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STUDIES ON ANTIVIRAL GLYCOSIDES

II. MODE OF ACTION FOR VIRUCIDAL EFFECTS ON SENDAI VIRUS *

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Summary

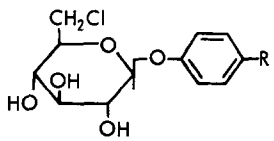
Treatment of Sendai virus with *p*-(*sec*-butyl)-phenyl-6-chloro-6-deoxy-β-D-glucopyranoside, followed by freezing and thawing resulted in a loss of hemolytic and cell fusion activities as well as infectivity without affecting hemagglutinating and neuraminidase activities. The anti-hemolytic activity of this compound was reversed by the addition of phosphatidyl choline to the virus samples. *p*-Azidophenyl-6-chloro-6-deoxy-β-D-[^{3}H]glucopyranoside was successfully used for photoaffinity labeling of a specific virion site, and we confirmed the affected site of the glucoside to be the lipid components in the viral envelopes.

Introduction

In a previous paper, the present authors have reported that some phenyl glucosides exhibited potent virucidal effects specifically on enveloped viruses. Studies on the relationships between structure and virucidal activity revealed that *p*-alkylphenyl 6-halogeno-6-deoxy-β-D-glucosides were the most effective, and removal of aglycones from these glycosides resulted in a loss of virucidal activity [1]. In order to obtain information on the mechanism of the virucidal activity, we examined for the action mechanism of these glycosides on Sendai virus, which possesses some notable biological activities in its envelopes [12] — such as hemagglutinating, neuraminidase, hemolytic and cell fusion activities. These activities are known to be indispensable for the viral infection of host cells [2]. Treatment of the Sendai virus with *p*-(*sec*-butyl)phenyl-6-chloro-6-

* Part I: see Ref. 1.

deoxy-β-D-glucopyranoside (Glucoside I), one of the most potent antiviral agents in this series, followed by freezing and thawing resulted in loss of hemolytic and cell fusion activities as well as infectivity without any effect on hemagglutinating and neuraminidase activities. Two functional glycoproteins (F and HN proteins, characterized on SDS-polyacrylamide gel electrophoresis) with the above biological properties have been identified in the envelopes of Sendai virus [3]. Hosaka and Shimizu have reported that F-protein and lipid components in the viral envelopes participate in the formation of active hemolysin and cell fusion [4,5]. Therefore, the virucidal activity of our glucoside on Sendai virus may be due to some effect on the viral envelopes that results in a loss of infectivity. To clarify this, we synthesized *p*-azidophenyl-6-chloro-6-deoxy-β-D-glucopyranoside (Glucoside II) and its radioactive derivative, which is a possible compound for photoaffinity labeling of a specific site in viral particles via an active nitrene intermediate. We used this compound to determine the virucidal mechanism and also to investigate the hemolytic and cell fusion activities of Sendai virus.



scheme I. Glucoside I, R = *sec*-butyl; Glucoside II, R = N_3.

Materials and Methods

Virus. Sendai virus (Z strain) was used. Infected chorioallantoic fluids were purified and concentrated with two cycles of alternating centrifugations at 5000 × *g* for 15 min and 43 000 × *g* for 30 min [6], then suspended in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline.

Assays of viral activities. Hemagglutinating activity was assayed by the usual Salk pattern method using 0.5% chicken red blood cell suspensions [7]. Neuraminidase activity was assayed by adding 0.2 ml of viral suspension to 0.3 ml of 0.01 M phosphate buffer (pH 5.0) supplemented with fetuin (2 mg/ml, Sigma) and the mixture was incubated at 37°C for 10 min. The liberated *N*-acetylneuraminic acid was measured by the method of Warren [8]. Hemolytic activity was measured according to the method of Homma et al. [9]. Cell fusion activity was determined with a slight modification of Hosaka's method [10]. A virus sample (0.2 ml, $1 \cdot 10^4$ HAU/ml) was added to $LLCMK_2$ cell monolayers grown for 3—4 days in Falcon plastic bottles (25 cm^2) and allowed to be adsorbed for 30 min at 4°C, then 5 ml of pre-warmed Eagle's Minimum Essential Medium was added to the culture, which was incubated at 37°C for 2 h then fixed to be stained. The fusion index was determined by counting the number of cells and nuclei within them according to the following equation [11].

$$\text{Fusion index} = \frac{\text{number of nuclei}}{\text{number of cells}} - 1$$

Infectivity was assayed by plaque assay in $LLCMK_2$ cells trypsin containing

overlay medium according to a method described previously [12].

Synthesis of p-azidophenyl-6-chloro-6-deoxy-β-D-glucopyranoside (Glucoside II). Glucoside II was prepared from *p*-nitrophenyl-β-D-glucopyranoside (purchased from Sigma) according to a method described in a previous paper [13]. *p*-Azidophenyl-6-chloro-6-deoxy-β-D-[1-^{3}H]glucopyranoside was prepared from D-[1-^{3}H]glucose by mixing 5 mCi of D-[1-^{3}H]glucose (3000 Ci/mol, Amersham) with non-labeled D-glucose (300 mg) and acetylating it with acetic anhydride in anhydrous pyridine to obtain 1,2,3,4,6-penta-*O*-acetyl-D-[1-^{3}H]glucose (450 mg). Treatment of the acetate with HBr in acetic acid [14] gave acetobromo-D-[1-^{3}H]glucose, which subsequently led to *p*-nitrophenyl-β-D-[1-^{3}H]-glucopyranoside by reaction with sodium *p*-nitrophenolate in acetone [15]. *p*-Azidophenyl-6-chloro-6-deoxy-β-D-[1-^{3}H]glucopyranoside ($7 \cdot 10^5$ cpm/μmol) was finally obtained by purification with a charcoal column chromatography, and showed a single spot on thin-layer chromatography (TLC) developed with butanol/ethanol/water (3 : 1 : 1, by vol.).

Ultraviolet irradiation. Sterilizing ultraviolet light (12 W, Toshiba) was used to irradiate Sendai virus in the presence of Glucoside II. The virus sample (approx. $1 \cdot 10^4$ HAU) in 0.5 ml of phosphate-buffered saline in a glass Petri dish (60 mm in diameter) was ultraviolet-irradiated from a distance of 30 cm for various periods and with various drug concentrations. Next, the virus suspension was centrifuged at 45 000 × *g* for 30 min, and the pellet was washed with phosphate-buffered saline three times then resuspended in 1 ml of phosphate-buffered saline and examined for biological activity.

Preparation of liposome. Purified phosphatidyl choline (lecithin) from chicken egg yolk was dissolved in chloroform, then dried in vacuo. Phosphate-buffered saline was added to the residue with vigorous mixing, followed by sonication for 30 min in an icewater bath (Ohtake, Sonicator-150).

Extraction of lipid components from Sendai virus. Freeze-dried viral particles (approx. $2 \cdot 10^4$ HAU) were suspended in 5 ml of chloroform/methanol (2 : 1, by vol.) and stirred for 30 min at room temperature. Insoluble substances were removed by filtration with a filter paper and the filtrate was dried in vacuo. The residual syrup was analyzed by TLC developed with chloroform/methanol/water (65 : 25 : 4, by vol.).

Density gradient centrifugation of the solubilized envelopes of Sendai virus [4]. A virus sample ($1 \cdot 10^5$ HAU) in 5 ml of phosphate-buffered saline containing 0.25% Nonidet P-40 was stirred for 15 min at room temperature and kept for another 15 h at 4° C. The suspension was centrifuged at 100 000 × *g* for 1 h to remove nucleocapsid proteins and CsCl was added to the supernatant to a concentration of 25%. Equilibrium centrifugation in CsCl was carried out at 42 000 rev./min for about 45 h (Hitachi, RPS-65 A).

Results

Effect of p-(sec-butyl)phenyl-6-chloro-6-deoxy-β-D-glucopyranoside (Glucoside I) on biological activities of Sendai virus

The effect of this compound on the respective biological activities was examined by freezing and thawing the virions in its presence. All biological activities were not affected by this treatment without the compound, except

hemolytic activity which was enhanced a little as reported previously [16]. As shown in Fig. 1, it strongly affected hemolytic and cell fusion activities as well as infectivity at 0.2 mM, but not hemagglutinating and neuraminidase activities even at higher concentrations. As for control experiments, *p-sec*-butylphenol and 6-chloro-6-deoxy-D-glucose, the respective components of Glucoside I, were found not to have any appreciable effects on Sendai virus around these concentrations. The hemolytic activity was enhanced up to the concentration of 0.1 mM of the glucoside, and this phenomenon might be explained to be an acceleration of the physical damage of viral particles by freezing and thawing [17] in the presence of the glucoside in lower concentrations. It is well known that hemagglutinating and neuraminidase activities originate in the HN-protein [18–20], and hemolytic and cell fusion activities depend on a complex of F-protein and lipids [4]. These two glycoproteins are also known to exist in the viral envelopes as spikes. Therefore, the glucoside may act specifically on F-spikes or lipid components in the envelopes, resulting in inactivation of hemolysin and cell fusion and infectivity. On the other hand, the virucidal effects of the glucoside were much weaker when the viral particles were not frozen and thawed. The meaning of this phenomenon will be discussed later.

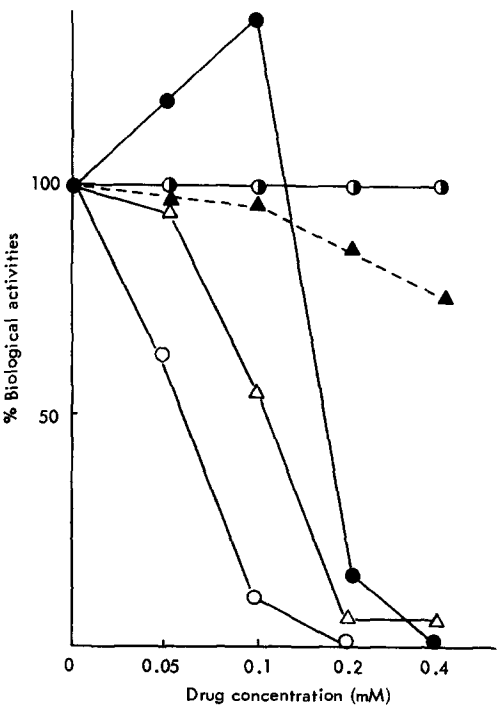


Fig. 1. Effect of Glucoside I on biological activities of Sendai virus. A virus sample (0.25 ml, 5120 HAU/ml) was mixed with the equal volume of various concentrations of the glucoside, and frozen at −20°C for 2 h. After rapid thawing at 37°C, the samples were diluted with phosphate-buffered saline for an assay of biological activities as described in Materials and Methods. Each plot was expressed as percentage of the control, whose titers were hemagglutinating activity: 5120 HAU, neuraminidase activity: 17.8 NAU, hemolytic activity: 20 HLU, cell fusion activity: fusion index 0.52. ○———○, hemagglutinating activity; ▲- - - -▲, neuramidase activity; ●———●, hemolytic activity; △———△, cell fusion activity; ○———○, infectivity.

Reversal of anti-hemolytic activity of p-(sec-butyl)phenyl-6-chloro-6-deoxy-β-D-glucopyranoside (Glucoside I) by liposome

As shown in Fig. 1, Glucoside I had anti-hemolytic activity at 0.1—0.2 mM. However, hemolytic activity of the viral particles was recovered by adding an appropriate amount of phosphatidyl choline to the virus sample, even in the presence of a higher concentration of Glucoside I as seen in Fig. 2. This reversal of hemolytic activity may be due to superior affinity of Glucoside I to lipids, which would decrease the drug concentration in the virus suspension. As a consequence of this experiment, we can assume that the anti-hemolytic activity of Glucoside I may cause this compound to adhere to lipid components in the viral envelopes, producing disorder in the lipid-protein interaction.

Effect of p-azidophenyl-6-chloro-6-deoxy-β-D-glucopyranoside (Glucoside II) on viral activities

To investigate further the action of Glucoside I, we prepared Glucoside II, a possible compound for photoaffinity labeling of a specific site in the viral

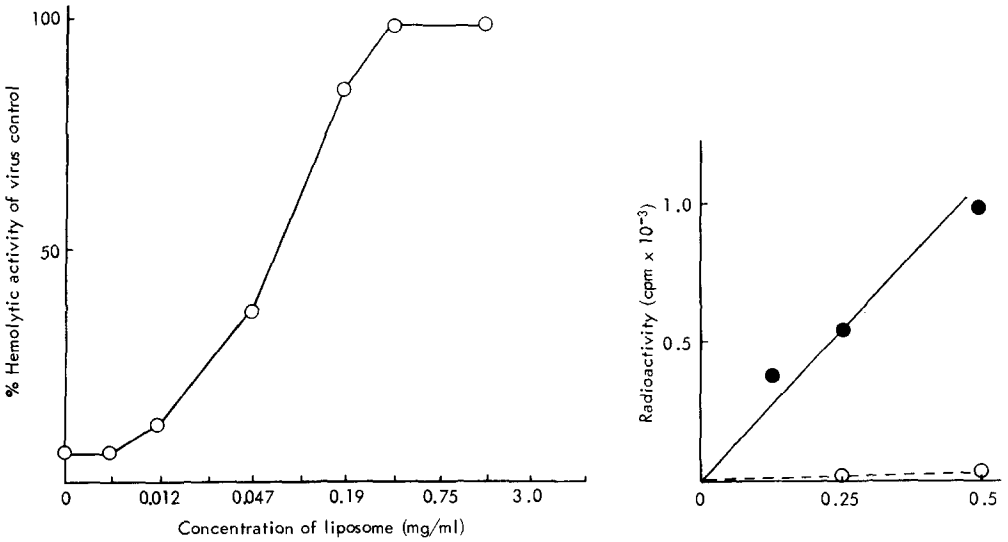


Fig. 2. Reversal of anti-hemolytic activity of Glucoside I by liposome. A solution of liposome (0.5 ml) with various concentrations of phosphatidyl choline was mixed with the equal volume of Glucoside I (1 mM), and incubated at 37°C for 1 h. A virus sample (0.25 ml, 5120 HAU/ml) was added to 0.25 ml of the solution and the mixture was assayed for hemolytic activity as in Fig. 1. The longitudinal axis; the percentage of hemolytic activity of the control without drug, whose titer was 19.5 HAU. The latitudinal axis; the concentration of liposome with serial 2-fold dilutions.

Fig. 3. Binding of [^{3}H]Glucoside II to Sendai virus. To a virus sample (0.5 ml, $1 \cdot 10^4$ HAU) was added tritium-labeled Glucoside II with various concentrations and kept for 10 min at room temperature. Ultraviolet irradiation was then performed for 10 min and the sample was washed three times with phosphate-buffered saline containing 10 mM non-labeled Glucoside II by repeated centrifugations at 23 000 rev./min. The pellets were resuspended in phosphate-buffered saline (0.5 ml) and 50 μl of the sample was solubilized with PROTOSOL and the radioactivity counted in toluene-based scintillation fluid. In the control experiment, the virus sample was incubated for 20 min with 0.5 mM [^{3}H]Glucoside II without ultraviolet irradiation, after which it was washed with phosphate-buffered saline by the same treatment and counted radioactivity. ●———●, radioactivity of virus sample with ultraviolet irradiation; ○- - - - -○, radioactivity of virus sample with no ultraviolet irradiation.

particles. This compound, without ultraviolet irradiation, affected the biological activities of Sendai virus like Glucoside I (data not shown). Therefore, we examined the effect of ultraviolet irradiation on the viral particles in the presence of Glucoside II. The data of Fig. 3 show that the binding of Glucoside II to the viral particles turned out to be irreversible by ultraviolet irradiation; more compounds bound to the virions with an increase in the glucoside concentration.

Next, optimum irradiation periods and drug concentrations were examined and determined to be 20 min in the presence of 0.5 mM of Glucoside II with no freezing and thawing of the virus. Under these conditions, Glucoside II abolished hemolytic and cell fusion activities completely without any effect on hemagglutinating and neuraminidase activities. The effect of ultraviolet irradiation on the virus in the absence of Glucoside II caused about 10% reduction in the biological activities during 20 min. The results of this experiment are shown in Fig. 4.

Analysis of solubilized envelopes of Sendai virus made to react with [^{3}H]-Glucoside II by density gradient centrifugation in CsCl

The action manner of ultraviolet-irradiated Glucoside II on viral particles was considered to be the same as that of Glucoside I as indicated by the results in Figs. 1 and 4. Reversal of the anti-hemolytic activity of Glucoside I by addition of liposome suggested that the affected site was the lipid component in the envelope (Fig. 2). To check this possibility, we measured the radioactivity of protein and lipid regions fractionated from viral particles reacted with tritium-labeled Glucoside II by density gradient centrifugation. As seen in Fig. 5, most

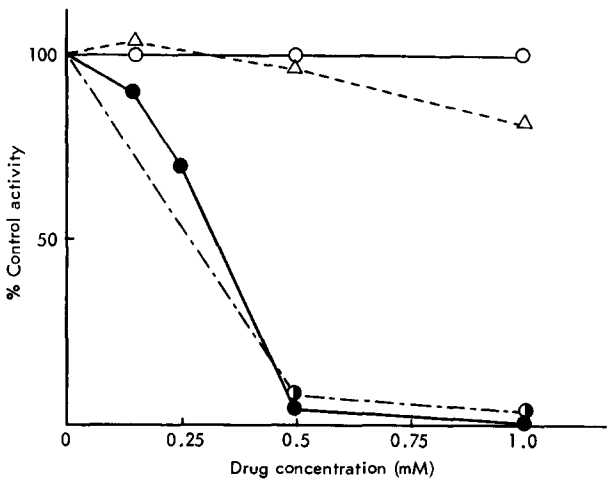
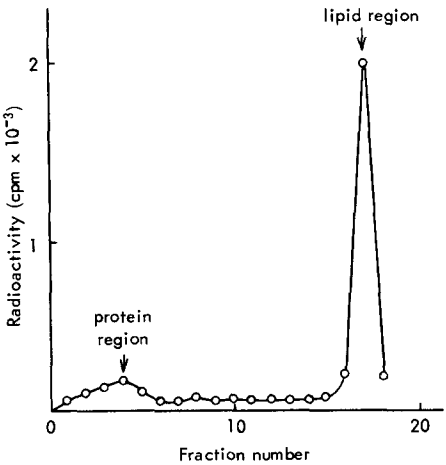


Fig. 4. Effects of Glucoside II on biological activities of Sendai virus under ultraviolet irradiation. The virus sample (12 800 HAU/ml) was used, and ultraviolet irradiation was performed after the drug was added to the virus solution. Assay of biological activities was done in the same manner as described in Fig. 1. The control activities with ultraviolet irradiation and with no drug were as follows; hemagglutinating activity: 9600 HAU, neuraminidase activity: 5.0 NAU, hemolytic activity: 10.5 HLU, cell fusion activity: fusion index = 3.4, ○———○, hemagglutinating activity; △- - - - -△, neuraminidase activity; ●———●, hemolytic activity; ○- · - · -○, cell fusion activity.

of the radioactivity was recovered from the lipid region with a little from the protein region. Some obscurity remains on the small recovery of radioactivity in the protein fraction. To examine whether the radioactivity in the protein region was recovered from specific polypeptides of Sendai varus, virions labeled with [^{3}H]Glucoside II were analyzed by SDS-polyacrylamide-gel electrophoresis



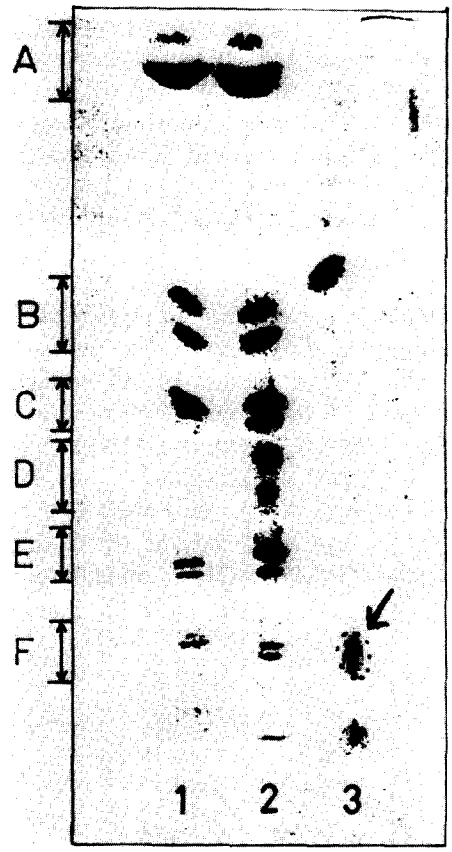


Fig. 5. Analysis for the solubilized envelopes of Sendai virus reacted with [^{3}H]Glucoside II by density gradient centrifugation in CsCl. A virus sample in phosphate-buffered saline (3 ml, $1 \cdot 10^5$ HAU) was ultraviolet-irradiated for 15 min in the presence of 0.5 mM tritium-labeled Glucoside II, and washed three times with phosphate-buffered saline by repeated centrifugations at 23 000 rev./min. The pellet was resuspended in 5 ml of the same buffer, solubilized with Nonidet P-40 and centrifuged as described in Materials and Methods. The gradient was fractionated every 20 drops by puncturing the bottom of the centrifuge tube. The radioactivity of each fraction (50 µl) was measured in Bray's scintillation fluid. The density of the protein region was measured to be about 1.16; the lipid region was at the top of the gradient.

Fig. 6. Analysis for the lipid components of Sendai virus reacted with [^{3}H]Glucoside II by TLC. The plate was developed with chloroform/methanol/water (65 : 25 : 4, by vol.) and colored with sulfuric acid. Sample 1: lipids from control virions (alphabetical notation for the regions is as follows: a, neutral lipids and cholesterol; B, ceramide monohexoside; C, phosphatidyl ethanolamine; E, phosphatidyl choline). Sample 2: lipids from [^{3}H]Glucoside II treated virions. Sample 3: Glucoside II ultraviolet-irradiated for 10 min (decomposed product by ultraviolet irradiation is indicated by an arrow). Each alphabetical region in sample 2 was scraped off using a razor blade and counted for radioactivity in toluene-based scintillation fluid. The recovery of radioactivity was A: 2.2%, B: 9.0%, C: 9.5%, D: 61.0%, E: 10.5%, F: 7.8%. Chemical properties of fraction D have not yet been identified.

and radioactivity for respective polypeptides were measured, however failed to detect any significant labels in each polypeptide. Therefore, radioactivity in the protein region was considered to be a non-specific. This means that the main site affected by Glucoside II may be the lipid component in the viral envelope.

Analysis of the lipid components of Sendai virus made to react with [^{3}H]-Glucoside II by TLC

Although a higher recovery of radioactivity was observed in the lipid fraction, unreacted or decomposed Glucoside II may have been contaminating this region. To check this, we extracted lipid components from viral particles which had been reacted with radioactive Glucoside II and analyzed them by TLC as described in Materials and Methods. As shown in Fig. 6, with ultraviolet irradiation, radioactivity was mainly detected between phosphatidylethanolamine and phosphatidylcholine which has an R_f value different from that of intact Glucoside II and its decomposed substance. This radioactive region was also colored by Molish reagent [21], but was undetectable in the lipid components isolated from virions not drug-treated. It is not yet known whether Glucoside II binds to a specific lipid or nonspecifically binds with lipid componts in the viral envelopes. However, our results showed that the glucoside may specifically act on a lipid bilayer in viral envelopes and disturb lipid-protein interaction, thus affecting some biological activities as well as infectivity.

Discussion

Previous studies on relationships between structure and virucidal activity revealed that *p*-alkylphenyl-6-halogeno-6-deoxy-β-D-glucopyranoside was the most potent anti-viral agent against enveloped viruses such as those of influenza and herpes simplex [1]. During assays of biological activities, we found that virucidal activity was extraordinarily enhanced by freezing and thawing of the virus samples in the presence of the glucoside. Two possible mechanisms can be thought of for the freezing effect on virucidal activity, one is an increase in solute concentration [22] and the other is changes of membrane functions by phase transition of the lipid bilayer [23]. An increase in the solute concentration, which occurs as pure ice separates from solution, can cause changes in the secondary and tertiary structures of macromolecules and can remove lipids from cell membranes. In a biological membrane containing proteins and lipids, as the membrane lipids freeze and the bilayer thickens, proteins normally exposed at the membrane surface may become buried, or they may be extruded due to exclusion from the frozen lipid region. Therefore, drugs may be able to act more easily on the biological membranes through the lipid bilayer. Based on observations from electron microscopy, Shimizu et al. reported morphological changes in the envelopes of Sendai virus which had undergone freezing and thawing, resulting in a change of permeability for uranyl acetate [24]. Figs. 5 and 6 show that the site affected by the glucoside in Sendai virus was considered to be the lipid component in the envelopes. Freezing and thawing of the viral particles may have increased the permeation efficiency into the lipid bilayer. There are some reports on the inactivation of hemolytic activity of Sendai virus by treatment with organic solvents, which

may solubilize membrane lipids [25,26]. However, the action of our glucosides on viral particles differs from that of organic solvents; its binding to viral particles (Fig. 3) as well as animal cells [13] is reversible and the structural specificity of the sugar moiety to exhibit higher activity [1]. Therefore, we consider the action of our glucosides to be based on obstruction of lipid-protein interaction by causing weak binding to the lipid bilayer thus exposing the sugar moiety outside of the envelopes, as with glycolipids [27]. The cell fusion activity of Sendai virus was first thought to result from the ability of the virus to cause hemolysis [28—30]. Recent studies have indicated that cell fusion and hemolytic activities are not always correlated [31], although they may be the same for a process of envelope-cell membrane fusion in an initial period, which is essential for the expression of infectivity [32]. Consequently, in the virucidal mechanism of our glucosides against enveloped viruses, the drug-treated virions first adsorb onto the surface of the host cell membranes. The virions cannot penetrate the cells because envelope-cell fusion is not possible. Further investigation of membrane fluidity and other physical properties affected by addition of the glucoside, and comparison with other drugs which act on membranes [33—35] are now in progress using spin labeling.

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