DEPENDENCE OF SALIVARY EPITHELIAL MORPHOLOGY

AND BRANCHING MORPHOGENESIS UPON

ACID MUCOPOLYSACCHARIDE-PROTEIN

(PROTEOGLYCAN) AT THE EPITHELIAL SURFACE

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ABSTRACT

The morphogenetic role of the acid mucopolysaccharide (glycosaminoglycan) at the epithelial surface of mouse embryo submandibular glands has been studied by comparing the in vitro morphogenesis of epithelia from which the mucopolysaccharide was removed with that of those that retained the mucopolysaccharide. Epithelia isolated free of mesenchyme by procedures which retain the bulk of surface mucopolysaccharide maintain their lobular shape and undergo uninterrupted branching morphogenesis in culture in direct combination with fresh mesenchyme. Under identical culture conditions, epithelia from which surface mucopolysaccharide was removed lose their lobules and become spherical masses of tissue. During continued culture, the spherical epithelia produce outgrowths from which branching morphogenesis resumes. The morphogenetically active mucopolysaccharide is localized within the basal lamina of the epithelial basement membrane and appears to be bound to protein. During culture in combination with mesenchyme, epithelia undergoing uninterrupted morphogenesis show maximal accumulation of newly synthesized surface mucopolysaccharide at the distal ends of the lobules, the sites of incipient branching. In contrast, the material accumulates nearly equivalently over the surface of the spherical epithelia, with the exception that there is greater accumulation of the material at the surfaces of the budding outgrowths, the sites where morphogenesis will resume. Rapidly proliferating cells 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 localization of proliferating cells. It is concluded that normal salivary epithelial morphology and branching morphogenesis require the presence of acid mucopolysaccharide-protein within the epithelial basal lamina.

INTRODUCTION

Extracellular materials such as collagen and mucopolysaccharide have frequently been identified in developing organs, but the morphogenetic role of these materials is not well defined (1). For example, collagen is at the interface between developing epithelia and their investing mesenchyme (2–4). When certain epithelia (salivary, lung, or ureteric bud) are cultured across a membrane filter from

mesenchyme and are treated with collagenase, the morphogenesis of the epithelia is altered (5, 6). These studies imply that materials susceptible to collagenase (presumably collagen) are required for the formation of epithelial structures. However, electron microscopy of these collagenase-treated rudiments (6) reveals, in addition to fewer collagen fibers, disruption of the basal lamina and dissolution of the extracellular matrix adjacent to the basal lamina, suggesting that the collagenase treatment affects materials other than collagen. Indeed, collagenase preparations similar to those used in these studies contain appreciable nonspecific protease and mucopolysaccharidase activities. Although evidence from other experiments suggests that collagen is involved in the morphogenetic process (1, 7), collagen per se is apparently not sufficient to support normal epithelial morphogenesis (4, 8, 9).

We have recently demonstrated that acid mucopolysaccharide is present at the interface between salivary epithelium and its investing mesenchyme (10). The acid mucopolysaccharide is distributed over the epithelial surface in nearly equivalent amounts, but newly synthesized mucopolysaccharide accumulates in greatest amounts at the sites of incipient cleft formation and branching, areas where collagen fibers are minimal. On the basis of these studies, we proposed a mechanism that accounts for the observed distribution near the epithelial surface of total and newly synthesized acid mucopolysaccharide, as well as that of collagen fibers. The mechanism proposes that acid mucopolysaccharide–protein complexes (proteoglycans) at the sites of new cleft formation initiate the fibrogenesis of collagen, which ultimately acts to stabilize epithelial morphology. The proposal suggests that salivary morphogenesis may be dependent upon the presence of acid mucopolysaccharide–protein complexes associated with the epithelial surface.

The present communication presents studies on embryonic salivary glands indicating that acid mucopolysaccharide within the epithelial basal lamina is required for the development of characteristic salivary epithelial structures. Salivary morphogenesis is dependent upon the presence of the acid mucopolysaccharide; loss of acid mucopolysaccharide correlates with loss of characteristic morphology, while reappearance of the material is associated with the resumption of morphogenesis.

MATERIALS AND METHODS

Chemicals and Enzymes

Glucosamine-^{3}H (1150 and 1300 mCi/mmole) was obtained from New England Nuclear Corp., Boston, Mass., and thymidine-^{3}H (10,000 mCi/mmole) from Schwarz Bio Research, Orangeburg, N. Y. Crude trypsin-pancreatin was prepared from three parts trypsin 1:250 (Difco Laboratories, Detroit, Mich.) and one part pancreatin N.F. (Difco). Crystalline trypsin (TRL50S), Clostridium histolyticum collagenase type A (CLSPA, lots 9BB, 9BA, 9EA, and 9EB) and C. perfringens sialidase (NEUP, lot 81A) were obtained from Worthington Biochemical Corp., Freehold, N. J., Clostridial histolyticum collagenase type A (designated 1SS), prepared by a modification of the procedure of Harper et al. (11), was kindly provided by Dr. Sam Seifter, Albert Einstein College of Medicine. Bovine testicular hyaluronidase (type I, lot 18B-0840) was obtained from Sigma Chemical Co., St. Louis, Mo. Proteus vulgaris chondroitinase ABC (lot 901) and Flavobacterium heparinum chondroitinase AC (lot 903) were preparations of Seikaguko Kogyo Co. Ltd. obtained from Miles Laboratories Inc., Research Products Div., Kankakee, Ill.

Culture Techniques

Mouse embryo submandibular salivary glands were obtained as described elsewhere (3), except that the 13-day pregnant females were sacrificed in the late afternoon to insure multilobularity of the epithelia (referred to as 13¼-day glands).

Isolated epithelia were prepared by removing as much mesenchyme as possible (without injuring the epithelium) by microdissection in a 1:1 (vol:vol) mixture of horse serum and Tyrode's solution (HST). Large mesenchyme pieces were stored in HST (at 23°C and 5% CO_2 in air) for later use. The epithelia with adherent mesenchyme were washed in 10 ml calcium- and magnesium-free Tyrode's solution (CMFT) and placed in 1.0 ml of a freshly prepared and gassed (5% CO_2 in air) enzyme solution in CMFT at 23°C. The epithelia were freed of mesenchyme by rapid and repeated pipetting of the rudiment with an orally controlled pipette (0.25–0.3 mm bore) and intermittently teasing off the adherent cells. After 5–15 min of flushing and teasing, depending on the enzyme, the epithelia were visually free of mesenchyme and were placed in HST. Epithelia isolated free of mesenchyme by collagenase treatment were washed in magnesium-free Tyrode's solution before flushing, and the collagenase was dissolved in this same solution.

For two-stage enzyme treatment, epithelia which were freed of mesenchyme with low collagenase concentrations were treated with a second enzyme. After

removal of mesenchyme with collagenase, these epithelia were rinsed in HST and washed five times in CMFT. The second enzyme treatment was carried out *without flushing* in pregassed CMFT at 23°C for 10 min, after which the epithelia were placed in HST.

Intact glands and isolated epithelia recombined with mesenchyme were cultured without a clot on the upper surface of a Millipore filter culture platform (Millipore Corporation, Bedford, Mass.). Epithelia recombined with mesenchyme were cultured by placing the mesenchyme pieces on the filter, then placing the epithelium on top of the mesenchyme. During reculture, the mesenchyme surrounds the epithelium while the epithelium grows into the mesenchyme. Incubation conditions were as previously described (3). Living cultures were observed and photographed with a Zeiss photomicroscope (Carl Zeiss, Inc., New York) using a bright field optical system.

Enzyme Assays

Enzymes were assayed in duplicate at a minimum of four concentrations in each assay. In all cases, enzyme activity was linearly proportional to enzyme concentration. In those instances where no enzyme activity was detected, the assays were repeated at least once with freshly prepared substrate and enzyme solutions.

MUCOPOLYSACCHARIDASE : The turbidometric method described by Mathews (12) was followed, except that crude chondroitin sulfate (Calbiochem, Los Angeles, Calif.) was used as substrate. Mucopolysaccharidase activity was also assessed by the enzymatic formation of α,β-unsaturated uronides (13) at pH 7.4, using crude chondroitin sulfate as substrate. Activity is expressed as change in absorbancy min^{-1} mg^{-1}.

PROTEASE : Nonspecific proteolytic activity was assessed with denaturated hemoglobin and with solubilized casein as substrates (14). Reactions were stopped by the addition of 0.3 M trichloracetic acid. After chilling and centrifugation (10,000 g, 20 min), the supernatant was analyzed for digestion products by a modified Folin procedure (15) using tyrosine as a standard. Activity is expressed as nmole min^{-1} mg^{-1} tyrosine solubilized. Tryptic esterase activity was measured by determining the rate of hydrolysis of p-tosyl-L-arginine methyl ester (TAME) (Mann Research Laboratories, Inc., New York), according to Hummel (16).

Collagenase activity was measured by the appearance of the chromophore dipeptide derived from the hydrolysis of a collagenase-specific acyl peptide (4 - phenylazobenzyloxycarbonyl - L - prolyl - L - leucyl-glycyl-L-prolyl-D-arginine, Mann Laboratories), according to Wünsch and Heidrich (17). Activity is

expressed as μmole min^{-1} mg^{-1} 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucine formed.

RIBONUCLEASE : The procedure described by Zimmerman and Sandeen (18) was used.

Labeling and Radioautographic Techniques

For glucosamine-^{3}H labeling, glands were trimmed of excess mesenchyme immediately after removal from the embryo and incubated in 1.0 ml nutrient medium containing 100 μCi/ml glucosamine-^{3}H. After 2 hr, the glands were washed five times for 5 min in 10 ml portions of cold Tyrode's solution. Epithelia were then isolated free of mesenchyme with various enzymes by the procedure described above, washed again in cold Tyrode's solution, and fixed in Carnoy's solution for radioautography. Epithelia subjected to two-stage enzyme treatment were labeled as described above, isolated in low concentrations of collagenase, and exposed to various enzymes before washing in cold Tyrode's solution and fixing. Glucosamine radioautograms were exposed for 14 days at 4°C before developing.

For thymidine-^{3}H labeling, glands trimmed of mesenchyme or cultures of epithelia combined with mesenchyme were incubated for 2 hr in 1.0 ml nutrient medium containing 20 μCi/ml thymidine-^{3}H. The tissues were washed by the procedure described above and fixed. Thymidine radioautograms were exposed for 3 days at 4°C. Other histologic and radioautographic techniques were as previously described (10).

Electron Microscopy

Ruthenium red staining and electron microscope methods were as described in the accompanying paper (10).

RESULTS

Submandibular salivary glands of the 13 1/4 day mouse embryo contain three to six lobes, which in vitro branch progressively and form multiple lobules with characteristic acini. These glands are not sufficiently differentiated to produce the mucins and sulfated glycoproteins characteristic of the mature gland (10) and are not developed to the extent that removal of mesenchyme is difficult. When epithelia isolated free of mesenchyme are recombined with fresh mesenchyme and placed in organ culture, the mesenchyme rapidly surrounds the epithelium, reconstituting the original relationship between the tissues. To demonstrate morphogenetically active materials at the epithelial-mesenchymal interface, epithelia isolated free of mesenchyme with various enzymes were grown
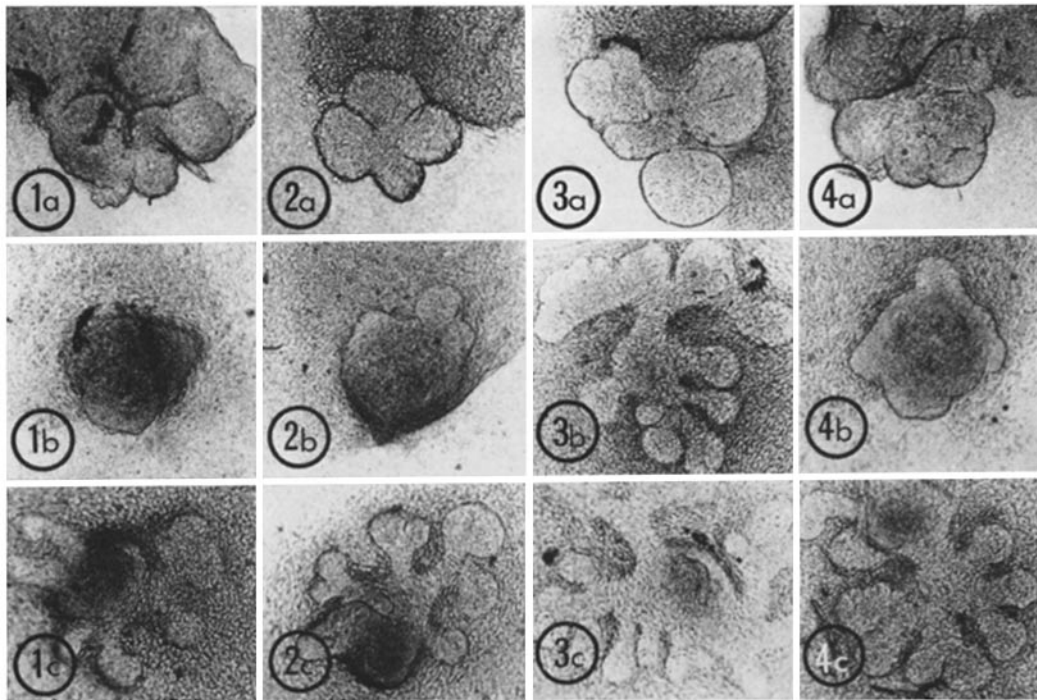
Figures 1–4 Living salivary epithelia isolated by various enzyme treatments and cultured in direct combination with fresh salivary mesenchyme. (a) At time of explantation, (b) after 24 hr in culture, and (c) after 48 hr in culture (× 50). Fig. 1: Isolated in crude trypsin-pancreatin (3.0 mg/ml). Fig. 2: Isolated in crystalline trypsin (0.01 mg/ml). Fig. 3: Isolated in collagenase 9EA (0.20 mg/ml). Fig. 4: Isolated in collagenase 9EA (0.60 mg/ml). Epithelia isolated with crude trypsin-pancreatin (Fig. 1), crystalline trypsin (Fig. 2), or high concentrations of collagenase (Fig. 4) lose their lobules and form ball-like rudiments, despite the continued presence of mesenchyme which surrounds the epithelia. At 24 hr, outgrowths have formed from these epithelia, and at 48 hr, these outgrowths have formed new lobules. Epithelia isolated with low concentrations of collagenase (Fig. 3) maintain their morphology and continue morphogenesis unabated.

in organ culture in direct combination with fresh mesenchyme. The capacity of these epithelia to maintain their morphology and to undergo morphogenesis was correlated with the presence of materials at the surface of the isolated epithelia.

Epithelial Morphogenesis after Removal of Mesenchyme

Attempts to isolate epithelia by flushing the glands in the presence of ethylenediaminetetra-acetate (EDTA) were unsuccessful, since concentrations of EDTA that completely removed mesenchymal cells also removed cells from the epithelium. Treatment of the glands with hyaluronidase over a wide range of concentrations did not remove the investing mesenchyme. Intact epithelia

could be isolated free of mesenchymal cells by flushing and teasing the glands in the presence of crude trypsin-pancreatin, crystalline trypsin, and purified collagenase (Table I, Figs. 1–4). During culture in direct combination with fresh mesenchyme, epithelia isolated in trypsin-pancreatin solutions (3–30 mg/ml) lost their characteristic shape (Figs. 1 a–c). The clefts between developing lobules disappeared, and within 16–20 hr the epithelium formed a spherical mass of tissue. With continued culture, the spherical epithelium produced budding outgrowths which underwent progressive branching. The distal ends of these branches ultimately become bulbous structures in which acini formed. Epithelia isolated with crystalline trypsin solutions (0.01–0.50 mg/ml) and cultured with fresh mesenchyme underwent similar changes; the

TABLE I

Epithelial Morphogenesis after Enzymatic Removal of Mesenchyme and Culture in Combination with Fresh Mesenchyme

Culture characteristic	Enzyme utilized for removal of mesenchyme			
	Crude trypsin/pancreatin	Crystalline trypsin	Collagenase	
			Low	High
Total No. cultures	41	45	79	44
24 hr				
Continued morphogenesis	0	0	67	0
Loss of lobular shape	41	45	12	44
48 hr				
Continued morphogenesis	34	41	64	35
Absence of morphogenesis	7	4	15	9

Enzyme	Concentration (mg/ml)	
	Low	High
Collagenase: 1SS	0.04	0.10
9EA	0.20	0.60
9BB	0.30	0.70
9BA	—	0.05–0.70
Crystalline trypsin	0.01	
Crude trypsin/pancreatin	3.0	

original morphology was not maintained, a spherical epithelium formed, and morphogenesis was delayed (Figs. 2 *a–c*).

When epithelia isolated by collagenase treatment were cultured in combination with fresh mesenchyme, the results varied with both the enzyme preparation and the enzyme concentration (Table I, Figs. 3 and 4). With three collagenase preparations (1SS, 9EA, and 9BB), concentrations were found (0.04, 0.20, and 0.30 mg/ml, respectively) that yielded epithelia which maintained their shape, retained the interlobular clefts, and continued morphogenesis (Figs. 3 *a–c*). Despite the presence of mesenchyme, epithelia isolated with higher concentrations of these preparations or any concentration tested of collagenase 9BA or 9EB (0.05–0.70 mg/ml) rapidly lost their lobular structure and formed a spherical mass (Figs. 4 *a–c*). At about 20–24 hr of culture, these "ball-like" epithelia began to produce outgrowths and branching morphogenesis resumed.

Epithelia isolated in high concentrations of collagenase lost their characteristic contour during culture more rapidly than those prepared in trypsin-pancreatin or crystalline trypsin. Although changes in epithelial morphology were generally not observed until 4–6 hr of culture, some epithelia prepared in high collagenase concentrations changed their shape within several minutes after isolation.

The epithelia isolated with low concentrations of collagenase were distinct from the other isolated epithelia in that a branched morphology was maintained in culture combined with mesenchyme. Moreover, these rudiments continued to undergo morphogenesis, whereas development of the other epithelia was delayed. This difference was not due to less nonspecific toxicity of the low collagenase concentrations, since glands subjected to the other enzymes, but not flushed to remove mesenchyme, underwent normal and equivalent morphogenesis (Table II). Regardless of the type or concentration of enzyme used to remove mesenchyme, all epithelia failed to develop when cultured in the absence of mesenchyme (Table II), suggesting that the difference between isolated epithelia was not due to a difference in their requirement for mesenchyme. Electron microscopy of epithelia

TABLE II

Epithelial Morphogenesis after Enzyme Treatment

Culture characteristic at 24 hr	Enzyme treatment of intact rudiment*			
	Crude trypsin/ pancreatin	Crystalline trypsin	Collagenase‡	
			Low	High*
Mesenchyme not removed				
Total No. cultures	8	8	8	9
Continued morphogenesis	8	8	8	9
Loss of lobular shape	0	0	0	0
Mesenchyme removed; isolated epithelia cultured				
Total No. cultures	4	4	7	7
Continued morphogenesis	0	0	0	0
Absence of morphogenesis	4	4	7	7

* Enzyme concentrations are as in Table I.
‡ Collagenase 9BB was used.

isolated by low collagenase treatment revealed extremely few residual mesenchymal cells at the epithelial surface. Additionally, no differences in the number of adhering mesenchymal cells were observed between epithelia isolated by the various enzyme treatments.

## Materials Associated with the Epithelial Surface after Removal of Mesenchyme

Retention of materials at the epithelial surface could be responsible for the maintenance of morphology of epithelia isolated in low concentrations of collagenase. Similarly, removal of such materials during isolation of epithelia in trypsin-pancreatin, crystalline trypsin, or high collagenase concentrations might be the cause of the loss of morphology. Since acid mucopolysaccharide is at the epithelial surface, the presence of acid mucopolysaccharide was investigated in epithelia isolated in an identical fashion as those used for the culture studies.

Intact glands were incubated with glucosamine-³H for 2 hr. Mesenchyme was removed by flushing in the presence of the various enzymes, and the isolated epithelia were examined by radioautography (Figs. 5–7). Epithelia isolated in low concentrations of collagenase showed substantial radioactivity adjacent to the epithelial surface of the distal ends of the lobules (Fig. 5). This surface-associated label was susceptible to

protease-free hyaluronidase digestion. Epithelia isolated in high concentrations of collagenase (Fig. 6) or in trypsin-pancreatin (Fig. 7) demonstrated considerably less glucosamine radioactivity at the epithelial surface, although incorporation was noted within the epithelium. Identical results were observed with epithelia isolated in crystalline trypsin. Thus, epithelia isolated with low concentrations of collagenase retain glucosamine-³H-labeled, hyaluronidase-susceptible materials at their surface, maintain branched morphology, and continue morphogenesis.

Epithelial Morphogenesis after Two-Stage Enzyme Treatment

To further characterize the materials at the epithelial surface involved in morphogenesis, epithelia isolated in low concentrations of collagenase were exposed (without flushing) to crystalline trypsin, protease-free hyaluronidase, sialidase, chondroitinase AC, chondroitinase ABC, and the enzyme diluent, CMFT. None of these treatments fragmented the isolated epithelia or were toxic as determined by darkening of the tissue during culture. The epithelia subjected to this two-stage enzyme treatment were cultured in direct combination with fresh mesenchyme (Table III, Figs. 8–11). Of the epithelia thus treated, only the rudiments exposed to CMFT and those exposed to sialidase (even at twice the indicated concentra-
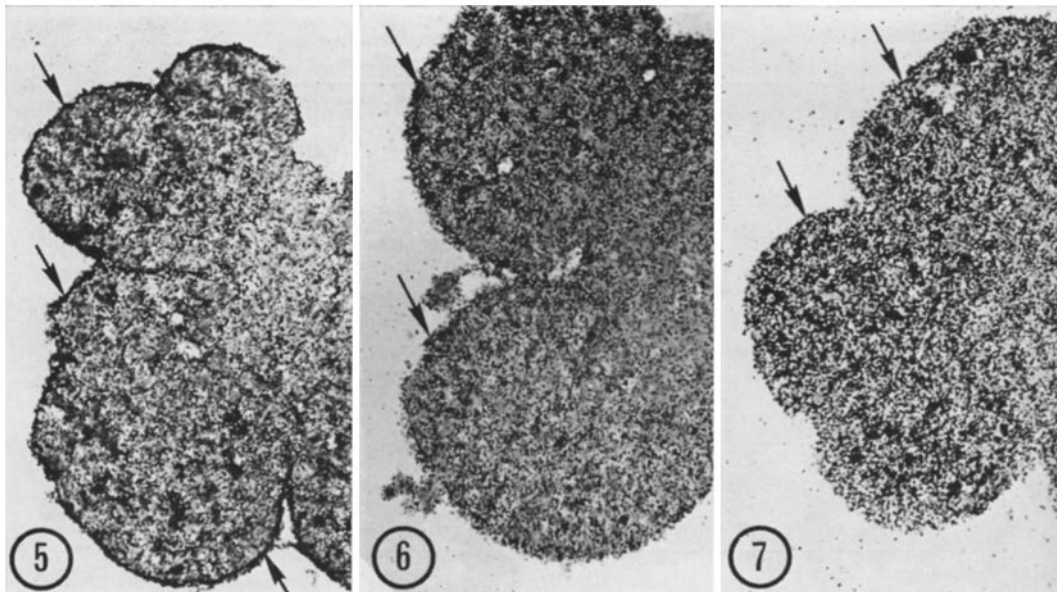
FIGURES 5-7 Radioautographs of salivary epithelia incubated as intact glands for 2 hr in glucosamine-^{3}H and then isolated free of mesenchyme with various enzymes ($\times$ 390). Fig. 5: Epithelium isolated with a low concentration of collagenase (collagenase 9EA, 0.2 mg/ml). Radioactivity is at the surface of the epithelium (arrows) and within the lobules. Fig. 6: Epithelium isolated with a high concentration of collagenase (collagenase 9EA, 0.6 mg/ml). Label is within the tissue, but there is substantially less radioactivity at the surface of the epithelium compared to Fig. 5. Fig. 7: Epithelium isolated with crude trypsin-pancreatin (3.0 mg/ml). The epithelial surface is essentially devoid of radioactivity, while label remains within the gland.

tion) maintained their original contour and continued morphogenesis, whereas epithelia exposed to the other enzymes lost their lobules. Depending on the degree of original lobulation of the epithelium, initial change of shape could be noted within 1–2 hr. With continued culture, the epithelia formed a spherical mass. Outgrowths from the ball-like structure began to form at 12–16 hr of culture, and branching morphogenesis from the outgrowths was generally not seen until after 24 hr of culture.

Materials at the Surface of Epithelia Exposed to a Second Enzyme

Intact glands were incubated with glucosamine-^{3}H for 2 hr, and then mesenchyme was removed with low concentrations of collagenase. The isolated epithelia were exposed to a second enzyme (or CMFT) in the same manner as for the culture studies and then fixed for radioautography. Glucosamine radioactivity remained at the epithelial surface in the rudiments exposed to CMFT

and in those exposed to sialidase (Fig. 12). However, very little radioactivity was present near the surface of the epithelia exposed to hyaluronidase (Fig. 13), crystalline trypsin (Fig. 14), and the chondroitinases (Fig. 15). Thus, there is a strong reciprocal relationship between the presence of glucosamine-^{3}H–labeled material associated with the epithelial surface, as indicated by radioautography, and the maintenance of lobular shape as well as the continuation of uninterrupted morphogenesis.

Materials at the Surface of Epithelia Cultured in Combination with Mesenchyme

The localization of surface-associated glucosamine radioactivity was examined in recombined cultures of epithelia undergoing uninterrupted morphogenesis and of epithelia that formed ball-like rudiments. Epithelia were isolated in low concentrations of collagenase, exposed to either CMFT or hyaluronidase, and then cultured in combination with fresh mesenchyme. At 14 hr of culture,

TABLE III

Epithelial Morphogenesis after Two-Stage Enzyme Treatment and Culture in Combination with Fresh Mesenchyme

Culture characteristic	Enzyme utilized for treatment of isolated epithelia					
	None	Hyaluronidase	Chondroitinase ABC	Chondroitinase AC	Crystalline trypsin	Sialidase
Total No. cultures	21	23	13	12	12	16
24 hr						
Continued morphogenesis	20	0	2	0	1	16
Loss of lobular shape	1	23	11	12	11	0
48 hr						
Continued morphogenesis	17	19	10	10	9	15
Absence of morphogenesis	4	4	3	2	3	1

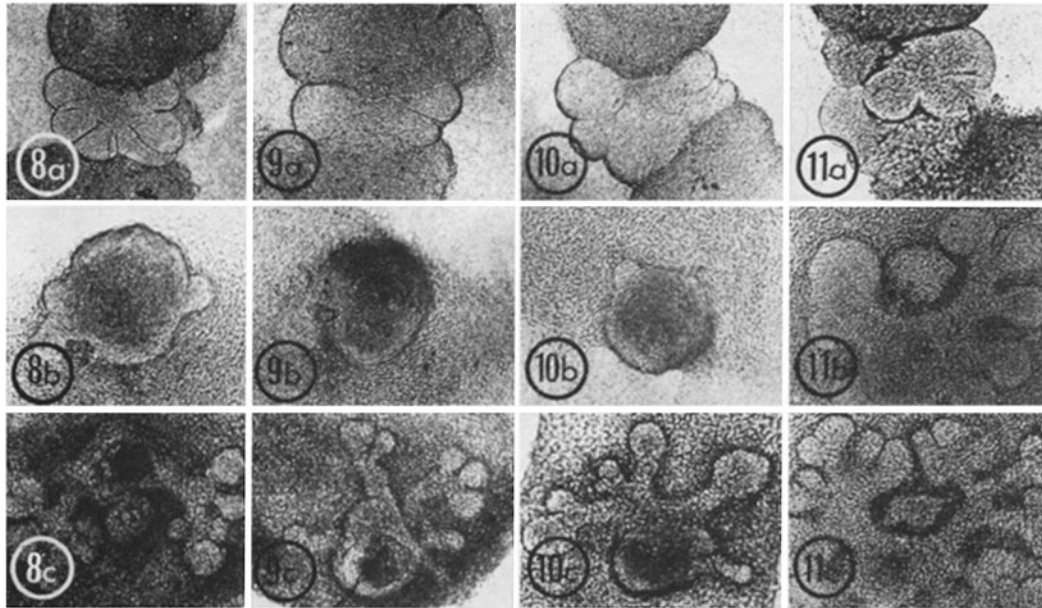| Enzyme	Concentration
	mg/ml
Stage 1: removal of mesenchyme	
Collagenase: 1SS	0.04
9EA	0.20
Stage 2: enzyme treatment of isolated epithelia	
Hyaluronidase	0.003
Chondroitinase ABC	0.05 units/ml
Chondroitinase AC	0.05 units/ml
Crystalline trypsin	0.001
Sialidase	0.03

the *low collagenase–treated epithelia* were lobulated while the *low collagenase plus hyaluronidase–treated epithelia* were nearly spherical and were beginning to produce outgrowths. The tissues were incubated with glucosamine-^{3}H from 14 to 16 hr of culture and then fixed for radioautography.

The sections of the recombined rudiments after 16 hr in culture illustrate the manner in which the original relationship between epithelium and mesenchyme was reconstituted during culture; the epithelium was completely enveloped by the mesenchyme. Epithelia retaining surface-associated materials during isolation (low collagenase–treated) continued morphogenesis and showed glucosamine radioactivity over the entire epithelial surface, with maximal accumulation of label at the surface of the lobules (Fig. 16). There was substantial incorporation within the lobules, and appreciably less radioactivity within the remainder of the epithelium. This distribution of glucosamine-^{3}H is similar to that observed in intact

glands in organ culture (10). Hyaluronidase treatment of such sections removed almost all of the surface-associated radioactivity.

Epithelia from which the surface-associated material was removed during isolation (low collagenase plus hyaluronidase–treated) became rounded masses of tissue. The glucosamine-^{3}H label at the surface of these ball-like structures was somewhat less, but was nearly uniformly distributed except for the surface of the budding outgrowths which showed a greater amount of label (Fig. 17). There was more glucosamine radioactivity within the outgrowth than within the remainder of the epithelium. Hyaluronidase treatment of such sections almost completely removed the surface-associated radioactivity. These studies suggest that accumulation of glucosamine-^{3}H at the surface of the ball-like epithelia precedes the formation of lobules and is maximal at the site where lobule formation and morphogenesis will resume.

FIGURES 8–11 Living salivary epithelia isolated with low concentrations of collagenase (collagenase 1SS, 0.04 mg/ml) and exposed to various enzymes before culturing in direct combination with fresh salivary mesenchyme ($\times$ 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.

Ruthenium Red Staining of Intact and Enzymatically Isolated Epithelia

Ruthenium red staining has been suggested to be an ultrastructural indicator of acid mucopolysaccharide (19). Intact epithelia, stained with ruthenium red, showed a uniform dense layer at the epithelial surface, coinciding with the basal lamina (Fig. 18). An appreciably thinner layer located at or over the plasma membrane was stained to a lesser degree. Fibrillar and amorphous materials external to the basal lamina were also stained, as were some materials within the intercellular spaces of the epithelium and mesenchyme.

The low collagenase–treated epithelia (Fig. 19) showed a densely stained basal lamina, similar in appearance to that of intact rudiments. However, the dye-positive materials external to the basal lamina were almost completely removed, whereas the materials within the intercellular spaces of the epithelium were retained. Treatment of such

epithelia with hyaluronidase (low collagenase plus hyaluronidase–treated) removed the basal lamina, but did not affect the dye-positive material associated with the plasma membrane or in the intercellular spaces of the epithelium (Fig. 20). Low collagenase plus hyaluronidase–treated epithelia showed occasional long slender evaginations of the plasma membranes of the surface epithelial cells. These ultrastructural observations of ruthenium red–staining material are analogous with the light microscope observations of glucosamine-^{3}H radioautograms and suggest that the materials responsible for the maintenance of morphology are within the basal lamina, and not within the associated fibrillar and amorphous material.

Cell Proliferation in Cultured Glands

The incorporation of thymidine-^{3}H during a 2 hr labeling period was used to assess sites of cell
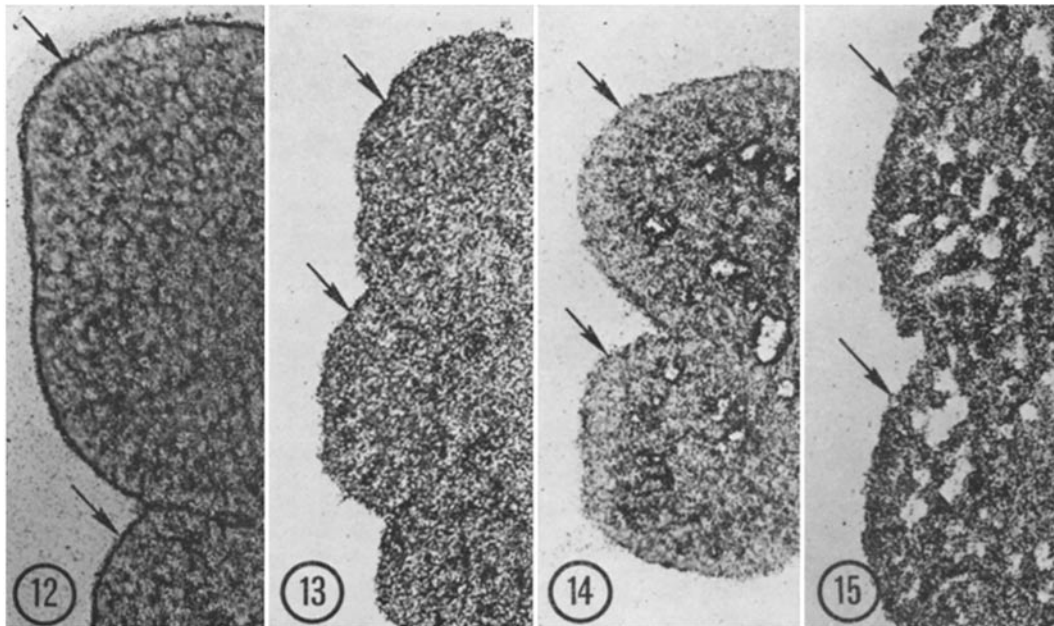
FIGURES 12–15 Radioautographs of salivary epithelia incubated as intact glands for 2 hr in glucosamine-^{3}H, isolated free of mesenchyme with a low concentration of collagenase (collagenase 9EA 0.2 mg/ml) and then exposed to various enzymes ($\times$ 390). Fig. 12: Exposed to clostridial sialidase (0.03 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).

proliferation in intact glands and recombined cultures. Intact rudiments were labeled immediately after explantation, and epithelia recombined with mesenchyme were labeled after 14 hr of culture. Intact glands demonstrated substantial incorporation of thymidine-^{3}H in the epithelium and meager incorporation in the mesenchyme (Fig. 21). Labeling in the epithelium was largely in the nuclei of cells at the epithelial surface, and these cells were predominantly within the lobules. This pattern of nuclear labeling was observed in sections at several levels of the glands, and the density of the labeled nuclei did not vary appreciably. It is unlikely that this labeling pattern is due to differential availability of thymidine-^{3}H to the central and peripheral epithelial cells, since a similar labeling pattern was not observed in the mesenchymal cells.

Low collagenase–treated epithelia cultured for 16 hr in combination with mesenchyme showed a labeling pattern similar to that observed in the

intact rudiments (Fig. 22). Dense labeling of nuclei was seen in the lobules, substantially less in the interlobular areas, and almost none in the center of the epithelium. During recombined culture, low collagenase plus hyaluronidase–treated epithelia showed labeled nuclei distributed nearly uniformly beneath the epithelial surface, and no focal areas of growth were seen (Fig. 23). In such epithelia which had formed outgrowths, thymidine incorporation was greater within the outgrowth. Again, very little label was seen within the mesenchymal cells.

These radioautograms indicate that cells proliferating at the greatest rate are within the lobules, and that in the absence of defined lobules, proliferation is not random, but is predominantly in the cortical region of the epithelium. The areas of most rapid cell proliferation are associated with the sites where mucopolysaccharide accumulates at the greatest rate.
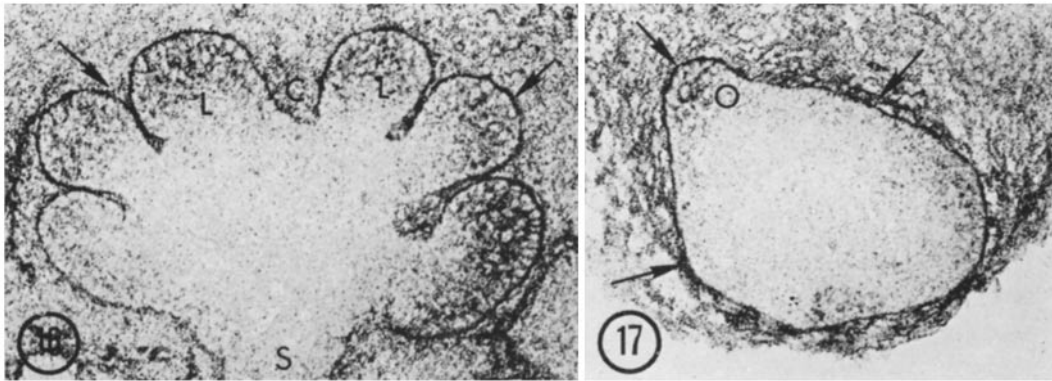
FIGURES 16 and 17   Glucosamine-³H radioautographs of epithelia cultured for 16 hr in direct combination with mesenchyme (× 189). Epithelia were isolated with a low concentration of collagenase (1SS, 0.04 mg/ml), exposed to either CMFT or to testicular hyaluronidase (0.003 mg/ml), and then cultured in combination with fresh mesenchyme. Tissue was labeled with glucosamine-³H from 14 to 16 hr of incubation and then fixed. Fig. 16: Isolated epithelium exposed to CMFT. After this treatment, the epithelium maintains normal morphology. Label is heavily distributed on the surface of the epithelium with maximal incorporation (arrows) at the surfaces of the lobules (L). Reduced incorporation is seen at the surfaces of the clefts (C) and the stalk (S). Within the epithelium, label is concentrated within the lobules. Fig. 17: Isolated epithelium exposed to testicular hyaluronidase. After hyaluronidase treatment, the epithelium becomes ball-like. Label is seen evenly distributed over the surface of the epithelium (arrows), being slightly more concentrated at a point where an outgrowth is starting to form (O). Label is also concentrated within the outgrowth.

Degradative Activities of Enzymes

The enzymes used for the removal of mesenchyme from epithelia, for the treatment of isolated epithelia and for the treatment of radioautographic sections were examined for contaminating activities (Table IV). Tryptic esterase activity was not demonstrated in the collagenase preparations, confirming the results of Grobstein and Cohen (5), and Wessells and Cohen (6). However, substantial noncollagen proteolytic activity was detected in the commercial preparations of collagenase (9EA, 9BB, 9BA). All of the collagenase preparations revealed appreciable mucopolysaccharidase activity, whether measured by degradation of mucopolysaccharide (turbidometric assay) or formation of unsaturated disaccharides (β-elimination assay). The mucopolysaccharidase activities of these preparations were not proportional to their collagenase activities.

Crystalline trypsin was devoid of mucopolysaccharidase and collagenase activities, but contained measurable ribonuclease activity. The preparations of testicular hyaluronidase and of the bacterial mucopolysaccharidases, chondroitinase ABC, and chondroitinase AC, revealed no detectable

protease, collagenase, or ribonuclease activity. The sialidase preparation demonstrated minimal protease activity and was free of detectable mucopolysaccharidase and collagenase activity.

DISCUSSION

The concept that extracellular materials are involved in the morphogenetic process is supported by substantial circumstantial evidence, but essential morphogenetically active extracellular substances have not been previously identified. The present report establishes that materials within the epithelial basal lamina which are, at least in part, acid mucopolysaccharide–protein complexes are required for the maintenance of lobulated morphology, continuation of morphogenesis, and the formation of new lobules.

Localization and Characterization of the Morphogenetically Active Material

The epithelial-mesenchymal junction of intact glands demonstrates acid mucopolysaccharide by staining and radioautographic procedures (10), and ultrastructurally shows a well-defined epithelial basement membrane consisting of a narrow
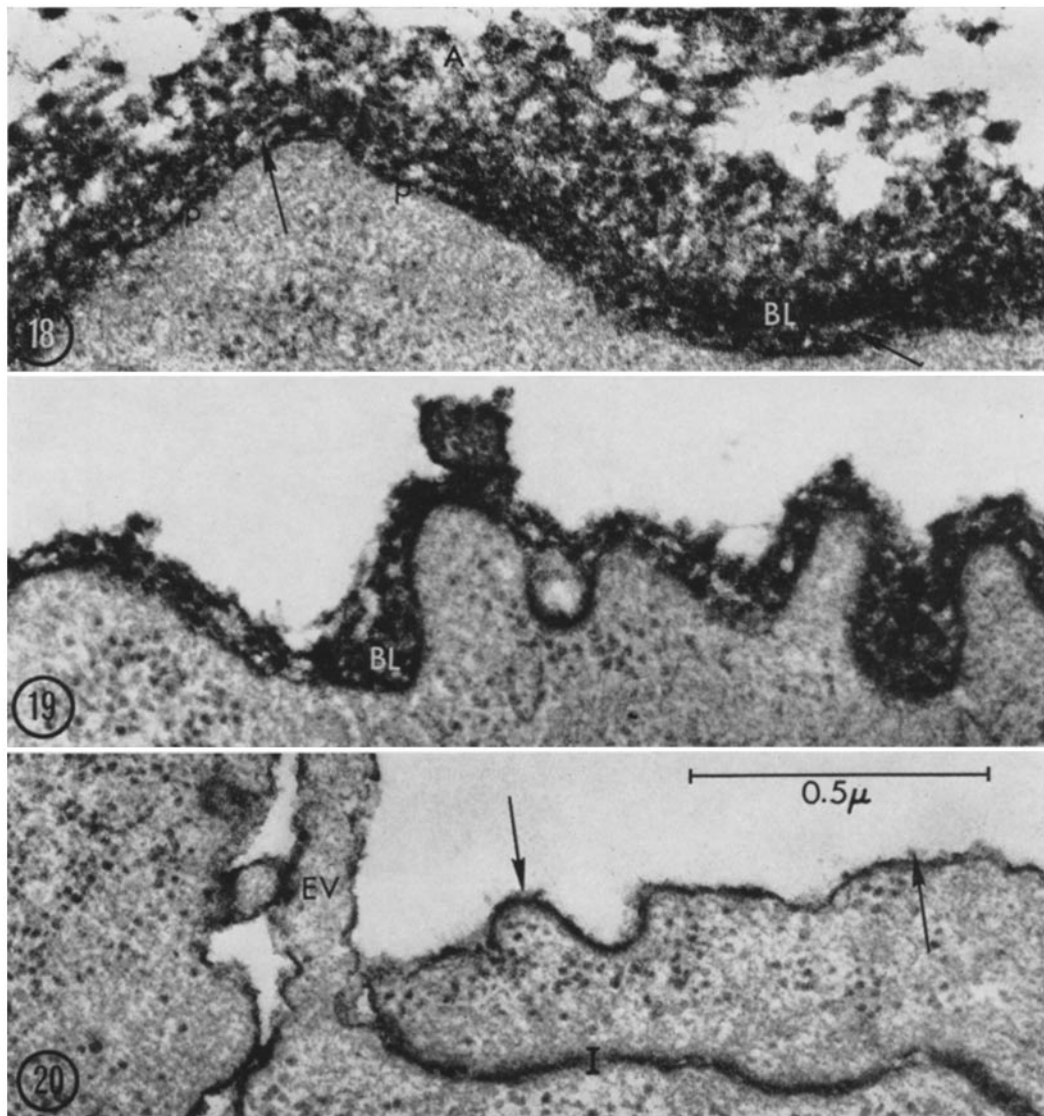
FIGURES 18–20 Electron micrographs of typical views near the epithelial surface of an intact gland and of isolated epithelia stained with ruthenium red in combination with osmium tetroxide (× 78,000). Fig. 18: Intact gland. The dye intensely stains the basal lamina (BL) and the plasmalemma (p). Amorphous material (A) external to the basal lamina stains to a lesser degree. Material between the plasmalemma and the basal lamina (arrows) also shows less stain than the basal lamina. Fig. 19: Epithelium after isolation with low concentrations of collagenase (1SS, 0.04 mg/ml). The ruthenium red–staining amorphous material external to the basal lamina seen in Fig. 18 is removed by this treatment. The basal lamina (BL) remains intact and maintains its ruthenium red–staining properties. Fig. 20: Epithelium isolated as in Fig. 19, and then exposed to testicular hyaluronidase (0.003 mg/ml). The amorphous material external to the basal lamina and the basal lamina are removed by this treatment. A small amount of dye-staining material remains adhered to the plasmalemma of the epithelial surface (arrows). The intercellular spaces (I) retain their ability to stain as seen in normal salivary rudiments stained with ruthenium red (see reference 10). Long slender evaginations (EV) are frequently seen at the epithelial surface after hyaluronidase treatment.
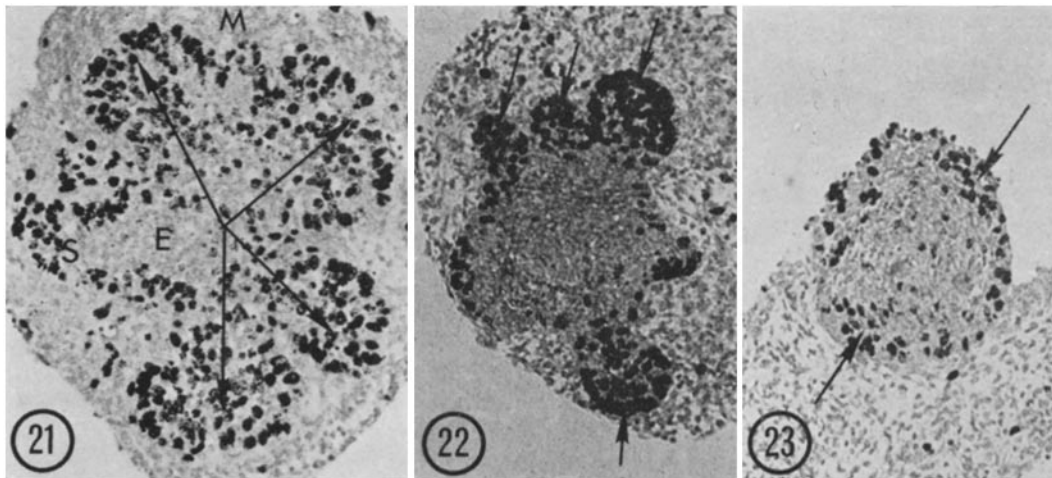
FIGURES 21–23 Thymidine-³H radioautographs of an intact gland at time of explantation and of epithelia cultured for 16 hr in direct combination with mesenchyme (× 140). Fig. 21: Intact glands labeled for 2 hr with thymidine-³H immediately after explantation. Labeled nuclei are seen primarily within lobules (arrows), with some labeled nuclei also present within the stalk (S) of the epithelium (E). Almost no label is seen in the mesenchyme (M). Figs. 22 and 23: Epithelia isolated with a low concentration of collagenase (9EA, 0.2 mg/ml), exposed to either CMFT (Fig. 22) or to testicular hyaluronidase (Fig. 23) and cultured in combination with fresh mesenchyme. Tissue labeled with thymidine-³H from 14 to 16 hr of incubation and then fixed. Figg. 22. Labeled nuclei are seen primarily within lobules (arrows), and substantially less label is present in the interlobular areas and the center of the epithelium, in a fashion similar to Fig. 21, indicating that morphogenesis is progressing in a normal manner. Fig. 23: This ball-like epithelium has labeled nuclei nearly uniformly distributed near its surface (arrows), with no focal areas of growth.

electron-lucent zone, a dense basal lamina, and variable amounts of fibrillar and amorphous materials (21). Epithelia isolated with low collagenase concentrations retain the bulk of surface-associated mucopolysaccharide and a distinct basal lamina, but only sparse patches of the associated materials. The surface mucopolysaccharide and the basal lamina are nearly completely removed by hyaluronidase treatment of such epithelia, but during culture combined with mesenchyme, surface-associated mucopolysaccharide reaccumulates and a nearly complete basal lamina is again seen (21). These studies, together with the results of ruthenium red staining, indicate that the morphogenetically active mucopolysaccharide resides within the epithelial basal lamina and not to a significant degree within the associated fibrillar or amorphous materials.

Despite the fact that acid mucopolysaccharides are generally absent from basement membranes of adult epithelia, these basement membranes are similar to the embryonic salivary epithelial basement membrane in ultrastructural appearance and certain staining properties (19). The nature of the

basement membrane may change during development; for example, ³⁵SO₄ incorporation into chick limb ectodermal basement membranes is limited to early developmental stages (22), and the relative amounts of glucosamine-³H and ³⁵SO₄ incorporated into embryonic salivary basement membrane vary with in vitro morphogenesis (10). These considerations suggest that mucopolysaccharide may be present in epithelial basement membranes only during periods when it plays a role in organogenesis.

Conclusions derived from studies of enzymatically treated tissues are heavily dependent upon the purity of the enzymes. Although the mucopolysaccharidase preparations revealed no detectable protease, collagenase, or ribonuclease activities, the possibility that another contaminating activity (e.g., any of several sulfatases, lipases, glycosidases, or a collagenase incapable of attacking the chromophore substrate) contributes to the loss of surface mucopolysaccharide or the interruption of morphogenesis cannot be rigorously excluded. This is an unlikely possibility since three distinct mucopolysaccharidases, which would be expected

TABLE IV

Degradative Activities of Enzymes*

Enzyme	Protease $\dfrac{nmole\ddagger}{min\ mg}$	Protease $\dfrac{nmole\S}{min\ mg}$	Esterase $\dfrac{\Delta A_{247}}{min\ mg}$	Mucopolysaccharidase Turbidometric $\dfrac{\Delta A_{600} \times 10^3}{min\ mg}$	Mucopolysaccharidase β-Elimination $\dfrac{\Delta A_{235} \times 10^2}{min\ mg}$	Collagenase $\dfrac{\mu mole}{min\ mg}$	Ribonuclease $\dfrac{\Delta A_{262}}{min\ mg}$
Collagenase: 1SS	0.0	—	—	41.6	28.5	2.2	0.00
9EA	16.3	22	<0.001	12.9	9.41	1.4	0.00
9BB	11.7	—	<0.001	14.7	7.98	3.3	0.00
9BA	75.7	—	<0.001	43.6	29.4	3.2	0.00
Crystalline trypsin	610	3120	150	0.0	—	0.000	0.038
Crude trypsin/pancreatin	108	534	0.67	106	—	0.0022	3.68
Hyaluronidase	0.0	0	—	194	—	0.000	0.00
Chondroitinase ABC‖	0.0	0	—	53.0	32.1	0.000	0.00
Chondroitinase AC‖	0.0	0	—	125	85.7	<0.001	0.00
Clostridial sialidase	14.9	—	—	0.0	0.0	0.000	0.00

* Protease, tryptic esterase, mucopolysaccharidase, collagenase, and ribonuclease assays of the enzymes used for isolation of epithelia and for treatment of isolated epithelia. Assays were performed as described in Materials and Methods

‡ Denatured hemoglobin as substrate

§ Casein as substrate

‖ Activities for the chondroitinases are calculated per unit enzyme (20)

to contain different contaminating activities, gave identical results. All of the collagenase preparations demonstrated appreciable mucopolysaccharidase activity, and with the exception of one preparation, showed nonspecific protease activity. Prior studies of developing epithelia treated with collagenase (5, 6) suggested that morphogenesis of salivary, lung, and ureteric bud epithelium is dependent upon collagenase-susceptible materials, presumably collagen. The basal lamina and adjacent materials at the surface of these collagenase-treated epithelia were disrupted and mostly removed, and the enzymes used were commercial preparations, undoubtedly containing mucopolysaccharidase and/or protease activity. These contaminating activities, together with the presence of acid mucopolysaccharide in the basal lamina, make it clear that *the dependence of morphogenesis on collagenase-susceptible materials does not necessarily implicate collagen.*

Most animal mucopolysaccharides occur as protein complexes (23), and the removal of surface radioactivity and the interruption of morphogenesis by trypsin suggest that the acid mucopolysaccharide in the basal lamina is linked to protein. Trypsin treatment of cells is known to solubilize

sialic acid (24), but the lack of a sialidase effect on either morphogenesis or surface radioactivity implies that sialic acid–containing glycoproteins are not involved. Since acid mucopolysaccharides show considerable molecular heterogeneity (23) and the substrate specificities of the mucopolysaccharidases used overlap, the nature of the surface mucopolysaccharide cannot be clearly discerned. However, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin do not appear to be involved, since these mucopolysaccharides are resistant to both testicular hyaluronidase (25) and chondroitinase AC (20). Therefore, the possibilities include the nonsulfated mucopolysaccharides (chondroitin and hyaluronic acid) and the chondroitin sulfates (chondroitin-4-sulfate and chondroitin-6-sulfate).

Acid Mucopolysaccharide–Protein Complexes in Morphogenesis

In the accompanying paper (10), a morphogenetic mechanism was proposed based upon the distribution of total and newly synthesized mucopolysaccharide as well as that of collagen fibers. In this scheme, mucopolysaccharide-protein

complexes are involved in the initiation and localization of collagen fibrogenesis. The resultant collagen fibers accumulate within newly formed clefts as they deepen in association with growth of the lobules adjacent to the new cleft, acting to stabilize the epithelial morphology. The proposal predicts that branching morphogenesis is dependent upon the presence of surface-associated mucopolysaccharide and that mucopolysaccharide accumulates most rapidly at the areas growing at the greatest rate.

During branching morphogenesis, the cells showing the greatest thymidine incorporation (presumably proliferating at the greatest rate) are within the lobules, the surfaces of which are the sites of most rapid mucopolysaccharide accumulation. In ball-like epithelia, the proliferating cells are distributed in a nearly uniform zone beneath the epithelial surface, and newly synthesized mucopolysaccharide accumulates over the surface in equivalent amounts. Cells within the budding outgrowths from ball-like epithelia proliferate at a greater rate and the surface of the outgrowth accumulates greater amounts of newly synthesized mucopolysaccharide. Thus, the areas showing the greatest rate of surface mucopolysaccharide accumulation *are* associated with the sites of most rapid cell proliferation. It is not known whether rapidly proliferating cells accumulate or synthesize more of the surface material or whether the surface material influences the adjacent epithelial cells. It is apparent, however, that an increased rate of localized cell proliferation accompanies the formation of lobules.

It is difficult to account for all the changes in morphology associated with loss and reaccumulation of surface mucopolysaccharide on the basis of the proposed role of this material in collagen fibrogenesis. These changes may result, in part, from the action of intracellular microfilaments, which in embryonic salivary epithelia are in fibrillar-like bundles beneath the plasma membrane of virtually every cell (26, 27). Contractility of the microfilaments has been suggested to cause cells to change shape (26), and in developing salivary glands, to initiate the formation of clefts (21, 26). Clefts are lost after removal of surface mucopolysaccharide and reappear coincident with the accumulation of newly synthesized material at specific sites.

The factors determining where and when microfilaments act are almost wholly unknown. Evidence suggesting that cellular contractility is dependent upon Ca^{++} has been obtained by Gingell (28) and extended to involve microfilaments by Ash and Wessells (27 and personal communication). Of the polyvalent cations with high affinity for mucopolysaccharide (29), only Ca^{++} is present in substantial concentrations extracellularly, the concentration being at least 10^2 higher than the intracellular concentration (30). Surface-associated mucopolysaccharide may be involved in regulating Ca^{++} access to the plasma membrane and/or the cell interior, and thus be involved in promoting cleft formation. Loss of surface mucopolysaccharide would result in removal of the materials controlling local or compartmentalized Ca^{++} availability. Epithelia freed of surface mucopolysaccharide lose their clefts and, during reculture, slowly form a ball-like structure. Newly synthesized mucopolysaccharide reaccumulates nonspecifically during reculture, and this distribution may induce nonspecific, uniform contraction of the microfilaments, resulting in the formation of a spherical epithelium. Removal of surface mucopolysaccharide does not alter microfilament structure, and microfilament bundles are present in ball-like epithelia (21) but their state of contractility or organization is not known.

Although the precise way in which the surface-associated material influences morphogenetic cell behavior remains to be determined, characteristic salivary epithelial morphology and branching morphogenesis require the presence of mucopolysaccharide (probably as mucopolysaccharide-protein complexes) within the epithelial basal lamina. Any mechanism proposed for the molecular basis of this requirement must account for the selective distribution of newly synthesized mucopolysaccharide, and for its capacity to maintain normal morphology as well as its apparent ability to promote the formation of lobules.

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