BBA 75 363

LOCALIZATION OF RABBIT INTESTINAL SUCRASE WITH FERRITIN–ANTIBODY CONJUGATES

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(Received July 3rd, 1969)

SUMMARY

Precipitating antisera to rabbit small intestinal sucrase were obtained from guinea pigs. Immunoglobulin G of such sera and of control sera were conjugated with horse spleen ferritin. Rabbit small-intestinal mucosa was reacted with these ferritin–antibody conjugates.

Specific ferritin-labeling of microvilli occurred at the luminal surface of the enterocytes. However, labeling was more pronounced after removal of the enteric surface coat by careful trypsin digestion. Ferritin cores were found at a minimal distance of 120 Å from microvillus membranes, indicating that the sucrase antigen was located at the membrane surface and did not project into the intermicrovillous space.

INTRODUCTION

Recognition of sucrase–isomaltase deficiency as the underlying genetic defect in sucrose–isomaltose intolerance has stirred much interest in this enzyme complex[1]. Early immunological studies using fluorescent antibodies to partially purified enzyme of rats pointed to the apical portion of enterocytes as the cellular site of the enzyme[2]. It has also been known for some time that after tissue fractionation procedures 75–80% of intestinal sucrase are recovered in a heavy fraction containing brush borders[3,4]. Functional evidence indicated close spacial arrangement of sucrase and of the active sugar transport system in the microvilli of intestinal epithelial cells[5,6]. Thus, sucrase was expected to reside either within or immediately adjacent to the surface of the microvillus membrane. In fact, with a suitable fractionation technique it has become possible to disrupt epithelial brush borders of hamster intestine into two main components of microvilli: cores and membranes, the latter containing the bulk of sucrase and of other enzymatic activities originally present in the brush borders[7–9].

Recently, precipitating noninhibitory antisera to isolated rabbit intestinal sucrase–isomaltase were prepared from guinea pigs[10]. In order to localize sucrase of epithelial cells with the use of the electron microscope, we have now reacted rabbit intestinal mucosa with antibodies purified from such sera and conjugated with fer-

Abbreviation: IgG, immunoglobulin G.

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ritin. Attempts to remove the enteric surface coat of microvilli, known to be remarkably resistant against various chemicals and enzymes[11,24], have now met with success. As will be shown, sucrase was demonstrated at the membrane of undisrupted microvilli.

METHODS

Isolation of rabbit intestinal sucrase–isomaltase

Rabbit small intestine was rinsed in cold saline, cut into 20–30-cm segments which were tied, filled with Krebs–Henseleit buffer (pH 7.5) containing 1 mM EDTA and 100 mg papain (twice crystallized, Sigma, St. Louis) and 100 mg L-cysteine per 100 ml, and placed in Krebs–Henseleit buffer at 37° for 45 min. The contents of the bags were then chilled and centrifuged at 100 000 × *g* for 90 min. The supernatants were concentrated with polyacrylamide P-200, dialyzed and chromatographed on Sephadex G-200 with 0.01 M potassium phosphate buffer (pH 6.8). Eluates were concentrated *in vacuo*, dialyzed against buffer and stored at −20° (refs. 10 and 12). Specific activity was 15 units/mg. Sucrase–isomaltase was homogeneous as judged by disc electrophoresis, immunodiffusion–precipitation, immunoelectrophoresis, and centrifugation in a linear mannitol gradient (5–20%, w/v) followed by tube precipitation, with guinea pig antiserum to rabbit small-intestinal sucrase[10].

Preparation of antisera

Adult guinea pigs were immunized by intracutaneous injections of an emulsion of equal parts of sucrase in saline and of Freund's complete (first injection) or incomplete adjuvant. Animals received 1 mg of sucrase each, in three injections spaced 18 and 17 days, and were bled by decapitation 1 week after the third injection. Sera were sterilized by filtration and kept on ice.

Purification of immunoglobulin G (IgG)

Globulins were precipitated from antisera and from control sera with $(NH_4)_2SO_4$ in three consecutive steps (0.3, 0.4 and 0.5 satn.) in the cold. The combined precipitates were dissolved in a small volume of water and dialyzed against cold 0.015 M potassium phosphate buffer (pH 8.0) for 90 min. After removal of a small precipitate by centrifugation at 3500 rev./min (4 min), the protein solution was applied to a 2.8 cm × 20 cm column of DEAE-cellulose and eluted with a discontinuous gradient of the same buffer (0.015–0.25 M)[13]. The first fraction containing most of the IgG was lyophilized, dissolved in a small volume of water, dialyzed against 0.015 M potassium phosphate buffer (pH 7.5) at 4° and stored at −20°.

Ferritin conjugation of immunoglobulin G

Specific and nonspecific IgG were reacted with ferritin separately. Horse spleen ferritin (Fluka, Buchs, Switzerland) was recrystallized[14] 8 times, dissolved in 0.05 M potassium phosphate buffer (pH 7.5), sterilized by filtration and kept at 4°. Antibody–ferritin conjugates were prepared according to SRI RAM *et al.*[15]. Prior to conjugation, ferritin was centrifuged at 165 000 × *g* for 2.5 h in order to remove apoferritin. Approx. 920 mg of ferritin and 320 mg of IgG, brought to a 2% protein concentration in 2%

sodium carbonate buffer (pH 10.7), were reacted with 10 mg of *p,p'*-difluoro-*m,m'*-dinitrodiphenylsulfone (Eastman Organic Chemicals, Rochester, N.Y.) dissolved in 1 ml of cold acetone. The mixture was stirred for 24 h at 2–4°. A precipitate was removed at 3500 rev./min and the supernatant dialyzed against 0.015 M potassium phosphate buffer (pH 7.4). Another small precipitate was removed and the supernatant centrifuged at 165 000 × *g* for 2.5 h. The precipitate was dissolved in a small volume of buffer, and centrifugation was repeated twice in order to remove most of the unreacted IgG. The final precipitate was suspended in buffer and filtered through a 2.5 cm × 35 cm column of Sepharose 4B (Pharmacia, Uppsala) with buffered saline in the cold. Early and late eluting fractions were discarded. The main ferritin fraction still contained unconjugated IgG as revealed by immunodiffusion and immunoelectrophoresis against rabbit antiserum to guinea pig γ-globulin (Hyland, Los Angeles, Calif.), and against rabbit antiserum to horse spleen ferritin. It was concentrated by negative pressure dialysis and centrifuged in a sucrose gradient (30–60%, w/v; Spinco SW 25.1 swinging bucket rotor, 63 000 × *g* for 15 h). The ferritin-containing fraction was collected, dialyzed against buffer, concentrated by negative pressure dialysis, sterilized by filtration and kept at 4°.

Immunoreaction of small intestinal mucosa

Small intestine of adult rabbits was excised rapidly, rinsed in cold saline, dissected into 5 mm × 5 mm pieces which were prefixed in cold 5% formalin in 0.015 M potassium phosphate buffer (pH 7.2) for 30 min, and washed thoroughly in cold Krebs–Henseleit buffer (pH 7.4). Mucosa fragments containing one to three villi were blotted slightly and reacted with ferritin–antibody conjugates in 0.01 M potassium phosphate buffer (pH 7.2) at room temperature for 30 min. After immunoreaction, the fragments were rinsed extensively with large volumes of cold Krebs–Henseleit buffer for approx. 1 h, postfixed in 0.05 M sodium cacodylate buffer (pH 7.2) containing 3% glutaraldehyde and 3% acrolein at 0–2° for 30 min and washed in sodium cacodylate buffer containing 0.18 M sucrose for 90 min.

In one series of experiments, after prefixation, specimens were washed and exposed to various concentrations (0.1–0.001%) of trypsin (Fluka, from beef pancreas trypsinogen b, twice crystallized) in 0.08 M sodium phosphate buffer (pH 7.0) for 7–18 min at 37°, washed thoroughly in cold Krebs–Henseleit buffer and further processed as indicated above.

Preparation of specimens for electron microscopy

Specimens were then postfixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2) for 30 min at 0–2°, rinsed with water, kept in 2% uranyl acetate overnight, dehydrated and embedded in Epon–Araldite[16]. Thin sections were stained with lead citrate[17] and examined with a Siemens Elmiskop I or a Philips EM 200.

Immunodiffusion methods

Double diffusion was carried out in 1.0% agar, immunoelectrophoresis in 1.5% agar (sodium barbiturate buffer, ionic strength 0.1, pH 8.6). Precipitation lines were stained with amido black for proteins or by the prussian blue reaction (equal volumes of 2% $K_4Fe(CN)_6$ and of 2% HCl) for ferritin.

RESULTS AND DISCUSSION

The ferritin–antibody conjugates

Upon immunoelectrophoresis, sera of immunized guinea pigs contained precipitins only in the IgG fraction. When IgG was purified and examined by a tube-precipitation technique[10], it had retained its precipitating capacity and was non-inhibitory. Ferritin–IgG conjugates used for immunoreaction with small intestinal mucosa of rabbits contained free ferritin but were free of unconjugated antibody; they reacted with anti-horse spleen ferritin sera from rabbits as well as with anti-guinea pig γ-globulin serum from sheep. As was expected[14], the electrophoretic mobility of ferritin-conjugated globulins was more cathodal than that of ferritin but more anodal than that of IgG. When tested by immunoelectrophoresis and by double diffusion and tube-precipitation technique, the precipitating capacity of conjugates against rabbit sucrase was markedly reduced. Ferritin–antibody conjugates from non-immunized guinea pigs were comparable to those from immunized animals except for their lack of immunoreactivity with rabbit sucrase[18].

Immunoreaction of rabbit intestinal mucosa with ferritin–antibody conjugates

Sucrase activity of pieces of small intestine was measured *in situ* and found unimpaired by prefixation of specimens in 5% buffered formalin for 30 min. After prefixation and immunoreaction, electron microscopic examination of mucosa fragments revealed that the cells though not ideally fixed from the morphological viewpoint, were unbroken and that the microvillus membranes were intact. The enteric surface coat* was prominent, but its thickness varied between individual cells and from specimen to specimen. Ferritin was apposed or imbedded into the surface coat and appeared as single molecules or in clusters (Figs. 1 and 2). Minimal distance of the edges of the ferritin cores from the microvillus membrane was 120 Å. Usually, concentration of ferritin was highest at the apical portions of microvilli and appeared thinned out towards their base. In unfixed specimens, ferritin was distributed throughout the apical tenth of epithelial cells, but at a much lesser concentration than that at the microvillous surface. Concentration of ferritin on the cell surfaces was independent of the thickness of the surface coat.

Specificity of ferritin-labeling of mucosal cells was demonstrated. When fragments were reacted after the ferritin–antibody conjugates had been preabsorbed with suitable concentrations of rabbit sucrase, no labeling of enterocytes was observed, and the intensity of labeling increased as the concentrations of sucrase used during preabsorption was decreased. No labeling occurred when fragments were reacted with horse spleen ferritin alone or with ferritin conjugates of antibody obtained from non-immunized animals. However, an attempt at preventing labeling of enterocyte surfaces by prereacting fragments with purified IgG from immunized animals failed; it was speculated that this failure was due to either insufficient concentrations of IgG with regard to the antigen at the cell surface, or to shortness of exposure of fragments to IgG, or possibly to a combination of unfavorable circumstances.

* The term "enteric surface coat" is used in this paper for the part of the enterocyte glycocalyx covering the microvilli (see ref. 24).

Occasionally, neighboring enterocytes would differ widely in the degree of ferritin labeling. Fig. 3 shows such an example. As can be seen, four adjoining cells reacted with markedly different amounts of ferritin–antibody conjugates. It was speculated that these cells were observed at various stages of cell surface differentiation. The "dark" cells[19] were more heavily labeled than were the lighter ones and may have been at a later stage of differentiation.

Thus, it was evident that anti-rabbit sucrase IgG bound to ferritin recognized an antigenic site at the epithelial cell surface. However, the question remained whether sucrase was localized in or at the membrane of microvilli or resided within the enteric surface coat. Therefore, we decided to attempt the removal of the coat prior to immunoreaction of mucosa fragments.

Effect of removal of enteric surface coat on the immunoreaction

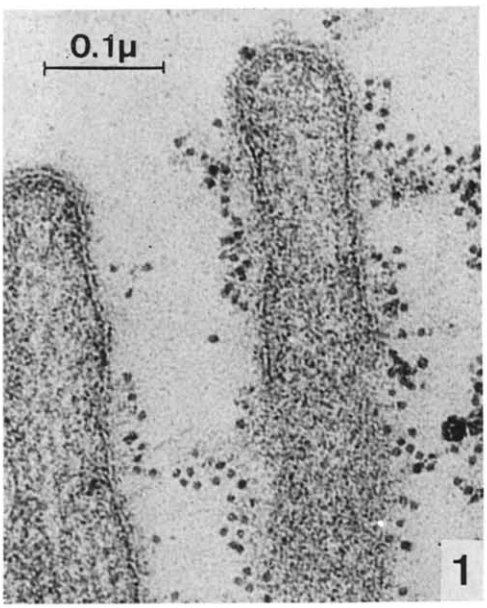
Resistance of the surface coat of absorbing cells to mucolytic agents and to proteolytic enzymes was stressed by FAWCETT[11,24]; cell coating was reported resistant as long as the integrity of brush borders was preserved. Our attempts to remove the surface coat prior to formalin prefixation by using different concentrations of papain (this enzyme is known to solubilize rabbit sucrase and was used in the isolation procedure) and of trypsin, which does not solubilize sucrase[6], again confirmed such resistance: as long as *in vitro* digestion conditions were mild enough to preserve brush borders, the surface coat remained in place; when the concentration of proteolytic enzymes was raised and the digestion time prolonged, epithelial cells would desintegrate. At this point it occurred to us that prefixation of specimens with formalin prior to proteolytic digestion might help preserve cell integrity and possibly render the cell coating more susceptible to proteolysis. In fact, prefixation in 5% buffered formalin at 0–2° for 30 min followed by digestion in 0.001% trypsin at 37° for 7–8 min left the epithelium of mucosa fragments and enterocytes reasonably well preserved; the microvillus membranes were left intact, but the surface coat was removed. Furthermore, such treatment did not solubilize or inactivate sucrase.

Incubation with ferritin–antibody conjugates of fragments treated in this way caused more prominent and more consistent labeling of enterocyte surfaces (Fig. 4). Not only was the number of ferritin particles at the microvilli higher, but ferritin was frequently found to be in regular arrangements along considerable portions of the microvillus membranes (Figs. 5, 6 and 7). Minimal distance of the edges of the ferritin cores from the microvillus membranes was again 120 Å, and a much greater proportion of ferritin molecules were observed at minimal distance from the membrane than had been seen in undigested specimens.

Fig. 1. Longitudinal thin section of microvilli after immunoreaction with ferritin–antibody conjugates. × 160000.

Fig. 2. Microvilli after immunoreaction in cross section. Note presence of surface coat. × 160000.

Fig. 3. Apical portions of four neighboring enterocytes after immunoreaction. Note differences in degree of ferritin labeling. × 70000.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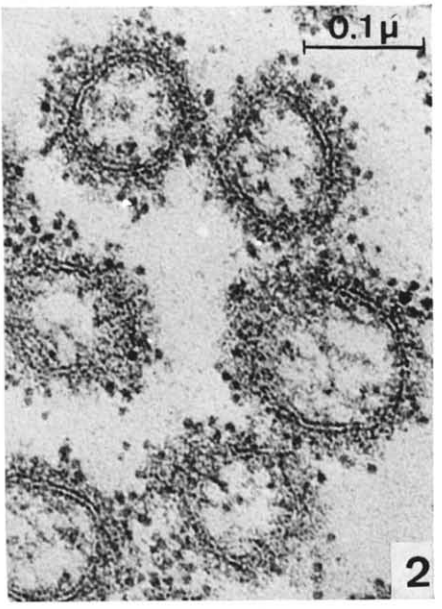
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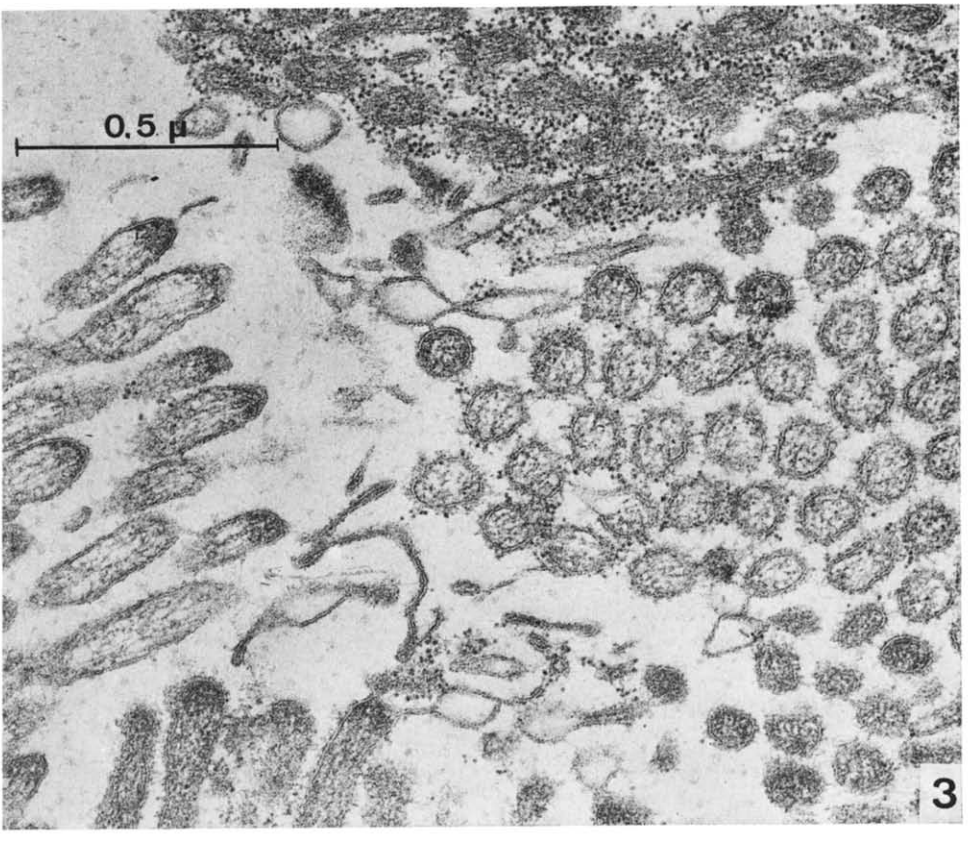
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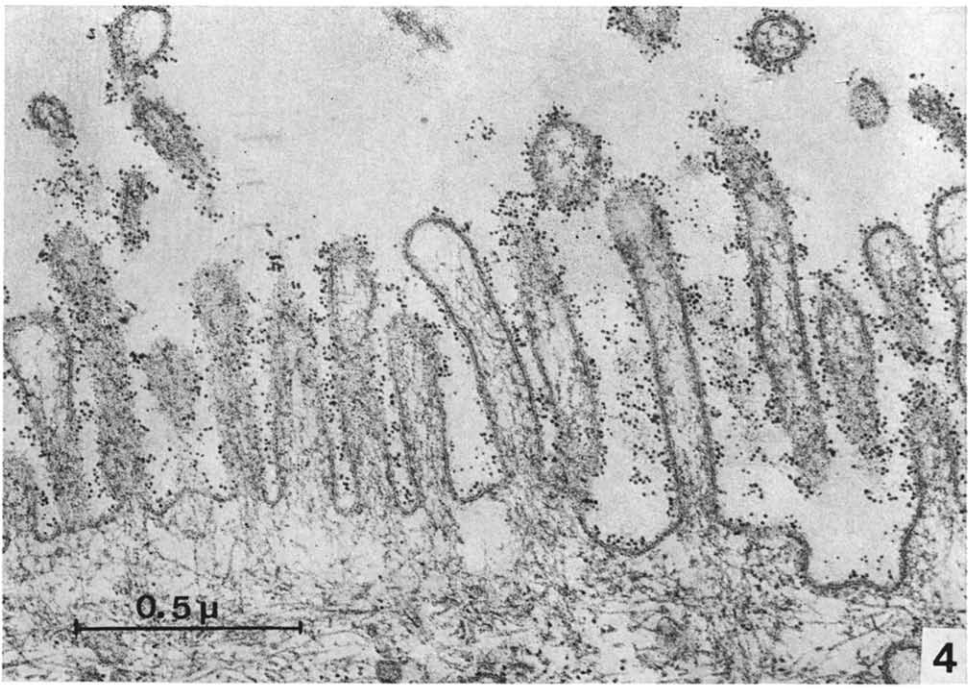
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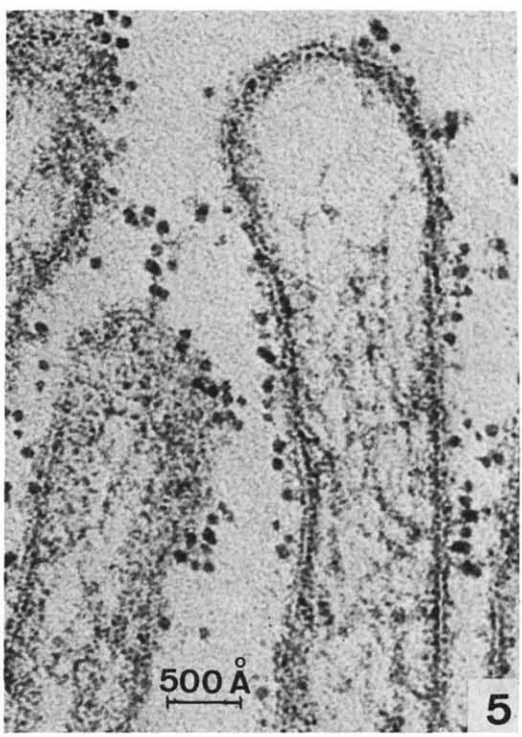
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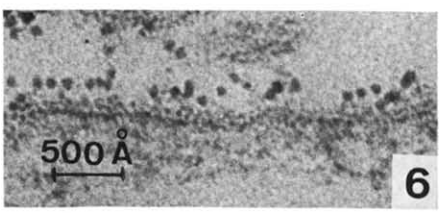
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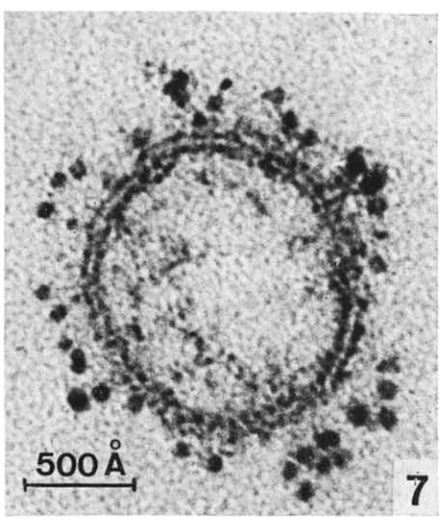
500 Å
7

CONCLUSIONS

It can be concluded that sucrase was not located in the enteric surface coat since after removal of the coat, (a) labeling was more prominent rather than weaker, and (b) many more ferritin cores were regularly arranged at the minimum distance of 120 Å along the brush border membrane.

The same conclusion is reached when the minimum ferritin to membrane distance is considered. Ferritin is a spherical protein, 120 Å in diameter, of which only the 50 Å iron core is visible on electron micrographs but not the 35 Å protein coat[14]. In both the trypsinized and the untreated preparations, the minimum distance between the ferritin cores and the outer leaflet of the brush border membranes measured 120 Å. The true distance could thus be calculated as 85 Å. Since IgG is an elongated ellipsoid of 240 Å × 50 Å (ref. 20), the ferritin to membrane space would just accommodate one IgG molecule reacted with its antigen. Thus, the antigen—the sucrase-isomaltase complex—must have been located at or within the brush border membrane. (Thereoretically, if attachment of the ferritin molecule to the antibody were close to the antibody combining site, the iron core could be expected as close as 35 Å to the membrane; it is reasonable, however, to assume that in this instance ferritin would sterically hinder the specific binding of the antibody to its antigen.)

If, on the other hand, sucrase had been apposed to the microvillus membrane and thus was projecting into the intermicrovillous space, the minimal distance of 120 Å might have been attained occasionally by single ferritin molecules situated between invisible membrane projections containing sucrase and arranged at considerable intervals. The fact, however, that unbroken rows of ferritin molecules at minimal distance were frequently visible over considerable portions, *i.e.*, several hundreds of Å of membrane surface (Figs. 5 and 6) precluded the view that sucrase was contained in projecting particles ("knobs") arranged in close vicinity of each other. Such an arrangement had been postulated from electron micrographs of isolated microvilli obtained with the negative staining technique by JOHNSON[21]. When he subjected hamster microvilli to this procedure, numerous projections measuring 60 Å studded the surfaces of the microvilli. After papain treatment of microvilli, no particles became visible, but sucrase and plasma membranes could be recovered separately by centrifugation; negative staining of the sucrase fraction again produced globular particles of about the same size. Interestingly, purified rabbit sucrase when negatively stained by NISHI *et al.*[22] appeared to consist of doughnut-shaped 110-Å structures and of 45 Å × 65 Å subunits. Similar structures were seen by these workers on the surface

Fig. 4. Longitudinal thin section of a brush border region. Trypsin treatment preceded immunoreaction. Ferritin molecules are seen at close distance from the microvillus membranes. Molecules apparently situated in the intermicrovillus spaces were adsorbed onto tangentially sectioned neighboring microvilli. × 60000.

Fig. 5. Detail from Fig. 4. Note absence of surface coat. × 200000.

Fig. 6. Detail of a thin-sectioned microvillus membrane studded with ferritin molecules. Specimen was treated with trypsin. × 180000.

Fig. 7. Cross section through a microvillus after trypsin treatment and immunoreaction. × 300000

of microvillous fragments and only few of them were visible after papain treatment of the specimens. Up to date, no such structures have been reported in fixed and thin-sectioned microvilli, and no attempts seem to have been made to demonstrate membrane-bound particles by the negative staining procedure after fixation of the fresh material. The fact that in negatively stained preparations no particles were seen after papain digestion does not prove that papain had removed naturally formed sucrase-containing particles from the membrane surface. Papain could have removed proteins from within the membranes thus preventing the appearance of an artifact during the subsequent negative staining procedure, *i.e.*, a similar "sweating out" of proteins as that invoked by SJÖSTRAND *et al.*[23] for mitochondrial membranes undergoing the negative staining process.

From the described arrangement of ferritin molecules in our own fixed and trypsin-treated specimens the conclusion seemed justified that sucrase was in or near level with the membrane at the microvillus surface, *i.e.*, that it formed an integral part of the outermost layer of the cell membrane.

ACKNOWLEDGMENT

Supported by Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung (Grants 5074.3 and 5193.3).

REFERENCES

1 A. PRADER AND S. AURICCHIO, *Ann. Rev. Med.*, 16 (1965) 345.
2 R. G. DOELL, G. ROSEN AND N. KRETCHMER, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1268.
3 D. MILLER AND R. K. CRANE, *Biochim. Biophys. Acta*, 52 (1961) 293.
4 R. GITZELMANN, E. A. DAVIDSON AND J. OSINCHAK, *Biochim. Biophys. Acta*, 85 (1964) 69.
5 R. K. CRANE, *Ann. Rev. Med.*, 19 (1968) 57.
6 G. SEMENZA, in C. F. CODE, *Handbook of Physiology—Alimentary Canal V*, Waverly Press, Baltimore, 1968, p. 2543.
7 J. OVERTON, A. EICHHOLZ AND R. K. CRANE, *J. Cell Biol.*, 26 (1965) 693.
8 A. EICHHOLZ, *Federation Proc.*, 28 (1969) 30.
9 G. G. FORSTNER, S. M. SABESIN AND K. J. ISSELBACHER, *Biochem. J.*, 106 (1968) 381.
10 D. L. CUMMINS, R. GITZELMANN, J. LINDENMANN AND G. SEMENZA, *Biochim. Biophys. Acta*, 160 (1968) 396.
11 D. W. FAWCETT, *J. Histochem. Cytochem.*, 13 (1965) 75.
12 J. KOLÍNSKÁ AND G. SEMENZA, *Biochim. Biophys. Acta*, 146 (1967) 181.
13 J. L. FAHEY, in C. A. WILLIAMS AND M. W. CHASE, *Methods in Immunology and Immunochemistry*, Vol. 1, Academic Press, New York–London, 1967, p. 321.
14 K. C. HSU, in C. A. WILLIAMS AND M. W. CHASE, *Methods in Immunology and Immunochemistry*, Vol. 1, Academic Press, New York–London, 1967, p. 397.
15 J. SRI RAM, S. S. TAWDE, G. B. PIERCE, Jr. AND A. R. MIDGLEY, *J. Cell Biol.*, 17 (1963) 673.
16 H. H. MOLLENHAUER, *Stain Technol.*, 39 (1964) 111.
17 E. S. REYNOLDS, *J. Cell Biol.*, 17 (1963) 208.
18 H. BINZ, *Pathol. Microbiol.*, in the press.
19 M. SHINER, *Modern Problems Pediat.*, 11 (1968) 5.
20 B. D. DAVIS, R. DULBECCO, H. N. EISEN, H. S. GINSBERG AND W. B. WOOD, Jr., *Principles of Microbiology and Immunology*, Harper and Row and Wheatherhill, New York–Tokyo, 1968, p. 418.
21 C. F. JOHNSON, *Federation Proc.*, 28 (1969) 26.
22 Y. NISHI, T. O. YOSHIDA AND Y. TAKESUE, *J. Mol. Biol.*, 37 (1968) 441.
23 F. S. SJÖSTRAND, E. ANDERSSON CEDERGREN AND U. KARLSSON, *Nature*, 202 (1964) 1075.
24 S. ITO, *Federation Proc.*, 28 (1969) 12.