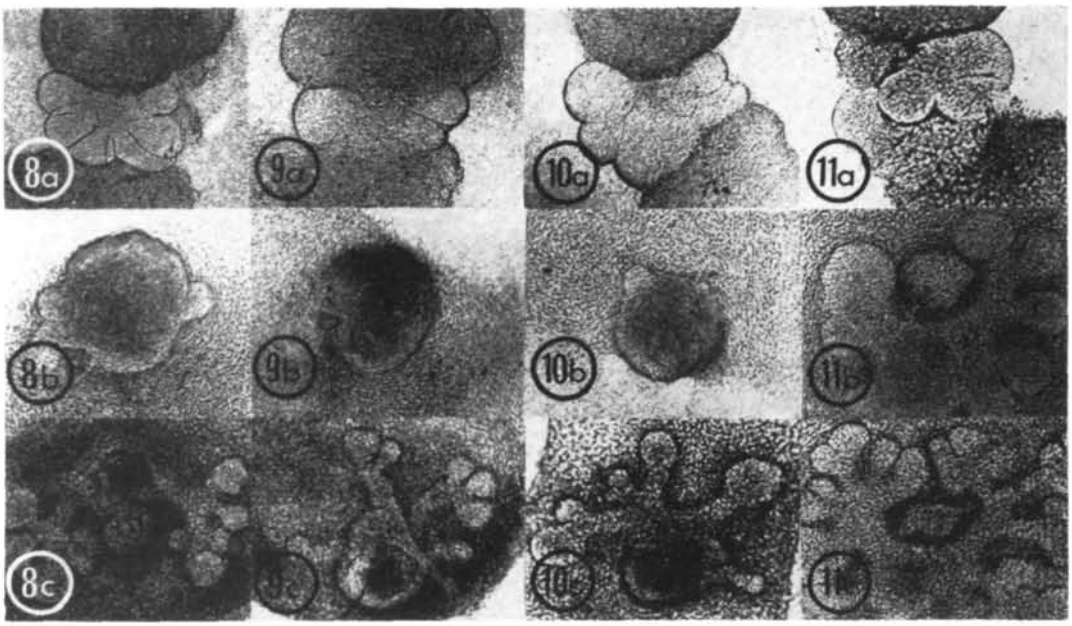


FIG. 7. Living salivary epithelium isolated with crude trypsin-pancreatin and then cultured in contact with fresh salivary mesenchyme (0 hr). The epithelium loses its lobules and forms a "ball-like" rudiment despite the continued presence of mesenchyme (24 hr). With continuing culture, outgrowths form from the "ball-like" mass and branching morphogenesis resumes (48 hr and 72 hr). ×48.

morphology.

During the reculture period, epithelia retaining a basal lamina undergo uninterrupted morphogenesis and continue to show maximal accumulation of newly synthesized GAG at their lobular surfaces. In contrast, the spherical epithelia accumulate labeled material nearly uniformly despite the reappearance of a nearly complete basal lamina (Bernfield and Wessells, 1970)—there is, however, a greater deposition of new GAG at the surfaces of the budding outgrowths, where morphogenesis will resume (Bernfield et al., 1972). Rapidly proliferating cells (assessed by ^{3}H-thymidine autoradiography) are localized within the lobules of epithelia undergoing uninterrupted morphogenesis, but are distributed uniformly in the cortex of the spherical epithelia, except for the outgrowths which show a greater concentration of proliferating cells. Thus, even under circumstances when a new basal lamina forms, the sites of maximal accumulation of newly synthesized GAG are those which subsequently show branching morphogenesis. This distribution may be a consequence or a cause of the change in epithelial shape. It is clear, however, that other factors are involved in the morphogenetic process. For example, epithelia containing a basal lamina cultured in the absence of



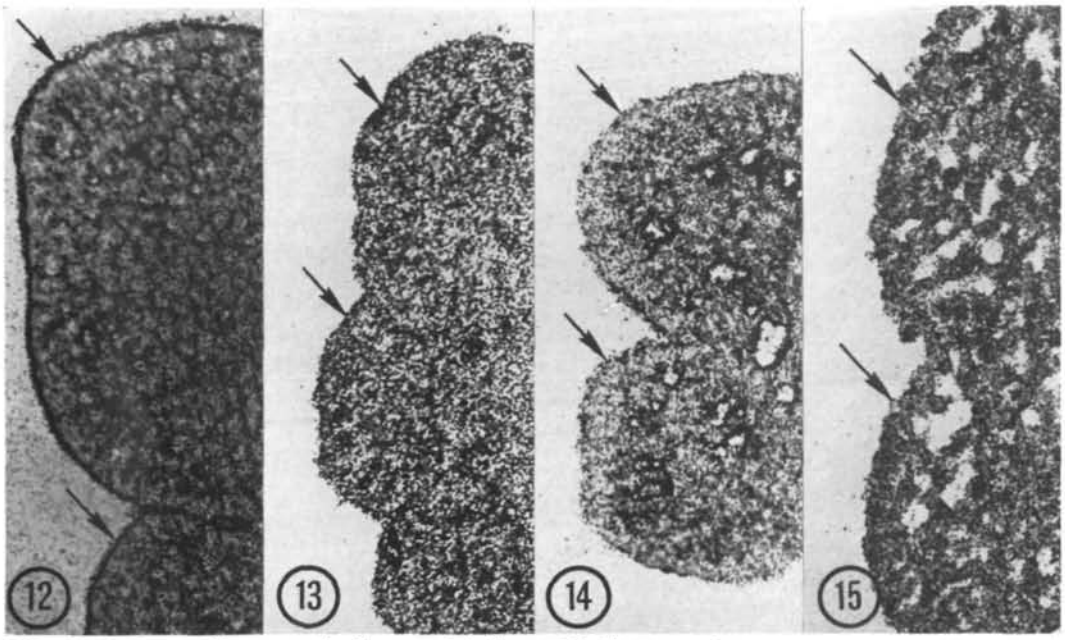
FIGS. 8-11. Living salivary epithelia isolated with low concentrations of collagenase and exposed to various enzymes before culturing in direct combination with fresh salivary mesenchyme. ×50. *a*, At time of explantation; *b*, At 24 hr of culture; and *c*, At 48 hr of culture. FIG. 8: Exposed to testicular hyaluronidase (0.003 mg/ml). FIG. 9: Exposed to crystalline trypsin (0.001 mg/ml). FIG. 10: Exposed to chondroitinase ABC (0.05 units/ml). FIG. 11: Exposed to clostridial sialidase (0.03 mg/ml). Epithelia exposed to hyaluronidase (Fig. 8), crystalline trypsin (Fig. 9), and chondroitinase ABC (Fig. 10) lose their lobules and form ball-like epithelia which at 24 hr have produced outgrowths. By 48 hr, branching morphogenesis has resumed from the outgrowths. Epithelia exposed to sialidase (Fig. 11) maintain their morphology and undergo uninterrupted morphogenesis. (From Bernfield et al., 1972.)

mesenchyme do not retain their morphology, but lose their lobules and round up (Fig. 16).

The enzymatic purity of the enzymes used in these studies is vital to their interpretation. All of the mucopolysaccharidase preparations were free of detectable protease, collagenase, or ribonuclease activities (Bernfield et al., 1972). Small amounts of non-collagen proteolytic activity were detected in four of five highly purified collagenase preparations but this activity is readily removed by treatment of the enzymes with N-ethyl maleimide (Peterkofsky and Diegelmann, 1971). Of greater importance is that all the collagenase preparations tested contained appreciable mucopolysaccharidase activity by two distinct types of assays. This contaminating activity, together with the presence of GAG in the basal lamina, indicates that dependence of morphogenesis on "collagenase-susceptible materials" does not necessarily implicate collagen.

Origin and characteristics of basal laminar GAG

Basal laminar GAG is derived from the epithelial cells. Substantial label accumulates at the surface of collagenase- and collagenase plus hyaluronidase-isolated epithelia incubated for 2 hr with ^{3}H-glucosamine in the absence of mesenchyme (Fig. 17). In contrast, when unlabeled epithelia are cultured for 2 hr in close association with pre-labeled mesenchyme, no localized radioactivity is at the epithelial surface (Fig. 18). Since surface GAG may turn over fairly rapidly, it must be shown that this lack of surface label is not due to rapid turnover in the absence of precursor. Therefore, epithelia pre-labeled as intact glands were isolated and incubated in me-



FIGS. 12-15. Autoradiograms of salivary epithelia incubated as intact glands for 2 hr in ^{3}H-glucosamine, isolated free of mesenchyme with a low concentration of collagenase (0.2 mg/ml) and then exposed to various enzymes. ×390. FIG. 12: Exposed to clostridial sialidase (0.03 mg/ml). Radioactivity remains at the epithelial surface (arrows). FIG. 13: Exposed to testicular hyaluronidase (0.003 mg/ml). The surface is nearly devoid of label (arrows). FIG. 14: Exposed to crystalline trypsin (0.001 mg/ml). The surface is free of label (arrows). FIG. 15: Exposed to chondroitinase AC (0.05 units/ml). No radioactivity remains at the surface (arrows). (From Bernfield et al., 1972.)

dium containing cold glucosamine. Collagenase-isolated epithelia retained their surface label and the pre-labeled hyaluronidase-treated epithelia produced surface label both in the presence and absence of mesenchyme (Fig. 19). Although the reappearance of surface GAG on hyaluronidase-treated epithelia precedes the resumption of morphogenesis by these epithelia, it remains to be proven that deposition of surface GAG is a prerequisite for branching morphogenesis.

Not only does the epithelium synthesize its own surface GAG, but it also produces a basal lamina (Fig. 20). Collagenase-isolated epithelia show a well-defined basal lamina by both ruthenium red and standard ultrastructural methods. Hyaluronidase treatment removes this basal lamina. However, when such denuded epithelia are incubated in submerged culture for 2 hr in the absence of mesenchyme, they display a basal lamina over most of their surface. This newly deposited lamina stains intensely with ruthenium red and appears similar to that of uncultured collagenase-isolated rudiments except that it shows occasional clusters of fibrillar material. These studies confirm our autoradiographic results and agree with the previous findings that embryonic corneal epithelia and neuroepithelia can produce a morphologically normal basal lamina (Dod-

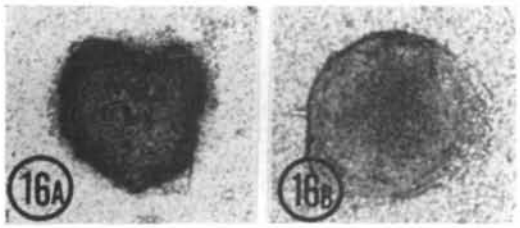
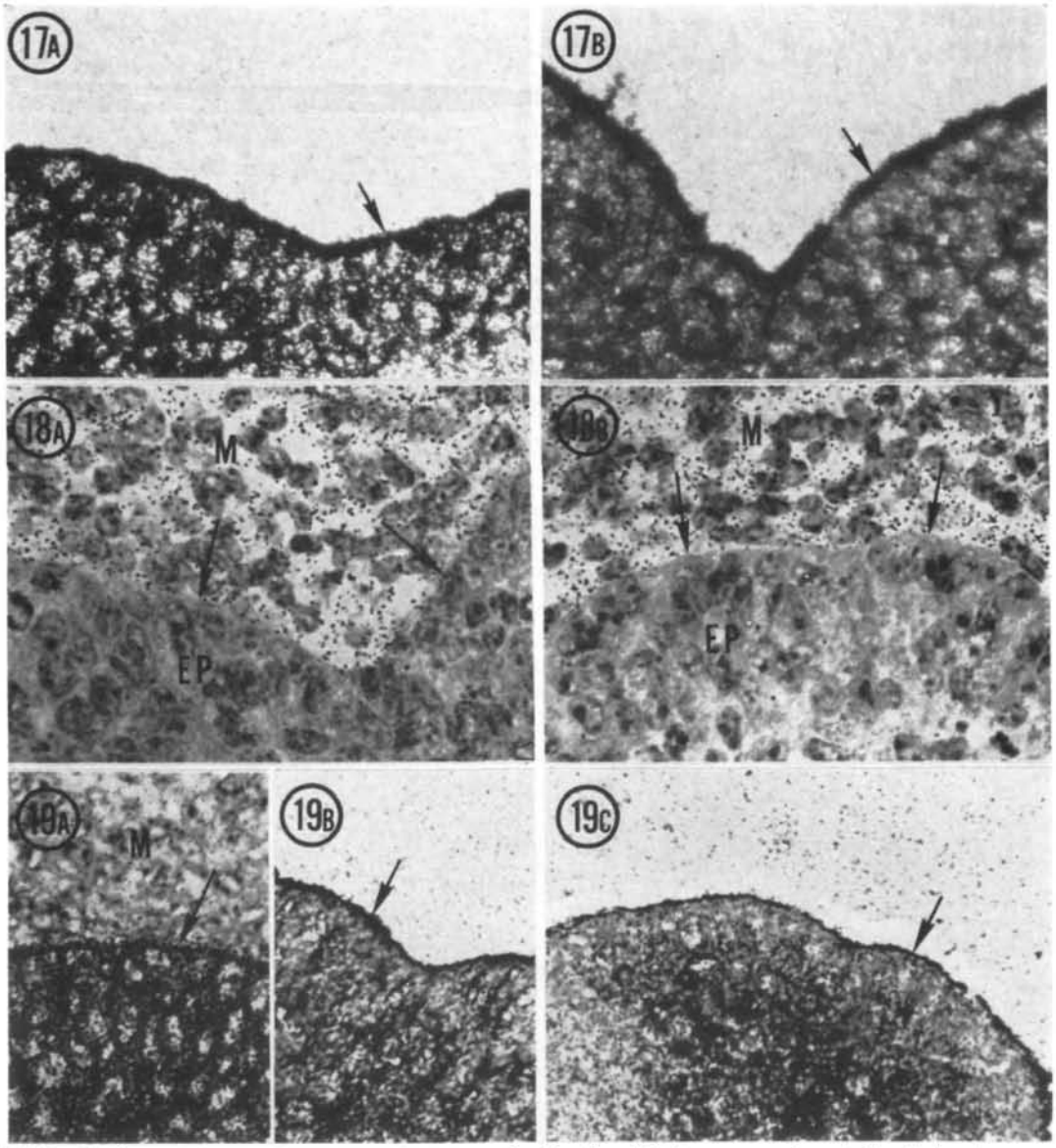


FIG. 16. Salivary epithelia, isolated with (*A*) low-collagenase and (*B*) crystalline trypsin and maintained in culture *without* mesenchyme for 48 hr. Whether GAG and a basal lamina have been (*A*) retained or (*B*) removed, the epithelia round up, lose morphology and discontinue morphogenesis. ×50.



FIGS. 17-19. ^{3}H-glucosamine autoradiograms of 13¼-day salivary epithelia isolated in low-collagenase (right column) or in low-collagenase and then exposed to hyaluronidase (left column). (M) mesenchyme, (EP) epithelium. ×640. FIG. 17: *A*, Collagenase plus hyaluronidase or, *B*, collagenase isolated epithelia were incubated in ^{3}H-glucosamine for 2 hr without mesenchyme. Substantial label accumulates at the surfaces of both types of epithelia (arrows). FIG. 18: Unlabeled (*A*) collagenase plus hyaluronidase or, (*B*), collagenase isolated epithelia were cultured for 2 hr in combination with ^{3}H-glucosamine-prelabeled mesenchyme. No labeled material is seen on the epithelial surfaces (arrows). FIG. 19: Epithelia were prelabeled in ^{3}H-glucosamine as whole glands. Prelabeled hyaluronidase treated epithelia produce localized surface label (arrows) after 2 hr (*A*) with mesenchyme or (*B*) without mesenchyme. *C*, Collagenase isolated, prelabeled epithelia retain localized surface label after 2 hr in culture (arrows).

son and Hay, 1971; Cohen and Hay, 1971). The salivary results are distinct, however, in that no killed tissue substratum containing a pre-formed basal lamina is required.

Some characteristics of the basal laminar

GAG can be deduced from the enzyme treatments which remove it. Dermatan, keratan, and heparan sulfate do not appear to be involved, since they are resistant to both testicular hyaluronidase and chondroitinase AC, enzymes which remove the surface GAG (Yamagata et al., 1968). Removal of GAG by crystalline trypsin implies linkage to protein, and this trypsin solubility was used to prepare newly synthesized GAG for preliminary characterization studies. Epithelia isolated by low collagenase treatment from salivary glands labeled with ^{3}H-glucose for 2 hr were briefly treated with crystalline trypsin to solubilize the basal lamina. Authenic GAG was isolated from mesenchyme, basal lamina, and epithelium. This material was hydrolyzed with chondroitinase ABC, yielding unsaturated disaccharides characteristic of the various GAGs (Yamagata et al., 1968): ΔDi-6S for chondroitin 6-SO_4, ΔDi-4S for chondroitin 4-SO_4, ΔDi-0S for chondroitin, and ΔDi-0HA for hyaluronic acid (Table 1). Mesenchyme produces nearly

TABLE 1. *Salivary gland glycosaminoglycans.*

	2-hr ^{3}H-glucose labeling		
Disaccharide	Mesenchyme	Basal lamina	Epithelium
	Percent of total		
ΔDi-6S	28	11	22
ΔDi-4S	31	22	26
ΔDi-OS	10	4.3	7.1
ΔDi-OHA	31	63	45

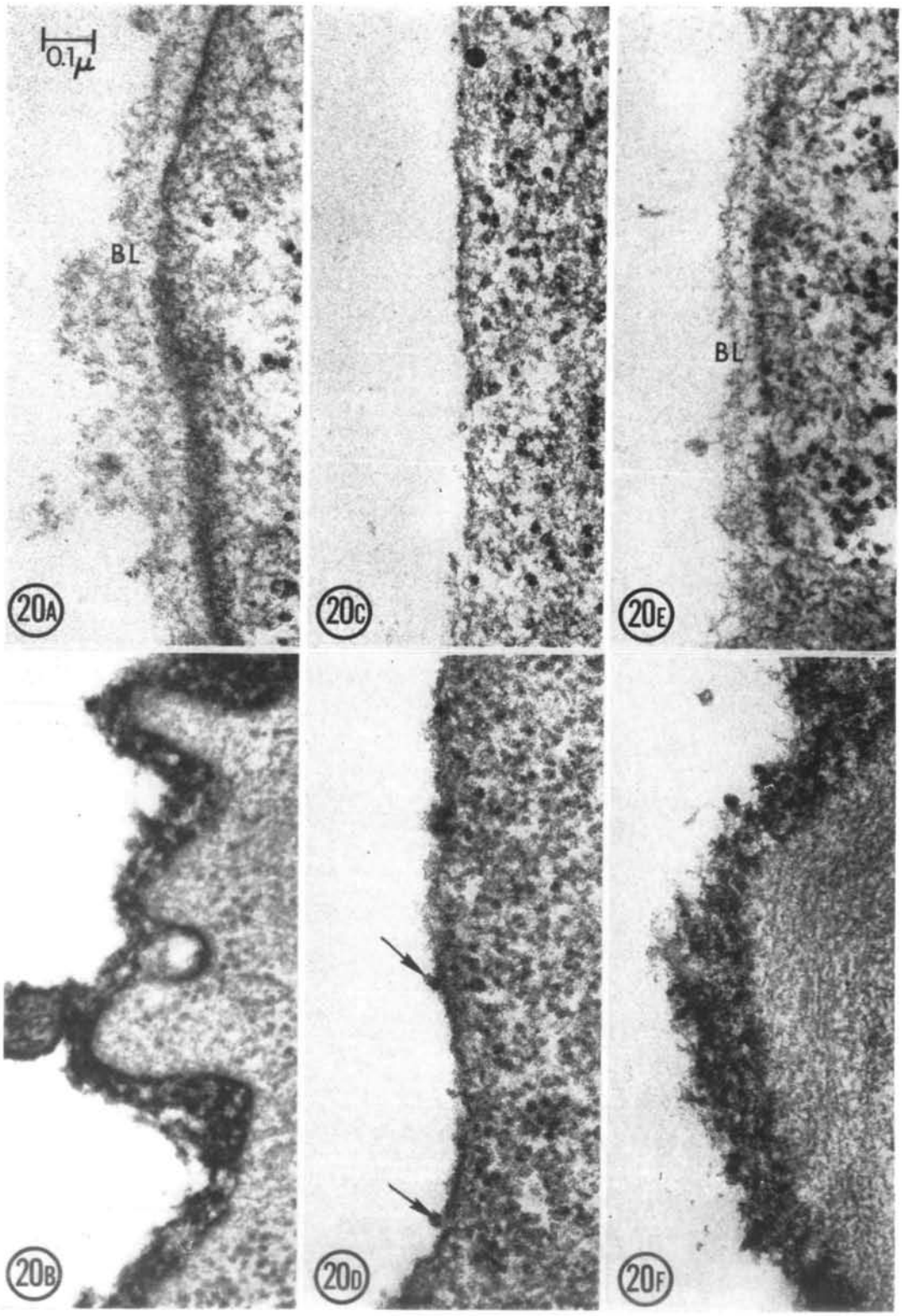
equivalent amounts of hyaluronic acid, chondroitin-6, and chondroitin 4-SO_4, whereas epithelium produces similar amounts of chondroitin sulfates, and substantially more hyaluronic acid. The newly synthesized GAG in the basal lamina is distinct from either tissue, having predominantly hyaluronic acid and twice as much chondroitin 4-SO_4 as chondroitin 6-SO_4. It must be emphasized that only newly synthesized GAG has been characterized and that fibrillar collagen (with any associated GAG) was removed during the isolation of the epithelium.

Morphogenetic function of basal laminar GAG

Epithelial basal laminae contain GAG, and the greatest rate of accumulation of newly synthesized GAG is at the morphogenetically most active sites. Transient loss of the basal lamina, but not of fibrillar collagen, alters epithelial morphology and interrupts branching morphogenesis. Prior to the resumption of morphogenesis, newly synthesized GAG is deposited at the epithelial surface and is maximally localized at sites of subsequent lobular outgrowth. The change in shape is not duplicated by the drug cytochalasin B (Spooner and Wessells, 1970), which interferes with microfilament function (Wessells et al., 1971), and indeed, cytochalasin has no effect on GAG synthesis or on GAG deposition in the basal lamina (Cohn et al., 1972). It appears that establishing and maintaining normal branching morphogenesis require both mesenchyme and a basal lamina containing newly synthesized GAG.

The experiments have not examined whether nor do they suggest that surface GAG *mediates* the epithelio-mesenchymal inductive interaction or *causes* specific changes in epithelial shape. Although maximal accumulation of newly synthesized GAG is associated with sites of subsequent change in shape in various tissues, the presence of newly synthesized glycan at an epithelial surface may not mean branching morphogenesis will occur. It is possible, even likely, that other components of the basal lamina, as well as materials on the cell surface, have a morphogenetic role.

How GAG may function morphogenetically is unknown. They are large, flexible, generally randomly oriented molecules which occupy very large domains consisting principally of solvent. Strong intermolecular interactions are suggested by their high viscosity and concentration dependence of sedimentation and diffusion constants. These characteristics imply that GAG may alter solute permeability as a type of filter, or may serve a mechanical or supportive function. These molecules bind various cations,



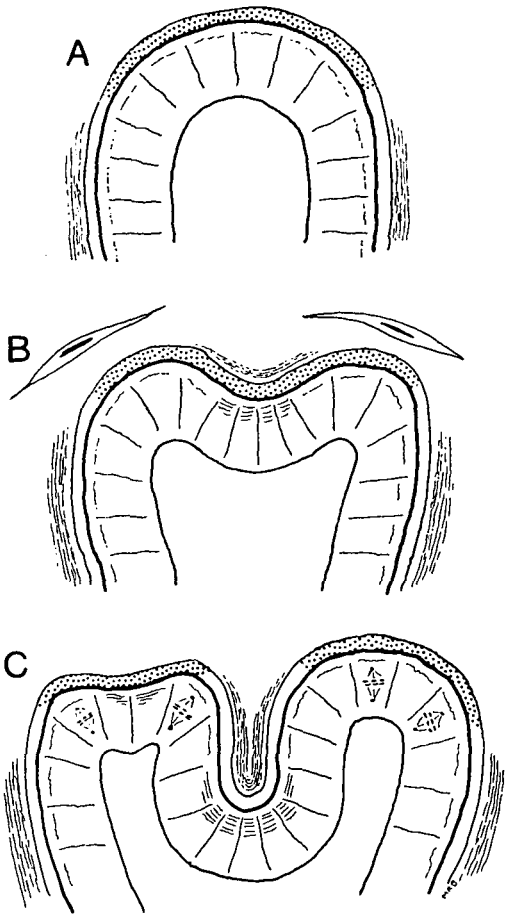


FIG. 21. Schematic model depicting the relationship between cleft formation and the distribution of extracellular materials. *A,* A primary lobule. Newly synthesized GAG (stippled areas) accumulates within the basal lamina at the distal end of the lobule. The basal laminae at the lateral aspects of the lobule are sites of low rates of new GAG accumulation and are associated with bundles of collagen fibres. Intracellular microfilaments are present at the basal end of each cell. *B,* Early cleft formation in a primary lobule. Contraction of microfilament bands within cells at the top of the lobule causes the tissue to bulge inward, initiating the formation of secondary lobules. Tropocollagen derived in part from the mesenchymal cells begins fibrogenesis near the surface of the incipient cleft. This cleft would be sensitive to the action of cytochalasin. *C,* Deepening of the cleft and growth of secondary lobules. Mitotic activity of cells within the secondary lobules and continued microfilament contractility cause the cleft to deepen. As the secondary lobules grow, extracellular materials originally at the surface of the primary lobule are incorporated into the cleft, where bundles of collagen fibers become organized and confer stability to the branch point. This cleft would be insensitive to the action of cytochalasin. The basal laminae at the distal ends of the growing secondary lobules show the bulk of newly synthesized GAG, and with further growth undergo cleft formation.

and, of particular physiologic significance, have high affinity for calcium ion (Scott, 1965). In addition, certain proteoglycans accelerate, while others inhibit tropocollagen fibrillogenesis, suggesting a possible role in the control of collagen fibril formation (Toole and Lowther, 1968).

A model for branching morphogenesis

A hypothetical model can be proposed to explain the formation of branch points and clefts, the pattern of which ultimately determines organ form (Fig. 21). Surrounding epithelial buds is a continuous basal lamina containing GAG. Newly synthesized GAG accumulates at specific regions, unique to the type of epithelium. These regions are the sites where branching will take place and where collagen fibers are minimal. The GAG is synthesized by the epithelial cells and its composition is distinctive from the newly synthesized GAG found in either mesenchyme or epithelium. Within the basal end of each cell are contractile microfilaments. Near the sites of new GAG deposition, the intracel-

FIG. 20. Electron micrographs of typical views at the surfaces of isolated salivary epithelia, shown with standard glutaraldehyde-osmium fixation (top row) and with ruthenium red (bottom row). (BL) basal lamina. ×80,000. *A,* Epithelium after isolation with a low concentration of collagenase. The epithelial surface retains a basal lamina which (*B*) demonstrates ruthenium red staining properties. No collagen fibrils are seen at the surfaces of these epithelia. *C,* Epithelia isolated in low-collagenase and then treated with hyaluronidase are devoid of a basal lamina and (*D*) show small amounts of ruthenium red-positive material (arrows) adhered to the plasmalemma. *E,* Epithelia nuded as in *C* and then cultured for 2 hr in the absence of mesenchyme, display a prominent basal lamina over most of the surface which (*F*) stains intensely with ruthenium red.

lular microfilaments contract, causing the tissue to bulge inward. Newly synthesized collagen, derived in part from the adjacent mesenchyme, begins to undergo fibrogenesis within the forming cleft. As the branching process progresses, extracellular materials originally at the surface of the bud are now pulled into the deepening cleft. Here, bundles of collagen fibrils organize and confer stability to the branch point. Because of the prior deposition of GAG at the surface of what is now the cleft, relatively little newly synthesized GAG accumulates within the cleft. Maximal accumulation is at the distal ends of the new buds, the sites of subsequent cleft formation and of most rapid cell proliferation.

This model accounts for the distribution of GAG (total and newly synthesized), of collagen fibrils, and for the resistance of older clefts and the susceptibility of recently formed clefts to cytochalasin treatment. It does not account for the loss of morphology after removal of the basal lamina, but raises several speculative possibilities, including the idea that basal laminar GAG may be involved in regulating microfilament function. Removal of GAG results in normal epithelial morphology being lost, yielding a "ball-like" rudiment surrounded by newly synthesized GAG and containing microfilament arrays (Bernfield et al., 1972). The uniformly distributed new GAG might induce non-specific microfilament contractility, resulting in formation of a spherical structure. Cytochalasin treatment flattens these "ball-like" epithelia in a similar manner as normal epithelia. The attractiveness of an hypothesis involving calcium-binding materials in the control of microfilament function is augmented by work implying that microfilament contractility is calcium-mediated (Wessells et al., 1971) and by the studies demonstrating that microfilaments may be the ultrastructural correlates of the muscle-like proteins isolated from non-muscle cultured and embryonic cells (Bray, 1972; Adelstein et al., 1972).

REFERENCES

Adelstein, R. S., M. A. Conti, G. S. Johnson, I. Pastan, and T. D. Pollard. 1972. Isolation and characterization of myosin from cloned mouse fibroblasts. Proc. Nat. Acad. Sci. U.S.A. 69:3693-3697.

Bernfield, M. R. 1970. Collagen synthesis during epitheliomesenchymal interactions. Develop. Biol. 22:213-231.

Bernfield, M. R., and S. D. Banerjee. 1972. Acid mucopolysaccharide (glycosaminoglycan) at the epithelio-mesenchymal interface of mouse embryo salivary glands. J. Cell Biol. 52:664-673.

Bernfield, M. R., S. D. Banerjee, and R. H. Cohn. 1972. Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide-protein (proteoglycan) at the epithelial surface. J. Cell Biol. 52:674-689.

Bernfield, M. R., and N. K. Wessells. 1970. Intra- and extracellular control of epithelial morphogenesis. Develop. Biol. 4 (Suppl.):195-249.

Bray, D. 1973, Cytoplasmic actin: a comparative study. Cold Spring Harbor Symp. Quant. Biol. 37:567-571.

Cohen, A. M., and E. D. Hay. 1971. Secretion of collagen by embryonic neuroepithelium at the time of spinal cord-somite interaction. Develop. Biol. 26:578-605.

Cohn, R. H., S. D. Banerjee, E. R. Shelton, and M. R. Bernfield. 1972. Cytochalasin B: Lack of effect on mucopolysaccharide synthesis and selective alterations in precursor uptake. Proc. Nat. Acad. Sci. U.S.A. 69:2865-2869.

Dodson, J. W., and E. D. Hay. 1971. Secretion of collagenous stroma by isolated epithelium grown in vitro. Exp. Cell Res. 65:215-220.

Fukushi, S., and Spiro, R. G. 1969. The lens capsule: sugar and amino acid composition. J. Biol. Chem. 244:2041-2048.

Grobstein, C. 1967. Mechanism of organogenetic tissue interaction. Nat. Cancer Inst. Monogr. 26: 279-299.

Grobstein, C., and J. Cohen. 1965. Collagenase: effect on the morphogenesis of embryonic salivary epithelium in vitro. Science 150:626-628.

Kallman, F., J. Evans, and N. K. Wessells. 1967. Normal epidermal basal cell behavior in the absence of basement membrane. J. Cell Biol. 32: 231-236.

Kallman, F., and C. Grobstein. 1966. Localization of glucosamine-incorporating materials at epithelial surfaces during salivary epitheliomesenchymal interaction in vitro. Develop. Biol. 14: 52-67.

Kefalides, N. A. 1970. Comparative biochemistry of mammalian basement membranes, p. 535-573. *In* E. A. Balazs [ed.], Chemistry and molecular biology of the intercellular matrix. Academic Press, New York.

Konigsberg, I. R. 1970. The relationship of collagen to the clonal development of embryonic skeletal muscle, p. 1779-1810. *In* E. A. Balazs [ed.], Chemistry and molecular biology of the intercellular matrix. Academic Press, New York.

Luft, J. H. 1971*a*. Ruthenium red and violet I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anat. Rec. 171:347-368.

Luft, J. H. 1971*b*. Ruthenium red and violet II. Fine structural localization in animal tissues. Anat. Rec. 171:369-416.

Peterkofsky, B., and R. Diegelmann. 1971. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10:988-994.

Quintarelli, G., and M. C. Dellovo. 1965. The chemical and histochemical properties of Alcian Blue. IV. Further studies on the methods for the identification of acid glycosaminoglycans. Histochemie 5:196-209.

Rifkind, R. A., D. Chui, and H. Epler. 1969. An ultrastructural study of early morphogenetic events during the establishment of fetal hepatic erythropoiesis. J. Cell Biol. 40:343-365.

Scott, J. E. 1965. Ion binding in solutions containing acid mucopolysaccharides, p. 171. *In* G. Quintarelli [ed.], The chemical physiology of mucopolysaccharides. Little, Brown, and Company, Boston, Mass.

Scott, J. E., and J. Dorling. 1969. Periodate oxidation of acid polysaccharides. III. A PAS method for chondroitin sulphates and other glycosaminoglycuronans. Histochemie 19:295-301.

Slavkin, H. C. 1971. The dynamics of extracellular and cell surface protein interactions, p. 221-276. *In* I. L. Cameron and J. D. Thrasher [ed.], Cellular and molecular renewal in the mammalian body. Academic Press, New York.

Slavkin, H. C., P. Bringas, J. Cameron, R. LeBaron, and L. A. Bavetta. 1969. Epithelial and mesenchymal cell interactions with extracellular matrix material in vitro. J. Embryol. Exp. Morphol. 22:395-405.

Spiro, R. G. 1970. The carbohydrate of collagens, p. 195-215. *In* E. A. Balazs [ed.], Chemistry and molecular biology of the intercellular matrix. Academic Press, New York.

Spiro, R. G., and S. Fukushi. 1969. The lens capsule: studies on the carbohydrate units. J. Biol. Chem. 244:2049-2058.

Spooner, B. S., and N. K. Wessells. 1970. Effects of cytochalasin B upon microfilaments involved in morphogenesis of salivary epithelium. Proc. Nat. Acad. Sci. U.S.A. 66:360-364.

Stuart, E. S., and A. A. Moscona. 1967. Embryonic morphogenesis: role of fibrous lattice in the development of feathers and feather patterns. Science 157:947-948.

Toole, B. P., and D. A. Lowther. 1968. The effect of chondroitin sulphate-protein on the formation of collagen fibrils in vitro. Biochem. J. 109:857-866.

Wessells, N. K. 1970. Mammalian lung development: interactions in formation and morphogenesis of tracheal buds. J. Exp. Zool. 175:455-466.

Wessells, N. K., and J. H. Cohen. 1968. Effects of collagenase on developing epithelia in vitro: lung, ureteric bud, and pancreas. Develop. Biol. 18:294-309.

Wessells, N. K., B. S. Spooner, J. F. Ash, M. O. Bradley, M. A. Ludueña, E. L. Taylor, J. T. Wrenn, and K. M. Yamada. 1971. Microfilaments in cellular and developmental processes. Science 171:135-143.

Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki. 1968. Purification and properties of bacterial chondoitinases and chondrosulfates. J. Biol. Chem. 243:1523-1535.