Relation of Calcium to Mucosal Structure And Vitamin B_{12} Absorption in the Canine Intestine

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THE ABSORPTION of vitamin B_{12} (B_{12}) in the intestine is known to proceed in several stages.[1,2] In most animal species, absorption begins with the formation of a complex between intrinsic factor (IF) and ingested B_{12} in the lumen of the gastrointestinal tract. In a second stage, the IF-B_{12} complex adheres to the surface of the mucosa by a calcium-dependent process of adsorption. This stage can be reversed experimentally by depleting the calcium ions at the mucosal surface. In a final stage, B_{12} penetrates the mucosa and enters the bloodstream.

In a recent study of the pathway for B_{12} absorption in the canine intestine,[3] surface and goblet cell mucus was found to bind labeled B_{12} *in vivo*. This finding suggested that the stage of B_{12} surface adsorption corresponds structurally to the attachment of B_{12} to the mucus coating the surface of the intestine. It further suggested that the reversal of B_{12} surface adsorption and the decrease in B_{12} absorption after experimental calcium depletion might be due to some interference with the capacity of intestinal mucus to bind B_{12}. This view is consistent with evidence in at least two types of human mucus secretions—submaxillary and tracheobronchial—that alterations in calcium content are associated with alterations in the solubility properties of the mucus.[4]

The present study was undertaken to test this hypothesis. The effect of intraluminal calcium depletion on the uptake and distribution of $^{57}CoB_{12}$ *in vivo* in the canine ileum was studied by scintillation counting, light and electron microscopy and by autoradiography. The results indicate that calcium depletion causes widespread separation of B_{12}-laden epithelial cells, especially goblet cells, from the surface of the mucosa. No alterations were observed in either the ultrastructural appearance of surface or goblet cell mucus, or in their capacity, as judged from autoradiographs, to bind labeled B_{12}. The decreased up-

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take of B_{12} by intestinal tissue, in these conditions, is apparently due to the widespread separation of B_{12}-laden epithelial cells, especially goblet cells from the surface of the mucosa.

Materials and Methods

Under pentobarbital anesthesia, the abdomen of each of 3 mongrel dogs was opened, the terminal ileum was identified, and a ligature was placed at the ileocecal value. The ileum was then divided with double silk ligatures into 5 cm-long sacs *in situ*. Care was taken to leave the mesentery intact and thus preserve the blood supply to the intestine.

The lumen of each sac was washed three times with 5–7 ml of either warm, neutralized 0.9% saline or a 1, 3 or 4% solution of di- or trisodium ethylenediaminetetraacetate (EDTA) injected transmurally. After washing, the content of each sac was withdrawn and replaced with 0.75 μg (~10 μCi) of $^{57}CoB_{12}$ dissolved in either neutralized 0.9% saline or a 1, 3 or 4% solution of EDTA. The washing and filling of each sac was so alternated that after washing with either saline or EDTA, adjacent sacs contained $^{57}CoB_{12}$ dissolved in either saline or EDTA.

The intestine was then carefully replaced into the abdomen, the abdominal wall closed with hemostats, and the entire abdomen covered with sterile towels that were kept continuously moist with warmed 0.9% saline. At intervals of 15 minutes and at 1, 2, and 3 hours, the abdomen was reopened by releasing the hemostats, one pair of test sacs was carefully mobilized, and its mesentery divided between the two silk ligatures. The content of each sac was then rapidly withdrawn and replaced with 5 ml of a 3% solution of phosphate-buffered glutaraldehyde [5] at room temperature. The ends of each sac were then immediately cut between the ligatures to separate them from the remainder of the intestine. The removed sacs were quickly opened and drained. Small tissue samples were excised from their centers and placed immediately in glutaraldehyde. The remainder of each sac was washed five times with 10 mM $CaCl_2$ blotted on filter paper, weighted on a torsion balance, and counted for radioactivity in a well-type scintillation detector. In each animal, just prior to sacrifice a sample of ileal mucosa from a sac in which no incubations were performed was also removed and placed in glutaraldehyde.

After 5 minutes in glutaraldehyde, the excised tissue samples were placed in a drop of glutaraldehyde solution on a pad of dental wax and carefully divided into smaller blocks. These smaller blocks were returned to the main glutaraldehyde solution for 1 hour and postfixed in a 1% solution of phosphate-buffered osmium tetroxide [6] for 1 hour at room temperature. They were then dehydrated in graded alcohols and propylene oxide and embedded in Epon 812 according to the method of Luft.[7] Specimens were placed into embedding medium under a dissecting microscope or hand lens so that orientation of the specimen during the embedding process could be controlled.

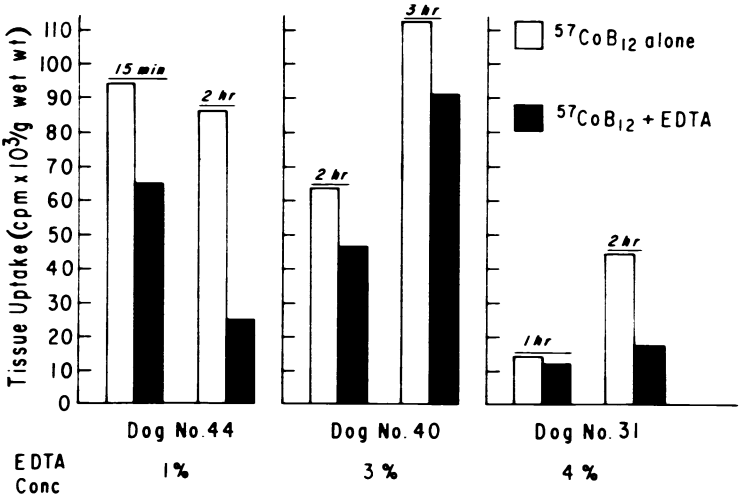
Thick sections of 1–2 μ and thin sections displaying gold interference colors [8] were cut on a Huxley microtome with a diamond knife. Sectioning was carried out in a well-oriented specimen parallel to the plane of the intestinal villi. One group of thick sections was stained with a 1% solution of toluidine blue and examined directly under the light microscope. Another group of thick sections was processed for light microscope autoradiography by the dipping technic [9] with NTB2 or NTB3 nuclear emulsion (Eastman Kodak Company, Rochester, NY) and stored in black, light-tight boxes for 3–8 weeks at 4 C. After exposure, the slides were developed in a 1:2 dilution of Dektol (Eastman Kodak Company, Rochester, NY), washed, and fixed in acid-fix for 10 minutes. After a second washing, the sections were stained with toluidine blue and examined.

Thin sections were picked up on Formvar-coated, 75-mesh, copper grids. One group of thin sections was stained with uranyl acetate [10] and lead citrate [11] and examined directly in a Siemens-Elmiskop 1A electron microscope. Another group of thin sections was processed for electron microscope autoradiography by coating with L-4 nuclear emulsion (Ilford, Essex, England), according to the method of Caro and Von Tubergen.[12] Coated grids were stored for 3–6 months in black, light-tight boxes at 4 C. After exposure, grids were developed in a 1:1 dilution of Kodak D-19, fixed in acid-fix, double stained with uranyl acetate and lead citrate, and examined in the electron microscope.

Results

Scintillation Counting

Chelation depletion of calcium by EDTA consistently lowered the uptake of $^{57}CoB_{12}$ by intestinal tissue (Text-fig 1). This was noted at each



TEXT-FIG 1. Uptake of radioactivity by mucosa of adjacent pairs of ileal sacs *in vivo*. Individual sac pairs incubated for time periods indicated after intraluminal instillation of 0.75 μg $^{57}CoB_{12}$ dissolved in either neutralized saline or EDTA.

EDTA concentration and incubation period tested. The decrease in tissue B_{12} uptake, however, was not proportional to either the concentration of EDTA or the duration of incubation.

Light Microscopy

The histologic appearance of the intestinal mucosa in tissue samples from control sacs and all sacs incubated with $^{57}CoB_{12}$ dissolved in neutralized saline was similar. This appearance is illustrated in Fig 1, which shows the mucosa of a sac incubated *in vivo* for 3 hours, the

longest incubation period of this study. The appearance is that of normal canine intestinal mucosa.

The histologic appearance of the intestinal mucosa in all sacs incubated with $^{57}CoB_{12}$ dissolved in EDTA was also similar but markedly different from that of sacs incubated in saline. This appearance is illustrated in Fig 2–4, which show the mucosa of a sac incubated *in vivo* for 15 minutes, the shortest incubation period in this study, after the intraluminal instillation of $^{57}CoB_{12}$ dissolved in 1% EDTA. The most striking feature in these preparations was the widespread separation of epithelial cells from the cores of the villi. This separation occurred chiefly in the upper one third to one half of the villus along the plane of attachment of epithelial cells to the lamina propria. At these sites, capillaries of the lamina propria could be seen to be denuded of their epithelial covering and were bulging into the intestinal lumen (Fig 2 and 4). This histologic lesion was observed in all EDTA-filled sacs and affected the majority of villi. The extent of the lesion did not appear to be proportional to either the concentration of EDTA or the duration of incubation. The ultrastructural details of this altered mucosa, as well as that of control sacs and all sacs incubated in saline, were then further examined with the electron microscope.

Electron Microscopy

The ultrastructural appearance of villi in control biopsies and tissue samples from all sacs containing $^{57}CoB_{12}$ dissolved in saline was identical. It conformed in all essential features to earlier descriptions of normal canine intestinal mucosa [3, 13] and will, therefore, not be described in detail.

The characteristic ultrastructural appearance of all villi denuded of their surface epithelium in sacs containing EDTA was also similar and is shown at low magnification in Fig 5. This specimen was taken from a sac containing $^{57}CoB_{12}$ in a 3% solution of EDTA after 1 hour of incubation. In this case, the loss of surface epithelium is complete, except for two cells in a final stage of detachment (*arrows*). At this stage (Fig 5 inset), the epithelial cells are rounded, frequently vacuolated, and appear to be loosely adherent to the underlying basement membrane. With the loss of its epithelial cover, the capillary network of the lamina propria bulges conspicuously into the intestinal lumen. At higher magnifications (Fig 6), these capillaries are seen to be separated from the lumen by a thin layer of connective tissue elements and the residual basement membrane, which is now the lim-

iting envelope of the villus. Although largely intact, the basement membrane shows occasional small discontinuities (Fig 6, *arrows*).

The separation of epithelial cells from each other and from their underlying basement membrane occurred in several stages (Fig 7–9). In the earliest stage, as seen in a sac incubated with 1% EDTA for 1 hour (Fig 7), the interdigitating plasma membranes of adjacent epithelial cells gradually retracted with widening of the intercellular space. Successive stages of detachment were characterized by the separation of epithelial cell surface membranes from each other and from the basement membrane, and a gradual change in the shape of epithelial cells from columnar to round (Fig 8). At this stage, the microvilli of each cell were seen to shorten and absorb into the body of the cell (Fig 8–11). Final loss of contact between the epithelial cells and their basement membrane was most often associated with deep infoldings of the basement membrane, which interdigitated with elongated cytoplasmic processes extended from the detaching epithelial cells (Fig 9).

Finally, epithelial cells in great numbers were seen floating freely in the lumen (Fig 10 and 11). Except for their rounded shape, vacuolizations and markedly shortened microvilli, they showed good ultrastructural preservation and could be easily classified as either goblet cells (Fig 10) or absorbing cells (Fig 11). The mucus granules of goblet cells, in particular, appeared normal ultrastructurally.

Light and Electron Microscopic Autoradiography

The distribution of radioactive label in sacs incubated with $^{57}CoB_{12}$ dissolved in neutralized saline was identical with that described in an earlier study.[3] During the first 2 hours, the label was concentrated chiefly in surface mucus and in goblet cells (Fig 12) and during the third hour was seen primarily in absorbing cells (Fig 13).

In sacs incubated with $^{57}CoB_{12}$ dissolved in EDTA, the distribution of label was somewhat different. Surface mucus and goblet cells were again heavily labeled at each time period, but this included goblet cells both attached to and detached from the villus. (Fig 14A and B). Thus, a portion of instilled B_{12} label was lost to the absorptive process because it was contained within goblet cells that had detached from the mucosal surface. Penetration of the label into absorbing cells was a relatively uncommon finding. When present, it was found most frequently within absorbing cells that had also detached from the surface of the mucosa (Fig 15).

Discussion

The principal finding of this study was that EDTA-induced calcium depletion produced a marked structural disorganization of the intestinal mucosa. This consisted of a widespread separation of mucosal epithelial cells from one another, and from their basement membrane, and led to extensive denudation of intestinal villi (Fig 2). The process of denudation included epithelial cells laden with radioactive B_{12} (Fig 14A and B, 15) and was associated with a decrease in radioactive B_{12} uptake by the tissues.

In view of these findings, the role of calcium ion in the process of intestinal B_{12} absorption must be reconsidered. In most past studies, both *in vivo* [14–18] and *in vitro*,[19–22] the decrease in mucosal B_{12} uptake after calcium depletion was taken to indicate that the IF-B_{12} complex failed to adhere to mucosal receptors by the calcium-dependent process of surface adsorption. In each of these studies, however, only the measurement of radioactive B_{12} by scintillation counting was used to determine B_{12} uptake in the absence of any histologic control. Changes in uptake of radioactivity were, therefore, thought to reflect only the movement of B_{12} alone or of IF-B_{12} complex. The present results clearly indicate that radioactive B_{12}, lost to the intact intestinal mucosa after EDTA-induced calcium depletion, is contained within mucosal epithelial cells that have themselves separated from the mucosa (Fig 14A and B, 15).

These results agree with numerous observations by others on the effect of comparable concentrations of EDTA in the mucosa of, among others, the toad bladder,[23] frog stomach,[24] and the rat [25] or dog intestine.[26] Chelation depletion of calcium in these studies produced both a loss of adhesiveness between adjacent epithelial cells [24,25] and complete separation of epithelial cells from the surface of the mucosa.[23,26] It is of interest to note that the effects of EDTA on both radioactive B_{12} uptake and the mucosal epithelium were not observed to vary directly with either the concentration of EDTA used or the time during which the mucosa was exposed to this agent. Although tissue calcium was not measured in this study *in vivo*, the range of concentrations of EDTA used was up to five times that known to produce calcium depletion in intestinal tissue.[25] It appears likely, therefore, that while overall calcium depletion occurs in all tissues treated with EDTA, the local effects on any given villus or villus segment must vary considerably. One of the variables most difficult to control or to measure in these circumstances is the supply of calcium ion arriving via the bloodstream over a given period of time.

Both the concentrations of EDTA used and the periods of incubation

in vivo, in the present study, were selected so as to be comparable to those used by others who studied the mechanism of intestinal B_{12} absorption.[14,16–22] Comparison of our findings with those of earlier studies, however, is limited to intact intestinal mucosal preparations. This is important because B_{12} uptake has been shown to be dependent upon the presence of calcium ion in homogenates of rat [22] and human [27] intestinal mucosa and brush border, and microvillous membrane preparations of hamster intestine.[28,29] Although the structural and physiologic properties of the normal intestine are greatly distorted in these preparations and the composition of intestinal mucosal homogenates, in particular, is extremely heterogenous,[30] the results of these studies point to a possible calcium-dependent step in the physiologic pathway for intestinal B_{12} absorption. In resolving this question, however, it is important that future experiments distinguish between two separate requirements for calcium ion (1) in maintaining the structural organization of normal mucosal epithelium and (2) in the possible attachment of a substrate, such as B_{12}, to mucosal receptors just prior to absorption.

In conclusion, it is pertinent to note that the present results confirm earlier observations from this laboratory on the binding of B_{12} to intestinal mucus during B_{12} absorption in the canine intestine.[3] In the present experiments, however, we were not able to alter either the structural appearance of surface or goblet cell mucus, or its capacity to bind radioactive B_{12}. Thus the role of surface and goblet cell mucus in the absorptive pathway for B_{12} is still not entirely clear. In particular, the applicability of this finding to the pathway for classical IF-mediated intestinal B_{12} absorption is uncertain, since B_{12} absorption in the canine intestine is apparently not IF-dependent.[31,32] We therefore suggest the possibility that B_{12} binders, originating in goblet cell mucus, may facilitate intestinal B_{12} absorption in the dog in a manner similar to that of gastric IF in other species.

Summary

The purpose of this study was to further define the role of calcium in the intestinal absorption of vitamin B_{12}. This was accomplished by comparing the normal uptake and morphologic distribution of radiolabeled vitamin B_{12} *in vivo* in the canine intestine with that which occurs after the chelation depletion of intraluminal calcium. The terminal ileum of mongrel dogs was divided into pairs of sacs *in situ*, which were then filled with 0.75 μg of $^{57}CoB_{12}$ dissolved in either neutralized saline or a solution of ethylenediaminetetraacetate. After incubation *in vivo* for periods of 15 minutes to 3 hours, the sacs were excised and

tissue samples prepared for measurement of radioactive B_{12} uptake, light and electron microscopy, and light and electron microscope autoradiography. Tissue B_{12} uptake was consistently lower in sacs containing B_{12} dissolved in ethylenediaminetetraacetate. All of the villi in these sacs had been extensively denuded of their surface epithelium. Ultrastructurally, denudation was seen to follow the progressive separation of epithelial cell surface membranes from each other and from their underlying basement membrane. Their final separation from the villus left the basement membrane an intact and continuous structural envelope covering the villus core. Autoradiography demonstrated large concentrations of labeled B_{12} within detached epithelial cells, especially goblet cells. Labeled B_{12} contained within this detached epithelium was thus lost to the absorptive process. None of these changes was seen in control sacs containing $^{57}CoB_{12}$ dissolved in saline. These results clearly indicate that chelation-depletion of intraluminal calcium in the intact intestine produces the extensive separation of epithelial cells from the mucosa. Lowered B_{12} uptake in these conditions, therefore, is caused, at least in part, by the loss of B_{12}-laden epithelial cells from the mucosa.

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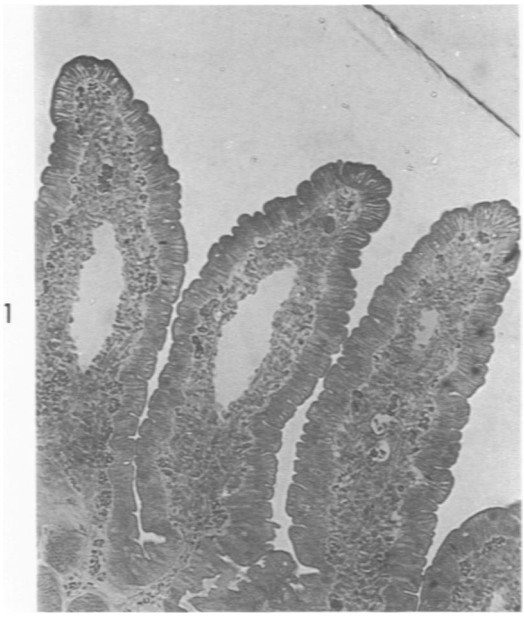
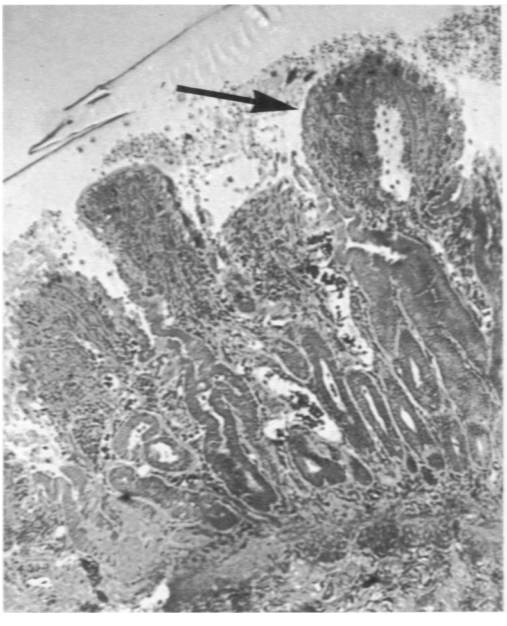
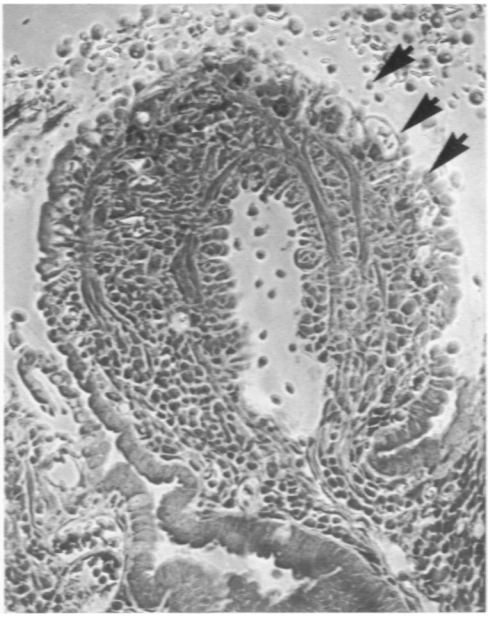
Legends for Figures

All sections for electron microscopy double stained with lead phosphate and uranyl acetate. All sections for light microscopy stained with toluidine blue.







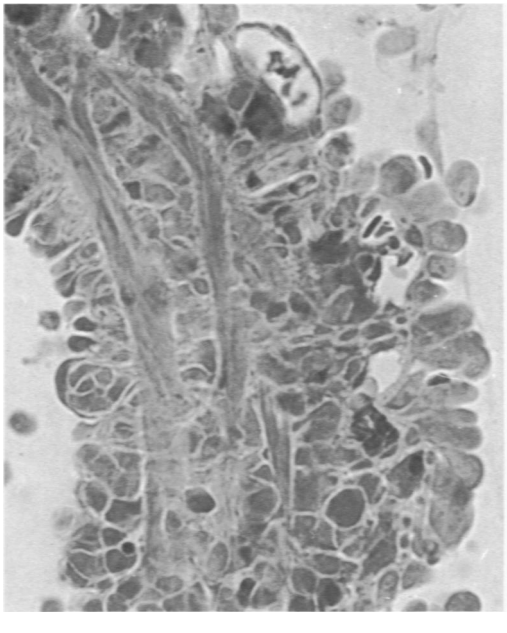


Fig 1.—Light microscopic appearance of the mucosa of an ileal sac incubated *in vivo* for 3 hr after intraluminal instillation of $^{57}CoB_{12}$ dissolved in neutralized saline. Histologic structure essentially normal. × 60.

Fig 2.—Light microscopic appearance of mucosa of an ileal sac incubated *in vivo* for 15 minutes after intraluminal instillation of $^{57}CoB_{12}$ dissolved in 1% EDTA. All villi show extensive denudation of surface epithelium. To illustrate this in greater detail, villus indicated by *arrow* is shown enlarged in Fig 3. × 75.

Fig 3.—Enlargement of villus indicated by arrow in Fig 2 showing widespread detachment of epithelial cells from upper portion of villus. For greater detail, enlargement of area indicated by *arrows* is shown in Fig 4. × 200.

Fig 4.—Enlargement of area indicated by arrows in Fig 3, showing detachment of surface epithelium. Stripped of surface epithelium, capillaries of lamina propria are seen to protrude directly into lumen. × 400.

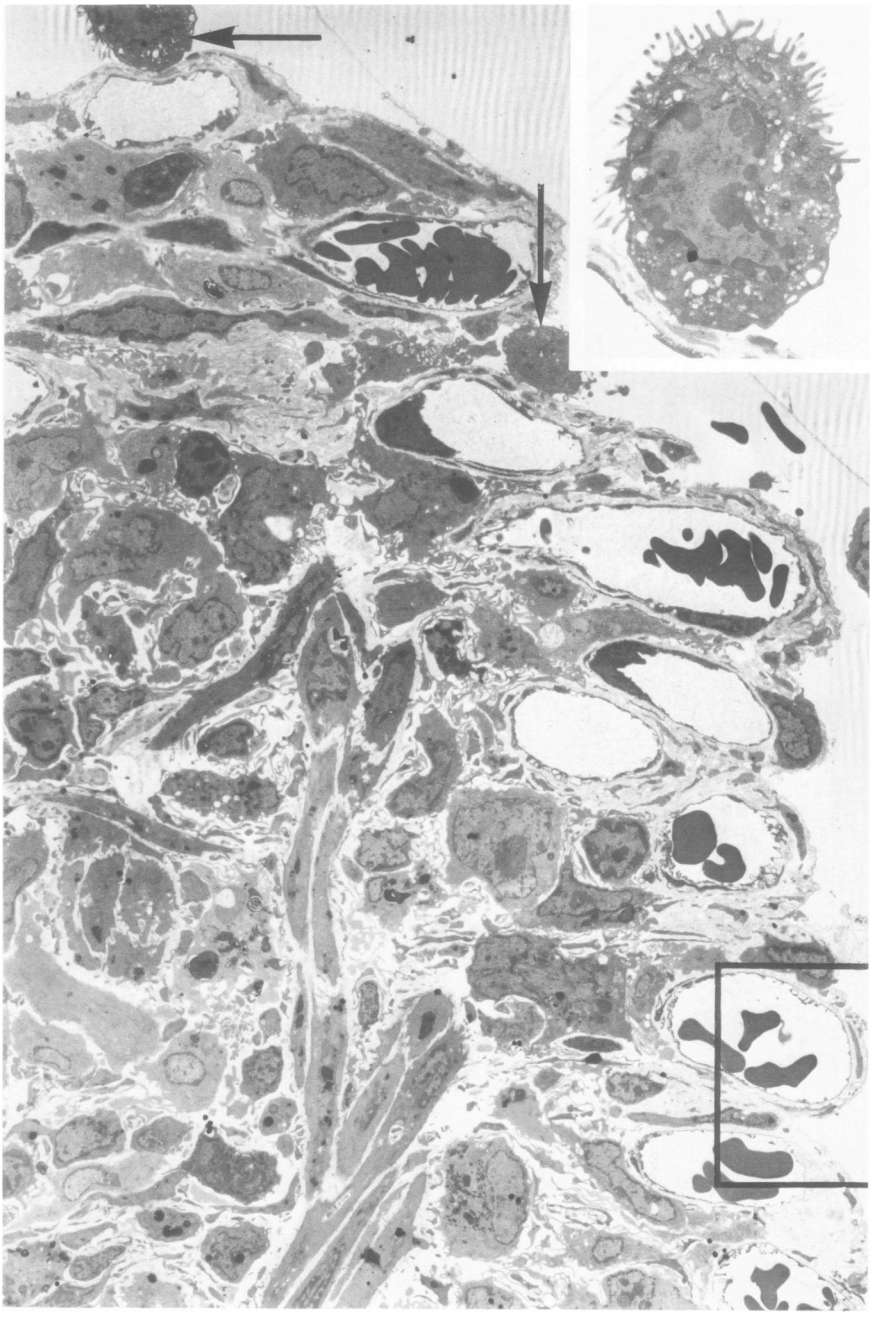


Fig 5.—Electron photomicrograph of area of villus from sac incubated for 1 hour after intraluminal instillation of $^{57}CoB_{12}$ dissolved in 3% EDTA. Complete loss of surface epithelium, except for two cells in final stages of detachment *(arrows)*. **Inset** shows cell indicated by uppermost arrow. Note rounded appearance, numerous vacuolizations and loose attachment of cell to underlying basement membrane. Capillaries of lamina propria bulge conspicuously into intestinal lumen protected by a thin membranous cover. To show this cover in greater detail, area enclosed by *rectangle* is enlarged in Fig 6. × 1300. Inset, × 3500.

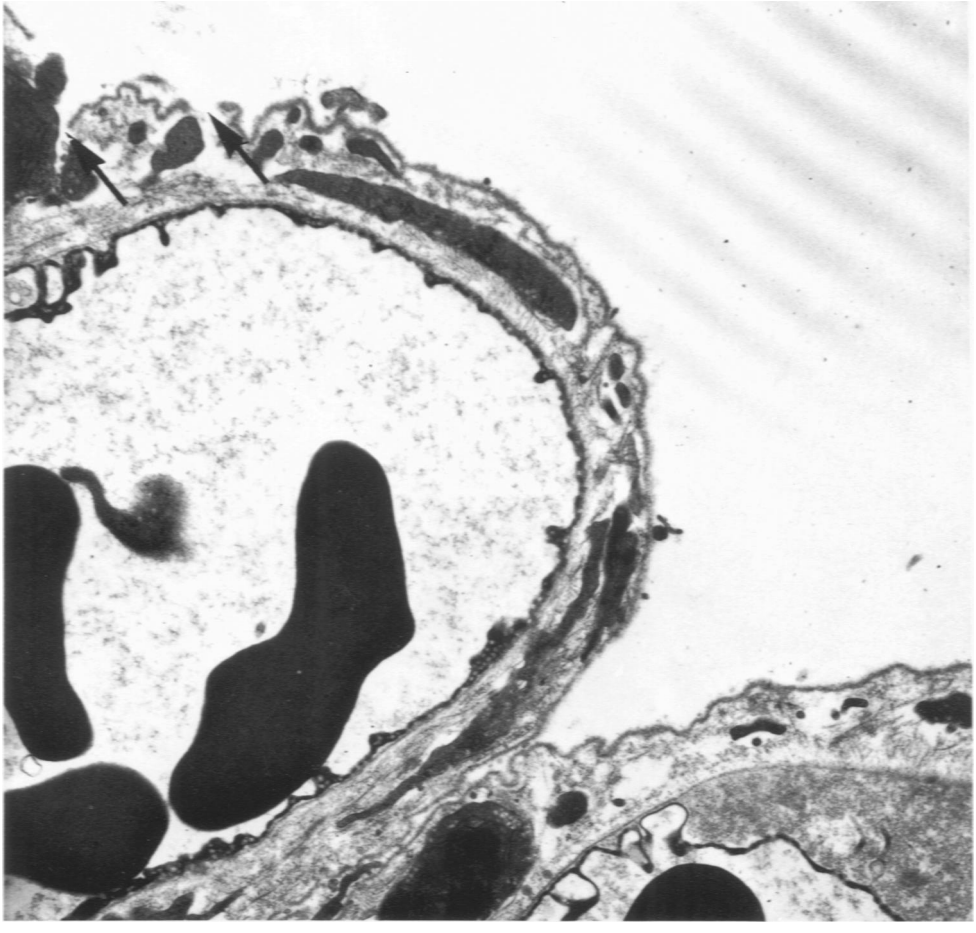


Fig 6.—Detail of area enclosed by rectangle in Fig 5. Basement membrane now forms outermost limit of villus. It is unaltered ultrastructurally, except for occasional small breaks *(arrows)*. Capillaries of lamina propria are now separated from lumen by a thin layer of connective tissue and basement membrane. × 18,500.

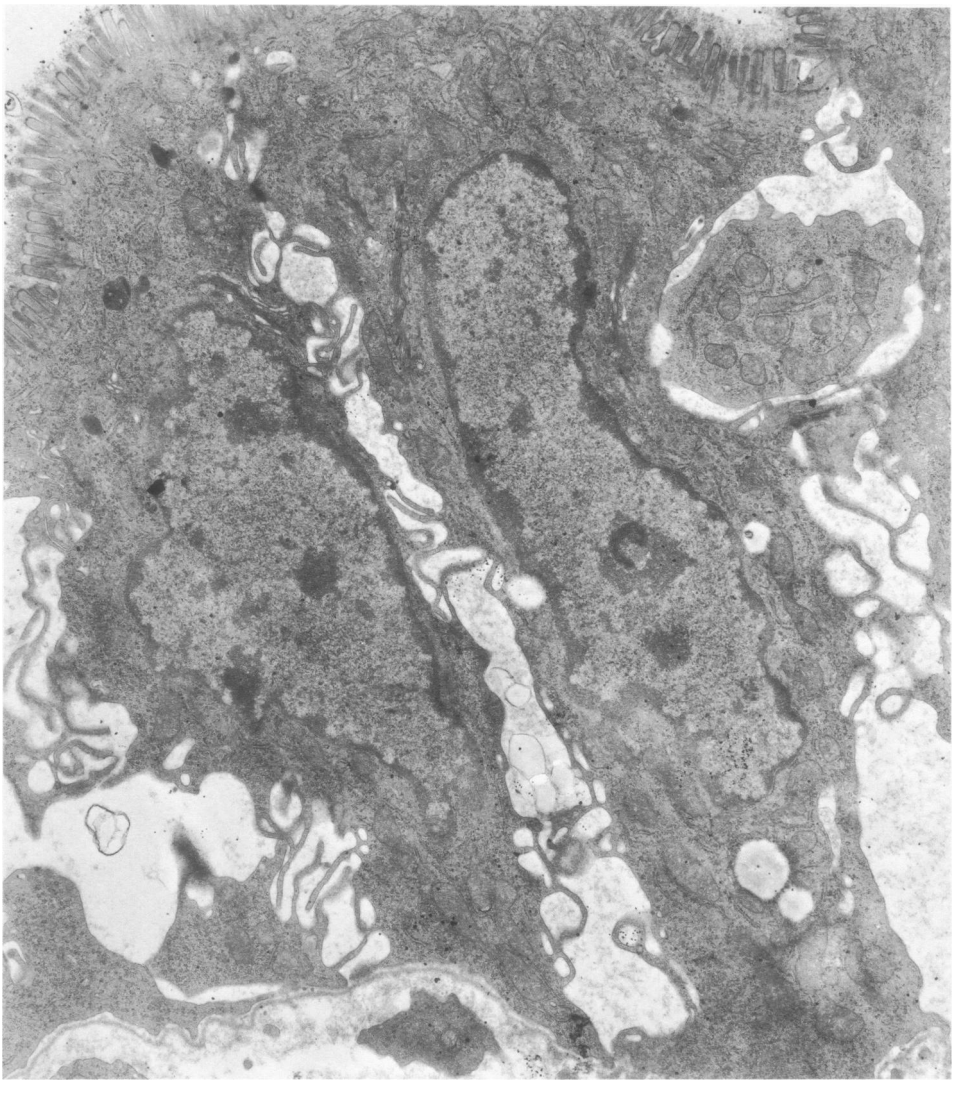


Fig 7.—Electron photomicrograph of villus epithelial cells from a sac incubated *in vivo* for 1 hour after intraluminal instillation of $^{57}CoB_{12}$ dissolved in 1% EDTA. Normally interdigitating lateral plasma membranes of adjacent epithelial cells now loosened and retracted from one another, resulting in considerable widening of intercellular space. × 9000.

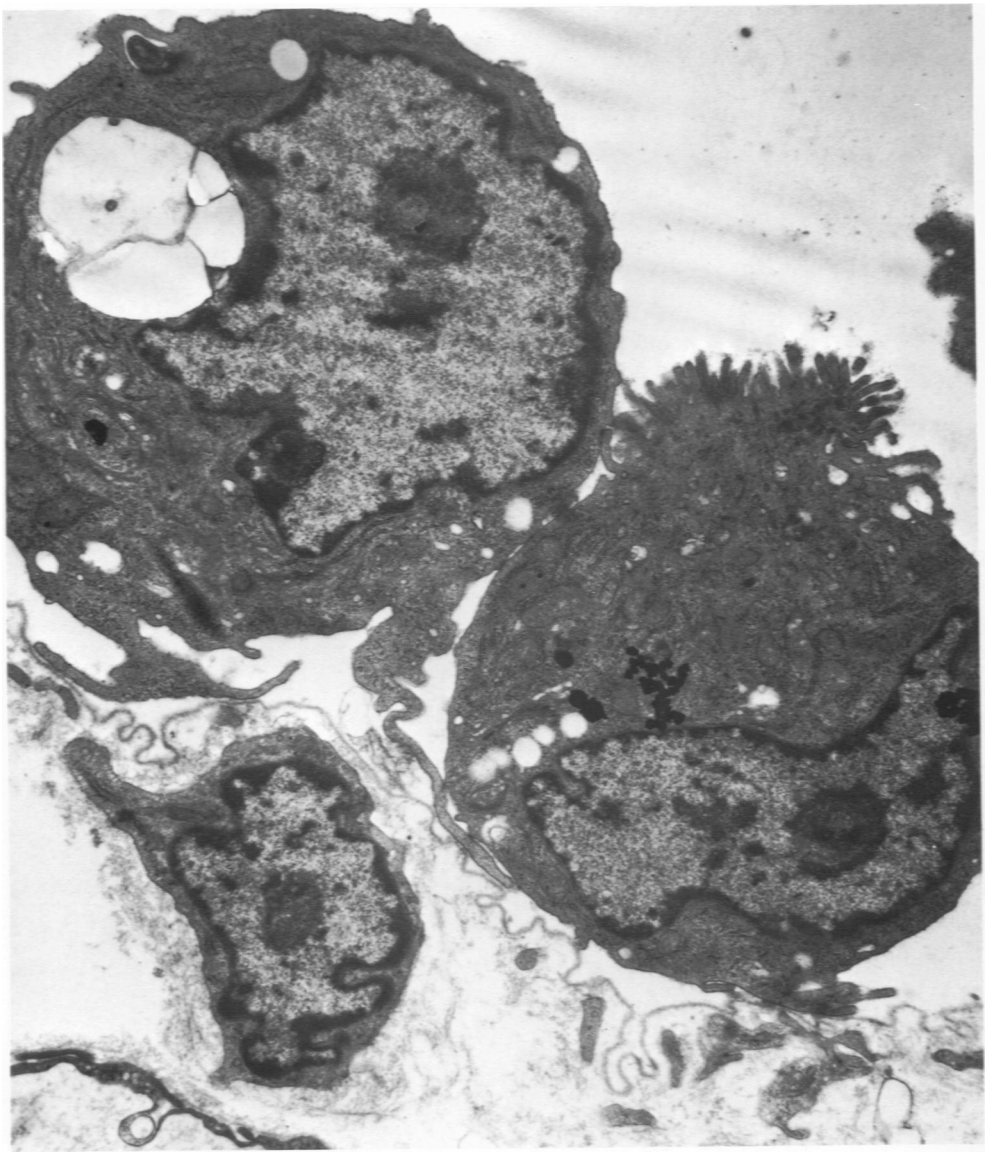


Fig 8.—Electron photomicrograph of two adjacent villus epithelial cells from same sac as in Fig 7. Separation between adjacent cells more advanced. Both cells have rounded appearance and, due to alterations of their plasma membranes, are only loosely adherent to one another and to underlying basement membrane. Microvillous border of cell on left appears to have been partly absorbed into body of the cell. × 8000.

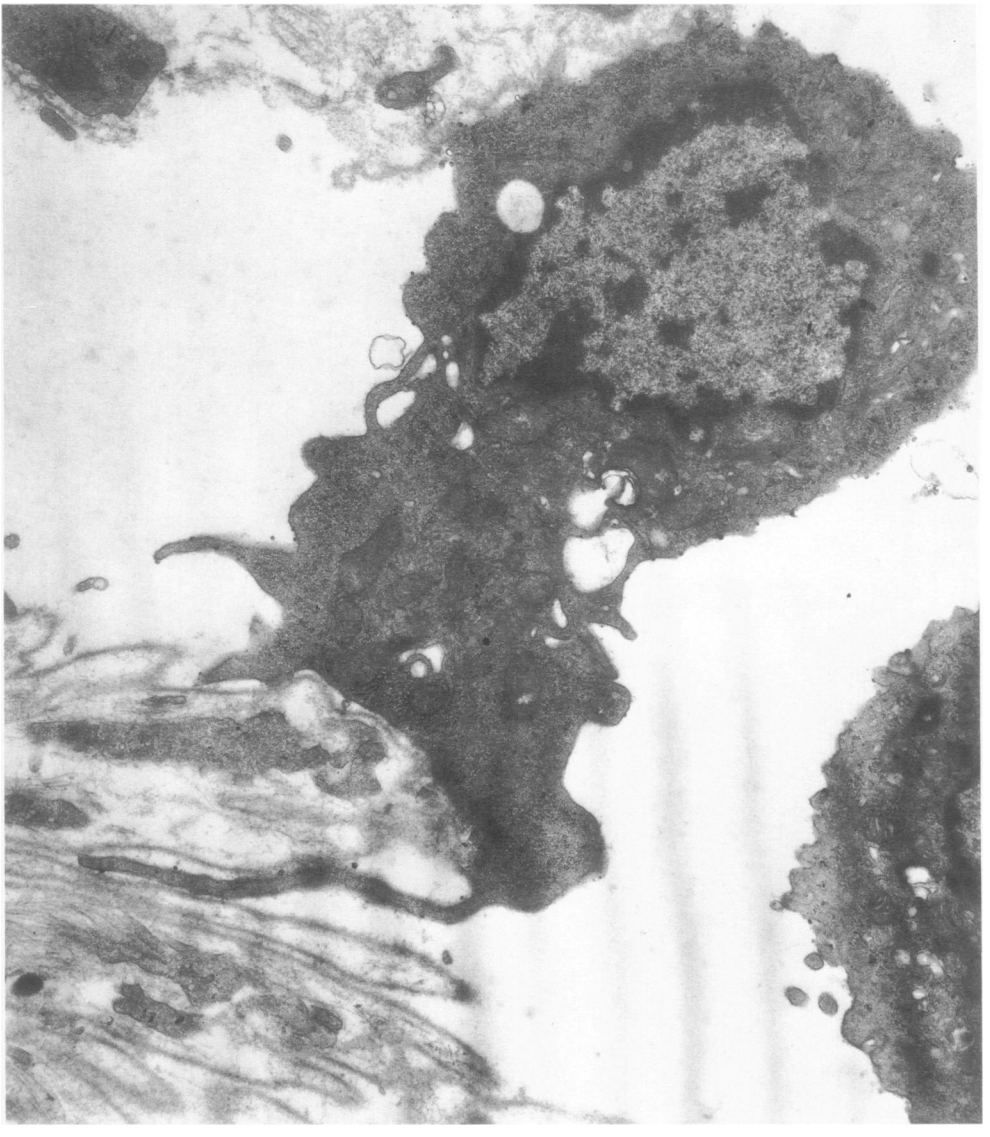


Fig 9.—Electron photomicrograph of villus epithelial cell from sac incubated *in vivo* for 15 minutes after instillation of $^{57}CoB_{12}$ dissolved in 1% EDTA. Cell has lost all lateral contacts and is attached to basement membrane at only two points. At lowermost point of contact, basement membrane is thrown into folds that interdigitate with processes extending from cell. × 10,500.

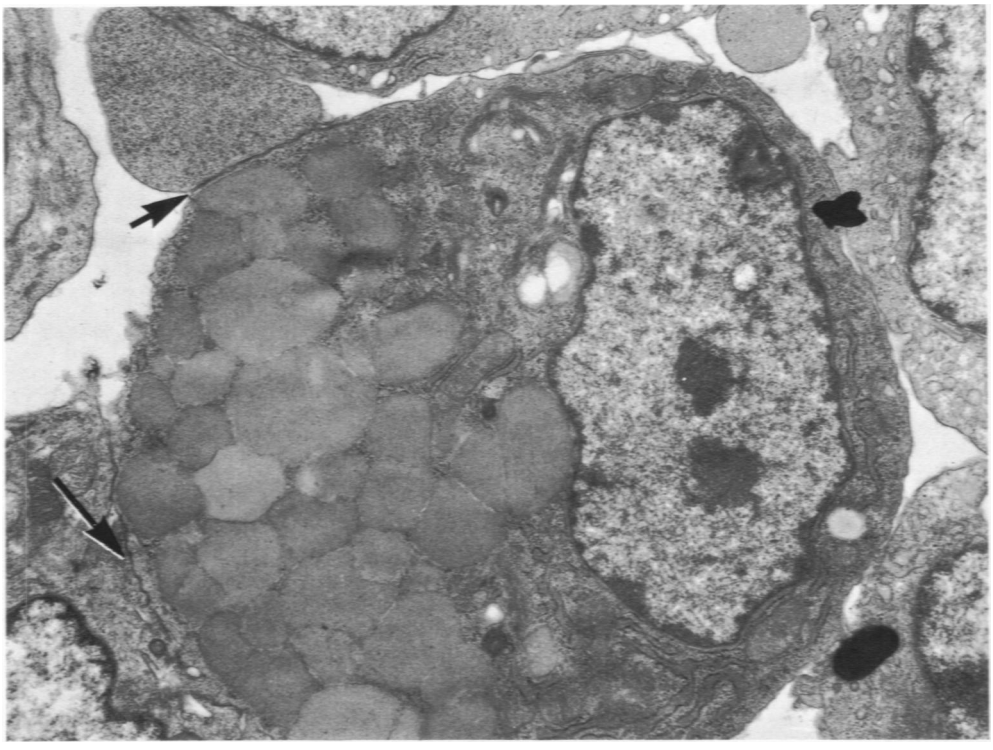
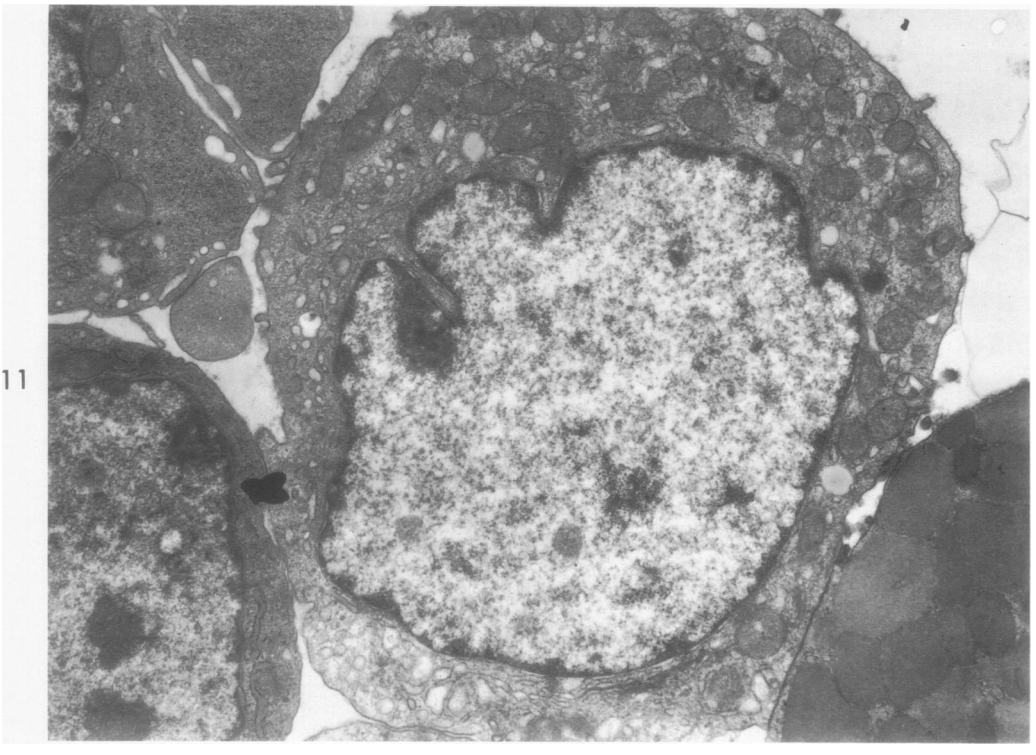


Fig 10.—Electron photomicrograph of goblet cell floating free in lumen of same sac shown in Fig 9. Endoplasmic reticulum, Golgi apparatus and mucus granules appear normal ultrastructurally. Note persisting points of attachment *(arrows)*. × 8000.



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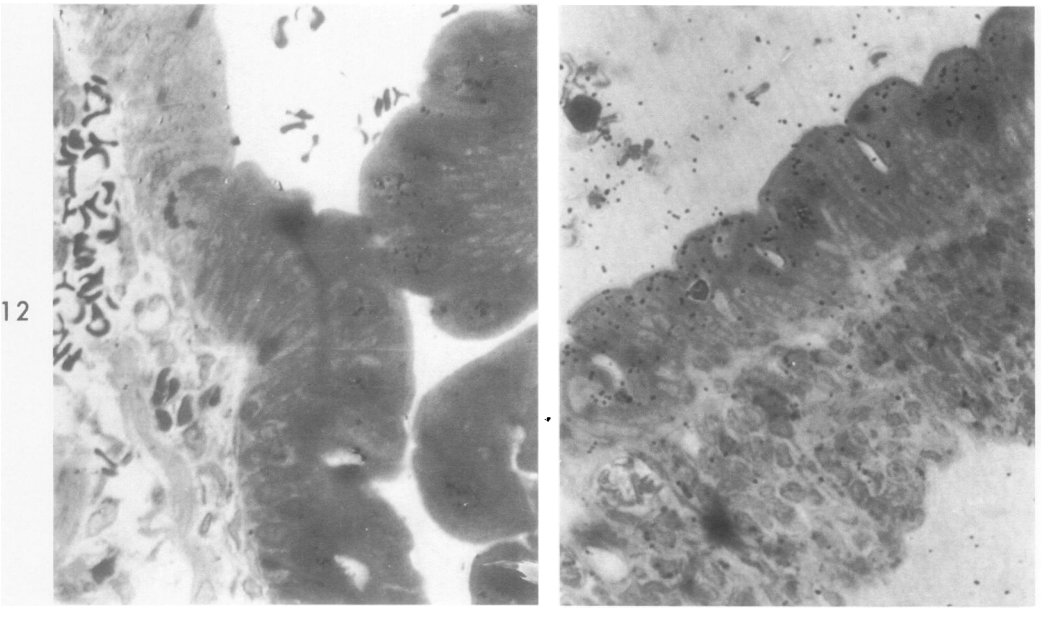

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Fig 11.—Electron photomicrograph of villus absorbing cell floating free in lumen of same sac shown in Fig 9. Well-preserved mitochondria, vesicular profiles of endoplasmic reticulum, and somewhat shortened microvilli facilitate identification. × 8000.

Fig 12.—Light microscopic autoradiograph of two adjacent villi from sac incubated *in vivo* for 1 hour after intraluminal instillation of $^{57}CoB_{12}$ dissolved in neutralized saline. Note concentration of developed grains over mucus granules of goblet cells. × 375.

Fig 13.—Light microscopic autoradiograph of villus from sac incubated *in vivo* for 3 hours after intraluminal instillation of $^{57}CoB_{12}$ dissolved in neutralized saline. Developed grains now overlie mucosal absorbing cells. × 3500.

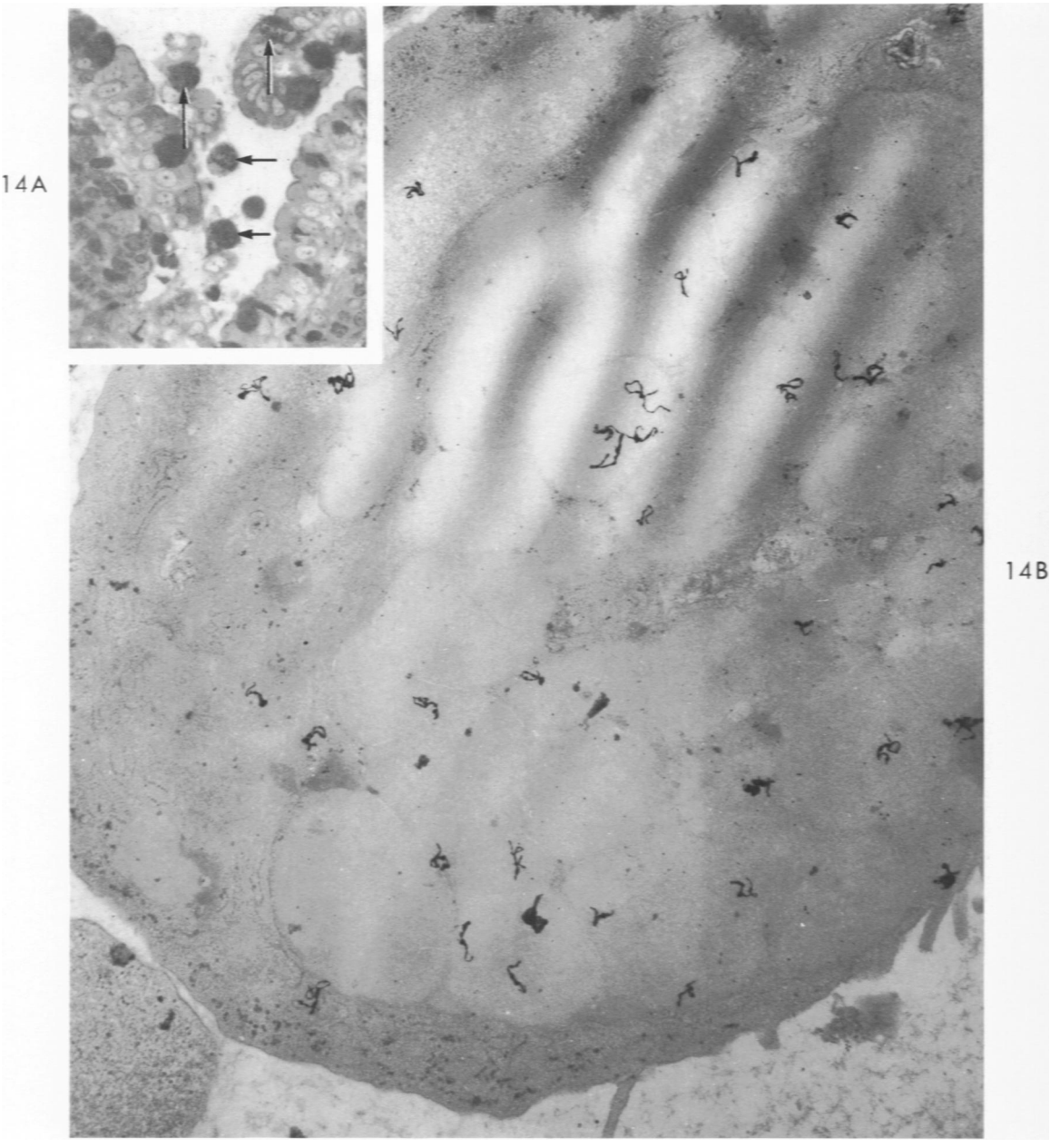

Fig 14A *(Inset).*—Light microscopic autoradiograph of two adjacent villi from sac incubated *in vivo* for 2 hours after intraluminal instillation of $^{57}CoB_{12}$ dissolved in 3% EDTA. Concentrations of developed grains overlie mucosal goblet cells, which are both attached to *(long arrow)* and detached from *(short arrows)* villi. × 130. **B.**—Electron microscopic autoradiograph of detached mucosal goblet cell from same sac as in inset showing concentration of developed grains over mucus granules. × 18,000.

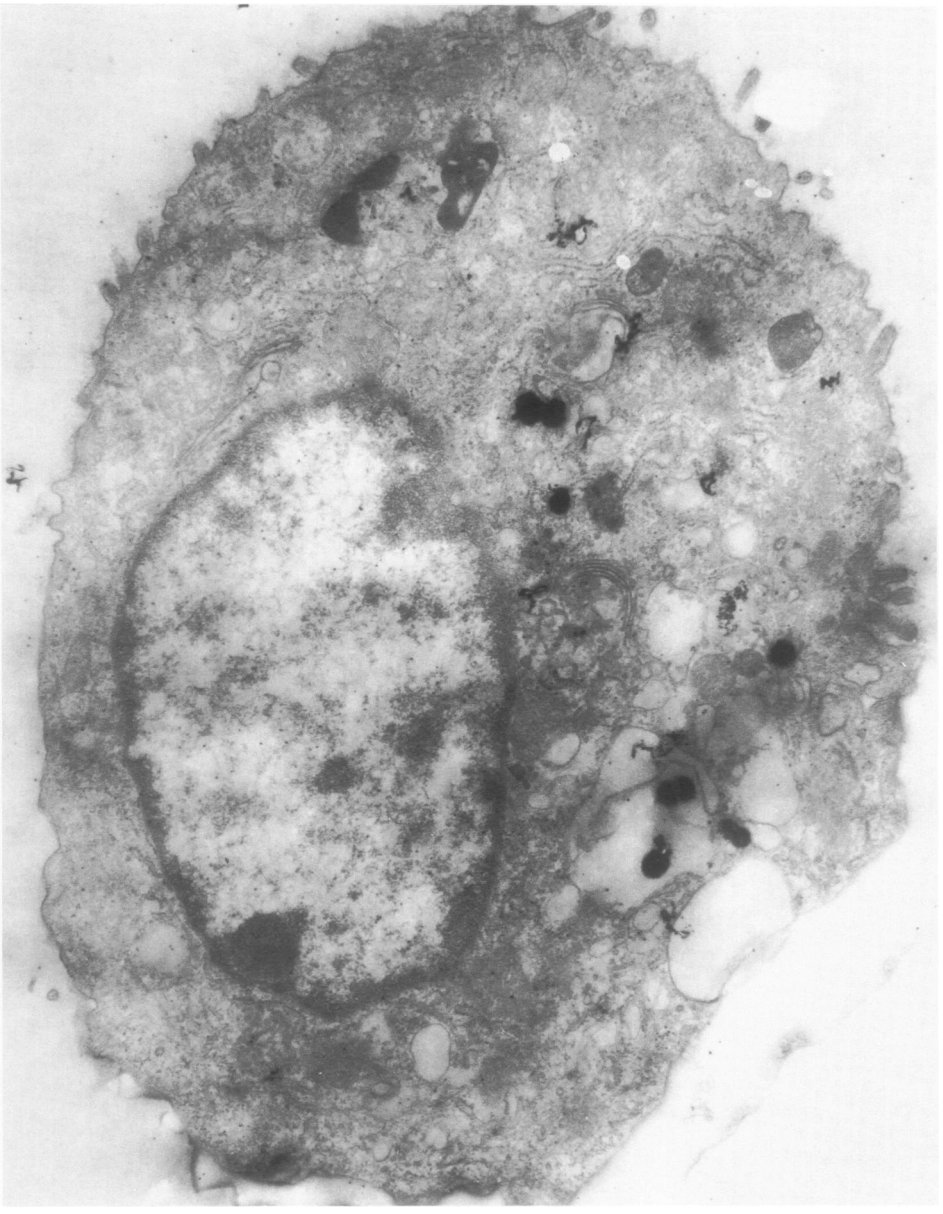

Fig 15.—Electron microscopic autoradiograph of detached mucosal absorbing cell from a sac incubated *in vivo* for 3 hours after intraluminal instillation of $^{57}CoB_{12}$ dissolved in 4% EDTA. Developed grains overlie dilated profiles of endoplasmic reticulum. Localization of radioactive B_{12} within these cells was an uncommon finding. × 12,000.