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Inhibition of protein synthesis and intestinal absorptive cell ultrastructure in cycloheximide or puromycin-treated rats

A. BERNARD, Hélène CARLIER

with the technical assistance of Marie-France GIRARDIER and Marie-Claude MONNOT

Laboratoire de Physiologie de la Nutrition, L.A. au C.N.R.S. n° 273 et U.E.R. ENS.BANA, (Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation) Campus Universitaire Montmuzard, 21100 Dijon, France.

Summary. Inhibitors of protein synthesis are useful for the investigation of some aspects of intestinal fatty acid absorption. The aim of the present study was to investigate both the biochemical and morphological effects of two inhibitors of protein synthesis on the intestinal mucosa of adult rats. Our results showed that 4 or 4 1/2 h after a single dose of cycloheximide or multiple doses of puromycin, the inhibition of protein synthesis in the jejunal mucosa, measured by the incorporation of ^{14}C-leucine, stabilized at 60 % of the controls. At the same time, these two drugs had different effects on absorptive cell morphology : cycloheximide definitely altered the apical surface of these cells, disorganizing their cytoplasm ; puromycin did not modify brush border morphology but remodeled the mitochondria and the Golgi complexes.

Introduction.

Both enzymatic and structural proteins are involved in the absorption of fatty acids in the epithelial cells of the intestine. As soon as they enter the absorptive cells, long-chain fatty acids bind to a protein (FABP) (Ockner *et al.* 1972 ; Ockner and Manning, 1974, 1976) which carries them into the smooth endoplasmic reticulum (SER) where a multienzyme system forms triglycerides. These triglycerides are then associated with various other molecules such as phospholipids, cholesterol and apoproteins to constitute lipoprotein particles. After restructuration and major glycosylation in the Golgi complexes, these particles (chylomicrons and VLDL) are secreted by the absorptive cells (Sabesin and Frase, 1977).

Many authors have used protein synthesis inhibitors to try to determine the roles of the various cell organelles during absorption. The main inhibitors that have been used are puromycin (Redgrave and Zilversmit, 1969 ; Kayden and Medick, 1969 ; Allen *et al.*, 1971 ; Friedman and Cardell, 1972 ; Yousef and Kuksis, 1972 ; Vahouny *et al.*, 1977) and cycloheximide (Sabesin and Isselbacher,

1965 ; Greenberger *et al.,* 1966 ; Vodovar *et al.,* 1968, 1969 ; Redgrave, 1969 ; Glickman *et al.,* 1970, 1972, 1973, 1978). Unfortunately, the inhibitor doses used have varied considerably. Also, the experimental animals often belonged to different species and were investigated under diverse experimental conditions. It has thus been difficult to obtain a valid comparison of the results. To illustrate, Allen *et al.* (1971), using a dose of 30 mg/kg of cycloheximide in rats, found no ultrastructural changes in the intestinal epithelium during lipid absorption, while Vodovar *et al.* (1969), using a dose of 100 mg/kg of the same drug in rats and pigs, observed lipid accumulation in the absorptive cells in the same conditions of absorption.

In previous work, we reported ultrastructural changes in the absorptive cells of fasted rats after cycloheximide treatment (Bernard and Carlier, 1980). This treatment had considerable effect on lipid absorption, causing a deficiency in that absorption. Cycloheximide perturbed lymphatic lipid absorption (1) by decreasing the amount of lipid absorbed by this route (Bernard and Carlier, 1981a) and (2) by increasing lipoprotein particle size (Glickman *et al.,* 1972 ; Bernard *et al.,* 1980a). Puromycin also caused biochemical changes in esterification or in lymphatic absorption during intestinal lipid absorption (Kayden and Medick, 1969 ; Redgrave and Zilversmit, 1969 ; Yousef and Kuksis, 1972 ; Vahouny *et al.,* 1977 ; Bernard *et al.,* 1980a) ; these perturbations paralleled ultrastructural changes induced by cycloheximide during the same process (Friedman and Cardell, 1972).

After the inhibition of protein synthesis by a given antibiotic has been quantified, the characteristics of absorptive cell ultrastructure in fasted animals should provide us with data for interpreting the changes occurring during long-chain fatty acid absorption.

We carried out biochemical studies to estimate the degree of protein synthesis after treating fasted rats with cycloheximide or puromycin. In parallel, we compared the action of these two drugs on absorptive cell morphology and ultrastructure at the same degree of protein synthesis inhibition induced by these drugs in the intestine. In the second case, we particularly completed the observations recorded after the cycloheximide treatment (Bernard and Carlier, 1980). The results give a clear picture of the consequences of the treatments on lipid absorption.

Material and methods.

Animals. — 200-g adult male Wistar rats were used. They were fasted for 12 h.

Treatment. — 1.6 mg/kg body weight of actidione cycloheximide (Boehringer-Mannheim GmBH or Upjohn Company, Kalamazoo, Michigan 49001, USA) in saline was given intraperitoneally in a single injection to the first batch of rats.

10 mg of crystallized dihydrochloride puromycin (Boehringer-Mannheim) were dissolved in 9 ml of phosphate buffer 0.04 M, pH 7.4. Four intraperitoneal

injections of 2 ml each were given at 1-hour intervals to the second batch of rats. 30 min or 1 h after the fourth injection, a last injection of 1 ml was given together with the labeled amino acid. The control rats received the same volumes of corresponding solutions by intraperitoneal injection.

Measurement of ^{14}C-leucine incorporation into absorptive cell soluble proteins. — The rats treated with each of the inhibitors were divided into two groups receiving a simple injection (12.5 μCi) of ^{14}C-leucine (specific activity : 318 mCi/mmole) 3 1/2 or 4 h after the onset of the inhibitor treatment. Twenty-five min after the ^{14}C-leucine injection, each rat was lightly anesthetized with ether, the abdominal wall was opened and the intestine was severed at the junction of the common (pancreato-biliary) duct and cecum. Exactly 30 min after the ^{14}C-leucine injection, the intestine was washed in 50 ml of cold saline kept on melting ice for 2 h before. For biochemical analysis, the duodeno-jejunal part of the intestine was opened longitudinally. The mucosa, scraped on a cold glass plate, was homogenized with 10 ml of physiological serum at 0 °C using a Thomas potter. The homogenate was centrifuged at 12 300 × g for 10 min using a refrigerated Sorvall centrifuge. In two cases, the ^{14}C-leucine injection was given 1 1/2 h after the actidione-cycloheximide treatment.

Assay methods. — The method of Mans and Novelli (1961) was used to measure ^{14}C-leucine incorporation into proteins. For each experiment, six aliquots of 10 μl of supernatant were each placed on a disk (diameter : 20 mm) of Whatman chromatographic paper n° 3 MM. Each disk was mounted on a support consisting of the heads of three upright pins. A cork disk of the same diameter, placed on the pin heads and under the paper disk, made it easier to handle the samples.

The disks were put under a stream of hot air and then immersed for 60 min in 10 % glacial trichloracetic acid ; they were dried using a vacuum filter, washed, extracted and placed in a scintillation vial. The background was determined on a blank disk treated according to the method described.

^{14}C-leucine incorporation was expressed in dpm/mg of protein (Lowry *et al.*, 1951) and represented in each experiment the mean of the radioactivity of six disks.

Preparation of tissue for electron microscopy. — The jejunal mucosa of control and treated rats was fixed by infusion into the intestinal lumen of 0.1 M phosphate buffered with 4.16 % glutaraldehyde. Small blocks of mucosa were then immersed in the same fixative for 16 h at 4 °C, washed in the buffer with 0.33 M sucrose added, postfixed for 1 h in phosphate buffer with 2 % osmium tetroxide, and dehydrated.

For transmission electron microscopy (TEM), the samples were embedded in Epon (Luft, 1961). The ultrathin sections were obtained on a Reichert OMU_2 ultramicrotome using glass knives, placed on formwar and carbon-coated copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and observed with an Hitachi HU 11E microscope. We analysed only the cells of the distal third of the villus, excluding the apical exfoliating cells. Microvillus height and number were measured on several cells of different villi and of different samples.

For scanning electron microscopy (SEM), the dried tissues were gold-coated and then studied directly under the microscope.

Results.

Inhibition of protein synthesis.

Protein synthesis inhibition was expressed as the percentage of decrease in ^{14}C-leucine incorporation in the treated rats compared to the controls.

$$X\ \% = 100 - \frac{\text{dpm treated/mg protein}}{\text{dpm control/mg protein}} \times 100$$

Table 1 shows the percentages of protein synthesis inhibition in rats treated with either cycloheximide or puromycin 2, 4 or 4 1/2 h before sacrifice. Two hours after the drug treatment, protein synthesis inhibition reached 81.9 % ; 4 h after the treatment it fell to 62.2 %. The inhibition was not significantly different between 4 and 4 1/2 hours after treatment or between the two drugs at the same time interval.

The stabilization of protein synthesis inhibition, observed in the 4-4 1/2-hour period, seems compatible with a study of absorption processes : it is preferable that this parameter, causing changes in the absorption process, does not vary.

TABLE 1

Percentages of inhibition of ^{14}C-leucine incorporation into total proteins of the duodenum and jejunum of rats treated with cycloheximide or puromycin 2, 4 or 4 1/2 h after treatment onset.

	2 hours	4 hours	4 h 30 min
Actidione-cycloheximide	n = 2 81.9 (0.3)	n = 6 62.2 (2.3)	n = 4 61.8 (1.6)
Puromycin		n = 2 57.2 (4.4)	n = 2 56.5 (2.1)

Mean ± SD ; n = number of rats.

Morphology and ultrastructure.

The cycloheximide treatment modified the apical surface of the jejunal absorptive cells (plate 1, fig. B and plate 3), compared with the controls (plates 1 and 2, figs. A). The height of the microvilli was reduced by 50 % (1 to 1.5 μm in controls to 0.5 to 0.8 μm in treated rats). Their number per μm^2 decreased from 80 to 100 microvilli in control rats to 40 to 50 in treated ones. The supranuclear cytoplasm was disorganized with degranulation of the rough endoplasmic reticulum (RER) and changes in the aspect of the Golgi complexes (Bernard and Carlier, 1980). Moreover, we observed a significant decrease in lateral membrane infoldings.

None of these observations were made after puromycin treatment (plate 1, fig. C ; plate 4). However, the mitochondria were elongated (3 to 4 μm *vs.* 1 to 1.5 μm in controls) and the flat Golgi cisternae were crescent-shaped and more densely packed than in the control rats (plate 2, fig. C).

Compared to the controls, the overall effect of puromycin on the ultrastructure was less drastic than that of cycloheximide.

Discussion and conclusion.

Inhibition of protein synthesis.

The reversibility of cycloheximide action, reported by several authors (Ennis and Lubin, 1964 ; Colombo *et al.,* 1966 ; Verbin *et al.,* 1971), was clear 4 h after the onset of treatment ; the dose of 4.5 mg/kg proved to be close to DL_{100}. Lieberman *et al.* (1970) showed that there was no irreversible damage when doses of less than 1.5 mg/kg of cycloheximide were given. Reversibility, which began early, gradually disappeared since the inhibition seemed to be stabilized within 4 to 4 1/2 h after treatment.

Lieberman *et al.* (1970) showed a 40, 70 and 80 % inhibition in rats with injections of 0.1, 0.25 and 0.5 mg/kg, respectively, of cycloheximide. We could have used the latter dose in the present study, but Glickman *et al.* (1970) emphasized the differences in protein synthesis inhibition observed in the small intestine, depending on the protocol used : while a dose of 0.25 mg/kg of cycloheximide in rats caused a 90 % inhibition of protein synthesis after 3 h, this inhibition was about 80 % at the end of a study of oleic acid absorption ; on the other hand, in rats fitted with a lymphatic fistula, 90 % protein synthesis inhibition was obtained with two injections of a double dose (0.5 mg/kg at a 1 1/2-hour interval). Thus, we chose the dose of 1.6 mg/kg which, in our experimental conditions, permitted the stabilization of protein synthesis inhibition at 62 % in the duodenum and jejunum of the small intestine of fasted rats 4 h after injection.

Several authors, including Allen *et al.* (1971) and O'Doherty *et al.* (1973), have studied the inhibition of ^{14}C-leucine incorporation into intestinal or absorptive cell protein *in vivo* and *in vitro* in rats when this label was injected intraperitoneally or infused into an isolated intestinal loop *in situ.* Inhibition was higher than 85 % in treatments using successive injections of puromycin for 8 to 24 h, depending on the authors. The differences among these various methods of treatment and ours explain the lower inhibition in the present work. The dose of puromycin and the methods of its injection induced a 57 % inhibition of protein synthesis which was also stabilized in 4 to 4 1/2 h after the onset of treatment.

Considering the standard deviations, the inhibition seemed to be of the same order of magnitude for both the inhibitors used. These comparable degrees of inhibition permitted us to compare the effects of the two inhibitors on absorptive cell ultrastructure in fasted rats and the influence of the inhibitors on changes induced during lipid absorption.

Cycloheximide inhibited protein synthesis in peptide synethases, while puromycin, a structural analogue of t-RNA aminocyls, bound to the polypeptide chain, thus interrupting protein synthesis and releasing polypeptides of various lengths. These different modes of action could explain the different effects observed in fasted rats as well as during lipid absorption.

Inhibition of protein synthesis and ultrastructure.

Our observations during lipid absorption showed that the ultrastructure was more or less modified and altered depending on absorptive activity, that is, on the nature of the fatty acids absorbed (Bernard and Carlier, 1981b, 1983) and on the amount of lipid and the form of lipid administered (Bernard *et al.,* 1980a ; Bernard and Carlier, 1983).

In fasted rats, clear, specific differences were found in the respective effects of cycloheximide and puromycin on ultrastructure, although the values of protein synthesis inhibition, observed at the same times after treatment onset, were not significantly different. As concerns protein involved in long-chain fatty acid absorption, the inhibition of protein synthesis might affect, among other processes, the synthesis of enzymes responsible for the resynthesis of triglycerides and of other components of intestinal lipoproteins and for the synthesis of apoproteins, and it could also influence membrane renewal. Friedman and Cardell (1977), favor the renewal *de novo* of membranes implicated in the processes of lipid absorption rather than a putative recycling of these membrane systems. These different incidences agree with the changes we reported in rats treated during lipid absorption (Bernard *et al.,* 1980a, b).

Cycloheximide or its derivatives has been shown to have a wide spectrum of action on the processes of absorption. Not only is the incorporation of the protein necessary for the formation of chylomicrons and VLDL affected (Glickman *et al.,* 1972), but the processes of esterification are deeply influenced (Bernard *et al.,* 1980a) and membrane renewal is impaired. According to Friedman and Cardell (1977), it is the preexistent RER in fasted cells that determines good lipid absorption. However, already in normal conditions, the renewal of the RER does not equilibrate the formation from that reticulum of smooth endoplasmic reticulum (SER), in which lipid is esterified, and of Golgi saccules. The Golgi complexes are thus less implicated in the process of the absorption of long-chain fatty acids (Bernard and Carlier, 1983), not only because the turn-over of the fatty acids is lower, as that in other membrane systems, but also because they are less involved due to the upstream decrease of their steps of absorption. The considerable diminution in the microvillous absorptive surface, compared to controls (Anderson and Taylor, 1973), already reported in chickens (Lecount and Grey, 1972), explains the lower uptake of lipids by absorptive cells (Bernard *et al.,* 1980a ; Bernard and Carlier, 1981b, 1983). Altmann (1975), using a dose of 15 mg/kg, showed an arrest of epithelial cell renewal and progression along the villi with increased exfoliation of mature cells after epithelial cell cohesion declined. This dose proved to be lethal about 12 h after a single injection. The fact that 10 times less cycloheximide was administered in our study could explain why we did not find the differences reported by Altmann between the lower cells

of the villi and the mature cells in the distal end which is an important site of lipid absorption. Except for a few variations from one cell to another, the size and number of microvilli in the absorptive cells of the distal part of the villi decreased, and a more or less large number of membrane coils and autophagic vesicles were observed in the cytoplasm. These aspects were particularly evident during lipid absorption and at high doses. However, in the lower part of the villi, phospholipid synthesis was very active, justifying the observations of Altmann. Moreover, Hugon and Charuel (1971) in a cytochemical and biochemical study on mice showed the inhibition of absorptive cell alkaline phosphatase, confirming the biochemical results of Glickman *et al.* (1970) who noted the disappearance of this same enzyme in rats during lipid absorption in the lymph. The impairment of the ER and Golgi complexes and the changes observed in cytoplasmic organization (Bernard and Carlier, 1980), as well as the aspect of the cellular interdigitations (Altmann, 1975), indicate that the cytoskeleton was affected. These observations provide arguments to explain the defective formation and secretion of lipoprotein particles (Vodovar *et al.*, 1968, 1969 ; Glickman *et al.*, 1972 ; Dobbins, 1966).

Contrary to cycloheximide, puromycin did not markedly affect the ultrastructure of fasted rat absorptive cells. The absorptive surface appeared unchanged and the cytoplasm still contained much RER, suggesting that some protein synthesis continued, especially of apoprotein B (Christensen *et al.*, 1983). This seems to contradict the measurements of labelled leucine incorporated into absorptive cell protein and does not permit us to say if the puromycin treatment was compatible with the formation of lipoprotein particles. During lipid absorption, Friedman and Cardell (1972) noted a defective dunctioning of the RER due to deficient membrane renewal in rats treated with puromycin. Data on absorptive cell ultrastructure alone in fasted, treated rats does not permit us to make a hasty judgement concerning the notion that synthesis was deficit during absorption. However, this deficient lipid absorption agrees with the inhibition of the protein synthesis we observed in fasted rats. The aspect of the Golgi complexes could explain the failure of these organelles in the final structuration of lipoprotein particles and of their secretion during lipid absorption (Friedman and Cardell, 1977). The impairment of the cytoskeleton observed with cycloheximide was not apparent with puromycin since microfilaments could be seen on the micrographs, although the techniques of tissue preparation were not specific in this respect.

Conclusion.

Apparently, the effects of cycloheximide were very characteristic in fasted rat absorptive cells. On the contrary, the effects of puromycin were not as evident compared to the controls. Puromycin could be a more selective inhibitor than cycloheximide in relation to the enzyme protein implicated in absorptive cell esterification of fatty acids, as ultrastructural observations seem to indicate a possible esterification. This notion has been put forward by Sabesin and Isselbacher (1965), O'Doherty *et al.* (1973), O'Doherty and Kuksis (1974, 1975). However, our studies (Bernard *et al.*, 1980b) revealed a defect in lipid

esterification during lipid absorption, thus agreeing with the observations of Kayden and Medick (1969) and Redgrave and Zilversmit (1969).

At all levels, the degree of protein synthesis inhibition explains the deficiencies observed in the process of lipid absorption, but to determine at which levels protein synthesis is deficient, the effects of cycloheximide or puromycin on the absorptive cell ultrastructure in fasted rats must be completed by studies of changes in that ultrastructure during lipid absorption.

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Résumé. *Inhibition de la synthèse protéique et ultrastructure de l'entérocyte de rat traité par la cycloheximide ou la puromycine.*

Les inhibiteurs de la synthèse protéique s'avèrent précieux pour élucider certains aspects de l'absorption intestinale des acides gras. Selon les conditions expérimentales, il apparaît indispensable d'évaluer le degré d'inhibition de la synthèse protéique et, simultanément, d'étudier les effets de ce degré d'inhibition sur l'organisation ultrastructurale de la cellule épithéliale intestinale absorbante. Dans nos conditions expérimentales, l'inhibition de l'incorporation de ^{14}C-leucine dans les protéines de la muqueuse jéjunale, 4 ou 4 h 1/2 après le traitement par la puromycine ou la cycloheximide, se stabilise à 60 %.

Le traitement par la cycloheximide entraîne une diminution considérable de la surface microvillositaire des cellules absorbantes et affecte l'organisation cytoplasmique de ces cellules. Avec la puromycine, la surface luminale des cellules absorbantes n'est pas modifiée, mais alors que le réticulum endoplasmique granuleux apparaît pratiquement inchangé, les mitochondries et les corps de Golgi se présentent différemment.

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PLATE 1. — *Intestinal absorptive surface in fasted control* (A), *cycloheximide-treated* (B) *and puromycin-treated* (C) *rat.* SEM. × 22 500.
The intestinal absorptive surface of cycloheximide-treated rat is greatly altered. Many anfractuosities are visible, causing a decrease in the absorptive surface. The intestinal absorptive surface of puromycin-treated rat (C) is not different from that of the control (A).
Scale = 1 μm.

PLATE 2. — *Jejunal epithelial cells of fasted control rat.* × 35 000.
The absorptive surface (A) is composed of parallel microvilli (MV). Microvillous microfilaments penetrate (◀) the apical cytoplasm (AC) which has no cell organelles. The supranuclear cytoplasm contains many SER vesicles (⇐) (A) and RER saccules (⇝) (B). The Golgi complexes (C) are composed of 4 to 6 elongated saccules (⇐). The vesicles on the maturation facet may contain lipoprotein particles (⇒).
Scale = 1 μm.

PLATE 3. — *Jejunal absorptive epithelial cells of cycloheximide-treated rat.* × 35 000.
The alteration of the microvilli (MV) is evident ; they are more rare and significantly smaller than in the controls (0.5 to 0.8 μm *vs* 1 to 1.5 μm). The apical cytoplasm (AC) is characterized by the unusual presence of ribosomes (r). The interdigitations of the lateral plasma membranes (pm) of the absorptive cells may show more or fewer modifications that probably lead to a looser cohesion among the epithelial cells.
Scale = 1 μm.

PLATE 4. — *Jejunal epithelial cells of puromycin-treated rat.* × 35 000.
The aspect of the absorptive surface (MV) and apical cytoplasm (AC) (A) is similar to that of the controls (Plate 1A). The supranuclear cytoplasm (B) containing many RER saccules (⇝) is characterized by exceptionally long mitochondria (M). The Golgi complexes are composed of saccules (⇒) that are beginning to coil.
Scale = 1 μm.

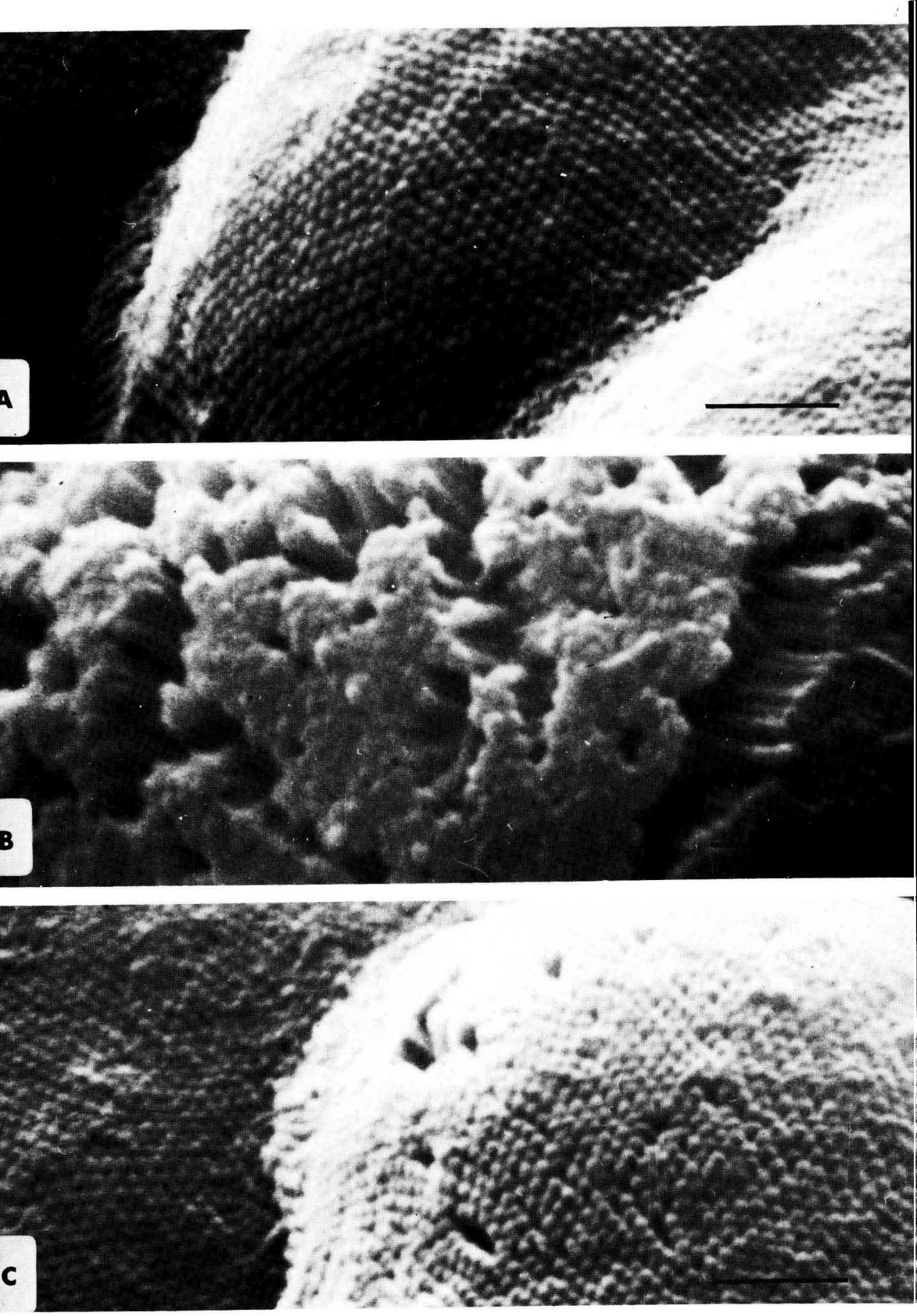

PLATE 1.

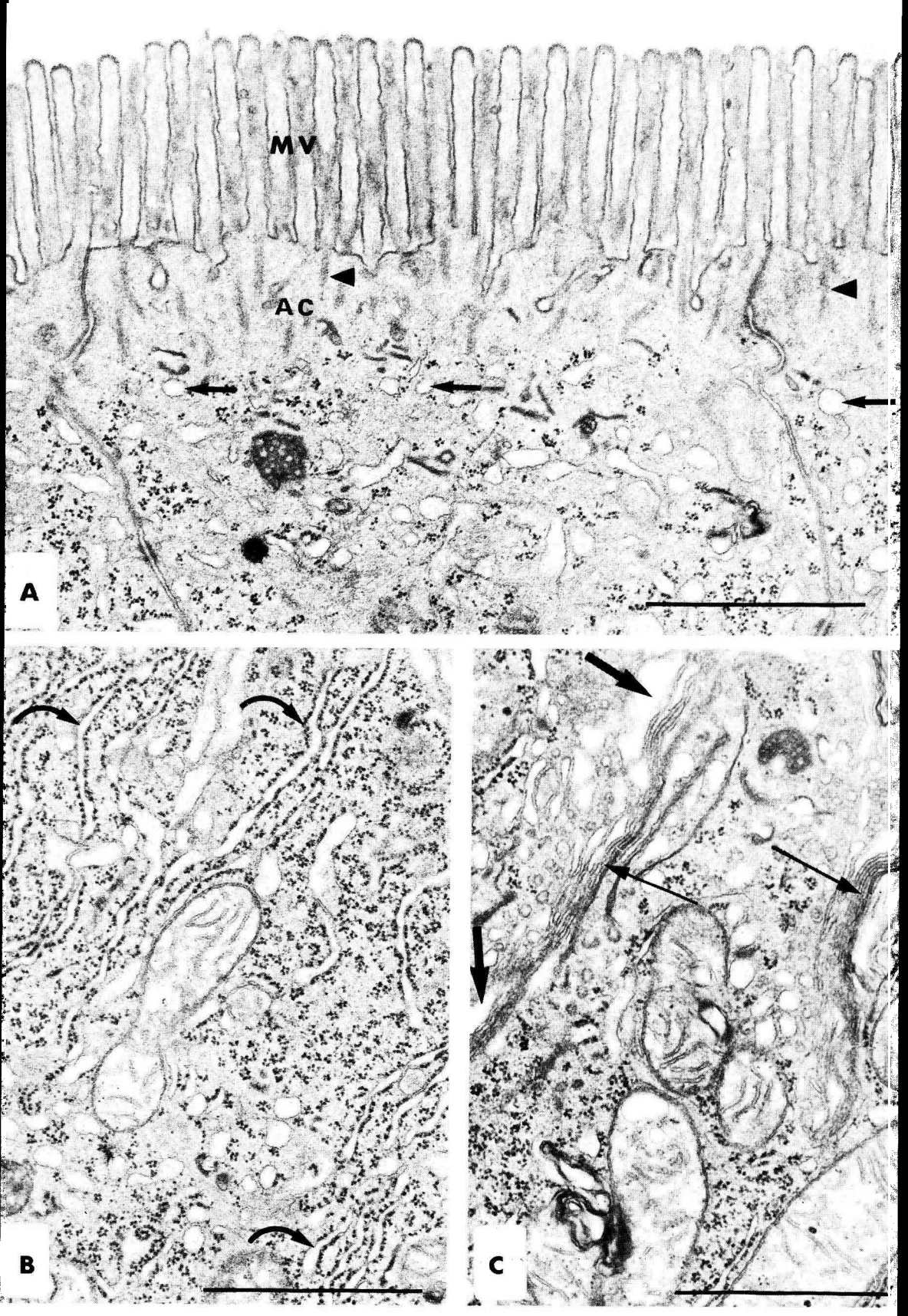

PLATE 2.

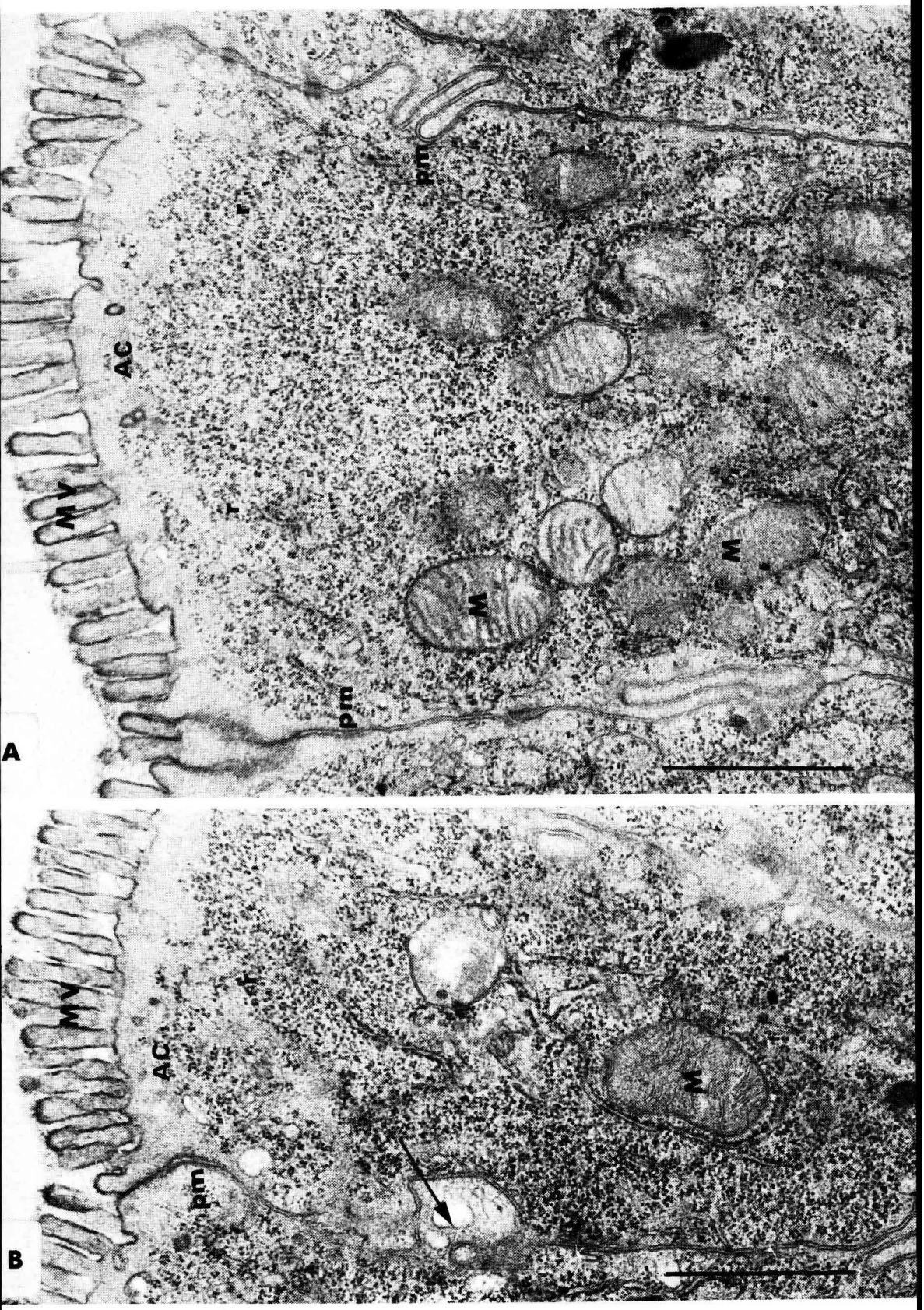

PLATE 3.

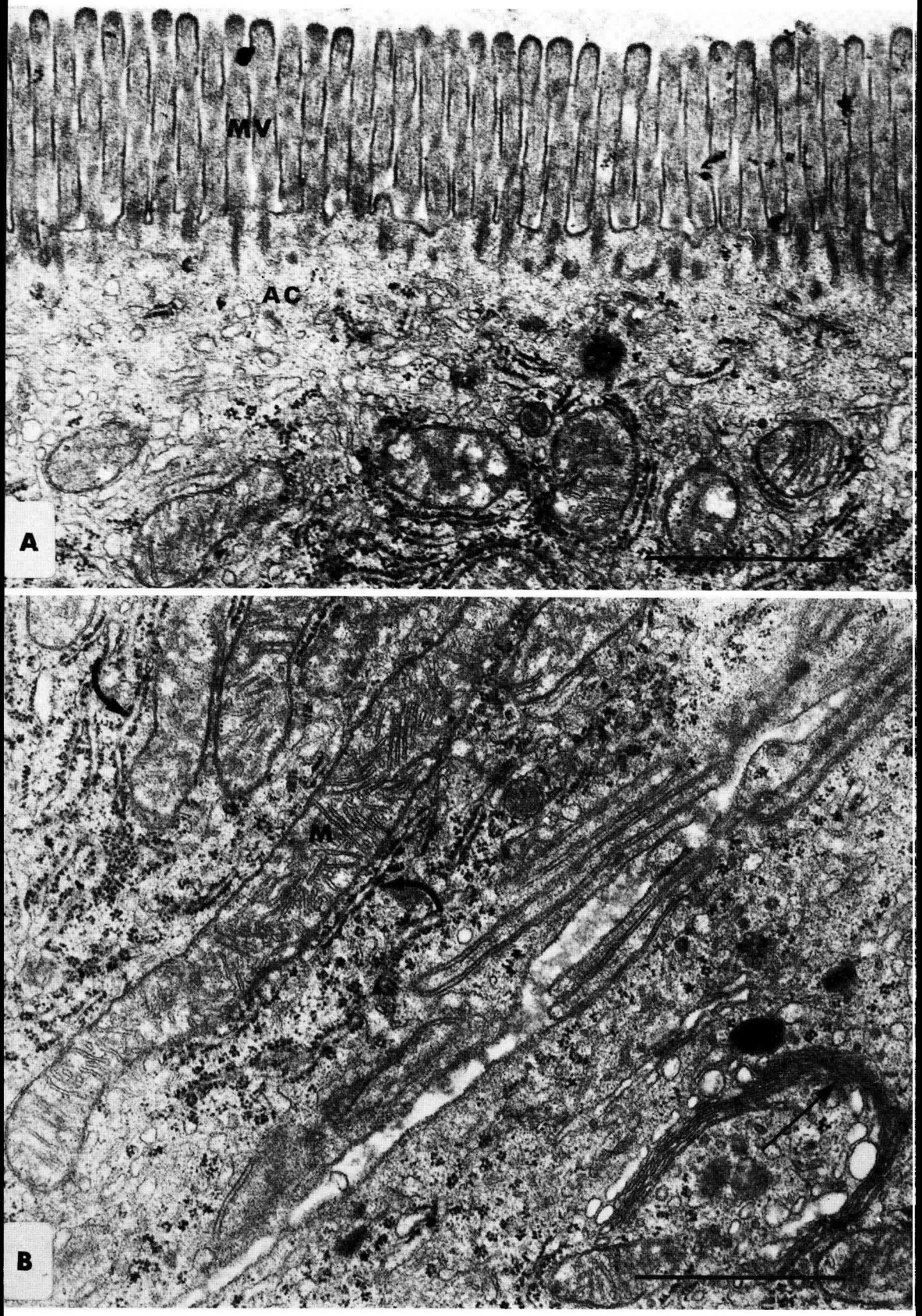

PLATE 4.