

INTERFERENCE WITH THYROID HISTOGENESIS BY INHIBITORS OF COLLAGEN SYNTHESIS

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ABSTRACT

Histogenesis of thyroid follicles in the chick embryo begins with a penetration by cells of the mesenchymal capsule into a solid epithelial primordium. Before penetration occurs, slits containing fibrillar material form between the epithelial cells. The fibrillar material is an epithelial cell product as shown by its formation within channels that form in cultures of isolated epithelial primordia. The drugs L-azetidine-2-carboxylic acid (LACA) and α, α' -dipyridyl, which interfere with collagen synthesis, prevent the formation of fibrils in cultured epithelial primordia and in cultures of whole thyroids. Furthermore, mesenchymal cells do not invade when whole thyroid primordia are cultured in the presence of either drug. The effects of α, α' -dipyridyl are reversed by washing out the drug; the effects of LACA are reversed by incubation with equimolar or greater amounts of L-proline added to the medium along with the drug. The results are interpreted to mean that the fibrillar material is collagen of epithelial origin, that the collagen in some way plays a role in mesenchymal penetration of the epithelial primordium, and that the epithelium is responsible for the pattern of lobulation within the developing gland.

KEY WORDS thyroid development · epithelial collagen · α, α' -dipyridyl · L-azetidine-2-carboxylic acid

Histogenesis of the embryonic chick thyroid begins with the penetration of cells from the mesenchymal capsule into a solid ball of epithelial cells. As a result, the epithelial mass becomes subdivided into cords which will further subdivide to form follicles (6, 22). Before the mesenchymal cells invade, channels which contain fibrillar material appear between the epithelial cells toward the center of the primordium (manuscript in preparation, for preliminary report see reference 14). These fibrils have a morphology similar to that of the collagen that is found beneath the basal lamina of many epithelial sheets (11, 23). Evidence has been presented that collagen is produced by epi-

thelial cell layers before periods of morphogenetic activity in the surrounding mesenchyme (7, 12, 28). It can be asked whether collagen secreted by an epithelium also can be involved in the differentiation of that same epithelium, as in the branching of a tubular organ or the subdivision of a solid primordium.

The experiments reported here were done to determine whether the fibrillar material within the developing thyroid primordium is produced by the epithelial cells, whether it is collagenous, and whether it plays a role in histogenesis. The drugs L-azetidine-2-carboxylic acid (LACA) and α, α' -dipyridyl were used to interfere with collagen synthesis. LACA substitutes for proline (25, 26), whereas α, α' -dipyridyl chelates the iron cofactor of prolyl hydroxylase (16). Both drugs prevent a normal degree of hydroxylation in the protocolla-

gen molecule, resulting in secretion of reduced amounts of the molecule (18, 19, 24). Thus, α , α' -dipyridyl has a more specific effect on collagen synthesis, since LACA will depress synthesis of all proteins that contain proline. Thyroids were explanted to organ culture before mesenchymal cells had invaded the epithelial primordium. When the epithelial component was explanted without its capsule, in medium containing either of the drugs, fibrillar material was not formed, whereas in the absence of the drug, fibrillar material did form. Explants which included capsule did not become invaded by mesenchyme in the presence of inhibitors, but invasion did occur in their absence.

MATERIALS AND METHODS

Thyroids were removed from stage 24 or 25 (10) Rhode Island Red chick embryos (Hardy's Hatchery, Essex, Mass.) and placed in Nutrient Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum and 100 U/ml penicillin and 100 μ g/ml streptomycin (Grand Island Biological Co.). The epithelial portion of the primordium could be freed of its capsule by soaking in ice-cold 2.0% trypsin (Bactotrypsin, Difco Laboratories, Detroit, Mich.) in calcium- and magnesium-free Hanks' saline for 5 min followed by gentle pipetting in and out of a capillary pipette of the same diameter as the primordium. Cleaned epithelial primordia were quickly transferred to the above-described culture medium and inspected at magnifications of $\times 50$ to $\times 100$ for adherent mesenchymal cells. Any contaminating cells either were removed by additional pipetting or the primordium was rejected.

Intact primordia, primordia stripped of capsule, and cleaned primordia with the capsule replaced were cultured on Millipore TH rafts (Millipore Corp., Bedford, Mass.) in Falcon organ culture dishes (BioQuest, BBL & Falcon Products, Cockeysville, Md.). The tissue was covered with a thin layer of 0.5% agar (agar agar BioQuest, BBL & Falcon Products, Cockeysville, Md.) and the well was filled with culture medium. Medium 199 containing 10% fetal calf serum was used for control cultures; for experimental cultures, LACA at concentrations of 20, 33, 50, or 100 μ g/ml (2×10^{-4} M to 10^{-3} M) or α , α' -dipyridyl at concentrations of 5, 10, 20, or 50 μ g/ml (3.2×10^{-5} M to 3.2×10^{-4} M) was added to the medium. The cultures were maintained in a humidified incubator gassed with a mixture of 95% O_2 /5% CO_2 . The medium was changed daily to assure that the supply of drugs was not diminished.

The cultures were fixed at intervals while still attached to the Millipore filters. The fixative used was 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2-7.4. The tissues were washed in buffer, postfixed in 1% osmium tetroxide in the same buffer, and detached from the filters for embedding in Araldite (R. P. Cargille Labs., Inc., Cedar Grove, N. J.). This procedure is essentially

the same as that used in the past (15). Thin sections for electron microscopy were stained with alcoholic uranyl acetate (30) and lead citrate (29) and observed with a Philips 300 electron microscope. Thick sections (0.5-1.0 μ m) were stained with azure II and methylene blue (20) and photographed with a Zeiss photomicroscope.

RESULTS

In Ovo

An example of a channel *in ovo* (Fig. 1) is included for comparison with the channels that are formed in culture. By stage 29, just before the mesenchymal cells invade, these extracellular spaces are extensive. They contain fine fibrils that tend to lie parallel to one another and to fill the channels.

Isolated Epithelium

Isolated epithelial primordia, free of adherent mesenchymal cells, were cultured in nutrient medium alone or in nutrient medium containing an inhibitor of collagen synthesis. The results are summarized in Table I. Control and experimental cultures were fixed at daily intervals from 24 h to 6 days of incubation. The primordia underwent maturation more rapidly in culture than they would have *in ovo* whether or not they were treated with the drugs.

CONTROL CULTURES: Epithelial primordia underwent small increases in size as judged by increased surface area of the living cultures and height in 1- μ m plastic sections. Primordia fixed after several days in culture contained small islands of dying cells, often in the same regions as division figures (Fig. 2). Channels formed between the healthy cells as early as 24 h after explantation. Many of the channels stretched for considerable distances through the sections.

Electron microscope preparations revealed that the contiguity of the cell surfaces lining the channels was interrupted by side branches that penetrated between the cells (Fig. 3). The shape and extent of the channels after 1 day of culture (total age, 5 days) were similar to those of the channels found after 6 days *in ovo* (stage 29; Fig. 1). The channels *in vitro* contained more extracellular material than *in ovo*, even after 24 h of culture. The extracellular material consisted of fibrils 20-30 nm in diam as well as particulate material, fine beaded strands 3-6 nm in diam, truly amorphous material, and a few densely staining granules 20-35 nm in diam. With the exception of the fibers, these materials comprise the amorphous ground substance of light

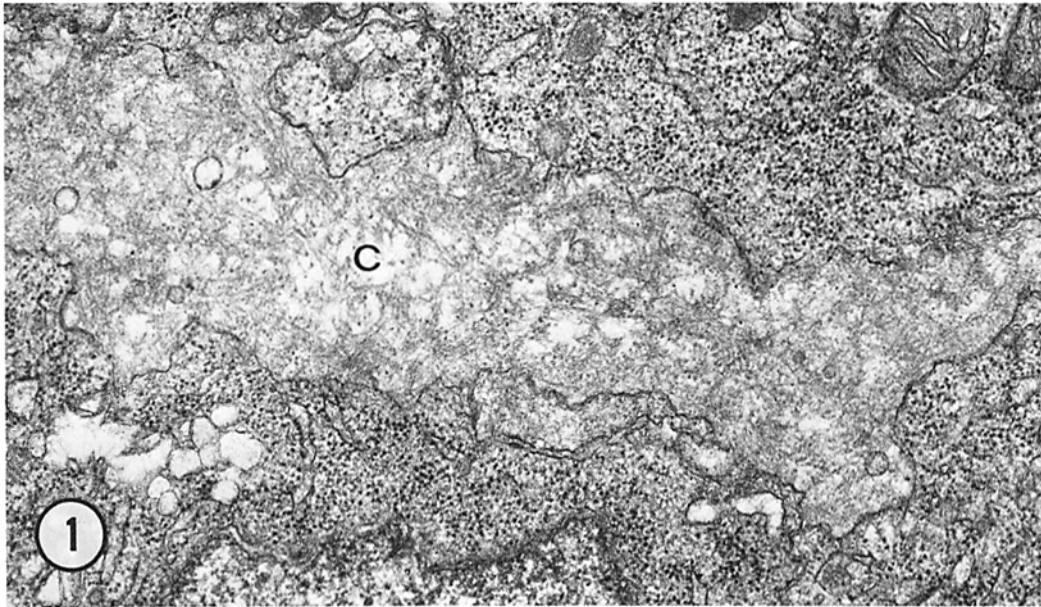


FIGURE 1 Electron micrograph of a portion of a channel (C) within a stage 29 thyroid *in ovo*. Note the meshwork of fine fibrils. $\times 31,400$.

TABLE I
Isolated Epithelial Primordia Cultured in the Presence of Inhibitors of Collagen Synthesis: Results of Light and Electron Microscope Examination

Treatment	No. cases examined	Fibrillar material	Amorphous material	Follicles
Control	21	21	21	21
20-100 $\mu\text{g/ml}$ LACA	8	0	8	8
20-50 $\mu\text{g/ml}$ α, α' -dipyridyl	5*	0	0	0
10 $\mu\text{g/ml}$ α, α' -dipyridyl	12	0	12	12
5 $\mu\text{g/ml}$ α, α' -dipyridyl	3	3	3	3

* All five samples were dead; additional samples were not sectioned because they also appeared necrotic.

microscopy and are thought to contain glycosaminoglycans (see reference 21 for discussion). All of these components of the ground substance were not found in all locations; for convenience, however, these components of the ground substance will be referred to collectively as "amorphous material."

By 2 or 3 days of culture, many of the thyroids contained wide, anastomosing passageways (Fig. 2) that were packed with extracellular material. The organization of the fibrils changed with length

of time in culture. During the first day or two, the extracellular spaces contained loose meshworks of fine fibrils; after 2 or more days in culture, the fibrils tended to align into loosely organized bundles (Fig. 4). Cells in the superficial regions of the cultures tended to be more tightly packed than towards the center, and few channels were seen to communicate with the surface. Clearly, the extracellular material was made by the epithelium.

DRUG TREATMENT OF ISOLATED EPITHELIUM: Addition of 20-100 $\mu\text{g/ml}$ of LACA to the medium did not affect the viability of the cultures although they underwent no appreciable increase in size. On the other hand, cultures treated with 20 $\mu\text{g/ml}$ or more of α, α' -dipyridyl became necrotic even after 1 day of incubation. At 10 $\mu\text{g/ml}$, the cultures remained viable and even underwent a modest increase in size. Small spaces identified only by electron microscopy formed between the cells both in the presence of LACA (Fig. 5) and in the presence of α, α' -dipyridyl (Fig. 6). The spaces were narrow in cross section, and in longitudinal section they rarely stretched across more than two cell diameters. Thus, the central cells did not assume the cord-like arrangements that characterized the control cultures or the primordium *in ovo* (Figs. 7 and 8). Fibrils were not found in any of these spaces. The spaces did contain varying amounts of amorphous mate-

rial, which often fixed as beaded strands (Figs. 5 and 6). With 5 $\mu\text{g/ml}$ of α, α' -dipyridyl in the medium, long channels did form and contained fibrillar material.

Although both LACA and α, α' -dipyridyl had the same effect of preventing the epithelial cells from forming fibrils, there were some differences in the appearance of the cultures. Incubation with LACA resulted in the formation of small extracellular spaces containing variable amounts of extracellular material. The cytoplasm appeared paler than in untreated cultures as a result of the clumping of ribosomes, absence of larger (glycogen?) particles, and reduced numbers of profiles of endoplasmic reticulum. In cultures treated with 10 $\mu\text{g/ml}$ of α, α' -dipyridyl, the spaces tended to contain more extracellular material than in cultures treated with LACA, the ribosomes appeared not to be as clumped, and some "glycogen" particles were present. There may have been a slight reduction in the amount of rough endoplasmic reticulum in comparison to control cultures. Treatment with either drug seemed to have little effect on either the Golgi region or follicle formation (see below).

Whole Thyroids

The results were the same whether intact thyroid primordia were placed in culture or whether the capsule was first removed and then replaced on the epithelial component in the culture dish. Therefore, these samples will be treated as a single group. The results are summarized in Table II.

CONTROL CULTURES: Whole thyroids in the control medium underwent a rapid increase in size. Few dying cells were found and the cultures contained many epithelial cells in mitosis (Fig. 9). Spaces were present between the epithelial cells after 24 h of culture (Fig. 10). After longer periods of incubation, the cultures became subdivided into masses of epithelial cells separated by connective tissue tongues containing sinusoid-like spaces (Fig. 9). Within any section, several of these broad channels communicated with the investing capsule. Channels within these epithelial subdivisions were difficult to recognize because they tended to be filled with cells. After 1 day, the channels contained a feltwork of fine fibrils (Fig. 10). After longer periods of culture, fibrils were found in the broader channels that contained fibroblasts (Fig. 11). Within the more compact masses of epithelial cells, fibers were seen only in fortuitous sections that passed parallel to a cell

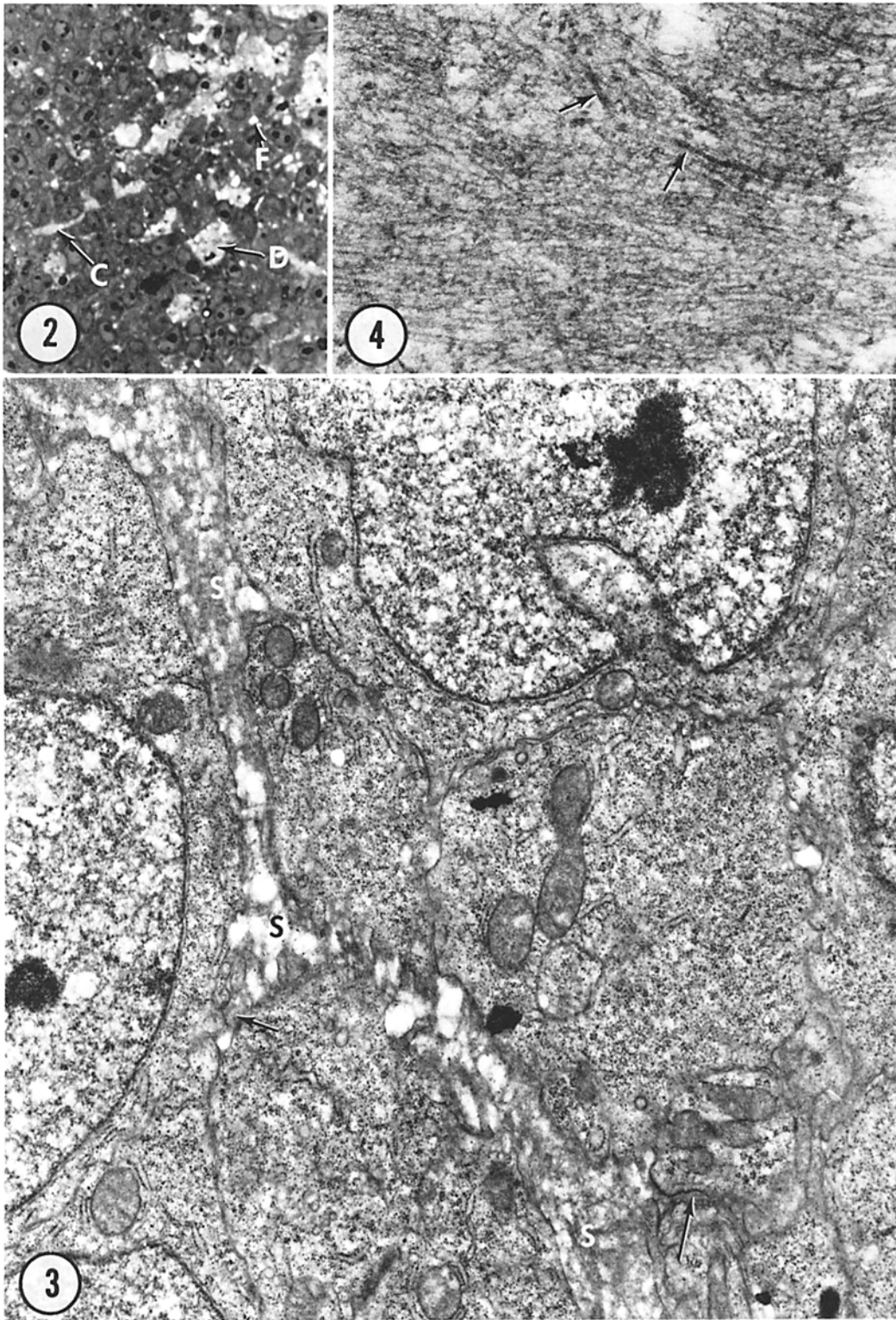
surface and exposed a regular array of fine fibrils. The major criterion for identification of a mesenchyme cell between closely apposed epithelial cells was identification of the same cell in adjacent thick and thin sections. Identification with any degree of assurance could be made only for an elongate cell at a break in the epithelial contour.

DRUG TREATMENT OF WHOLE THYROIDES: The same dosages of LACA (20–100 $\mu\text{g/ml}$) and α, α' -dipyridyl (10 $\mu\text{g/ml}$) were found as effective for the cultures of whole thyroids as for the isolated epithelia (Figs. 12 and 13). The cultures underwent minimal increases in size. The spaces that appeared between the epithelial cells contained amorphous material but fibrils did not form (Figs. 14 and 15). The cytoplasm contained clumped ribosomes, fewer glycogen particles, and fewer channels of rough endoplasmic reticulum than in control cultures. Treatment with LACA had more of an effect upon these characteristics than treatment with α, α' -dipyridyl.

The internal organization of the epithelial component was virtually identical to that of cultures prepared from isolated epithelial primordia. No evidence could be found of penetration of mesenchyme cells into the epithelial component. Large expanses of the epithelial surface exhibited an unbroken contour (Figs. 12, 13, and 16); there was no indication of extension of mesenchymal cell processes between the epithelial cells. The basal cytoplasm of the epithelial cells was clearly marked with a band of microfilaments that lay parallel to the plasmalemma (Fig. 17). Since the filaments of one cell ended at the plasmalemma adjacent to the location of filaments in the adjoining cell, an interruption of the surface would have been obvious. In addition, the mesenchyme was separated from the epithelial surface by a distinct basal lamina.

The basal microfilament bundles were a constant feature of drug-treated cultures with or without the mesenchyme. Occasionally, filament bundles were found in the basal region of surface cells in epithelial primordia in control medium. Filaments have never been found in this location in thyroid primordia *in ovo* nor in cultures of whole primordia. The presence of microfilament bundles in the basal cytoplasm of epithelial cells at the surface of a primordium, then, seems to be unique to cultures in which there is no invasion of mesenchyme.

The mesenchyme cells at the periphery of the epithelial mass were surrounded by cross-striated



collagen fibrils. Some or all of these fibrils may have been synthesized before the thyroids were placed in culture, since thyroids *in ovo* at stage 25 are surrounded by a capsule which contains collagen. Since collagen which might have been synthesized after the primordia were placed in culture could not be distinguished from pre-existing fibrils, it is impossible to tell from these experiments whether the mesenchyme and epithelium respond differently to the same dose of these drugs.

REVERSAL OF INHIBITION: It is possible that the failure of mesenchymal cells to penetrate the epithelium in cultures containing the drugs was a result of greater sensitivity of the mesenchyme toward some toxic effect of the drugs. The cells may have failed to penetrate the epithelium because they were poisoned. The effect of LACA on mesenchyme cells was tested on cultures of whole thyroids by two different procedures based upon the competition between proline and its analogue for insertion into polypeptide chains (3). First, equimolar concentrations of LACA and L-proline were added to the nutrient medium at the start of an experiment. Fibrils formed within the epithelial portion and mesenchymal cells invaded. Since the presence of proline in the medium might have protected the mesenchymal cells from toxic effects of the drug, a second approach also was used. Cultures were incubated for 2 days in medium containing LACA. Then the medium was changed and the same cultures were incubated for 3 additional days in medium containing LACA plus equimolar or greater amounts of L-proline. Cultures fixed after 2 days contained neither fibrils nor invading mesenchymal cells, as described above. Cultures fixed 3 days after the medium had been changed showed a complete reversal of the

drug effect. Elongate channels were filled with fibrils, and mesenchymal cells were found both in broad channels and between closely apposed cells. As early as 24 h after changing the medium, there was evidence of mesenchymal penetration of the epithelial mass (Fig. 18).

The effects of α , α' -dipyridyl could be reversed in a similar fashion. Cultures were incubated with medium containing α , α' -dipyridyl for 2 days. The medium then was replaced with control medium and the cultures were fixed 3 days later. Again, there was no indication of selective mesenchymal cell death, and the mesenchymal cells were able to migrate into the epithelial mass.

Follicle Formation

All of the cultures formed many follicles. The largest follicles were formed in the control cultures of the whole thyroids (Fig. 9). The sections contained numerous examples of follicular lumina having a scalloped appearance (Fig. 19), a shape that has been taken to indicate follicles in the process of enlarging (13). In some cases the lumen was filled with dense colloid, while in other follicles the space contained only wisps of dense particulate material. All of the cultures—whether they were of whole primordia, the epithelial component alone, drug treated, or in nutrient medium—contained many small follicles and places where follicle formation was being initiated. The apical surfaces of two adjacent cells were joined by tight junctions (Fig. 20). The apical cytoplasm contained a well-developed Golgi region consisting of five or more stacked lamellae and many small vesicles. The apical cytoplasm was characterized also by its content of many dense bodies that have been shown to be lysosomes. The follic-

FIGURE 2 Portion of a thyroid primordium that was isolated from its capsule and fixed after 3 days of culture. This light micrograph shows that numerous spaces existed between the cordlike arrangements of epithelial cells. Electron micrographs of the same specimens show that the larger spaces (*D*) contained fibrillar material and cell debris while the elongate channels (*C*) contained only fibrils and amorphous material. Follicular spaces (*F*) were smaller and appeared less dense than in cultures of intact primordia. $\times 560$.

FIGURE 3 Electron micrograph of an epithelial primordium fixed after 24 h in control medium. The extracellular channel (*S*) which coursed from upper left to lower right had several side branches (arrows). Fibrillar material was unevenly dispersed within the channel. The cells lining the extracellular space contained narrow profiles of rough endoplasmic reticulum associated with mitochondria. $\times 15,000$.

FIGURE 4 A small portion of an extracellular channel from an epithelial primordium after 3 days in culture. The fibrils tended to align in loose, parallel bundles. The thicker fibrils show some indications of cross banding (arrows). $\times 54,200$.

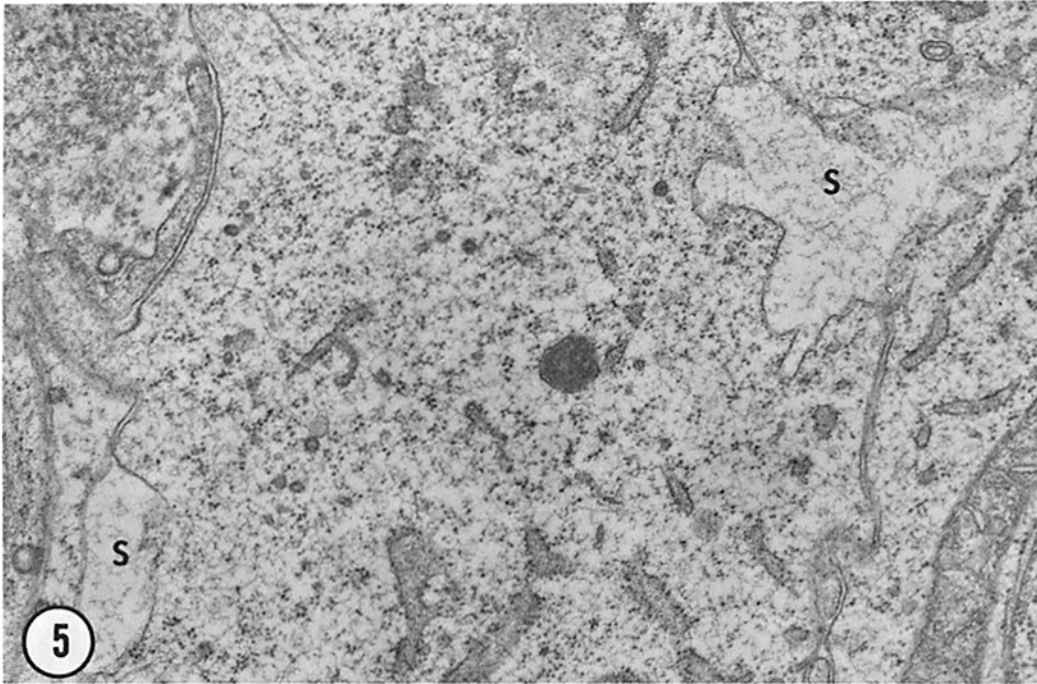


FIGURE 5 Small portion of an epithelial primordium after culture for 2 days in medium containing 20 $\mu\text{g}/\text{ml}$ LACA. The cells contained normal channels of rough endoplasmic reticulum but many of the free ribosomes had clumped. The extracellular spaces (S) were small and contained only the amorphous component of the matrix. $\times 39,900$.

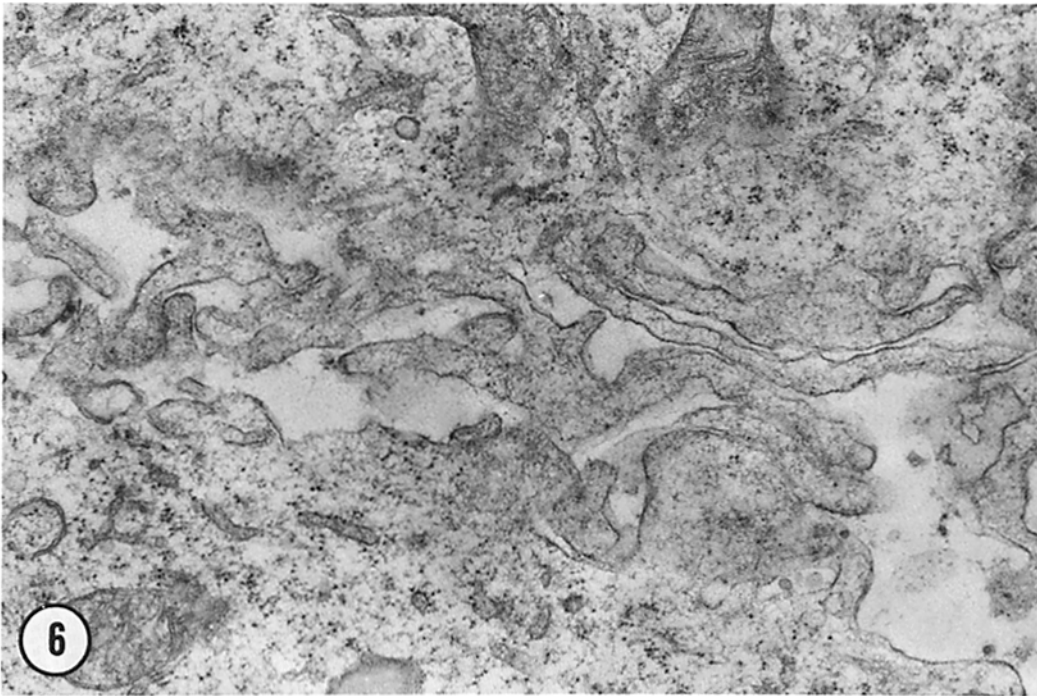


FIGURE 6 Portion of two cell surfaces delineating a channel in an epithelial primordium after culture for 3 days in medium containing 10 $\mu\text{g}/\text{ml}$ α, α' -dipyridyl. The extracellular space was relatively large and the cell surfaces were covered with projections. The cytoplasmic matrix appears slightly denser than that in cultures treated with LACA, but the distribution and content of cytoplasmic organelles was similar. $\times 26,800$.

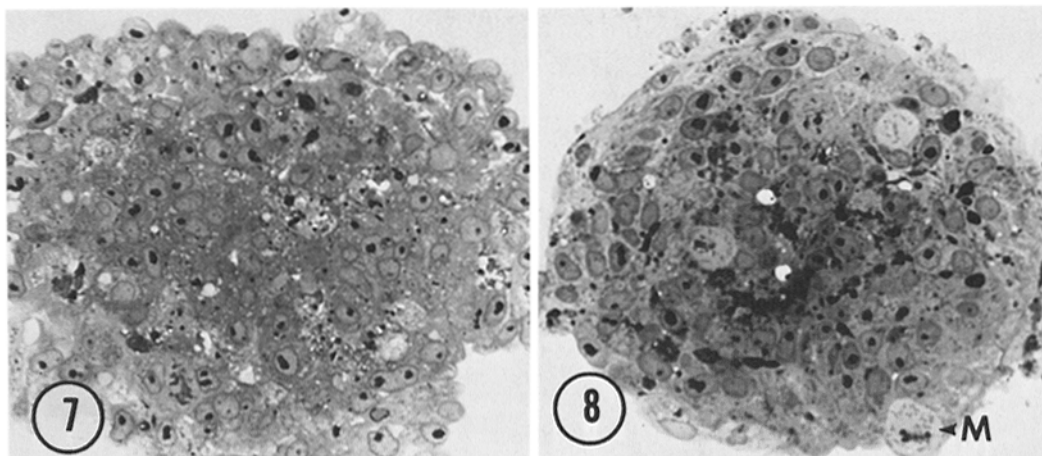


FIGURE 7 Light microscope preparation of a stage 25 thyroid epithelial primordium after 2 days in culture with 100 $\mu\text{g/ml}$ LACA. The cells form a compact mass. The spaces visible at this magnification were small follicles. A few of the cells were pycnotic. $\times 550$.

FIGURE 8 Light microscope preparation of the epithelial portion of a thyroid primordium after 4 days of culture with 10 $\mu\text{g/ml}$ α, α' -dipyridyl. The cells formed a compact mass with little indication of cell death. Note the absence of channels and the presence of division figures (*M*). $\times 550$.

TABLE II
Intact Thyroid Primordia Cultured in the Presence of Inhibitors of Collagen Synthesis: Results of Light and Electron Microscope Examination

Treatment	No. cases examined	Fibrillar material	Mesenchymal penetration	Amorphous material	Follicles
Control	14	14	14	14	14
20–100 $\mu\text{g/ml}$ LACA	15	0	0	15	15
10 $\mu\text{g/ml}$ α, α' -dipyridyl	8	0	0	8	8
LACA + Proline*	3	3	3	3	3
LACA followed by LACA + Proline‡	5	5	5	5	5
α, α' -dipyridyl followed by medium§	6	6	6	6	6

* Medium contained 100 $\mu\text{g/ml}$ LACA and 100 $\mu\text{g/ml}$ L-proline.

‡ Medium contained 50 $\mu\text{g/ml}$ LACA for the first 2 days, then 50 $\mu\text{g/ml}$ LACA and 100 $\mu\text{g/ml}$ L-proline for 3 additional days.

§ Medium contained 10 $\mu\text{g/ml}$ α, α' -dipyridyl for 2 days. This was replaced with control medium for 3 days.

ular space between the cell apices was filled with cytoplasmic folds at the earliest stages (Fig. 20) and coarse microvilli and dense, particulate material at later developmental stages (Fig. 21).

Epithelial primordia in control medium formed fewer large follicles than cultures of whole primordia. The majority of the profiles in the epithelial primordia were of small follicles but many initial stages of follicle formation also were present. In cultures treated with LACA or α, α' -dipyridyl, few follicles developed beyond the stage of interdigitation of the plasma membrane (Fig. 20). Only occasionally was a larger follicle seen (Fig. 21). In

the presence of the drugs, a similar proportion of interdigitated apices and small follicles was found whether the epithelium was cultured alone or with its capsule. In spite of the effect on enlargement of the follicles, the drugs had no apparent effect on the organization of the Golgi region or the number of lysosomes.

DISCUSSION

The results of this study show that the extracellular fibrils which accumulate within the developing thyroid primordium of the chick embryo are of epithelial origin, that the fibrils probably are colla-

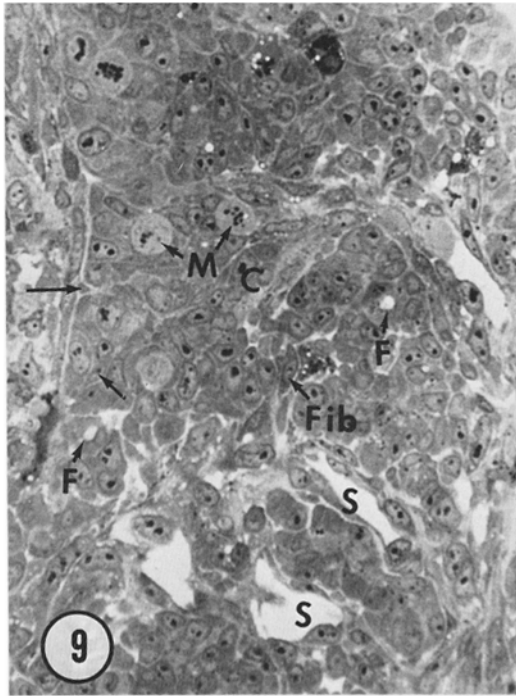
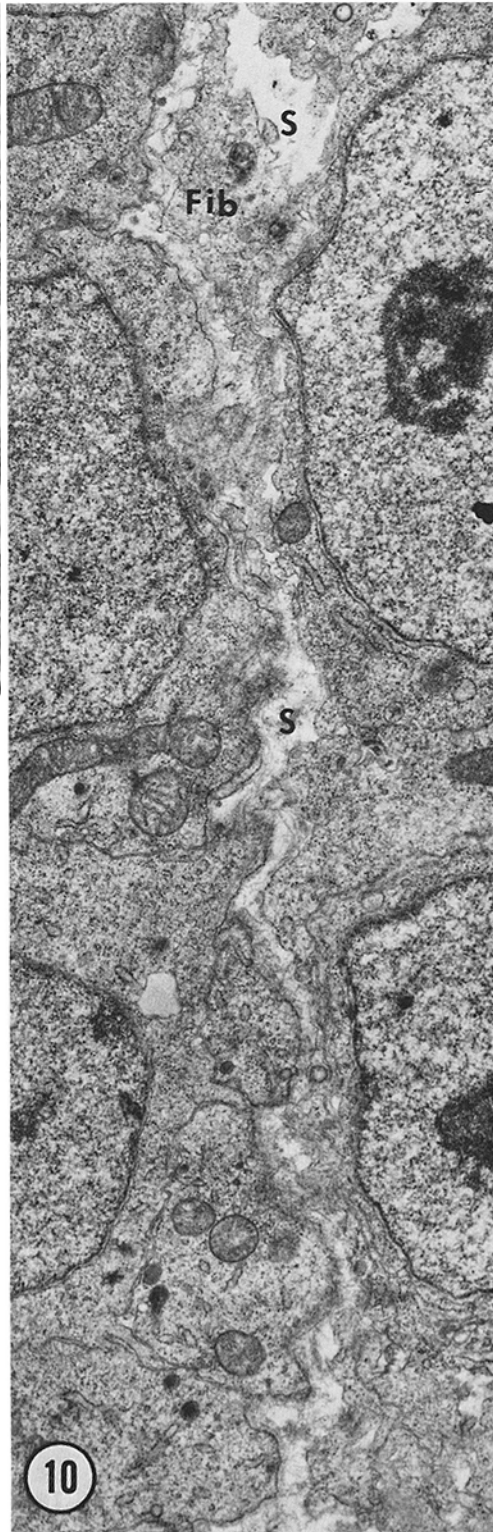


FIGURE 9 Light micrograph of an intact thyroid primordium after 3 days of culture in control medium. The epithelial cells formed islands and cords that were separated from each other by elongate (fibroblastic) cells (*Fib*) and spaces resembling sinusoids (*S*). The organization of the mesenchymal cells varied from wider tongues in some areas (*C*) to single cells interposed between epithelial cell surfaces (arrows). The follicles (*F*) tended to be larger than in cultures of the epithelial portion of the primordium. Note the division figures (*M*). $\times 550$.

FIGURE 10 Portion of a channel deep within a whole thyroid after 2 days of culture in control medium. The space (*S*) contained fine fibrillar and amorphous material as well as processes of elongate cells (*Fib*), probably of mesenchymal origin. $\times 15,000$.



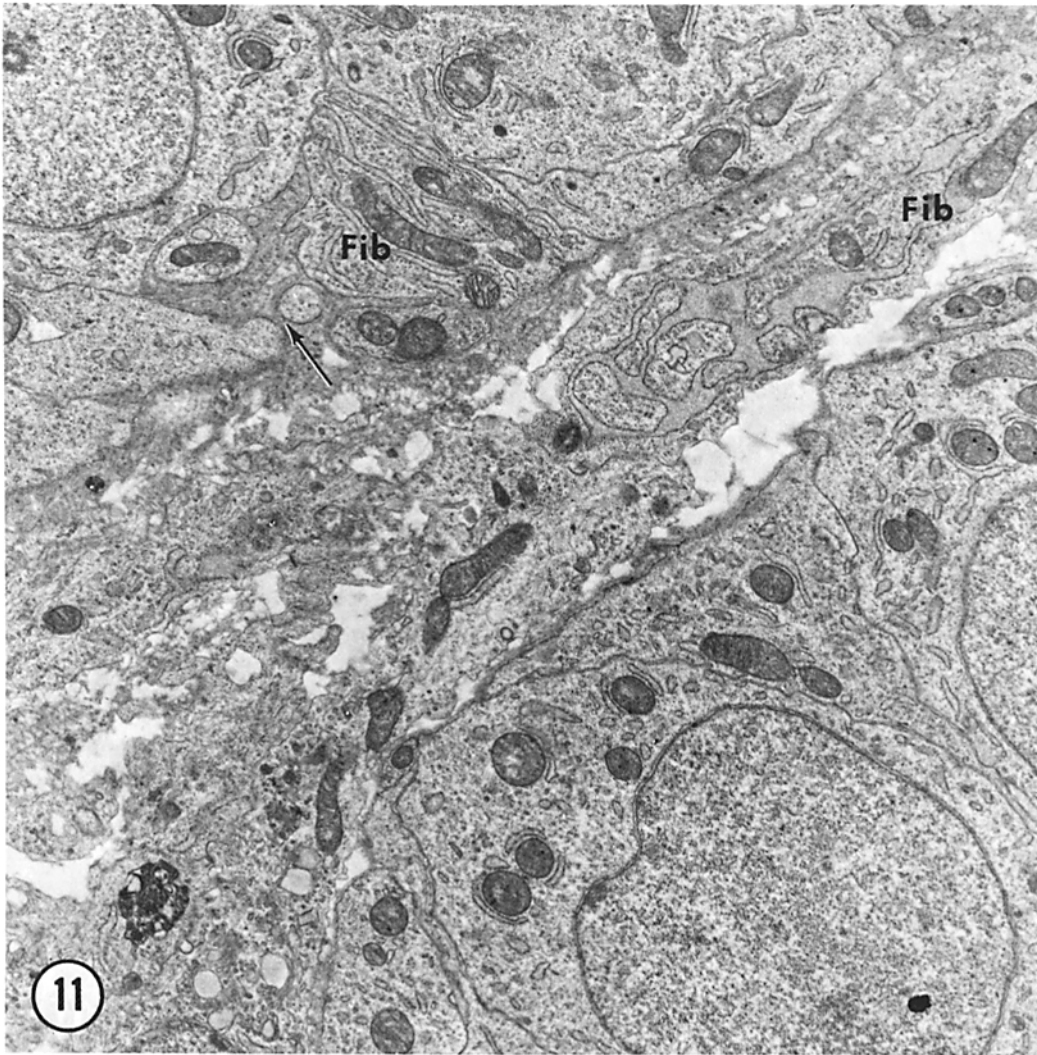


FIGURE 11 A channel broader than that shown in Figure 10, within a whole thyroid primordium after 3 days in control medium. The fibroblasts (*Fib*) had well-developed channels of rough endoplasmic reticulum that contained electron-dense product. The fibroblasts tended to align along the basal lamina of the epithelial cells. A break in the continuous epithelial cell surface (arrow) was filled with extracellular material and fibroblastic cell processes (*Fib*). This organization is characteristic of the region labeled C in Fig. 9. $\times 10,400$.

gen, and that they may play a role in normal thyroid histogenesis. The epithelial origin of the fibrils is established by the fact that fibrillar material accumulated between the epithelial cells of cultured primordia from which the mesenchymal component had been completely removed. The probable collagenous nature of the fibrils can be concluded from the absence of fibrils in cultures treated with drugs that interfere with collagen synthesis (LACA or α, α' -dipyridyl). A possible

involvement of the fibrillar material in histogenesis is indicated by the establishment of normal epithelial-mesenchymal relationships in cultures of whole thyroids under conditions in which the fibrils were present and by the absence of a normal relationship when formation of fibrils was inhibited by the drug treatment.

We have found no single study in which both morphological and biochemical data have been collected after treatment with either of these two

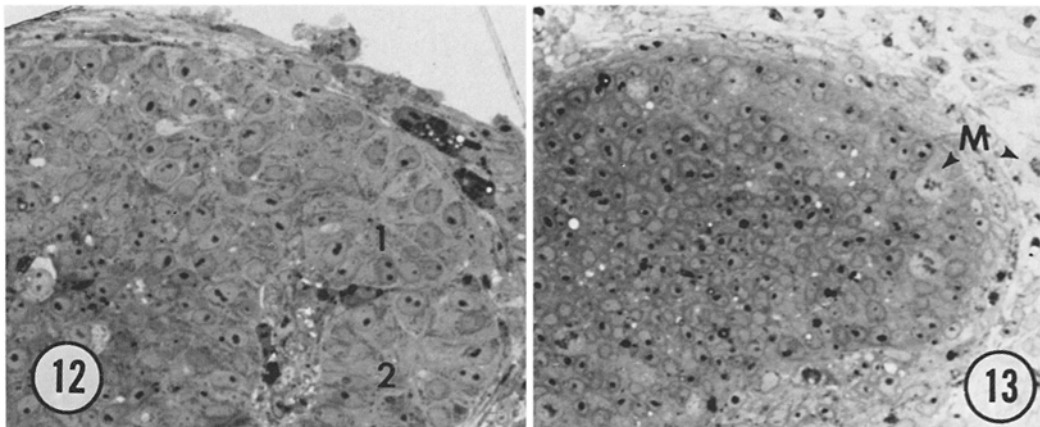


FIGURE 12 Light microscope preparation of several pooled intact thyroid primordia cultured for 3 days with 100 $\mu\text{g}/\text{ml}$ LACA. Parts of two primordia (1 and 2) are shown, including the capsule at the surface of the culture. There was a distinct separation between the epithelial and mesenchymal cell types, with no indication of invasion by the mesenchyme cells. The epithelium was free of channels, but some of the cells appeared to form circular arrangements. $\times 430$.

FIGURE 13 Light microscope preparation of a single intact thyroid primordium cultured for 3 days with 10 $\mu\text{g}/\text{ml}$ α, α' -dipyridyl. The compact epithelial mass made a smooth border with the peripherally located mesenchymal cells. Note the division figures (M) in both the epithelium and mesenchyme. $\times 430$.

drugs. However, the specificity, effectiveness in blocking collagen secretion, and mechanism of action have been studied thoroughly for the drug, LACA. In vitro studies of cartilage cells, using radioactively labeled LACA, have shown that LACA is incorporated into the collagen chain in place of some proline residues (25, 26). LACA remains unhydroxylated and its presence within the polypeptide chain reduces hydroxylation of proline and lysine residues within the chain. The cells can secrete the resultant abnormal molecule only at reduced levels, if at all (19). The addition of LACA to cells in culture or to a purified enzyme system had no effect on prolyl hydroxylase, but did reduce both the rate and final degree of hydroxylation of cartilage procollagen. Thus, the inhibitory effect of the drug is not directly on the hydroxylating enzymes, but possibly is concerned with chain conformation and accessibility of the proper residues to the hydroxylating enzymes.

LACA is known to have nonspecific effects as well as its specific effect on collagen synthesis. The drug is incorporated into other proteins, presumably all proteins that contain proline (3, 19, 31). An effect of LACA on growth parameters has been noted in tissues (1, 2, 19) and in bacteria (17). The cultures that we treated with LACA

also did not increase in size, admittedly as judged by a rather qualitative method. However, there was no inordinate amount of cell death, and cellular ultrastructure was affected only slightly. These effects included a minimal reduction in the channels of endoplasmic reticulum and some clumping of free ribosomes. Furthermore, the action of the drug was reversed by proline. Reversal involved not only the formation of fibrillar material in channels but also the increase in size of the cultures. Nevertheless, interference with general cellular functions as represented by inhibition of protein synthesis other than collagen might have been the basis for the restricted morphogenesis in our cultures. Therefore, we compared the results obtained with LACA with those obtained by using an inhibitor of collagen synthesis that has a different mode of action.

The drug α, α' -dipyridyl acts as an inhibitor of prolyl hydroxylase by chelating one of its cofactors, the ferrous ion; the underhydroxylated molecule cannot be secreted in normal amounts by the cell (18, 24). If the drug is washed out, hydroxylation of monomer within the cell apparently occurs rapidly (24). There appears to be no published account of the effect of α, α' -dipyridyl on the morphology of treated tissues. The drug should interfere with any cellular process that re-

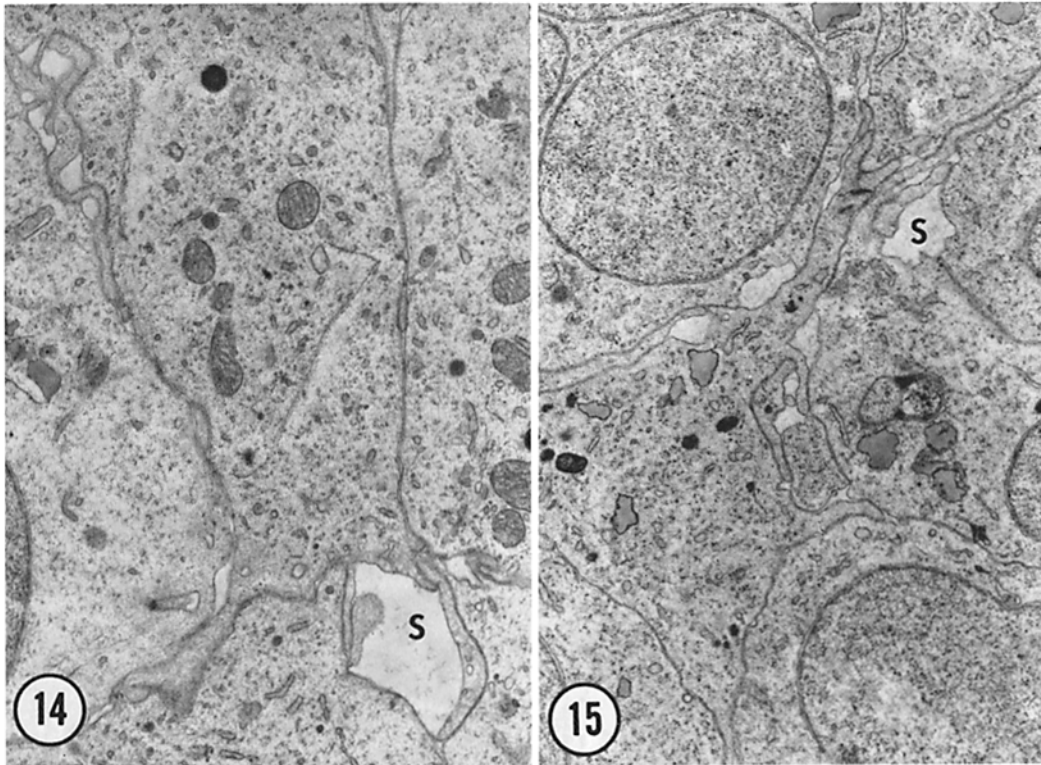


FIGURE 14 Portion of a whole thyroid primordium after 3 days in medium containing 50 $\mu\text{g/ml}$ LACA. The organization of the intracellular and extracellular (S) compartments is similar to that in cultures of the isolated epithelium treated with the drug. The spaces were small, contained only amorphous material, and there was no evidence of mesenchymal invasion. These cells possessed more surface projections than were ordinarily seen after treatment with LACA. $\times 8,800$.

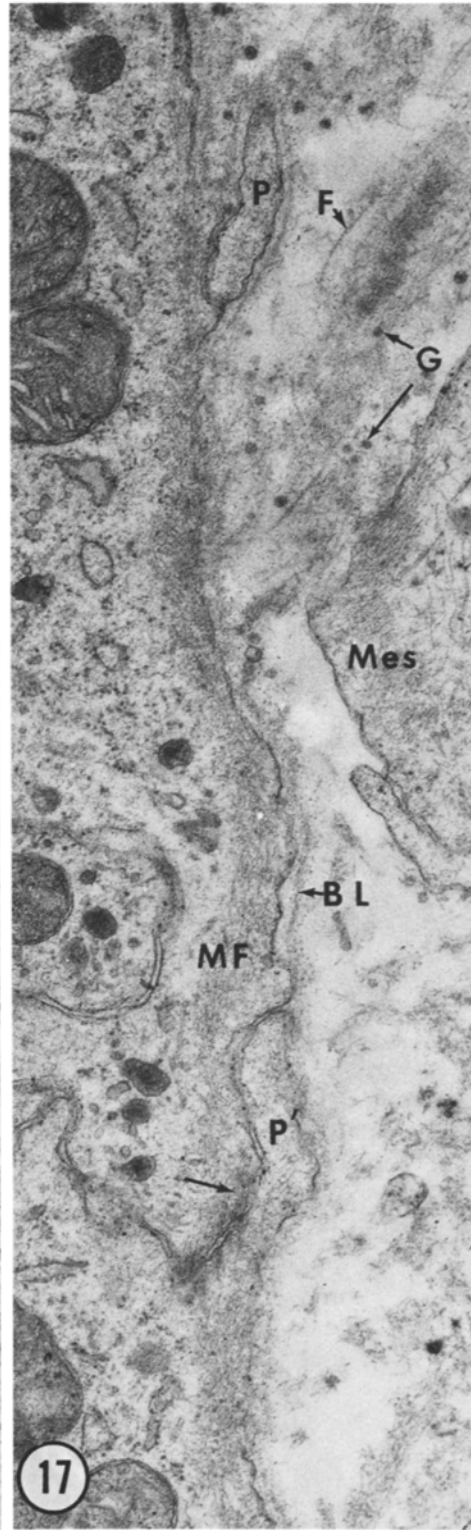
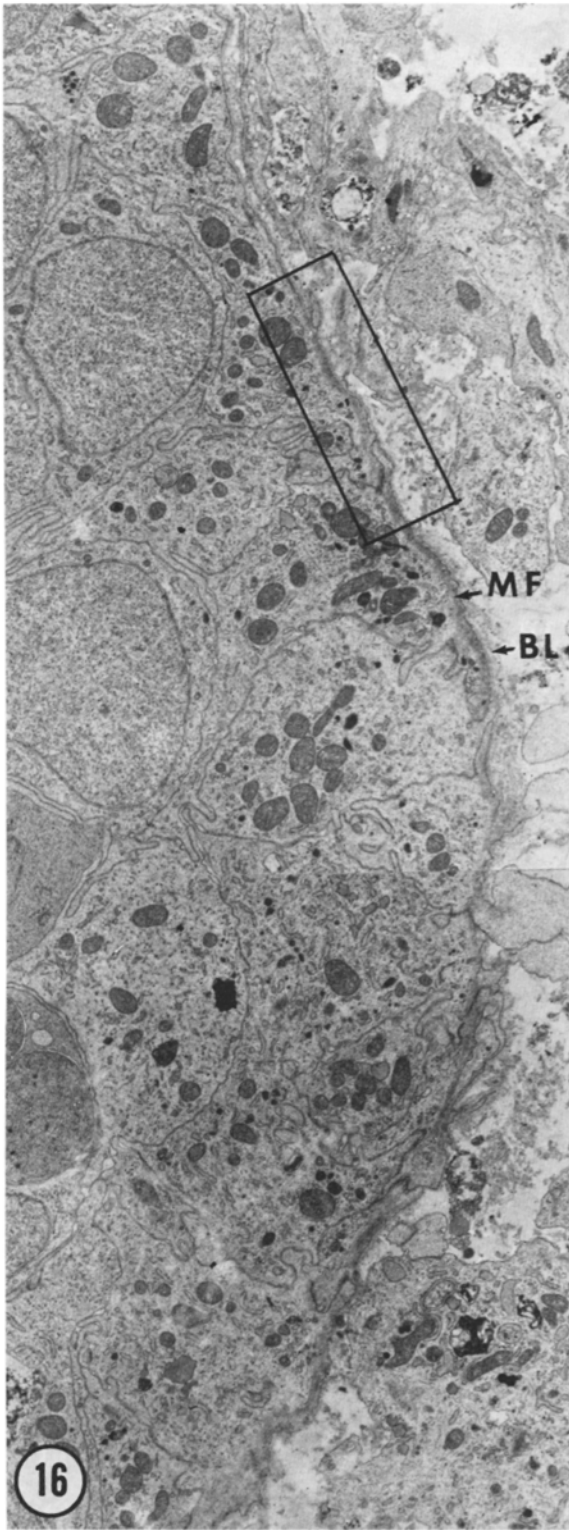
FIGURE 15 Central portion of a whole thyroid after 3 days in medium containing 10 $\mu\text{g/ml}$ α, α' -dipyridyl. There is essentially no difference in morphology between this culture and those treated with the drug in the absence of mesenchyme. $\times 8,800$.

quires ferrous ions; however, our cultures increased in size in the presence of the drug and contained some division figures. The drug also had no apparent inhibitory effect on the synthesis of sulfated mucopolysaccharides by embryonic chick cartilage cells in culture (5). In the thyroids, there was even less indication of an effect on ultrastructural organization after α, α' -dipyridyl treatment than after LACA treatment. The effects on organization of rough endoplasmic reticulum and clumping of free ribosomes were slight. Thus, it is unlikely that the effects we have obtained were caused by inhibition of any process other than collagen synthesis.

Whatever influence either drug may have had on both the epithelium and mesenchyme, it was not permanent. Incubation with subthreshold

doses of α, α' -dipyridyl or incubation in medium containing both LACA and proline resulted in fibril formation and morphogenesis of the cultures. Similarly, fibrils formed and invasion of mesenchyme occurred after the drugs were washed out. Since there was no indication of any serious generalized inhibitory effect by either drug, the absence of fibrils probably was a result of inhibition of collagen synthesis. This conclusion is in agreement with the data obtained from the biochemical experiments already cited.

It has been well documented that not only fibroblastic cell types but also epithelial cells synthesize and secrete collagen (7, 12). Epithelial collagen may act as a substratum to guide cell movements during morphogenesis (7, 8, 9, 11, 28). In these systems, epithelial collagen may be involved in the



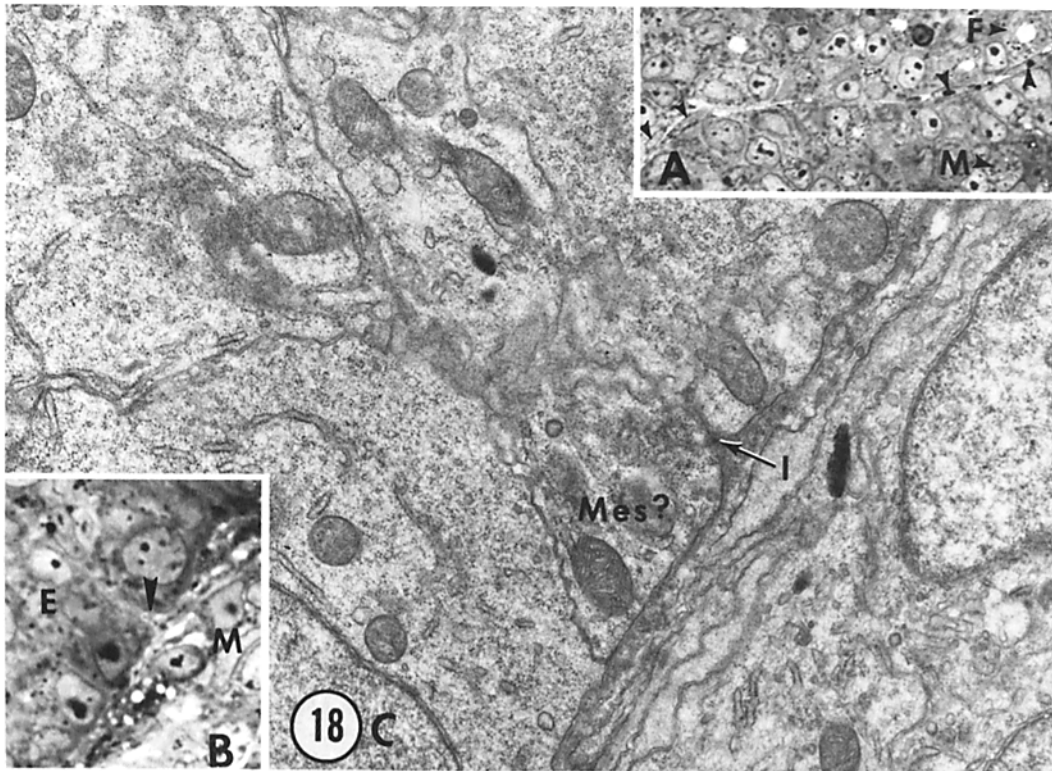
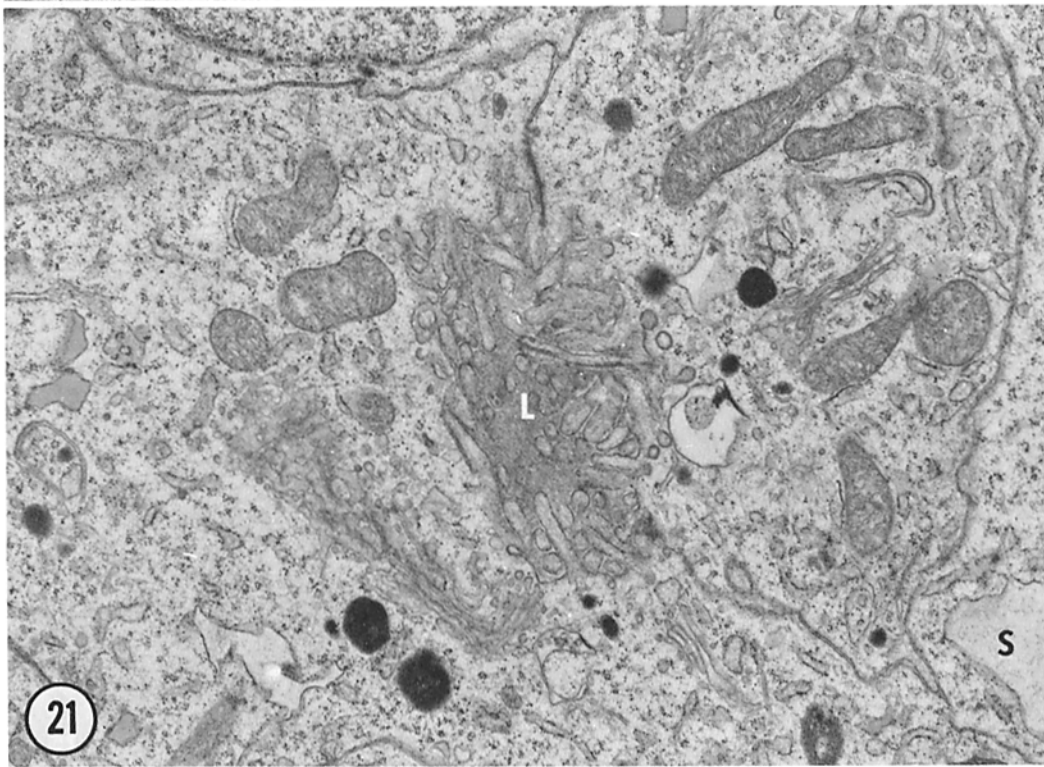
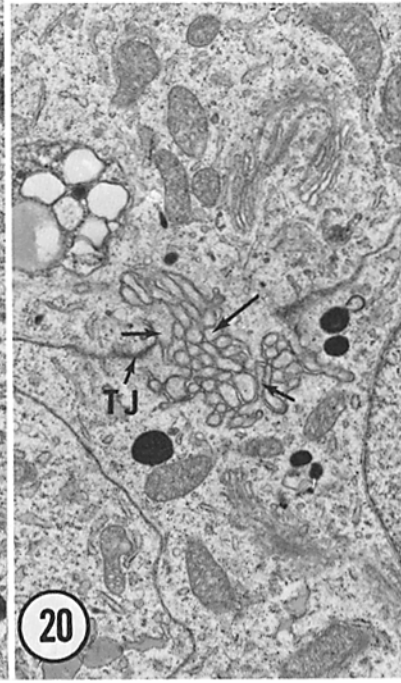
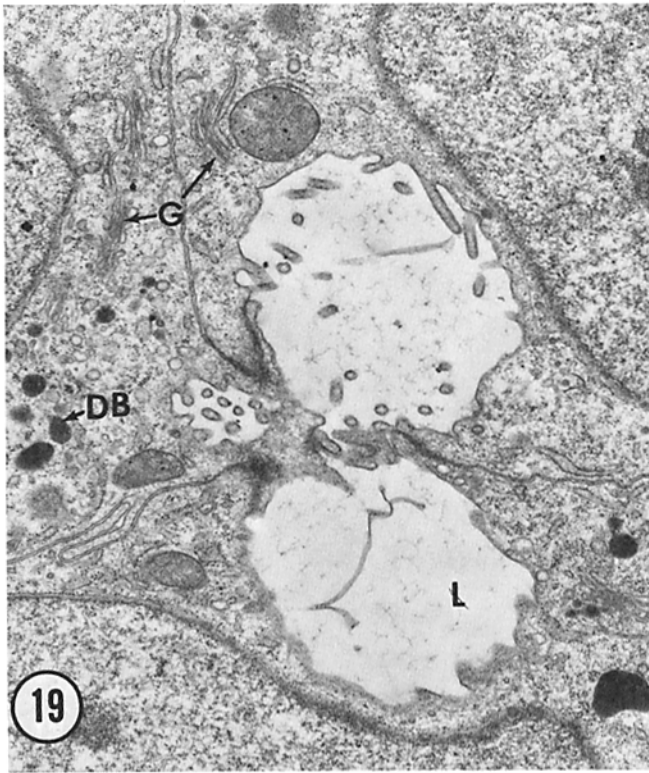


FIGURE 18 Light (*A* and *B*) and electron (*C*) micrographs of sections through the same culture of a whole thyroid primordium, treated for 2 days with 0.5 mM (50 $\mu\text{g}/\text{ml}$) LACA followed by an additional day in medium containing 0.5 mM LACA and 1 mM L-proline. (*A*) The epithelial mass contained a few narrow channels, such as this one, that were penetrated by a row of elongate cells (arrowheads). Note the division figure (*M*) and follicles (*F*). $\times 700$. (*B*) In the same section, indentations (arrowhead) in the epithelial surface (*E*) appeared to be filled by mesenchymal cell processes. $\times 1,280$. (*C*) The same region as in (*B*) from a thin section $<5 \mu\text{m}$ deeper in the block. Cytoplasmic organelles lay within the cortical region of the epithelial cell bases; no microfilament bundle was visible. The epithelial surface was indented (*I*) and the adjacent cell (*Mes?*) may represent a mesenchymal cell process as in (*B*). $\times 15,600$.

FIGURE 16 Surface of a whole primordium after 3 days in nutrient medium containing 10 $\mu\text{g}/\text{ml}$ α, α' -dipyridyl. This large expanse of epithelial surface was covered by a continuous basal lamina (*BL*). There is no evidence of interruption by invading mesenchymal cells, either at the epithelial cell bases or at their lateral surfaces. Bands of microfilaments (*MF*) lay parallel to the basal cell surface in the cortical cytoplasm. The mesenchyme cells were healthy and were surrounded by matrix containing collagen fibrils. The box encloses the region enlarged in Fig. 17. $\times 5,200$.

FIGURE 17 Higher magnification of the basal cytoplasm of an epithelial cell and the adjacent extracellular matrix. The bundles of microfilaments (*MF*) 7 nm in diam were associated at the plasma membrane with a region of greater density (arrow). However, junctional complexes of the sort existing at cell apices were not found. The matrix external to the basal lamina (*BL*) contained some fibrils (*F*) and granules (*G*). The mesenchymal cell processes (*Mes*) contained a microfilament web; processes did not touch the epithelial surface, however. The process *P* is probably from an overlapping epithelial cell, similar to process *P'*. $\times 30,800$.



morphogenesis of a mesenchymal component. In the thyroid, the presence of epithelial collagen is correlated with the establishment of the histological organization characteristic of the mature gland. This correlation could have several bases. Although there is no proof as yet, it is possible that secretion of collagen is directly involved in the formation of intraepithelial channels; perhaps its secretion is the mechanism by which smaller spaces between cells become linked into longer channels. Second, secretion of collagen could be involved in the stabilization of a channel that is formed by some other process. Such a role for collagen has been suggested for the stabilization of the salivary branching pattern (4). On the other hand, collagen secretion may not play any direct role in channel formation but might merely be an indicator of epithelial specialization, as for example the establishment of an apical-basal polarity. Thus, the release of a combination of extracellular macromolecules including collagen and glycosaminoglycans (27) might stabilize the cell bases and provide a channel as well as a substratum to guide the penetration of the connective tissue (mesenchymal) component of the gland. At the present time, the data do not exist to select from these or other alternatives.

Whether or not collagen synthesis proves to have a role in thyroid morphogenesis, this study shows that the pattern of mesenchymal penetration into the epithelial mass of the thyroid can be thought of as directed by the epithelial cells rather than by the mesenchymal cells. If the specific sites for penetration of the mesenchyme were selected by the invading cells, the pattern of cords and the subsequent lobular arrangement of the follicles would be under mesenchymal control. Then the

mesenchyme would play a decisive role in histogenesis, determining polarity of the secretory cells as well as arrangement of the follicles. The results of these experiments, on the other hand, suggest that the mesenchyme plays a passive role in histogenesis. The points of mesenchymal entry seem to depend upon the establishment of contiguity between the intraepithelial channels and the surface of the epithelial primordium. Before mesenchymal entry, the epithelial cells have established an apical-basal polarity as evidenced by the basal lamina lining the channels and follicle formation occurring at the opposite cell surface. These channels are formed *in vitro* in the complete absence of mesenchyme. Thus, inhibitors of collagen synthesis seem to limit the extent of the intraepithelial channels and to prevent the channels from reaching the epithelial periphery.

A question that this study does not answer concerns the stimulus that leads to penetration of the mesenchymal cells. From stage 26 until stage 29, the thyroid consists of a peripheral layer of tightly joined, cuboidal cells and a central region of polygonal cells. At stage 26, the polygonal cells have an apparent random orientation, but as the longer channels begin to form, more and more of the cells exhibit an apical-basal polarity (Hilfer, unpublished observations). Even at stage 29, when the central cells are oriented along extensive channels, the peripheral cells form a compact border for the epithelial portion of the gland. This observation raises the possibility that there are two distinct populations of cells within the epithelial mass. At stage 29, the integrity of this peripheral layer is destroyed and within a period of an hour or so the mesenchyme with accompanying blood vessels penetrates to the center of the primordium. Al-

FIGURE 19 The cell apices of one of the larger follicles in a whole primordium after 3 days in control medium. The luminal surfaces were deeply scalloped and contained microvilli. Adjacent cells were joined by tight junctions typical of immature follicles. The follicular lumen (*L*) contained a sparse amount of particulate material. The apical cytoplasm contained organized Golgi regions (*G*) and dense bodies (*DB*). $\times 15,300$.

FIGURE 20 Epithelial cell apices toward the center of a whole thyroid primordium after 3 days of culture in nutrient medium containing 100 $\mu\text{g/ml}$ LACA. The apical cell surfaces were joined by tight junctions (*TJ*) and were highly interdigitated (arrows). The apical cytoplasm contained extensive Golgi lamellae and dense bodies. $\times 9,300$.

FIGURE 21 A small follicle in a whole primordium after 3 days of culture in medium containing 100 $\mu\text{g/ml}$ LACA. The luminal surface was covered with microvilli, and the lumen (*L*) was filled with a dense particulate material. Note the close association between the Golgi regions and the apical cell surfaces. A space (*S*) containing amorphous material is seen at the bottom right corner. $\times 15,300$.

though it is clear that the cords of cells are formed before the invasion occurs, the source of the stimulus that initiates invasion is unclear. The experiments do not allow us to distinguish between an epithelium-controlled event and a mesenchyme-controlled event, because we cannot distinguish between the drug effects on the two cell types. Penetration of the surface layer could result simply by the continuation of the internal channels until they reach the outside, or it could result from activation of the mesenchyme cells to penetrate the epithelium at stage 29. It might be possible to select from these two alternatives by treating with the drugs after the channels have been initiated but before they reach the periphery. This seems a rather simpleminded approach, however.

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