Organ Culture of Mucosal Biopsies of Human Small Intestine

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ABSTRACT In vitro experiments of small intestinal mucosal function and metabolism utilizing excised tissue have been limited to a few hours by rapid epithelial cell necrosis which occurs with current incubation methods. We describe a method for culturing human mucosal biopsies for up to 24 hr employing organ culture methodology and demonstrate its potential application to studies of mucosal function. Peroral biopsies were placed in organ culture plates and maintained with modified Trowell's medium in 95% O_2–5% CO_2 at 37°C for 6–24 hr. To study cell proliferation, 2 μc of thymidine-^{3}H was added per ml of medium. To study fat absorption, biopsies were exposed to micellar solutions of linolenic acid, monoolein, and taurodeoxycholate in Krebs-Ringer buffer for 15 min after culture in vitro for 24 hr. After 24 hr of culture, villi were shorter and wider. Cells in the lamina were reduced in number. Light and electron microscopic morphology of epithelial cells compared favorably to those of control biopsies except in occasional areas of partial necrosis. Some absorptive cells were more cuboidal and contained more lysosomes; many appeared entirely normal. Most crypt cells appeared normal; some contained increased glycogen and lysosomes. Mitoses were present, and labeled cells were abundant in crypts of biopsies after 6 hr of incubation with thymidine-^{3}H–containing medium. By 24 hr, labeled cells migrated to the base of the villi. When biopsies cultured in vitro were subsequently exposed to micellar lipid, numerous lipid droplets were identified in the cytoplasm of absorptive cells. Thus, after 24 hr in vitro under these culture conditions, many human small intestinal epithelial cells maintain near normal morphology, epithelial cell proliferation proceeds, and fat absorption occurs.

INTRODUCTION

Preservation of small intestinal tissue in vitro has been a serious problem in the past clearly limiting the duration of in vitro experiments of mucosal function and metabolism. Many short-term experiments (2 hr or less) have been satisfactorily conducted by maintaining open intestinal segments or everted rings and sacs of intestinal tissue in suitable oxygenated buffer solutions (1, 2). Careful morphological studies have shown that epithelial cell integrity is maintained in these short-term experiments (3–5). However, marked epithelial cell necrosis and disintegration occurs if incubation of intestinal tissue is prolonged much beyond 2 or 3 hr.[1]

More recently, cell suspensions of epithelial cells have been isolated from rat small intestine (6, 7), but there is question whether these cell suspensions remain viable for much more than an hour or 2 after isolation. Others have attempted, with some success, to apply recent improvements in vascular perfusion techniques coupled with storage in hyperbaric oxygen to the preservation in vitro of large segments of canine small intestine (8, 9), but application of perfusion methods to the study of human tissue would be limited by the need for large segments of fresh tissue.

In this report, we describe a method for maintaining human peroral mucosal biopsies in vitro for a 24 hr period by applying to the biopsies modifications of culture techniques utilized previously for the maintainance in

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vitro of fragments of other mature organs (10). We also describe initial studies of cell proliferation and fat absorption by the cultured biopsies which suggest that these parameters of organ function procede in the in vitro environment.

METHODS

Peroral biopsies were obtained from young adult male volunteers by use of a multipurpose biopsy tube (11) equipped with a modified capsule with four ports which permitted the excision of four biopsy samples with a single intubation. Biopsies were obtained after a 12-hr fast from the duodenojejunal junction by fluoroscopic placement of the biopsy capsule at the ligament of Treitz. Written, informed consent for the biopsy procedure was obtained from all subjects.

Biopsies were placed in the organ culture system within 1–5 min after their excision. Sterile, plastic culture dishes (Falcon Plastics, Los Angeles, Calif.) 5.5 cm in diameter were used. These had a central 2 cm well in which the medium was placed and which supported a triangular piece of stainless steel mesh. The central well was surrounded by an outer well which contained a cotton pad which was saturated with 0.9% NaCl solution. Biopsies were oriented with villi up and cut surface down on the mesh, and approximately 1 ml of medium was added to the central well until a thin layer was drawn over the villous surface of the biopsy by capillary action. The medium employed in these studies consisted of 9 parts of Trowell T-8 medium (12) (GIBCO, Inc., Grand Island, N. Y.) and 1 part of fetal calf serum (Hyland Laboratories, Los Angeles, Calif.). In addition, 10,000 U of crystalline penicillin G and 0.01 g of streptomycin sulfate were added to each 100 ml of medium. The dishes were covered, placed on a steel rack which was set in a McIntosh jar (Scientific Products, Inc., Los Angeles, Calif.), maintained at 37°C in a constant temperature oven. The jar was gassed with 95% O_2 and 5% CO_2 for 20 min and was then sealed. The medium was renewed and the jar regassed for 20 min after 6 and 12 hr had elapsed.

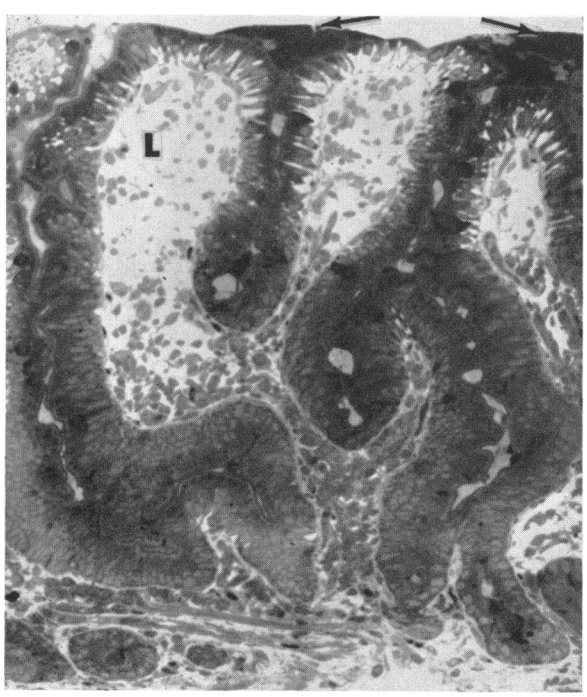

FIGURE 1. Light micrograph of a duodenojejunal biopsy maintained in vitro in organ culture for 24 hr. The villi are somewhat shortened, and the cellularity of the lamina propria (*L*) is decreased. Heterogeneous material (arrows) covers some of the well-preserved epithelium. Toluidine blue stain, × 150.

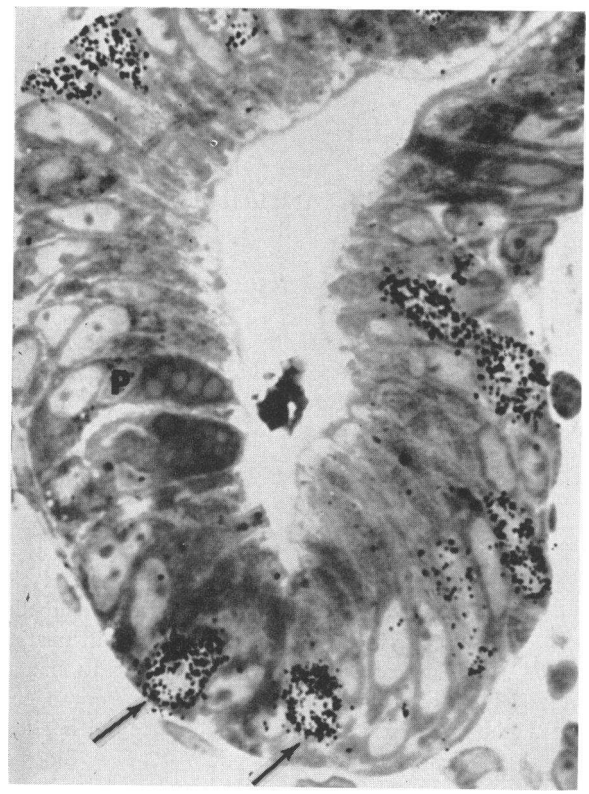

FIGURE 2 Light micrograph of a radioautograph of a crypt from a normal biopsy cultured in vitro for 12 hr in media to which thymidine-^{3}H has been added. The nuclei of many of the undifferentiated crypt cells are labeled (arrows). Paneth cells (*P*) are not labeled. Toluidine blue stain, × 1200.

To study cell proliferation in vitro, 2 μc of thymidine-^{3}H (New England Nuclear Corp., Boston, Mass., SA 6.7 c/mmole) was added to each ml of medium. Biopsies which were to be fixed for study after 6 and 12 hr of in vitro culture were exposed to thymidine-^{3}H for the duration of the culture period. Biopsies cultured in vitro for 24 hr were maintained in thymidine-^{3}H–containing medium for the 1st 12 hr and in medium without thymidine-^{3}H for the final 12 hr.

To test the ability of the absorptive cells of the cultured

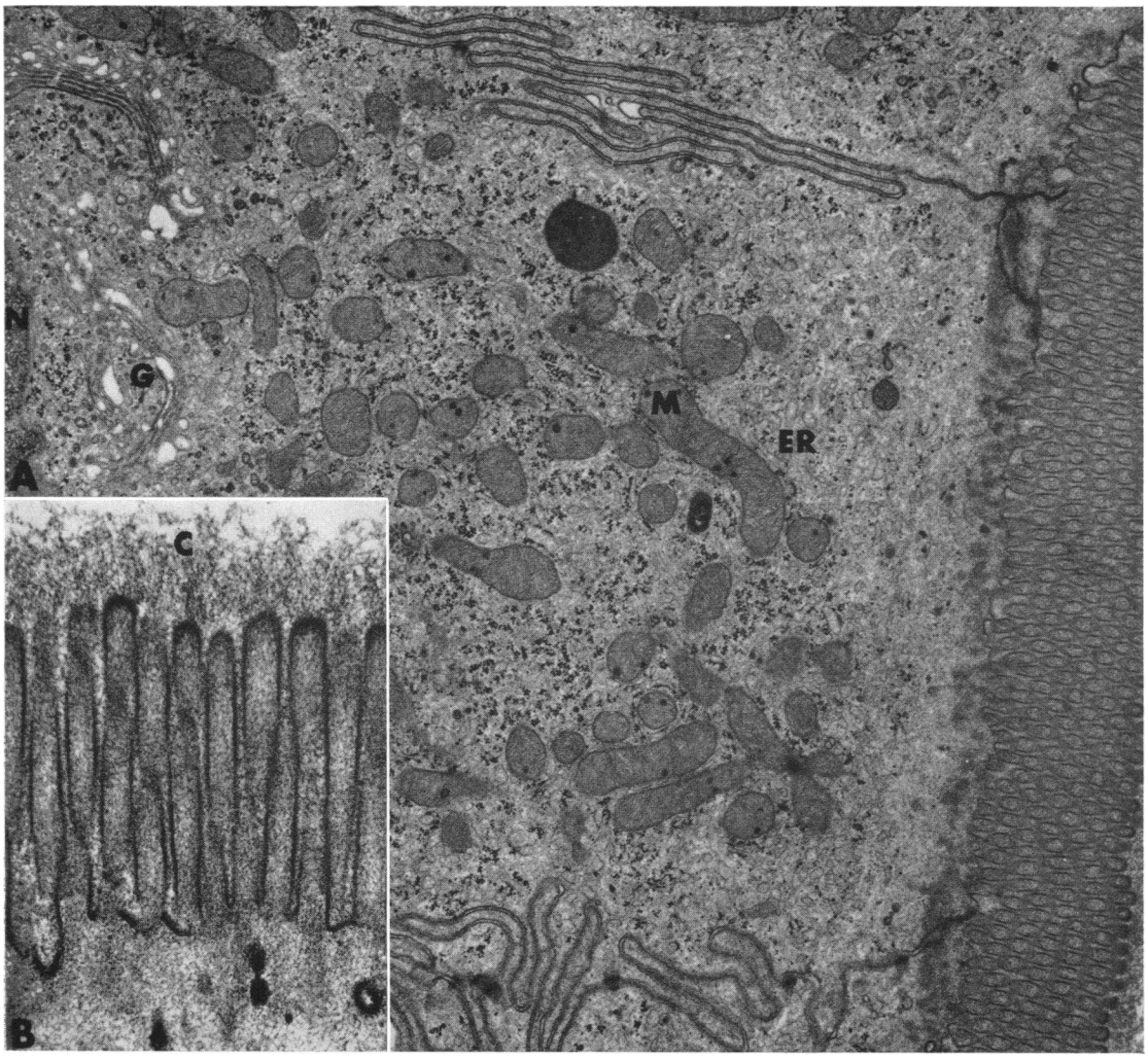

FIGURE 3 Electron micrograph of villous absorptive cells from a normal biopsy cultured in vitro for 24 hr. A. Cytoplasmic organelles including mitochondria (*M*), endoplasmic reticulum (*ER*), and Golgi material (*G*) are well preserved. *N*, nucleus, × 14,000. B. The microvilli as well as their fibrillar glycoprotein surface coat (*C*) are well preserved. × 30,000.

biopsies to absorb micellar fat, a micellar solution was prepared as described by Strauss (4). Briefly, for each 5 ml of micellar solution, 3.0 μmoles of linolenic acid (Applied Science Laboratories, Inc., State College, Pa.) and 1.5 μmoles of monoolein (Geo. A. Hormel & Co., Austin, Minn.) were dissolved in reagent-grade diethyl ether in the same container. The ether was removed by evaporation under nitrogen in a vacuum chamber. To this 0.3 ml of 0.04 M taurodeoxycholate (Calbiochem, Los Angeles, Calif.) was then added, and the mixture was shaken gently for 2 min. Finally, 4.7 ml of Krebs-Ringer phosphate solution without calcium or magnesium ions (KRP) was added resulting in a final concentration of 0.6 μmole of linolenic acid, 0.3 μmole of monoolein, and 2.4 μmoles of sodium taurodeoxycholate per ml of solution. After biopsies were cultured in vitro for 24 hr by the organ culture techniques described above, they were transferred to 25-ml Erlenmeyer flasks which contained 5 ml of micellar solution in KRP and were incubated for 15 min at 37°C in an environment of 95% O_2 and 5% CO_2 before fixation.

All cultured biopsy specimens were placed in chilled chrome-osmium fixative (13) upon completion of the culture or incubation period. After 4 min they were removed and carefully cut into slices approximately 1 mm in width and

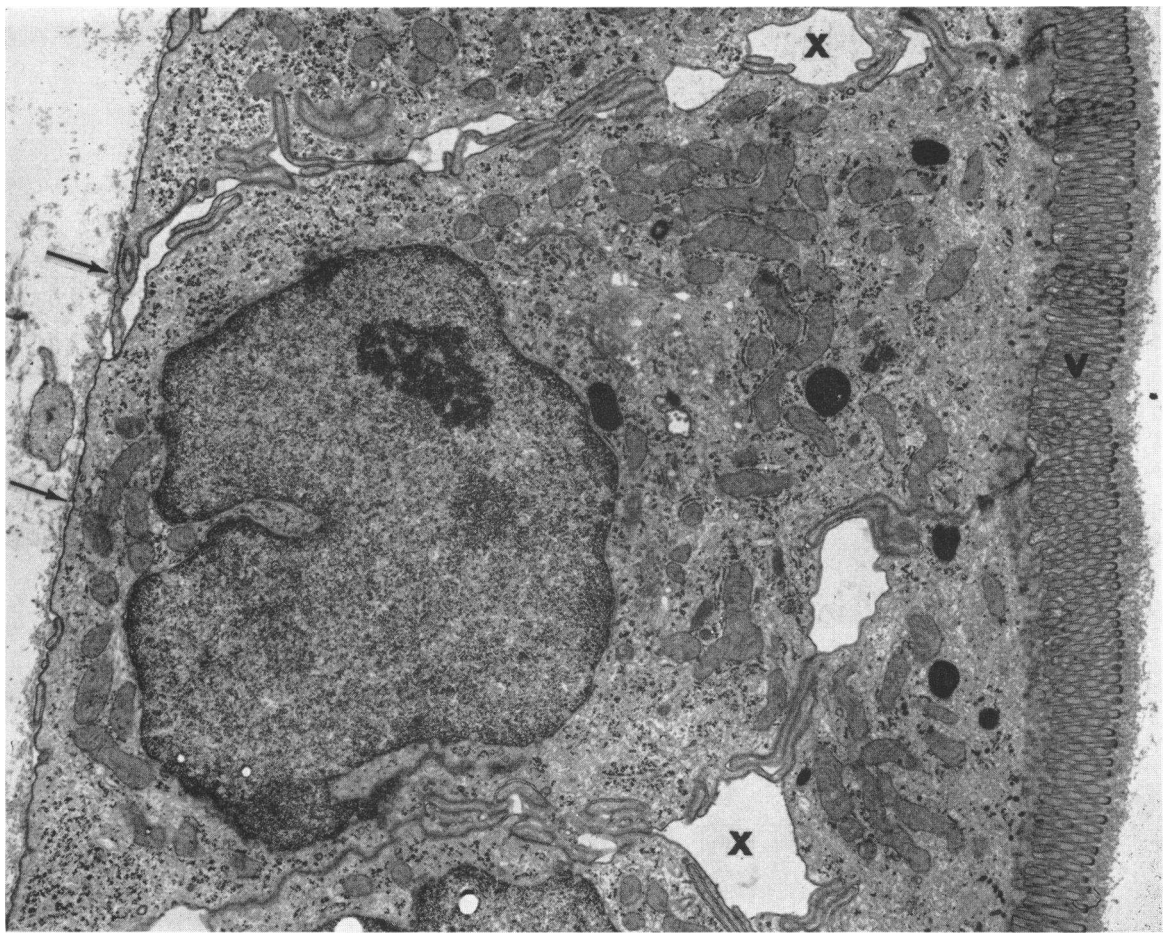

FIGURE 4 Electron micrograph of a villous absorptive cell nearer the villous tip from the same biopsy as Fig. 3. This cell is more cuboidal than normal, and relatively wide intercellular spaces (X) are seen between it and the adjacent cells. However, the cytoplasmic organelles themselves appear normal as does the microvillous border (V) and the basement membrane (arrows). × 7000.

returned to the fixative for 1 hr. Tissue was then placed in 10% neutral buffered formalin for ½ hr, dehydrated in increasing concentrations of ethyl alcohol, and embedded in epoxy resin (14). The embedded tissue slices were then cut out of the epoxy block, carefully oriented, and mounted with epoxy cement on short aluminum rods machined to fit the microtome chuck. Serial sections of the entire width of the biopsy slice were then cut 1 μ thick with glass knives with a Sorvall MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.). Sections not used for radioautography were then stained with toluidine blue (15).

For radioautographic studies, the slides with unstained 1 μ sections were dipped in Ilford L-4 emulsion (Ilford, Ltd., Essex, England), diluted 1:1 with distilled H_2O and, after appropriate drying, were stored in light-proof boxes in the presence of anhydrous calcium sulfate as previously described (16, 17). Slides were incubated in the refrigerator for 6-8 wk and then developed with Kodak D-19 developer for 4 min, fixed for 5 min, washed, dried, and then stained with toluidine blue.

Suitable areas for electron microscopic study were selected from the toluidine blue–stained 1 μ sections, and tissue blocks were appropriately trimmed for thin sectioning. Thin sections were cut with a diamond knife on an LKB microtome, mounted on uncoated, copper mesh grids, stained with uranyl acetate (18) and lead citrate (19), and examined with a Philips EM-300 electron microscope.

RESULTS

Villous architecture was reasonably well maintained in biopsies subjected to in vitro organ culture. Fig. 1 is a relatively low magnification, light micrograph of a representative area of normal human intestinal mucosa

which was maintained in culture for 24 hr. The villi were somewhat shorter and wider than those of biopsies fixed immediately after excision, and there were fewer cells in the lamina propria, but those present appeared normal. Variable amounts of heterogeneous material, presumably consisting of mucus and cellular debris, were noted on the surface epithelium of some villi (Fig. 1) and in the lumina of some crypts. Except in occasional areas of partial necrosis, the epithelium was intact, and its cellular elements appeared normal by light microscopy (Figs. 1 and 2). Both the columnar structure of the epithelial cells and their striated border were maintained. Many mitoses were present in the crypts, even after 24 hr of culture. Areas of necorsis were less common in biopsies fixed after 12 hr of culture and rare in biopsies fixed after 6 hr of culture.

Electron microscopy confirmed the impression gained with the light microscope that near normal epithelial cell morphology was maintained in most areas during in vitro culture for 24 hr. In many areas, the villous absorptive cells, such as the one shown in Fig. 3 A, appeared indistinguishable from those seen in freshly fixed biopsies (20). Their cytoplasm was compact, and the cytoplasmic organelles, including mitochondria, elements of endoplasmic reticulum, Golgi material and lysosomes were normal in appearance and distribution. In many areas, the relationship of adjacent cells to one another was undisturbed (Fig. 3 A). Both the microvillous surface and its delicate glycoprotein surface coat were maintained (Fig. 3 B). In other areas, particularly near the tip of the villi, absorptive cells were shorter than normal (Fig. 4), and the number of lysosomes in the cytoplasm was moderately increased. However, both the apical surface and the basal lamina of all absorptive cells examined from nonnecrotic areas were structurely intact (Fig. 4).

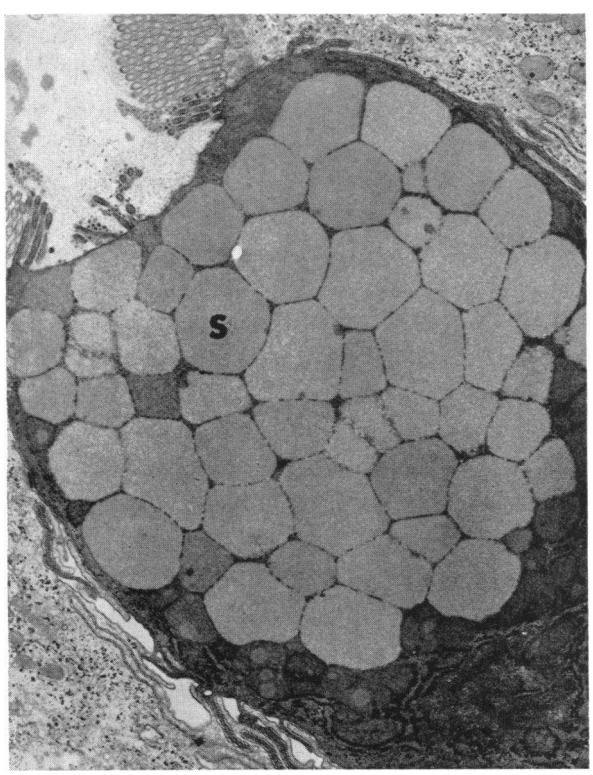

FIGURE 5 Electron micrograph of a villous goblet cell from a normal biopsy maintained in organ culture for 24 hr. The cell appears morphologically normal and contains many typical mucous secretory granules (*S*). × 7000.

Goblet cells, both on the villi and in the crypts, were filled with large mucous granules and appeared normal morphologically when viewed with the electron microscope (Fig. 5). Many goblet cells were actively secreting mucus.

Most undifferentiated cells were normal even after culture for 24 hr, except that the number of secretory granules in the apical cytoplasm appeared decreased (Fig. 6). In a few, lysosomes were more numerous and larger in size, and the amount of glycogen in the cytoplasm was increased over that normally found (Fig. 7).

Paneth cells in the base of the crypts were normal after 24 hr of culture and were characterized by large homogeneous membrane-bounded secretory granules in the apical cytoplasm, an extensive supranuclear accumulation of Golgi material, and abundant cytoplasmic granular endoplasmic reticulum (Fig. 8). Like the Paneth cells, the differentiated enterochromaffin cells remained normal and contained characteristic cytoplasmic granules of varying densities. A few enterochromaffin cells contained unusually large accumulations of glycogen (Fig. 9).

The fine structure of most cellular elements in the lamina propria, including plasma cells, lymphocytes, eosinophiles, fibroblasts, blood and lymph vessels, unmyelinated nerve fibers, and smooth muscle cells also appeared normal in biopsies cultured in vitro for 24 hr.

When thymidine-^{3}H was added to the culture medium, the nuclei of many undifferentiated crypt cells were labeled after 6 hr of incubation in vitro. After 12 hr of incubation in thymidine-^{3}H–containing medium, labeling was more extensive but still confined to the lower two-thirds of the crypts (Fig. 2). In biopsies cultured in vitro for a total of 24 hr with 12 hr of exposure to thymidine-^{3}H–containing medium followed by 12 hr of exposure to medium without isotope, labeled cells were seen not only in the lower crypt but also in the upper crypt and the lower third of the villi.

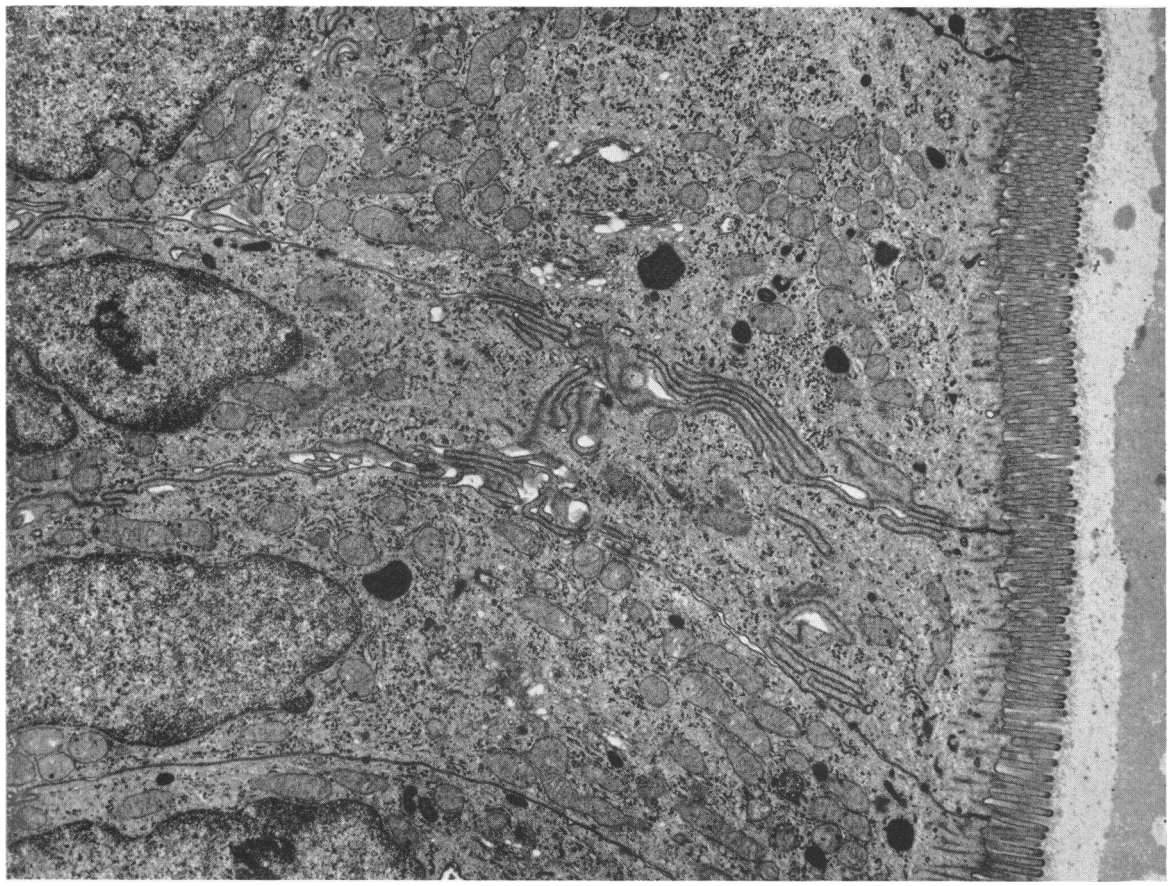

FIGURE 6 Electron micrograph of crypt cells at the mouth of a crypt of a normal biopsy maintained in vitro for 24 hr. The fine structure of these cells compares favorably with that of similar cells in freshly fixed tissue, except that the number of secretory granules in the undifferentiated cells is reduced. × 6000.

When biopsies were exposed for 15 min to a micellar solution of linolenic acid, monoolein, and sodium deoxycholate in KRP after first having been maintained in culture in vitro for 24 hr, relatively normal morphology of the epithelial cells persisted. In addition, absorptive cells on the upper third of the villi contained many recognizable lipid droplets in their cytoplasm (Fig. 10 A). These were distributed throughout the cytoplasm but were most abundant in the supranuclear region, and they were often enclosed by elements of the endoplasmic reticulum and by Golgi membranes (Fig. 10 B). Control biopsies did not contain identifiable droplets in the absorptive cell cytoplasm.

DISCUSSION

In our initial efforts to maintain mucosal biopsies from the small intestine in vitro, we used methods similar to those applied previously, with some success, to the in vitro culture of human rectal mucosa (21–23). These methods actually resembled tissue culture techniques in which biopsies were immersed in tissue culture medium such as Eagle's basic salt solution and were then oxygenated by agitation in a metabolic shaker after they were gassed with 95% O_2 and 5% CO_2. By use of this method, Deschner, Lewis, and Lipkin (21) reported survival of 50% of rectal biopsies after 19 hr of in vitro culture. In contrast to their findings with rectal mucosa, we noted necrosis of all tissue samples by 6 hr when this method was applied to in vitro culture of small intestinal mucosa. However, a simple and inexpensive organ culture method, in contrast to tissue culture methods, proved successful for the in vitro maintainance of intestinal mucosa for at least a 24 hr period.

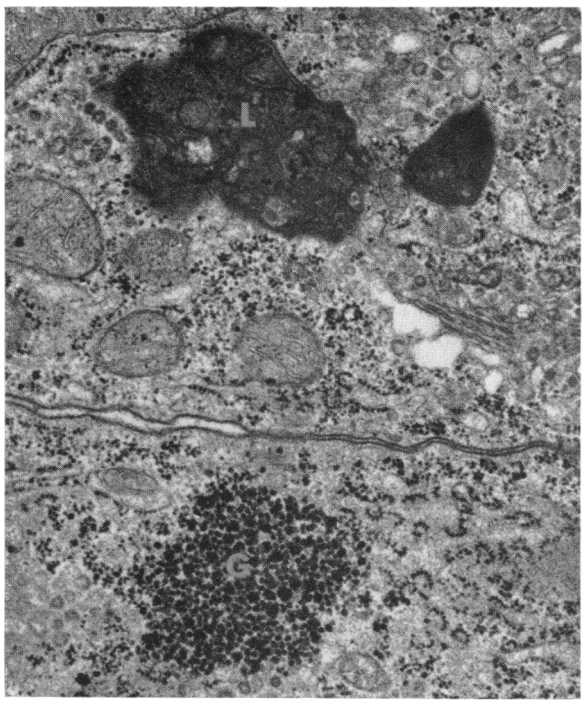

FIGURE 7 Higher magnification micrograph of undifferentiated crypt cell cytoplasm from the same biopsy as Fig. 6. A large lysosome (*L*) is seen above and a prominent accumulation of glycogen (*G*) below. × 22,000.

There are critical differences between organ and tissue culture methodology (10, 24) which may account for the greater success of the organ culture technique. When organ culture methods are used, the tissue sample is not immersed; rather, the tissue is placed on the surface of the medium, and the medium is allowed to seep over the specimen by capillary action. In all likelihood, this technique permits far better oxygenation of the tissue sample than can be achieved during immersion, yet exposure to the medium is satisfactory for the nutritional needs of the tissue sample. Also, agitation, such as occurs with a metabolic shaker which is often used in tissue culture, is carefully avoided during organ culture, since it may severely traumatize the tissue sample and contribute to its disintegration. Finally, organ culture and tissue culture media differ significantly. For example, Trowell's T-8 medium used in these studies contains insulin and 4 g of glucose per liter, whereas Eagle's tissue culture medium contains no insulin and only 1 g of glucose per liter.

Others have successfully organ-cultured small intestine obtained from chick embryos (*25–27*) but, to our knowledge, successful culture of small intestinal mucosa obtained from adult mammals has not been reported before. Unlike chick embryo tissue which is sterile, intestinal mucosal samples obtained by peroral biopsy are grossly contaminated by bacteria. Therefore, while media, culture plates, and instruments used for the orienting and handling the tissue were sterile in our studies, no attempt was made to maintain strict aseptic technique in the initial handling of the biopsies. The addition of penicillin and streptomycin to the media undoubtedly helped retard further growth of those organisms present, since bacterial overgrowth did not appear to interfere with the culture of mucosa for the 24 hr period. It may be that bacterial proliferation might become a serious problem should longer survival of the culture be desired.

There seems little doubt that intestinal mucosa remains viable after 24 hr of in vitro culture by the method described. As is illustrated above, the fine structure of the mucosa after 24 hr of culture compares favorably with fresh tissue processed immediately after excision. The incorporation of thymidine-^{3}H into the nuclei of undifferentiated crypt cells provides evidence that the epithelial cells were actively synthesizing deoxyribonucleic acid (DNA). The presence of mitoses in crypts of biopsies maintained in vitro for 24 hr and the migration of cells with labeled nuclei from the crypts onto the base of the villi indicate that epithelial cell proliferation and migration occurred as well. Moreover, the rate of migration in normal biopsies cultured for 24 hr was the same as that observed in vivo in biopsies obtained from volunteers without intestinal disease 24 hr after injection with thymidine-^{3}H (28). In both situations, labeled cells had migrated to the base of the villi.

The studies of fat absorption provide further evidence that the epithelial cells remain viable after in vitro culture for 24 hr. Many fat droplets were present in the absorptive cells of biopsies cultured for 24 hr and then postincubated in micellar lipid solutions. Strauss (4) has shown that the morphological detection of the absorbed micellar lipid requires not only absorption of monoglyceride and fatty acid but their subsequent synthesis into triglyceride within the absorptive cell cytoplasm. Thus it appears that both lipid uptake by the absorptive cells and triglyceride synthesis within the absorptive cells occurred in biopsies cultured in vitro for 24 hr.

Previous in vitro studies of the function and metabolism of intestinal mucosa have been limited by the rapid epithelial cell necrosis which occurs after excision of intestinal mucosal samples. It is hoped that the organ culture technique described here, with which peroral mucosal biopsies can be maintained in vitro for 24 hr, will permit studies of normal mucosal function and metabolism not previously possible. Moreover, the utilization of in vitro organ culture of biopsies obtained from pa-

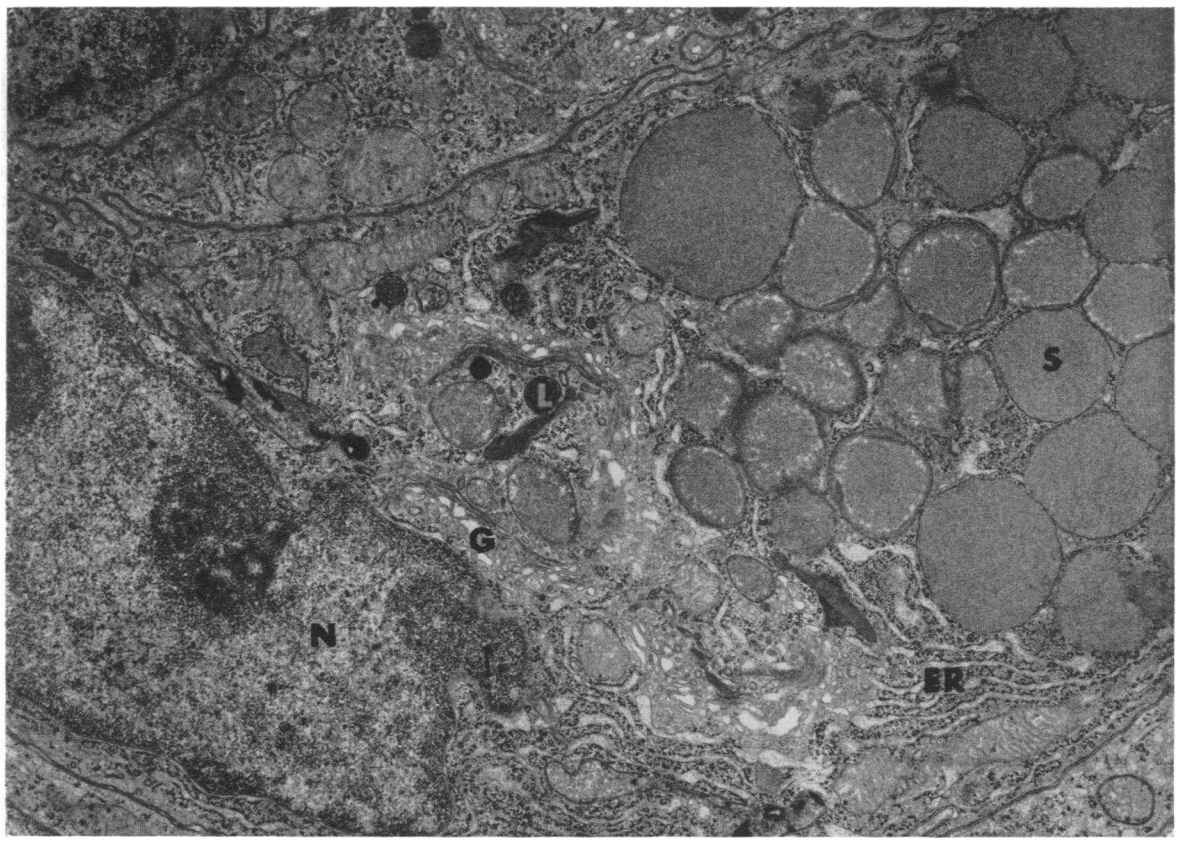

FIGURE 8 Paneth cell cytoplasm from a biopsy maintained in vitro for 24 hr. Again, cell structure is well preserved and indistinguishable from that seen in biopsies fixed after excision. *N*, nucleus; *ER*, granular endoplasmic reticulum; *G*, Golgi material; *L*, lysosome; *S*, secretory granules. × 12,000.

tients with diffuse mucosal diseases, such as celiac sprue, should permit studies not possible in vivo which might help clarify the pathogenesis of such disorders.

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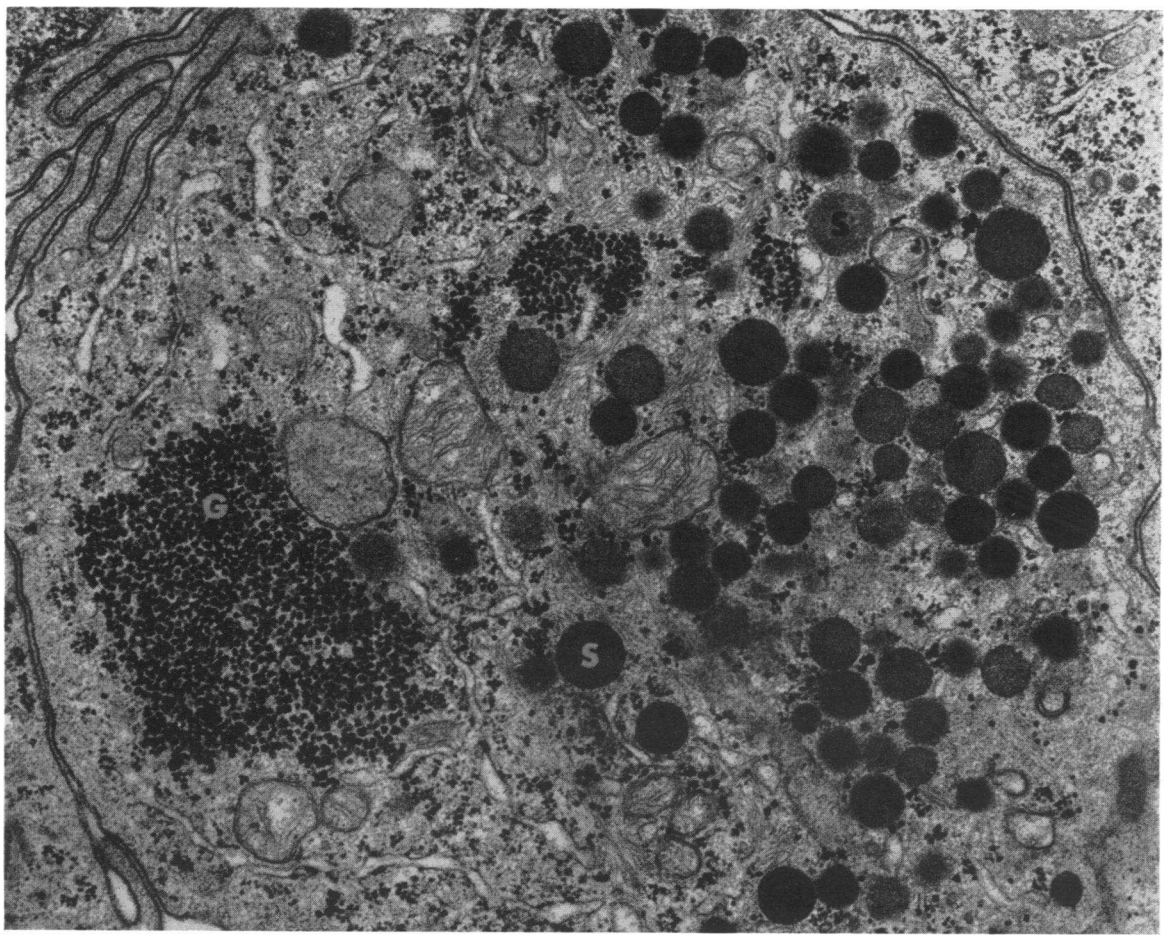

FIGURE 9 Electron micrograph of cytoplasm of an enterochromaffin cell from a biopsy maintained in vitro for 24 hr. Cell fine structure is normal except that there are excessive accumulations of glycogen (*G*) in the cytoplasm. Typical enterochromaffin granules (*S*) of varying density are abundant. × 22,000.

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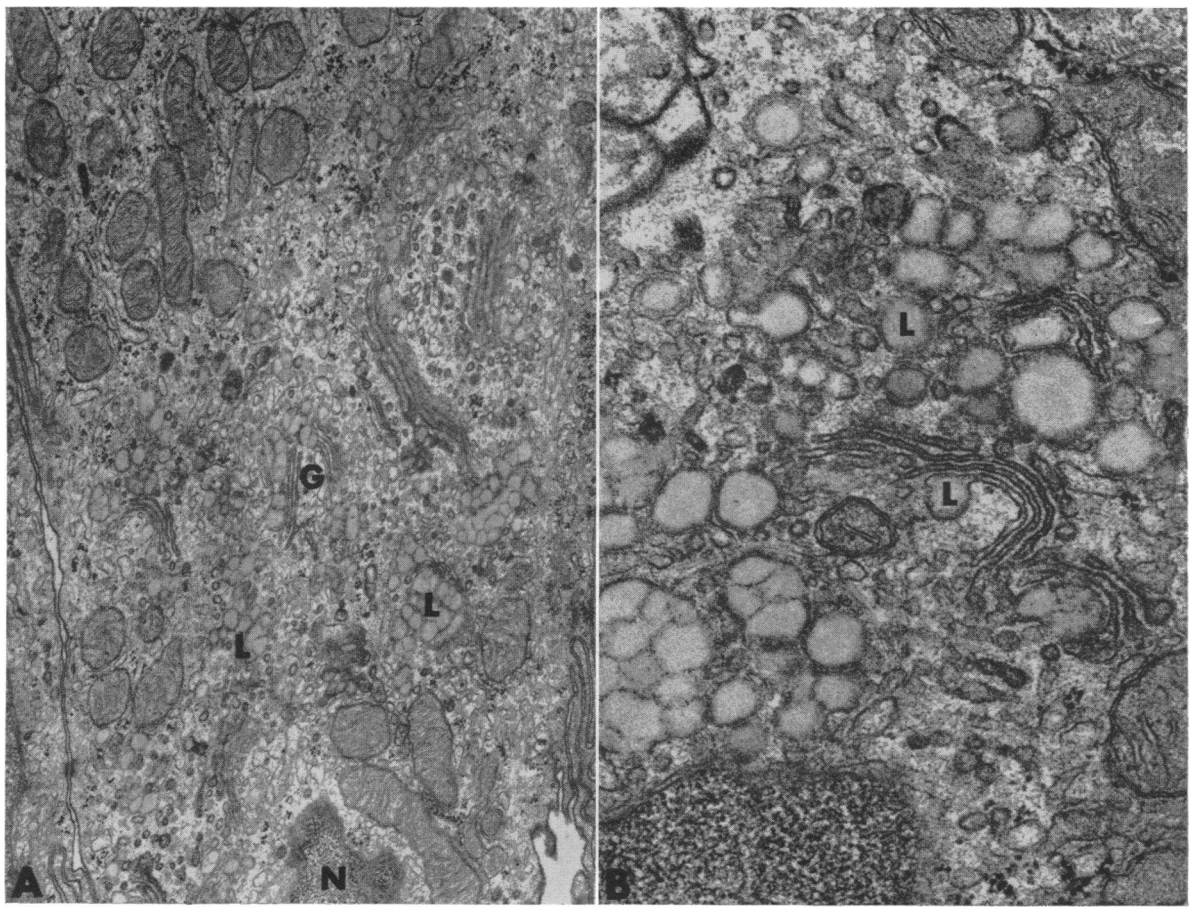

FIGURE 10 Electron micrograph of supranuclear cytoplasm of absorptive cells from a biopsy cultured in vitro for 24 hr and then postincubated in a micellar solution as described in the text. A. Many droplets of absorbed lipid (*L*) are present in the cytoplasm. *N*, nucleus, *G*, golgi material. × 14,000. B. At higher magnification, absorbed lipid (*L*) can be seen within Golgi vesicles and cysterns. × 33,000.

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