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Topographical Studies on Intestinal Microvillous Leucine β-Naphthylamidase on the Outer Membrane Surface

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Summary. The location of leucine β-naphthylamidase on the outer surface of the microvillous membrane of rabbit small intestine was examined by analyzing the interaction of antibodies against leucine β-naphthylamidase or another microvillous enzyme, sucrase-isomaltase complex, with microvillous vesicles having different relative amounts of these enzymes, in respect to vesicle agglutination, inhibition of enzyme activity, and electron-microscopic morphology. The results obtained indicate that leucine β-naphthylamidase, or at least its antigenic sites, protrude about 10 nm from the outer surface of the microvillous membrane.

The microvillous membrane of small-intestinal mucosal cells contains a number of hydrolases, e.g., sucrase-isomaltase complex and leucine β-naphthylamidase (LNAase), which have been implicated in absorption as well as in the final step of digestion (Hagihira, 1964; Semenza, 1968; Ugolev, 1972). Sucrase-isomaltase complex and LNAase are located close to each other on the outer membrane surface (Takesue, Yoshida, Akaza & Nishi, 1973; Takesue & Nishi, 1976). Based on the immunoelectron microscopic observations obtained using nonlabeled antibodies against sucrase-isomaltase complex, we have concluded that the complex protrudes about 15 nm from the outer membrane surface, having its hydrophobic portion buried in the membrane (Nishi & Takesue, 1975). Like sucrase-isomaltase complex (Sigrist, Ronner & Semenza, 1975; Nishi & Takesue, 1977), LNAase consists of a hydrophilic and enzymatically active part and a smaller hydrophobic part acting as the membrane anchor of this enzyme (Maroux & Louvard, 1976); the latter part spans the membrane and is exposed on the inner surface (Louvard, Semeriv &

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Maroux, 1976). LNAase has at least 80% of its antigenic determinants accessible on the external face of microvillous vesicles (Louvard, Maroux & Desnuelle, 1975; Louvard *et al.*, 1976). The same results have been obtained with the renal LNAase (Vannier, Louvard, Maroux & Desnuelle, 1976). These results, together with our data (Takesue & Nishi, 1976), indicate the localization of LNAase from the outer surface of the microvillous membrane. In the present work we have investigated how distant the LNAase molecule protrudes from the outer membrane surface using an immunological technique combined with electron microscopy.

Materials and Methods

Microvillous vesicles of rabbit small intestines were purified from microsomes by centrifugation on a sucrose density gradient (Takesue *et al.*, 1973) or from isolated brush borders as follows: Brush borders isolated by the EDTA method (Takesue & Sato, 1968) were suspended in 0.01 M sodium phosphate (pH 7.0), sonicated at 10 kc and a power of 125 W for 2 min at 4 °C in a sonifier (Kubota Seisakusho, Ltd., model KMS-250), made 0.01 M in $MgCl_2$, and stored at 4 °C overnight. The resulting precipitates were removed by centrifugation at $10{,}000 \times g$ for 60 min, and the supernatant was further centrifuged at $78{,}000 \times g$ for 60 min to yield microvillous vesicles, which were suspended in phosphate-buffered saline. A number of vesicle preparations differing in the LNAase:sucrase (L:S) ratio, which will be defined below, were obtained from different rabbits over a period of about one year. Variation in the L:S ratio was possibly caused by uncontrolled feeding conditions, but not by the preparation methods used.

Preparations of immunoglobulin G against sucrase-isomaltase complex (anti-SI IgG) and LNAase (anti-L IgG) were the same as described previously (Takesue *et al.*, 1973; Takesue, 1975). In experiments such as in this work it is important to use specific antibodies which are directed toward antigenic sites widely distributed over the protein surface. The specificity for the respective antigens of the IgG used has been confirmed (Yoshida, Akaza, Nishi & Takesue, 1968; Takesue *et al.*, 1973; Takesue, 1975; Takesue & Nishi, 1976). The molar ratio of the IgG to the respective antigen in an immunoprecipitate is calculated to be at least 4 and 6 for sucrase-isomaltase complex and LNAase, respectively, from the curves obtained in the quantitative precipitation experiment (*see* Fig. 2 of Takesue *et al.*, 1973, and Fig. 13 of Takesue, 1975). Electron microscope observations have demonstrated that monovalent (Fab) fragments from anti-SI IgG are bound to different sites widely distributed over the Triton-solubilized sucrase-isomaltase molecule, except for the presumed hydrophobic part (Nishi & Takesue, 1977). Although such electron microscope observation has not been done with the anti-L IgG-LNAase system, it is not unreasonable to assume that anti-L IgG molecules also widely cover the LNAase molecule surface, judging from its molar ratio in the immunoprecipitate (*see also* Louvard *et al.*, 1976).

Sucrase and LNAase assays and protein determination were performed as described previously (Takesue & Nishi, 1976). One unit of enzyme activity was defined as the quantity of enzyme hydrolyzing 1 μmole of substrate per min under the conditions used. Vesicle agglutination induced by antibodies was followed as described previously (Ta-

kesue & Nishi, 1976). In brief, microvillous vesicles were incubated with various amounts of antibodies at 37 °C for 60 min and then at 4 °C for 20 hr or 3 days, after which agglutinated vesicles were precipitated by low-speed centrifugation. The whole mixtures and supernatants were assayed for sucrase and LNAase. The extent of vesicle agglutination was calculated by the following equation:

$$\% \text{ extent of agglutination} = \frac{a-x}{a} \times 100$$

where a is the activity in the supernatant without antibodies added and x is the activity in supernatant with antibodies added. Since LNAase was inhibited by anti-L IgG, its activity in supernatants was corrected for the inhibition (Takesue, 1975).

Microvillous vesicles, incubated or not with antibodies, were collected by centrifugation and divided into two portions, one for thin sections and the other for negative staining. For thin sections, vesicles in pellet were fixed for 60 min at 4 °C in 2.5% glutaraldehyde-2% paraformaldehyde in 0.05 M phosphate (pH 7.4), postfixed for 2 hr at 4 °C in 1% osmium tetroxide (pH 7.4), dehydrated in graded ethanols, and embedded in Epon 812 (Luft, 1961). Sections approximately 60 nm thick were cut with an ultramicrotome (LKB Ultrotome type 4801-A). The sections were doubly stained with aqueous uranyl acetate and lead citrate (Reynolds, 1963; Sato, 1968). For negative staining, precipitated vesicles were suspended in 0.4% sucrose, at a protein concentration suited for electron microscopic observation, and processed as described previously (Nishi & Takesue, 1977). The specimens were examined in an electron microscope (Hitachi HU-11B) equipped with an anticontamination device, operating at 75 kV and at a direct magnification of 50,000. The protrusion of electron-dense structures from the outer membrane surface was measured on the region of vesicles where the trilaminar membrane structure was clearly made visible. The minimum distance between two vesicles linked by antibodies was measured on the regions where their electron-dense structures were in contact with each other as well as where their trilaminar membrane structure was clearly seen. These measurements were performed on prints magnified four times with a scale magnifier of 0.1-mm subdivision. The protrusion was measured on 50 different regions, but not the distance between the paired vesicles on 30 different regions because such regions could not be frequently observed.

Leucine β-naphthylamidase as substrate for LNAase was purchased from Nutritional Biochemical Corp.; osmium tetroxide from E. Merck, Darmstadt; glutaraldehyde from TAAB Laboratories; Epon 812 from Shell Chemical Co.

As described in *Results*, vesicle agglutination induced by anti-L IgG varied greatly with the vesicle preparation. In order to explore any possible relationship between this variation in agglutination and the amount of LNAase contained in the membrane, we have plotted the extent of agglutination against the L:S ratio, not against the specific activity of LNAase, of the vesicle preparation used; here, the L:S ratio of a vesicle or a vesicle preparation is defined as the specific activity ratio of LNAase to sucrase of the vesicle or preparation. At present we cannot determine the density, i.e., the number of molecules per unit area of membrane, of sucrase or LNAase of the microvillous membrane, especially because microvillous vesicles are more or less filled with filamentous material (Louvard *et al.*, 1973; Nishi & Takesue, *unpublished data*) which would contribute variably to the protein content of the vesicles. On the other hand, we can estimate their relative densities from the specific activities of the vesicles, because the specific activities and the molecular weights of purified sucrase-isomaltase complex and LNAase are known (Takesue, 1969; Takesue, 1975). For example, when sucrase-isomaltase complex and LNAase are present in the same number on a vesicle, its L:S ratio is calculated to be about 1.3.

Results

Microvillous vesicles with the L:S ratio of 1.62 were quantitatively agglutinated by an adequate amount of anti-L IgG during 20-hr incubation at 4 °C (Fig. 1 *a*). However, agglutination of vesicles with a much lower L:S ratio of 0.23 was not completed even after a 3-day incubation (Fig. 1 *b*). As seen from Fig. 1, the extent of vesicle agglutination was almost the same, whether it was determined by the LNAase or sucrase assay method, though the difference between the two determinations seemed to be a little more with the vesicle preparation having a lower L:S ratio. This indicates that the L:S ratio of vesicles remaining not to be agglutinated was nearly the same as that of agglutinated ones; that is, roughly speaking, each vesicle in a given preparation had nearly the same L:S ratio as that of the preparation itself. The LNAase activity was inhibited by anti-L IgG, but this LNAase inhibition was quite the same with the two preparations, in contrast to vesicle agglutination (Fig. 1).

Similar experiments were carried out with other vesicle preparations having various L:S ratio, and the extent of agglutination and inhibition attained by 15 µl of anti-L IgG per unit of LNAase after 20-hr in-

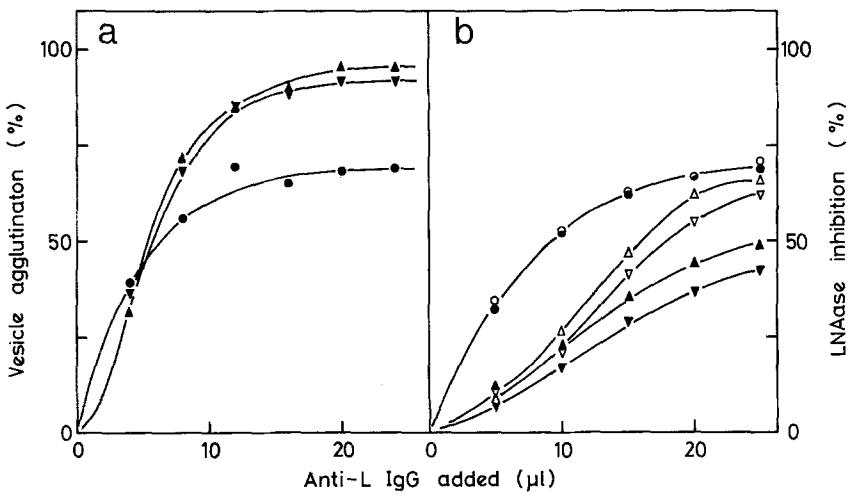

Fig. 1. Vesicle agglutination and LNAase inhibition induced by anti-L IgG. Microvillous vesicles with the L:S ratio of 1.62 (*a*) or 0.32 (*b*), containing 1 unit LNAase, were incubated with various amounts of anti-L IgG at 37 °C for 60 min and then at 4 °C for 20 hr (solid symbols) or 3 days (open symbols), after which vesicle agglutination was determined by the LNAase (▲, △) or sucrase (▼, ▽) assay method as described in the text. At the same time the inhibition of LNAase activity by anti-L IgG (●, ○) was measured. Each point represents the mean of duplicate measurements

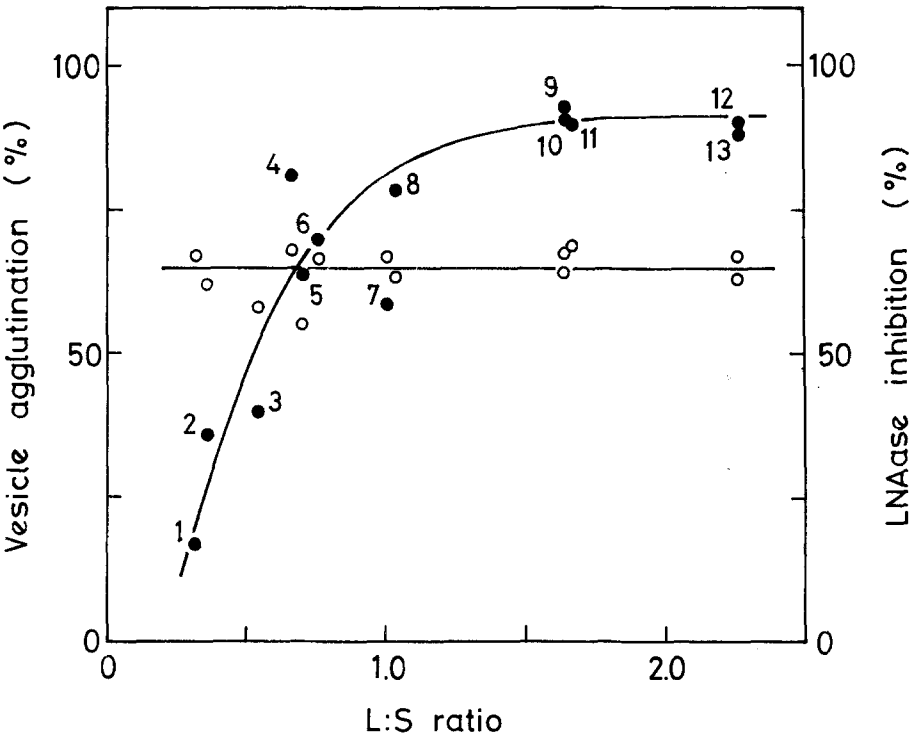

Fig. 2. Relationship between vesicle agglutination or LNAase inhibition by anti-L IgG and the L:S ratio. A number of microvillous vesicle preparations differing in the L:S ratio were incubated with various amounts of anti-L IgG under the same conditions as in Fig. 1. The extent of vesicle agglutination and LNAase inhibition attained by 15 µl of anti-L IgG per unit of LNAase after 20-hr incubation at 4 °C were determined and plotted against the L:S ratio of the preparations examined. The specific activity of LNAase (U/mg protein) of the vesicle preparations examined were as follows. 1, 0.176; 2, 1.06; 3, 1.10; 4, 0.252; 5, 1.25; 6, 0.944; 7, 1.25; 8, 1.78; 9 & 10, 0.869; 11, 0.899; 12 & 13, 0.890. ●, vesicle agglutination; ○, LNAase inhibition

cubation at 4 °C were plotted against the L:S ratio of vesicle preparations examined (Fig. 2). About 90% or more agglutination was observed with vesicles having the L:S ratio higher than 1.4. When the L:S ratio decreased below this value, however, agglutination decreased hyperbolically. On the other hand, as was expected, the inhibition of LNAase activity by anti-L IgG was quite independent on the L:S ratio and was about 65% over the range of the L:S ratio examined. Interestingly, the L:S ratio of 1.4 approximates the ratio of vesicles having LNAase and sucrase-isomaltase complex molecules in the same number, as mentioned earlier. If the specific activity of LNAase were used as the abscissa

Table 1. Agglutination by anti-SI IgG of microvillous vesicles differing in the L:S ratio

No.	Vesicle preparation (U/mg protein)			Vesicle agglutination (%)
	Specific activity		L:S ratio	
	Sucrase	LNAase		
1	0.395	0.890	2.25	89.9
2	0.545	0.178	0.32	89.6

Microvillous vesicles, containing 0.22 (No. *1*) or 0.25 (No. *2*) unit sucrase, were incubated with 15 µl of anti-SI IgG at 37 °C for 60 min and then at 4 °C for 20 hr, after which vesicle agglutination was determined by the sucrase assay method as described in the text.

instead of the L:S ratio, we would have a very puzzling figure, as will easily be seen from the values given in the legend to Fig. 2.

In contrast to anti-L IgG, anti-SI IgG quantitatively agglutinated vesicles with a high L:S ratio of 2.26, i.e., rich in LNAase and poor in sucrase-isomaltase complex, as well as those with a low L:S ratio of 0.32 (Table 1).

Figure 3 shows electron micrographs of microvillous vesicles having the L:S ratio of 1.0, incubated or not with anti-L IgG. In thin-section (Fig. 3*a* and *b*), nontreated vesicles were closed by the trilaminar membrane with a rather smooth surface (Fig. 3*a*), but when they were incubated with anti-L IgG, continuous or bunch-like structures, appeared on the outer membrane surface (Fig. 3*b*). They protruded 16 nm (2.1 SD and 50 measurements) from the membrane surface. The minimum distance between the membrane surface of paired vesicles approximated 23 nm (2.1 SD and 30 measurements) and was shorter, as compared with about 33 nm of vesicles paired by anti-SI IgG (Nishi & Takesue, *unpublished data*). In negative staining (Fig. 3*c* and *d*) particulate layer 15 nm (1.6 SD and 50 measurements) wide was seen on the edge of vesicles before incubation with antibodies (Fig. 3*c*). Its width was not broadened by anti-L IgG (Fig. 3*d*), in contrast to anti-SI IgG, which increases the width of the layer to 20 nm (2.5 SD and 50 measurements) (Nishi & Takesue, 1975). These results indicate that LNAase, or at least its antigenic sites, are located nearer the outer membrane surface than sucrase-isomaltase complex or its antigenic sites.

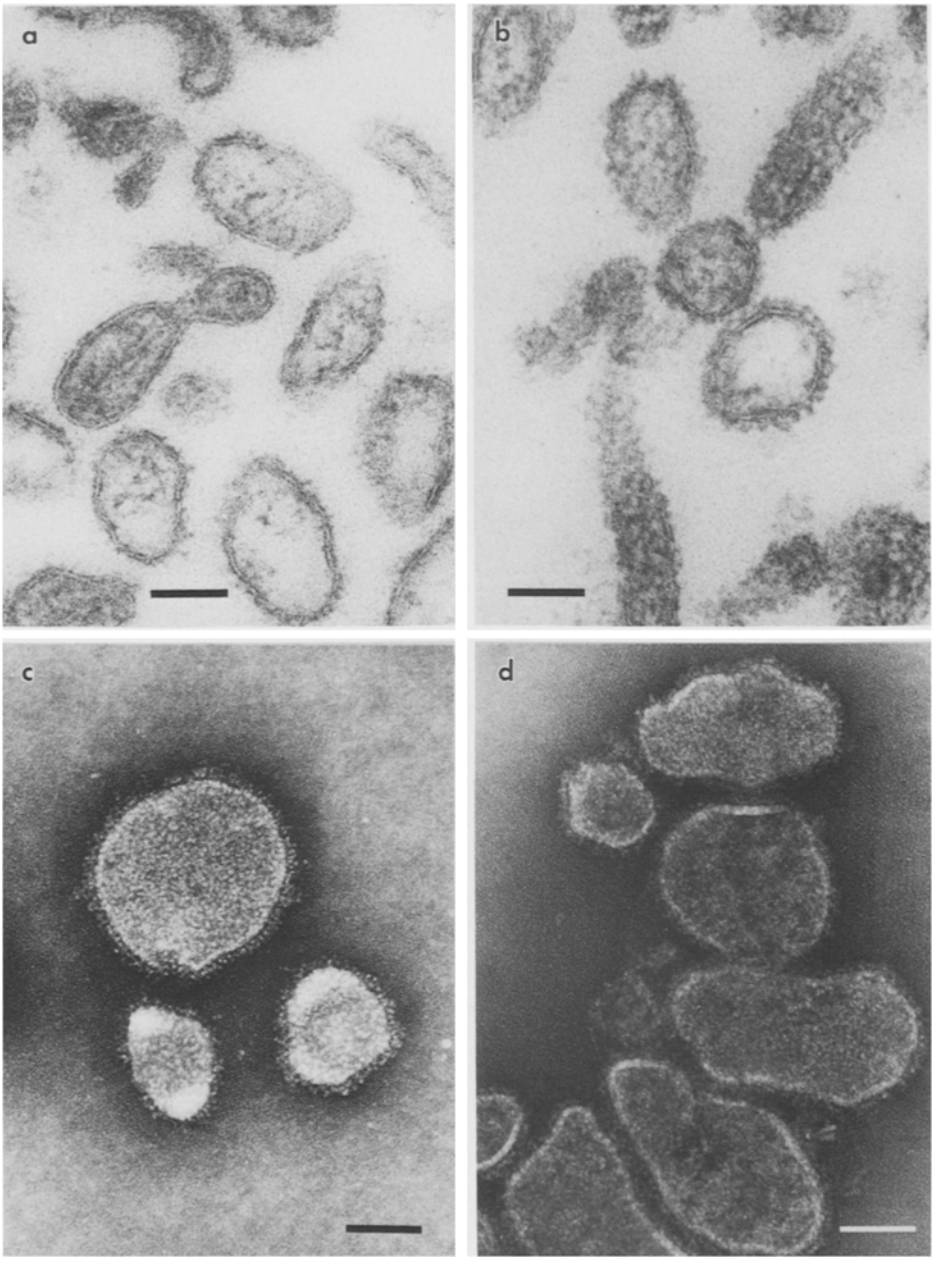

Fig. 3. Thin-section (*a*, *b*) and negative-staining (*c*, *d*) electron micrographs of microvillous vesicles before and after agglutinated by anti-L IgG. Microvillous vesicles with the L:S ratio of 1.0, containing 1 U LNAase, were incubated with 25 μl of anti-L IgG at 37 °C for 60 min and then at 4 °C for 20 hr, after which agglutinated vesicles were collected by low-speed centrifugation. *a* and *c*, vesicles before incubation; *b* and *d*, agglutinated vesicles. Bar = 100 nm

Discussion

Microvillous vesicles are agglutinated by antibodies, most probably in the same mechanism as that for cell agglutination induced by antibodies and lectins (Rutishauser & Sachs, 1975). As the critical parameters in cell and vesicle agglutination, we must consider longitudinal factors as discussed in a previous paper (Takesue & Nishi, 1976), as well as well-studied lateral factors such as the number and mobility of receptors. No dependency of the LNAase inhibition by anti-L IgG on the L:S ratio indicates that the interaction of the first site of anti-L IgG with LNAase antigens is not interfered with by neighboring sucrase-isomaltase complexes. Therefore, the L:S ratio-dependent vesicle agglutination by anti-L IgG suggests that neighboring sucrase-isomaltase complexes exhibit their inhibitory effect at the step when anti-L IgG molecule bound to an LNAase antigen on one vesicle is about to bind another LNAase antigen on a second vesicle. As in the case of monovalent fragments of anti-SI IgG which also inhibit vesicle agglutination by anti-L IgG (Takesue & Nishi, 1976), the inhibitory effect is also probably due to steric hindrance. In this respect the electron microscope observations provide much useful information.

We have investigated electron-microscopically the interaction of microvillous vesicles with anti-SI IgG and concluded that sucrase-isomaltase complex protrudes about 15 nm from the outer membrane surface (Nishi & Takesue, 1975; *see also* Fig. 4). By comparing anti-L IgG with anti-SI IgG in electron-microscopically observed effects, such as the width of antibody-induced electron-dense structures extending from the outer surface (16 *vs.* 18 nm), antibody-induced broadening of the particulate layer seen on the edge of the negatively stained membrane (0 *vs.* 5 nm), and the minimum distance between the outer surface of vesicles paired by antibodies (23 *vs.* 33 nm), we have concluded that LNAase, or at least its antigenic sites, protrude about 10 nm from the outer membrane surface.

On the basis of these observations and conclusion, the L:S ratio-dependency of vesicle agglutination by anti-L IgG can be explained by taking into consideration the effective height as defined in a previous paper (Takesue & Nishi, 1976). The distance between the outer surface of two vesicles linked by anti-L IgG is about 23 nm, while sucrase-isomaltase complex protrudes about 15 nm from the outer surface. Therefore, in order for anti-L IgG to tightly crosslink two vesicles, either the vesicles must align themselves so that their sucrase-isomaltase complexes do not face each other or the complexes must be removed from the portions of

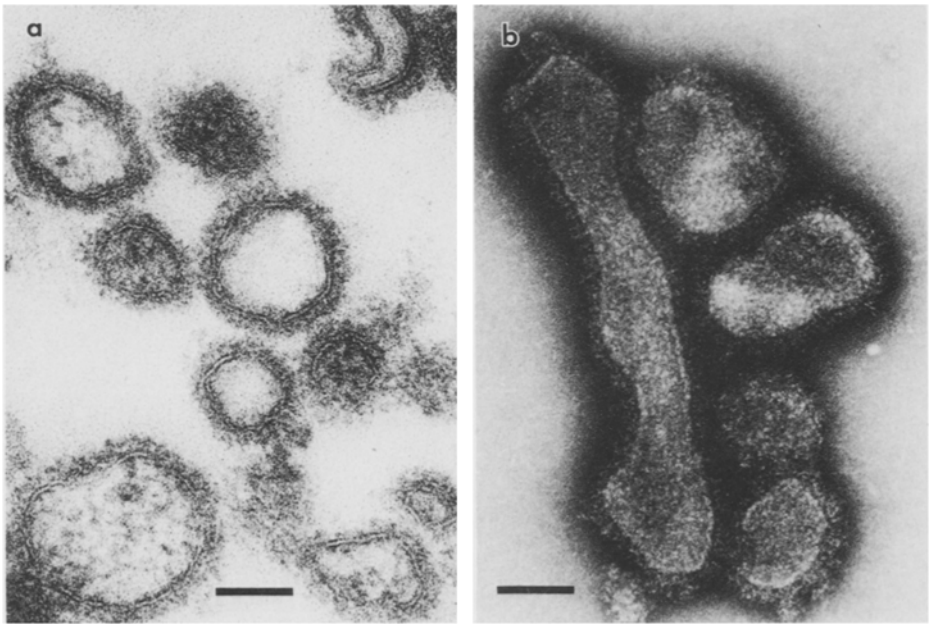

Fig. 4. Microvillous vesicles (L:S ratio, 1.0) agglutinated with anti-SI IgG. (*a*) Thin section: In contrast to a rather smooth outer surface of nontreated vesicles, a new electron-dense layer of rather uniform thickness (about 18 nm) is seen surrounding the whole external surface of each vesicle. (*b*) Negative staining: Compared with that of nontreated vesicles, the fine particulate structure of the membrane surface and of the peripheral layer are more obscured in agglutinated vesicles and, furthermore, the width of the layer is increased to about 20 nm. Bar = 100 nm

the vesicles facing each other; it would take a longer time for vesicles having a lower L:S ratio to establish such situation. On the other hand, the crosslinkage of two vesicles by anti-SI IgG would not be interfered with by neighboring LNAases, which protrude 10 nm from the outer surface. The possibility cannot be completely excluded that sucrase-isomaltase complex, alone or complexed with anti-SI IgG, might move on the membrane much faster than LNAase or LNAase-antibody complex. We have just discussed the membrane surface architecture, based on the data obtained after prolonged incubation. Some membrane proteins might be dislocated during this treatment. For example, the progressive, though very slow, agglutination of vesicles with a very low L:S ratio might be due to such presumed movement of LNAase or other membrane proteins during incubation. This possibility could be checked by shorter time experiments.

If the above-mentioned conclusion is valid, sucrase-isomaltase complex should protrude about 5 nm higher than LNAase, in spite of the fact that the two enzymes have similar molecular weights (Maroux & Louvard, 1976; Sigrist *et al.*, 1975; Takesue, 1969; Takesue, 1975). Sucrase-isomaltase complex consists of two subunits, sucrase and isomaltase moieties, almost similar in molecular weight (mol wt 130,000) (Semenza, 1976). The two subunits are linked at their long axes, and the complex molecule has a total length of about 15 nm, except for the presumed hydrophobic tail (Nishi & Takesue, 1977). The isomaltase moiety has a hydrophobic tail and is directly bound to the membrane (Nishi & Takesue, 1976; J. Brunner & G. Semenza, *personal communication*). Therefore, the location of sucrase-isomaltase complex on the outer surface of the membrane can be depicted schematically as in Fig. 5*a*. It is not known whether the sucrase moiety has some connection with the membrane continuum, either transitory or constantly. On the other hand, LNAase is composed of three subunits which are very different from one another in molecular weight (mol wt: 130,000, 96,000, 49,000) (Vannier *et al.*, 1976). Its hydrophobic tail is located in one of the

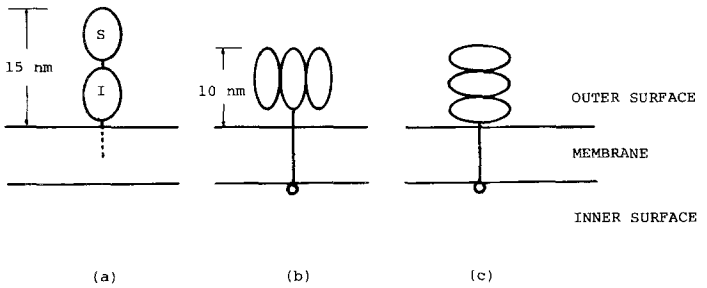

Fig. 5. The schematic representation of the location of sucrase-isomaltase complex (*a*) and LNAase (*b*, *c*) on the outer membrane surface. (*a*) Since it has not been clarified whether or not the hydrophobic tail of sucrase-isomaltase complex spans the membrane, it is expressed in a broken line. The two subunits are linked to each other at their long axes, the isomaltase moiety is directly associated with the membrane, and the whole molecule protrudes about 15 nm from the outer surface of the membrane. (*b*) and (*c*): The three subunits are tentatively expressed in the same shape and size, except for the one carrying the hydrophobic tail. The tail spans the membrane and is exposed on the inner surface (Louvard *et al.*, 1976). The whole molecule, in which the anchoring subunit may be associated with each (*b*) or only one (*c*) of the other two subunits, protrudes about 10 nm from the outer surface. We must keep in mind a possibility that LNAase may consist of two subunits (*see text*)

subunits, though the molecular weight of the subunit has not been specified (Maroux & Louvard, 1976; Vannier *et al.*, 1976). This subunit seems to anchor the whole molecule to the membrane. Therefore, although we have no data on the spatial arrangement of the three subunits in the LNAase molecule, we can imagine the two cases as to the location of the two other subunits relative to this anchoring subunit; i.e., each (Fig. 5*b*) or only one (Fig. 5*c*) of the former two subunits is associated with the latter one. At present we cannot decide which of the locations (*b* or *c*) is more probable. Furthermore, there is preliminary evidence indicating that LNAase consists of two very similar, if not identical, subunits, not three (H. Wacker, *personal communication*). Therefore, in order to discuss further the spatial arrangement of subunits in the whole molecule protruding from the membrane surface, we have to await the more concrete and detailed data concerning the subunit structure of purified LNAase.

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