and therefore of the genome, in the control of antibody specificity, depends on the availability of RNA with high template activity, devoid of significant protein contamination and obtained in an undegraded form. The characteristics of RNA from fraction III seem to fulfill these requirements.

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13 August 1965

Collagenase: Effect on the Morphogenesis of Embryonic Salivary Epithelium in vitro

Abstract. *Salivary epithelium in culture, under conditions which promote morphogenetic branching, grows as a simple disc in the presence of collagenase, or is "depatterned" midway in the morphogenetic course by a short exposure to a collagenase.*

The characteristic morphogenesis of salivary epithelium of mouse embryos in vitro is dependent upon closely associated salivary mesenchyme, either in direct combination or across a membrane filter (*1*). In the trans-filter situation collagen fibers appear at the epithelial surface, and evidence has been presented which is in conformity with the hypothesis that the collagen is synthesized by the mesenchyme and polymerized on the epithelial side after crossing the filter in soluble form (*2*). The question arises whether collagen at epithelial surfaces has morphogenetic significance. The question is fortified by the observation that collagen fibers of characteristic periodicity are particularly abundant in association with the surface of the morphogenetically inactive stalk of the 13-day rudiment; they are much less abundant in the bulb region and seem to be absent at the ends of morphogenetically active adenomeres (*3*). Further, autoradiographically demonstrable label, introduced into the mesenchyme as tritiated proline and conforming in distribution to ultrastructurally identifiable collagen, is removed by collagenase (*2*). Experiments were performed, therefore, to test whether collagenase treatment would affect the morphogenetic pattern of the epithelium.

Two kinds of experiments were carried out: (i) continuous treatment by inclusion of collagenase in the medium; (ii) treatment for a short period during morphogenesis, followed by subsequent culturing in the absence of collagenase. Both procedures gave results suggesting that a collagenase-sensitive material, presumably collagen, plays a role in salivary morphogenesis.

The procedure for obtaining salivary rudiments from 13-day mouse embryos, and for separating their epithelial and mesenchymal components by means of trypsin, has been described (*4*). The epithelia were clotted in the cup of a membrane filter assembly (*5*), and a piece of salivary capsular mesenchyme was placed immediately opposite on the upper or platform side of the filter. The whole assembly was in contact with a subjacent drop of nutrient medium (Eagle's plus 10 percent horse serum and 10 percent chick embryo juice) in the well of a culture dish (*5*). The cultures were incubated at 38°C in a 5 percent CO_2-gassed, high-humidity incubator.

Three collagenase preparations were used: (i) Worthington crude ("CLS"); (ii) Worthington crystalline A (CLSP-A, monomer); (iii) Worthington crystalline B (CLSP-B, dimer). In the continuous exposure experiments crude collagenase was incorporated into the medium of standard cultures, including mesenchyme, at concentrations ranging from 0.006 to 0.4 mg/ml. The highest concentration proved toxic as judged by darkening and fragmentation of the epithelium; the lowest was weakly active in affecting morphogenesis. Intermediate concentrations in the range of 0.025 to 0.1 mg/ml were most effective. Crys-

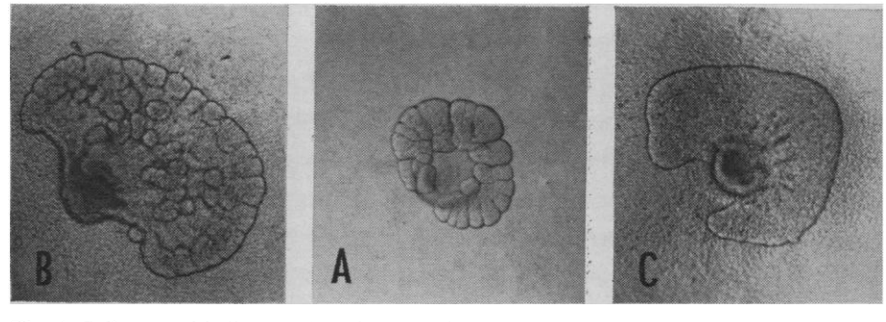

Fig. 1. Salivary epithelium undergoing morphogenesis in vitro. (*A*, center) At 48 hours, untreated; (*B*) at 72 hours, treated with Tyrode's solution at 48 hours; (*C*) at 72 hours, treated with collagenase at 48 hours.

talline collagenase at 0.002 mg/ml was about equally effective. For short-term exposures the mesenchyme was removed from the upper surface of the filter, and the assembly with the clotted epithelium was transferred to 0.5 mg/ml collagenase in Tyrode's solution preincubated at 38°C. Exposure at this temperature for 20 minutes proved effective. The assembly was washed through one change of medium, and fresh mesenchyme was added to its upper surface.

Hyaluronidase (Sigma Chemical Co., bovine type III, salt-free, purified; Worthington, bovine, chromatographically purified, "HSE-P") was tested at 1.0 mg/ml in Tyrode's solution for 20 minutes at 38°C and continuously at 0.028 to 0.44 mg/ml. Elastase (Worthington, electrophoretically purified, "ESFF") and lipase (Worthington, pancreatic I, PLI, lyophilized) were tested at 0.5 mg/ml in Tyrode's solution for 20 minutes at 38°C.

The cultures were photographed immediately before enzyme treatment, and 18 to 24 and 40 to 48 hours later. Tracings of the several sequential photographs were superimposed and compared to determine morphogenetic effects.

At 40 to 48 hours of culture the epithelium of untreated cultures has undergone considerable branching to form from 6 to 20 adenomeres (Fig. 1*A*). Exposure to collagenase has no immediate effect on the appearance of the epithelium. Twenty-four hours later, however, the epithelium not only has failed to continue its branching behavior [controls treated with Tyrode's solution (Fig. 1*B*) do continue branching], but has been "depatterned" to a flat, smoothly contoured mass (Fig. 1*C*). This occurs despite the fact that the epithelium appears healthy and continues to increase in area (Fig. 2*B*, compare 48 and 72 hours). Moreover, on continued culturing in the presence of mesenchyme the epithelium resumes its normal morphogenetic behavior, the border gradually scalloping and forming adenomeres (Fig. 2*B*, 96 hours). The results described were obtained in repeated experiments, in which a total of 29 of 31 cultures treated with crude collagenase, and 14 of 14 treated with crystalline collagenase, showed depatterning. As controls, 27 of 29 cultures treated with Tyrode's solution underwent continued normal morphogenesis.

The activity is believed to be enzymatic and at least primarily collagenolytic on the following grounds. Dialysis of the crude collagenase against Tyrode's solution for 24 hours failed to alter the activity, but heating for 15 minutes at 60°C significantly reduced the activity, and heating to 95°C for 5 minutes eliminated it. No significant tryptic or chymotryptic activity could be detected (Worthington "Dermatube") in the crude collagenase. Purified collagenase gave qualitatively identical results at concentrations an order of magnitude lower than crude collagenase. Calcium- and magnesium-free Tyrode's solution, ethyldiaminetetraacetate (0.5 mg/ml in calcium- and magnesium-free Tyrode's solution), hyaluronidase, lipase, and elastase failed to give comparable results, though hyaluronidase treatment yielded somewhat flattened adenomeres with more rounded contours.

Continuous treatment with collagenase from the beginning of culturing (47 cultures) gave results similar to those obtained after exposure at 48 hours, in that the epithelium expanded as a flat disc without any evidence of peripheral scalloping or adenomere formation (32 cases), or spread as a thin sheet (ten cases). The condition persisted to 96 hours if collagenase was maintained in the medium. When collagenase was withdrawn at 48 hours, however, recovery occurred in the flat discs (but not in spread sheets) by 96 hours in the same fashion as in cultures briefly treated at 48 hours. Continuous exposure to hyaluronidase (six cases) failed to give comparable results.

In general, the factors governing morphogenesis of the kind described are poorly understood. It is likely that important among them are cell-to-cell and cell-to-substrate interrelationships, since these must underlie integrated behavior at the multicellular level. It is not surprising, therefore, that collagenase should affect the morphogenetic pattern, especially since the enzyme is known to be effective in dissociating some tissues to cells (*6*). Nonetheless, the mechanism of the effect, either in dissociating tissue or in altering morphogenesis, is not clear.

The only known action of purified collagenase is the splitting of collagen to peptides (*7*). The assumption that it is acting in this fashion in the present instance suggests that collagen plays some role in at least maintaining, and possibly in initiating, the indentations of epithelial contour which

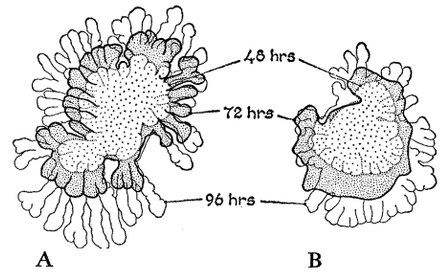

Fig. 2. Superimposed contours of salivary epithelium undergoing morphogenesis in vitro, at 48, 72, and 96 hours. (*A*) Treated with Tyrode's solution at 48 hours; (*B*) treated with collagenase at 48 hours.

characterize not only salivary morphogenesis but the development of many other glandular rudiments. The fact that ultrastructurally identifiable collagen fibers are present in the indentations, as well as more abundantly in the vicinity of the morphogenetically quiescent stalk (*3*), raises the possibility that the collagen is a stabilizing component "jacketing" certain regions, whereas others remain unjacketed and morphogenetically active (Fig. 3). On the further assumption that the collagen is provided in soluble form by the mesenchyme and becomes fibrous in the presence of an epithelial polymerizer (*2*), it would be expected that morphogenetically active and inactive epithelial surfaces might have different amounts or kinds of polymerizer. It is further to be considered that localized collagenolytic activity by the epithelium may be involved (*8*).

It is important to note that attributing a morphogenetic role to collagen does not suggest that it is the active agent in the specific effect of salivary mesenchyme on salivary epithelium (*4*). Nonsalivary mesenchyme labeled with tritiated amino acids

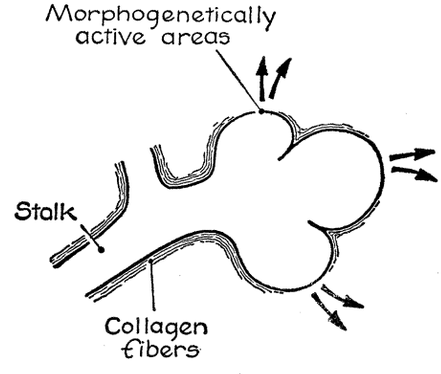

Fig. 3. Possible relation of collagen distribution to morphogenetically active and inactive areas.

transfers large-molecular label to the epithelial surface where ultrastructurally visible collagen occurs (*4*), and soluble tropocollagen does not replace salivary mesenchyme in its morphogenetic effect (*9*). These facts suggest that while the developmental influence of salivary mesenchyme on salivary epithelium may include the provision of soluble collagen, it involves additional components as well.

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Protein Synthesis in Rat Liver: Influence of Amino Acids in Diet on Microsomes and Polysomes

Abstract. *Rats were killed 1 hour after having been fed by stomach tube with amino acid mixtures that were either nutritionally complete or lacked tryptophan. Microsomes isolated from the livers of animals fed the incomplete mixture showed a reduced capacity to incorporate ^{14}C-leucine in vitro, and polysomes prepared from the same livers showed an increased proportion of monosomes and disomes. Prior treatment with actinomycin D did not prevent these differences in response to the two amino acid mixtures, an indication that synthesis of messenger RNA may not be involved in the cell mechanism which recognizes differences in amino acid pattern.*

The protein content of the liver changes rapidly when the intake of dietary protein is varied (*1*). The amounts of RNA and phospholipid in the liver cell vary with protein content of the cell (*2*), and structural changes have also been demonstrated in the nucleus (*3*) and in the endoplasmic reticulum (*4*). Consequently, these observations point to a complex regulation of protein synthesis in liver in relation to the supply of amino acids.

Clark, Naismith, and Munro (*5*) showed that there are considerable differences in uptake of precursors into liver RNA within a few hours of feeding protein to fasting rats. In later experiments the effect of feeding an amino acid mixture on labeling of liver RNA depended on the nutritional properties of the amino acids in the mixture; deletion of tryptophan from the mixture led to reduced incorporation of ^{14}C-orotic acid into the pyrimidine bases and of ^{14}C-glycine into the purine bases (*6*). The most likely explanation for these observations is that the absorption of a complete mixture of amino acids stimulates synthesis of messenger RNA (mRNA), whereas the incomplete mixture fails to do so. Consequently, the formation of polysomes in the cytoplasm may be favored by the additional messenger, and this should be reflected in greater protein synthesizing activity of the microsome fraction in the livers of such animals. We have accordingly fed rats with an amino acid mixture that was either nutritionally complete or was deficient in tryptophan. The microsomes prepared from the livers of each group of animals were tested for their capacity to incorporate labeled amino acids, and the pattern of polysomes in the microsomes was also examined.

Male albino rats (150 g) were fasted overnight. Groups of rats were fed by stomach tube with mixtures of amino acids (*6*) in which the complete mixture provided all ten essential amino acids, and also cystine, tyrosine, aspartic acid, glutamic acid, proline, and alanine. One group of rats received the nutritionally complete mixture, whereas a second group were given the mixture with tryptophan omitted. The complete mixture of amino acids, administered in 3 ml of water to each rat, consisted of the L-isomers of valine, 100 mg; leucine, 120 mg; isoleucine, 80 mg; cystine, 20 mg; methionine, 80 mg; threonine, 70 mg; phenylalanine, 120 mg; tyrosine, 60 mg; lysine (HCl), 25 mg; histidine (HCl), 95 mg; arginine (HCl), 50 mg; alanine, 20 mg; proline, 20 mg; aspartic acid, 20 mg; glutamic acid, 200 mg; and tryptophan, 50 mg; together with $NaHCO_3$, 127 mg. One hour after feeding, the animals were stunned, and the livers were removed and homogenized in two volumes of medium A (*7*). Cell debris, nuclei, and mitochondria were precipitated and removed by centrifugation at 19,000*g* for 10 minutes; the remaining supernatant was divided into two portions. The first was used for studies of amino acid incorporation into microsomes, and the second for preparation of polysomes.

Microsomes were prepared from the first portion of this supernatant by centrifugation at 135,000*g* for 70 minutes. The final supernatant, after removal of the microsomes, was recovered from the homogenate from both groups and mixed to provide the cell-sap fraction used for incorporation studies. The microsome pellets were suspended in 0.25*M* sucrose buffer and recovered by centrifugation. The new pellet was suspended in a small volume of sucrose buffer, and heavy aggregated particles were removed by centrifugation at 850*g* for 10 minutes. The microsomes remaining in suspension were used for the study of amino acid incorporation in vitro. Samples of the microsomes containing 2 mg protein were incubated at 37°C with cell sap (7.5 mg protein) which had been passed through Sephadex G25 to remove inhibitors (*8*). The incubation medium consisted of 0.25*M* sucrose-tris buffer at *p*H 7.6 and contained 2 μmole adenosine triphosphate, 0.5 μmole guanidine triphosphate, 14.8 μmole creatine phosphate, 20 μg creatine phosphokinase, and 1 μc ^{14}C-DL-leucine. At the end of the incubation, the tubes were chilled and treated with an equal volume of cold 0.6*N* $HClO_4$ containing 1 mg of ^{12}C-DL-leucine. After two washings with 0.3*N* $HClO_4$, the RNA was hydrolyzed by incubation in 0.3*N* KOH for 1 hour at 37°C. The protein was then recovered quantitatively by acidification of the digest, separated by centrifugation, and redissolved in alkali for measurement of radioactivity in a gas-flow counter. The results were calculated as counts per minute per milligram of microsome protein in the sample.

In order to obtain polysomes, the second portion of the supernatant fraction (from which mitochondria had been removed) was treated with deoxycholate to a final concentration of 1 percent, and C-ribosomes (polysomes) were prepared by the procedure of Wettstein *et al.* (*9*). The deoxycholate-treated fraction (2.3 ml) was layered on to an equal volume of 0.5*M* sucrose in medium-A buffer, which in turn had been layered over the same volume of 2.0*M* sucrose in the same buffer. This