Acta Medica Okayama

Volume 23, *Issue* 5 1969 *Article* 2

OCTOBER 1969

Molecular basis of structure and function of the microvillus membrane of intestinal epithelial cells

Takuzo Oda* Shuji Seki† Sekiko Watanabe‡

*Okayama University,
†Okayama University,
‡Okayama University,

Copyright ©1999 OKAYAMA UNIVERSITY MEDICAL SCHOOL. All rights reserved.

Molecular basis of structure and function of the microvillus membrane of intestinal epithelial cells*

Takuzo Oda, Shuji Seki, and Sekiko Watanabe

Abstract

Correlation of molecular structure with biochemical functions of the plasma membrane of the microvilli of intestinal epithelial cells has been investigated by biochemical and electron microscopic procedures. Repeating particles, measuring approximately 60 Åin diameter, were found on the surface of the microvilli membrane which had been isolated or purified from rabbit intestinal epithelial cells and negatively stained with phosphotungstic acid. These particles were proved to be inherent components of the microvillus membrane, attached to the outer surface of its trilaminar structure, and were designated as the elementary particles of the microvilli of intestinal epithelial cells. Biochemical and electron microscopic identification of these elementary particles has been carried out by isolation of the elementary particles with papain from the isolated microvillus membrane, followed by purification of the particles by chromatographies on DEAE-cellulose and Sephadex columns. The partially purified particles containing invertase and leucine aminopeptidase are similar in size and structure to those of the elementary particles in the microvillus membrane. Evidence indicates that each of the elementary particles coincide with or include an enzyme molecule such as disaccharidase or peptidase, which carry out the terminal hydrolytic digestion of carbohydrates and proteins, respectively, on the surface of the microvillus membrane. Magnesium ionactivated adenosine triphosphatase and alkaline phosphatase cannot be solubilized with papain but remains in the smooth-surface membrane after the elementary particles have been removed. Cytochemical electron microscopic observation revealed that the active site of magnesium ion-activated adenosine triphosphatase is localized predominantly in the inner surface of the trilaminar structure of the microvillus membrane.

*PMID: 4244045 [PubMed - indexed for MEDLINE] Copyright ©OKAYAMA UNIVERSITY MEDICAL SCHOOL

Acta Med. Okayama **23**, 357—376 (1969)

MOLECULAR BASIS OF STRUCTURE AND FUNCTION OF THE MICROVILLUS MEMBRANE OF INTESTINAL EPITHELIAL CELLS

Takuzo ODA, Shuji SEKI, and Sekiko WATANABE

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. T. Oda)

Received for publication, July 10, 1969

Available evidence suggests that the microvilli of intestinal epithelial cells carry out two principal functions: one is the terminal hydrolytic digestion of carbohydrates and proteins by the action of disaccharidases, peptidases, and some other enzymes; and the other is the absorption, including active transport, of various ions and molecules liberated by enzymic digestion, such as certain monosaccharides, amino acids, and others (1—12). The intestinal epithelial microvilli are unique not only for the former function, but also are the prototype for the latter function in all biological membrane systems. These principal functions of the microvilli have been assumed to be closely correlated with and to be carried out by the enzymes or enzyme systems arranged on or in the microvilli. It is the purpose of the present investigation to correlate these functions with the molecular structure of the membrane of the microvilli. Preliminary reports on this work have already been published (4—7).

MATERIALS AND METHODS

Materials

Microvilli of small intestinal epithelial cells from rabbits were mainly used, but in some experiments those of rat, mouse and human origins also served as materials.

Isolation of Microvilli (Brush Border)

For the isolation of microvillus border the method of MILLER and CRANE (1961) (2) was used with a slight modification. Male adult rabbits (1~5 animals/group) were fasted for 1~2 days, killed by bleeding after stunning by a blow on the head, and the small intestine was taken out. The following procedure was carried out at 1~4°C. The intestine was everted and washed several times with an ice cold washing solution containing 140 mM NaCl and 4 mM KCl, pH 7.4. The mucosa was stripped off on the edge of a slide glass, placed in an ice cold homogenizing solution containing 5 mM ethylenediamine tetraacetate (EDTA), pH 7.4, about one liter for one animal, and then homogenized gently in a teflon

Produced by The Berkeley Electronic Press, 1969

homogenizer at 1,000 rpm for 2 minutes. The homogenate was filtered through a double layer of tetoron (polyester synthetic fiber) cloth and the filtrate was centrifuged at 1,000 × g for 10 minutes. The sediment was collected, and filtration and centrifugation were repeated as above several times to recover the microvilli in the form of brush borders.

Purification of the Microvillus Membrane by Density Gradient Centrifugation After Disruption of the Isolated Brush Borders by Sonic Oscillation

The microvilli isolated in the form of brush borders were disrupted by sonic oscillation at 20 Kc for 5 minutes, and the separated microvillus membranes were isolated by density gradient centrifugation on 20, 40, 60, 80, 100 per cent glycerol layers at 160,000×g for 60 minutes in a Beckman L-2 ultracentrifuge using an SW 39 swinging rotor.

Isolation and Purification of Invertase and Leucine Aminopeptidase from the Isolated Microvilli

An aliquot of the isolated intestinal epithelial cell microvilli or separated microvillus membranes suspended in a 5 mM EDTA solution, pH 7.4, was diluted to twice the original volume so as to contain 10 mM potassium phosphate buffer, 150 mM KCl, 5 mM EDTA, and 2∼5 mg protein per ml in the final concentration. The sample was incubated either with trypsin or papain (0.2 mg/mg protein in each case) at 37°C for 30 or 60 minutes (13). After the incubation, the sample was immediately chilled in ice water and centrifuged at 160,000×g for 60 minutes in a No. 50 Rotor of the Beckman L-2 preparative ultracentrifuge at 4°C. The supernatant was decanted and the residue was suspended in one half the original volume of 5 mM EDTA, pH 7.4, centrifuged again for washing, and the supernatant decanted and the residue resuspended in a 5 mM EDTA solution, pH 7.4. The volume of each sample was measured and a small amount of each sample was drawn in each step for enzyme assay and calculation of recovery.

The collected supernatant was dialyzed against 1 mM potassium phosphate buffer, pH 6.8, concentrated, dialyzed again, and centrifuged at 160,000×g for 60 minutes. The supernatant was decanted, and fractionated by chromatography on a DEAE-cellulose column (14). The peak fractions for invertase activity were collected and further fractionated by Sephadex G-75, and G-200 column chromatographies (14).

Assay of Enzyme Activities

The activity of invertase was determined by the method of SUMNER (15—16) with sucrose as substrate, and the reducing sugar formed was assayed with 3,5-dinitrosalicilic acid; the enzyme activity was expressed in μ moles reducing sugar produced per mg protein per min. The activity of leucine aminopeptidase (strictly speaking leucyl naphthylamide hydrolase) was determined by the method of NACHLAS *et al.* (17) with some modifications. The azo compounds of β-naphthylamine, liberated with *L*-leucyl-β-naphthylamide hydrochloride as substrate, and Fast blue B was extracted with an ether-acetone solution (1 : 1). This extract was subjected to colorimetric determination at the maximum absorption wave length of 520 mμ. As for the activity of alkaline phosphatase the method of BESSEY and

LOWRY (18) was used ; the nitrophenol liberated with *p*-nitrophenylphosphate as substrate was determined colorimetrically at the maximum absorption wave length of 410 mμ. Magnesium ion-activated ATPase activity was determined by KIELLEY's method (19) with slight modifications; the liberated inorganic phosphate was measured by the method of MARTIN and DOTY (20).

Protein Determination

Protein concentration was determined by a biuret method (21) as well as spectrophotometrically by the absorption of light at 280 mμ and 260 mμ (22).

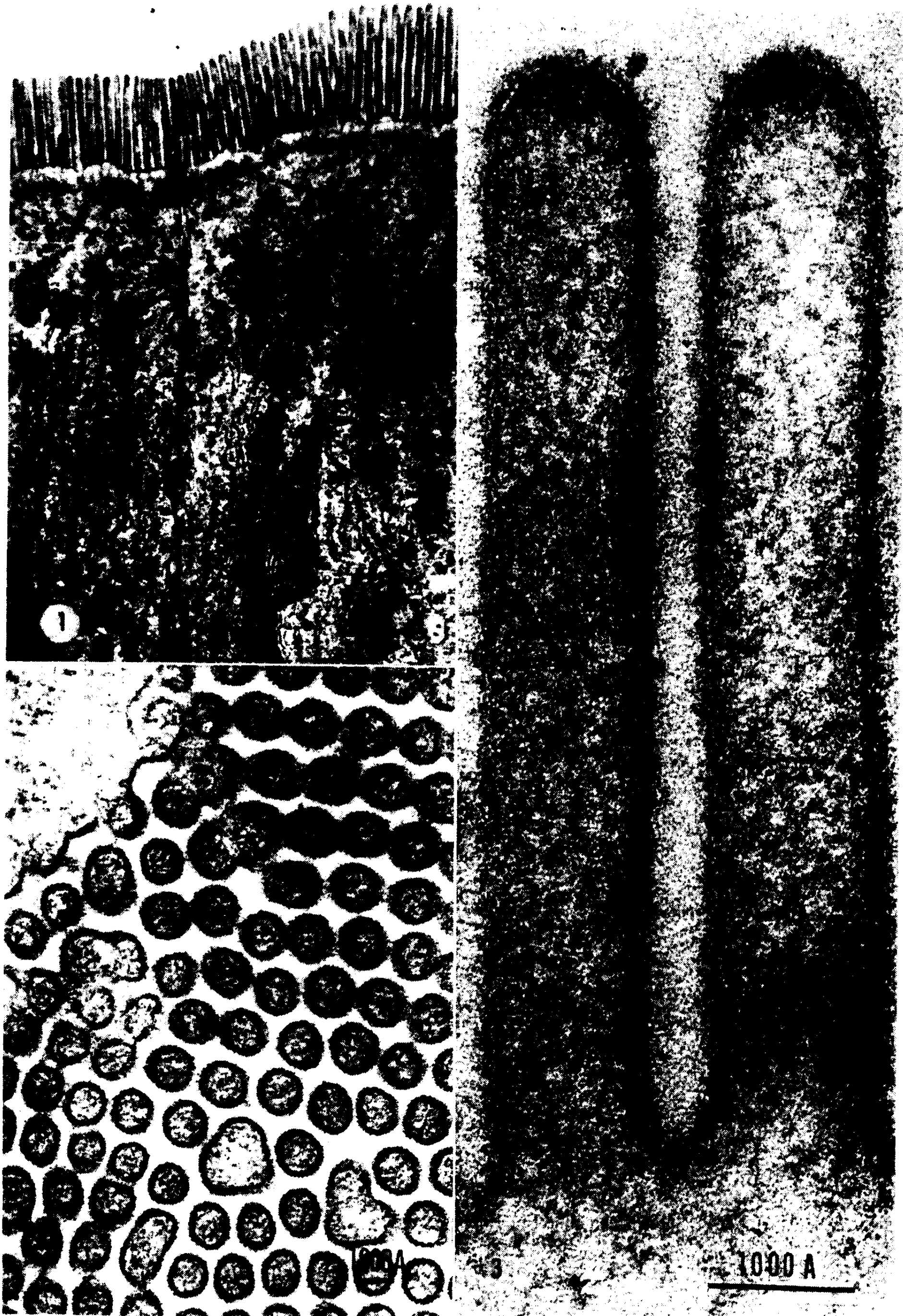
Electron Microscope Observation

Samples were fixed either in 2 % osmium tetroxide in 0.1 M phosphate buffer at pH 7.4 (23), or 2 % potassium permanganate (24), or 5 % glutaraldehyde and then 2 % osmium tetroxide (25), dehydrated with a graded series of alcohol, and embedded in Epon 812 (26). Ultrathin sections were stained with ethanolic uranyl acetate (27). For negative staining, 1 % phosphotungstic acid (PTA) adjusted to pH 7.0 with N KOH (28) or 0.5∼1 % uranyl acetate, pH 4.3, was used (29). Some of the samples were negatively stained by various procedures previously described (30—35), but the majority of samples were stained by either of the following simple procedures. (a) *Mixed dropping method*- A sample suspension was mixed in a 1 % PTA solution and dropped on a collodion coated specimen grid; excess fluid was removed with filter papers. (b) *Floating method*-A drop of a sample suspension was placed on a collodion-coated specimen grid, and excess fluid was removed with filter papers. The grid was then floated on the surface of a drop of 1 % PTA solution, so as to make the sample attach to the surface of the PTA solution. The grid was taken out with a droplet of PTA solution, and excess fluid was removed with filter papers. In both procedures, the specimen was immediately dried by shaking the grid in the air and by keeping it in a desiccator. The dried specimens were coated with a thin layer of carbon and examined in a HU-11 or JEM-7 electron microscope at 75 KV or 80 KV, respectively. The cytochemical-electron microscopic demonstration of ATPase was carried out by the method of WACHSTEIN-MEISEL (25, 36) except that Tris buffer was replaced with histidine buffer as previously described (37).

RESULTS

General Structure of the Microvilli

The mucosa of the small intestine is lined with a monolayer of epithelial cells, and the apical surface of the epithelial cells is composed of numerous tubular protrusions of plasma membrane (approximately 500∼1,000 Å in width and 1∼2 μ in length), which are known as microvilli (Figs. 1—3). The membrane of the microvilli presents a distinct trilaminar image, with so-called outer and inner leaflets (unit membrane), the total thickness of which is about 90∼110 Å (Fig. 3). The core of the microvilli contains many longitudinal microfibers (30∼50 in number), which appear


1
1000 A

to be the supporting fibers, and which communicate with the terminal web at the base. These structures, comprising the membranes and cores of the microvilli and the terminal webs, have been called the brush borders or microvillus borders. All of these structures are similar to those reported earlier (38—42).

Enzyme Activity of the Isolated Brush Borders

When the intestinal mucasa is homogenized in a 5 mM EDTA solution, the components other than the brush borders are practically all destroyed and they can readily be eliminated by centrifugation, leaving only the brush borders with their structural integrity. This was confirmed by the interference phase-contrast microscope (Fig. 4a) and by the electron microscope (Figs. 4b, 5).

In the assay, conducted at each step of this fractionation as well as with the brush border fractions, of invertase, leucine aminopiptidase, and alkaline phosphatase, it has been shown that the major part of these enzymes is localized in the brush border fraction. Furthermore, on the basis of the increase in the specific activity of these enzymes, it follows that most of these enzymes are localized in the microvilli (Table 1). Since the magnesium ion-activated ATPase is located in various membrane structures, the increase in activity during fractionation was not so high, but it was demonstrated that the highest concentration of this enzyme is found in the microvilli.

Table 1 Results of enzyme assay in each step of isolation of the epithelial microvilli from rabbit small intestinal mucosa

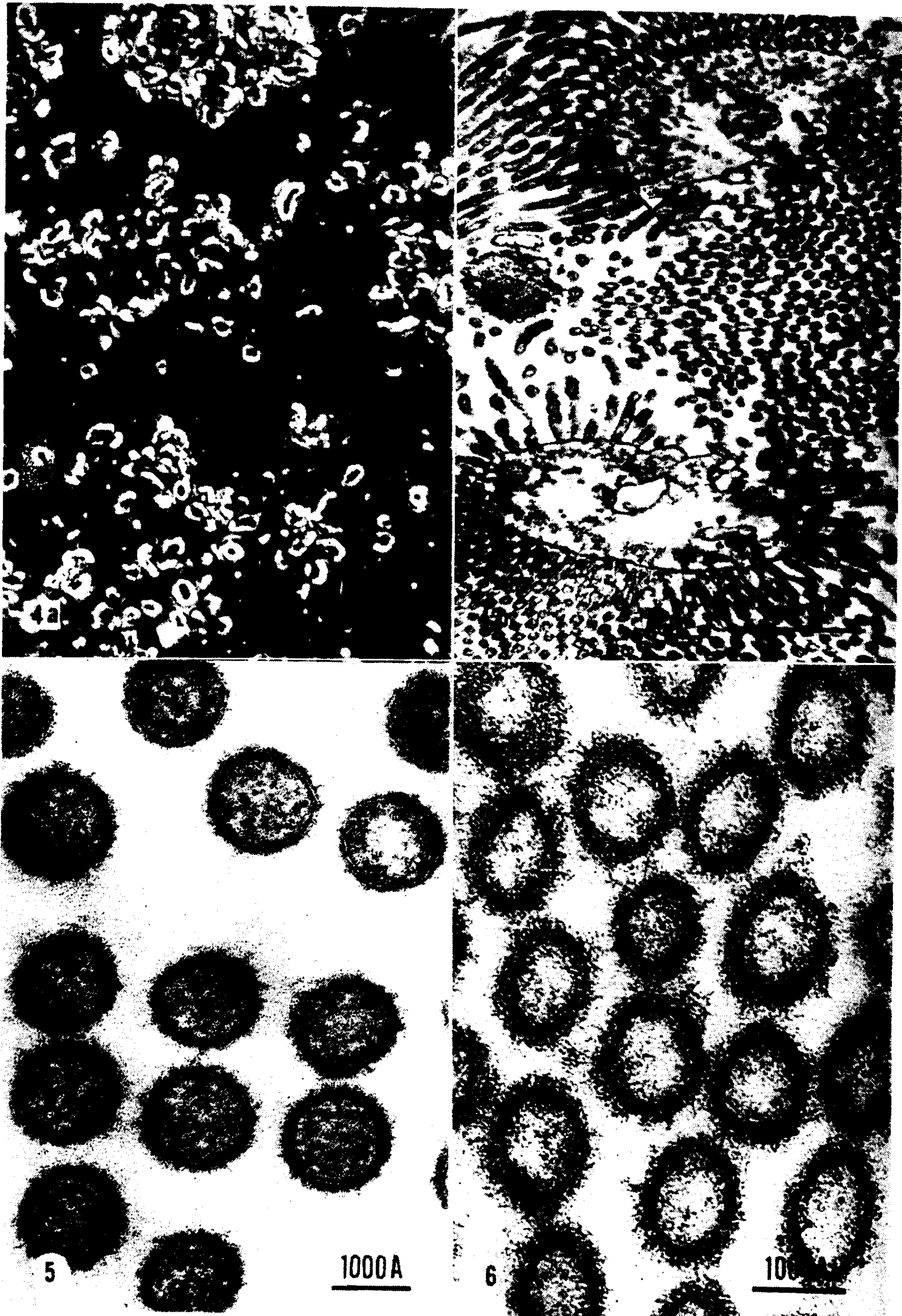
Fractions	Recovery of total protein(%)	Enzyme activity							
		Invertase		Leucine amino-peptidase		Alkaline phos-phatase		ATPase	
		TA*	SA**	TA	SA	TA	SA	TA	SA
Original	100	100	1.0	100	1.0	100	1.0	100	1.0
R-1	26	80	3.1	53	2.0	84	3.1	52	2.0
R-2	14	66	4.8	40	2.9	63	4.6	27	2.0
R-3	8.6	56	6.5	35	4.0	52	6.0	16	1.8

* TA: Recovery of total activity expressed in percentage.
** SA: Increasing rate of specific activity.

Figure 1 An electron micrograph of rat intestinal epithelial cells fixed with osmium tetroxide, showing microvilli on the apical surface of the cells. × 12,200.

Figure 2 A transverse section of the microvilli of the intestinal epithelial cells fixed with osmium tetroxide. × 55,400.

Figure 3 A magnified picture of the longitudinal section of the microvilli fixed with osmium tetroxide, showing asymmetrical trilaminar structure of the membrane. × 203,000.


5
1000A
6

Elementary Particles of the Microvilli

When the ultrathin sections of the isolated brush borders fixed in potassium permanganate and stained with uranylacetate were observed under the electron microscope at a high resolution, the microvillus membrane presents distinct outer and inner leaflet image, which has been considered as a unit membrane. On the external surface of the so-called outer leaflet, another array of particles could be observed (Fig. 6). In the specimens negatively stained with phosphotungstic acid distinct, neatly-arranged repeating particles of approximately 60 Å in diameter with the center-to-center distance of 60～80 Å were discovered on the surface of the microvillus membrane (Fig. 7). Since these particles remain attached to the microvillus membrane both in frozen and thawed samples and even in repeatedly-washed samples, and also on the basis of the data to be mentioned below, these were considered to be the proper components that perform the inherent functions of the microvillus membrane. Therefore, they were designated as "elementary particles" of the microvilli of intestinal epithelial cells. Judging from the size of individual particles each was assumed to be a single protein molecule.

Enzymes Localized in the Microvillus Membrane

When the isolated brush borders were disrupted by sonic oscillation and fractionated by glycerol density gradient centrifugation, the isolated microvillus membranes were collected mostly in 40—60 % glycerol layers. The elementary particles were still attached to the isolated microvillus membrane (Fig. 8), in which the activities of invertase, leucine amino-peptidase, alkaline phosphatase, and ATPase were concentrated (Table 2). Thus it has been confirmed that most of these enzymes are localized in the membrane of the microvilli.

Isolation of the Elementary Particles from the Isolated Brush Borders or Isolated Microvillus Membranes

An attempt was made to isolate these elementary particles from the microvillus membrane without destroying the membrane structure for the

---

Figure 4 An interference phase contrast micrograph (a) and an electron micrograph (b) of the brush borders isolated from rabbit intestinal epithelial cells. The specimen for electron microscopy was fixed with glutaraldehyde and osmium tetroxide, sectioned, and stained with uranyl acetate. The brush borders show morphological integrity of the basic structures, a, × 2,400 ; b, × 16,000.

Figure 5 A transverse section of the isolated microvilli, fixed with osmium tetroxide. × 108,000.

Figure 6 A transverse section of the isolated microvilli, fixed with potassium permanganate. × 133,000.

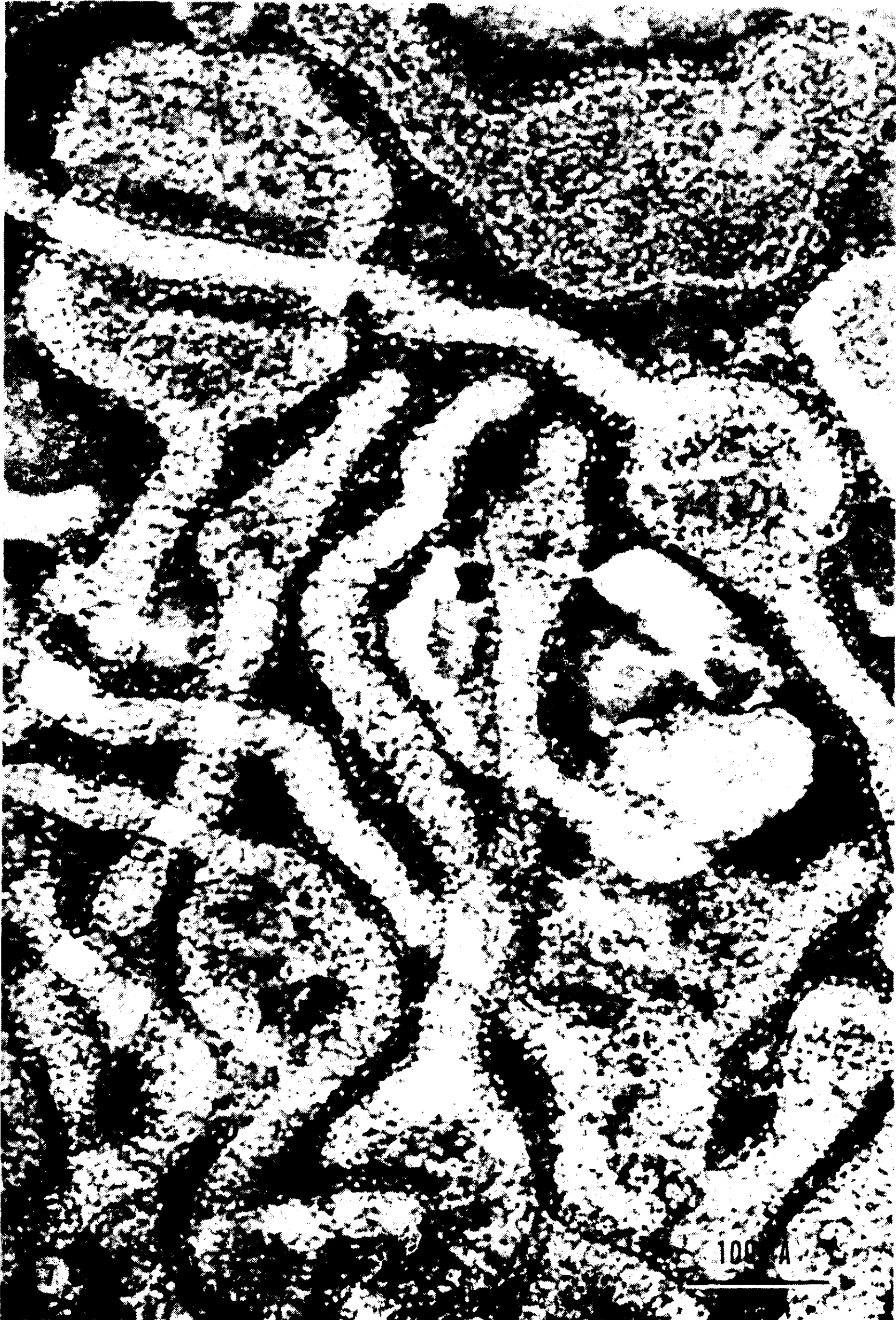


Table 2 Distribution of invertase in the fractions obtained by glycerol density gradient centrifugation after sonic disruption of the isolated microvillus borders

Samples	Total protein (mg)	Specific activity*	Total activity**	Increase in specific activity
Mucosal sheet	6970	0.092	640	1.0
Isolated microvilli sonicated	522	0.474	247	5.2
Layer of glycerol				
0 %	247	0.152	38	1.7
20 %	56	0.33	19	3.6
40 %	52	1.01	53	11.0
60 %	31	1.39	51	15.1
80 % upper	14	0.91	13	9.9
80 % and 100 %	32	0.63	17	6.8

* μ moles of reducing sugar formed per mg protein per min.
** μ moles of reducing suger formed per min.

purpose of enzymic identification of these particles. Treatment of the isolated brush borders or microvillus membranes with trypsin or chymotrypsin at 37°C for 60 minutes did not significantly solubilize any of invertase, leucine aminopeptidase, alkaline phosphatase, or ATPase from the microvillus membrane, but these enzymes were recovered in the insoluble membrane fraction (Table 3). Electron microscopy of the membrane

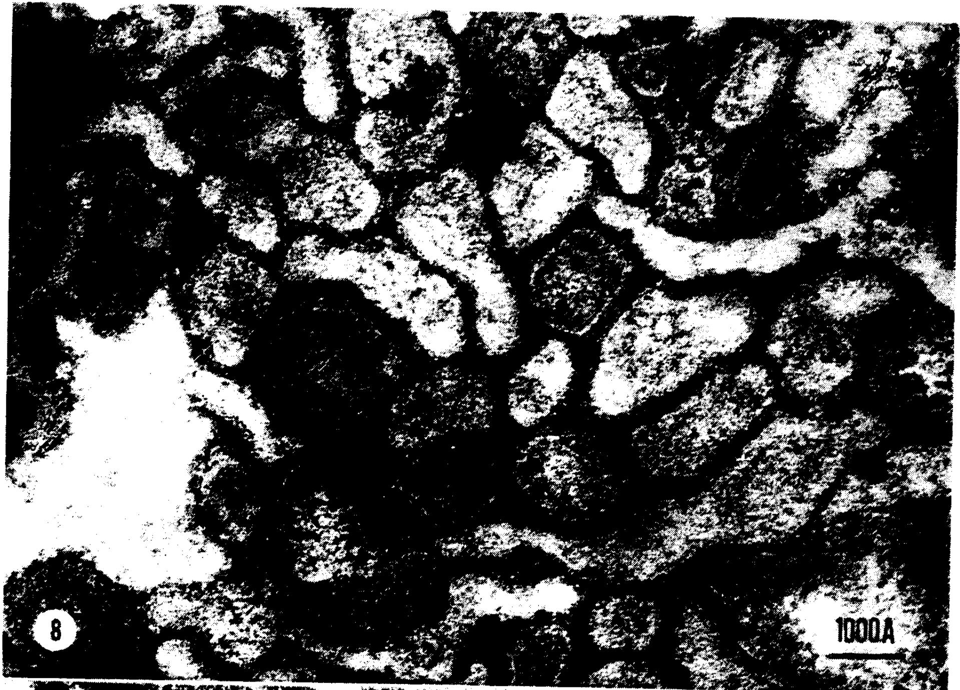
Table 3 Results of solubilization of enzymes from the isolated rabbit intestinal epithelial microvilli by treatment with trypsin*

Enzyme	Recovery of enzyme activity (percentage of original)		
	Soluble	Insoluble	Total
Invertase	0.5	90.0	90.5
Leucine aminopeptidase	16.0	84.0	100
Alkaline phosphatase	1.0	83.0	84.0
ATPase	0	77.0	77.0

* The microvilli were incubated with trypsin at 37°C for 60 min.

fraction confirmed that the elementary particles were still attached to the surface of the insoluble microvillus membrane (Fig. 9). On the other hand, treatment of the isolated brush borders or microvillus membranes with papain for 30—60 minutes solubilized practically all of the invertase and leucine aminopeptidase, which were recovered in the supernatant fraction

Figure 7 Isolated microvilli, showing the elementary particles on the surface of the membrane, negatively stained with phosphotungstate. × 200,000.


8
1000A

9
1000A

Table 4 Results of solubilization of enzymes from the isolated rabbit intestinal epithelial microvilli by treatment with papain*

Enzyme	Incubation time	Recovery of enzyme activity (percentage of original)		
		Soluble	Insoluble	Total
Invertase	30 min	68.6	9.4	78.0
	60 min	68.1	7.7	75.8
	60 min**	98.8	1.0	99.8
Leucine aminopeptidase	30 min	59.7	13.9	73.6
	60 min	68.6	12.5	81.1
Alkaline phosphatase	30 min	2.4	62.7	65.1
	60 min	3.5	60.5	64.0
ATPase	30 min	0.8	72.0	72.8
	60 min	0.5	58.0	58.5

* The microvilli were incubated with papain at 37°C for the period of time indicated.
** In this case, the microvillus membrane purified by density gradient centrifugation after sonication of isolated microvilli were incubated with papain.

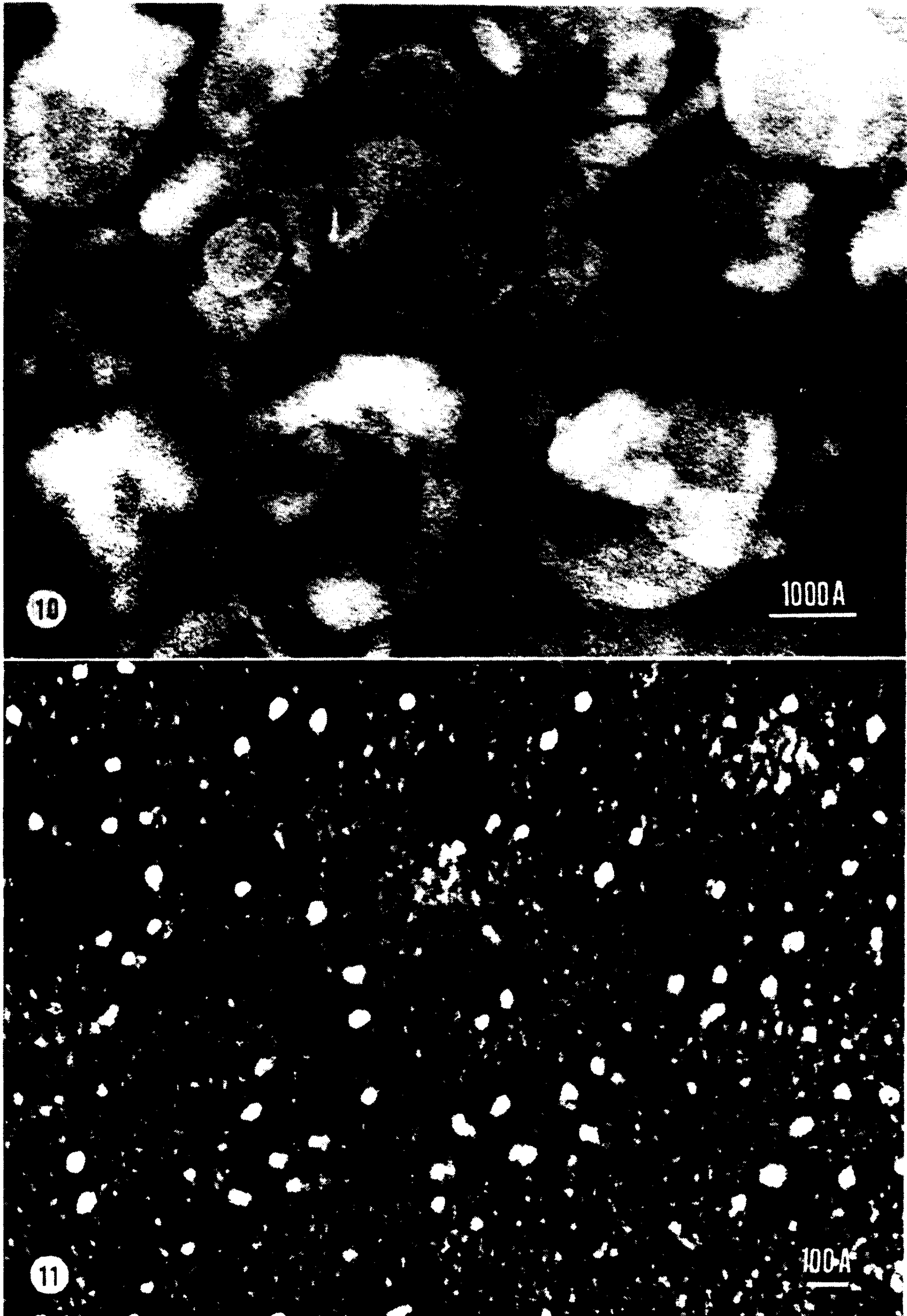
on centrifugation at 160,000×g for 60 minutes (Table 4). This soluble fraction contains particles of approximately 60 Å in size but no membrane structures. However, neither alkaline phosphatase nor ATPase could be solubilized in any significant amounts by the papain treatment. The majority of these enzyme activities were found to remain in the insoluble membranes, which sustained their unit membrane image, but from which the elementary particles had been detached, leaving a smooth surface membrane structure (Fig. 10). When the amount of papain was diminished, the rates of solubilization of invertase and of the detachment of the elementary particles from the microvillus membrane decreased proportionately.

Purification and Electron Microscopy of the Elementary Particles

As the supernatant fraction containing invertase and leucine aminopeptidase solubilized by papain treatment also contains papain and other crude proteins, the purification of the elementary particles was attempted. It was found that dialysis, condensation, and further dialysis procedures made about one half of the total crude protein insoluble, and these crude

Figure 8 Isolated microvillus membrane, obtained by glycerol density gradient centrifugation after sonication of the isolated microvilli, negatively stained with phosphotungstate. The elementary particles remain attached to the surface of the microvillus membrane. ×97,300.

Figure 9 Membrane fractions of the isolated microvilli after treatment with trypsin, negatively stained with phosphotungatate. The elementary particles still remain attached to the surface of the microvillus membrane. ×98,200.


10
1000 A
11
100 A

proteins could be eliminated by centrifugation. When the supernatant thus obtained was subjected to the step-wise purification for invertase through DEAE-cellulose (Fig. 12) and Sephadex G-75 (Fig. 13) and G-200

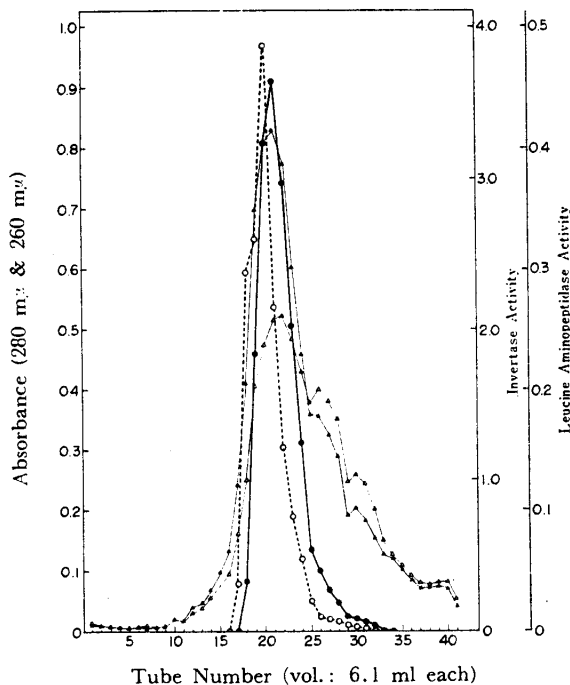


Figure 12 Elution pattern of solubilized invertase fraction on DEAE-cellulose column. Solid circles, invertase activity expressed as μ moles of reducing sugar formed per ml of eluates per min.; Open circles, leucine amino-peptidase activity experssed as μ moles of β-naphthylamine formed per ml of eluates per min.; Solid triangles and open triangles, absorbance at 280 mμ and 260 mμ, respectively.

(Fig. 14) column chromatographies, the specific activity of invertase increased with each step of purification and finally in the peak fraction reached 92 times (11.2 μ moles reducing sugar produced/mg protein/min) the original level (Table 5). This fraction contained also leucine amino-peptidase but no papain. The electron micrograph of this fraction negatively stained with uranyl acetate revealed protein molecules of appro-

---

Figure 10 Membrane fraction of the isolated microvilli after treatment with papain, negatively stained with phosphotungstate. The elementary particles are removed from the microvillus membrane and the membrane shows a particle free smooth surface. ×118,000.

Figure 11 Elementary particles isolated from the isolated microvilli and purified for invertase by chromatographies on DEAE-cellulose and Sephadex columns, negatively stained with uranyl acetate. ×527,000.

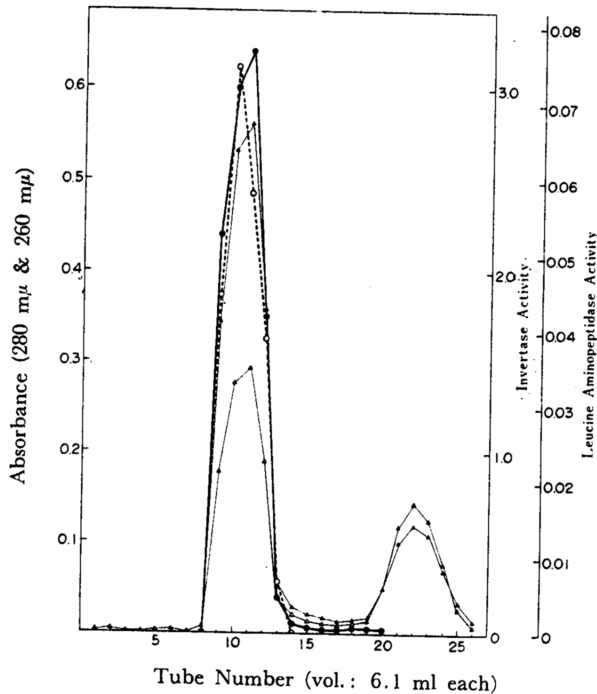


Figure 13 Elution pattern on Sephadex G-75 column of invertase fraction collected from the tube No. 20—23 on DEAE-cellulose column. Explanation of the figure is as same as that of Fig. 12.

Table 5 Results of purification of invertase from the microvilli of intestinal epithelial cells

Step	Total protein (mg)	Specific activity*	Total activity**	Recovery (%)	Increase in specific activity
Mucosal sheet	3120	0.122	384	100	1
R3 (Brush border fraction)	342	0.673	230	60	5.5
Papain treated supernatant	95	2.40	228	59	19.7
Concentration and dialysis	57.2	3.67	219	55	30.1
DEAE cellulose chromatography	19.7	4.70	92.6	24	38.6
Sephadex G-75	14.2	5.40	76.7	20	44.3
Sephadex G-200		8.21, (11.2)			67.0, (92.0)

* μ moles of reducing sugar formed per mg protein per min.

** μ moles of reducing sugar formed per min. The values in the parenthesis show those in a peak fraction tube.

ximately 60 Å in diameter (Fig. 11), that were similar in size and structure to the elementary particles attached to the surface of the microvillus membrane.

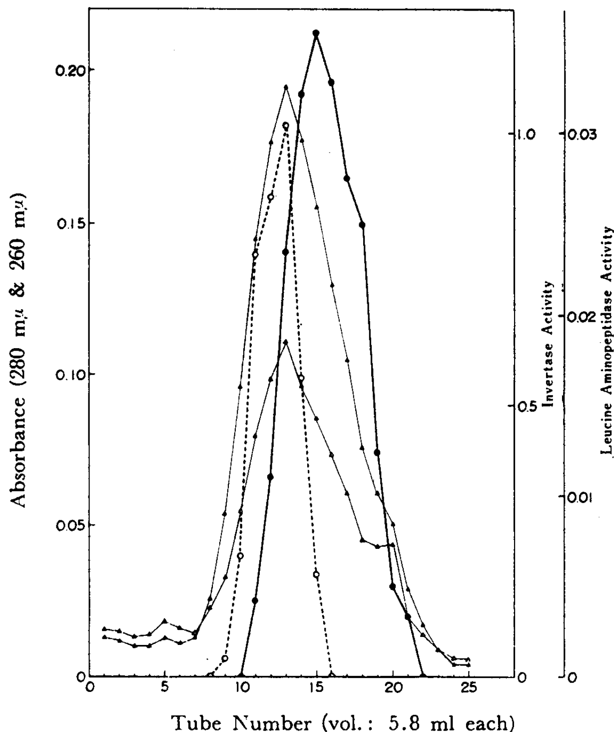


Figure 14 Elution pattern on Sephadex G-200 column of invertase fraction collected from the tube No. 9—12 on Sephadex G-75 column. Explanation of the figure is as same as that of Fig. 12.

Site of ATPase Localization in the Microvillus Membrane

On the basis of the findings relative to the isolation of the elementary particles it is obvious that magnesium ion-activated ATPase is not contained in the elementary particles but is localized in the unit membrane structure of the microvillus membrane. In the further observation of ATPase activity by an improved method of histochemical and electron microscopic demonstration procedures, it has been confirmed that this enzyme is localized in the unit membrane structure and that the active site of the enzyme faces the inner side of the microvilli (Fig. 15).

DISCUSSION

Since our preliminary report (4—7) on the finding of the repeating particles on the outer surface of the microvillus membrane isolated from intestinal epithelial cells it was also examined and verified by several other investigators (43—45). On the nature of these particles, judging from the

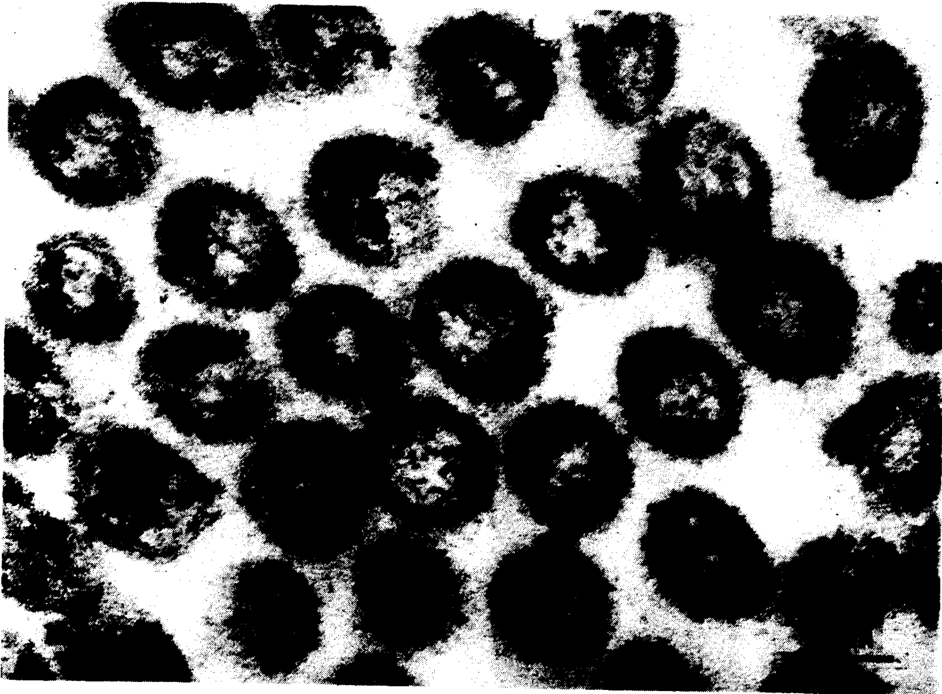


Figure 15 Cytochemical demonstration of magnesium ion activated adenosine triphosphatase with the electron microscope in the inner leaflet of trilaminar structure of the microvillus membrane. × 140, 000.

size of individual particles we have assumed that each particle corresponds to a single protein molecule. Among various kinds of enzymes to be localized in the microvillus membrane, we have examined invertase (sucrase) as a maker enzyme for the terminal hydrolytic digestion of carbohydrates in the microvillus membrane and leucine aminopeptidase as an example of enzyme for the terminal hydrolytic digestion of proteins. Data on the systematic and stepwise isolation of the microvillus membrane and quantitative solubilization of these enzymes with papain indicate that the elementary particles contain these enzymes.

Although invertase and leucine aminopeptidase did not clearly separated by the present column chromatography, it will be no doubt that these enzymes are different enzyme molecules and should be separated by the improvement of the method of chromatography, since the peaks of these enzyme fractions in the present chromatography are already partially separated. As invertase fraction contaminated with leucine aminopeptidase is active even in a highly diluted state, in which the sample for electron microscopic observation is prepared, each particle observed in the

electron micrograph is considered to correspond to each enzyme molecule such as invertase and leucine aminopeptidase. Possibility that the elementary particles also contain some other enzyme molecules can not be excluded.

The partially purified invertase fraction also presents maltase activity. As the correlation of maltase, isomaltase, and invertase (sucrase) are not fully understood in the present knowledge on the their specifity and structure we have just examined mainly invertase among various disaccharidases, and detailed characterization of disaccharidases have to await further study.

SUMMARY

Correlation of molecular structure with biochemical functions of the plasma membrane of the microvilli of intestinal epithelial cells has been investigated by biochemical and electron microscopic procedures. Repeating particles, measuring approximately 60 Å in diameter, were found on the surface of the microvilli membrane which had been isolated or purified from rabbit intestinal epithelial cells and negatively stained with phosphotungstic acid. These particles were proved to be inherent components of the microvillus membrane, attached to the outer surface of its trilaminar structure, and were designated as the elementary particles of the microvilli of intestinal epithelial cells. Biochemical and electron microscopic identification of these elementary particles has been carried out by isolation of the elementary particles with papain from the isolated microvillus membrane, followed by purification of the particles by chromatographies on DEAE-cellulose and Sephadex columns. The partially purified particles containing invertase and leucine aminopeptidase are similar in size and structure to those of the elementary particles in the microvillus membrane. Evidence indicates that each of the elementary particles coincide with or include an enzyme molecule such as disaccharidase or peptidase, which carry out the terminal hydrolytic digestion of carbohydrates and proteins, respectively, on the surface of the microvillus membrane. Magnesium ion-activated adenosine triphosphatase and alkaline phosphatase cannot be solubilized with papain but remains in the smooth-surface membrane after the elementary particles have been removed. Cytochemical electron microscopic observation revealed that the active site of magnesium ion-activated adenosine triphosphatase is localized predominantly in the inner surface of the trilaminar structure of the microvillus membrane.

ACKNOWLEDGEMENT

This investigation was supported by a PHS research grant GM 10538 from the National Institutes of Health, U. S. A., and a research grant GA MNS 63114 from the Rockefeller Foundation.

The authors are grateful for the advice of Prof. D. E. GREEN, Institute for Enzyme Research, University of Wisconsin, and Prof. R. SATO, Institute for Protein Research, Osaka University.

REFERENCES

1. MILLER, D., and CRANE, R. K.: The digestive function of the epithelium of the small intestine. I. An intracellular locus of disaccharide and sugar phosphate ester hydrolysis. *Biochim. Biophys. Acta* **52**, 281, 1961
2. MILLER, D., and CRANE, R. K.: The digestive function of the epithelium of the small intestine. II. Localization of disaccharide hydrolysis in the isolated brush border protein of intestinal epithelial cells. *Biochim. Biophys. Acta* **52**, 293, 1961
3. HOLT, J. H., and MILLER, D.: The localization of phosphomonoesterase and aminopeptidase in brush borders isolated from intestinal epithelial cells. *Biochim. Biophys. Acta* **58**, 239, 1962
4. ODA, T., and SATO, R.: Elementary particles of the microvilli of intestinal epithelial cells. Invited lecture at the Symp. of the 4th Ann. Meeting of the Amer. Soc. for Cell Biol., Cleveland, 1964
5. ODA, T., SEKI, S., TAKESUE, Y., and SATO, R.: Molecular structure and biochemical function of the microvilli of intestinal epithelial cells. Proc. of the 17th Symp. for Enzyme Chem., 389, 1965
6. ODA, T., and SEKI, S.: Molecular structure and biochemical function of the microvilli membrane of intestinal epithelial cells with special emphasis on the elementary particles. *J. Electron Microscopy* **14**, 210, 1965
7. ODA, T., and SEKI, S.: Molecular basis of structure and function of the plasma membrane of the microvilli of intestinal epithelial cells. Electron Microscopy, 6th International Congress for Electron Microscopy, Kyoto, 1966, edited by R. UEDA, Tokyo, Maruzen Co., Ltd, **2**, 387, 1966
8. SEMENZA, G., TOSI, R., VALLOTTON-DELACHAUX, M. C., and MÜLHAUPT, E.: Sodium activation of human intestinal sucrase and its possible significance in the enzymic organization of brush borders. *Biochim. Biophys. Acta* **89**, 109, 1964
9. UGOLEV, A. M., JESUITOVA, N. N., TIMOFEEVA, N. M., and FEDIUSHINA, I. N.: Location of hydrolysis of certain disaccharides and peptides in the small intestine. *Nature* **202**, 807, 1964
10. ALVARADO, F., and CRANE, R. K.: Studies on the mechanism of intestinal absorption of sugars. VII. Phenylglycoside transport and its possible relationship to phlorizin inhibition of the active transport of sugars by the small intestine. *Biochim. Biophys. Acta* **93** 116, 1964
11. EICHHOLZ, A., and CRANE, R. K.: Studies on the organization of the brush border in intestinal epithelial cells. I. Tris disruption of isolated hamster brush borders and density gradient separation of fractions. *J. Cell Biol.* **26**, 687, 1965
12. UGOLEV, A. M., JESUITOVA, N. N., and LEAY, P. DE.: Localization of invertase activity in small intestinal cells. *Nature* **203**, 879, 1964
13. AURICCHIO, S., DAHLQVIST, A., and SEMENZA, G.: Solubilization of the human intestinal disaccharidases. *Biochim. Biophys. Acta* **73**, 582, 1963

14. GITZELMANN, R., DAVIDSON, E. A., and OSINCHAK, J.: Disaccharidase of rabbit small intestine: intracellular distribution, solubilization, purification and specificity. *Biochim. Biophys. Acta* **85**, 69, 1964
15. SUMNER, J. B.: The estimation of sugar in diabetic urine, using dinitrosalicylic acid. *J. Biol. Chem.* **62**, 287, 1924—25
16. BERNFELD, P.: Amylases, α and β. In Method in Enzymology, edited by S. P. COLOWICK and N. O. KAPLAN: New York, Academic Press Inc., **1**, 149, 1955
17. NACHLAS, M. M., CRAWFORD, D. T., and SELIGMAN, A. M.: The histochemical demonstration of leucine aminopeptidase *J. Histochem. Cytochem.* **5**, 264, 1957
18. BESSEY, O. A., LOWRY, O. H., and BROCK, M. J.: A method for the rapid determination of alkaline phosphatase with fine cubic millimeters of serum. *J. Biol. Chem.* **164**, 321, 1946; In Method in Enzymology, edited by S. P. COLOWICK and N. O. KAPLAN, New York, Academic Fress Inc., **4**, 371, 1957
19. KIELLEY, W. W.: Mg-activated muscle ATPases, In Method in Enzymology, edited by S. P. COLOWICK and N. O. KAPLAN: New York, Academic Press Inc., **2**, 588, 1955
20. MARTIN, J. B., and DOTY, D. M.: Determination of inorganic phosphate modification of the isobutyl alcohol procedure. *Anal. Chem.* **21**, 965, 1949
21. GORNALL, A. G., BARDAWILL, C. J., and DAVID, M. M.: Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751, 1949
22. KALCKER, H. M.: Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. *J. Biol. Chem.* **167**, 461, 1947
23. MILLONIG, G.: Further observations on a phosphate buffer for osmium solutions in fixation. Electron Microscopy, 5th International Congress for Electron Microscopy. Academic Fress **2**, 8, 1962
24. LUFT, J. H.: A new fixative for electron microscopy. *J. Biophys. Biochem. Cytol.* **2**, 799, 1956
25. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J.: Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**, 19, 1963
26. LUFT, J. H.: Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**, 409, 1961
27. WATSON, M. L.: Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.* **4**, 475, 1958
28. BRENNER, S., and HORNE, R. W.: A negative staining method for high resolution electron microscopy of viruses. *Biochim. Biophys. Acta* **34** 103, 1959
29. VAN BRUGGEN, E. F. J., WIEBENGA, E. H., and GRUBER, M.: Negative-staining electron microscopy of proteins at pH values below their isoelectric points. Its application to hemocyanin. *Biochim. Biophys. Acta* **42**, 171, 1960
30. FERNANDEZ-MORAN, H., ODA, T., BLAIR, P. V., and GREEN, D. E.: A macromolecular repeating unit of mitochondrial structure and function. *J. Cell Biol.* **22**, 63, 1964
31. ODA, T., and NISHI, Y.: Fundamental structure and function of mitochondrial membrane. *J. Electron Microscopy* **12**, 290, 1963
32. ODA, T., and HORIO, T.: Electron microscope observation of chromatophore of *Rhodospirillum rubrum. Exptl. Cell Res.* **34**, 414, 1964
33. ODA, T., and HUZISIGE, H.: Macromolecular repeating particles in the chloroplast membrane. *Exptl. Cell Res.* **37**, 481, 1965
34. ODA, T., SEKI, S., and HAYASHI, H.: Analytical studies on the molecular structure of the mitochondrial membrane by selective extraction of the components of the electron transfer chain. *J. Electron Microscopy* **14**, 354, 1965

35. ODA, T., and SEKI, S.: Molecular organization of the energy transducing system in the mitochondrial membrane. Electron Microscopy, 6th International Congress for Electron Microscopy, Kyoto, 1966, edited by R. UEDA, Tokyo, Maruzen Co., Ltd, **2**, 369, 1966
36. WACHSTEIN, M., and MEISEL, E.: Histochemistry of hepatic phosphatases at a physiological pH with special reference to the demonstration of bile canaliculi. *Amer. J. Clin. Pathol.* **27**, 13, 1957
37. ODA, T.: Cytochemical electron microscopic demonstration of adenosine triphosphatase in unfixed mitochondria. *J. Electron Microscopy* **14**, 343, 1965
38. FARQUHAR, M., and PALADE, G. E.: Junctional complexes in various epithelia. *J. Cell Biol.* **17**, 375, 1963
39. YAMAMOTO, T.: On the thickness of the unit membrane *J. Cell Biol.* **17**, 413, 1963
40. MILLINGTON, P. F.: Comparison of the thickness of the lateral wall membrane and the microvillus membrane of intestinal epithelial cells from rat and mouse. *J. Cell Biol.* **20**, 514. 1964
41. OVERTON, J., and SHOUP, J.: Fine structure of cell surface specializations in the maturing duodenal mucosa in the chick. *J. Cell Biol.* **21**, 75, 1964
42. MCNABB, J. D., and SANDBORN, E.: Filaments in the microvillous border of intestinal cells. *J. Cell Biol.* **22**, 701, 1964
43. OVERTON, J., EICHHOLZ, A., and CRANE, R. K.: Studies on the organization of the brush border in intestinal epithelial cells. II. Fine structure of fractions of tris-disrupted hamster brush borders. *J. Cell Biol.* **26**, 693, 1965
44. JOHNSON, C. F.: Disaccharidase; localization in hamster intestine brush borders. *Science* **155**, 1670, 1967
45. EICHHOLZ, A.: Studies on the organization of the brush border in intestinal epithelial cells. V. Subfractionation of enzymatic activities of the microvillus membrane. *Biochim. Biophys. Acta.* **163**, 101, 1968