Inhibition of the Multiplication of Vesicular Stomatitis and Newcastle Disease Virus by 2-Deoxy-D-Glucose

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The production of infectious vesicular stomatitis (VSV) and Newcastle disease virus can be completely inhibited by 2-deoxy-d-glucose in pyruvate-containing medium, if virus either grown in pyruvate-containing medium or dialyzed against phosphate-buffered saline is used for infection. Under these conditions, the synthesis of all VSV proteins is reduced. VSV RNA, which is synthesized at reduced rates, seems to be unstable. The effect is completely reversible. If virus grown in glucose-containing medium is used for infection, the production of both viruses is not significantly inhibited by 2-deoxy-d-glucose. Under these conditions the production of the VSV glycoprotein is specifically impaired, but does not lead to a marked reduction of the yield of infectious virus.

Recently it was shown that the multiplication of most enveloped viruses can be inhibited by 2-deoxy-D-glucose or glucosamine (3, 4, 6-8, 10) through interference with viral glycoprotein synthesis. The only exceptions so far tested were vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) (7). Since all of these viruses contain glycoproteins as constituents of their envelope, it is not understood why the production of some of these viruses is resistant to the action of the sugar derivatives.

2-Deoxy-D-glucose is incorporated into viral glycoproteins mainly in place of mannose and interferes with the processing of viral precursor proteins (6, 10). Studies on the uptake and incorporation of the labeled sugar antimeabolite have revealed that 2-deoxy-D-glucose is taken up and incorporated best into those cells which were infected with viruses the multiplication of which is inhibited most efficiently (14). Thus it was thought that the difference in the uptake and activation of the antimeabolite in the various virus cell systems might be responsible for the different action on virus multiplication.

As far as the multiplication of Semliki Forest virus is concerned, it was found that 2-deoxy-d-glucose is about 10 times more effective in media which do not contain sugars interfering with the uptake of the antimeabolite (6). Therefore, it was tested whether the synthesis of viruses which are not inhibited under normal conditions is sensitive against the sugar antimeabolite in media containing pyruvate as the energy source instead of glucose.

MATERIALS AND METHODS

Tissue cultures and virus infection. Primary chicken embryo cells in plastic petri dishes of 9 cm in diameter were used 24 h after seeding at a cell density of 2 x 10⁶ cells per culture. VSV, strain Indiana, or NDV, strain Italien, were propagated in primary chicken embryo cells, starting with a multiplicity below 1, either in Earle medium containing 10 mM glucose or in Earle medium with 10 mM pyruvate, instead of glucose, and buffer containing N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid (HEPES), pH 7.4, (5) instead of bicarbonate. The supernatant medium of these cultures was used 15 h after infection for the single cycle experiments at a multiplicity of about 100 PFU/cell.

Virus quantitation. Virus infectivity was titrated by the common plaque assay in primary chicken embryo cells. NDV was determined also by the hemagglutination test at 4 C.

Determination of viral RNA. Synthesis of viral RNA in vivo was followed by pulse labeling cells in the presence of 0.5 μg of actinomycin D per ml with 1.25 μCl of [³H]uridine per culture for up to 1.5 h starting at the times indicated in the tables and figures. The radioactive acid-soluble intermediates were determined after three washings with phosphate-buffered saline by two repeated extractions of the cells with 5 ml of 6% trichloroacetic acid each, and counting a portion in the Tri-Carb scintillation counter (Packard). After two further washings with trichloroacetic acid, ethanol, and methanol, the dried cell layers were dissolved in 1 ml of 0.2 M NaOH for 10 min at 60 C and assayed for radioactivity (15).

In another experiment cells were extracted by phenol after three washings with phosphate-buffered saline. The labeled RNA was precipitated with ethanol and dissolved in 2 ml of 5 mM Tris-hydrochloride (pH 8) containing 1 mM EDTA. Portions of 0.1 ml
were hybridized with a surplus of RNA isolated from virus particles as described (16).

The determination of the viral RNA polymerase was essentially the same as described for the corresponding influenza enzyme using a cytoplasmic extract. The incorporation of \(^{3}H\)GTP into acid-precipitable material was followed with time. Portions were used for phenol extraction and hybridization as described above (12).

**Polyacrylamide gel electrophoresis of VSV proteins synthesized in vivo.** At the times after infection as indicated, a pulse with \(^{3}H\)tyrosine, \(^{3}H\)valine, and \(^{3}H\)leucine, in total 20 \(\muCi\) per culture, was started. One hour later the cells were processed. Proteins were dissolved with sodium dodecyl sulfate and mercaptoethanol and separated by electrophoresis on 10\% polyacrylamide gels as described by Lämmli (11). The slicing and processing of gels for the determination of radioactivity by liquid scintillation has been reported previously by Klenk et al. (9). The nomenclature of the various peaks was adopted from Wagner et al. (17).

For the labeling of viral particles, a pulse with 50 \(\muCi\) of a \(^{3}H\)amino acid mixture per culture as above was started 2 h after infection. The virus particles released into the medium were collected 8 h after infection. They were mixed with nonlabeled virus concentrate and pelleted at 50,000 rpm for 1 h in an SW56 rotor (Spinco), and after resuspension the sample was centrifuged in a 10 to 45\% sucrose gradient for 45 min at 32,000 rpm in an SW41 rotor (Spinco). According to Cartwright et al. (2), the A particles and B particles were collected and worked up for the gel electrophoresis separately. Infectivity was found only with the A particles.

**Chemicals and isotopes.** 2-Deoxy-D-glucose was purchased from Serva, Heidelberg, Germany. [5-\(^{3}H\)]uridine (29 Ci/mol), L-[4,5-\(^{3}H\)]leucine (38.0 Ci/mol), L-[2,3-\(^{3}H\)]valine (5.3 Ci/mol), L-[3,5-\(^{3}H\)]tyrosine (1.0 Ci/mol), and [U-\(^{14}C\)]protein hydrolysate (57.0 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. [8-\(^{3}H\)]GTP (9 Ci/mmol) was obtained from Schwarz/Mann, Orangeburg, N.Y.

**RESULTS**

**Dose response curve of 2-deoxy-d-glucose on the multiplication of VSV and NDV.** Recently it was demonstrated that 2-deoxy-D-glucose does not inhibit the multiplication of VSV and NDV significantly when glucose-containing Earle medium is used (7). Figure 1 shows that rather low doses of the metabolite interfere with the production of both viruses in pyruvate-containing medium if glucose is completely omitted, even during the infection period. This was achieved by using virus for infection that either had been dialyzed overnight against phosphate-buffered saline or had been grown in pyruvate-containing medium. In preliminary experiments it was shown that passage of VSV in pyruvate medium had no influence on the yield of infectious virus. Since the infectivity titer was higher when the virus was grown in the presence of pyruvate than when glucose-grown virus was dialyzed overnight, the former preparations were used throughout.

It has to be stressed that, for these studies, 24-h-old tissue cultures were used. If the age of the cultures was 48 h, glucose-grown VSV could also be inhibited to some degree by 2-deoxy-D-glucose in pyruvate-containing medium. In this case the difference between pyruvate- and glucose-grown VSV was much less pronounced. Concerning the effect of glucosamine on the UTP pool and on the production of UDP-sugar derivatives, 24-h-old chicken embryo cells and 48-h-old cultures also behave differently (13).

**Effect of 2-deoxy-D-glucose on the multiplication of VSV added or counteracted at different times after infection.** When cells were infected with pyruvate-grown VSV, the inhibition of multiplication by 0.5 mM 2-deoxy-D-glucose can be counteracted by glucose as well as mannose at any time after infection. As shown in Fig. 2, addition of 10 mM glucose at 4 h after infection leads to an immediate rise of the titer of infectious virus without any lag. This means that the early steps of virus multiplication like adsorption and penetration should proceed even in the presence of the antimetabolite.

Addition of 2-deoxy-D-glucose at different
After infection the cells were incubated in pyruvate-containing medium. The cultures grown in pyruvate-containing medium received 0.5 mM 2-deoxy-D-glucose. To some of the latter cultures 10 mM glucose was added 4 h after infection (see arrow). The infectivity was assayed at times as indicated on the abscissa. Essentially the same results were obtained when mannose was investigated instead of glucose. Symbols: ×, glucose-containing medium, no 2-deoxy-D-glucose; ○, pyruvate-containing medium, 0.5 mM 2-deoxy-D-glucose; ▲, same as ○, but 10 mM glucose was added 4 h after infection.

times after infection with pyruvate-grown VSV influences the multiplication at any time after infection (Fig. 3). There is only some additional virus produced after addition of the antimetabolite. The leakage is probably due to the time needed for the uptake and activation of 2-deoxy-D-glucose. Virus release is not inhibited.

Synthesis of VSV proteins in the presence of 2-deoxy-D-glucose. The polyacrylamide gel patterns shown in Fig. 4 (left) demonstrate that, under conditions which inhibit virus multiplication by 3 to 4 log units, the synthesis of all virus proteins is inhibited. At relatively low doses of the antimetabolite, the viral glycoprotein G appears at the same position as in the uninhibited system. The same pattern was found also when the pulse length with the labeled amino acids was only 3 min (not shown). A corresponding inhibition of all viral proteins was found also if 2-deoxy-D-glucose and the label were added 4 or 6 h after infection (not shown). These polyacrylamide gel patterns appear identical to those presented in Fig. 4 (left). It is interesting to note that, although virus production is completely inhibited by 2-deoxy-D-glucose, the effect of the infection on cellular protein synthesis is the same as in the uninhibited system. This is shown in Fig. 4D, where the background of incorporation of labeled amino acids into cellular proteins is negligible.

If virus propagated in pyruvate medium is used for infection, in the presence of 0.5 mM...
FIG. 4. Polyacrylamide gel electrophoresis of VSV-infected chicken embryo cells labeled with amino acids and treated with different doses of 2-deoxy-D-glucose. Cells were infected with VSV grown in the presence of either glucose (right) or pyruvate (left). After infection all cultures were incubated in pyruvate medium containing 0.5 µg of actinomycin D per ml. The cultures received no (A and E), 0.05 mM (B), 0.2 mM (C), 0.5 mM (D and F), 2 mM (G), and 5 mM (H) 2-deoxy-D-glucose. Four hours after infection a pulse with a mixture of equal amounts of 3H-labeled tyrosine, leucine, and lysine (total of 20 µCi per culture) was started. The cells were processed 1 h later (●). For comparison samples B and G were subjected to coelectrophoresis with proteins of cells which were infected with glucose-grown VSV and were preincubated with glucose-containing medium. The cells were pulsed with [14C]protein hydrolysate (1 µCi per culture) from 4.5 to 5.5 h after infection (○).
2-deoxy-D-glucose no virus particles could be seen by electron microscopy within or on the periphery of the cells.

In Fig. 4 (right) data are included concerning the effect of 2-deoxy-D-glucose on viral protein synthesis, when glucose-grown VSV is used for infection. It can be seen that, under conditions when there is only little or no effect on the yield of infectious particles, the synthesis of viral glycoprotein G is specifically inhibited. Several additional peaks with higher and lower molecular weights than the glycoprotein can be seen which were observed either only as minor peaks or not at all, when 2-deoxy-D-glucose was omitted. The production of unglycosylated proteins like N and L is not significantly impaired.

The radioactivity in the NS peak relative to the other peaks is increased. It cannot be excluded that some faultily translated or processed G glycoprotein appears in this region of the gel under the conditions employed.

Thus the antimeatabolite seems to have two completely different effects, depending on the virus preparation used for infection. The latter effect is comparable to that on the production of Semliki Forest virus proteins (6).

When virus particles labeled in vivo and released into the medium were analyzed by gel electrophoresis, the patterns shown in Fig. 5 were obtained. If glucose-grown VSV is used for infection and if 0.5 mM 2-deoxy-D-glucose is present in the pyruvate medium, the A particles as well as the B particles released into the medium contain at least some false glycoprotein, which does not migrate into the same position as the glycoprotein of particles of untreated cells. The pattern, however, is not as heterogeneous as the corresponding one of infected cells in the presence of 2-deoxy-D-glucose (Fig. 4F and G).

**Effect of 2-deoxy-D-glucose on RNA synthesis of VSV and on the induction of the viral RNA polymerase.** Synthesis of VSV RNA in vivo has been studied by inhibiting the production of cellular RNA by actinomycin D and starting a pulse with [3H]uridine at 5 h after infection. Since VSV particles already contain RNA polymerase activity (1), as a control cells were pulsed were also 1 h after infection, when only the RNA polymerase introduced by the invading particles used for infection could be active. As shown in Fig. 6 (right), in the presence of inhibiting doses of 2-deoxy-D-glucose, some viral RNA is still being synthesized which cannot be due exclusively to the particle polymerase. At the relatively low multiplicity of infection employed, the particle polymerase is not active enough to give an incor-

**Fig. 5.** Polyacrylamide gel electrophoresis of VSV particles labeled with [3H]amino acids in the presence of 0.5 mM 2-deoxy-D-glucose. Cells were infected with glucose-grown VSV and incubated in pyruvate-containing medium in the presence of 0.5 mM 2-deoxy-D-glucose. Two hours after infection, 50 µCi of [3H]amino acid mixture per culture was added and the released particles were harvested 8 h after infection. The upper panel represents the pattern of the A particles, the lower panel represents that of the B particles. For comparison, infected cultures without 2-deoxy-D-glucose were labeled with [3H]amino acids. The corresponding particles were subjected to coelectrophoresis with the 3H-labeled particles. Symbols: ●, 3H label; ○, 14C label.

poration of labeled uridine above that of noninfected cells. Since the incorporation of the label into infected cells treated with 2-deoxy-D-glucose does not proceed linearly with time, it is suggested that the product is not stable in vivo. After about 45 min, the system seems to come to an equilibrium between synthesis of viral RNA and breakdown of the newly synthesized product. The values presented in Fig. 6 can be compared directly, since the uptake of labeled uridine into the acid-soluble pool was in all cases almost the same. In another experiment the newly synthesized RNA was characterized further by specific hybridization with a surplus of unlabeled virus particle RNA (Table 1). Most
of the radioactive RNA has a base sequence complementary to particle RNA.

When cells were tested for viral RNA polymerase in vitro 5 h after infection, some polymerase activity could also be detected in the presence of 2-deoxy-D-glucose (Fig. 6, left). By a chase with a surplus of nonlabeled GTP, it is demonstrated that the product synthesized by

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**Table 1. Hybridization of VSV RNA synthesized either in vitro or in vivo**

<table>
<thead>
<tr>
<th>Treatment of sample</th>
<th>Hybridization of RNA (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro RNA</td>
</tr>
<tr>
<td></td>
<td>Control VSV</td>
</tr>
<tr>
<td>Total RNA</td>
<td>232</td>
</tr>
<tr>
<td>Immediately treated with RNase</td>
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</tr>
<tr>
<td>Self annealed</td>
<td>50</td>
</tr>
<tr>
<td>Annealed with 5 μg of VSV-particle RNA</td>
<td>43</td>
</tr>
<tr>
<td>Annealed with 12 μg of VSV-particle RNA</td>
<td>40</td>
</tr>
</tbody>
</table>

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*To cells infected with pyruvate-grown VSV or to noninfected cells, Earle medium containing 10 mM pyruvate was added. All cultures received 0.5 μg of actinomycin D per ml immediately after infection. To the cultures as indicated, 0.5 mM 2-deoxy-D-glucose was added also immediately after infection. For the in vitro RNA synthesis 5 h postinfection a cytoplasmic fraction was prepared and incubated with [3H]GTP plus cofactors for 15 min at 32 C as described (12). For the in vivo RNA synthesis a 30-min pulse with 10 μCi of [3H]uridine per culture was started 5 h after infection. The RNA was extracted with phenol and dissolved in 2 ml of Tris(hydrochloride) buffer (1 mM, pH 8.0) containing 0.5 mM EDTA. Portions of 0.1 ml were used for hybridization as described (16).
extracts of 2-deoxy-D-glucose-treated cells is unstable in vitro. These kinds of results have been obtained in three independent experiments. Although the in vitro product in general does not hybridize as well to nonlabeled particle RNA as the in vivo synthesized RNA, the data presented in Table 1 demonstrate that some of the in vitro product is viral complementary RNA.

Effect of 2-deoxy-D-glucose on cellular protein synthesis. The results of Fig. 4 demonstrate that 2-deoxy-D-glucose has an effect on the production of all viral proteins, not only on glycoprotein, if virus grown in pyruvate medium is used for infection. Therefore it was checked how far 2-deoxy-D-glucose under comparable conditions affects cellular protein synthesis. Figure 7 shows that the incorporation of [3H]leucine proceeds almost linearly with time up to 20 min independently of whether the cells were preincubated with 2-deoxy-D-glucose or not. Later on the incorporation of the labeled amino acid proceeds with a slower rate in the pretreated cells. By a chase with a surplus of nonlabeled leucine, it is shown that the newly synthesized proteins in the control cells are rather stable whereas those of the pretreated cells are partially broken down. These results indicate that 2-deoxy-D-glucose does not significantly influence cellular protein synthesis; however, it seems to have a significant effect on protein turnover.

DISCUSSION

The multiplication of VSV and NDV can be inhibited by 2-deoxy-D-glucose only if virus grown in pyruvate-containing medium or dialyzed overnight against phosphate-buffered saline is used for infection and if the infected cells are further incubated in pyruvate-containing medium. Under these conditions the early steps of virus multiplication occur undisturbed, since, after counteracting the block at later times after infection by addition of either glucose or mannose, infectious particles are produced without any significant lag. Virus multiplication is inhibited at any time during the infectious cycle by the antimetabolite. In contrast to influenza (4, 10) and Semliki Forest viruses (6), 2-deoxy-D-glucose interferes not only with the production of glycoproteins, but with the synthesis of all proteins of VSV, if pyruvate-grown virus is used for infection. The electrophoretic mobility of the glycoprotein of VSV is not measurably changed by incubating infected cells with 2-deoxy-D-glucose. Under the conditions described, virus particles could not be seen in the infected cells by electron microscopy.

It has been shown that in vivo some virus-specific RNA is synthesized in the presence of 2-deoxy-D-glucose which seems to be unstable. The in vitro synthesized RNA using a cytoplasmic fraction of 2-deoxy-D-glucose-treated cells also is unstable. Thus, one possible explanation of the effect of 2-deoxy-D-glucose on the production of infectious VSV might be that, because of the instability of viral RNA, fewer viral proteins and even fewer infectious particles are produced compared with nontreated controls. Why this effect is found only when pyruvate-grown virus is used for infection is completely obscure. How far the production of unstable proteins which can be demonstrated even in uninfected cells in the presence of 2-deoxy-D-glucose (Fig. 7) are involved in this effect cannot be said.

With glucose-grown VSV 2-deoxy-D-glucose has a specific effect on the synthesis and/or processing of viral glycoprotein(s). This observation is comparable to the effect of 2-deoxy-D-glucose on influenza and Semliki Forest virus.
multiplication (4, 6, 10). In contrast to the latter systems, however, the aberrant synthesis of the glycoprotein of VSV seems not to have such a dramatic effect on the production of infectious virus particles. The A particles produced under these conditions contain some aberrant glyco-
proteins. It cannot be said, however, whether these particles are still infectious, since there is also some residual correctly-synthesized glyco-
protein found in the A particle region. It could be that only those particles containing the correct glycoprotein are infectious.

In summary, 2-deoxy-D-glucose acts in two different ways on the multiplication of VSV, depending on the virus preparation used for infection. With glucose-grown virus the yield of infectious virus is almost unimpaired in spite of an aberrant viral glycoprotein synthesis. With pyruvate-grown virus or when glucose-grown virus was dialyzed against phosphate-buffered saline, the yield of infectious virus was strongly inhibited, probably because of an aberrant viral RNA synthesis. The metabolic state of the host cell seems to be quite important in clearly demonstrating the difference of these two ef-
fects. In contrast to 24-h-old chick embryo cells, in 48-h-old cells multiplication of VSV after infection with glucose-grown virus can be inhibited to a variable degree by 2-deoxy-D-glucose.

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LITERATURE CITED