Morphological Development of the Epithelium of the Embryonic Chick Intestine in Culture: Influence of Thyroxine and Hydrocortisone [1]

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ABSTRACT A culture system with defined medium has been utilized to elucidate the role of thyroxine (T4) and hydrocortisone (HC) in growth and differentiation of the epithelium of embryonic chick duodenum during the third week of development. Duodenal explants maintained a basically normal morphology for 48 to 72 hours, but during subsequent culture, muscle and mesenchyme deteriorated, previllous ridges shrank, the epithelial surface became pitted, and the tissue lost weight. These degenerative processes were retarded by addition of HC to the culture medium.

Previllous ridges of cultured duodenum failed to develop into true villi and ridge growth was subnormal. Epithelial mitotic counts were increased by T4 or HC, as compared to cultured control tissue, but dropped below values in vivo, by 72 hours in culture.

Epithelial differentiation proceeded more rapidly in duodenum cultured without hormones than in vivo. Increase in cell height, flattening of the epithelial surface, attainment of uniform staining of the brush border with periodic acid-Schiff, increase in microvillar density, and formation of a terminal web were accelerated by 24 to 48 hours in culture. In the presence of T4, cell height and microvillar growth were stimulated further, reaching the state found at 19 to 21 days in vivo.

During the week before hatching, the duodenum of the chick embryo undergoes important morphological changes. At 14 days no villi are present, but 16 previllous ridges run longitudinally along the luminal surface in a zig-zag pattern; indentations subsequently appear in these ridges, resulting in the formation of definitive villi which then elongate rapidly until hatching (Coulombre and Coulombre, '58; Grey, '72). The epithelial cells are pseudostratified at 14 days, and the epithelial surface appears "bubbly" (Grey, '72; Tsai and Overton, '76). Later, the epithelial surface flattens as a simple columnar epithelium forms (Grey, '72), and the cells increase rapidly in height between days 19 and 22 (Moog, '50; Hinni and Watterson, '63).

Microvilli are present at day 14, but increase in density between days 14 and 19 (Grey, '72). Their length remains constant from day 14 through day 16, doubles by day 20, and increases 3-fold between days 20 and 22 (Overton and Shoup, '64). Polysaccharides are first clearly demonstrable in the brush border by periodic acid-Schiff (PAS) staining at 17 days, after which the staining increases rapidly in intensity until hatching (Moog and Wenger, '52).

During the third week of development, proliferation of epithelial cells becomes restricted to the crypts. At day 14, mitotic figures can be seen occasionally at the previllous ridge crests, but the majority of dividing epithelial cells are found in the basal halves of ridges (Overton and Shoup, '64). As development continues, an increasing percentage of dividing cells are found near the villar base; by two days after hatching, mitosis occurs only within the crypts.

That glucocorticoids play a role in morphological development of chick intestine was first suggested by the finding that injection of

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cortisone into embryos at 10, 14, or 16 days elicited an acceleration of several aspects of differentiation, including increase in epithelial cell height a PAS-stainability of the brush border (Moog and Richardson, '55). That thyroxine (T4) is also required was indicated by the finding that thiourea, an inhibitor of thyroid function, caused severe retardation of villar elongation, of increase in epithelial cell height, and of intensification of brush-border staining with PAS (Moog, '61). In the thiourea-treated embryos, normal development was restored by injections of T4, but not of hydrocortisone (HC).

Further evidence for hormonal control of intestinal development has been provided by study of "hypophysectomized" chick embryos obtained by removing the anterior part of the developing brain at 33 hours of incubation. In such embryos the rate of duodenal development was retarded, and the normal 18-day state of morphological differentiation was not exceeded, even in embryos that remained alive in the shell up to day 26 (Hinni and Watterson, '63; Bellware and Betz, '70). The fact that the adrenal and thyroid glands of "hypophysectomized" embryos remained in an immature condition suggested that deficient secretion of glucocorticoid and T4 were responsible for the retarded development. In agreement with this interpretation, chorioallantoic grafts of pituitary fragments, known to secrete corticotropin and thyroid-stimulating hormone, restored normal duodenal development in "hypophysectomized" embryos (Hart and Betz, '72).

Although the hypothesis that both glucocorticoids and thyroid hormones are implicated in duodenal development is well supported, the way in which these hormones act is not clear. It is, in particular, uncertain whether T4 plays a merely permissive role, perhaps through alteration of growth and metabolism in the embryo as a whole, or whether it independently controls a basic step in differentiation. In the present study, I have cultured embryonic duodenum in defined medium in order to investigate the separate roles of HC and T4 in the growth of the embryonic duodenum and the differentiation of its absorptive epithelium.

MATERIALS AND METHODS

Preparation of tissue; culture conditions

Duodenal loops were removed from 14-day embryos and prepared for culture as described previously (Black and Moog, '77). Duodena were cut into 2- to 3-mm segments, opened by splitting along the longitudinal axis, and distributed to 25-ml Erlenmeyer flasks.

Culture conditions were similar to those described by Moog and Kirsch ('55). Each culture flask routinely contained 3 ml of Medium 199 (with glutamine and unmodified Earl's salts) plus 0.03 ml of penicillin-streptomycin solution (50 units/ml-50 μg/ml), both purchased from Grand Island Biological Company. When solutions of hormones or macromolecules were used, the medium was adjusted to maintain a total volume of 3 ml. Control cultures received a volume of solvent equal to the volume of hormonal or macromolecular solution added to experimental cultures (0.1-0.3 ml). After addition of tissue, all culture flasks were gassed with 95% oxygen-5% carbon dioxide, stoppered tightly, and incubated at 38°C.

Preparation of hormonal and macromolecular solutions

Thyroxine (3,3',5,5'-tetraiodo-DL-thyronine), obtained from Sigma Chemical Company, was dissolved in 0.01 M NaOH. Hydrocortisone-21-sodium succinate (Sigma Chemical Company) was dissolved in 0.9% NaCl. Crystalline bovine insulin (The Lilly Laboratories) was dissolved in 1 N HCl, then diluted with 0.9% NaCl. Gastrin pentapeptide, blocked (t-BOC-β-Ala-Trp-Met-Asp-Phe-NH_2), was obtained from Calbiochem, dissolved in several drops of dimethyl formamide, then diluted with 0.9% NaCl. All hormone solvents were autoclaved prior to use.

Dextran 500 (Pharmacia, Sweden), carboxymethylcellulose (Bios Laboratories), and high-molecular-weight polyvinyl pyrrolidone (K & K Laboratories) were dissolved in distilled water, dialyzed for five days against 0.9% NaCl, then adjusted to the desired concentration by adding 0.9% NaCl. All macromolecular solutions were autoclaved prior to use.

The concentrations referred to in RESULTS designate the final concentration of hormone or macromolecule after addition to the culture flask.

Histology

Uncultured duodena were removed from embryos, cut into 2- to 3-mm segments, and split open in culture medium prior to fixation. Both uncultured and cultured tissues were fixed for two to four hours in Carnoy's fixative

and embedded in paraffin. Serial sections 5 μm thick were made by cutting the embedded tissue along the longitudinal axis, perpendicular to the lumenal surface. Sections to be used for mitotic counts were stained by the Feulgen reaction and counterstained with fast green. Sections to be examined for brush-border mucopolysaccharides were treated with 0.5-1.0% amylase in phosphate buffer, pH 6.9, for 30 minutes, then stained by the periodic acid-Schiff procedure and counterstained with fast green.

Mitotic figures in the epithelial layer were counted in every third serial section on a slide until 100 villi (or previllous ridges) had been examined. All villi which were cut symmetrically from tip to base were used. Mitotic counts for each specimen were expressed as number of mitotic figures/100 villi.

Electron microscopy

For scanning electron microscopy (SEM), segments from the proximal and distal ends of uncultured duodena were split open in culture medium prior to fixation. Tissue from both uncultured and cultured duodena were fixed for 24 hours in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. The tissue was post-fixed for one hour in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), dehydrated in absolute alcohol, and transferred to liquefied carbon dioxide for critical-point drying. Dried specimens were coated, first with carbon, then with chromium in a Kinney vacuum evaporator, and viewed in a Cambridge scanning electron microscope.

For transmission electron microscopy (TEM), tissue was collected and fixed in the same manner as for SEM except that 0.2 M cacodylate buffer (pH 7.4) was used. Specimens were embedded in Epon 812 epoxy resin. Thin sections (80-90 nm) were poststained with uranyl acetate followed by lead citrate and examined in Hitachi HU-11C electron microscope.

Weight and protein determination; statistics

All tissue from a given culture flask was removed with forceps, rinsed with 0.9% NaCl, and drained briefly on filter paper prior to weighing. The tissue was then homogenized in 0.9% NaCl, and protein concentration was measured by the method of Lowry et al. ('51).

Results were evaluated by using the Student t-test (mitotic counts) or paired t-test (tissue weight and protein concentration).

RESULTS

General characteristics and integrity of cultured tissue

Immediately after being split open, duodenal segments curl along the longitudinal axis, assuming a tubular shape with previllous ridges on the outside (fig. 3). In a few cases, epithelium from the cut edges fused in culture to form a spherical structure of central muscle surrounded on all sides by ridges, but in most specimens the cut edges healed without fusion, and distinct mucosal and serosal sides were maintained throughout the culture period. Previllous ridge structure was maintained over most of the tissue surface during culture, but ridge-free regions of small to moderate size were observed in many specimens. Observations of living cultures suggested that such regions form where the tissue makes contact with the bottom of the culture flask.

Mesenchymal tissue of control cultures maintained a relatively normal appearance for 24 to 48 hours (figs. 4, 8), after which fibroblasts in the lamina propria became separated and cavities tended to form in the deep mucosal regions (figs. 5, 23). Both of these abnormalities, which have been described previously in duodenal cultures, are apparently the result of fluid uptake by the lamina propria and mucosal vascular channels (Hancox and Hyslop, '53; Hayes, '65a; Pedernera, '70). Attempts to reduce tissue edema by adding macromolecules to the culture medium (0.5% dextran, 1% dextran, 0.1% carboxymethylcellulose, or 0.2% polyvinyl pyrrolidone) were unsuccessful.

Circular muscle of control cultures maintained a normal morphology for 48 hours (fig. 4), although it tended to become vacuolated in those regions lacking ridges. The healthy appearance of muscle in paraffin sections was verified by observations of peristalic contractions in most 48-hour specimens. These contractions were of two to three seconds duration; frequency of contractions varied in different tissue fragments. By 72 hours in culture, few contractions were seen and peristalsis ceased completely by 96 to 120 hours. Paraffin sections of 72-hour cultures reveal a deterioration of muscle structure (fig. 5).

Changes in tissue weight and protein concentration during culture were measured by starting with flasks containing equal aliquots of pooled tissue and collecting one culture on each day of incubation. Tissue weight de-

TABLE 1

Change in weight and protein concentration of tissue during culture

Hours in culture	Control cultures	10^{-8} M T_4 cultures	Control cultures	10^{-6} M HC cultures
	Wet weight of tissue as a percentage of that at beginning of culture			
0	100%	100%	100%	100%
24	91.7±1.0	85.3±2.2	87.6±5.7	81.8±3.7
48	87.5±3.8	76.8±5.4	81.7±1.1	85.3±4.3
72	76.4±3.0	74.7±7.2	70.0±3.6	78.4±1.1
96	72.7±2.6	60.8±5.0 [1]	66.6±4.4	72.9±1.4
120	60.3±4.7	52.7±3.4	59.3±0.8	71.1±2.1 [2]
	Protein concentration of tissue as a percentage of that at beginning of culture			
0	100%	100%	100%	100%
24	101±5.2	107±6.9	94.3±6.2	96.3±7.9
48	111±5.0	103±5.0	97.7±4.3	97.3±7.3
72	105±4.2	106±7.0	94.7±4.3	95.0±2.9
96	102±4.7	104±6.3	96.3±4.5	87.7±2.7
120	104±1.5	103±8.4	99.7±7.4	101±5.8

Values represent the mean ± SEM of three experiments.
For each experiment, duodena from 10 to 12 embryos were pooled and divided between control and T_4 or HC flasks.
[1] $p < 0.05$ (T_4 vs. control).
[2] $p < 0.02$ (HC vs. control).

creased throughout the incubation period; 120-hour cultures retained 60% of their original weight (table 1). In contrast, protein concentration of the tissue (μg protein/mg wet weight) remained constant during culture (table 1).

A similar decrease in the weight of cultured 16-day duodenum, without change in nitrogen concentration, has been reported previously (Moog and Kirsch, '55). The weight loss is partly due to the detachment of epithelial cells that are recoverable intact from the medium (Black, '76).

The effect of hormones on the general condition of 24- to 120-hour cultures was ascertained from observations of a total of 77 cultures. For 48 hours, tissue cultured in 10^{-9} M T4 resembled control explants (fig. 6), but during subsequent culture, formation of "bare" regions on the mucosal surface, uptake of fluid by the lamina propria, and deterioration of muscle were somewhat increased by the hormone. In living cultures, peristalic contractions were rarely seen. Weight loss was somewhat accentuated by T4, although the differences between control and T4 cultures were significant only at 96 and 120 hours. The presence of 10^{-6} M HC retarded the formation of "bare" regions, reduced edema in the lamina propria, and prevented muscle deterioration for 72 to 96 hours in culture (fig. 7). Most tissue fragments in HC cultures contracted vigorously for 72 hours and a few specimens continued to contract up to 120 hours. Tissue cultured in HC formed cavities, apparently greatly dilated vascular spaces, in the deep mucosal region (fig. 9), as described previously by Pedernera ('70). Weight loss was less extreme than in control tissues, HC cultures retaining 72% of their original weight after 120 hours of incubation (table 1).

Growth of cultured tissue

The form of previllous ridges during five days of culture was examined in paraffin sections (280 specimens) and by SEM (70 specimens). Previllous ridges of control cultures increased in height during the first 48 hours of incubation, but usually failed to reach the 16-day-in-vivo height. Between 48 to 120 hours, the previllous ridges either maintained a constant height or shrank slightly. Unlike the narrow, pointed ridges of duodena in vivo, the ridges of cultured tissue assumed a broad blunt form. This increase in ridge width is clearly shown by a comparison of SEM specimens of 14-day duodenum in vivo and 14-day explants cultured for 24 to 72 hours (figs. 10-13). The zig-zag pattern of ridges was usually maintained for 120 hours of culture, although apparent villar formation occurred between 48 and 72 hours in a small percentage of specimens (fig. 14). Formation of villi in vivo was observed during the same time-period (16-17 days of development, fig. 17), consistent with previous SEM observations of embryonic chick duodenum (Grey, '72; Tsai and Overton, '76). Between 16 and 17 days, an additional

rank of villi appeared during normal development (fig. 17), but not in cultured tissue.

The previllous ridges of tissue treated with 10^{-9} M T4 tended to be slightly higher than those of control tissue at 24 and 48 hours in culture, but shrinkage of ridges was accelerated by T4 during subsequent culture. Shrinkage of ridges was more pronounced in 10^{-8} M T4; a complete loss of ridges by 120 hours was occasionally observed. The pattern of previllous ridges in T4 cultures was indistinguishable from that of control cultures.

In HC-treated tissue, previllous ridges remained narrow and somewhat higher than ridges of control cultures for 72 to 96 hours of incubation (figs. 7, 16). Such beneficial effects of HC on the morphology of cultured duodena have been noted previously (Moog and Kirsch, '55; Hayes, '65a). Previllous ridges tended to lose their regular zig-zag pattern after 48 to 72 hours of culture with HC, becoming convoluted and more compactly folded. No formation of villi occurred.

The observations that previllous ridges of cultured tissue increase in height for 48 hours and that mitotic figures are found in the epithelium (fig. 8) indicated that the ridges were growing. To quantitate the effect of hormones on growth of the epithelial layer, mitotic counts of epithelial cells were made in control and hormone-treated cultures and compared to mitotic counts of duodena in vivo. The number of mitotic figures/100 villi increased gradually between 14 and 18 days, then rapidly between days 18 and 19 (fig. 1). These results agree closely with previously reported mitotic counts of duodenal epithelium at 15 and 16 days, but the 19-day value is considerably higher than previously found (Hijmans, '72).

Both control and 10^{-9} M T4 cultures maintained mitotic counts within the range found at 14 to 15 days in vivo for 24 hours of incubation. After 24 hours, control mitotic counts dropped; T4 cultures maintained for 48 hours the counts found in vivo at 14 days, before dropping to the low control value at 72 hours of culture (fig. 1). In HC cultures, mitotic counts were lower than values at 14 to 15 days in vitro, after 24 hours of culture, but only a slight further decrease occurred during the 24- to 72-hour period. After 72 hours of culture, mitotic counts in HC-treated tissue were significantly higher than in control cultures, but only 66% of the 17-day value in vivo.

At day 14 in vivo, 70% of the mitotic figures were confined to the basal halves of villi (fig.

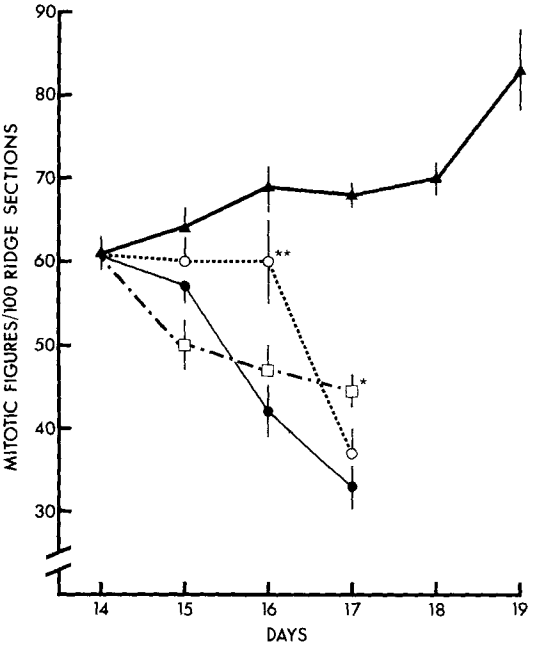

Fig. 1 Comparison of mitotic counts from duodena in vivo (▲) to duodena explanted at 14 days and cultured without hormones (●), with 10^{-9} M T4 (○), or with 10^{-6} M HC (□). For each day of development, each point represents the average number of mitotic figures per 100 ridge sections (MATERIALS AND METHODS) on 10 specimens from duodena in vivo; on 24 specimens from control cultures; and on 12 specimens, each, from T4 and HC cultures. Vertical lines indicate standard error of the mean. *$p < 0.01$ (HC vs. control), **$p < 0.001$ (T4 vs. control).

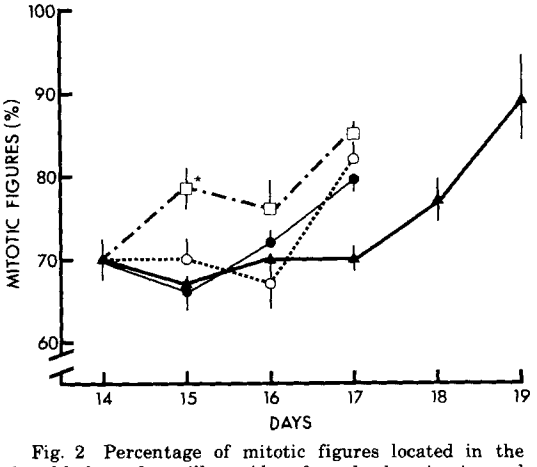

Fig. 2 Percentage of mitotic figures located in the basal halves of previllous ridges from duodena in vivo and duodena cultured with or without hormones. Lines and symbols as in figure 1. *$p < 0.001$ (HC vs. control).

2); a further segregation of dividing cells toward the villous base occurred between 17 and 19 days of development, consistent with previous reports (Overton and Shoup, '64; Dobbins et al., '67). For 48 hours, control and T4-treated tissue both maintained a distribution of mitotic figures from ridge base to tip, similar to that in vivo. At 72 hours, the percentage of mitotic figures in the basal halves of villi rose, reaching a value of 80%, as compared to a 17-day value of 70% in vivo (fig. 2). In HC cultures, the percentage of mitotic figures in the basal halves of villi increased to 80% in only 24 hours, and remained higher than values in vivo throughout the 72-hour culture period.

Pentagastrin, at a concentration (6.5×10^{-7} M) reported to stimulate mitosis in cultures of rat duodenal cells (Lichtenberger et al., '73), had no effect on mitotic counts in 72-hour cultures. Insulin had no effect in 48-hour cultures at concentrations of 2×10^{-7} M, 4×10^{-8} M, or 4×10^{-9} M.

Differentiation of the absorptive epithelium during culture

Changes in epithelial cell size and shape occurred in vivo between 14 and 17 days. At 14 and 15 days, the epithelial surface appeared "bubbly"; individual cells were somewhat round in shape and appeared to be loosely attached to the ridge surface (figs. 10, 30). Between 15 and 17 days of development, cells at the crests of ridges (or villi) acquired a flat surface and seemed to become more firmly attached to one another (figs. 17, 31). Such changes in the developing duodenal epithelium have been described previously (Grey, '72; Tsai and Overton, '76), and coincide with conversion of 14-day pseudostratified epithelium to a simple columnar layer.

Changes within the epithelium of a single duodenum during culture were examined by collecting tissue at the time of explantation at 14 days, and after 24, 48, and 72 hours in culture. SEM specimens from two such series of control cultures revealed that the epithelium of cultured tissue became smooth at ridge crests after only 24 hours of culture (figs. 11, 32). After 48 hours of culture, the "bubbly" appearance of the epithelium was completely lost, and "pits" frequently appeared on the epithelial surface. By 72 hours of culture, the entire ridge surface was pitted; the "pits" were typically hexagonal in shape and appeared to result from a sinking-in of epithelial cell surfaces to form concave regions (figs. 13-15). This interpretation is supported by transmission electron micrographs of tissue cultured 48 hours (fig. 20). "Pits" were never seen during development in vivo, in which the surface of epithelial cells remained flat or slightly convex (figs. 17, 31).

The epithelial surface of 10^{-9} M T4 cultures underwent the same changes as control cultures. In HC cultures, however, the "bubbly" epithelium flattened without the subsequent formation of "pits." Instead, the cells remained slightly convex, so that the epithelial surface resembled that of duodenum in vivo throughout the 72-hour culture period (figs. 16, 44, 48). The addition of 1% dextran, a concentration reported to possess the viscosity and osmotic properties of serum (Healy and Parker, '66), had no effect on the epithelial surface in 48- or 72-hour cultures.

The morphology of the epithelium was further studied by TEM and in paraffin sections. Electron micrographs of 48-hour cultures revealed that tissue cultured in the presence or absence of hormones maintained a normal ultrastructure, including rough endoplasmic reticulum, Golgi elements, numerous mitochondria, and well-formed junctional complexes (figs. 19-21). The epithelium of control, T4, and HC cultures appeared to have developed more rapidly than intact epithelium of the same chronological age (16 days), in terms of flattening of cell surface and attainment of a narrow, columnar cell shape (figs. 18-21).

Accelerated development of the epithelium during culture was verified in paraffin sections of duodenum cultured for 48 and 72 hours. The size and shape of epithelial cells in 72-hour control and HC cultures were similar; cell height was greater than that of 17-day duodenum in vivo, but did not reach the 20-day height seen in vivo (figs. 22-25). In the presence of 10^{-9} M T4, a further acceleration of development occurred, since epithelial height exceeded that of control cultures after 72 hours of incubation and often equalled the height of 20-day epithelium in vivo (figs. 25, 26).

The brush border of duodena in vivo first showed definite, uniform staining with PAS at day 17 of development, with staining intensity increasing thereafter, as reported by others (Moog and Wenger, '52; Hinni and Watterson, '63; Bellware and Betz, '70). In 14-day explants, staining of the brush border in-

creased more rapidly than in vivo, reaching the intensity seen at 17 to 18 days after only 48 hours in culture (figs. 27-29). A gradient in PAS-staining from ridge (or villar) tip to base was usually apparent both in culture and in vivo. Neither 10^{-9} M T4 nor 10^{-6} M HC had a clear-cut effect on staining of brush border with PAS.

Brush border ultrastructure was studied by TEM of 48-hour cultures and tissue taken at 16 days in vivo, as well as in the SEM specimen described previously. The SEM specimens of tissue in vivo revealed that microvilli increased in number between 15 and 17 days of development (figs. 30, 31), as also noted by Grey ('72). In vitro, this increase was accelerated, with most 24-hour control cultures attaining a concentration of microvilli greater than the condition at 15 days in vivo, and comparable to 16- to 17-day duodena in vivo (figs. 30, 31, 33). By 48 to 72 hours of culture, microvilli became too densely packed to be easily distinguished in SEM specimens. Examination of 48-hour cultures by TEM clearly revealed that the microvilli were not only more densely packed, but also were more uniform in shape than microvilli from uncultured duodenum of the same chronological age (figs. 34, 35). Rootlets extending from the microvillar cores were likewise more prominent in cultured tissue than in vivo (figs. 34, 35). In addition to this relatively uniform covering, exceptionally long microvilli occurred singly and randomly over the epithelial surface. They were seen occasionally after 24 hours of culture (fig. 33), but occurred more frequently after 48 to 72 hours. Small numbers of elongated microvilli have also been observed in fetal rat intestine cultured with defined medium (DeRitis et al., '75).

The microvilli of hormone-treated cultures were similar to those of controls, with two exceptions. First, T4 greatly enhanced the abundance of exceptionally long microvilli. In the presence of 10^{-9} M T4, the frequency of such microvilli reached a peak at 48 hours of culture, then decreased to a low level by 72 hours (figs. 36-38). These long microvilli were more numerous in T4-treated tissue than in their paired controls at 24 hours (4 specimens) and 48 hours (4 specimens). Examination of 48-hour T4 cultures by TEM further revealed that both short and very long microvilli had typical core structure and an abundant glycocalyx (figs. 39, 40). Exceptionally long microvilli were also present in large numbers after 24 and 48 hours of culture with 10^{-8} M T4 (figs. 41, 42, 45, 46). Tissue cultured with HC developed microvilli similar to those of control cultures, except that density of packing and length tended to be more uniform, showing little variation over the epithelium of a given specimen (figs. 43, 44, 47, 48). The microvilli of tissue cultured in 1% dextran for 48 or 72 hours were similar to those of control tissue.

DISCUSSION

Embryonic chick duodenum can be maintained in a culture system with defined medium for four to five days. During 48 to 72 hours of culture, the tissue maintains a basically normal morphology. The disruption of the mesenchymal core of previllous ridges, which becomes more pronounced after 48 to 72 hours, may lead to the subsequent shrinkage of villi and formation of "bare" regions. This reduction in ridge surface-area is accompanied by a loss of epithelial cells into the culture medium (Black, '76), and, together with deterioration of muscle and connective tissue, probably accounts for the decrease in tissue weight during culture. Maintenance of a constant protein concentration during culture might mean that the detached epithelial cells have a protein concentration similar to the intact tissue or that an increase in protein synthesis by the tissue balances the protein lost due to cell detachment and degeneration.

The "protective" effects exerted by HC on cultured duodenum are probably due primarily to the channeling of water into vascular spaces, thus preventing fluid from collecting in and disrupting lamina propria and muscle regions. The ability of HC to retard weight loss and formation of "bare regions" might also reflect non-specific effects such as have been demonstrated in cultures of Henle cells (a human embryonic intestinal cell line). In these cultures Prednisalone reduced leakage of intracellular components and increased cell survival under suboptimal nutritional conditions (Melnykovych et al., '67).

The failure of true villi to form in most specimens of cultured duodenum might also be related to disruption of mesenchymal regions by edema. That an intact connective tissue substrate might be required for normal villar morphogenesis is suggested by the finding that collagen fibers at the base of previllous ridges and between ridges change from a random to an oriented pattern during villar formation (Tsai and Overton, '76). Alter-

natively, formation of villi from previllous ridges may require an intact tubular duodenal structure as suggested by the mechanical pressure hypothesis (Coulombre and Coulombre, '58).

Mitotic counts in the epithelium of both control and hormone-treated cultures fall below values in vivo after 24 to 48 hours of incubation. This drop in mitotic counts might be due to depletion of a nutrient essential for cell division from the culture medium. Alternatively, disruption of the normal epithelial-mesenchymal relationship by edema in the lamina propria might interfere with completion of the cell cycle. The importance of epithelial-mesenchymal interactions has been demonstrated in cultured embryonic chick skin, in which synthesis of DNA and mitosis in the epidermis is dependent upon both an adequate nutrient supply and a "suitable physical substrate" (Wessels, '64). The subnormal growth of cultured duodenum might also reflect a lack of endogenous growth factors. Although neither pentagastrin nor insulin increased mitotic counts when added to the culture medium, other hormones known to have trophic effects (such as prolactin and growth hormone) were not tested.

In this culture system, T4 increases cell division over control values between 24 and 48 hours of incubation. Stimulation of mitosis is accompanied by a normal distribution of mitotic figures on the villus and occurs at a T4 concentration (10^{-9} M) which is well within the range of circulating thyroid hormones in 14- to 21-day chick embryos (King et al., '77; Thommes and Hylka, '77). Thus the effect of T4 on growth in vitro may reflect an action of the hormone in vivo. This interpretation is consistent with the findings that "hypophysectomy" (Bellware and Betz, '70) or thiourea treatment (Moog, '61) of chick embryos retards the growth of duodenal villi, whereas pituitary grafts to the chorioallantois, or T4 injections, restore growth to normal. Furthermore, T4 injection has been reported to increase the mitotic index of duodenal epithelium in both "hypophysectomized" and intact chick embryos (Betz and Mallon, '70). That T4 may be required for normal growth of mammalian duodenum is indicated by the finding that the low mitotic index in rats hypophysectomized or thyroidectomized at six days of age can be raised by T4 injection (Yeh and Moog, '75, '77).

Mitotic counts in cultured duodenum are significantly increased over control values by HC at 72 hours of incubation, but whether this response represents a physiological action of the hormone is uncertain. For one thing, HC increases the percentage of mitotic figures in the proximal halves of villi to the 18-day value in vivo, after only 24 hours of culture. Although this distributional change could represent an acceleration of the normal process, such an interpretation is complicated by the fact that HC fails to maintain mitotic counts at the level found in vivo. Furthermore, 10^{-6} M HC is probably not physiological, since the corticosterone concentration of 14- to 17-day embryonic chick plasma is approximately 10^{-8} M (Wise and Frye, '73; Siegel and Gould, '76). Previous studies of embryonic chick duodenal cultures have indicated both a stimulatory and an inhibitory action of 10^{-6} M HC on epithelial mitosis (Dobbins et al., '67; Hijmans, '72).

As differentiation of cultured duodenum proceeds, the epithelium loses its "bubbly" appearance, microvilli increase in number and assume a consistent shape and orientation perpendicular to the cell surface. Since the terminal web also becomes more pronounced during culture, it seems likely that an enhancement of the terminal web brings about the flattening of cell apex and regularity of microvillar orientation. Such changes in the intestinal epithelium have been attributed to terminal web formation during brush border regeneration in salamanders and in microvillar formation during metamorphosis of *Xenopus* tadpoles (Bonneville and Weinstock, '70; Tilney and Cardell, '70). The rapid increase in intensity of PAS staining in brush border during 48 hours of culture probably reflects the increase in microvillar surface area during this period.

The major effect of T4 on morphological differentiation of the epithelium is a stimulation of microvillar growth, as revealed by the abundance of exceptionally long microvilli during 24 to 48 hours of culture. Thus, T4 may elicit in 14-day tissue the rapid increase in microvillar length that occurs in vivo between 20 and 22 days of development. It should be noted, however, that some of the microvilli of T4 cultures reach lengths much greater than seen in vivo. Microvilli in hatched chick and adult chicken duodenum are approximately 2 μm long and 0.08 μm in diameter (Overton

and Shoup, '64; Michael and Hodges, '73). In T4-treated tissue, microvilli maintain the normal 0.08-μm diameter, but lengths as great as 4 μm occur (fig. 40). Cytochalasin B can elicit growth of microvilli to similar lengths in embryonic chick duodenal cultures (Burgess and Grey, '74), but these microvilli become highly branched, with multiple constrictions along their length. Such abnormal microvilli were never seen in the present work.

The disappearance of long microvilli from T4-treated tissue after 48 hours of incubation is apparently due to loss of microvillar membrane into the culture medium. This interpretation is suggested by the finding that enzymes normally located in the brush border membrane accumulate in the medium during 24 to 120 hours of incubation (Black, '76; Black and Moog, '78). Additionally, membranous vesicles of apparent microvillar origin may be isolated from the culture medium (Black, '76). Since this enzyme activity of the culture medium is much higher in T4 than in control cultures, it is unlikely that the small number of very long microvilli in the controls reflects a more rapid microvillar loss from the tissue.

The epithelial surface of cultures containing HC differs from that of control cultures in that "pits" do not form, and microvilli tend to have a more uniform appearance over large areas of a given specimen. Both of these HC actions might be produced through a stabilization of the apical plasma membrane, or by strengthening of the terminal web, but there is no direct evidence for either of these possibilities.

Comparison of cultured duodenum with that in vivo clearly indicates that morphological differentiation of the absorptive epithelium is accelerated in control cultures. Increase in epithelial cell height, flattening of the epithelial surface, attainment of uniform PAS staining of the brush border, increase in microvillar density, and development of the terminal web are all accelerated by 24 to 48 hours in culture. That a normal developmental pattern is maintained during culture is indicated by the initiation of epithelial flattening at ridge crests, by the presence of a gradient in PAS staining of brush border from ridge crest to base, and by the normal structure of newly formed microvilli. An acceleration of goblet cell differentiation (Black and Moog, '77) and of increase in alkaline phosphatase and maltase activity (Black and Moog, '78) also occur in control cultures. Thus, explanting the tissue apparently elicits an increase in developmental rate.

Few previous observations have been made regarding such acceleration of intestinal development in vitro. Using 16-day explants, Moog and Nehari ('54) noted that an increase in epithelial height occurred more rapidly in cultured control tissue than in vivo. Maturation of argentaffin cells from argyrophil precursors was also found to occur at an accelerated rate in cultures of 13-day embryonic chick intestine (Monesi, '60). However, both of these culture systems contained serum and embryo extract.

The cause of the increased rate of development in this duodenal culture system is obscure. It is conceivable that a nutrient present in the culture medium, but limiting in vivo, stimulates development. Indeed, vitamin A in cultures of embryonic chick skin alters the pattern of keratin synthesis, while vitamin C stimulates mucus production and secretion (Smith, '73). Physical properties of the culture medium can also stimulate development of chick skin, since T4-induced acceleration of epidermal differentiation is mimicked by addition of polyvinyl pyrrolidone (Wessels, '61). In the present work, the coordinated acceleration of several diverse aspects of morphological differentiation, and the lack of response to change in osmotic conditions, suggest that the stimulus is not a nutrient or a physical property of the culture system. It seems more likely that the developmental rate is restricted in vivo by the presence of an inhibitor of duodenal differentiation. Such a circulating inhibitor has been suggested previously to explain the precocious differentiation of embryonic chick liver during culture in defined medium (Skea and Nemeth, '69).

Although all aspects of morphological differentiation studied proceed during culture in the absence of hormones, the addition of T4 further accelerates the increase in epithelial cell height and stimulates microvillar growth. These results suggest that thyroid hormones directly affect differentiation during normal development. Indeed, a state of epithelial differentiation similar to that induced by T4 is attained in vivo between days 19 and 21, a period during which circulating triiodothyronine increases rapidly to a maximum of approximately 5×10^{-9} M at hatching (King et

al., '77). A direct stimulatory effect of T4 on developing embryonic intestine has been reported previously only in relation to goblet cell differentiation (Black and Moog, '77). In neonatal rat intestine, however, T4 has been found to influence morphological differentiation of the absorptive epithelium. Injections of T4 into 6-day-old hypophysectomized rats elicits an increase in rough endoplasmic reticulum, restores Golgi complexes to their normal appearance, and intensifies brush border staining with PAS (Yeh and Moog, '75).

The failure of HC to accelerate duodenal development in the present study is consistent with previous reports in which glucocorticoids had no clear-cut effect on epithelial cell height or microvillar dimensions in 13-, 15-, or 16-day duodenal explants (Hayes, '65b; Dobbins et al., '67). The present results seem to be in conflict with reports on the action of HC in vivo, since injection of HC into 14-day embryos advanced morphological differentiation by 24 hours in duodena examined at 17 days of development (Moog and Richardson, '55). Lack of an HC effect in vitro might be due to the fact that development is already accelerated by 24 to 48 hours in control cultures. Alternatively, glucocorticoid action might require the presence of endogenous hormones. A failure of HC to stimulate epithelial differentiation in vitro is consistent with the finding that HC has no effect on duodenal differentiation when injected into embryos rendered hypothyroid by thiourea treatment (Moog, '61). Finally, these studies in vitro do suggest that glucocorticoids might exert an indirect, stabilizing effect on the epithelial surface during the third week of duodenal development.

ACKNOWLEDGMENTS

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PLATE 1

EXPLANATION OF FIGURES

3 Duodenal segment explanted at 14 days and cultured for 48 hours without hormones. Segments of split duodenum curl into a tubular shape with previllous ridges external. SEM. × 84.

4 Duodenal segment explanted at 14 days and cultured for 48 hours without hormones. Previllous ridges, mesenchyme, and circular muscle (CM) maintain a relatively normal morphology during 48 hours of culture. Feulgen and fast green. × 84.

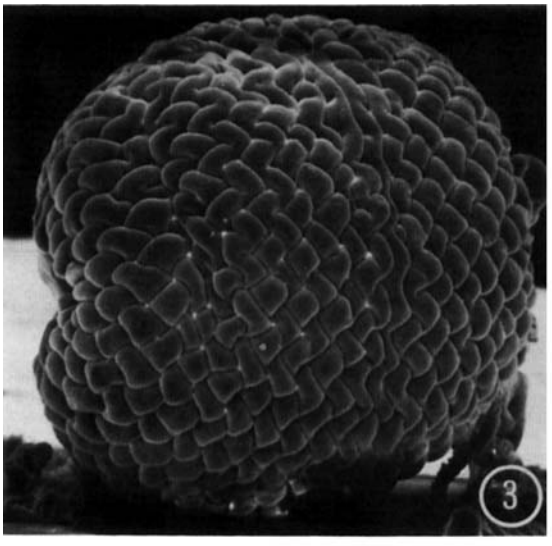
5 Duodenal segment explanted at 14 days and cultured for 72 hours without hormones. Previllous ridges are broader and fibroblasts within the lamina propria tend to be less compact than in tissue cultured for 48 hours (also fig. 23). Circular muscle (CM) is vacuolated. Feulgen and fast green. × 236.

6 Duodenal segment explanted at 14 days and cultured for 72 hours with 10^{-9} M T4. Morphology is similar to that of tissue cultured without hormones. CM, circular muscle. Feulgen and fast green. × 236.

7 Duodenal segment from same embryo as tissue in figure 5, but cultured for 72 hours with 10^{-6} M HC. Previllous ridges are narrower, fibroblasts within the lamina propria are more compact, and circular muscle (CM) is better preserved than in tissue cultured for 72 hours without hormones. Feulgen and fast green. × 236.

8 Previllous ridges of specimen in figure 4. Fibroblasts maintain a compact arrangement within the lamina propria for 48 hours of culture. Note mitotic figure in the epithelial layer (arrow). Feulgen and fast green. × 1,150.

9 Duodenal segment explanted at 14 days and cultured for 72 hours with 10^{-6} M HC. Large cavities, apparently dilated vascular spaces, are present in the mucosa. Feulgen and fast green. × 460.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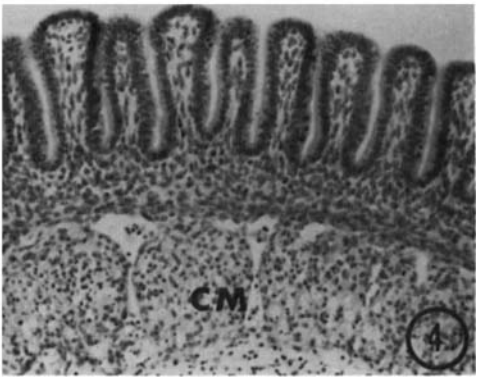
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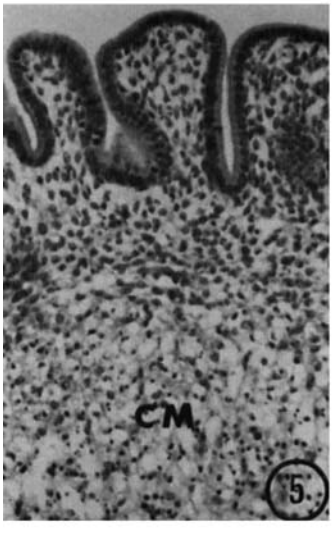
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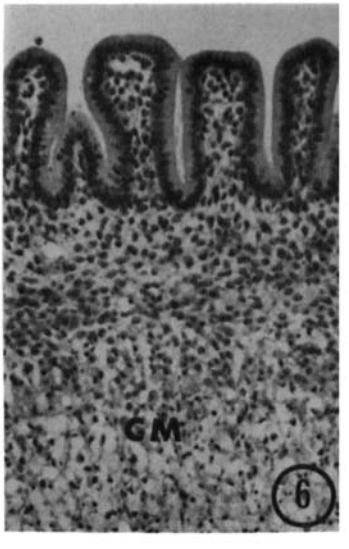
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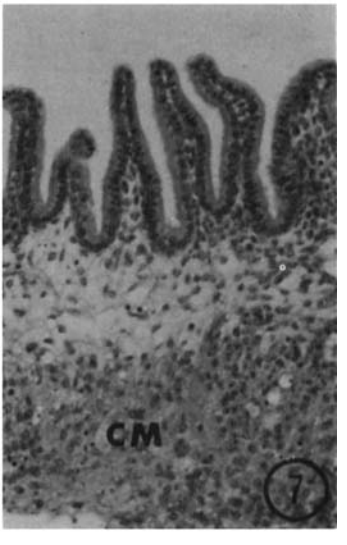
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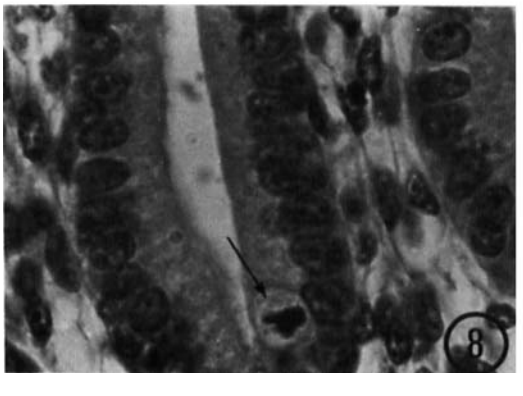
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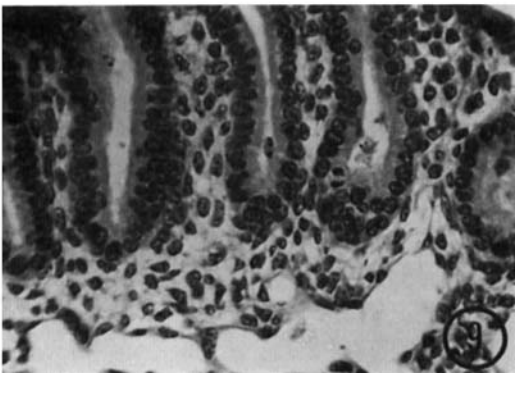
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PLATE 2

EXPLANATION OF FIGURES

10 Uncultured duodenum from a 14-day embryo. Previllous ridges are narrow and epithelium has a "bubbly" appearance. SEM. × 441.

11 Duodenal segment explanted at 14 days and cultured for 24 hours without hormones. Ridges are broader than at 14 days in vivo and epithelium has begun to flatten at ridge crests. SEM. × 438.

12 Duodenal segment explanted at 14 days and cultured for 48 hours without hormones. Previllous ridges are broader and epithelial flattening is more extensive than at 24 hours of culture. SEM. × 420.

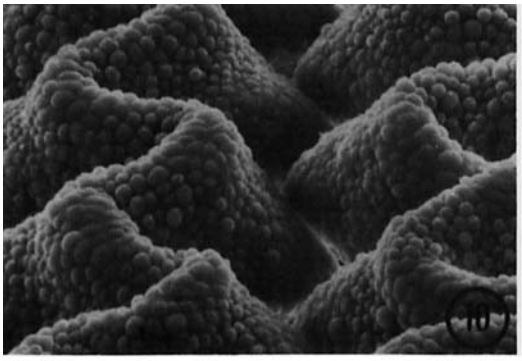
13 Duodenal segment explanted at 14 days and cultured for 72 hours without hormones. The apical regions of most epithelial cells have become concave, creating a "pitted" epithelial surface. SEM. × 476.

14 Duodenal segment explanted at 14 days and cultured for 72 hours without hormones. Although previllous ridges usually maintain a zig-zag pattern during culture, a villus-like pattern occasionally forms between 48 and 72 hours. SEM. × 476.

15 Epithelial surface of a duodenal segment explanted at 14 days and cultured for 72 hours without hormones. "Pits" in the epithelium have a characteristic hexagonal shape. SEM. × 4,340.

16 Duodenal segment explanted at 14 days and cultured for 72 hours with 10^{-6} M HC. Previllous ridges remain narrow during culture, but the zig-zag pattern tends to become convoluted. The epithelial surface flattens without the formation of "pits" and resembles the surface of 17-day duodenum in vivo. SEM. × 427.

17 Uncultured duodenum from a 17-day embryo. Villi have formed from the previllous ridges and an additional rank of villi (arrow) is forming. The epithelial surface has flattened at the tips of most villi. SEM. × 413.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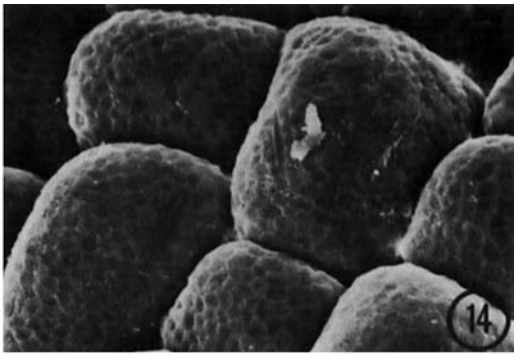
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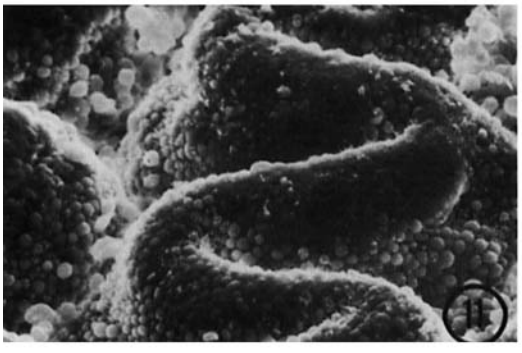
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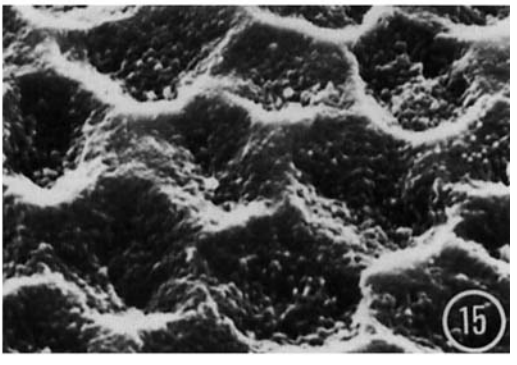
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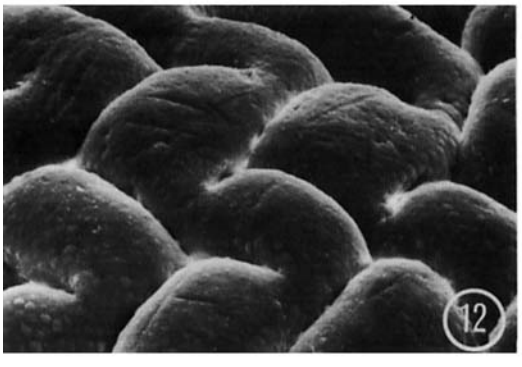
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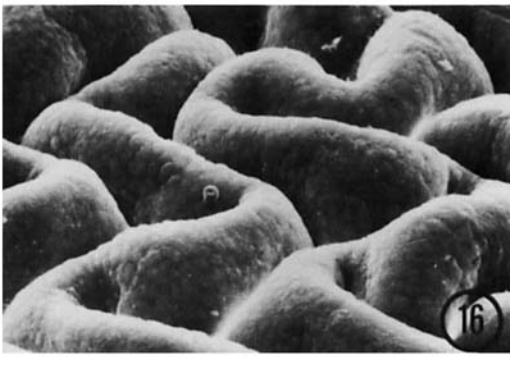
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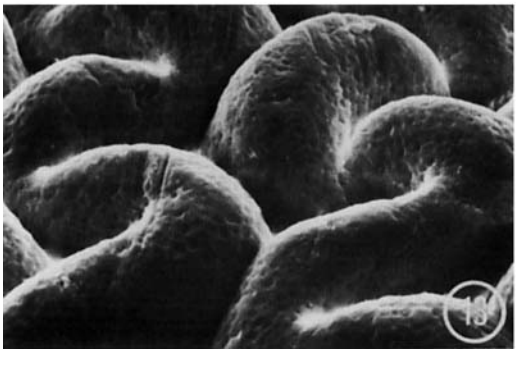
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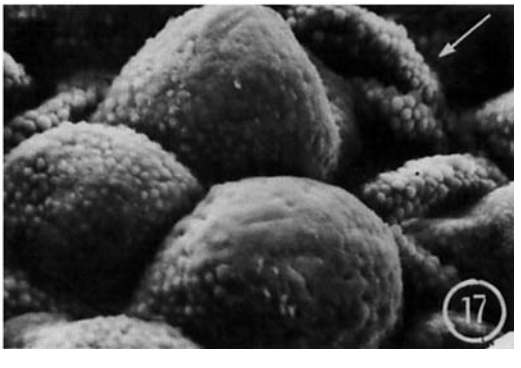
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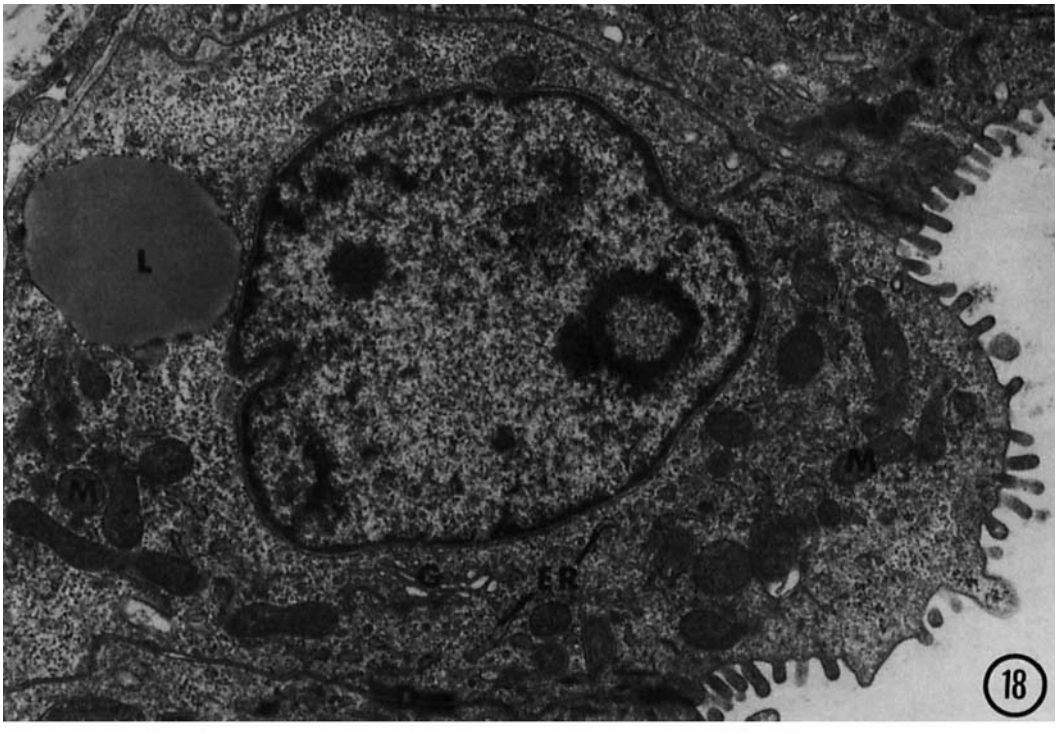
PLATE 3

EXPLANATION OF FIGURES

18 Epithelial cell of uncultured duodenum from a 16-day embryo. Rough endoplasmic reticulum (ER), Golgi elements (G), mitochondria (M), and a lipid droplet (L) are visible. D, desmosomes. TEM. × 18,900.

19 Epithelial cells from duodenal segment explanted at 14 days and cultured for 48 hours without hormones. The epithelium of cultured duodenum maintains a normal ultrastructure for at least 48 hours, including rough endoplasmic reticulum (ER), Golgi elements (G), numerous mitochondria (M), and well formed junctional complexes with desmosomes (D). Glycogen deposits (GL) are also present. The epithelial cells have developed more rapidly than those of the same chronological age in vivo, in terms of flattening of the cell surface and attainment of a narrow, columnar shape. TEM. × 12,150.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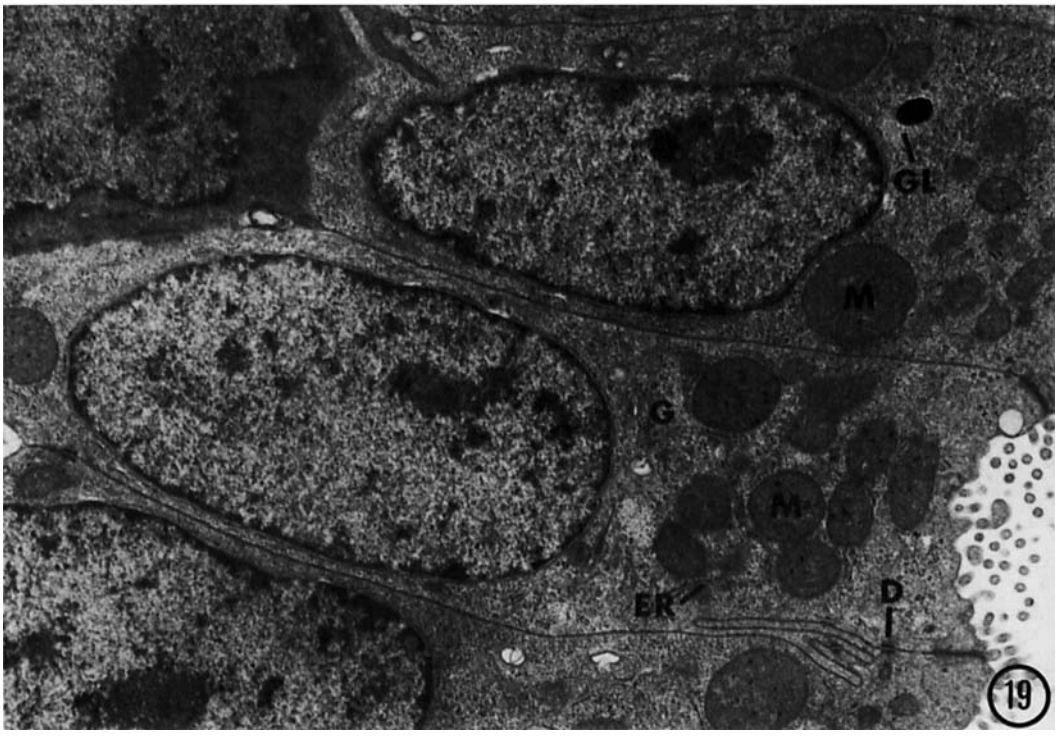
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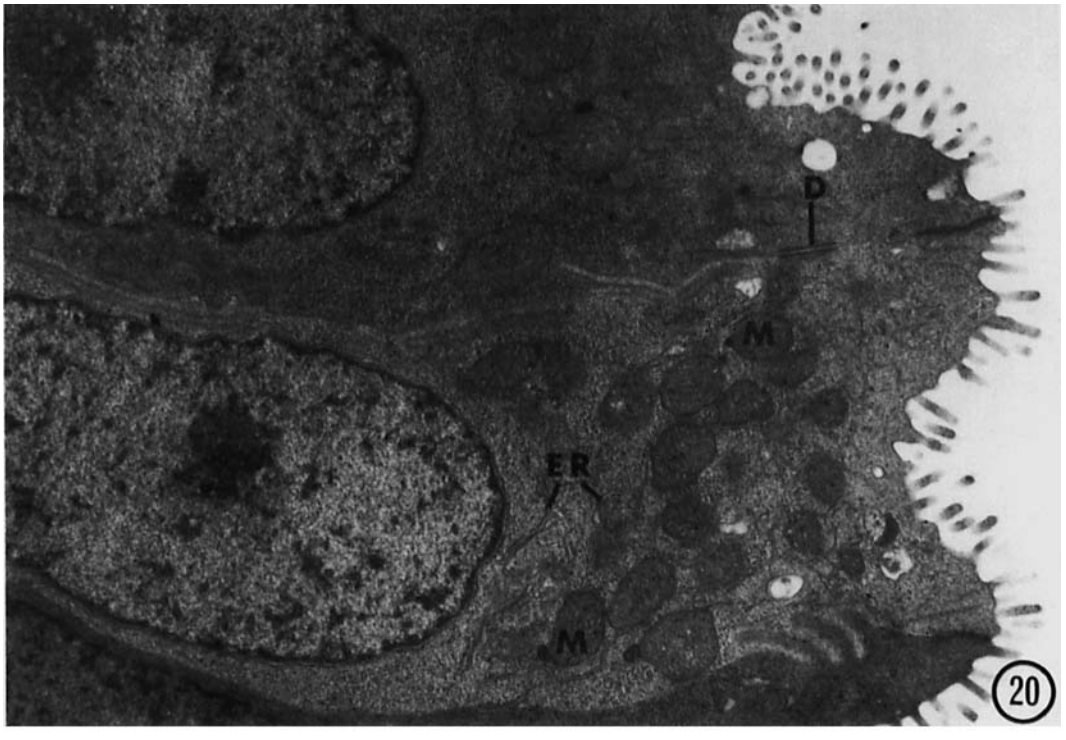
PLATE 4

EXPLANATION OF FIGURES

20 Epithelial cells from a duodenal segment explanted at 14 days and cultured for 48 hours with 10^{-9} M T4. Ultrastructure resembles that of tissue cultured without hormones (fig. 19). The apex of the cell has become concave, forming a "pit" in the epithelial surface. TEM. × 13,500.

21 Epithelial cells from a duodenal segment explanted at 14 days and cultured for 48 hours with 10^{-6} M HC. Ultrastructure resembles that of tissue cultured without hormones (fig. 19). TEM. × 16,200.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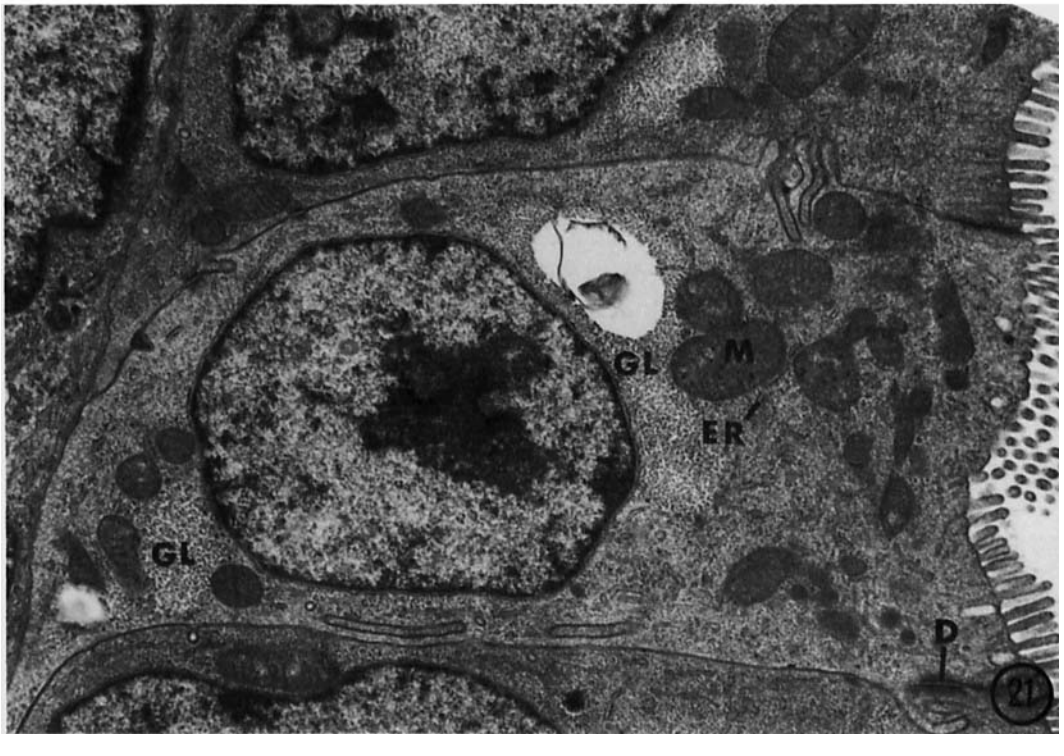
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PLATE 5

EXPLANATION OF FIGURES

22 Villi of uncultured duodenum from a 17-day embryo. Feulgen and fast green. × 1,150.

23 Previllous ridges of a duodenal segment explanted at 14 days and cultured for 72 hours without hormones. Epithelial cell height of tissue cultured 72 hours exceeds that of 17-day duodenum in vivo. Feulgen and fast green. × 1,150.

24 Villi of a duodenal segment explanted at 14 days and cultured for 72 hours with 10^{-6} M HC. Epithelial cell height is similar to that of tissue cultured without hormones. Feulgen and fast green. × 1,150.

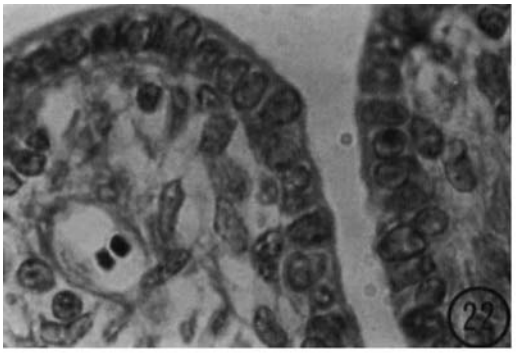
25 Villi of uncultured duodenum from a 20-day embryo. Epithelial height is greater than at 17 days. Feulgen and fast green. × 1,150.

26 Villi of a duodenal segment from the same embryo as tissue in figure 23, but cultured for 72 hours with 10^{-9} M T4. Epithelial height is greater than in tissue cultured without hormones and equals the height of epithelium from 20-day duodenum in vivo. Feulgen and fast green. × 1,150.

27 Villi of uncultured duodenum from a 16-day embryo. The brush border of villar tips stains lightly with PAS. PAS and fast green. × 589.

28 Villi of uncultured duodenum from an 18-day embryo. Brush border stains with PAS darker and more uniformly than at 16 days. PAS and fast green. × 589.

29 Villi of duodenal segment explanted at 14 days and cultured for 48 hours without hormones. Brush border stains with PAS darker in 48-hour cultured tissue than in 16-day duodenum in vivo and is similar in staining to the brush border of 18-day duodenum. PAS and fast green. × 589.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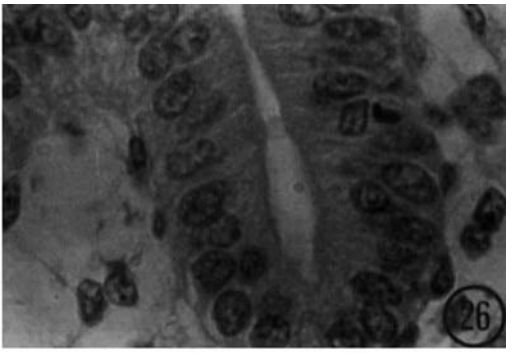
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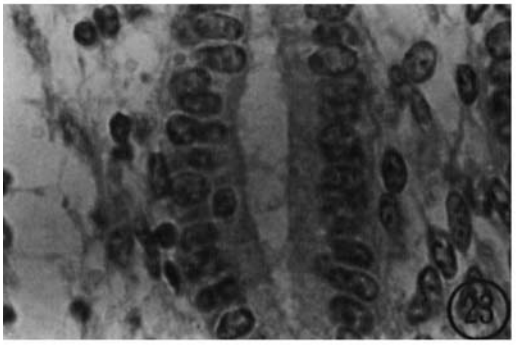
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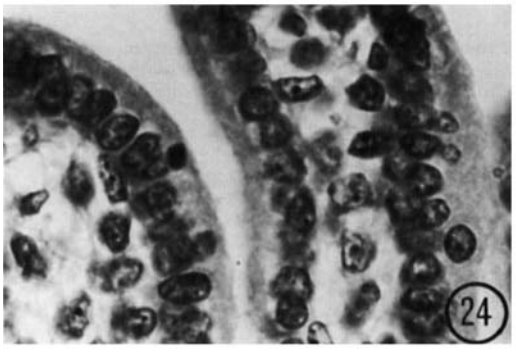
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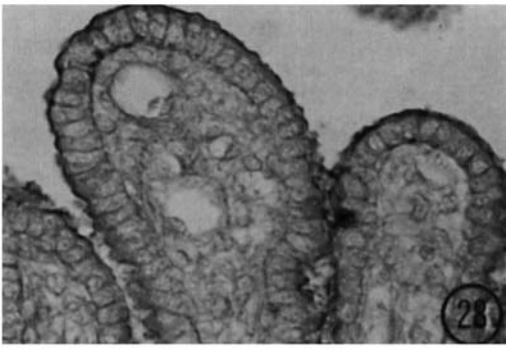
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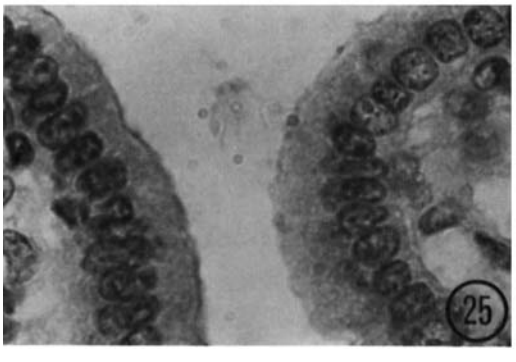
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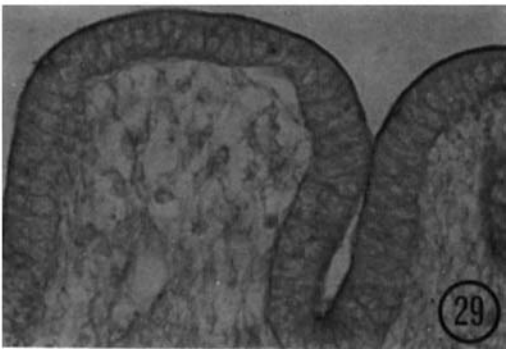
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PLATE 6

EXPLANATION OF FIGURES

30 Previllous ridge crest of uncultured duodenum from a 15-day embryo. Epithelium has a "bubbly" appearance and individual cells appear to be loosely attached to the tissue. Scattered microvilli are present; between microvilli the cell surface is visible. SEM. × 4,690.

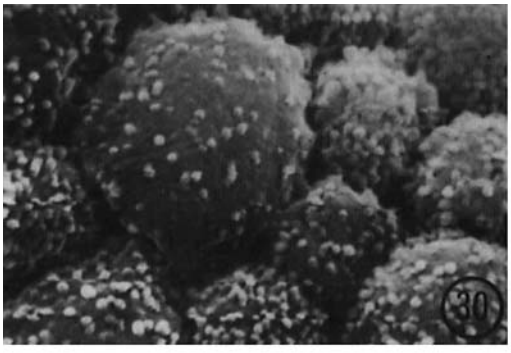
31 Villus tip of uncultured duodenum from a 17-day embryo. Epithelium has flattened and the cells appear to be tightly attached to the tissue. Microvilli, which are much more densely packed than at 15 days, obscure the cell surface. SEM. × 4,200.

32 Previllous ridge of a duodenal segment explanted at 14 days and cultured for 24 hours without hormones. Epithelium at the ridge crest has flattened, but some cells along the side of the ridge still appear to be loosely attached to the tissue. SEM. × 1,680.

33 Ridge crest of the specimen in figure 32. Microvilli of tissue cultured 24 hours are more numerous than in 15-day duodenum in vivo and the epithelial surface resembles that of 17-day tissue. A small number of exceptionally long microvilli (arrow) also occur in the brush border of cultured duodenum. SEM. × 4,200.

34 Brush border of uncultured duodenum from a 16-day embryo. TEM. × 22,950.

35 Brush border of a duodenal segment explanted at 14 days and cultured for 48 hours without hormones. Microvilli of cultured tissue are more numerous and more uniform in shape than those of duodenum of the same chronological age in vivo. Likewise, microvillar rootlets (R) of cultured tissue are more prominent and tend to penetrate more deeply into the apical cytoplasm. TEM. × 24,300.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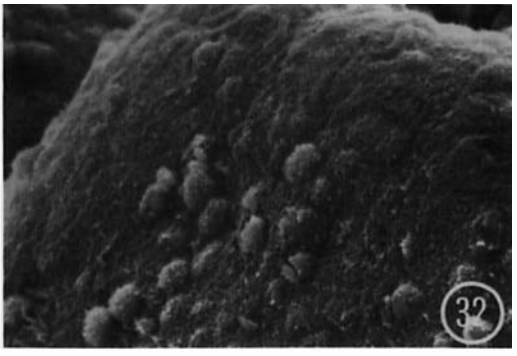
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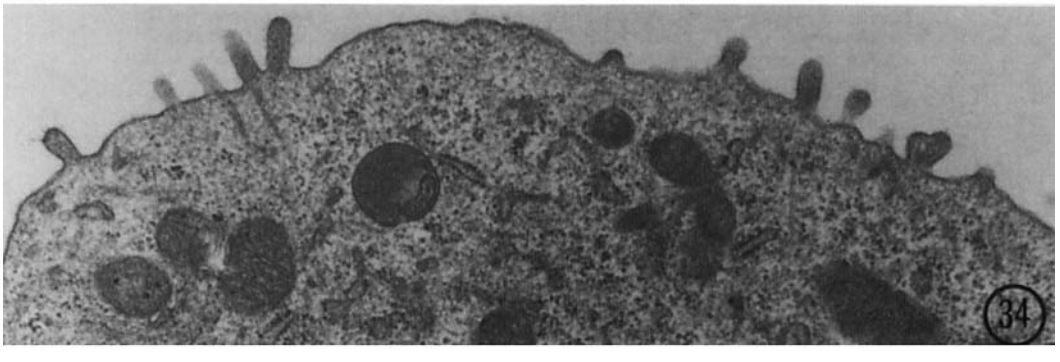
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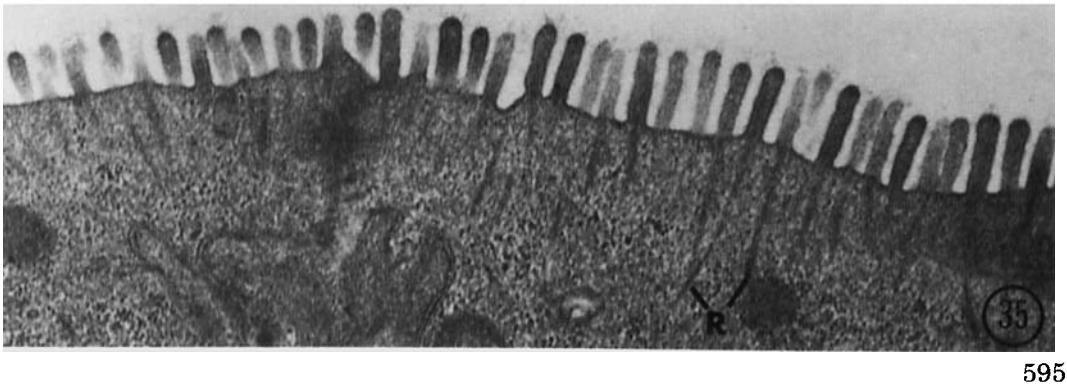
R
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PLATE 7

EXPLANATION OF FIGURES

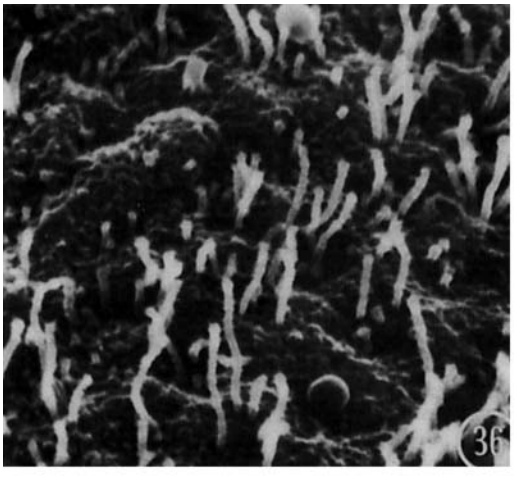
36 Previllous ridge crest of duodenal segment explanted at 14 days and cultured for 24 hours with 10^{-9} M T4. Short, densely packed microvilli obscure the surfaces of epithelial cells. A moderate number of exceptionally long microvilli are also present. SEM. × 4,340.

37 Previllous ridge crest of duodenal segment explanted at 14 days and cultured for 48 hours with 10^{-9} M T4. A large number of exceptionally long microvilli are present. SEM. × 4,200.

38 Previllous ridge crest of duodenal segment explanted at 14 days and cultured for 72 hours with 10^{-9} M T4. Short, densely packed microvilli are still present, but only a few long microvilli remain. SEM. × 4,200.

39 Brush border of a duodenal segment explanted at 14 days and cultured for 48 hours with 10^{-9} M T4. Short, densely packed microvilli are present in addition to scattered long microvilli. Microvilli have an abundant glycocalyx (GX). TEM. × 32,400.

40 Same specimen as figure 39. The exceptionally long microvilli of T4-treated tissue have an apparently normal core structure and an abundant glycocalyx (GX). TEM. × 32,400.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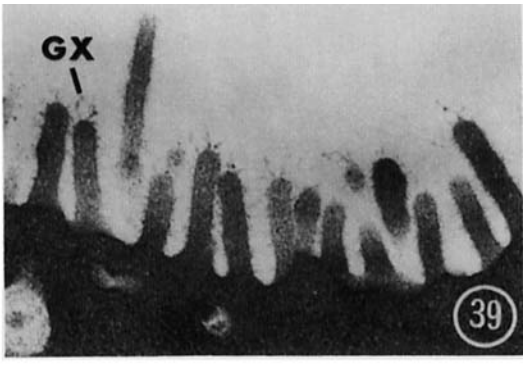
GX
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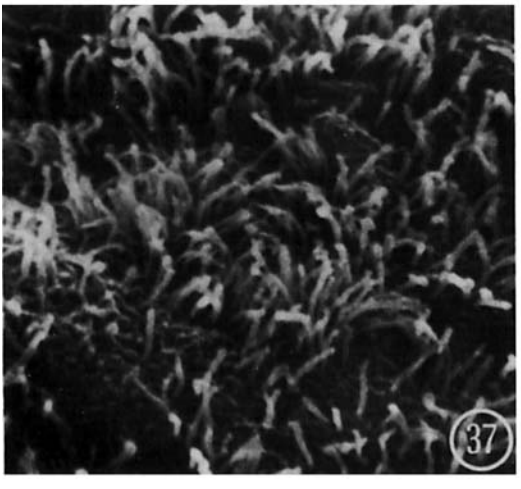
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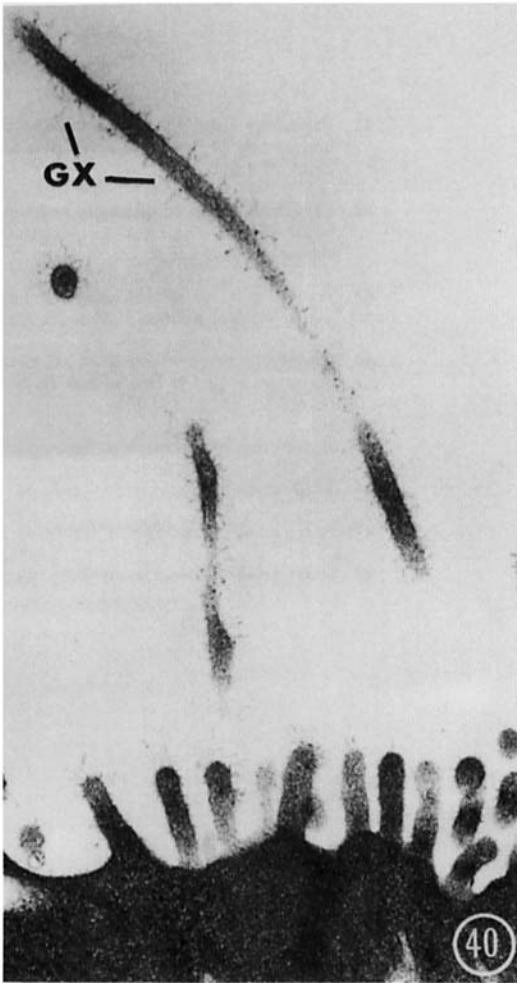
GX
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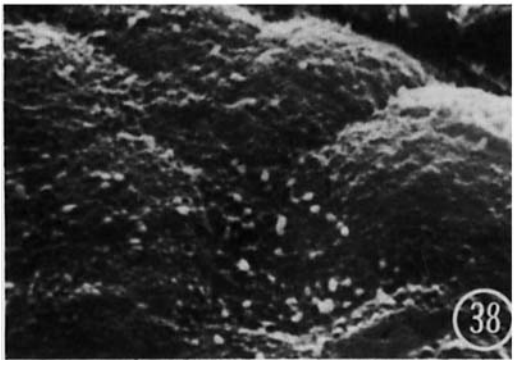
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PLATE 8

EXPLANATION OF FIGURES

41 Previllous ridge of duodenal segment explanted at 14 days and cultured for 24 hours with 10^{-8} M T4. A large number of exceptionally long microvilli are present. SEM. × 1,680.

42 Previllous ridge of duodenal segment explanted at 14 days and cultured for 48 hours with 10^{-8} M T4. More exceptionally long microvilli are present than found in tissue cultured for 48 hours without hormones (fig. 43). SEM. × 1,680.

43 Previllous ridge of duodenal segment explanted at 14 days and cultured for 48 hours without hormones. Few long microvilli are present. SEM. × 1,680.

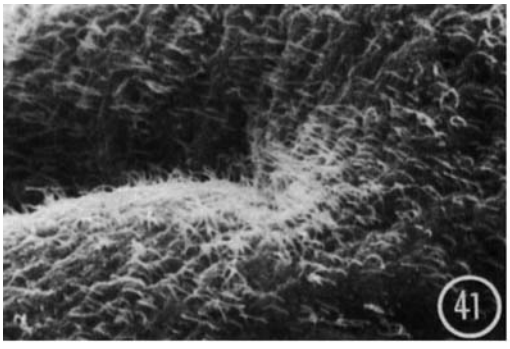
44 Previllous ridge of duodenal segment explanted at 14 days and cultured for 48 hours with 10^{-6} M HC. Microvilli are similar to those of tissue cultured without hormones. SEM. × 1,715.

45 Ridge crest of specimen in figure 41. SEM. × 4,200.

46 Ridge crest of specimen in figure 42. SEM. × 4,200.

47 Ridge crest of specimen in figure 43. SEM. × 4,200.

48 Ridge crest of specimen in figure 44. SEM. × 4,200.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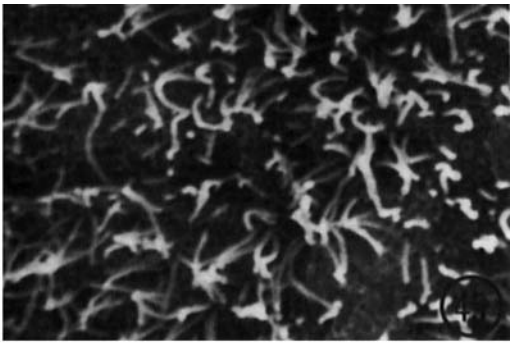

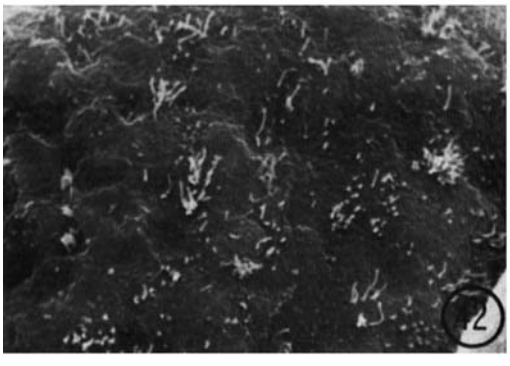

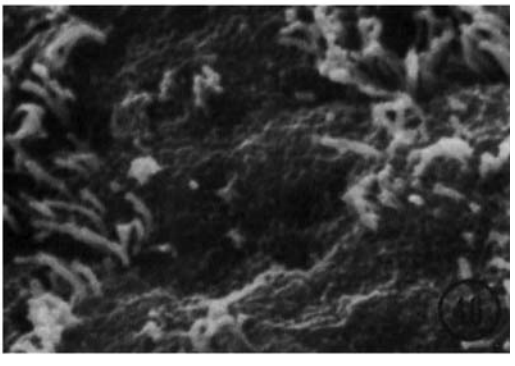

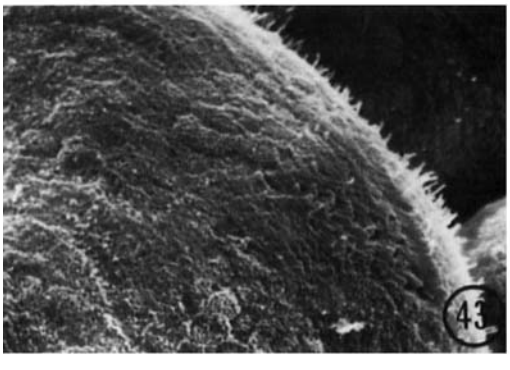
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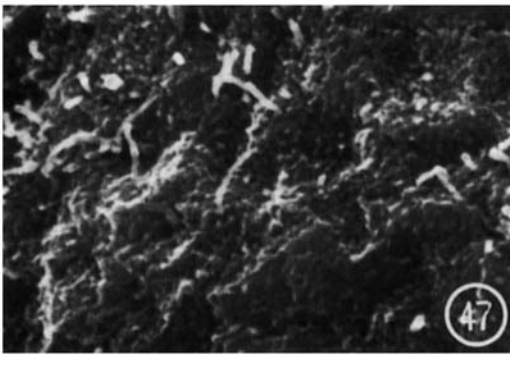
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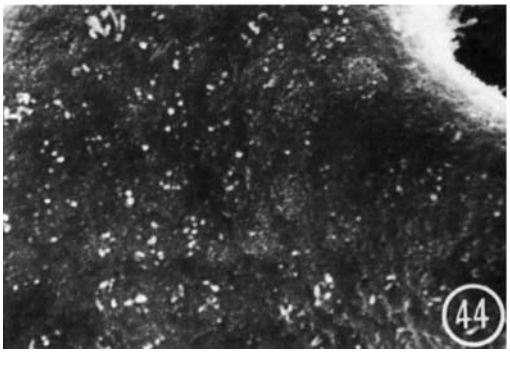
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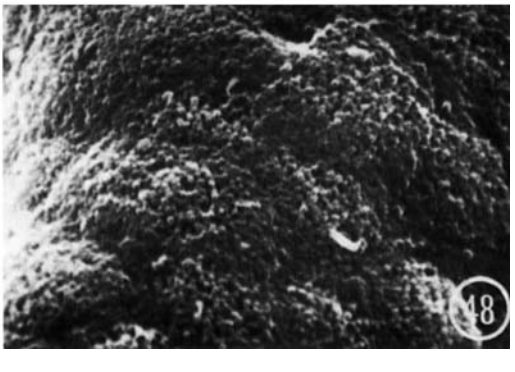
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