ALTERATIONS IN MORPHOLOGY OF DEVELOPING MICROVILLI ELICITED BY CYTOCHALASIN B

Studies of Embryonic Chick Intestine in Organ Culture

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INTRODUCTION

The ultrastructure of microvilli comprising the brush border of cells of the intestinal epithelium has been extensively studied (e.g., Ito, 1965; Overton et al., 1965; Brunser and Luft, 1970; Mukherjee and Staehelin, 1971), and the sequence of events that occurs during normal development of these organelles has been described for several species (Overton and Shoup, 1964; Overton, 1965; van der Starre-van der Molen and dePriester, 1972; and Bonneville and Weinstock, 1970). Nevertheless, the processes that regulate the morphogenesis of microvilli are, for the most part, still enigmatic.

Cytochalasin B (CB) somehow interferes with mechanisms that regulate morphogenetic processes in a variety of developing systems (Wessells, et al., 1971; Spooner and Wessells, 1972; Wrenn, 1971; Yamada et al., 1971; Schroeder, 1969, 1970; Cloney, 1969, 1972). During the course of a study of the effects of CB on morphogenetic folding of the intestinal epithelium in the chick embryo, we discovered that the drug exerted a profound and unusual effect upon the formation of microvilli at the cell surface. CB therefore suggests itself as a potentially useful tool with which to analyze some aspects of microvillar morphogenesis. This report describes the cytological events that result from exposure of embryonic intestine to CB in organ culture.

MATERIALS AND METHODS

Eggs from a hybrid line of white leghorn chickens were incubated in a forced draft incubator. All stages were counted as days postincubation. 11-day old embryos were sacrificed by decapitation. The duodenal loop was removed and slit open lengthwise, and 2-mm pieces were cut from the proximal end for culture. Only tissues showing eight distinct previllous ridges (cf. Grey, 1972) were used in these experiments. Tissues were cultured, serosal surface down, in chambers positioned on the chorioallantoic membrane (CAM) of 10–11-day old host embryos (Kato, 1970). Usually, five explants could be made with the tissue from one donor. At least one piece of tissue from each donor served as a control. Explants (about 80%) were defined as being successful if the tissue became vascularized, exhibited rhythmic contractions, and had all previllous ridges present. No significant differences could be detected between the ultrastructure of duodenal epithelium from 12-day embryos and that of tissue from 11-day embryos that had been cultured on the CAM for 24 h.

The culture medium consisted of nine parts Trowell T8 plus one part fetal calf serum and contained 100 U/ml of

penicillin and 100 μg/ml streptomycin (Grand Island Biological Co., Grand Island, N. Y.). For control cultures we first used medium containing 1% dimethylsulfoxide (DMSO), the solvent for CB. DMSO had no effect on the tissue; it was therefore omitted from most control cultures. Experimental cultures were exposed for 15 min–6 h to culture medium to which cytochalasin B (CB) (Imperial Chemical Industries Ltd.) was added to achieve a final concentration in the medium of 0.1, 1.0, 2.5, and 10.0 μg/ml.

Tissues were fixed in cacodylate-buffered 2% glutaraldehyde (pH 7.2), postfixed in osmium tetroxide, and embedded in Epon. Thin sections were stained in 2% aqueous uranyl acetate and lead citrate and examined with a Hitachi HU-11E electron microscope. Some explants were prepared for scanning electron microscopy and examined in a Cambridge Stereoscan electron microscope (Grey, 1972).

RESULTS

Control Cultures

Fig. 1 illustrates the apical region of the epithelium in control cultures and serves as a reference for the alterations evoked by CB. As will be described later, CB alters the following properties: (*a*) length, shape, and distribution of microvilli; (*b*) length of microvillar core filaments; (*c*) cell shape; and (*d*) frequency and size of vesicles in the apical cytoplasm.

In control tissues, the apical surfaces of the epithelial cells are studded with many short, straight microvilli, about 0.4 μm long and 0.08–0.1 μm in diameter (Fig. 1). (In mature cells, the comparable dimensions are 2 μm in length and 0.1 μm in diameter, cf. Overton and Shoup, 1964; LeCount and Grey, 1972.) Although they are rather evenly distributed over the apical surface (Grey, 1972), there are fewer microvilli per cell in the 12-day embryonic epithelium than in the adult (Overton and Shoup, 1964). The microvilli usually appear as singular structures; branching is rarely observed. The apical membrane at this stage is already coated with a thin, wispy glycocalyx (Fig. 1).

In the interior of each microvillus, the typical 40–60-Å core filaments are present at the 12-day stage (Overton and Shoup, 1964) and are organized into a compact bundle that inserts into an electron-dense region beneath the membrane at the tip of the microvillus (Fig. 1, inset). At the opposite end, each bundle of filaments extends about 0.2 μm beyond the base of the microvillus into the terminal web region of the cell.

The terminal web of 12-day embryonic epithelium is only partially developed and lacks the dense granular or fibrillar appearance seen in mature epithelial cells (Brunser and Luft, 1970; Mukherjee and Staehelin, 1971). Most large organelles such as mitochondria, endoplasmic reticulum, or Golgi apparatus are, however, excluded from this area (Fig. 1).

Membrane-bound vesicles, suggested to be the source of the membranous component of developing microvilli (Bonneville and Weinstock, 1970; van der Starre-van der Molen and dePriester, 1972), are present in the apical cytoplasm and are filled with a flocculent, fibrillar material that is closely associated with the inner component of the vesicle membrane (Fig. 1).

General Effects of CB on Cultured Intestine

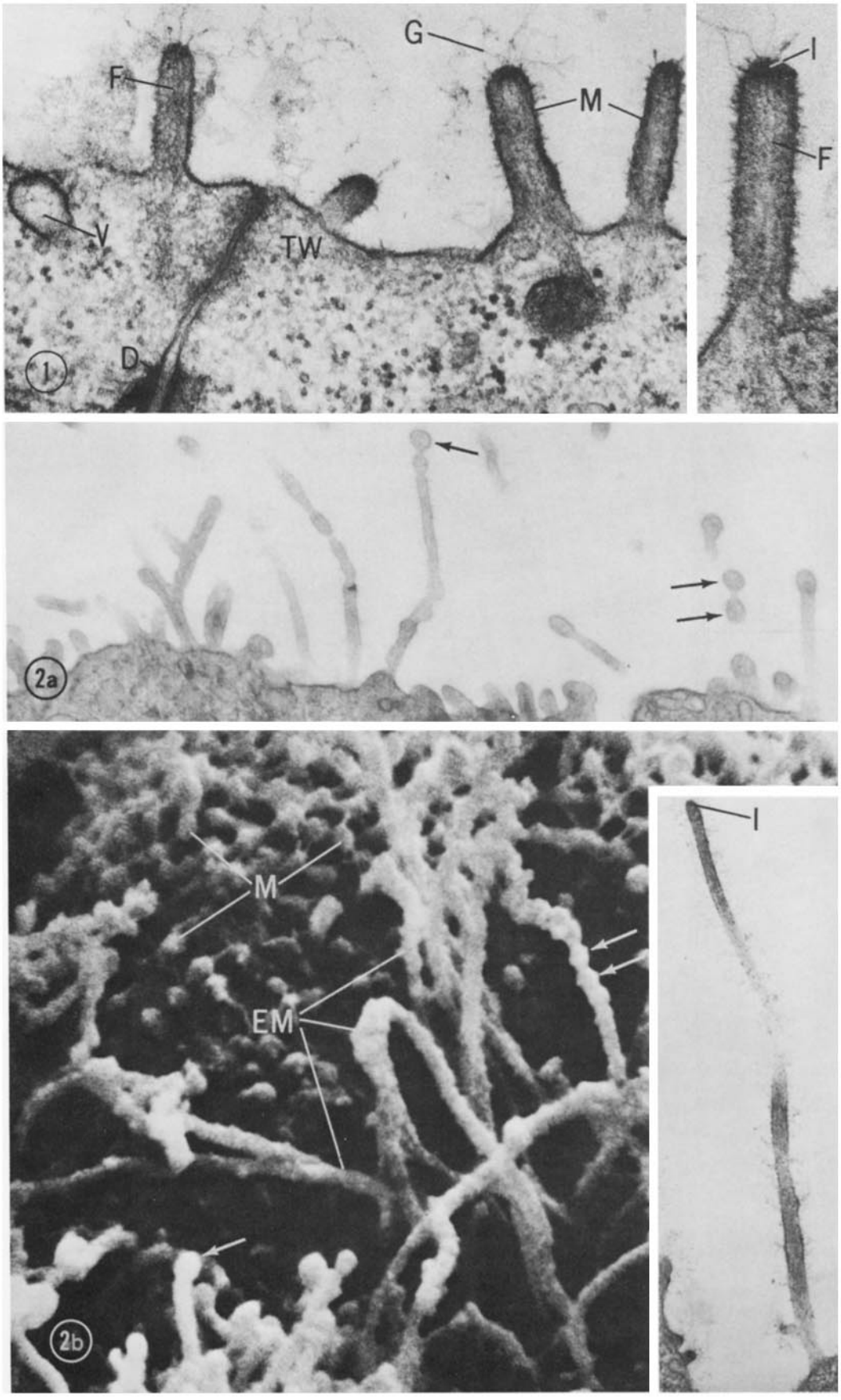
At the concentrations of CB employed for most of our experiments almost all cultured tissues appeared to be healthy. The cytoplasm of CB-treated tissues was indistinguishable from that of controls; desmosomal tonofilaments, 90–120 Å in diameter, maintained proper orientation and were not disrupted by CB treatment at any dose level.

Concentrations of CB of 10 μg/ml produced erratic results. Many of the cultures exposed to this high concentration appeared healthy ultrastructurally; the effects of CB in these cases were confined primarily to the brush border, as discussed in the next section. In some cases, CB concentrations of 10 μg/ml caused the cultured tissue to die. All obviously damaged explants were excluded from consideration in the results to be presented.

Effects of CB on Length and Shape of Microvilli

Exposure of cultured 12-day duodenal fragments to CB at concentrations of 5 or 10 μg/ml for 6 h resulted in the formation of microvilli that were extremely long and, in many cases, branched (Figs. 2, 3). Typically, the length of elongated microvilli was about 3 μm, i.e., the length of microvilli in the adult duodenum. The maximum length was 4 μm.

Except for the bifurcations described below, the structure of most of the exceptionally long microvilli appeared normal. The long microvilli were generally of the same diameter as normal-sized microvilli, although elongated microvilli were oc-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casionally observed to exhibit multiple constrictions (Fig. 2 *a*, *b*). The bundle of filaments in the core was normal in appearance; the filaments inserted as usual in an electron-dense cap at the tip of the organelle (Fig. 2).

The branched segments of the elongated microvilli were usually of the same diameter as the normal microvilli and each ramification contained a bundle of core filaments of normal dimensions. The bundles inserted in a typical electron-dense cap at the terminus of the segment (Fig. 3).

The elongation effect was dependent upon the concentration of CB in the medium. At exposure times of 6 h, maximum elongations of microvilli were evoked with CB concentrations of 10 μg/ml; at 5 μg/ml, CB also promoted elongation, but less effectively. Concentrations of 0.1–2.5 μg/ml rarely evoked elongation of microvilli. Doses from 0.1 to 5.0 μg/ml did, however, result in the appearance of exceptionally long (up to 1.7 μm) bundles of filaments that protruded from microvilli deep into the cytoplasm (Fig. 4). In most cases the filaments were associated with microvilli that were normal in appearance for the 12-day stage, although some were associated with branched microvilli. The bundles were usually very straight, of the same diameter as the bundles in normal microvilli, and tapered to a fine point in the cytoplasm. The combination of elongated filaments and normal-sized microvilli was also observed after brief exposures (e.g., 15 min) of cultures to 5 μg/ml and in a few cases, after exposure to 10 μg/ml.

We examined the kinetics of elongation at CB concentrations of either 5 or 10 μg/ml of culture medium (Fig. 5). The effect of the drug was extremely rapid. Lengths of 1 μm were observed in some cultures after only 15 min. At 1 h, a five-fold increase in length was common. The frequency of branching also increased with time. Exposure periods of 15 or 30 min (10 μg CB/ml) produced microvilli that were branched at only one point, and all of these were short branches near the bases of the microvilli. After 1–6 h, the number of branches on some individual microvilli also increased, sometimes producing cactus-shaped structures with numerous side arms (Fig. 3 *b*). Branching was less frequent with CB concentrations of 5 μg/ml.

All cultures treated with CB at 5 or 10 μg/ml exhibited elongated microvilli, but the effect within any one fragment was erratic, in that not all cells were affected. The number of cells that exhibited elongated or branched microvilli was difficult to determine, but we estimate that the response involved about 50% of the cells in each explant. In some cases, only a portion of the surface of a cell seemed to be affected by CB, since elongated microvilli were observed on cells bearing microvilli of normal dimensions (Fig. 2).

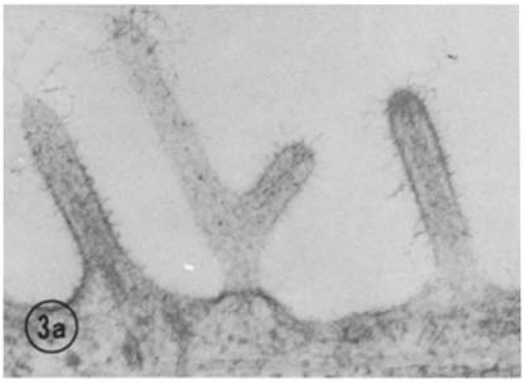
Cell Shape and the Distribution of Microvilli

CB, particularly at concentrations of 5 and 10 μg/ml, sometimes exaggerated the bulging of the

FIGURE 1 Apical region of epithelial cells of a control explant showing typical microvilli (M) containing core filaments (F) inserting into a dense plaque (I) at the apex of the microvilli (see inset). The luminal plasma membrane is coated with a filamentous glycocalyx (G). Adjacent to the luminal membrane is a cytoplasmic vesicle (V). The terminal web (TW) at this stage is a loosely organized filamentous zone distinguished primarily by the presence of microvillar core filaments. Desmosome, (D). × 58,000. Inset × 80,000.

FIGURE 2 *a* Luminal surface of one epithelial cell after exposure to 10 μg CB/ml for 6 h showing numerous elongated microvilli and a branching microvillus adjacent to normal-sized microvilli. Some of the elongated microvilli have a beaded appearance (arrows), seen rarely, that may be due to degeneration. × 24,000.

FIGURE 2*b* Scanning electron micrograph of the surface of several epithelial cells from an explant that had been exposed to 5 μg CB/ml for 6 h. The elongated microvilli (EM) are about 3 μm long. Microvilli (M) of normal dimensions are partially obscured by extracellular material adhering to the cell surface. Some of the elongated microvilli have a beaded appearance, especially near their tips (arrows). × 20,000. *Inset.* An extremely long microvillus extending from an epithelial cell in an explant cultured for 6 h in the presence of 10 μg CB/ml. This microvillus, about 4 μm in length, has a dense plaque (I) in the tip and a short branch near its base. × 20,000.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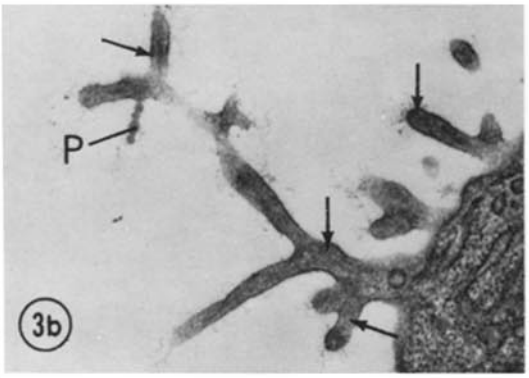
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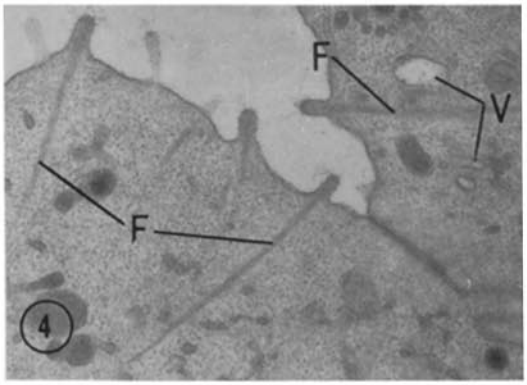
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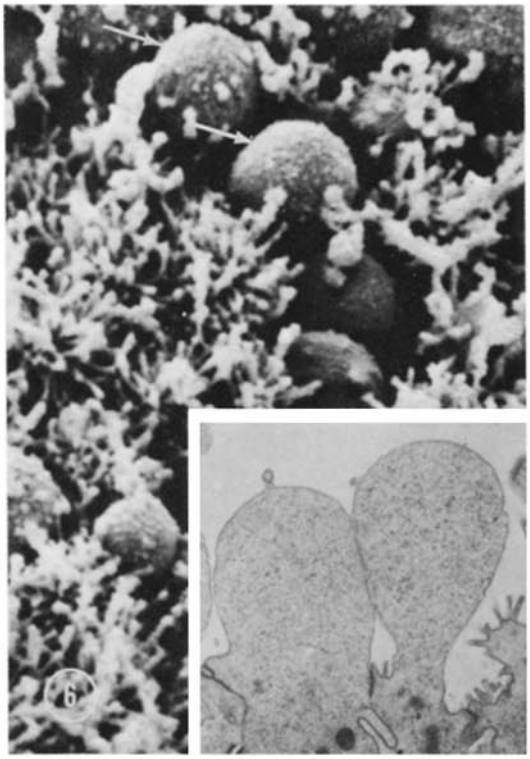
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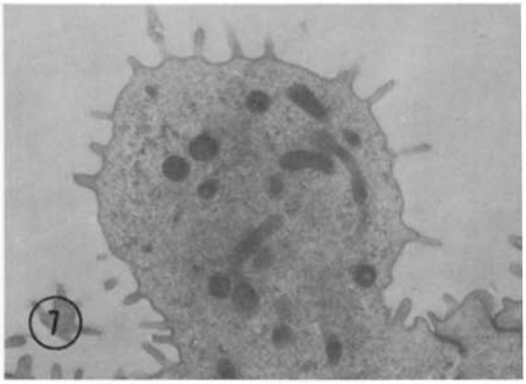
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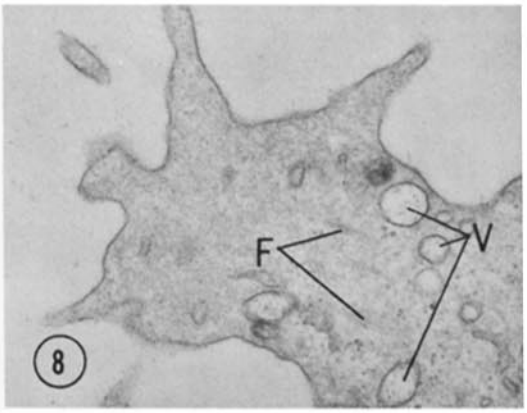
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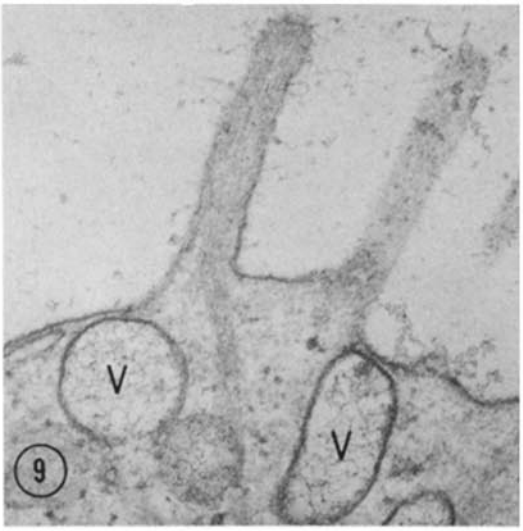
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apical surface, especially in cells lining the valleys between previllous ridges. Although not common, these distortions are described because they were usually associated with an alteration in the distribution of microvilli. The altered distribution of microvilli, in turn, seemed to be associated with a change in the appearance of the cytoplasm in the region of the terminal web. In affected cells, either part or all of the apical surface showed bulging (Figs. 6–8). In cases where the bulged surface lacked microvilli, the region within the bulge was devoid of organelles and was filled with a finely granular material (Fig. 8). In those cases where microvilli were present on the bulged region, the cells resembled those cells normally found in the valleys between previllous ridges (Fig. 7).

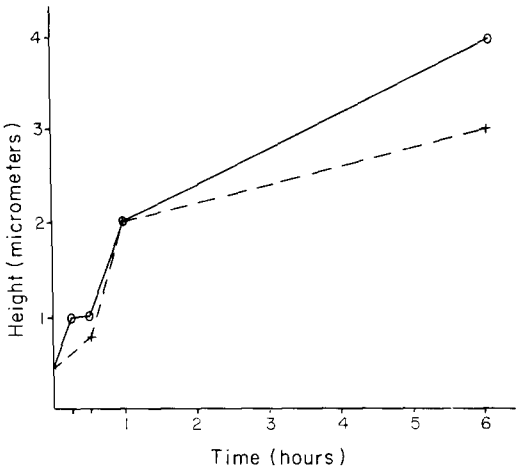

FIGURE 5 Maximum height of microvilli observed after culturing explants in CB at either 10 μg/ml (—) or 5 μg/ml (---). Only those microvilli in which the dense plaque in the apex could be observed were measured. Each point represents the longest microvillus observed at any one dose and exposure period.

Effects of CB on Cytoplasmic Vesicles

The membrane-bound vesicles of the apical cell cytoplasm appeared to be larger and more abundant after CB treatment (Figs. 4, 8, 9). The increased frequency was especially noticeable since many of the vesicles were larger than those present in controls (up to 0.4 μm in diameter compared to 0.1 μm in controls).

DISCUSSION

The two major features of microvillar morphogenesis that are most dramatically perturbed

FIGURE 3 *a* A single branch on a microvillus of an explant treated for 15 min with 10 μg CB/ml. Normal-sized microvilli flank the branching microvillus. A dense cap can be seen in the unbranched microvillus at the right. × 56,800.

FIGURE 3 *b* Complex branching microvilli, produced after 6 h of treatment with 5 μg CB/ml. Filaments (arrows) can be identified at several locations in the core regions of the microvilli. A rarely observed narrow protrusion (*p*) is present on the highly branched microvillus. × 37,500.

FIGURE 4 Exceptionally long bundles of core filaments (F) from microvilli of normal length. Explant was exposed to 5 μg CB/ml for 6 h. These cells in this region of the explant bulged into the lumen. Vesicles (V) are present in the apical cytoplasm. × 36,000.

FIGURE 6 Scanning electron micrograph of the surface of epithelial cells from an explant cultured for 3 h in 5 μg CB/ml. The apical surface of some of the cells has bulged into the lumen (arrows). These cells lack microvilli on their surface. Most of the other cells in this region of the explant possess elongated and branching microvilli which obscure their apical surfaces. × 5,000. *Inset*. Transmission micrograph of the bulged apical regions of cells exposed to CB. No microvilli are seen; the cytoplasm within the bulges is devoid of large organelles and is finely granular. (10 μg CB/ml, 15-min exposure). × 5,700.

FIGURE 7 Ballooned apical region of a cell that still has normal microvilli after treatment with 10 μg CB/ml for 30 min. The bulging of the surface is very similar to that of normal cells in the valleys between previllous ridges of the embryonic intestine. × 12,200.

FIGURE 8 A small, localized region of bulging on the surface of a cell after treatment with 10 μg CB/ml for 1 h. No normal microvilli are present and the cytoplasm within the bulge is finely granular with some arrays of fine filaments (F) present. Numerous cytoplasmic vesicles (V) are also seen. × 29,000.

FIGURE 9 Apical region of a cell from tissue exposed to 10 μg CB/ml for 1 h. The apical cytoplasm contains exceptionally large vesicles (V) filled with a flocculent material. × 61,000.

by CB are: (*a*) elongation of microvilli and/or core filaments; and (*b*) spatial distribution of microvilli over the apical surface of the cell.

Just how CB accentuates the elongation of microvilli is not known. A prominent effect of the drug in many other cell types is its inhibition of contractile functions (Carter, 1967, 1972) and the concomitant dissociation of 40–70-Å actin-like microfilaments that accompanies this inhibition in many cases (Cloney, 1972; deLaat et al., 1973; Schroeder, 1970; Spooner and Wessells, 1970, 1972; Wrenn and Wessells, 1970; Yamada et al., 1971). CB also alters significant properties of the plasma membrane (Cohen et al., 1972; Estensen and Plagemann, 1972; Kletzien and Perdue, 1973; Lieberman et al., 1973; Mizel and Wilson, 1972; Plagemann and Estensen, 1972), and may even exert its effect on microfilaments at this site (cf. discussion by Spooner, 1973), although it does alter the properties of purified actin (Spudich, 1972).

Cytoplasmic microfilaments that are susceptible to CB resemble actin in several of their characteristics (Fine and Bray, 1971; Schroeder, 1973; Spooner et al., 1973; Yang and Perdue, 1972). It is perhaps not surprising that the morphogenesis of microvilli in the chick intestine should be susceptible to CB, since actin is a component of the core filaments of these organelles (Ishikawa et al., 1969, Tilney and Mooseker, 1971).

The notion that actin may serve to elongate structures, in addition to its better-known function of contraction, is supported by recent studies of acrosome elongation in sperm (Tilney et al., 1973; Jessen et al., 1973). Such a function was also suggested for the core filaments of intestinal microvilli by Tilney and Cardell (1970), who observed reformation of microvilli in the adult salamander intestine after release from hydrostatic pressure. Since microvilli do not begin to elongate until the terminal web (which is disrupted by pressure) reforms, those authors suggested that the terminal web serves as a platform for the growing core filaments to push against. Many of the CB-treated cells resemble cells immediately after release from hydrostatic pressure, in that bulging of the luminal surface occurred in both cases (Figs. 6–8). This similarity suggests that CB may alter or disrupt the terminal web, a possibility further supported by the anomalous penetration of the exaggerated bundles of filaments into the cytoplasm (Fig. 4).

The conclusion that CB alters the normal spatial distribution of microvilli on cells is derived from two observations. First, elongated microvilli are often positioned beside microvilli of normal length or adjacent to regions of cell surface on which no microvilli are present at all (Fig. 2). The second observation is the branching phenomenon, which occurs with extreme rarity in normal development. Branching of microvilli has, however, been induced experimentally in the intestinal epithelium of the adult salamander. In that case, branching occurs as microvilli reform after the tissue is released from hydrostatic pressure (Tilney and Cardell, 1970).

These alterations in spatial distribution suggest that CB affects the spatial arrangement in the plane of the plasma membrane of the sites responsible for the initiation and/or formation of microvilli. Tilney and Cardell (1970) suggested that the electron-dense region at the apex of each microvillus may serve as a nucleation center for assembly of the core filaments. They observed such regions on the *sides* of reforming microvilli and interpreted these regions as sites of incipient branches. We observed similar electron-dense caps at the apices of branched segments in CB-treated tissues. The suggested alteration of microvillar initiation sites is consistent with a direct effect of CB on the plasma membrane.

Since the major effect reported in this study involves an organelle concerned with transport, it is of obvious importance to determine the effects of CB on transport processes. This problem is difficult to approach with a culture technique that utilizes a vascular supply from a host embryo. Preliminary studies with alternative culture methods show, however, that CB will elicit elongation and branching of microvilli when pieces of intestine are suspended in defined medium for short time periods. Experiments are in progress to determine whether CB alters microvilli by blocking transport of components from the medium.

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