JOURNAL OF VIROLOGY, May 1976, p. 644-651
Vol. 18, No. 2

Printed in U.S.A.

Effects of 2-Deoxyglucose, Glucosamine, and Mannose on Cell Fusion and the Glycoproteins of Herpes Simplex Virus

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Received for publication 10 December 1975

2-Deoxyglucose and glucosamine were found to inhibit cell fusion caused by a syncytial mutant of herpes simplex virus and to inhibit the glycosylation of viral glycoproteins in the infected cells. The inhibition of fusion and the inhibition of glycosylation caused by 2-deoxyglucose were substantially prevented when mannose was also present during infection. When glycosylation was inhibited, three new bands were found in the major glycoprotein region on sodium dodecyl sulfate-polyacrylamide gels. These bands may be precursors to the normal glycoproteins. The correlation between fusion and glycosylation in the presence of 2-deoxyglucose, glucosamine, and mannose suggests that the cells cannot fuse if their glycoproteins have a considerably reduced carbohydrate content.

Membrane fusion occurs in a number of biological processes (19). One example of membrane fusion is the process of cell fusion that occurs during a productive viral infection with mutants of herpes simplex virus (HSV). We have isolated a number of *syn* mutants that cause extensive cell fusion starting several hours after the infection of human embryonic lung (HEL) cells. They were isolated from KOS, a type 1 HSV strain, which only causes a small amount of cell fusion (18). Fusion induced by these *syn* mutants can occur at a low multiplicity of infection and requires viral protein synthesis. It has been termed fusion from within to distinguish it from fusion from without, which only occurs at a high multiplicity of infection and does not require protein synthesis (1).

The sugar analogue, 2-deoxyglucose, has been shown to inhibit fusion from within caused by several viruses including a fusion-causing strain of HSV (5, 9, 17). 2-Deoxyglucose has also been shown to inhibit the glycosylation of HSV glycoproteins (3). Glycosylation of influenza glycoproteins has been shown to be inhibited by glucosamine as well as by 2-deoxyglucose (16, 23). The multiplication of Semliki forest virus is inhibited by 2-deoxyglucose, but mannose has been shown to prevent this inhibition (12).

The purpose of these experiments was to further examine the apparent correlation between cell fusion and the glycosylation of viral glycoproteins using one of our HSV *syn* mutants. We have examined the effects of 2-deoxyglucose and glucosamine on fusion and glycosylation and have examined the ability of mannose to prevent these effects.

MATERIALS AND METHODS

Cell cultures and virus stocks. The growth and maintenance of HEL cell cultures and virus stocks were described previously (18). The HSV type 1 strain, KOS, was kindly provided by Priscilla Schaffer (Baylor College of Medicine, Houston, Tex). The mutant of KOS used here, *syn* 20, causes extensive syncytia formation. The procedure for mutant isolation was described previously (18).

The growth medium used in these experiments was a modified F12 containing 10% fetal bovine serum. The saline solution used to wash cells and dilute virus suspensions was a tricine-buffered saline (TBS). Both were described previously (18).

Coulter counter fusion assay. The procedures for virus infection and the assay for cell fusion have been described previously (18). At various times after infection, cells were harvested with trypsin and EDTA and counted using a Coulter counter. The extent of fusion was determined by measuring the number of cells with pulse heights terminating in the threshold interval 10 to 15, which corresponds to the size of small single cells. Cells disappear from this interval as they fuse with other cells and shift to a larger threshold interval.

Radioactive labeling of viral proteins and glycoproteins. The procedures for virus infection were similar to those used with the Coulter counter fusion assay. HEL cells were grown in 2-ounce prescription bottles to approximately 1.2×10^6 cells per bottle. The cells were rinsed twice with TBS and infected by adding 10^7 PFU in 0.2 ml of TBS for 1 h. After attachment, unadsorbed virus were removed and 3 ml of growth medium was added. The infected cells were incubated at 34 C for 4 h to allow for the shutoff of most of the host protein synthesis (10) before

adding ^{14}C-labeled amino acids or sugars. To label proteins, 1 μCi each of alanine, histidine, leucine, proline, and threonine was added to each bottle (average specific activity 200 mCi/mmol, at an average concentration of 0.2 μg/ml). To label glycoproteins, 5 μCi of glucosamine-hydrochloride (specific activity 200 mCi/mmol, at a concentration of 2 μg/ml) or 5 μCi of galactose (specific activity 50 mCi/mmol, at a concentration of 5 μg/ml) was added. Incubation at 34 C was then continued for 20 h before harvesting. Infected cells were scraped from the bottles, pelleted by low-speed centrifugation, and prepared for electrophoresis. To study the effects of 2-deoxyglucose, glucosamine, and mannose, the appropriate sugars were present in the medium throughout the infection.

SDS-polyacrylamide slab gel electrophoresis. Pellets of infected cells were resuspended in 0.1 ml of a sample solution. The sample solution contained 2% (wt/wt) sodium dodecyl sulfate (SDS), 5% (wt/wt) 2-mercaptoethanol, 20% (wt/wt) sucrose, and 0.2 mg of bromophenol blue per ml and was buffered with 0.05 M Tris, adjusted to pH 7.0 with hydrochloric acid. Samples were heated in a boiling-water bath for 5 min.

The slab gel apparatus described by Studier (25) was obtained from Aquebogue Machine and Repair Shop (Aquebogue, N.Y.) and was used with a continuous Tris-borate buffer system at pH 7.1. This included an electrode buffer with 12 g of Tris per liter, 27 g of boric acid per liter, 1 g of SDS per liter, and 1 g of EDTA per liter (D. Spencer, personal communication). Each slab gel was prepared from 30 ml of the electrode buffer containing 5% (wt/vol) acrylamide and 0.13% (wt/vol) *N,N'*-methylenebisacrylamide and polymerized with 0.1 ml of *N,N,N',N'*-tetramethylethylenediamine and 0.2 ml of 10% (wt/wt) ammonium persulfate. Small sample volumes of 0.005 ml were layered into wells at the top of the slab gel under electrode buffer. The voltage was set at 20 V until the marker dye entered the gel and then increased to 100 V for either 2 or 4 h. The slab was then dried and Kodak Blue Brand Medical X-ray film was used to obtain an autoradiogram of the gel.

Chemicals and isotopes. The sugars, 2-deoxy-D-glucose, D-glucosamine-hydrochloride, and D-mannose were obtained from Sigma Chemical Co. (St. Louis); acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and 2-mercaptoethanol from Eastman Kodak Co. (Rochester, N.Y.); 99% pure SDS from Pierce Chemical Co. (Rockford, Ill.); ^{14}C-labeled amino acids, D-[^{14}C]-glucosamine·hydrochloride and D-[^{14}C]galactose from New England Nuclear (Boston, Mass.). 2-Deoxy-D-glucose, D-glucosamine, D-galactose, and D-mannose will be referred to here as 2-deoxyglucose, glucosamine, galactose, and mannose.

RESULTS

Inhibition of cell fusion and inhibition of the glycosylation of glycoproteins by 2-deoxyglucose. HEL cells were infected with the HSV syncytial mutant, *syn* 20, and fusion was measured using the Coulter counter assay described in Materials and Methods. In the absence of 2-deoxyglucose there is an exponential decrease in the number of unfused single cells after the onset of fusion, as described previously (18). Figure 1 shows the effect of adding various concentrations of 2-deoxyglucose to the medium during infection. The rate of fusion was unaffected by 0.1 mM 2-deoxyglucose but was significantly inhibited by a 1 mM concentration and completely inhibited when 10 mM was present.

To study the effect of 2-deoxyglucose on the glycosylation of glycoproteins, radioactive amino acids or glucosamine were added to the medium from 4 to 24 h after infection of HEL cells with *syn* 20 in the presence of various concentrations of 2-deoxyglucose. Infected cells were harvested and their proteins were separated by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. An autoradiogram of the resulting slab gel is shown in Fig. 2. When 0.1 mM 2-deoxyglucose

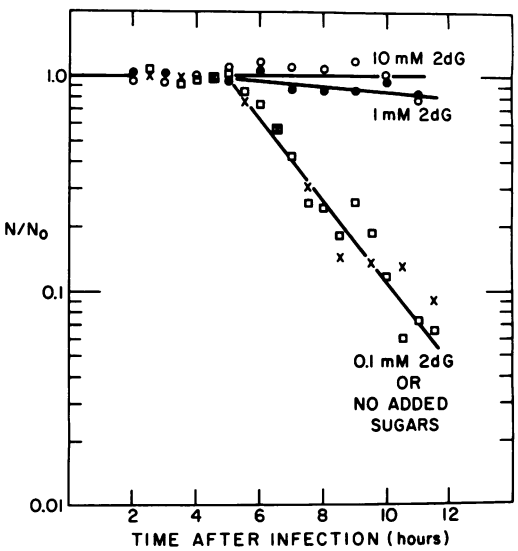

FIG. 1. *The effect of 2-deoxyglucose on cell fusion. HEL cells were infected with the HSV syncytial mutant, syn 20, and incubated in the presence of the indicated concentrations of 2-deoxyglucose (2dG). Cells were harvested at various times after infection and counted using a Coulter counter. Zero time corresponds to the time when the cells were incubated at 34 C after adsorption. The number of unfused single cells in the threshold interval 10 to 15, N, was measured and plotted as the ratio N/N_0, where N_0 was the average number of cells in the interval during the first 5 h after infection. The ratio N/N_0 represents the fraction of cells remaining unfused. No added sugars (□); 0.1 mM 2dG (×); 1 mM 2dG (●); 10 mM 2dG (○).*

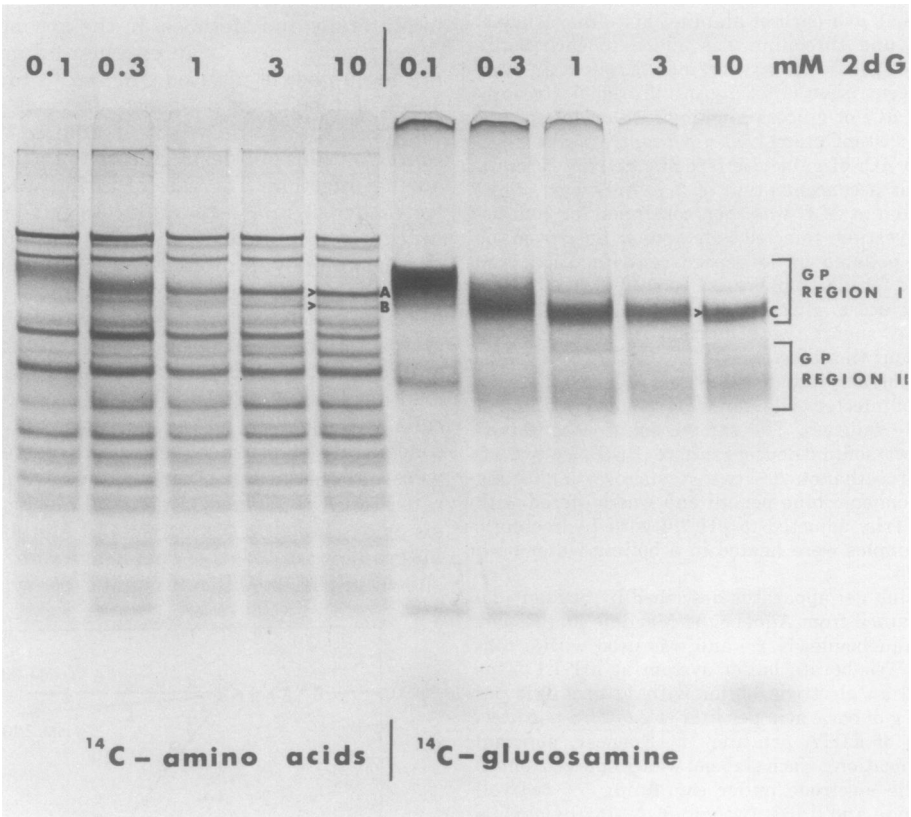

FIG. 2. *The effect of 2-deoxyglucose on the glycosylation of glycoproteins. HEL cells were infected with syn 20 and incubated in the presence of the indicated concentrations of 2-deoxyglucose. Infected cells were labeled with ^{14}C-labeled amino acids or [^{14}C]glucosamine from 4 to 24 h after infection. Their proteins were separated by SDS-polyacrylamide gel electrophoresis at 100 V for 2 h. An autoradiogram of a 5% slab gel is shown with the direction of electrophoresis from top to bottom. The two glycoprotein regions which appear in the absence of 2-deoxyglucose are designated here as GP region I and GP region II. A, B, and C refer to three bands found in GP region I in the presence of 10 mM 2-deoxyglucose.*

was present, the glycoproteins that incorporated labeled glucosamine were found mainly in two regions on the gel, designated GP region I and GP region II on the autoradiogram. Essentially the same pattern was obtained when no 2-deoxyglucose was added (not shown). As the 2-deoxyglucose concentration was increased from 0.1 to 10 mM, the amount of glucosamine label associated with the glycoproteins in GP region I decreased considerably. At the same time the glycoproteins migrated faster on the gel. The broad glycoprotein band containing the amino acid and the glucosamine label gradually shifted to two amino acid-labeled bands, designated A and B, and one glucosamine-labeled band, designated C, all with increased mobility. In contrast to these striking changes in the glycoproteins, the nonglycosylated polypeptides were synthesized in nearly normal amounts and migrated to the same positions on the gel regardless of the 2-deoxyglucose concentration. When these samples were run twice as long on 5% acrylamide gels (see Fig. 4) or on 7.5% acrylamide gels (not shown), the glucosamine band, C, migrated ahead of band B and had very little amino acid label associated with it. This would indicate that band C has a high glucosamine-to-amino acid ratio, even when 10 mM 2-deoxyglucose is present. When the autoradiogram was traced with a densitometer and the areas under the peaks in GP region I were measured, the overall ratio of labeled glucosamine to labeled amino acids incorporated in the presence of 0.1 mM 2-deoxyglucose was approximately 2:1. This ratio decreased to less than 1:1 in the presence of 10 mM 2-deoxyglucose, whereas the total amount of amino acid label remained nearly the same.

Inhibition of fusion and glycosylation by glucosamine. Figure 3 shows the effect of add-

ing various concentrations of glucosamine to the medium during infection with *syn* 20. The rate of fusion was unaffected by 0.1 mM glucosamine but was inhibited by 1 mM glucosamine. When 10 mM glucosamine was present, fusion was completely inhibited.

To compare the ability of glucosamine with the ability of 2-deoxyglucose to inhibit glycosylation, radioactive amino acids or sugars were added to the medium from 4 to 24 h after infection with *syn* 20 in the presence of 10 mM glucosamine, 10 mM 2-deoxyglucose, or with no added sugars. To label glycoproteins in the presence of glucosamine, [^{14}C]galactose was added to the medium, whereas [^{14}C]glucosamine was used in the presence of 2-deoxyglucose. Infected cells were harvested and their proteins were separated by SDS-polyacrylamide gel electrophoresis as before, except that the proteins were separated twice as long to improve the resolution of GP region I (Fig. 4).

Both [^{14}C]glucosamine and [^{14}C]galactose were incorporated into the glycoproteins in GP region I and gave similar patterns when no other sugars were added. The addition of glucosamine resulted in a pattern that was similar to that found when 2-deoxyglucose was added. The amount of sugar associated with the glycoproteins decreased and bands A, B, and C appeared. Peak C, however, did not migrate as fast when glucosamine was added as it did when 2-deoxyglucose was added. The relative decrease in galactose label in the presence of glucosamine did not appear to be as great as the decrease in the glucosamine label in the presence of 2-deoxyglucose. The amino acid-labeled pattern shows four bands in GP region I when glucosamine was added. Band A migrated slightly slower than when 2-deoxyglucose was used as the inhibitor, and a second band is resolved which also appears to be present when no sugars were added. In addition to band B, another faint band can be seen migrating just behind B which could also be present when no sugars were added. In addition, the glycoprotein pattern was altered less by glucosamine than by 2-deoxyglucose at lower concentrations of the inhibitors (not shown).

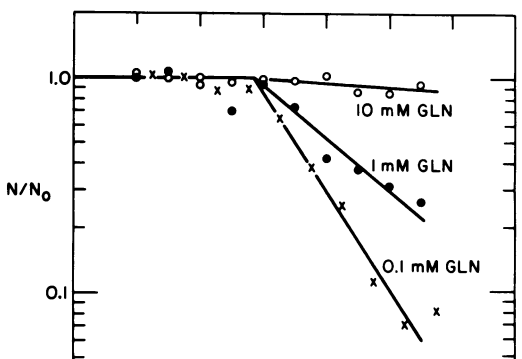

FIG. 3. *The effect of glucosamine on cell fusion. HEL cells were infected with syn 20 and incubated in the presence of the indicated concentrations of glucosamine (GLN). Cell fusion was assayed using a Coulter counter as in Fig. 1.*

Effect of mannose on the inhibition of fusion and glycosylation caused by 2-deoxyglucose and glucosamine. The inhibition of fusion by 2-deoxyglucose was prevented if mannose was added along with the 2-deoxyglucose (Fig. 5A). However, the addition of mannose did not prevent the inhibition of fusion by glucosamine (Fig. 5B).

Figure 6 shows the effect of mannose on the inhibition of glycosylation caused by 2-deoxyglucose or glucosamine. Proteins were labeled with radioactive amino acids or glucosamine from 4 to 24 h after infection with *syn* 20 in the presence of 1 mM 2-deoxyglucose, with or without 10 mM mannose; or they were labeled with radioactive amino acids or galactose during infection in the presence of 3 mM glucosamine, with or without 10 mM mannose. As a control, proteins were labeled with radioactive amino acids or glucosamine in the presence of 10 mM mannose alone. Infected cells were harvested and their proteins were separated by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the slab gel is shown in Fig. 6.

The patterns of the glycoproteins in GP region I which were found when either 1 mM 2-deoxyglucose or 3 mM glucosamine were present show that glycosylation had been inhibited. Mannose substantially prevented the inhibition due to 2-deoxyglucose but had no effect on the inhibition due to glucosamine. The pattern obtained in the presence of 10 mM mannose alone is included for comparison.

DISCUSSION

Analysis of the patterns of proteins and glycoproteins that are obtained by separation on SDS-polyacrylamide gels indicates that glycosylation of the glycoproteins in HSV-infected cells is inhibited by 2-deoxyglucose and by glucosamine. Whereas the patterns were nearly normal for the nonglycosylated proteins, a dra-

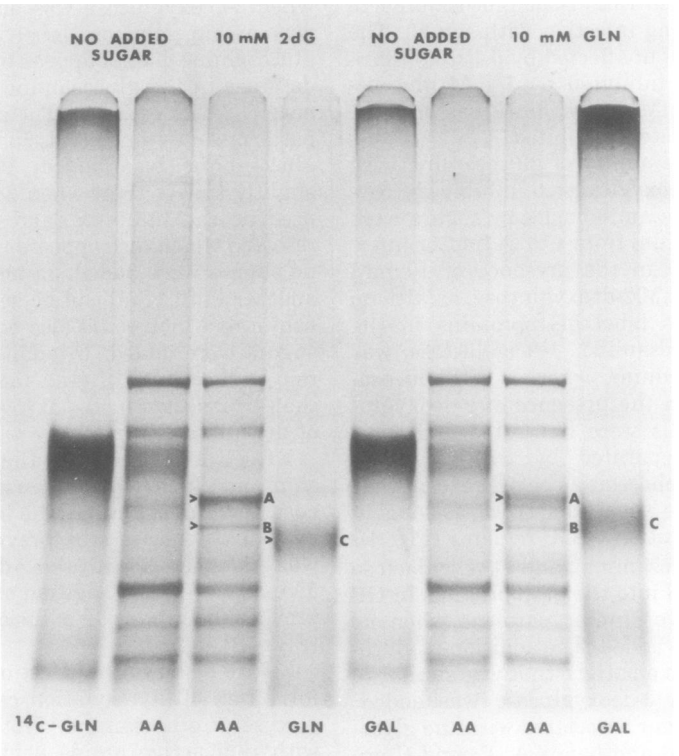

FIG. 4. *The effect of glucosamine on the glycosylation of glycoproteins in GP region I. HEL cells were infected with syn 20 and incubated in the presence of 10 mM glucosamine (GLN), 10 mM 2-deoxyglucose (2dG), or with no added sugar, as indicated. Infected cells were labeled with ^{14}C-labeled amino acids (AA), [^{14}C]galactose (GAL), or [^{14}C]glucosamine (GLN), as indicated, from 4 to 24 h after infection and then harvested. Their proteins were separated on a 5% slab gel at 100 V for 4 h to obtain better separation of the glycoproteins in GP region I. An autoradiogram of the gel is shown. A, B, and C refer to three bands found in GP region I when 10 mM 2-deoxyglucose or 10 mM glucosamine is present.*

matic change occurred in the major glycoprotein region, designated here as GP region I, which corresponds to the C5 region of Courtney et al. (3) and the ICP 9-12 region of Honess and Roizman (10). In the absence of 2-deoxyglucose the amino acid and sugar labels in this region were found as a broad band. As the 2-deoxyglucose concentration was increased from 0.1 to 10 mM, both the amino acid and glucosamine label gradually shifted and were resolved as three bands, designated here as A, B, and C. There was an overall decrease in the glucosamine-to-amino acid ratio in this region, with little decrease in the total amount of amino acid label as it shifted into bands A and B. In the presence of 10 mM 2-deoxyglucose, bands A and B contained essentially no sugar label, whereas band C contained the reduced amount of sugar label which was still incorporated into this region.

Although the relative mobility of glycoproteins on SDS-polyacrylamide gels is not a valid indication of their molecular weight (2, 20, 24), analysis of the mobility of the influenza hemagglutinin synthesized in the presence of 2-deoxyglucose and glucosamine indicates that a reduction in the carbohydrate content of the glycoprotein leads to an increase in its relative mobility (23). Therefore, the increase in relative mobility of the amino acid and sugar label in GP region I, which occurs as the sugar-to-amino acid ratio decreases, indicates that glycosylation is being inhibited. Since there is little decrease in the total amount of amino acid label as it shifts into bands A and B, and since A and B have essentially no sugar label associated with them, they appear to be precursors to the glycoproteins that normally comprise the broad band in GP region I. Band C has very little amino acid label associated with it, but its increased relative mobility indicates that a more fully glycosylated form of C is also found as part of the broad band in the absence of 2-deoxyglucose. Further studies would be needed to establish the precursor-product relationship between

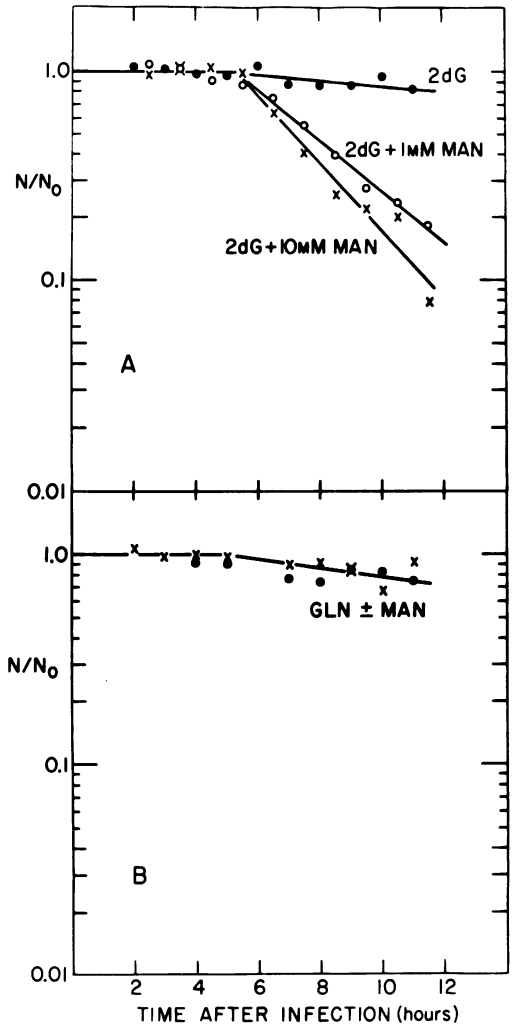

FIG. 5. *The effect of mannose on the inhibition of cell fusion. HEL cells were infected with syn 20 in the presence of (A) 1 mM 2-deoxyglucose (2dG) with or without mannose (MAN); or (B) 3 mM glucosamine (GLN) with (×) or without (●) 10 mM mannose. Cell fusion was assayed using the Coulter counter as in Fig. 1.*

the bands found in the presence and absence of 2-deoxyglucose.

Courtney et al. (3) showed that 2-deoxyglucose altered the pattern of HSV glycoproteins on SDS-polyacrylamide gels and inhibited virus multiplication. Similar effects have been found for several of the RNA viruses (6, 9, 11, 13, 15, 16, 22, 23). The observation reported here that glucosamine also inhibits glycosylation had not previously been demonstrated for HSV but is in agreement with the results in the RNA virus systems (4, 6, 11, 16, 23). The observation that mannose can partially prevent the 2-deoxyglucose inhibition of glycosylation had not been demonstrated previously in any virus system, but mannose had been shown to prevent the 2-deoxyglucose inhibition of the multiplication of several RNA viruses (13, 22). Mannose also prevents the 2-deoxyglucose inhibition of interferon production (7). Recently Courtney has obtained patterns similar to those presented here using slab gels and autoradiography with HEL cells infected with KOS in the presence of 2-deoxyglucose. In addition he has observed that mannose substantially prevents the 2-deoxyglucose inhibition of glycosylation (personal communication). The correlation between the inhibition of glycosylation and virus multiplication reported for HSV by Courtney et al. (3) has also been observed with our syncytial mutant in the presence of both 2-deoxyglucose and glucosamine, whereas mannose prevents the inhibition of virus multiplication caused by 2-deoxyglucose but has no effect on the inhibition caused by glucosamine (unpublished observations).

The inhibition of glycosylation by 2-deoxyglucose and glucosamine is not well understood. In a study on the inhibition of Semliki forest virus multiplication caused by 2-deoxyglucose, it was shown that radioactively labeled 2-deoxyglucose was incorporated into the viral glycoproteins (12). Since mannose prevented the inhibition of virus multiplication and mannose was also found in viral glycoproteins, the authors proposed that the inhibition resulted from the incorporation of 2-deoxyglucose into the glycoproteins in place of mannose. In a study with HSV, labeled 2-deoxyglucose was shown to be incorporated into the viral glycoproteins (3), and we have found that labeled mannose is also incorporated into HSV glycoproteins (unpublished observation). Since mannose prevents the inhibition of glycosylation caused by 2-deoxyglucose in our system, our observations are consistent with the proposal that 2-deoxyglucose is incorporated into the glycoproteins in place of mannose. Under our growth conditions, glucose is present at a concentration of 10 mM, which apparently reduces the antimetabolic effects of 2-deoxyglucose acting as an analogue for glucose, since the nonglycosylated proteins were made in nearly normal amounts. Further studies on the biosynthetic pathways of glycosylation should help to establish more directly the mechanisms for inhibition by 2-deoxyglucose and glucosamine.

Glycoproteins have been found in the plasma membranes of HSV-infected cells (8) and have been implicated previously in cell fusion (14, 17). Throughout this study the effects of 2-deoxy-

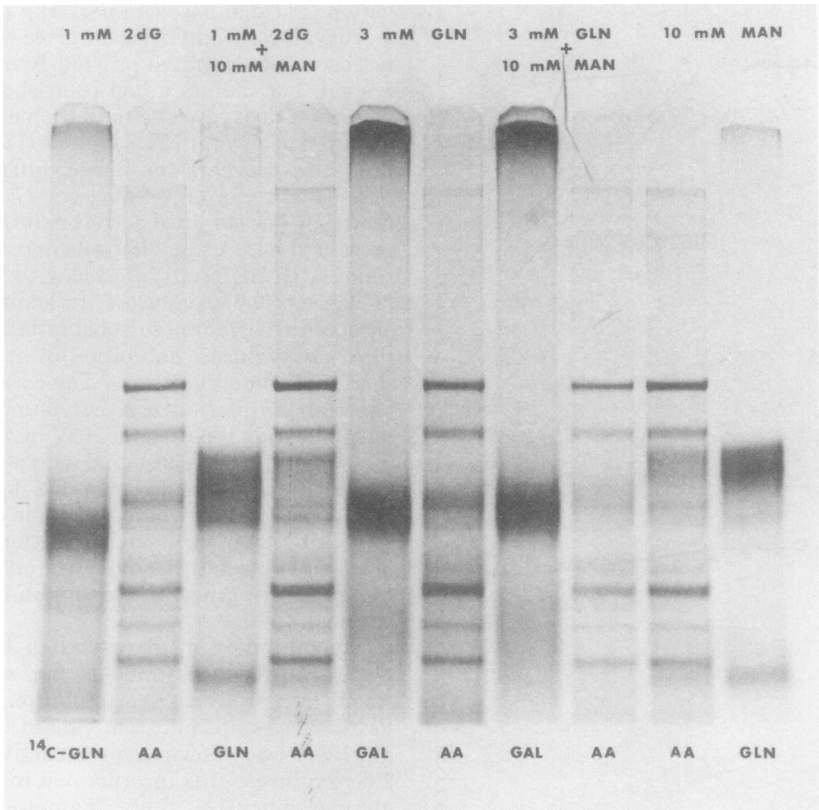

FIG. 6. *The effect of mannose on the inhibition of glycosylation caused by 2-deoxyglucose. HEL cells were infected with syn 20 and incubated in the presence of 1 mM 2-deoxyglucose (2dG) with or without 10 mM mannose (MAN); in the presence of 3 mM glucosamine (GLN) with or without 10 mM mannose; or in the presence of 10 mM mannose alone. Infected cells were labeled with ^{14}C-labeled amino acids (AA), [^{14}C]glucosamine (GLN), or [^{14}C]galactose (GAL), as indicated, from 4 to 24 h after infection and harvested. Their proteins were separated on a 5% slab gel at 100 V for 4 h. An autoradiogram of the gel is shown.*

glucose, glucosamine, and mannose on glycosylation have been compared to their effects on cell fusion. We have confirmed the correlation between the separate observations that 2-deoxyglucose inhibits glycosylation (3) and cell fusion (5). Furthermore, our results extend this correlation between fusion and glycosylation to include their inhibition by glucosamine and the ability of mannose to prevent their inhibition by 2-deoxyglucose. This correlation supports the hypothesis that the cells cannot fuse if their glycoproteins have a considerably reduced carbohydrate content. It should be noted, however, that it is the glycosylated forms of the glycoproteins that are synthesized during a wild-type virus infection in the absence of any inhibitors, even though the wild-type virus causes only a small amount of cell fusion (18). Therefore the difference in the amount of fusion caused by the wild-type virus and the *syn* mutant virus is not due to a major difference in the glycosylation of the glycoproteins comparable to the difference which was observed in the presence and absence of 2-deoxyglucose or glucosamine. Further studies are currently in progress to compare the glycoproteins and glycolipids from wild-type and *syn* mutant-infected cells.

ACKNOWLEDGMENTS

We appreciate the excellent technical assistance of Susan Warner and thank Thomas Holland, G. Sullivan Read, and Paul Keller for helpful suggestions and discussion of the results and manuscript. At the same time that we were pursuing these studies similar results on the effects of 2-deoxyglucose and mannose on the glycosylation process were independently obtained by R. Courtney. His results are being published elsewhere. This work was supported by U.S. Public Health Service grant AI-11513 from the National Institute of Allergy and Infectious Diseases.

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