

VITAMIN-A-INDUCED MUCOUS METAPLASIA

An In Vitro System for Modulating Tight and Gap Junction Differentiation

PETER M. ELIAS and DANIEL S. FRIEND

From the Departments of Pathology and Dermatology, University
of California School of Medicine, San Francisco, California 94143

ABSTRACT

Stratified squamous epithelia from 14-day chick embryo shank skin contain rare tight-junctional strands and only small gap junctions. Exposure of this tissue to retinoic acid (vitamin-A) (20 U/ml) in organ culture, however, induces mucous metaplasia, accompanied by tight-junction formation and gap-junction growth; untreated specimens continue to keratinize. To investigate sequential stages of junctional assembly and growth, we examined thin sections and freeze-fracture replicas at daily intervals for 3 days. During the metaplastic process, tight junctions assemble in midepidermal and upper regions, beginning on day 1 and becoming maximal on day 3. Two tight-junctional patterns could be tentatively identified as contributing to the emergence of fully formed zonulae occludentes: (a) the formation of individual ridges along the margins of gap junctions; (b) *de novo* generation of continuous ramifying strands by fusion of short strand segments and linear particulate aggregates near cellular apices. Gap junction enlargement, already maximal at day 1, occurs primarily three to four cell layers deep. Growth appears to occur by annexation of islands of 20–40 8.5-nm particles into larger lattices of islands separated by particle-free aisles. Eventually, a single gap junction may occupy much of the exposed membrane face in freeze-fractured tissue, but during apical migration of the cells such junctions disappear. The vitamin-A chick-skin system is presented as a responsive model for the controlled study of junction assembly.

The tight junction (zonula occludens) is a barrier to large molecule exchange between the lumina and intercellular spaces of epithelial tissues. Forming a continuous belt around the apices of polarized epithelia, it is visualized in thin section as a series of pentalaminar fusions between adjacent cells, and in freeze-fractured specimens as a network of fibrils or ridges and complementary furrows on the A and B faces, respectively (reviewed in references 23, 36, and 48). Whereas the

distribution of this junction (16, 19, 43), its architecture (9, 19, 25, 30), permeability properties (10, 19, 20), breakdown (48), and presumed mode of growth (37) have been described, a relatively simple laboratory model for inducing tight junctions in an epithelium normally deficient in such cellular components has been heretofore unavailable.

The gap junction (nexus) is a highly ordered plasma membrane specialization considered essen-

tial for electrotonic coupling (6, 21, 46), metabolic cooperation (22, 46), and the regulation of growth and differentiation (23, 31, 36). Gap junctions consist of rectilinear particle arrays packed into lattices which span the intercellular space, linking adjacent cells without impeding the movement of small tracers within that space (8, 35, 42). The development of gap junctions has been studied both *in vivo* (5, 11, 43) and *in vitro* (27). From these studies, the following picture of gap junction assembly has emerged. First, a relatively clear area of membrane appears (formation plaque), followed by loose aggregation of particles into rows or clusters within the membrane, and then by more characteristic packing into hexagonal arrays (5, 11, 27). The fully formed gap junction remains surrounded by a relatively particle-free zone.

Mucous metaplasia of several keratinizing epithelia, most notably embryonic avian skin (14, 17), can be induced by vitamin A compounds *in vivo* and, especially, *in vitro*. Without stimulating cell division *per se* (41),¹ vitamin A influences postmitotic suprabasilar cells to abandon their presumptive pathway toward keratinization in favor of differentiation into a glandular, mucus-secreting epithelium. After the addition of vitamin A to cultures of embryonic, featherless shank skin, this normally tight-junction-deficient epithelium becomes mucus secreting, and the progressive emergence of tight junctions between cells forming microscopic acini can then be observed. Concomitant florid proliferation, assembly, and growth of gap junctions occur as well.

MATERIALS AND METHODS

Materials and Organ Culture

Under sterile conditions, shank skin was removed from living 14-day old chick embryos, scraped clean of excess fat and dermis, and incubated dermis-side down in plastic Falcon organ-culture dishes, each containing approximately 1 ml of medium (see below), at 37°C in a humidified (5% CO₂/95% air) atmosphere. A standard concentration of 20 U/ml retinoic acid (Hoffman-LaRoche, Nutley, N. J.; *all-trans* retinoic acid, Sigma Chemical Co., St. Louis, Mo.), found to result reproducibly in mucous metaplasia, was utilized in all experiments. Retinoic acid (18 mg) was initially solubilized in 5 ml absolute ethanol; then 0.1 ml of this solution was added to 2.5 ml fetal calf serum with continuous stirring. A dilution of 1 ml vitamin-A-containing serum in 19 ml

¹ Peck, G. L., P. M. Elias, and B. Wetzel. Submitted for publication.

medium (Weymouth's Pacific Biological Co., Berkeley, Calif.) produces the desired concentration of retinoic acid. Weymouth's medium was selected for these experiments because it is enriched and contains no vitamin A. All solutions were prepared in semidarkness (the acid is light sensitive), freshly made on the first day of experiments, and subsequently stored in air- and light-tight containers at 4°C. Both treated and control solutions were changed after 24 h and again at 48 h. Parallel flasks of vitamin-A-treated and control preparations were removed at 0, 24, 48, and 72 h (1, 2, and 3 days). A total of eight separate experiments were performed, and all were terminated after 72 h. Material from each flask was then divided into three portions and processed as below for light-microscope histochemistry, thin-section microscopy, and freeze-fracture. As assessed histochemically and ultrastructurally, vitamin A elicited a similar sequence of events in every experiment.

Tissues maintained in organ culture evidenced no deterioration and differentiated in the same way as *in ovo* samples at comparable time periods.

Light and Electron Microscopy

For light-microscope histochemical procedures, samples were fixed in Bouin's solution, embedded in paraffin, and stained with colloidal iron. For electron microscope studies, tissue preparation was the same as that described previously for mammalian epidermis (15). For transmission electron microscopy, sample specimens were fixed in cacodylate-buffered glutaraldehyde, postfixed in Veronal-buffered osmium tetroxide, embedded in Epon, and stained with uranyl acetate and lead citrate. Samples for freeze-fracturing were fixed for 1–2 h in cacodylate-buffered glutaraldehyde, cryoprotected in 25% glycerol in cacodylate buffer, mounted on cardboard disks, fractured at –115°C in a Balzer's Freeze-Etch apparatus (Balzer's High Vacuum Corp., Santa Ana, Calif.), and replicated with platinum-carbon. Thin sections and replicas were examined and photographed with Siemens 1A and 101 electron microscopes at 80 kV.

OBSERVATIONS

Organization of the Epithelium in Control Cultures (Table I)

PERIDERM: The skin of the metatarsal shank of the 14-day old chick embryo (hatching occurs between days 20 and 21) consists of a two-cell layer-deep periderm overlying a four- to six-cell layer-deep stratified squamous epithelium (Fig. 1, cf. Fig. 2). Cells of the outer layer of periderm are linked by junctional complexes which encompass almost the entire lateral surfaces of these cells and consist of zonulae occludentes (tight junctions), zonulae adherentes (intermediate junctions), and

maculae adherentes (desmosomes) (Fig. 3). No gap junctions were recognized in our preparations.

Between cells of the second layer of periderm, only incomplete zonulae occludentes are present. As in the outer layer, these also enclose desmosomes, and gap junctions are absent. Here the periderm is loosely attached (Fig. 1) to the underlying epithelium (Fig. 4).

Periderm structural integrity and differentiation are retained during maintenance in organ culture, remaining comparable to periderm morphology in ovo. In both situations, the periderm exhibits few changes until it is finally shed subsequent to keratinization of the underlying epidermis (i.e., between days 18 and 19 in ovo, or between the 4th and 5th days of culture in 14-day embryonic skin).

EPIDERMIS (SAMPLES OBTAINED BEFORE CULTURE): In 14- and 15-day (14 days in ovo + 1 day in vitro) control preparations, cell junctions infrequently occur between apical squames. When present, these consist of small gap junctions abutting against short tight-junctional strands (Figs. 5, 6). By the 3rd day in vitro, with the onset of keratinization (Fig. 4), such junctional configurations disappear as the membranes of apical squames thicken (Fig. 8). The smooth plasma membrane faces of keratinizing cells are punctuated only by large, irregular desmosomal aggregates (Figs. 4, 8). Beneath keratinizing layers squames continue to be joined by desmosomes and small gap junctions (Fig. 7), although tight junction strands are absent (Table I). The morphological events accompanying keratinization in these experiments conformed to previous descriptions of thin sections of avian keratinization both in vitro (18) and in ovo (32, 38, 40).

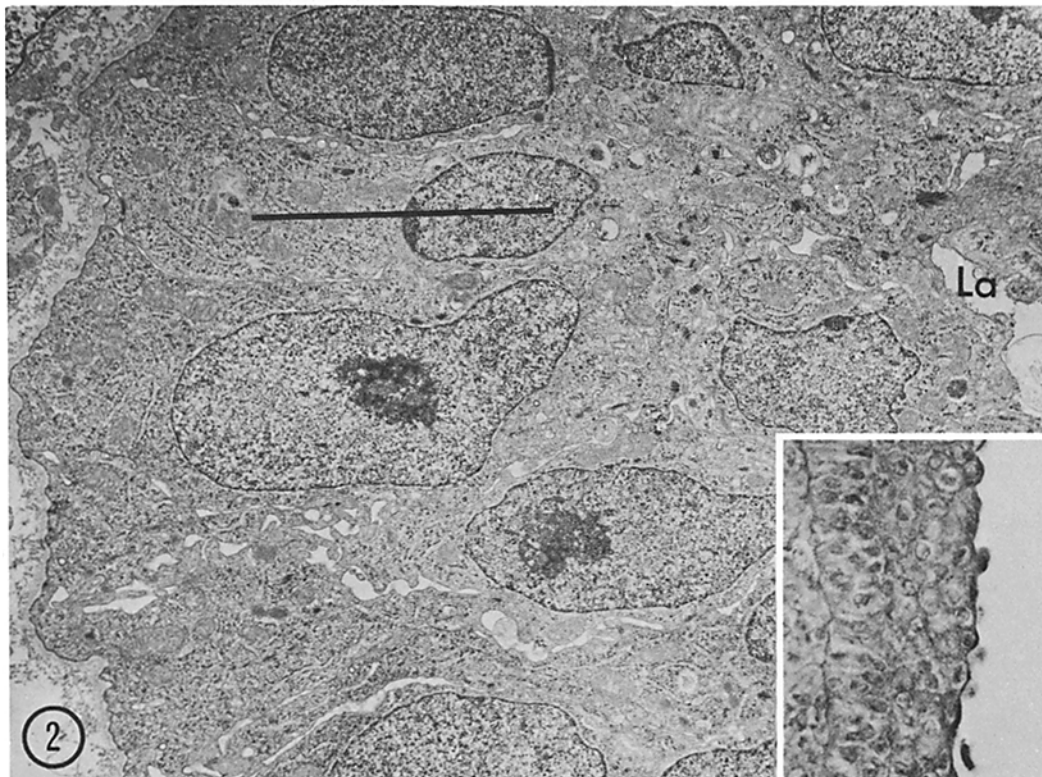
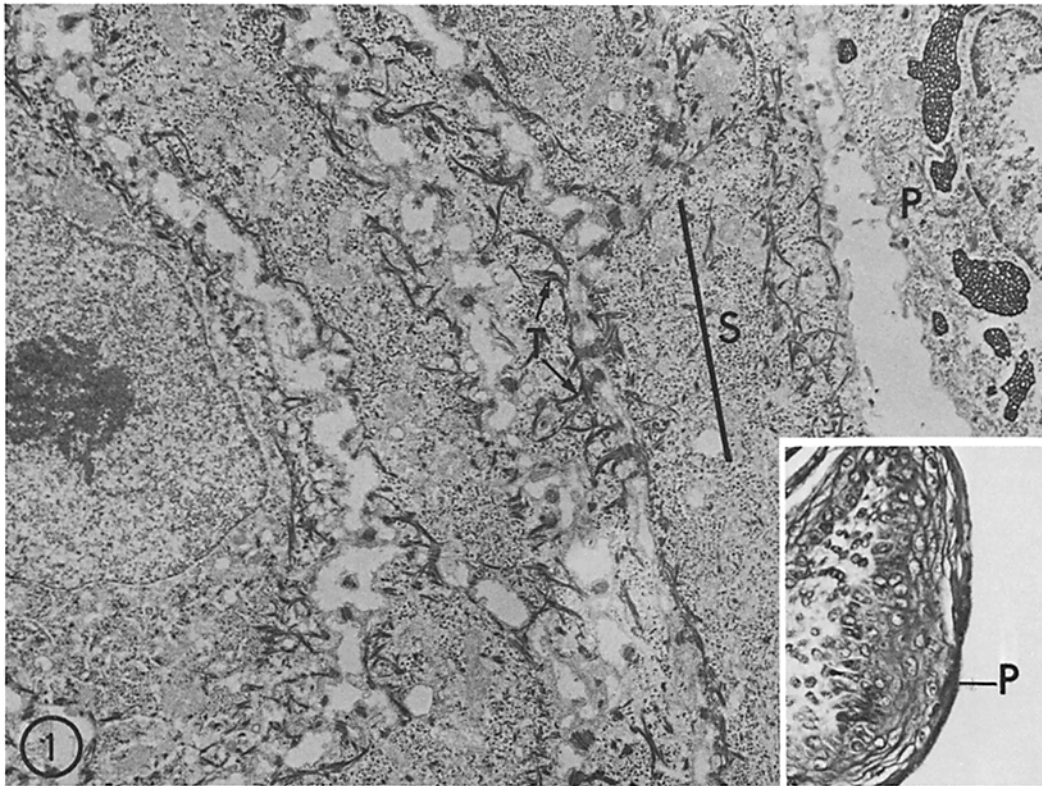
Differentiation in the Presence of Vitamin A

GENERAL ARCHITECTURE: As differentiation progresses, from the 1st to the 3rd day of organ-culture (days 14 through 16 in development), three striking differences become evident in sections of the above-described tissue. Initially, accelerated shedding of the periderm occurs, with sloughing between the 2nd and 3rd days (vs. 4 or 5 days in control cultures). Next, developing epidermal suprabasilar cells proliferate focally and undergo transformation from squamous to cuboidal contour, accompanied by a loss of distinct cell stratification (Fig. 2). Other investiga-

tors have shown that glycogen and mucus granules progressively replace tonofilaments, keratohyalin, and surface-coating granules in these metaplastic microvilli-bearing superficial cells (18, 40). After loss of the periderm (between 48 and 72 h), these apically located epidermal cells form abortive glands and/or desquamate individually from the outer surface. In contrast to development in control cultures, cornification does not take place. The third notable difference involves the type, distribution, and size of cell junctions (Table I). Zonulae occludentes emerge in the superficial layer of cells (Figs. 9-16), gap junctions enlarge (most predominantly at a depth of three to four cells from the top) to extremely broad proportions (Figs. 17, 18), and focal pentalaminar fusions appear in the region of gap junctions in subapical portions of the membranes (Figs. 14-16). The rate at which these features become evident is vitamin-A dose dependent as well as time dependent, but the events are not concurrent. Thus, early and late changes often occur at different times in adjacent areas of the same specimen.

Tight Junction Development (Figs. 9-16, cf. Fig. 19)

EARLY STAGES: Tight junction generation generally lagged behind gap junction proliferation, becoming maximal at 3 days, while large gap junctions abounded even in 1-day cultures (see below). Although two patterns of formation predominated, this interpretation is tentative. A more definitive presentation of the early events in tight junction formation thus far has been thwarted by a tendency for early junctional elements to fragment, leaving A-face particles adherent to the B face. As early as day 1, increased focal pentalaminar fusions are seen in thin sections, while short strands appear in apical zones of fractured specimens. In early cultures, these strands are not oriented with respect to cell polarity. In later cultures, short strands are still found, but ramifying networks of ridges begin to predominate. Axially, these networks are smooth, but basally and peripherally they consist of brief, discontinuous segments or lines of particles (Fig. 9). The B face often reveals particle-free grooves in relief, suggesting that the junctions enlarge by accretion of these discontinuous elements into adjacent, more continuous strings, and that junctions are



generated from linear particulate arrays arising *de novo* in the membrane (Fig. 9, *inset*).

A second pattern of strand formation occurs along the margins of proliferating gap junctions in midepithelial regions. Here strands often grow to encircle much of the gap junction. Gap junctions appear to provide the nidus for tight-junction strand assembly because: (a) gap-junction growth and proliferation precede the appearance of tight-junction elements; (b) gap junctions are often found without encircling strands in these regions; and (c) isolated tight-junction elements are not found in this area. As the cells migrate apically, the gap junctions disappear. Presumably, the pre-formed tight-junction strands persist, to be incorporated into forming tight junctions.

LATE STAGES: In 3-day specimens, multiple pentalaminar fusions typical of tight junctions lie at the apices of superficial cells (Figs. 10–13). Three to five ridges comprise the average array seen in fracture. Their disposition is now circumferential, dividing the heavily particulate microvillar portion of the plasma membrane and the less particulate lateral portion.

Gap Junction Proliferation (Table I)

On day 1 of organ culture, longer, seven-layered membrane appositions with periodic substructure appear between squamous cells (Fig. 18) in thin sections. In freeze-fractured preparations, loose aggregates of up to 50 8-nm particles (or complementary B-face pits) surrounded by relatively

particle-free “halos” (Fig. 17) occupy regions coincident with the heptalaminar membrane appositions seen in thin section. We noted no tails and strands, such as may attend the formation of gap junctions in other embryonic tissues (2, 5, 11) in this region, i.e. three to four cell layers deep. However, since this process was already at its zenith on day 1, at a time when tight junction formation was just commencing (Table I, Fig. 19), the earliest changes in gap junctions may have been missed.

The large gap junction comprises a latticework of angular islands consisting of 20–40 particles incompletely segregated by particle-free domains of rather constant width (Fig. 17). These particulate islands, however, are seldom wholly circumscribed by areas lacking particles—usually they span the smooth areas at some point via bridges of particles. Gap junctions appear to grow by the steady accretion of more islands and smaller island congregations until the junctions occupy much of the exposed fracture face and surface (Figs. 17, 18). Gap junctions between basal cells, apparently unaffected by the metaplastic process, can be distinguished from vitamin A-induced junctions by their diminutive size and their packing without islands and channels.

DESMOSOMES: Although thin sections convey the impression that desmosomes dwindle in number in response to vitamin A, in fractured specimens desmosomal profiles remain both plentiful and prominent (Figs. 10, 15). Desmo-

Abbreviations:

<i>A</i>	A face	<i>La</i>	lacuna
<i>B</i>	B face	<i>P</i>	periderm
<i>D</i>	desmosome	<i>t</i>	tonofilament
<i>G</i>	gap junction	<i>pa</i>	particle aggregates
<i>L</i>	lumen	<i>S</i>	squamous cell

FIGURE 1 Electron micrograph of chick embryo shank skin, 2-day control culture. The periderm, with its characteristic granules, is loosely attached to the underlying epithelium. Keratinization of flattened squames has not yet begun, and desmosomes, tonofilaments, and polyribosomes abound. The bar denotes horizontal orientation of the squames. *Inset:* Light micrograph showing the organization of the squamous epithelium and overlying periderm. $\times 7,500$; *inset*, $\times 800$.

FIGURE 2 Electron micrograph of chick embryo shank skin, 2-day vitamin-A-treated culture. Presumptive keratinizing squamous epithelium has been converted to a cuboidal/columnar arrangement with an attendant 90° shift in polarity, as represented by the bar (cf. Fig. 1). In apical regions, intercellular lacunae broaden and excavate. Tonofilaments diminish strikingly, but desmosomes are still plentiful. *Inset:* Light micrograph of colloidal iron-stained, paraffin-embedded section reveals hyperplasia, loss of distinct stratification, and rounding of apical cells, but no apparent gland formation. The periderm is absent, having been shed prematurely after vitamin-A treatment. $\times 6,500$; *inset*, $\times 1,000$.

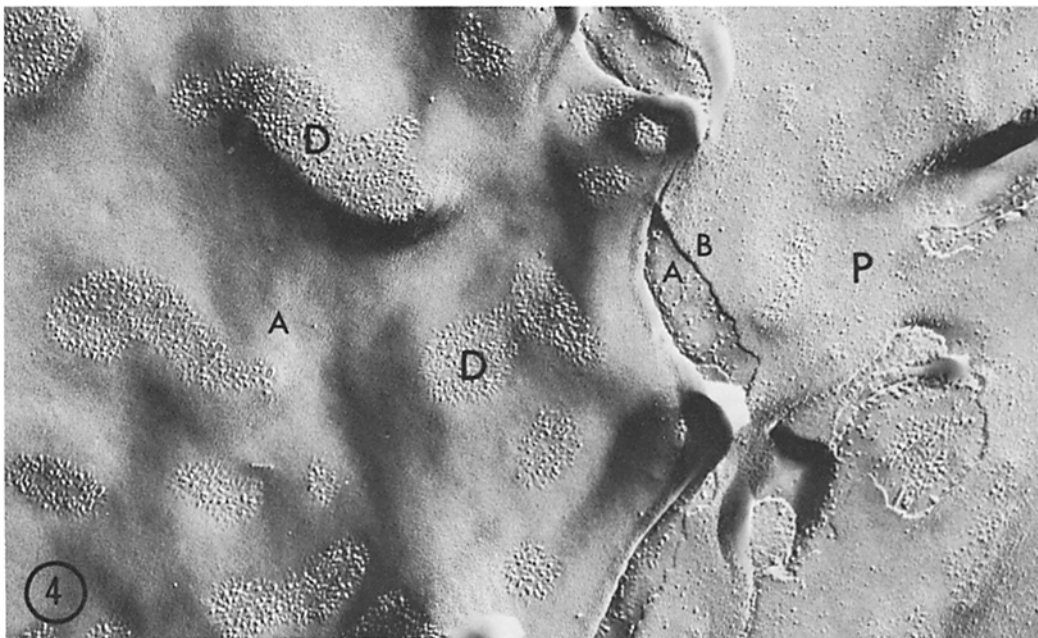
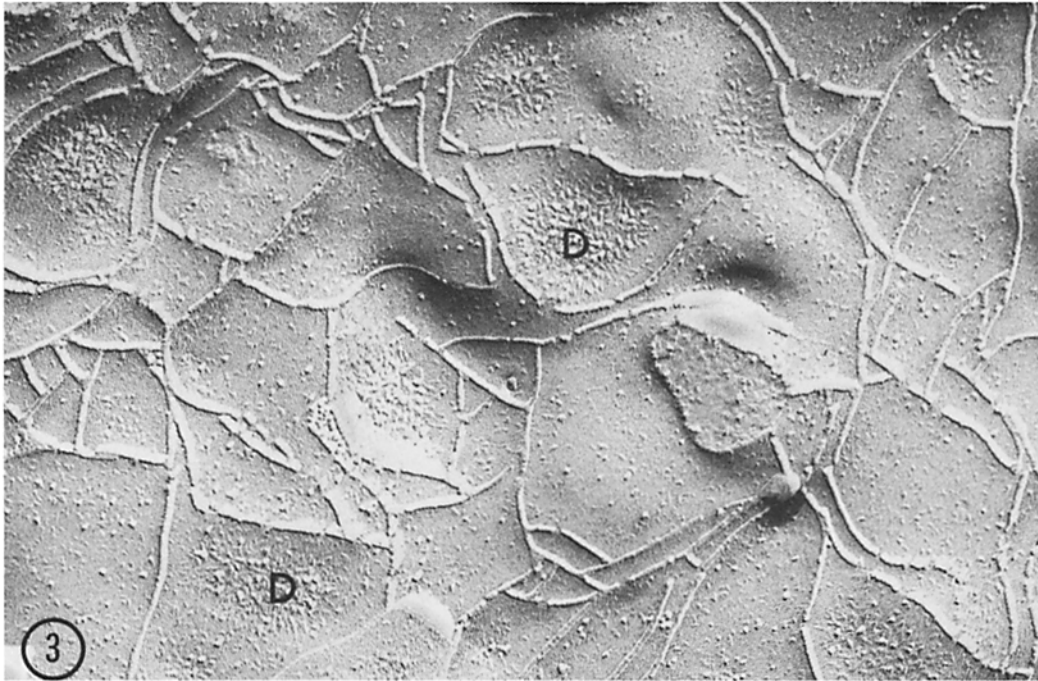


FIGURE 3 Freeze-fracture replica of chick embryo shank skin from the periderm, 2-day control culture. In this and in subsequent replicas, the direction of shadowing is upward. Adjacent cells of the apical layer of a two cell layer-thick periderm are linked by festooning tight-junctional elements deployed in a wreathlike radial arrangement around desmosomes. Most of the lateral surfaces are occupied by such tight junction-desmosomal elements; no gap junctions were noted in our material. $\times 64,000$.

FIGURE 4 Chick embryo shank skin from the epidermis, 3-day control culture. Keratinization occurs between days 2 and 3 in vitro. Membranes of the apical squames display desmosomal aggregates enveloped by particle-free membrane regions. To the right, the basal portion of the plasma membrane of an overlying peridermal cell contains more particles between desmosomes seen on the A and B faces. $\times 40,000$.

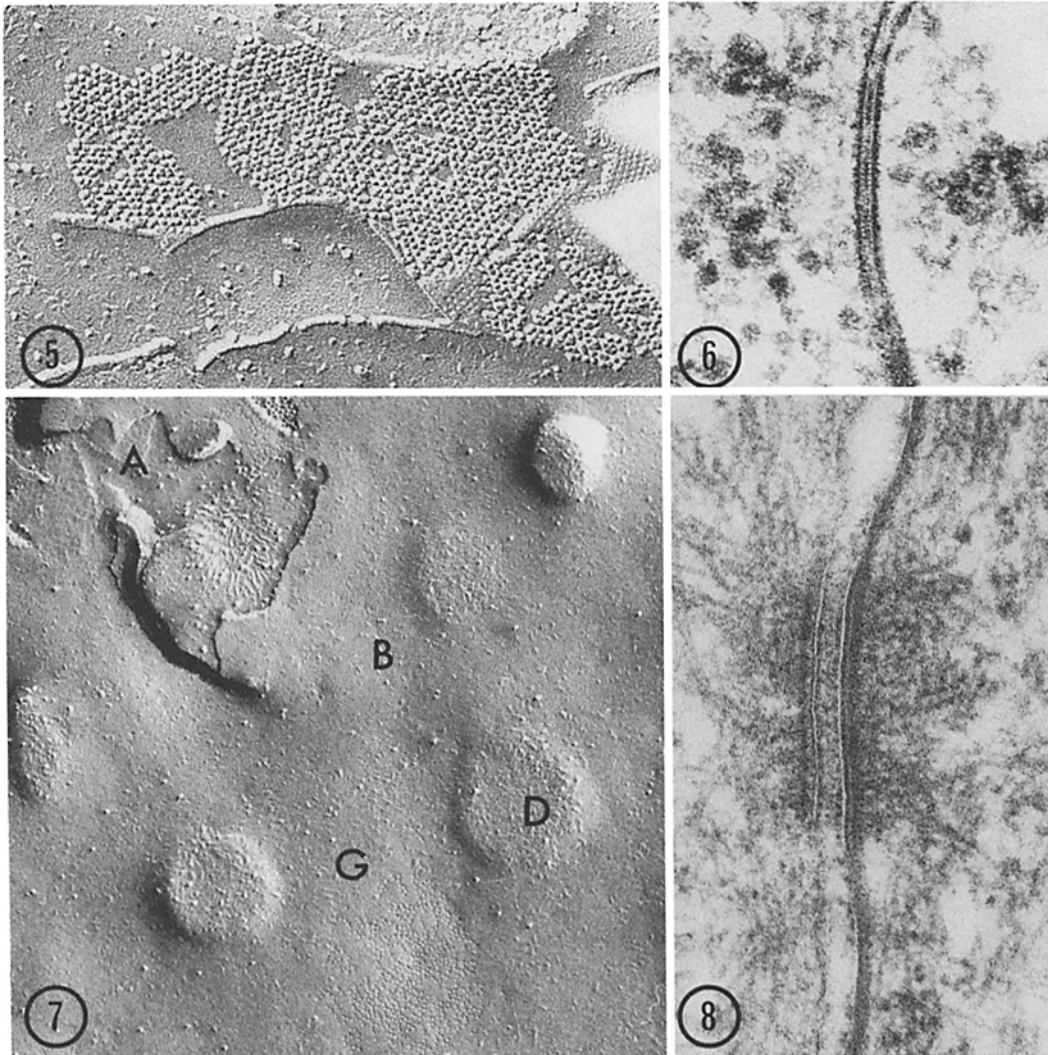


FIGURE 5 Chick embryo shank skin from the epidermis, 2-day control culture. Between apical squames, a gap junction reveals a few strands along the margin, and a typical particle-free halo (11, 24) near islands of hexagonally packed particles. Numerous small gap junctions nestle beneath apical zones, but they are not accompanied by ridges such as these. (Fully formed occluding zonules were not encountered.) $\times 87,000$.

FIGURE 6 Chick embryo shank skin from the epidermis, 1-day control culture. The cross section of a gap junction exhibits periodic, dark, intramembranous striations which correspond in dimension and spacing to gap-junction particles in freeze-fracture replicas. $\times 132,000$.

FIGURES 7 and 8 Chick embryo shank skin from the epidermis, 2-day control cultures. Desmosomes in replica (Fig. 7) reveal fairly flat A faces and protruding, particle-poor B faces. The adjacent thin section (Fig. 8) depicts similar asymmetrical bowing of the plasma membrane in the desmosomal region. Fig. 7, $\times 82,000$; Fig. 8, $\times 80,000$.

somes in control and vitamin A-treated specimens display identical structural features. Desmosomes, spaced at uniform intervals along fractured surfaces, consist of heterogeneous

clusters of irregularly sized particles and filamentous profiles (36). In these fixed specimens, A-face arrays are more particle rich than their B-face counterparts, but both become progres-

TABLE I
Junctional Alterations (Type and Quantity) during Incubation

Culture	Day	Tight junctions	Gap junctions
Vitamin-A-treated	0	Occasional apical strand	Small, widespread
	1	{ Strands along margins of expanding gap junctions only	{ Proliferating, greatly expanded, middle layers only
	2	{ Strands along margins of expanding gap junctions, occluding zonules forming apically	{ Proliferating, greatly expanded, middle layers only
	3	{ Strands along margins of expanding gap junctions, occluding zonules in all stages of formation	{ Proliferating, greatly expanded, middle layers only
Control (non-treated)	0-2	Rare tight-junctional strands apically	Small, widespread
	3	None	{ Small, primarily basal and midepidermal layers

sively more distinct during suprabasilar migration. Particulate clusters often surmount circular plateaus or lie in depressions on either the A or the B face. As we have related, desmosomes form a distinctive association with the short tight-junctional strands and enlarging gap junctions in mid-epithelial regions.

DISCUSSION

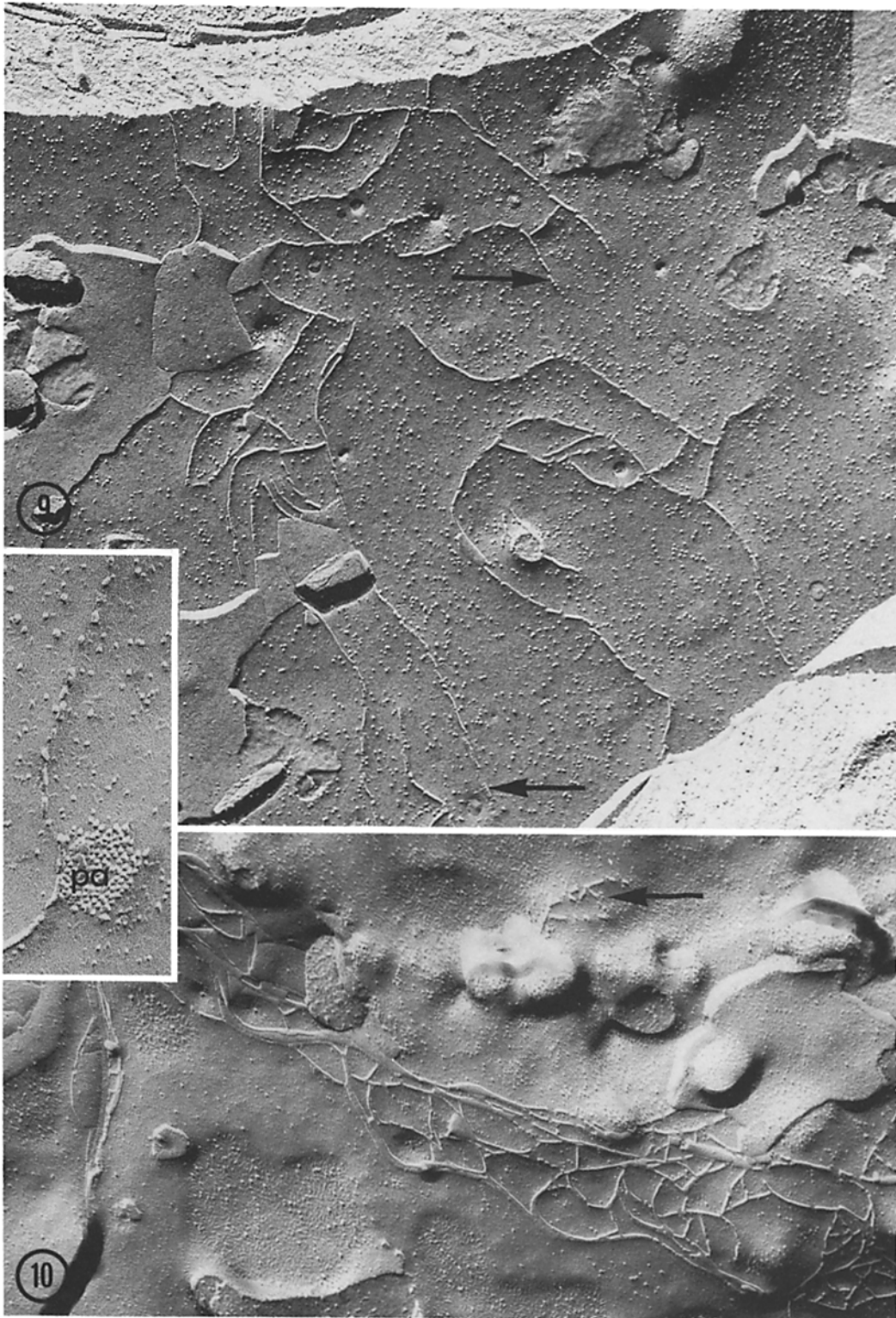
The Vitamin-A Model System

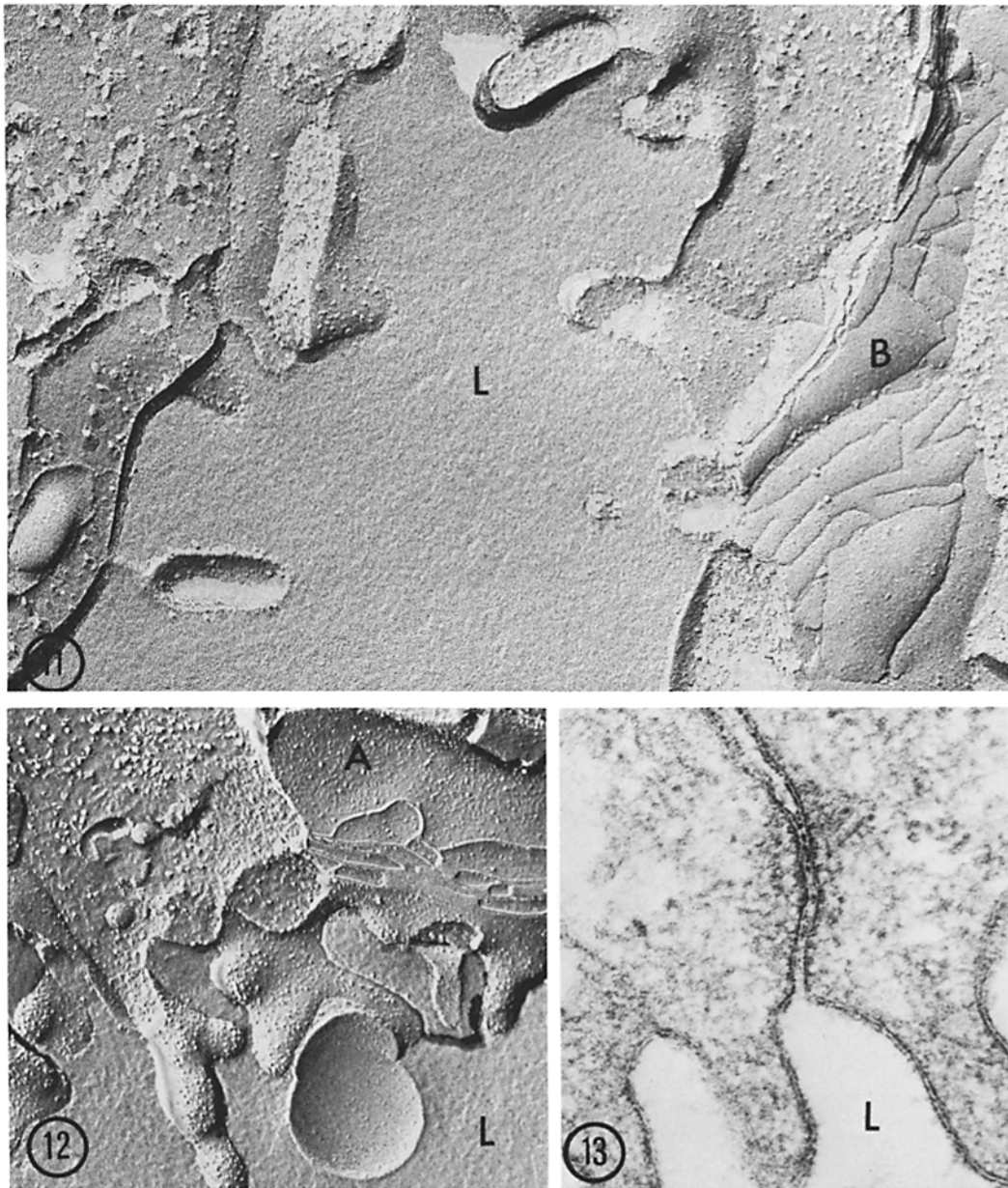
Although several experimental manipulations of cell junction assembly have been performed in this

study, the morphological details presented here are primarily intended to offer an *in vitro* model system for examining such a junctional assemblage rather than a definitive description of these processes. The apparent advantages of using chick embryonic skin are: (a) the tissue is readily obtained and easily kept in organ cultures; (b) tight junction formation evidently occurs *de novo*; (c) the various stages of tight-junction dilution and degradation can be observed as separate processes because of the known direction of migration of the cells involved, and because of the paucity of tight junctions in the starting tissue; (d) extensive areas

FIGURE 9 Chick embryo shank skin from the epidermis, 2-day vitamin-A culture. An extensive, incomplete tight junction with predominantly smooth, ramifying strands in the axial portion of the cell. Beaded linear arrays (arrows) are in continuity with the free ends of the strands. *Inset: De novo* generation of strands appears to occur in the vicinity of particle aggregates often lacking the characteristic hexagonal packing and B-face pits of gap junctions. Strands seem to form here by the confluence of short segments and particles arising from slight, ridge-like elevations of the A face: $\times 55,000$; *inset*, $\times 77,000$.

FIGURE 10 Chick embryo shank skin from the epidermis, 3-day vitamin-A culture. A completely formed occluding zonule at a three cell juncture. The A face *beneath* the tight junction contains large desmosomes, while above the junction a portion of the cell already in a state of desquamation reveals disintegrating tight-junctional elements. Dismemberment of strands into curt, disconnected fragments was encountered in apical sites where cells had partially or completely detached from the underlying epithelium. $\times 43,000$.





FIGURES 11-13 Chick embryo shank skin from the epidermis, mucus-secreting cells, 3-day vitamin-A cultures. Fully formed occluding zonules link apical, microvillar regions of metaplastic cells lining acini. A faces consist of three to five interconnected ridges (Fig. 12), while the B face presents a similar network of particle-free grooves (Fig. 11). Fig. 13 is a comparable region in thin section. Fig. 11, $\times 80,000$; Fig. 12, $\times 47,000$; Fig. 13, $\times 150,000$.

of cell membranes are composed of gap junctions; and (e) the system is amenable to monitoring and modulation by pharmacological, biochemical, and electrophysiological methods. Pertinent to the last point, it may be a good system for correlating gap-

junction size with such parameters as intercellular resistance, the nutritive requirements for junction development and growth, and the receptivity of the epithelium to pharmacologically active agents.

On a subcellular level, vitamin-A is known to

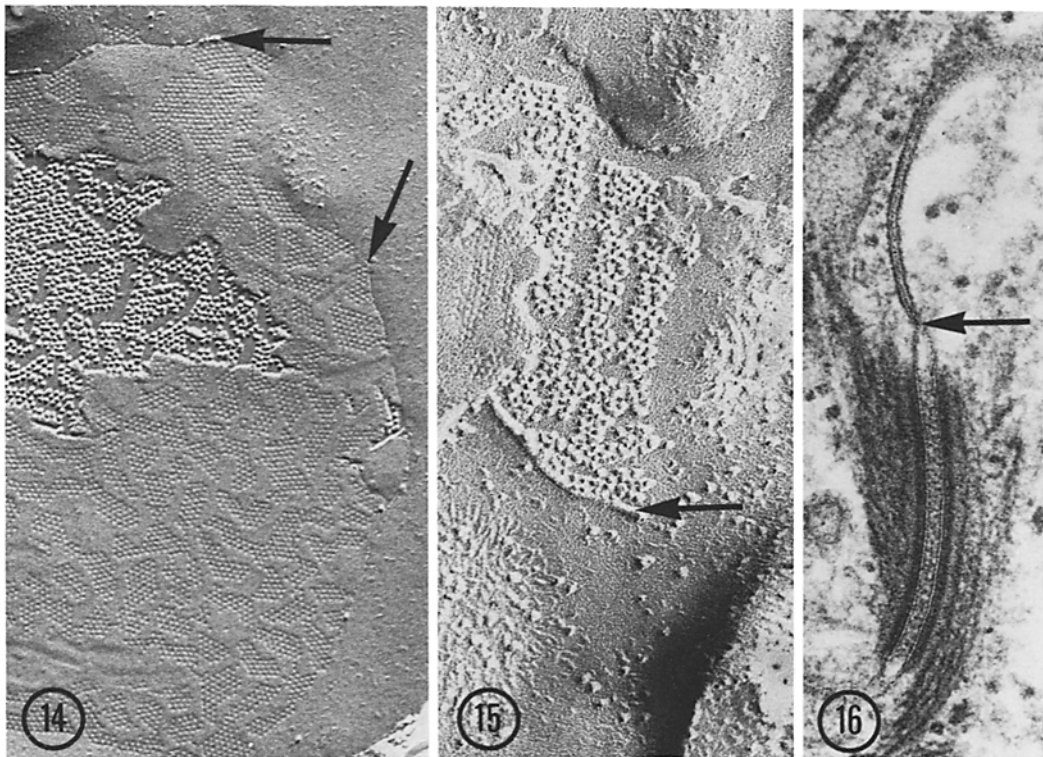


FIGURE 14 Chick embryo shank skin from the epidermis, several cells beneath the surface layer, 3-day vitamin-A culture. Large gap junctions consist of interjoined islands of 20–40 particles each. Continuous strands of varying length form along the margins (arrow). The strands presumably persist during apical migration as gap junctions involute. $\times 96,000$.

FIGURES 15 and 16 Chick embryo shank skin from the epidermis, several cells beneath the surface layer, 3-day vitamin-A cultures. Gap junctions, short strands, and desmosomes form a characteristic cluster of junctional elements. The arrows point to comparable areas seen in freeze-fracture and thin-section preparations. Fig. 15, $\times 88,000$; Fig. 16, $\times 80,000$.

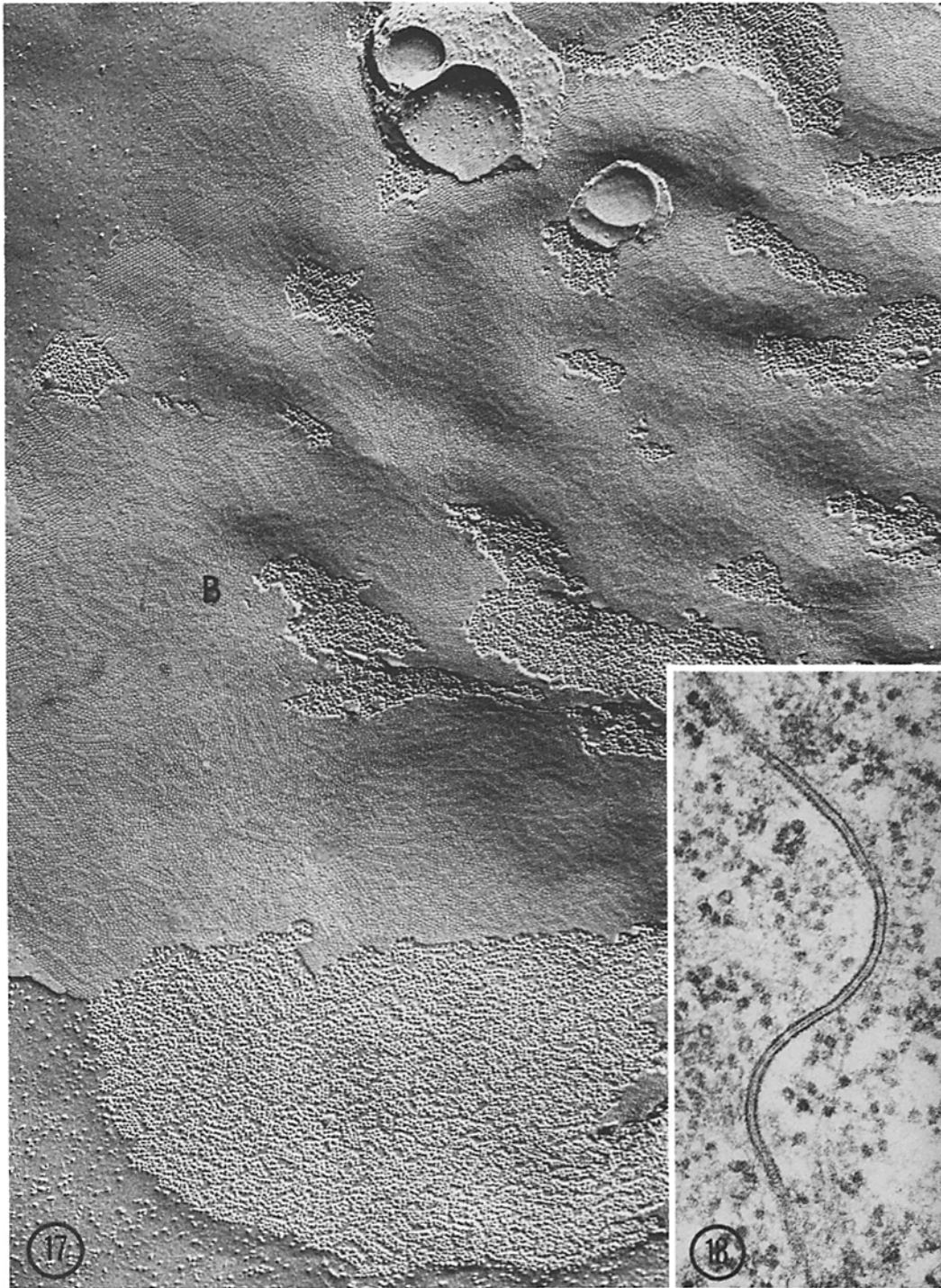
stimulate the synthesis of glycoprotein and RNA (7, 13, 29) and to bind to plasma proteins (28) and certain cytosol fractions (4, 44). No direct effect on the cell membrane has been demonstrated. Since gap-junction proliferation precedes tight-junction formation and impending secretion (Fig. 19), we are tempted to speculate that the vitamin influences postmitotic differentiation (12, 33) by means of an immediate membrane effect. More extensive observations between 0 and 24 h, utilizing ultrastructural and biochemical techniques, will be needed to determine the earlier cellular events and binding sites.

Within 1 day of treating embryonic chick skin with vitamin A, cellular polarity, gap junctional differentiation, and keratinization are profoundly altered. Yet histochemically detectible synthesis of

mucin, the occurrence of secretion, and tight-junction assembly generally trail other metaplastic happenings by an additional 1–2 days (18).¹ A similar sequence of increased secretion and tight-junction proliferation transpires when pancreatic beta cells are treated with pronase (39). It is enticing to consider that tight-junction formation in the vitamin-A system and in others (39) is “coupled” to synthesis of secretion products. The establishment of fully functional tight junctions may be the prelude to secretion.

Tight Junction Assembly

Some of our observations on tight-junction formation coincide with those involving other developmental systems (37, 39). In previously described models, the following sequence is con-



FIGURES 17 and 18 Chick embryo shank skin from the epidermis, 1-day vitamin-A cultures. Gap junction enlargement is not concurrent with development, even at 1 day, and is most prominent three to four cells beneath the surface. Fig. 17 illustrates only a portion of one such extensive junction, and Fig. 18 is a thin section of another ample junction from the same region. Proliferation appears to occur by accretion of small islands to the peripheries of gap junctions (not depicted). (Linear aggregates were not detected.) Fig. 17, $\times 40,000$; Fig. 18, $\times 65,000$.

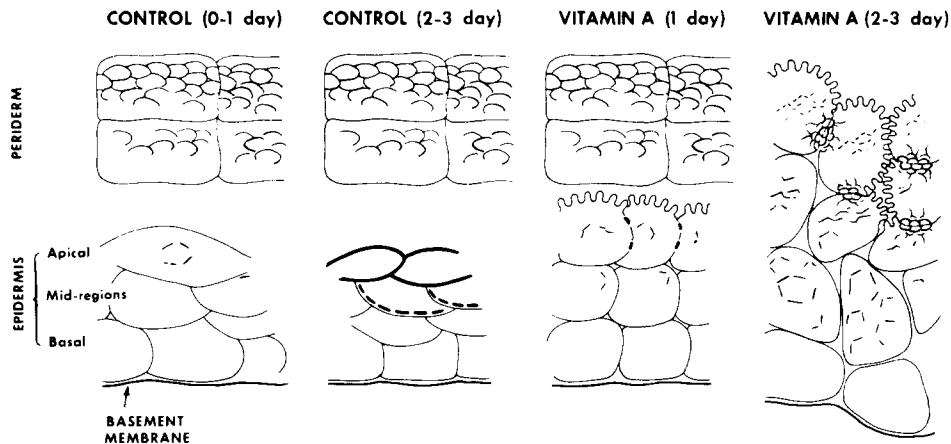


FIGURE 19 Schematic representation of epithelial development in vitamin-A-stimulated and control cultures. Note that the periderm is shed by the 2nd day in experimental cultures. Alterations in membrane structure are particularly emphasized. Only gap junctions (faintly stippled) and tight-junctional elements are diagrammed; desmosomes are not altered by vitamin A.

strued as a common mode of tight junction evolution. A clear area of membrane first develops, succeeded by the appearance of linear aggregates and clusters of small particles within this area. Next, the particles "fuse" or become coated to form discontinuous fibrils or ridges (37); ultimately, the ridges merge into continuity and orientation at the apical margins of the epithelial cells. A similar pattern of particle confluence and strand annexation along the basilar portions of expanding tight junctions was suggested here. However, some features of *in situ* strand assembly were difficult to delineate because fractured tight-junction fragments tend to adhere to the B face in early cultures.

Tight-junctional elements are also generated along the margins of pre-existing gap junctions, an association previously observed in mammalian liver (26) and ovary (3). But in the present study, during upward migration, the gap junctions disappeared, and these prefabricated tight-junction strands may plausibly be incorporated into pre-existent or newly developing tight junctions more apically.

Unlike the majority of epithelia, most vertebrate keratinizing epithelia investigated to date (15, 34) do not depend upon the tight junction as a basis for the permeability barrier. Instead, extruded lamellar material fills the lower regions of these epithelia, producing impervious intercellular seals (15, 34, 45, 47). Avian epidermis is similarly impoverished in tight junctions¹ but here the

morphological basis for impermeability has not been studied. With the stimulus of vitamin A and impending secretion, tight junctions emerge—thus their role in this system is seemingly not related to permeability alone.

Tight-Junction Turnover and Degradation

The vitamin-A system also affords the opportunity for tracing the fate of tight junctions during cellular proliferation, detachment, and degeneration. On the basis of its polarized structure and the enhanced upward migration and desquamation in response to vitamin A, we can reliably link the various cellular events to stages of assembly or decay. In most other embryonic and regenerating systems (11, 43, 48), the emergence, dilution, and decay of tight junctions are admixed, making more difficult the determination of whether a specific image represents assembly or degeneration. Although it is not the focus of this paper, we coincidentally witnessed a pattern of tight-junctional decay into short, disconnected segments during desquamation (Fig. 10); this disintegration very much resembled that observed in separated pancreatic acinar and intestinal epithelial cells (reviewed in reference 48). Furthermore, unlike other systems investigated, wherein endocytosis is influential in the removal of decomposing tight junctions (48), this system does not appear to possess the apparatus for endocytosis. Large coated vesicles are not seen (18),¹ nor are junc-

tional fragments found within lysosomal elements in desquamating cells. Junction disassembly can therefore be followed to a greater extent within the plasma membrane itself. Finally, since the vitamin A effect is reversible (17),¹ the process of junctional breakdown could be easily and comprehensively studied in orderly sequence.

Gap-Junction Proliferation

One of the most striking features of this study was the great proliferation of gap junctions in response to vitamin A. Gap junctions in the mammalian ovary are similarly responsive to hormonal stimulation (1-3), but they never expand to the proportions encountered in these experiments. As well as can be surmised from micrographs, in both systems growth seems to occur by peripheral accretion of smaller congregations of particles.

It is notable that gap-junction proliferation occurred during the course of early metaplasia, clearly preceding tight-junction generation by 1-2 days. Large gap junctions were also found in later cultures, but at these times many appeared to serve as sites of origin for tight-junction strand formation. During apical migration, the gap junctions disappear and tight-junction strands persist. This view of the gap junction as a nidus for tight-junction formation opposes the sequence formerly proposed by Decker and Friend (11), who observed that tight junctions provide a framework for gap-junction generation in neurulating amphibian embryos. Conceivably, a pool of membrane particles is shared by both types of junction, with redistribution toward either type, depending on the particular stimulus.

Some of the particle aggregates found within the angles of tight junctions, both here (Fig. 9, *inset*) and in other systems (3, 11), lack hexagonal packing of particles and B-face pits. The function of such aggregates is unknown. As recently noted by Albertini et al., particle aggregates do not invariably connote gap junctions (3). Again, these particulate groups could serve as a storehouse for tight-junction particles in the process of generation.

In conclusion, the vitamin-A system is a simple model for the detailed perusal of the evolution, growth, assembly, and involution of tight and gap junctions. Difficulties in distinguishing formation from breakdown are largely circumvented because of the intrinsic single-directional dynamics of the

metaplastic process—a feature which lends this paradigm advantage over a number of other developmental systems. We hope that our preliminary account of junctional evolution in embryonic chicken epithelium will provoke further exploration of vitamin-A-stimulated metaplasia.

We gratefully acknowledge the generous contributions of facilities and thought by Dr. Joel Elias, as well as the adroit editorial aid of Rosamond Michael.

Portions of this work were presented at the Fifteenth Annual Meeting of the American Society for Cell Biology, San Juan, Puerto Rico, November 11-14, 1975.

This study was supported by grants from the United States Public Health Service, National Institutes of Health (HD 06895-03 and GM 57053-01) to Dr. Friend and Dr. Elias, respectively, and a Dermatology Foundation Fellowship to Dr. Elias.

Received for publication 9 June 1975, and in revised form 18 September 1975.

REFERENCES

1. ALBERTINI, D. F., and E. ANDERSON. 1974. The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. *J. Cell Biol.* **63**:234-250.
2. ALBERTINI, D. F., and E. ANDERSON. 1975. Structural modifications of lutein gap junctions during pregnancy in the rat and the mouse. *Anat. Rec.* **181**:171-194.
3. ALBERTINI, D. F., D. W. FAWCETT, and P. J. OLDS. 1975. Morphological variations in gap junctions of ovarian granulosa cells. *Tissue Cell* **7**:389-405.
4. BASHOR, M. W., D. O. TOFT, and F. CHYTIK. 1974. In vitro binding of retinol to rat-tissue components. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3483-3487.
5. BENEDETTI, E. L., I. DUNIA, and H. BLOEMENDAL. 1974. Development of junctions during differentiation of lens fibers. *Proc. Natl. Acad. Sci. U. S. A.* **71**:5073-5077.
6. BENNET, M. V. L. 1973. Function of electrotonic junctions in embryonic and adult tissues. *Fed. Proc.* **32**:65-75.
7. BONANNI, F., and L. DELUCA. 1974. Vitamin A-dependent fucose-glycopeptide from rat tracheal epithelium. *Biochem. Biophys. Acta.* **343**:632-637.
8. BRIGHTMAN, M. W., and T. S. REESE. 1970. Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* **47**:666-688.
9. CHALCROFT, J. P., and S. BULLIVANT. 1970. An interpretation of liver cell membrane and junction structure based on observation of freeze-fracture replicas of both sides of the fracture. *J. Cell Biol.* **47**:49-60.
10. CLAUDE, P., and D. A. GOODENOUGH. 1973. Frac-

- ture faces of zonulae occludentes from "tight" and "leaky" epithelia. *J. Cell Biol.* **58**:390-400.
11. DECKER, R. S., and D. S. FRIEND. 1974. Assembly of gap junctions during amphibian neurulation. *J. Cell Biol.* **62**:32-47.
 12. DELUCA, L., and G. WOLF. 1972. Mechanism of action of vitamin A in differentiation of mucus-secreting epithelia. *Agriculture and Food Chemistry*. **20**:474-476.
 13. DELUCA, L., and S. H. YUSPA. 1974. Altered glycoprotein synthesis in mouse epidermal cells treated with retinyl acetate *in vitro*. *Exp. Cell Res.* **86**:106-110.
 14. DODSON, J. 1967. The differentiation of epidermis. II. Alternate pathways of differentiation of embryonic chicken epidermis in organ culture. *J. Embryol. Exp. Morphol.* **17**:107-117.
 15. ELIAS, P. M., and D. S. FRIEND. 1975. The permeability barrier and pathways of transport in mammalian epidermis. *J. Cell Biol.* **65**:180-191.
 16. FARQUHAR, M. G., and G. E. PALADE. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375-412.
 17. FELL, H. B., and E. MELLANBY. 1953. Metaplasia produced in cultures of chick ectoderm by high vitamin A. *J. Physiol.* **119**:470-488.
 18. FITTON-JACKSON, S., and H. B. FELL. 1963. Epidermal fine structure in embryonic chicken skin during atypical differentiation induced by vitamin A in culture. *Dev. Biol.* **7**:394-419.
 19. FRIEND, D. S., and N. B. GILULA. 1972. Variations in tight and gap junctions in mammalian tissues. *J. Cell Biol.* **53**:758-776.
 20. FRÖMTER, E., and J. DIAMOND. 1972. Route of passive ion permeation in epithelia. *Nat. New Biol.* **235**:9-13.
 21. FURSHPAN, E. J., and D. O. POTTER. 1968. Low resistance junctions between cells in embryos and tissue culture. In *Current Topics in Developmental Biology*. A. A. Moscona and A. Monroy, editors. Academic Press, Inc. New York. 3:95-127.
 22. GILULA, N. B., O. R. REEVES, and A. STEINBACH. 1971. Metabolic coupling, ionic coupling, and cell contacts. *Dev. Biol.* **26**:627-636.
 23. GILULA, N. B. 1974. Junctions between cells. In *Cell Communication*. R. P. Cox, editor. John Wiley & Sons, Inc., New York. 1-29.
 24. GOODENOUGH, D. A., and N. B. GILULA. 1974. The splitting of hepatocyte gap junctions and zonulae occludentes with hypertonic disaccharides. *J. Cell Biol.* **61**:575-590.
 25. GOODENOUGH, D. A., and J.-P. REVEL. 1970. A fine structural analysis of intercellular junctions in the mouse liver. *J. Cell Biol.* **45**:272-290.
 26. GOODENOUGH, D. A., and STOECKENIUS, W. 1972. The isolation of mouse hepatocyte gap junctions. *J. Cell Biol.* **54**:646-656.
 27. JOHNSON, R., M. HAMMER, J. SHERIDAN, and J.-P. REVEL. 1974. Gap junction formation between reaggregated Novikoff hepatoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4536-4540.
 28. KANAI, M., A. RAZ, and DE W. S. GOODMAN. 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47**:2025-2044.
 29. KAUFMAN, D. G., M. S. BAKER, J. M. SMITH, M. B. SPORN, F. E. JACKSON, and U. SAFFIOTTI. 1972. RNA metabolism in tracheal epithelium: alteration in hamsters deficient in vitamin A. *Science (Wash. D. C.)* **177**:1105-1108.
 30. KREUTZIGER, G. O. 1968. Freeze-etching of intercellular junctions of mouse liver. In *Proceedings of the 26th Annual Meeting of the Electron Microscopy Society of America*. Claitor's Publishing Division, Baton Rouge, La. 234-235.
 31. LOWENSTEIN, W. R. 1966. Permeability of membrane junctions. *Ann. N. Y. Acad. Sci.* **137**:441-472.
 32. MATOLTSY, A. G. 1969. Keratinization of the avian epidermis: an ultrastructural study of the newborn chick skin. *J. Ultrastruct. Res.* **29**:438-458.
 33. MCMAHON, D. 1974. Chemical messengers in development: a hypothesis. *Science (Wash. D. C.)* **185**:1012-1021.
 34. MCNUTT, N. S. 1973. Freeze-etch study of plasma membrane differentiation and junctions in a keratinized stratified squamous epithelium. *J. Cell Biol.* **59**(2,Pt.2):210 a. (Abstr.).
 35. MCNUTT, N. S., and R. S. WEINSTEIN. 1970. The ultrastructure of the nexus. A correlated thin section and freeze-cleave study. *J. Cell Biol.* **47**:666-688.
 36. MCNUTT, N. S., and R. S. WEINSTEIN. 1973. Membrane ultrastructure at mammalian intercellular junctions. *Progr. Biophys. Mol. Biol.* **26**:45-101.
 37. MONTESANO, R., D. FRIEND, A. PERRELET, and L. ORCI. 1975. In vivo assembly of tight junctions. *J. Cell Biol.* **67**:310-319.
 38. MOTTET, N. K., and H. M. JENSEN. 1968. The differentiation of chick embryonic skin. *Exp. Cell Res.* **52**:261-283.
 39. ORCI, L., M. AMHERDT, J. C. HENQUIN, A. C. LAMBERT, R. H. UNGER, and A. E. RENOLD. 1973. Pronase effect in pancreatic beta cell secretion and morphology. *Science (Wash. D. C.)* **180**:647-649.
 40. PARAKKAL, P. F., and A. G. MATOLTSY. 1968. An electron microscopic study of developing chick skin. *J. Ultrastruct. Res.* **23**:402-416.
 41. PELC, S. R., and H. B. FELL. 1960. The effect of excess vitamin A on the uptake of labelled compounds by embryonic skin in organ culture. *Exp. Cell Res.* **19**:99-113.
 42. REVEL, J.-P., and M. J. KARNOVSKY. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33**:C7-C12.
 43. REVEL, J.-P., P. YIP, and L. L. CHANG. 1973. Cell

- junctions in the early chick embryo—a freeze etch study. *Dev. Biol.* **35**:302–317.
44. SANI, B. P., and D. L. HILL. 1974. Retinoic acid: a binding protein in chick embryo metatarsal skin. *Biochem. Biophys. Res. Commun.* **61**:1276–1282.
45. SCHREINER, E., and K. WOLFF. 1969. Die permeabilität des epidermalen Intercellularräumens für kleinmolekulares Proteinen. *Arch. Klin. Exp. Dermatol.* **235**:78–88.
46. SHERIDAN, J. D. 1971. Dye movement and low resistance junctions between reaggregated embryonic cells. *Dev. Biol.* **26**:627–636.
47. SQUIER, C. A. 1973. The permeability of keratinized and non-keratinized oral epithelium to horseradish peroxidase. *J. Ultrastruct. Res.* **43**:160–177.
48. STAEHELIN, L. A. 1974. The structure and function of intercellular junctions. *Int. Rev. Cytol.* **39**:191–283.