Absence of Glycoproteins in Poliovirus Particles

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SUMMARY

Properly purified preparations of poliovirus particles are practically free of \( \alpha \)-galactose and \( N \)-acetyl-\( \alpha \)-glucosamine. These sugars, however, are found in crude virus preparations, obtained by high and low speed sedimentation and by CsCl-gradient fractionation from infected HeLa cells. They are separated from the virus particle by extraction with chloroform and subsequent isopycnic sedimentation of the virus preparation. Since \( \alpha \)-galactose and \( N \)-acetyl-\( \alpha \)-glucosamine are regular constituents of glycoproteins, the absence of significant amounts of these sugars demonstrates the lack of glycoproteins in poliovirus particles.

Radioactive monosaccharides are recommended for the detection of cellular impurities in carbohydrate-free viruses.

A simple and rapid method for the purification of large quantities of poliovirus by ‘precipitation’ with polyethylene glycol, resuspension in CsCl solutions, extraction with chloroform and isopycnic sedimentation is described.

INTRODUCTION

Glycoproteins are integral constituents of a number of different viruses. They were detected in virus particles of orthomyxovirus (Compans et al. 1970; Schulze, 1970), paramyxovirus (Klenk, Caliguiri & Choppin, 1970), oncornavirus (Hung, Robinson & Robinson, 1971), togavirus (Strauss, Burge & Darnell, 1970), herpesvirus (Ben-Porat & Kaplan, 1970), and poxvirus (Holowczak, 1970). All of these viruses belong to the group of enveloped (complex) viruses which incorporate carbohydrates and lipids from the host cell into the virus particle. No evidence has been presented demonstrating glycoproteins in non-enveloped virus particles. Attempts were made to determine the content of carbohydrates in them (for review, see Green, 1969). In earlier experiments, the amount of virus material was usually insufficient for the detection of small amounts of carbohydrates. Furthermore, the colorimetric procedures employed were not specific enough to distinguish between the nucleic acid’s pentose and other sugars. In 1957 Schwerdt, employing the anthrone test, reported the absence of carbohydrates in poliovirus other than the pentose of the nucleic acid (see Schaffer & Schwerdt, 1959). These results, however, are questionable due to the difficulties just mentioned.

Last year, Burness, Pardoe & Fox (1973) reported the absence of glycoproteins in encephalomyocarditis (EMC) virus particles grown in Krebs II ascites tumour cells. The EMC virus belongs to cardioviruses (Fenner, 1968), a subgroup of picornaviruses, differing

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in some respect from poliovirus which belongs to the group of enteroviruses (for review, see Rueckert, 1971). On the other hand, Halperen, Stone & Korant (1973) isolated glucosamine from the capsid of echovirus 12, a member of the enterovirus group. The carbohydrate, however, was not covalently bound to any of the four virus polypeptides. There are several other reports suggesting the presence of carbohydrates in capsids of picornaviruses (for review, see Rueckert, 1971).

The experiments presented in this paper demonstrate the lack of radioactive carbohydrates and thus the absence of glycoproteins in the capsid of poliovirus. They also demonstrate the fate of carbohydrates initially found in partially purified poliovirus preparations and the requirement for detergents and chloroform to separate material containing sugar from the virus particle.

METHODS

Chemicals. The following radioactive chemicals were purchased from New England Nuclear Corp., Boston, Massachusetts: [4,5-3H]-L-leucine (N), sp. act. 30 to 50 mCi/mmol; [1-14C]-D-galactose, sp. act. 5 to 10 mCi/mmol; [1-14C]-N-acetyl-D-glucosamine, sp. act. 40 mCi/mmoll. Triton N-101 was obtained from Sigma Chemical Company, St Louis, Missouri.

Minimum essential medium (MEM), Joklik-modified, for suspension cultures, foetal calf serum and other ingredients for tissue cultures were obtained from Grand Island Biological Company (GIBCO), Grand Island, New York. MEM devoid of all amino acids except for glutamine was used in experiments with radioactive leucine.

Actinomycin D was obtained from Merck, Sharp & Dohme, West Point, Pennsylvania. Reticulocyte standard buffer (RSB), pH 7.4, is composed of 0.01 M-tris-HCl, 0.01 M-NaCl and 0.05 M-MgCl2. Phosphate-buffered saline (PBS), pH 7.2, contains 0.02 M-PO4 -4- 0.72 % NaCl.

Virus. Poliovirus, type 1, strain Mahoney, was propagated at 37 °C in HeLa S3 cells and purified by three different procedures. In procedures 1 and 2 intracellular virus was released from cellular material using Triton N-101 in hypertonic sucrose (Koch, 1971) instead of SDS (Mandel, 1962) to avoid destruction of cell nuclei. In procedure 3 extracellular virus, harvested after cell damage about 22 to 24 h after infection, was concentrated and purified by 'precipitation' with polyethylene glycol 6000. For routine use, procedures 2 or 3 are recommended. Procedure 1 was only used to demonstrate the effect of SDS and chloroform on the purity of poliovirus.

Procedure 1. Poliovirus labelled with [3H]-leucine and [14C]-D-galactose was obtained by infecting HeLa S3 cells (1 × 10⁷ cells/ml) at an input multiplicity of 20 in the presence of 2 μg/ml of actinomycin D in MEM devoid of amino acids except L-glutamine. After 2.5 h radioactive leucine (0.3 μCi/ml) and D-galactose (0.3 μCi/ml) were added. Cells were sedimented after 5.5 h, resuspended in RSB (~ 2 × 10⁸ cells/ml), frozen and thawed. Sucrose and Triton N-101 were added to a final concentration of 0.3 M and 0.3 %, respectively. After 30 min the sample was purified by low speed sedimentation at 12000 g, 4 °C, for 20 min. The supernatant fluid was subjected to ultracentrifugation in the Beckman/Spinco Type 30 rotor for 2 h at 30000 rev/min at 6 °C. The pellet was resuspended in PBS. Then CsCl was added (0.45 g/ml) and the sample was centrifuged in the SW 41 rotor at 35000 rev/min for 22 h at 6 °C. The lower part of the gradient (ρ > 1.3 g/ml) was dialysed against PBS. SDS was added to a final concentration of 1 %, and the sample was subjected to low and high speed sedimentation. The pellet was resuspended in PBS + CsCl (see above) and extracted twice with chloroform. Five ml of CHCl₃ were added to 2 ml of sample. The
mixture was shaken vigorously for about 5 min and subsequently separated by short sedimentation. The water phase was centrifuged in the SW 41 rotor at 35000 rev/min for 35 h at 15 °C. The visible band containing the virus was collected.

Procedure 2. Virus labelled with [3H]-leucine and [14C]-N-acetyl-glucosamine ([14C]-GlcNAc) was obtained by infecting HeLa S3 cells (1 × 10⁷ cells/ml) at an input multiplicity of 10 in the presence of 0.5 μCi/ml of [3H]-leucine and 0.3 μCi/ml of [14C]-GlcNAc. No actinomycin was added. The cells were harvested after 5.5 h and treated with Triton N-101 and sucrose as described above. The low speed supernatant fluid was clarified by treatment with 0.25 % (final concentration) of SDS for 15 min and by a second low speed sedimentation. The slightly opalescent supernatant fluid was subjected to ultracentrifugation (see procedure 1). The pellet was resuspended in PBS; subsequently CsCl (0.5 g/ml) was added, and the sample was centrifuged in the SW 41 rotor at 35000 rev/min for 48 h at 15 °C. The visible band containing virus was extracted twice with CHCl₃, followed by isopycnic sedimentation at the same conditions as just described. The fraction containing virus was collected.

Procedure 3. 4.6 × 10⁸ cells in 1000 ml of MEM devoid of amino acids except glutamine were infected with 5 × 10¹⁰ p.f.u. of poliovirus at 37 °C. After 2 h [3H]-leucine and [14C]-N-acetyl-D-glucosamine were added (see above). Virus was harvested after 22 h. Cells and cell debris were removed by sedimentation at 3000 g (rₛₒ = 10.9 cm) for 10 min at 4 °C. NaCl (2.2 g per 100 ml) and solid polyethylene glycol 6000 (10 g per 100 ml) were added to the supernatant fluid. The solution was mixed for 1 h at room temperature, then stored for 24 h at 4 °C. The precipitate containing virus was collected by sedimentation at 3000 g for 20 min at 4 °C. The pellet was resuspended in 23 ml of PBS, and 0.5 g CsCl/ml were added. The suspension was sonicated for 1.5 min (MSE Sonicator, 6 mA), extracted twice with chloroform and subjected to ultracentrifugation as described in procedure 2. The visible virus band was collected. It contained a total of 6 × 10¹¹ p.f.u. of poliovirus. After a second isopycnic CsCl-gradient sedimentation, the ratio of [3H]-leucine to [14C]-N-acetyl-D-glucosamine radioactivity was determined.

Infectivity of poliovirus was determined in HeLa cells using the agar-cell suspension plaque assay (Koch, Quintrell & Bishop, 1966).

Radioactivity was measured in the Packard Tri Carb Scintillation Spectrometer with the aid of the Absolute Activity Analyzer (Packard Instrument Company, Inc., Downers Grove, Illinois). The samples were counted in a Triton-based toluene cocktail composed of 333 ml Triton X-100, 667 ml toluene, 5.5 g 2,5-diphenyl-oxazole (POPOP) and 100 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP).

RESULTS

In order to test for the possible presence of glycoproteins in poliovirus, the virus was propagated in HeLa cells in the presence of radioactive carbohydrates and amino acids. D-galactose and N-acetyl-D-glucosamine were chosen since these sugars were found in most virus glycoproteins (references: see Introduction), and they are metabolized more slowly into other carbohydrates or into amino acids than, for example, glucose (Melchers, 1971).
Table 1. Distribution of infectivity and of $[^3H]$-leucine and $[^{14}C]$-D-galactose in fractions containing virus

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Infectivity (p.f.u.)</th>
<th>$[^3H]$-leucine (ct/min)</th>
<th>$[^{14}C]$-D-galactose (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>$3.6 \times 10^{11}$</td>
<td>$4.4 \times 10^7$</td>
</tr>
<tr>
<td>B</td>
<td>Supernatant fluid after ultraseedimentation</td>
<td>$&lt; 1 \times 10^9$</td>
<td>$3.4 \times 10^6$</td>
</tr>
<tr>
<td>C</td>
<td>Pellet after ultraseedimentation</td>
<td>$2.2 \times 10^{11}$</td>
<td>$7.0 \times 10^5$</td>
</tr>
<tr>
<td>D</td>
<td>CsCl gradient, upper part ($\rho &lt; 1.3$ g/ml)</td>
<td>$1.6 \times 10^{10}$</td>
<td>$3.4 \times 10^6$</td>
</tr>
<tr>
<td>E</td>
<td>CsCl gradient, lower part ($\rho &gt; 1.3$ g/ml)</td>
<td>$2.9 \times 10^{11}$</td>
<td>$1.2 \times 10^7$</td>
</tr>
<tr>
<td>F</td>
<td>Supernatant fluid of low speed sedimentation after dialysis of the fractions containing virus of the CsCl gradient followed by 1 % SDS treatment</td>
<td>n.d.*</td>
<td>$7.0 \times 10^5$</td>
</tr>
<tr>
<td>G</td>
<td>Pellet resuspended in PBS after ultraseedimentation of the above supernatant fluid</td>
<td>$1.4 \times 10^{11}$</td>
<td>$5.2 \times 10^5$</td>
</tr>
<tr>
<td>H</td>
<td>Water phase after CHCl₃ extraction</td>
<td>$1.7 \times 10^{12}$</td>
<td>$2.3 \times 10^5$</td>
</tr>
<tr>
<td>I</td>
<td>Fraction containing virus after CsCl gradient sedimentation</td>
<td>$7.5 \times 10^{10}$</td>
<td>$2.7 \times 10^4$</td>
</tr>
</tbody>
</table>

* Not done.

The experimental procedure is given in Methods.

Distribution of infectivity, $[^3H]$-leucine and $[^{14}C]$-D-galactose in fractions containing poliovirus

HeLa cells were infected with poliovirus, type 1, strain Mahoney, in the presence of $[^3H]$-leucine and $[^{14}C]$-galactose. The virus was purified by procedure 1 as described in Methods. The low speed supernatant fluid (fraction A, Table 1) contained a total of $3.6 \times 10^{11}$ infectious particles and the bulk of radioactive leucine and galactose. This radioactivity represents the approximate uptake of the labelled compounds into the cells. In order to separate the infectious virus from low mol. wt. material, the sample was subjected to ultraseedimentation. The supernatant fluid (B) contained most of radioactive leucine and galactose but only negligible amounts of virus. Nearly all of the infectivity but 10 % of the radioactivity was recovered in the pellet (C). This material was further purified by isopycnic CsCl density gradient sedimentation. In contrast to material treated with SDS which forms a discrete band of virus (see the following section), Triton-treated preparations did not give a visible band at the position of the virus, i.e. at a density of 1.33 g/ml CsCl. However, a diffuse band was present at this and at higher densities. No band could be seen at the position ($\rho = 1.29$ g/ml) where empty capsids usually occur. The upper part of the gradient was removed down to a density of 1.3 g/ml. It contained about 5 % of infectivity and 0.5 to 1 % of radioactivity (D). Nearly all of the infectivity but 2.7 % of $[^3H]$-leucine and 1.3 % of $[^{14}C]$-galactose were found in the lower part (E) of the gradient.

The sedimentation of Triton-treated cell material had only a slight effect on the leucine to galactose ratio of the poliovirus fraction. Therefore, the lower part of the CsCl gradient (E) was treated for 10 min with 1 % SDS (final concentration) at room temperature, followed by low and high speed sedimentation. The pellet obtained after ultraseedimentation (G) was clear and contained less $[^{14}C]$-galactose than $[^3H]$-leucine. A drastic reduction of the $[^{14}C]$-
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Table 2. Distribution of infectivity and of $[^{3}H]$-leucine and $[^{14}C]$-N-acetyl-D-glucosamine in fractions containing virus

| Fraction |
|-----------------|-----------------|-----------------|
| Infectivity (p.f.u.) | $[^{3}H]$-leucine (d/min) | $[^{14}C]$-NAcGlc (d/min) |
| Supernatant fluid from infected HeLa cells after Triton N-101 and SDS treatment followed by low speed sedimentation | $1.8 \times 10^{12}$ | $3.7 \times 10^{7}$ | $2.2 \times 10^{7}$ |
| Supernatant fluid after ultracentrifugation | $1 \times 10^{9}$ | $3.5 \times 10^{7}$ | $1.5 \times 10^{7}$ |
| Pellet after ultracentrifugation | $5.2 \times 10^{11}$ | $7.1 \times 10^{6}$ | $1.2 \times 10^{6}$ |
| Fraction containing virus of the second CsCl gradient after extraction of the first CsCl gradient with chloroform | $6.3 \times 10^{11}$ | $7.6 \times 10^{6}$ | $3.1 \times 10^{6}$ |

The experimental procedure is described in Methods (procedure 2).

galactose content was achieved by extracting the virus material twice with chloroform at hypertonic conditions, i.e. in the CsCl solution (see Methods). The water phase (H) contained only a small part of $[^{14}C]$-galactose, the isotope being removed into the chloroform layer and into a strong interphase. Further removal of $[^{14}C]$-galactose was achieved by isopycnic sedimentation of the solution containing CsCl. The virus fraction (I) held about 20% of the infectivity of the starting material but only 0.6% of $[^{3}H]$-leucine and 0.003% of $[^{14}C]$-galactose.

The results indicate that it is necessary to use SDS and chloroform to purify poliovirus. They demonstrate the usefulness of radioactive galactose for checking the purification steps. From the very low content of $[^{14}C]$-galactose in purified poliovirus preparations (Table 1) and the successive decrease of this isotope in the virus fraction, it is concluded that poliovirus particles are essentially free of galactose containing carbohydrates, glycolipids and glycoproteins.

Distribution of $[^{3}H]$-leucine and $[^{14}C]$-N-acetyl-D-glucosamine in fractions containing poliovirus

To further verify the absence of glycoproteins in poliovirus, $[^{14}C]$-labelled N-acetyl-D-glucosamine was used as carbohydrate marker.

The amount of $[^{14}C]$-N-acetyl-D-glucosamine which entered the cells is given in the first line of Table 2. Most of this radioactivity was separated from the virus by ultracentrifugation (line 2). The virus was found in the pellet together with about 20% of radioactive leucine and 0.5% of radioactive N-acetyl-glucosamine (line 3). Compared to the pellet obtained by procedure 1 (see fraction C, Table 1), this crude virus contains much less labelled carbohydrate. This is attributed to the SDS-treatment of the virus prior to ultracentrifugation. It is evident from the preceding section that a large amount of radioactive galactose remained in the virus fraction even after isopycnic CsCl gradient sedimentation if the virus was not treated with SDS. From both purification procedures it is concluded that SDS-treatment removes large amounts of poliovirus-associated carbohydrates.

For further purification, isopycnic CsCl gradient sedimentation of crude virus, followed by extraction with chloroform and a second CsCl gradient sedimentation, was performed (see Methods). The visible band of the CsCl gradient contained 35% of the virus infectivity but only 2% of $[^{3}H]$-leucine and only 0.01% of the $[^{14}C]$-label of the original sample (Table 2). These data demonstrate a further elimination of $[^{14}C]$-N-acetyl-D-glucosamine
Table 3. Relation of $^{14}C$-labelled carbohydrates to $^{3}H$-labelled leucine in poliovirus particles

<table>
<thead>
<tr>
<th>Method of purification</th>
<th>Time of incubation (h)</th>
<th>Radioactive carbohydrate</th>
<th>$[^{14}C]$-radioactivity (d/min)</th>
<th>$[^{3}H]$-radioactivity (d/min) $\times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procedure 1</td>
<td>5.5</td>
<td>$[^{14}C]$-d-galactose</td>
<td>$\leq 0.5%$</td>
<td></td>
</tr>
<tr>
<td>Procedure 2</td>
<td>5.5</td>
<td>$[^{14}C]$-N-acetyl-d-glucosamine</td>
<td>$\leq 0.3%$</td>
<td></td>
</tr>
<tr>
<td>Procedure 3</td>
<td>22</td>
<td>$[^{14}C]$-N-acetyl-d-glucosamine</td>
<td>2.0 $\times 100$</td>
<td></td>
</tr>
</tbody>
</table>

and $[^{3}H]$-leucine from the fraction containing virus by chloroform treatment and isopycnic sedimentation.

The amount of the $[^{14}C]$-label found in the virus fraction is extremely small. It has to be pointed out that the $3 \times 10^{9}$ d/min of $[^{14}C]$-N-acetyl-d-glucosamine (Table 2) represent the $[^{14}C]$-radioactivity of the total sample (0.7 ml) of poliovirus obtained from $6 \times 10^9$ cells, i.e. $6 \times 10^{11}$ p.f.u. The $[^{14}C]$-radioactivity was measured on 10 µl samples which contained about 40 to 45 d/min. This value is just 1.5 to 2 times above the background of radioactivity detected in the CsCl gradient. No corrections of the measured radioactivity were made which were due to a shift of $[^{3}H]$-radioactivity into the $[^{14}C]$-channel. Therefore, this amount of $[^{14}C]$-radioactivity in the virus fraction is the maximal figure for $[^{14}C]$-radioactivity in the virus. In cases in which corrections for the spill-over were made, the $[^{14}C]$-radioactivity was just in the range of the background. This indicates that purified poliovirus samples were essentially free of N-acetyl-d-glucosamine.

Relation of $[^{14}C]$- to $[^{3}H]$-radioactivity in poliovirus particles

In order to determine the ratio of radioactive $[^{14}C]$ to $[^{3}H]$ in purified poliovirus preparations, the absolute radioactivity (d/min) of both isotopes was measured in purified virus preparations. The results are summarized in Table 3. In the experiments performed, the amount of radioactive carbohydrates was found to be in the range of 0.3 % to 0.5 % or less of the protein radioactivity. As mentioned above, this is a maximal figure.

There were, however, experiments in which about 2 % of $[^{14}C]$-radioactivity compared to $[^{3}H]$-radioactivity was found (Table 3). But in these cases the isotope was present for 22 h in the infected cells (see Methods). This time of incubation is evidently too long since the added carbohydrates can be converted to other cell constituents through cellular metabolism as recently reported (Burness et al. 1973).

The distribution of $[^{3}H]$- and $[^{14}C]$-label on the four virus polypeptides by electrophoresis in polyacrylamide gels could not be determined due to the extremely low $[^{14}C]$-radioactivity of purified poliovirus preparations.

DISCUSSION

Although a variety of enveloped viruses contain glycoproteins, it is noted that they are neither present in purified preparations of poliovirus as described here nor in EMC virus, another member of the picornavirus group as recently reported (Burness et al. 1973). Unfortunately, sufficient data are not available on the content of glycoproteins in adenovirus, papovavirus or other non-enveloped animal viruses. One can only conclude from the data published that non-enveloped viruses are free of measurable amounts of carbohydrates beyond the nucleic acid’s pentose (Green, 1969) and are therefore also free of glycoproteins. Publications suggesting the presence of carbohydrates in some picornaviruses were based on the periodate inactivation of the haemagglutinating activity of Coxsackie virus (Tillotson
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& Lerner, 1966), EMC virus (Kunin, 1967) or of the infectivity of ME virus (Mak, 1969; Rueckert, 1971). Periodate, however, not only destroys carbohydrates but also reacts with proteins, preferentially with their amino acids containing SH groups, as well as with serine and threonine.

The isolation of glucosamine (Halperen, Stone & Korant, 1970) from echovirus 12 could be due either to a contamination of the virus particle by cellular material firmly attached to the virus particle or it could be due to the trapping of free glucosamine during the assembly of the virus particle as suggested by Halperen et al. (1973).

Although Burness et al. (1973) found radioactive label of the employed carbohydrate in purified EMC virus particles, they clearly demonstrated that this radioactivity was not associated with the carbohydrate itself but was transferred to amino acids by metabolic pathways of the cell. Thus, in no case has a glycoprotein been found in picornavirus particles. Preliminary experiments also revealed that non-capsid virus proteins (NCVP's) of poliovirus were devoid of carbohydrates (G. Kaluza, personal communication).

From the chemical composition of poliovirus (Levintow & Darnell, 1960; Munyon & Salzman, 1962; Cooper & Bennett, 1973) it is evident that serine and threonine are present in a high proportion among the amino acids of the virus protein. These amino acids are the main anchorage points of carbohydrates to polypeptides (Gottschalk, 1972). At the moment it is not clear why these amino acids are not glycosylated during the replication of poliovirus. One possible reason could be that the biosynthesis of poliovirus polypeptides proceeds at sites of the cell lacking glycosylating enzymes. Another explanation could be a possible phosphorylation of serine and threonine of poliovirus polypeptides as described, for example, in adenovirus polypeptides (Russell et al. 1972; Tao & Doerfler, 1972).

The absence of carbohydrates in picornaviruses and probably in other virus groups makes it possible to use radioactive monosaccharides as markers of cellular components. We suggest that the use of radioactive carbohydrates should allow one to follow the biosynthesis of membranes containing carbohydrate in the course of virus infection. Thus, the increased biosynthesis of cellular membranes during picornavirus infection as revealed by electron microscopy (Dales et al. 1965; Amako & Dales, 1967a; Skinner, Halperen & Harkin, 1968) and by biochemical methods (Penman, 1965; Amako & Dales, 1967b; Caliguiri & Tamm, 1970; Mosser et al. 1972; Mosser, Caliguiri & Tamm, 1972) could be followed, using radioactive carbohydrates. It is hoped that a better understanding of the cellular sites of the biosynthesis of viruses will answer the question why certain proteins of some virus particles are glycosylated but others are not.

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