# STUDIES ON THE ORGANIZATION OF THE BRUSH BORDER IN INTESTINAL EPITHELIAL CELLS

## II. Fine Structure of Fractions of Tris-Disrupted Hamster Brush Borders

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ABSTRACT

Two of the fractions obtained by density gradient centrifugation of Tris-disrupted brush borders from hamster intestinal mucosa have been identified as the microvillus cores and their surrounding membranous coats, respectively. This identification has the following morphological basis. In shadowed preparations one fraction (cores) appears as rounded, compact rods, and the other fraction (coats) appears as flattened sheets. Both rods and sheets have dimensions appropriate to the identities assigned to them. In addition, negative staining shows that the rods are composed of aligned particles of roughly 60 A, consistent with the appearance of the core in tissue section, where 60-A fibrils are characteristic. The sheets are covered by non-aligned particles of approximately the same size. Sectioned preparations show that the core fraction contains predominantly fibrous material with some membranous contamination and that the coat fraction is apparently composed exclusively of elongated sacs with a unit membrane structure. Some details of the structure of the core are evident in cases where the compact rod appears to be loosened, revealing a doubled strand. The strand is approximately 350 A wide; the compact rod is roughly twice this width. With negative staining the strand shows a dense central region. The morphological identification presented here is consistent with the distribution of enzymic activity among the density gradient fractions described in the preceding paper.

There is at present very little information concerning the possible relationship of the elements of fine structure of the microvillus to the reputed digestive and absorptive functions of the microvillus. The development of this specialization of the cell surface has been followed in the intestinal brush border in the chick, and it has been observed that the microvilli develop synchronously and undergo their main increase in length after the terminal web and microvillus cores become established (23). Among the various enzymes known to be present in the brush border (10, 17), only phosphatase has been studied with the electron microscope and localized in the microvillus membrane (4, 27). Slight differences in microvillus membrane thickness between mice on various feeding regimes have been reported (28).

In the preceding publication (7), a procedure is described by means of which structural components of the microvilli may be isolated and identi-

fied by position on a density gradient and by enzymic assay. It is the purpose of the present study further to identify and to describe the main morphological features of two of the isolated components as a prelude to more extensive studies of their enzymatic, chemical, structural, and functional properties. A preliminary report of this work has been given (22).

## MATERIALS AND METHODS

Brush borders were isolated from the pooled jejunal and ileal mucosal scrapings of several animals by a modification of the method of Miller and Crane (16). The scrapings were homogenized in cold 0.005 M EDTA at low speed in a Waring Blendor, filtered through bolting silk, and collected by low speed centrifugation. These preparations were washed several times, disrupted by treatment with 1 M Tris (Tris(hydroxymethyl)aminoethane), dialyzed against 0.005 M EDTA, and separated into fractions by centrifugation on glycerol density gradients. Full details of these procedures are provided in the preceding paper (7). The two fractions which contained structures representing the main components of the microvilli (fractions C and D) will be described here. Other fractions contained globular particles of various sizes.

For this special study, a sample of fraction D was treated with deoxycholate to remove residual contaminating membranes. Fraction D obtained in the usual way was resuspended in 3 ml of 1 M Tris, pH 7.4, containing 0.25 per cent deoxycholate; it was mixed mechanically and subjected to glycerol density gradient fractionation. The suspension yielded some material corresponding to the A, B, and C bands and the expected quantity in the D fraction. The new fraction D contained less than 4 per cent of the original alkaline phosphatase activity of the isolated brush borders.

Pellets of brush border preparations were fixed in 1 per cent osmium tetroxide in 0.1 M phosphate buffer at pH 7.8 for ½ hour at 0–4°C, carried through a graded series of alcohols, brought to room temperature, and embedded in Epon (14). Pellets of fractions were fixed in the same way or fixed first for 4 hours in 6 per cent glutaraldehyde, then for ½ hour in osmium tetroxide (26). Sections were cut on a Porter-Blum ultramicrotome with a glass knife and stained in uranyl acetate (31) for 8 to 10 hours. To prepare isolated fractions, a drop of cold glycerol suspension was placed on a Parawax-covered microscope slide chilled on ice. A carbon-coated grid was touched to the surface of the drop, then immersed 30 seconds in 10 per cent formalin in phosphate buffer at pH 7.6. The grid was then rinsed briefly in distilled water, in 100 per cent ethanol, and finally in amyl acetate. After drying, the grids were either shadowed with 80 per cent platinum–20 per cent palladium from an angle of 20 degrees, or negatively stained (2). For negative staining, a 2 per cent solution of phosphotungstic acid was adjusted to pH 7.0 with normal KOH. Grids were immersed in the stain and then drained on filter paper. This procedure was followed because preliminary trials showed that preparations were considerably distorted by flattening if dried from water. Staining was somewhat variable. All preparations were viewed with an RCA EMU-3 microscope.

## RESULTS

### *The Isolated Brush Border*

Although variable amounts of apical cytoplasm remained attached to the isolated brush borders, preparations appeared to be otherwise largely uncontaminated. Characteristic of these isolates were an indentation in the region of the terminal bar (Fig. 1) and a bowing outward of the original free cell surface. Microvilli were consequently farther apart at the tip than at the base. The morphology of the microvilli themselves generally appeared normal in both longitudinal and cross-sections (Figs. 2 and 3), although occasionally microvilli were less dense centrally than laterally (Fig. 2). Membranes remained intact and rootlets were evenly spaced. Since the cells for these preparations are taken from the entire lining of the small intestine, there is, as expected, a considerable variation in microvillus size (3). The lengths of microvilli varied from 0.6 to 2.2 micra, the longest being slightly longer than have been observed in tissue sections. It is possible that these longest microvilli represent a distortion due to the osmotic shock to which the isolation procedure subjects them; increase in length is a typical response of microvilli to treatment with hypotonic solutions (18).

### *The Shadowed Fractions*

Fraction D from the density gradient contained rods in clusters or lying separately; fraction C contained sheets.

When in clusters, the rods were attached at one end and the free ends radiated out from the region of attachment (Figs. 4 and 5). This appearance suggests that the clusters may represent small portions of the "skeleton" of the free cell surface. It has been estimated in the rat that each cell bears about 1000 microvilli (24) and $OsO_4$-fixed and sectioned material (15, 18, 23) suggests that the central structure in the microvillus which emerges as a

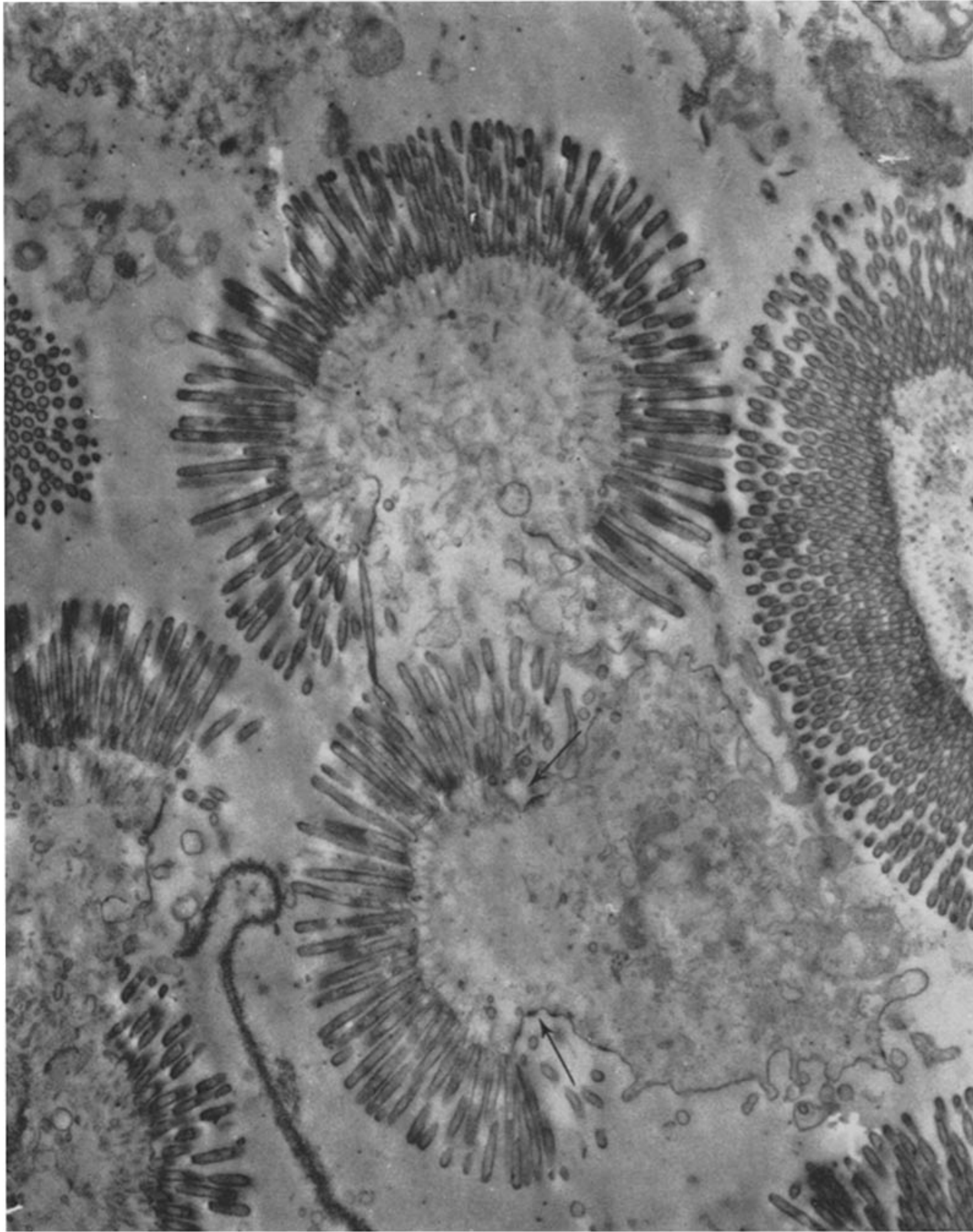
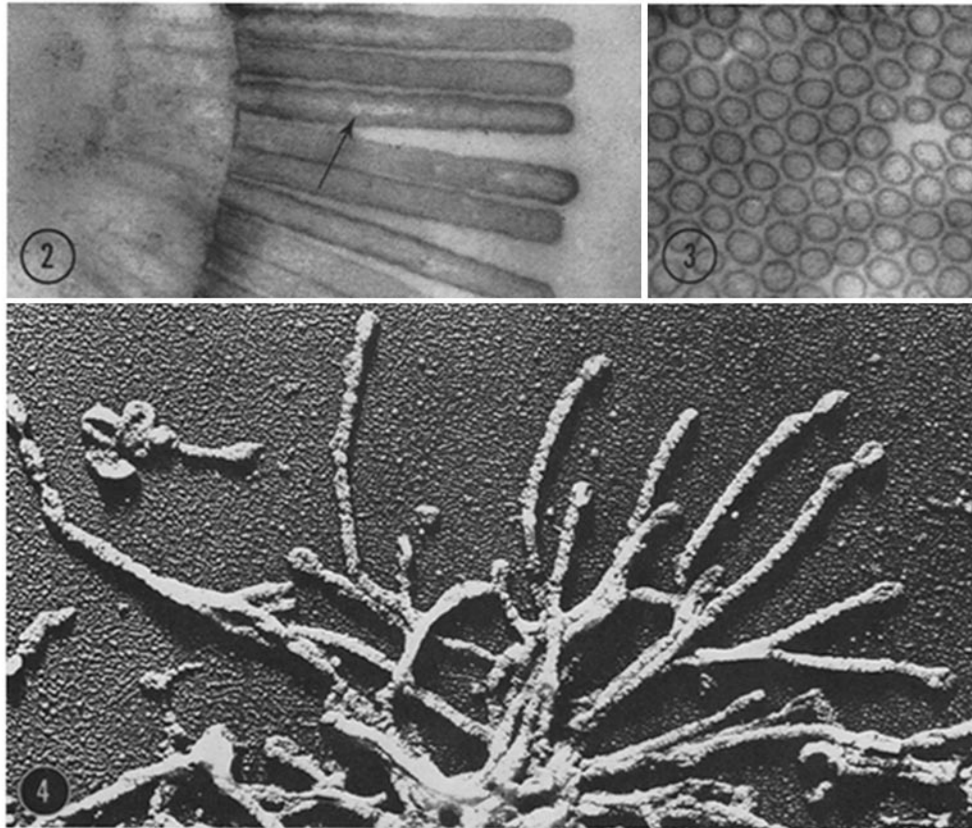


FIGURE 1 Sectioned pellet of a brush border preparation. Arrows indicate indentations at the terminal bar region. The only regular contaminant appears to be some components of the distal cytoplasm. Nuclei are extremely infrequent. × 9000.



FIGURES 2 and 3 Longitudinal and cross-sections of brush border preparations. Arrow (Fig. 2) indicates abnormal microvillus core in which the electron-opaque material is split in two. × 32,000.

FIGURES 4 and 5 Shadowed preparation of fraction D, showing cluster. *L*, loop. Loops may appear at the free ends of rods rather regularly. Cores may appear as a twisted doubled strand. Fig. 4, × 32,000; Fig. 5, × 56,000.

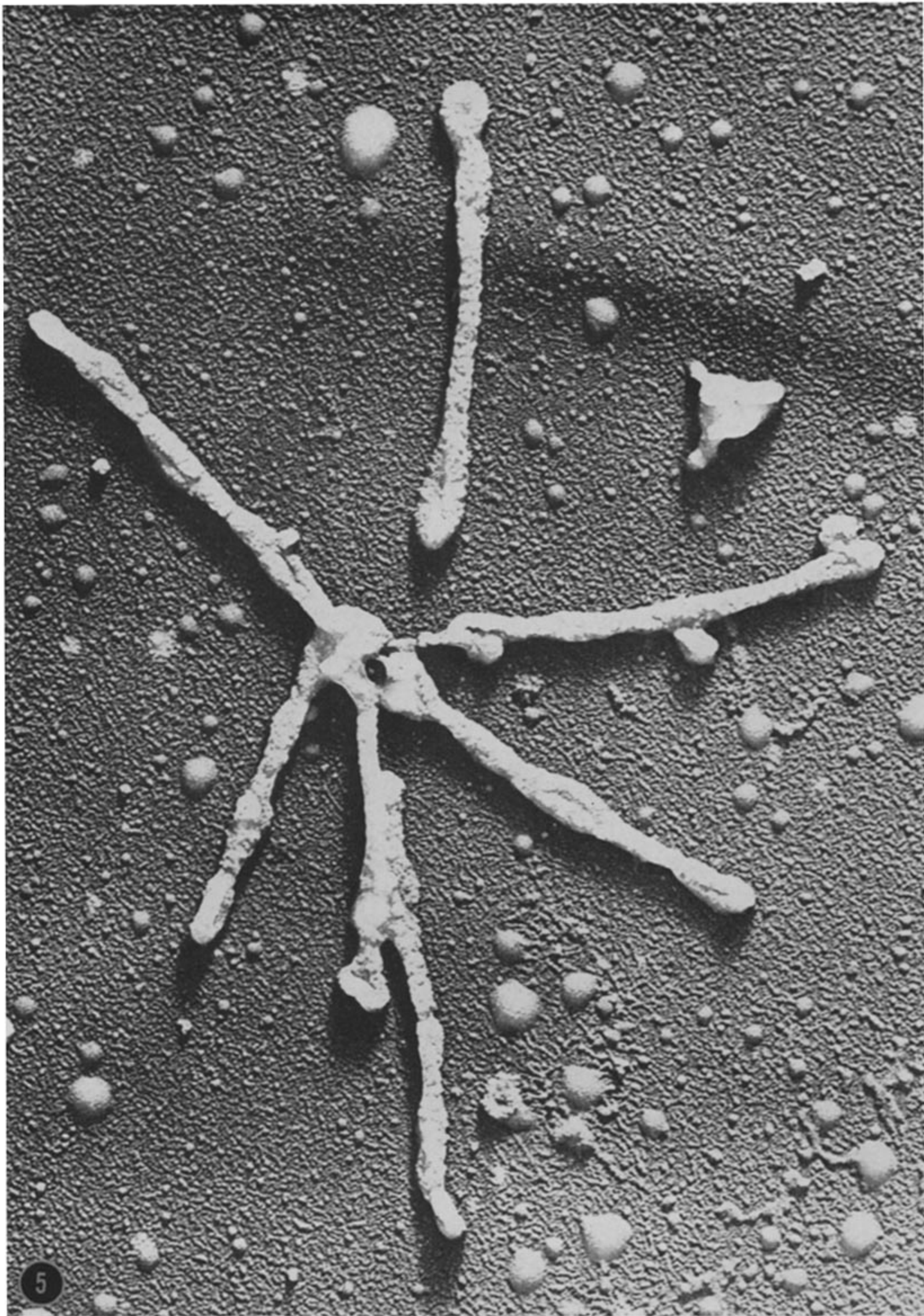
root may have some attachment to the terminal web. These shadowed preparations do not indicate the nature of the attachment of the rods to one another. The free ends of the rods frequently bear rounded enlargements evident in some cases as loops.

When lying separately, the compact rods (Fig. 8) are roughly 700 A in diameter and vary in length, much as the microvilli vary in length in the isolated brush border preparations. Also seen (Fig. 9, see also Fig. 5) are structures composed of a continuous double strand which is about 350 A in width, or half the width of a compact rod, and roughly equivalent to a rod in length. This doubled structure appears to represent a rod in a loosened state, and its formation could account for the loops often seen at the free tips of clustered rods. In some preparations, the doubled strand appears to be somewhat twisted (Fig. 5).

The elements contained in density gradient fraction C are considerably more flattened than the rods of fraction D; they are also somewhat more variable in width (Figs. 6 and 7). They are of appropriate dimensions to serve as envelopes for the rods (compare Figs. 7 and 8). Their appearance suggests that they are flattened, elongate vesicles, although in some regions they may be either considerably stretched or partially spread in a single layer.

### *The Negatively Stained Fractions*

The surface of the compact rods, when negatively stained, appears to be covered with particles which tend to be aligned in rows running parallel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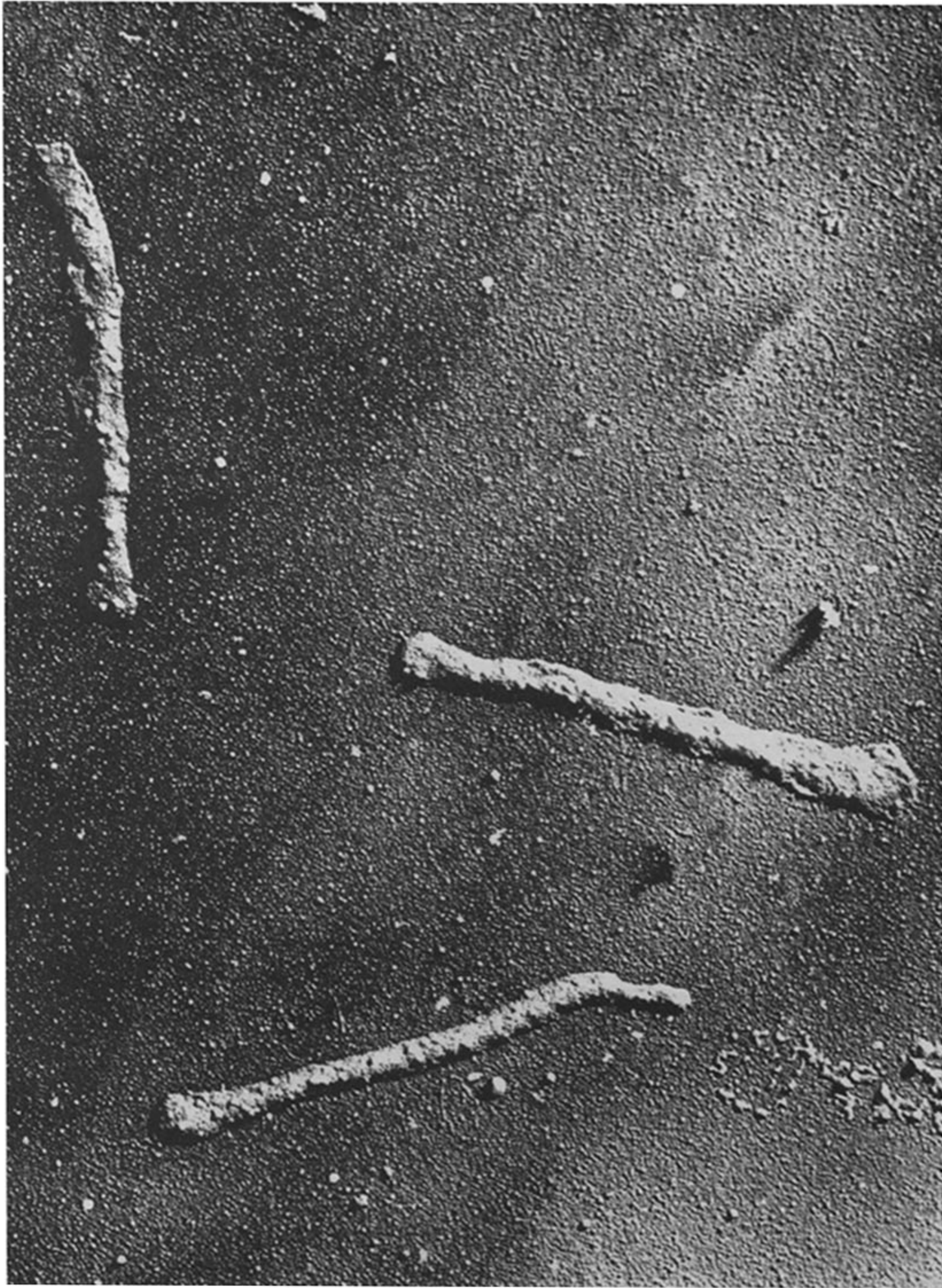

FIGURE 6 Shadowed preparation of fraction C (coats). × 40,000.

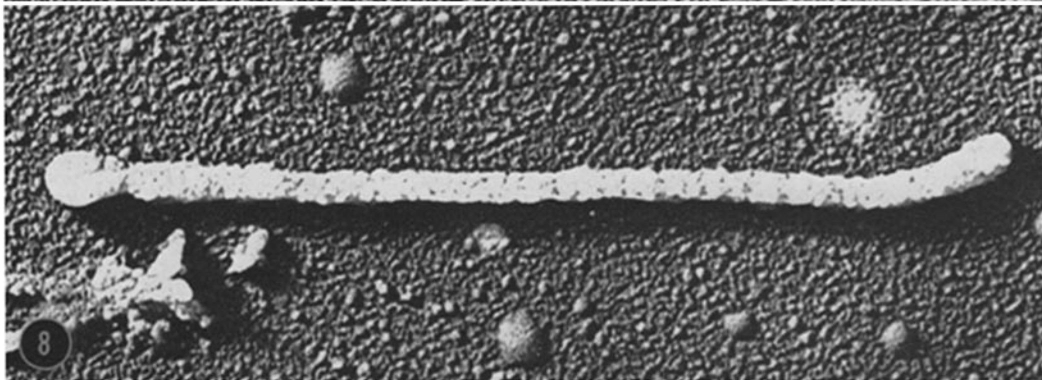

FIGURE 7 Shadowed coat. × 72,000.

FIGURE 8 Shadowed compact rod. × 72,000.

FIGURE 9 A shadowed rod which is loosened and shows a doubled strand. × 72,000.

to the long axis. This appearance is shown in a cluster of adjacent rods in Fig. 10, and in two single rods in Figs. 12 and 13.

Thus the appearance of the negatively stained compact rod is what may be expected of the isolated microvillus core, since the latter is seen in normal tissue sections (15) to be composed of longitudinally oriented, 60-A fibrils. Loosened rods in which the double strand is evident (Figs. 11 and 15) characteristically have a heavily stained central region running continuously throughout their length.

The elements in fraction C are seen, when negatively stained, to be covered with particles of *ca.* 60 A (Fig. 14) in which no alignment was observed. This appearance is similar to that which has been described for the surface of microvilli (20) and suggests that the elements of fraction C are the coats of the microvilli. The vesicular nature of these elements is very clear in sectioned pellets, as described below.

### *The Sectioned Fractions*

Examination of sectioned pellets of fractions C and D (Figs. 16 to 19) confirms the interpretation based on shadowing and negative staining. Fraction C is a substantially pure preparation of elongated, hollow vesicles. When it is cut normal to the surface, the unit membrane structure of the surface is clearly apparent (Fig. 16, insert). Vesicles cut in section appear empty after $OsO_4$ fixation, or after fixation in glutaraldehyde followed by $OsO_4$. Thus, these vesicles have an obvious identity with the membranous coat of microvilli, though they may be somewhat swollen or distorted (compare Fig. 16 with Fig. 2 and 3).

Fraction D is predominantly composed of fibrous material, though it is not so cleanly separated from other structural elements of the brush border as is fraction C; it shows an appreciable contamination (see Fig. 18). In favorable instances (Figs. 17 and 18), strands with dimensions appropriate for representing the microvillus core are evident, and they frequently appeared twisted (Fig. 18), but details of conformation cannot be made out. Contamination of this fraction with membranous material is eliminated by deoxycholate treatment (Fig. 19).

## DISCUSSION

The formed elements in two of the density gradient fractions of Tris-disrupted hamster brush borders appear to have been identified with structures present in intact microvilli. This identification is based on three kinds of evidence. The first is simply their separation on the density gradient, which depends upon size and average density. By far the greatest proportion of the total substance of isolated brush borders is recovered in fractions C and D, and it is reasonable to surmise that they respectively are derived from individual major structures of the microvilli. The second kind of evidence is morphological, as given in detail in this paper. The formed elements in fractions C and D are of appropriate size and shape to be, respectively, the coats and cores of microvilli. The microvilli in the brush border preparations average about 0.12 micron in width and are of variable length. Since the rods in fraction D are about 700 A in diameter, the maximal possible thickness of the coat is thus approximately 250 A, which is ample. Actually, as seen in section after conventional fixation, the coats in fraction C appear to have the thickness of the unit membrane only. It is of course entirely possible that they include additional unstained material on the inside of the unit membrane, or on the outside (11, 12). The diameter of the rod as described here is consistent with the diameter of the core as seen in cross-section in normal tissue (23), and it may also be noted that a double structure, resembling the loosened rod, has been clearly described in sections of tissue subjected to hypotonic saline solutions (18). A suggestion of a doubled structure has also been reported in normal tissue sections of microvilli of the kidney (9). Since brush borders are prepared in hypotonic media, such an appearance for the isolated core may be expected. The fibrils *ca.* 60 A in diameter running the length of the microvillus core, as viewed in tissue sections (15), are seen here as rows of *ca.* 60 A particles, similar to what has been found with secondary fibers in sperm tails (1). This would have been to some degree confusing were it an isolated observation, because such particles are usually thought to be found in association with membrane surfaces. However, the membrane surface together with its associated enzymic activities is found in an altogether different fraction, namely, fraction C, where the unit membrane structure is clearly evident and, when negatively stained, is seen to be covered with *ca.* 60 A particles, as also observed by Oda and Sato (20).

The third kind of evidence for the identification of the elements of fractions C and D is biochemical,

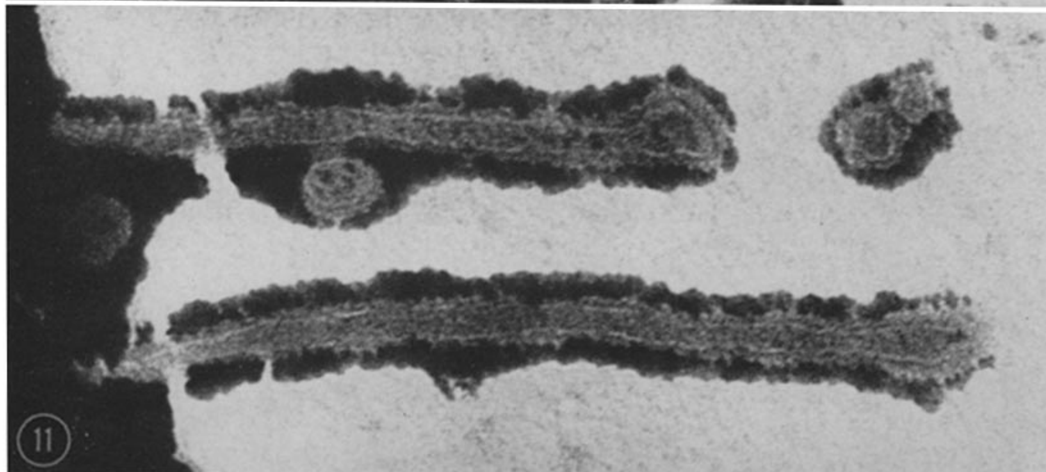

FIGURE 10 Cluster of compact rods negatively stained. In each rod a longitudinal alignment of particles is evident, particularly at points indicated by arrows. × 80,000.

FIGURE 11 Loosened rods showing a doubled strand with a densely staining central region. × 80,000.

as described in the preceding paper (7). Alkaline phosphatase has been localized in the brush border membrane by histochemical means (4), and the disaccharidases present in isolated brush borders have been shown to be external to the transport barrier for monosaccharides (6). Alkaline phosphatase and maltase activities are, as expected, found predominantly in fraction C.

In summary, the identification of the elements of fraction C as the membranous coats of the microvilli appears to be unequivocal, and, in view of this, the rods of fraction D, on the weight of the evidence cannot be other than the exposed cores of the microvilli. The rods cannot, for example, be intact but shrunken microvilli, for in such a case they should possess the enzymic activity of the microvilli. It is possible that their apparent structure has, to a greater or lesser extent, been altered by the procedures employed throughout their preparation for electron microscopy, but this need not be so; only further work with the isolated structures will provide an answer.

The morphological detail revealed here is of interest to consider in connection with some of the changes which microvilli undergo in the course of development. Studies of the intestinal brush border in the chick (23) and mouse (21) show that after hatching or birth and weaning the microvilli undergo a marked increase in length without change in volume. The resulting great increase in surface area is concurrent with increases in total alkaline phosphatase activity and in function. The conformation of the doubled strand in the microvillus core may possibly be an adaptation for this purpose (see reference 13); if the strand were coiled *in vivo*, loosening of the compact rod would increase its length. It would be of interest to know whether other sorts of cell surface extensions which have a fibrous core, such as microextensions from tissue culture cells (30) or the acrosomal filament of sperm (5), are similar to intestinal microvilli in the nature of the isolated core.

The particles associated with the surfaces of the coats and cores described here are of particular interest, since such particles have up to now been found only in association with enzymatically highly active surfaces such as the membranes of mitochondria (8, 25, 29). However, the particles in the microvillus fractions are preserved by formalin fixation, but surface-bound particles in mitochondria are reported as not preserved to any useful extent by this fixation (29).

Since there is ample biochemical evidence to suggest that the disaccharidases and the sugar transport system are intimately juxtaposed in the brush border (6), it is tempting to speculate on the possibility that the membrane surface particles visualized here are, in fact, subunits containing the digestive and absorptive components of the microvillus membrane in a fixed relationship which provides for efficient coupling between digestive hydrolysis and absorption. Further analysis of this membrane system may provide a model for understanding transport not only as it occurs in the intestine but also as it occurs in structurally similar surfaces (19) such as the kidney epithelium or the cortex of the oocyte.

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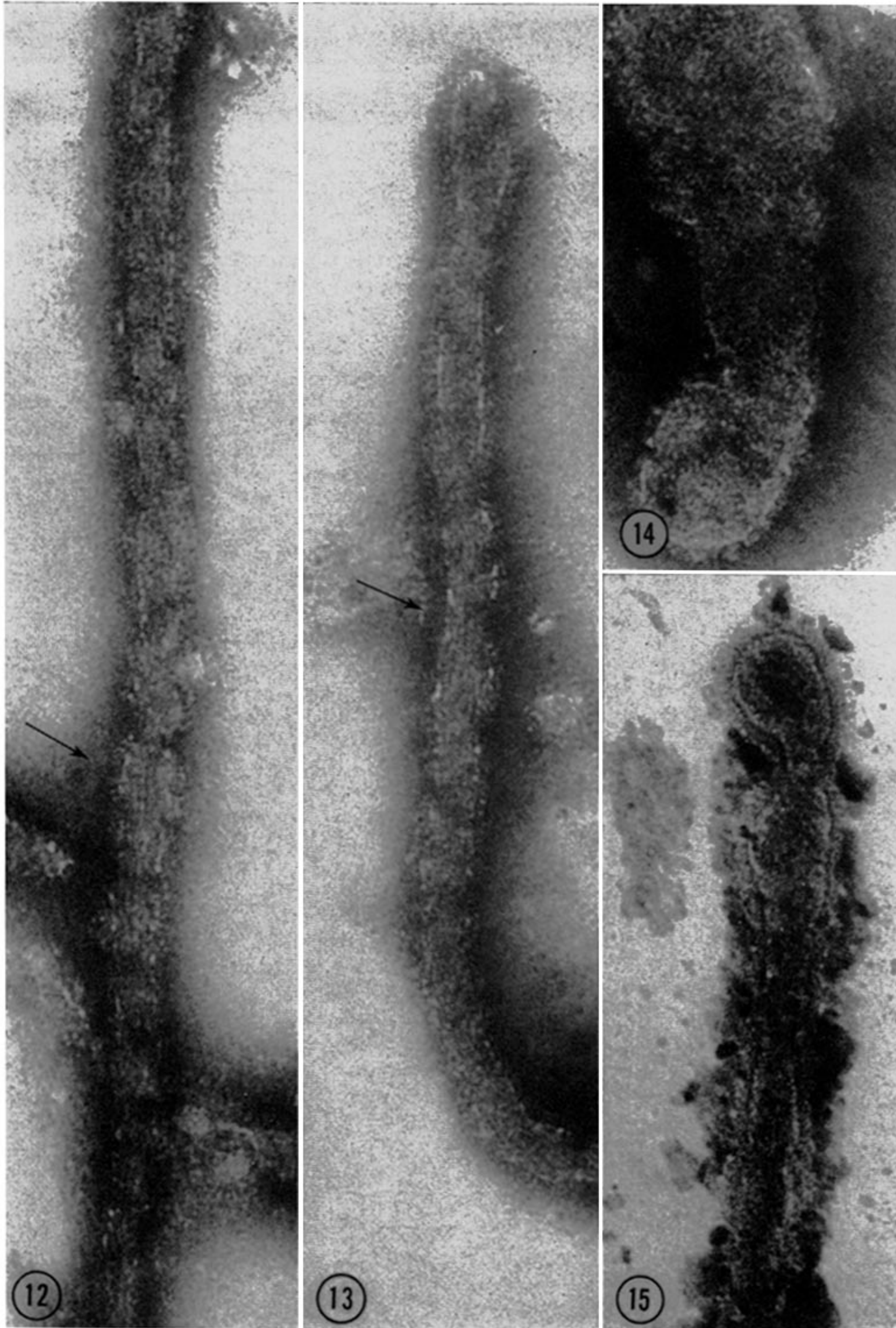


FIGURES 12 and 13 Negatively stained compact rods. The longitudinal alignment is particularly clear at points indicated by arrows. × 112,000.

FIGURE 14 Microvillus coat, negative stain. No alignment of particles is apparent. × 112,000.

FIGURE 15 Negatively stained loosened rod showing a double strand with a central dense region. × 112,000.

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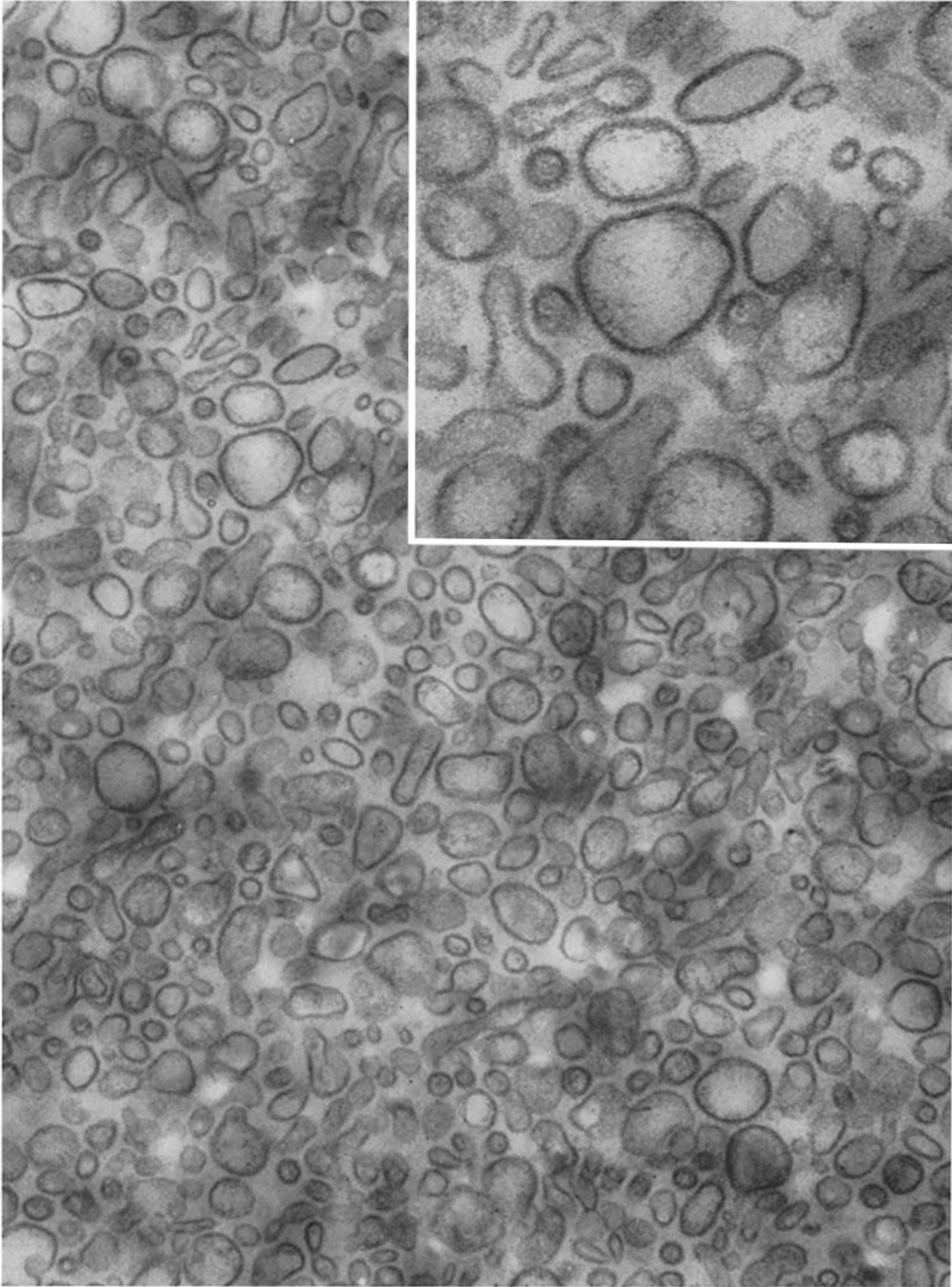
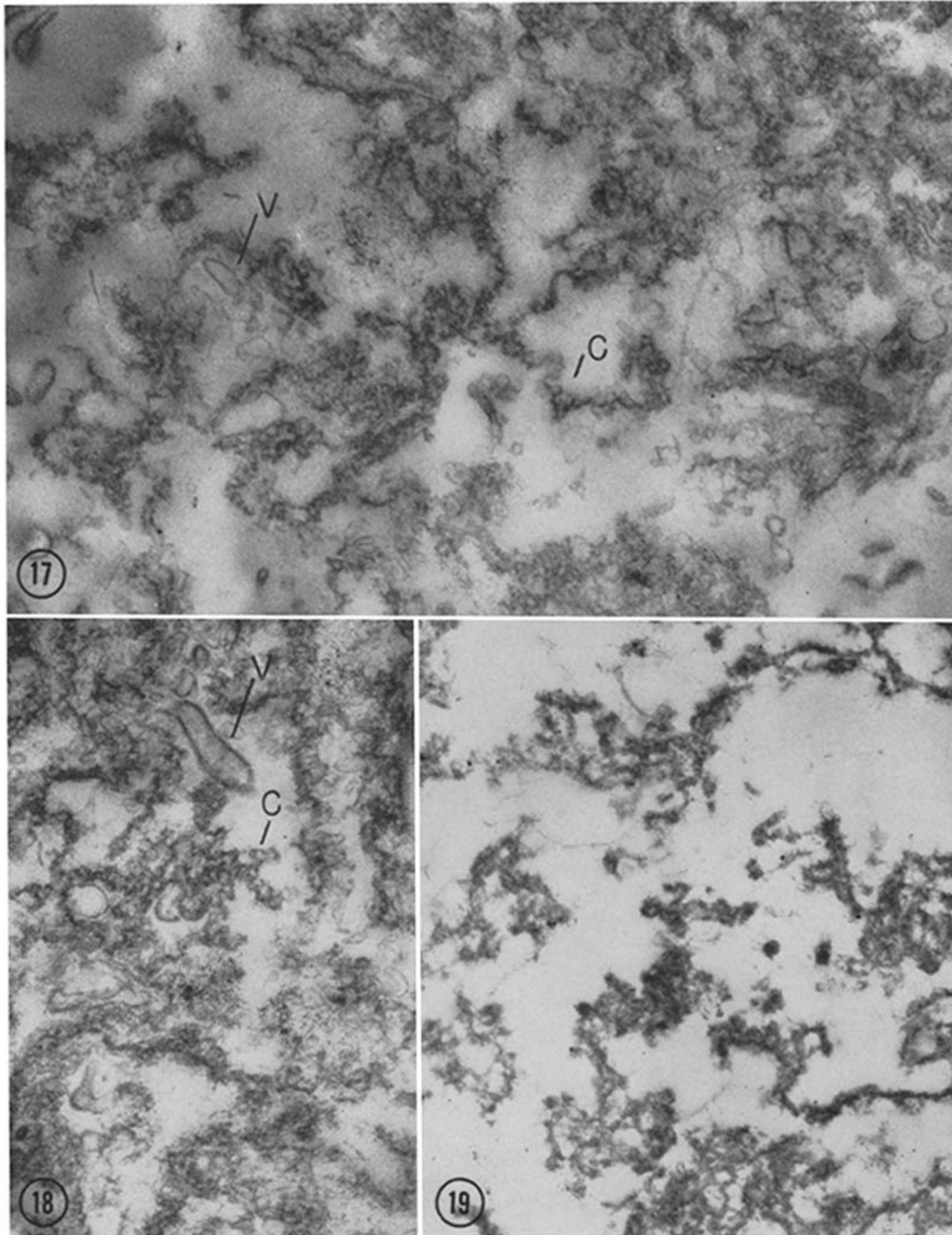


FIGURE 16 Sectioned pellet of fraction C (coats), osmium tetroxide fixation. This fraction appears to contain membranous vesicles exclusively, as seen in inset, glutaraldehyde fixation. × 32,000; inset, × 80,000.




FIGURES 17 and 18 Sectioned pellets of fraction D, glutaraldehyde fixation. *C*, fibrous strands; *V*, membranous vesicles. × 32,000.

FIGURE 19 Sectioned pellet of fraction D after deoxycholate treatment, glutaraldehyde fixation. Membranes are no longer evident. × 32,000.