[Chem. Pharm. Bull. 29(10)2928—2933(1981)]

# Studies on Antiviral Glycosides: V. Formation of Incomplete Sendai Virions in the Presence of Phenyl-6-chloro-6-deoxy-β-D-glucopyranoside[1)]

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(Received February 27, 1981)

Addition of phenyl-6-chloro-6-deoxy-β-D-glucopyranoside (PCG) to culture cells before infection with Sendai virus resulted in the production of incomplete virions defective in biological activities as well as infectivity. These noninfectious virions were characterized by chemical and physical methods. Electrophoretic analysis by SDS-PAGE showed differences in the M-protein region between the intact and incomplete virions, although no morphological differences were observed by electron microscopy.

**Keywords**——Sendai virus; antiviral glycosides; phenyl-6-chloro-6-deoxy-β-D-glucopyranoside; M-protein; incomplete virion

In previous papers, we reported that some phenyl glycosides had virucidal effects against enveloped viruses,[1a)] and also described a study on the mechanism of virucidal action against Sendai virus.[1b)]

During studies on the effect of these compounds on the growth of several viruses, we found that phenyl-6-chloro-6-deoxy-β-D-glucopyranoside (PCG) specifically inhibited the multiplication of paramyxoviruses, but did not restrict the growth of myxoviruses.[1c)] Many drugs have been reported to be effective for inhibiting the multiplication of myxo- and paramyxoviruses,[2,3)] but few specifically inhibit the growth of paramyxoviruses.[4)] In this respect, PCG is an interesting reagent. Some differences in multiplication between paramyxo- and myxoviruses have been elucidated, although much remains obscure.[5,6)]

To investigate the specific action of PCG on multiplication of paramyxoviruses, we examined its action on Sendai virus. As mentioned in the previous paper,[1c)] PCG inhibited the multiplication of all strains of Sendai virus tested (Fushimi, Z, RL and RS strains); we used the Fushimi strain in this paper. Addition of an appropriate amount of PCG (1—2 mM) in the initial period of viral infection resulted in production of incomplete virions with defective biological activities, such as hemagglutinating activity (HA), neuraminidase activity (NA) and hemolytic activity (HL), as well as infectivity.[1c)] These PCG-treated Sendai virions (PCG virions) were characterized by chemical and physical methods, and some differences were found between PCG and intact virions, although no morphological differences were observed by electron microscopy. We report here the analytical data for the PCG virions and also discuss the mode of PCG action against Sendai virus.

## Experimental

**Virus Multiplication and Purification**——The Fushimi strain of Sendai virus was used. $LLCMK_2$ cell monolayers were infected at a multiplicity of 1 to 10 plaque forming units (p.f.u.) per cell. After 90 min (for absorption), the infected cells were incubated at 37°C in Eagle's essential medium (MEM) for 24—48 h, then harvested and frozen and thawed three times. The viral particles were purified and concentrated by means of successive centrifugations at 5000 rpm for 15 min then at 23000 rpm for 30 min in a Hitachi RP-30A rotor, according to the method of Homma *et al.*[7)]

PCG virions were purified in the same manner with addition of PCG (1 mM) to the cells before viral infection. The final preparations of virus were suspended in $Ca^{2+}$, $Mg^{2+}$-free phosphate-buffered saline (PBS).

**Assays for Viral Activities**——Hemagglutinating and neuraminidase activities (HA and NA) were

measured according to the methods described elsewhere.[8] Hemolytic activity (HL) was assayed after treatment of the virious with trypsin.[9] Infectivity was assayed by plaque assay in $LLCMK_2$ cells.[10]

**Isotopic Labeling of Virus**——The virus was labeled by addition of L-[U-$^{14}$C] leucine (270 mCi/mmol, Amersham) at 1.2 μCi/ml or L-[4,5-$^{3}$H] leucine (40000—60000 mCi/mmol, Amersham) at 3 μCi/ml in MEM during 4—24 h after viral infection. The labeled viruses were purified by centrifugation as described above.

**Polyacrylamide-gel Electrophoresis**——Double-label difference analysis of Sendai virions was done by SDS-polyacrylamide disc-gel electrophoresis (SDS-PAGE) according to the system described by Maizel.[11] Samples were dissolved in PBS containing 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol and 10% sucrose then heated at 100°C for 2 min. Electrophoresis was performed with bromophenol blue as the tracking dye in 10% acrylamide gel at a constant current of 3 mA per tube for 18 h. The gels were sliced transversely with a razor blade into sections with 1 mm thickness, and the radioactivity of the slices was counted in toluene–based scintillation fluid after solubilization with PROTOZOL (New England Nuclear) at 50°C overnight. For further analysis of the virus polypeptides, high–resolution polyacrylamide slab-gel electrophoresis in Tris–glycine buffer with SDS was performed.[12] Gels were prepared between two glass plates, separated by 2 mm spacers, and subjected to electrophoresis in an apparatus supplied by Toyo Scientific Ltd., Osaka Japan.

The gels were subjected to electrophoresis for 18 h at a constant voltage of 40 V, then soaked for 10 min in 20% ethanol/7% glacial acetic acid/0.05% Coomassie blue. Designation of polypeptides of Sendai virus was according to Homma *et al.*[13]

**Carbohydrate Analysis**——The carbohydrates in intact and PCG virions were analyzed by gas–liquid chromatography (GLC) using alditol acetates.[14] Lyophilized virus sample ($2\times10^4$ HAU or 1 mg protein) was washed with chloroform–methanol (2: 1) to remove glycolipids, and the residue was dissolved in 0.6 ml of 0.5 N sulfuric acid in 95% acetic acid and heated in a sealed glass tube for 20 h at 80°C. The mixture was heated for another 5 h after addition of 0.6 ml water. The hydrolysate was then applied to a Dowex 1-X8 (acetate form) column ($0.5\times2$ cm) and eluted with methanol. The eluate was evoporated to dryness *in vacuo* the residue was dissolved in 0.6 ml of water. Sodium borohydride (5 mg) was added to the solution and the mixture was kept at room temperature overnight. Glacial acetic acid was added to neutralize the solution, which was then evaporated to dryness *in vacuo* and co-distilled with methanol several times to remove boric acid.

Acetic anhydride (1 ml) was added to the residue, which was then heated on a boiling bath for 2 h. Next, the solution was extracted with chloroform and washed three times with water. The chloroform layer was evaporated to dryness *in vacuo*. The alditol acetates were dissolved in acetone containing 0.7 μg/ml of methyl-2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside as an internal standard, and analyzed by gas chromatography with a Perkin–Elmer Model 881 apparatus using a 3% ECNSS-M glass column, which was kept at 195°C for 12 min, then raised to 210°C at a programmed rate of 6°C/min.

**Assay for Glycosyl Transferase Activity**[15]——$LLCMK_2$ cells at confluency were harvested by scraping with a rubber policeman and washing the cells once with PBS. Cells ($6\times10^6$) were suspended in 2 ml of Dulbecco's medium without serum, containing 0.25 ml of 0.1 M $MgCl_2$, 0.25 ml of 0.1 M $MnCl_2$ and 5 μCi of $^{3}$H-UDP precursors (Amersham) in the presence or absence of 0.2 mM PCG. The mixture were incubated at 37°C, and 1 ml aliquots of suspension were taken into glass tubes after 15 and 30 min. To sample was added three volumes of 1% phosphotungstic acid in 0.5 N HCl, and the precipitate was washed three times with cold 10% trichloroacetic acid then once with ethanol–diethyl ether (2: 1, by vol.). The residue was solubilized with Protozol and its radioactivity was counted in toluene-based scintillation fluid.

**Rate Zonal Centrifugation Analysis of Sendai Virus**——Rate zonal centrifugation was performed in a Hitachi RPS-40 rotor at 4°C. First, 4.6 ml of 15 to 60% (w/v) linear sucrose gradient in PBS was placed in the centrifuge tube. Next, a virus sample in 0.2 ml of PBS containing $^{3}$H-labeled PCG virious ($1.25\times10^4$ cpm), $^{14}$C-labeled intact virions ($5.40\times10^3$ cpm) and non-labeled virons ($1\times10^4$ HAU) was layered on the gradient and centrifuged at 24000 rpm for 60 min. The bottom of the centrifuge tube was punctured and 5-drop fractions were collected. The radioactivity of each fraction was measured in toluene-based scintillation fluid after placing the sample on Millipore filter paper, washing it twice with cold 5% trichloroacetic acid and once with ethanol–diethyl ether (2: 1, by vol), then drying it at 60°C.

**Electron Microscopy**——For electron microscopy, partially purified virions were mounted on carbon-coated micro-grids. Preparations were negatively stained with 2% phosphotungstic acid adjusted to pH 6.6.[16] Samples were examined in a Hitachi HU-11A equipped with a pointed cathode at an opening voltage of 75 kV.

## Results

### Double-label Difference Analysis by SDS-PAGE

The unsual weakness of all biological activities of PCG virions suggested that PCG caused the formation of incomplete virions in the process of viral multiplication. To compare the polypeptides in intact and PCG virions by SDS–PAGE, we labeled the former with $^{14}$C-leucine

and the latter with $^{3}$H-leucine as described in the "Experimental" section. As shown in Fig. 1A, polypeptides of both virions were apparently the same except for the M-protein region. The M-protein in Sendai virions exists as a matrix protein which connects nucleocapsid and viral envelope,[17,18] although this does not satisfactorily explain its biological function. Differences between other main polypeptides (P, HANA, and F) could not be distinguished by this electrophoresis. Homma and Ohuchi have successfully converted a biologically inactive precursor protein (F) of Sendai virus grown in mouse L cells into $F_1$-protein by regulated protease cleavage, accompanied by an increase in hemolytic activity as well infectivity.[19] To further discriminate the overlapping region (P, HANA, and F bands) in Fig. 1A, we treated a mixture of intact and PCG virions with trypsin in a similar manner. Fig. 1B shows that F bands of both intact and PCG virions were similarly altered to $F_1$-bands by trypsinization, which means that the F-proteins have the same susceptibility to trypsin.

**Analysis of Polypeptides in the Intact and PCG Virions by Polyacrylamide Slab-gel Electrophoresis**

The results of the electrophoretic experiment given in Fig. 1A and 1B showed differences in the M-protein region between PCG and intact virions. High-resolution polyacrylamide slab-gel electrophoresis further discriminated the mobility of the M-protein in the two virions, indicating that the PCG M-protein was more mobile than that of intact virions (Fig. 2). Some unidentified protein bands were observed on slab-gel electrophoresis of PCG-virions, but the virions could not be further purified because of their extreme agglutinability during centrifugation. The same phenomenon was also found in the Z-strain of Sendai virus.

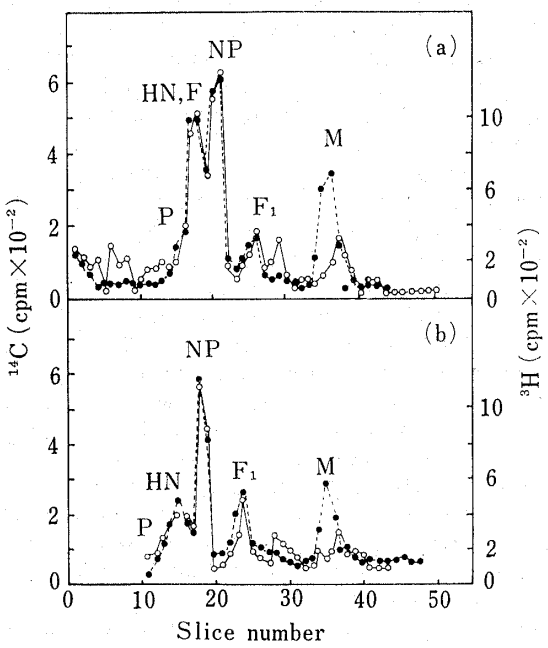


Fig. 1. Polypeptides Profiles of the Control and PCG Virions of Sendai Virus in SDS-PAGE. The procedures are described in "Materials and Methods"

$^{3}$H-labeled control (●) and $^{14}$C-labeled PCG virions (○) were mixed and not treated (a) or treated (b) with trypsin at a concentration of 10$\mu$g/ml for 10 min before co-electrophoresis.

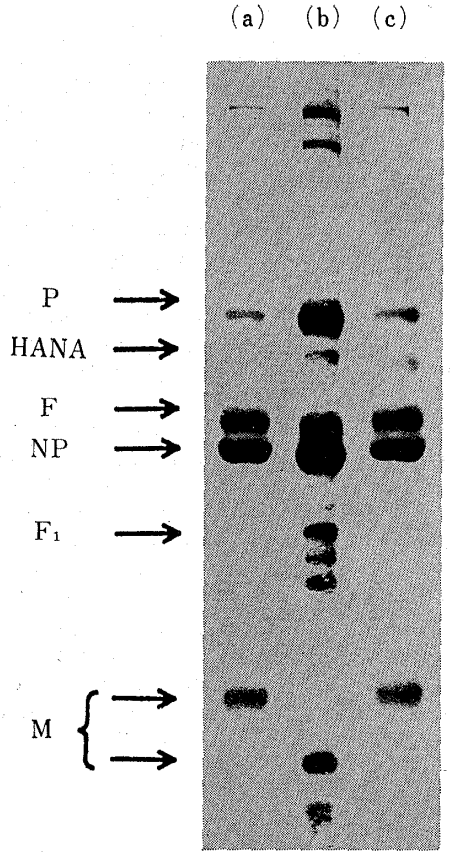


Fig. 2. Slab SDS-PAGE Analysis of the Polypeptides of Control (a, c) and PCG (b) Virions of Sendai Virus

Designation of polypeptides is according to Homma *et al.*[19]

## Analysis of Carbohydrates in the Virions by GLC

Various biological activities (HA, NA, and HL) were almost lost in PCG virions.[1c] These activities are known to depend on the glycoproteins (HANA and $F_1$-proteins) in the viral envelope. In general, complex carbohydrates are thought to exist in the lipid bilayer of biological membranes, with the carbohydrate moiety exposed outside the membrane;[20] such carbohydrates play important roles in biological functions. Inhibition of the glycosylation process in some enveloped viruses has produced incomplete virions with no biological activities.[21] Therefore, we analyzed the total carbohydrates in PCG virions and also examined the effect of PCG on glycosyl transferases in host cells. The ratios of neutral and amino sugars in both virions were determined by GLC analysis (Table I). No appreciable differences were observed, which suggests no variation of the carbohydrate sequences in glycoproteins. As expected, PCG had no effect on glucosyl transferase and galactosyl transferase in $LLCMK_2$ cells (data not shown).

TABLE I. Comparison of the Carbohydrate Contents of Control and PCG Virions

| Virions | Fuc | Man | Sugars[a] Gal | Glc | GlcNac |
|---|---|---|---|---|---|
| Control virions | D[b] | 0.25 | 1.34 | 0.20 | 1.16 |
| PCG virions | D | 0.25 | 1.17 | 0.11 | 1.20 |

*a*) Sugars were analyzed by GLC as alditol acetates. Each value is given as the comparative ratio to the internal standard (methyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside).
*b*) D: detected.

## Sedimentation Analysis

The results of rate zonal centrifugation analysis clearly showed that PCG virions had almost the same density ($\rho$=1.167) as intact virions (see Fig. 3). This means that the components (lipids, proteins, and ribonucleic acids) in both viral particles are similar.

## Effect of Time of Addition on the Multiplication of Sendai Virus

To determine the time relationship between the onset of insensitivity to PCG and the synthesis of progeny viruses during a single multiplication cycle of Sendai virus, infected

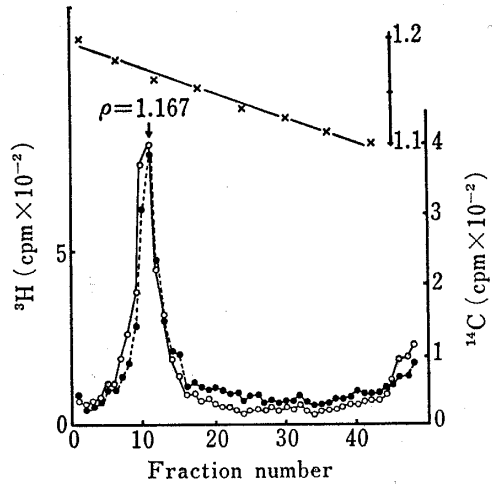


Fig. 3. Sedimentation Analysis of the Control and PCG Virions Procedures are described in "Materials and Methods."

$^3$H-labeled control (●) $^{14}$C-labeled PCG (○) virions were mixed before centrifugation.

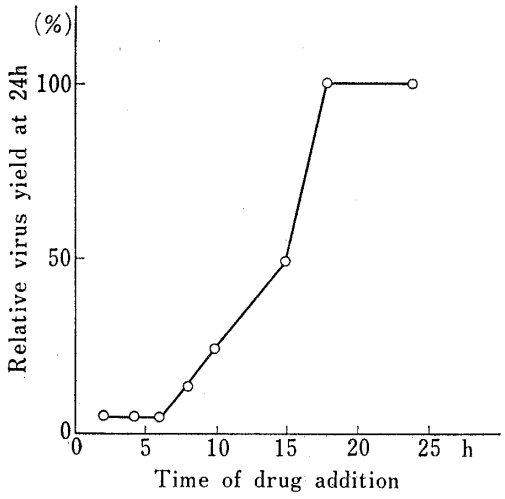


Fig. 4. Effect of Time of PCG Addition on the Multiplication of Sendai Virus

PCG was added at a concentration of 1 mM to the infected culture at various times after infection, and the virus yields were determined in terms of the HA activity at 24 h after virus infection.

cultures were treated with the drug (1 mM) at different times after infection. These cultures were then incubated at 37°C until 24 h after infection and assayed for the yield of infectious virus. The results of a typical experiment are shown in Fig. 4.

In cultures treated with PCG within 6 h after infection, virus production was completely inhibited. When PCG was added 8 h or more after infection, inhibition was still observed although the effect progressively decreased. The results suggested that PCG inhibited processes concerned with the production of infectionus virios during 6—15 h after viral infection.

## Discussion

PCG affected the growth of Sendai virus, producing incomplete virions lacking biological activities. As mentioned in the previous paper,[1c] the hemagglutinating activity (HA) and infectivity of the PCG virions were less than 10% of those of the intact virions. The neuraminidase (NA) and hemolytic (HL) activities per mg protein were 8.5 and 6.7%, respectively, of those of the intact virions. These biological activities originate in the glycoproteins or lipid components in the viral envelope,[22,23] so PCG virions may have some deficiency or modification in their envelopes. Sokol *et al.* reported the formation of incomplete Sendai virions with normal surface properties but without infectivity during undiluted passage of Sendai virus,[24] and these noninfectious virions with incomplete genomes have been separated by rate zonal centrifugation from infectious virions.[25]

In contrast to the above results with Sendai virus, PCG virions had some different characteristics. They had essentially the same density as intact virions on sedimentation analysis (see Fig. 3) but did not show appreciable biological activities, although glycoproteins in the envelope appeared in the same region as those of intact virions on SDS–PAGE. Electron microscopic observation by negative staining revealed no morphological differences between PCG and intact virions, although PCG virions showed extremely high agglutinability characteristics. Analysis of polypeptides by SDS–PAGE did not show any appreciable difference of HANA and F ($F_1$)-glycoproteins in intact and PCG virions, and on significant differences in the carbohydrate components were detected by GLC analysis. The results of double-isotopic label difference analysis and slab-gel electrophoresis indicated that the mobility of M-protein of the PCG virions was distinctly different from that of M-protein in intact virions. The M-protein, also known as matrix or membrane proteins, is a class of proteins which is found in influenza, parainfluenza and rhabdoviruses; these proteins are believed to just underlie the membrane of the virus.[26,27]

Investigation of the synthesis of virus-specific proteins in several cell types infected with Sendai virus suggested the synthesis of M-protein to be a rate-limiting step in virus assembly,[28] and it has also been shown that M-protein has some role in viral assembly in host cell membranes.[29] The synthesis of viral macromolecules in $LLCMK_2$ cells pretreated with actinomycin D has been confirmed not to be affected by PCG.[1c] However, the yield of PCG virions was less than one-third of that of intact virions.[1c] The decrease of viral production in the presence of PCG is probably due to the formation of M-protein with lower molecular weight, which may cause disorder of the virus assembly. It is not clear whether an incomplete M-protein in the viral particles directly results in the decreases of various biological activities of PCG virions. Other possibilities include some change in conformation or sequence of glycoproteins (F and HANA proteins), which might cause loss of the biological activities.

It is noteworthy that the growth of myxoviruses, which resemble paramyxoviruses in biological properties and morphology, is not affected by PCG. Though we cannot yet explain the narrow spectrum, there might be some differences in the environment of viral protein synthesis as well as in that of viral RNA synthesis. Further investigations on the selectivity of PCG against myxo- and paramyxoviruses are in progress.

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