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Inhibition of Glycosylation of the Influenza Virus Hemagglutinin

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D-Glucosamine and 2-deoxy-D-glucose interfere with the biosynthesis of the hemagglutinin glycoproteins. With increasing inhibitor concentrations a progressive decrease in size of the precursor HA and the cleavage products, HA_1 and HA_2 can be observed. The shift in molecular weight is paralleled by a decrease of the carbohydrate content. This was shown by labeling studies with radioactive sugars which revealed that the inhibitors block the incorporation into glycoproteins, whereas they have no or only slight effects on the uptake and activation of sugars. Under conditions of maximal inhibition, the hemagglutinin proteins lack all or most of their carbohydrates. These findings indicate that the inhibitory effect of D-glucosamine and 2-deoxy-D-glucose is due to an impairment of glycosylation. When glycosylation is inhibited, the precursor polypeptide is synthesized at normal rates. Its cleavage products, however, are very heterogeneous. This suggests that carbohydrate protects the hemagglutinin from proteolytic degradation.

The hemagglutinin of influenza virus consists of a glycoprotein with a molecular weight of about 76,000 which can exist as a single polypeptide chain (HA) or as a disulfide-bonded complex of two polypeptides with molecular weights of about 50,000 (HA_1) and about 30,000 (HA_2) (11, 17, 19, 23-25, 33). Glycoprotein HA is a primary viral gene product from which HA_1 and HA_2 are derived by proteolytic cleavage (17, 20, 24, 25, 34).

D-Glucosamine and 2-deoxy-D-glucose inhibit the formation of hemagglutinin, neuraminidase, and infectious influenza virus (12, 16). Biochemical studies have revealed that in the presence of these sugars glycoprotein synthesis is inhibited, whereas carbohydrate-free polypeptides are still being formed (9, 20). Furthermore, a novel protein (molecular weight 64,000) not present under normal conditions could be detected, if glycoprotein synthesis was impaired. This protein which had been designated HA_0 resembles glycoprotein HA in several respects. Both proteins have a similar amino acid composition. After release of the glucosamine block, HA_0 is converted into a protein with a lower electrophoretic mobility similar to that of HA. Like HA, HA_0 has a high affinity for the membranes of the host cell and migrates from the rough to the smooth endoplasmic reticulum (20, 21).

In the present report we show that HA_0 differs from HA by the lack of carbohydrate. This finding supports the concept that HA_0 is the unglycosylated or incompletely glycosylated polypeptide of glycoprotein HA and that the inhibitory effect of glucosamine and deoxyglucose is due to an impairment of glycosylation. We will also present evidence that the carbohydrates protect the hemagglutinin polypeptide from proteolytic degradation.

MATERIALS AND METHODS

Virus and cells. The Rostock strain of FPV was grown in secondary cultures of chicken embryo fibroblasts (37). Infected cells were maintained in minimal medium (7) with the twofold concentration of glucose (31). Virus stocks were grown in embryonated eggs.

Assays of viral activities. Plaque assays (37) and hemagglutination titrations (6), were carried out according to described procedures.

Chemicals and isotopes. Reagents for polyacrylamide gels, 2-deoxy-D-glucose (reagent grade), D(+)-glucosamine: HCl (reagent grade), *N*-α-*p*-tosyl-L-lysin chloromethyl keton (TLCK, reagent grade), *p*-tosyl-L-phenylalanin chloromethyl keton (TPCK, reagent grade), and phenylmethyl sulfonylfluoride (PHMS, reagent grade) were obtained from Serva, Heidelberg, Germany. Protein [U-^{14}C]hydrolysate, L-[4,5-^{3}H]leucine (55 Ci/mmol), L-[3,5-^{3}H]tyrosine (49 Ci/mmol), L-[2,3-^{3}H]valine (39 Ci/mmol), L-[1-^{3}H]fucose (2,6 Ci/mmol), D-[1-^{3}H]galactose (13 Ci/mmol), D-[1-^{3}H]glucosamine: HCl (3 Ci/mmol), D-[1-^{3}H]mannose (9.1 Ci/mmol), D-[1-^{14}C]glucosamine: HCl (58 mCi/mmol), and uridine diphospho-*N*-acetyl-D-[U-^{14}C]glucosamine (286 mCi/mmol) were purchased from Amersham Buchler, GmbH, Braunschweig, Germany.

Determination of the uptake, activation, and

incorporation into glycoprotein of glucosamine. Confluent monolayers on glass Petri dishes (5 cm diameter) were inoculated at a multiplicity of 10 to 50 PFU/cell. After a 30-min adsorption period, the cells were washed twice with phosphate-buffered saline (PBS). Maintenance medium (2 ml) was added containing the desired concentrations of deoxyglucose. After 4 h of infection [^{14}C]glucosamine (10 μCi per Petri dish) was added. After a 3-h pulse the cells were washed three times with ice cold PBS and extracted three times with 1 ml of 0.9 N perchloric acid in the cold. The amount of radioactivity in the perchloric acid-soluble fraction was determined by counting a portion in a Triton X-100 containing liquid scintillation mixture (27). The pooled extracts were then neutralized with 10 N potassium hydroxide. The precipitate was removed by centrifugation.

For further analysis of the intermediate metabolites, 200 μliters of the supernatant were subjected to paper chromatography according to the methods described by Harms et al. (10). The radioactivity spots were identified in a windowless paper chromatogram scanner (model 280, Berthold, Germany) and quantitated as described previously (M. F. G. Schmidt, R. T. Schwarz, and C. Scholtissek, Eur. J. Biochem., in press). After perchloric acid treatment, the cells were extracted three times with 1 ml of chloroform-methanol (2:1, vol/vol). The delipidized cells were hydrolyzed for 5 h at 100 C with 4 N HCl. The hydrolysate was dried in the vacuum with phosphorus pentoxide and sodium hydroxide. The residue was dissolved in water, and the hexosamines were purified by chromatography on a Dowex 50 column (6 by 1 cm) according to the method of Boas (2).

After reacetylation the amino sugars were examined by paper electrophoresis (8). Electrophoresis was carried out in 1% sodium tetraborate buffer (pH 8.2) (26) on Whatman no. 3 paper for 15 h at 4 V/cm. The radioactivity on the electropherograms was analyzed and quantitated as described above for the paper chromatograms.

Determination of the UDP-N-acetylhexosamine pool. Confluent monolayers in plastic Petri dishes (14 cm diameter) were infected as described above. Maintenance medium (25 ml) containing the desired concentration of deoxyglucose was added. The cells were washed twice with 20 ml of ice-cold PBS and extracted three times with 20 ml of ice-cold 0.9 N perchloric acid 7 h postinfection. To the combined extracts a defined amount of uridine 5'-diphosphate (UDP)-[^{14}C]*N*-acetylglucosamine (ca. 20,000 dpm) was added as an internal standard. After neutralization with 10 N potassium hydroxide, the precipitate was removed by centrifugation. To the supernatant, acetic acid was added to a final concentration of 0.01 M. The activated amino sugars were then adsorbed to charcoal and eluted as described by O'Brien and Neufeld (28). The eluate was concentrated in a flash evaporator and further purified by paper chromatography on Whatman no. 3 paper in ethanol-1 M ammonium acetate (pH 7.5) (5:2, vol/vol) (29) for 50 h. The UDP-*N*-acetylhexosamine region was identified by scanning of the radioactivity as described above and was eluted in water. After lyophilization the activated amino sugars were quantitated by the method of Keppler et al. (14, 15) using a double-beam photometer (DP-Digital, Leitz, Germany).

Labeling of infected cells for polyacrylamide gel electrophoresis. Confluent monolayers on plastic Petri dishes (5 cm diameter) were infected and incubated in maintenance medium containing the desired concentration of deoxyglucose or glucosamine as described above. Four hours after infection the medium was removed and replaced by medium containing radioactive isotopes. These were used at the following concentrations: [^{14}C]protein hydrolysate, 0.5 μCi/ml; [^{3}H]amino acids, 10 μCi/ml; [^{3}H]sugars, 50 μCi/ml. The medium containing the isotopes was left on the cells for the pulse periods indicated in the figure legends. When the pulse was followed by a chase, the radioactive medium was removed and the cells were washed three times with reinforced Eagle medium (1), and incubated in this medium. At the end of the incubation period the cells were washed three times with cold PBS, scraped off with a rubber policeman, and pelleted by low-speed centrifugation. The cells were resuspended in 0.5 ml of distilled water and disrupted by sonication. Ten percent mercaptoethanol (50 μliters) and 10% sodium dodecyl sulfate (50 μliters) were added. After heating for 1 min at 100 C the solution was stored at −20 C.

Polyacrylamide gel electrophoresis. Samples of 50 μliters were adjusted to a final concentration of 0.0625 M Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 0.001% bromophenol blue. Electrophoresis was carried out as described by Laemmli (22). Ten-centimeter gels were prepared in plastic tubes with an inside diameter of 4.5 mm. The stacking gel contained 3%, the resolving gel 10% acrylamide. Electrophoresis was carried out at 2 mA per gel for 1 h and then at 3 mA for about another 4 h. Gels were frozen and fractionated as described before (3). The slices were incubated in polyethylene tubes (2 by 5 cm) with 0.2 ml of Soluene 350 (Packard, Inc. Downers Grove, Ill.) at 65 C for 2 h. Two milliliters of toluene scintillation fluid was added and radioactivity was determined in a Packard Tricarb 3380 counter.

RESULTS

For the analysis of viral polypeptides, in the present study a discontinuous system of polyacrylamide gel electrophoresis has been used which offers a few advantages over the continuous system (36) used in our previous work on fowl plague virus proteins (17, 19, 20, 21). There is a lower background on the discontinuous gels and the bands of the carbohydrate-free polypeptides are sharper. In general, however, the results obtained by both methods are similar with a few exceptions. In the discontinuous system two P proteins can be detected as has been described before by Skehel and Schild (34). The NS protein presumably comigrates with the M protein and cannot be discriminated as a separate band. On all discontinuous gels a radioactivity peak is found in the region around

fraction 90. This peak comigrates with the Coomassie blue marker dye and presumably corresponds to low molecular weight material.

Effect of glucosamine and deoxyglucose on the electrophoretic mobility of the HA glycoproteins. By 4 h after infection in FPV-infected chicken embryo fibroblasts there is an almost complete shift in protein synthesis from host-specific to virus-specific proteins. Therefore, the viral protein pattern emerges clearly from a very low background of host cell proteins (20). Pulse-chase experiments with radioactive amino acids demonstrated that glycoprotein HA is synthesized as a primary viral gene product which subsequently yields by proteolytic cleavage glycoproteins HA_1 and HA_2 (17).

The effect of increasing doses of glucosamine on the precursor protein and the cleavage products has been analyzed in Fig. 1. The polypeptide profile obtained in the absence of the inhibitor after a 5-min pulse followed by a 5-min chase period shows three major virus-specific protein peaks representing the HA, NP, and M polypeptides. Glycoproteins HA_1 and HA_2 are absent. A fourth peak can be detected in the region of fraction 48. This protein migrates in the same position as the shoulder which is regularly found on the front slope of glycoprotein HA_1. It remains to be seen whether it corresponds to the viral neuraminidase, as has been suggested previously (19), or to the host protein described by Skehel (32), or to something else. The two P proteins migrate at fraction 22 and 25, respectively.

If the pulse is followed by a long chase period of 60 min, glycoproteins HA_1 and HA_2 can be detected, whereas HA is almost completely absent as has been shown before (17).

Inhibitory amounts (0.5 to 10 mM) of glucosamine or deoxyglucose (not shown) specifically alter the electrophoretic behavior of the hemagglutinin glycoproteins. These proteins gradually migrate faster as the doses of the inhibitor increase. It is known that the molecular weight

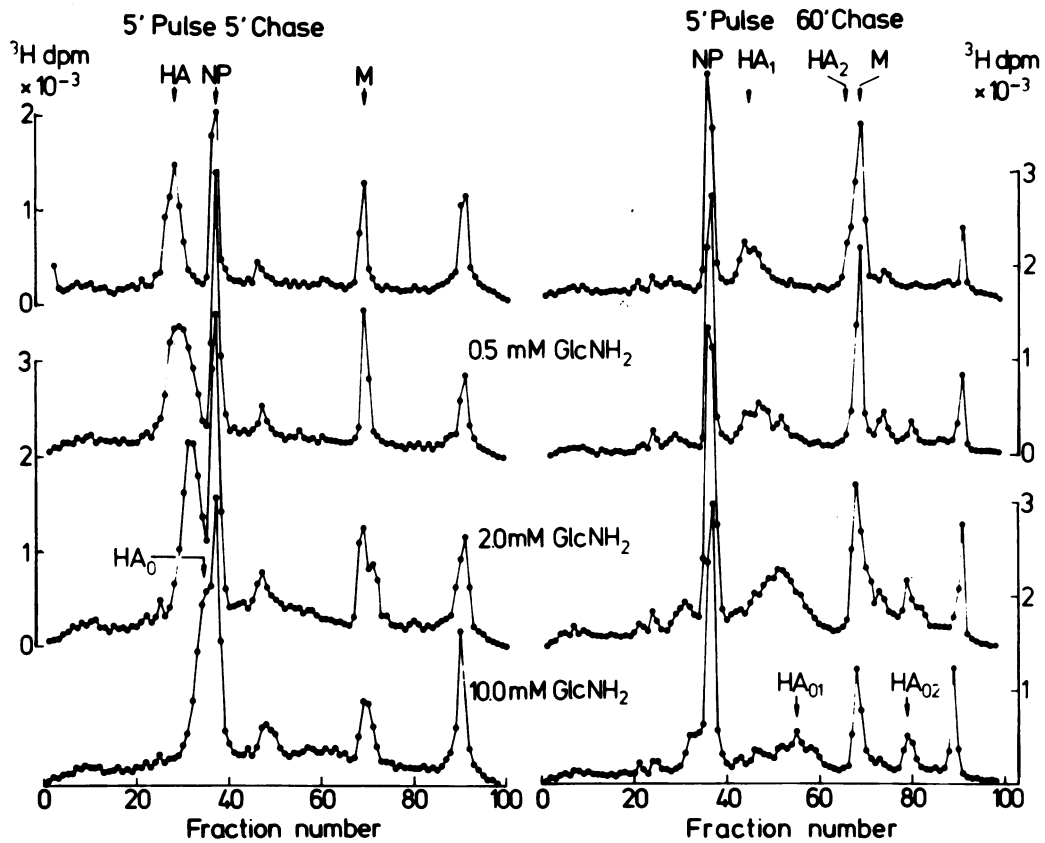

FIG. 1. *Effect of increasing doses of glucosamine on FPV proteins synthesized in chicken embryo fibroblasts. Infected cells were incubated in the absence (top profiles) and the presence of glucosamine and labeled by a 5-min pulse with a mixture of [^{3}H]leucine, [^{3}H]tyrosine, and [^{3}H]valine. The pulse was followed by either a 5- or a 60-min chase period. The proteins were analyzed by polyacrylamide gel electrophoresis. Migration in this and all subsequent gels is from the left to the right.*

of glycoproteins can be determined from the electrophoretic mobility on polyacrylamide gels only with caution. Unlike the proteins, the glycoproteins behave anomalously, their observed molecular weight varies with the gel concentration. However, with increasing acrylamide concentrations the apparent molecular weights of the influenza glycoproteins approach assymptotically constant values which most probably correspond to the real molecular weights (30). The conditions under which our experiments have been carried out (10% polyacrylamide gels) meet these criteria. The data shown in Fig. 1 indicate, therefore, that the molecular weight of HA is gradually shifted from 76,000 over 74,000 (0.5 mM glucosamine) and 70,000 (2 mM glucosamine) to 64,000. This is the molecular weight of HA_0 which is obtained under the conditions of maximal inhibition at a glucosamine concentration of 10 to 20 mM.

A change in the electrophoretic mobility similar to that of glycoprotein HA is also observed in the case of the cleavage products HA_1 and HA_2. Figure 1 shows that glycoprotein HA_1 is gradually shifted from fraction 44 to fraction 55, a position which roughly corresponds to a molecular weight of 40,000. Such a protein peak could be detected before on a cell fraction containing smooth membranes and has been designated HA_{01} (21). In the case of the hemagglutinin protein HA_2 the stepwise shift is not so obvious. However, it is clear that in the presence of the inhibitor, HA_2 can no longer be detected in its original position and that a novel peak appears with a molecular weight of about 23,000 (Fig. 1 and 2). This protein has been detected on smooth membrane fractions, too, and designated HA_{02} (21).

With increasing amounts of glucosamine, the HA_1 peak not only migrates faster but also becomes significantly broader. On some gels it is so broad that it can hardly be discriminated from the background (Fig. 7). This finding indicates that, as HA_1 becomes smaller, it also becomes more heterogeneous in size. The precursor, in contrast, is quite homogeneous at the various stages on inhibition.

Previously we have obtained evidence that protein HA_0 is the precursor of proteins HA_{01} and HA_{02} (21). As has been shown above, the gradual increase in the migration rate of HA is paralleled by an increase in the electrophoretic mobility of HA_1 and HA_2. Both findings are compatible with the concept that HA_0, like HA, is cleaved by proteolytic enzymes to yield HA_{01} and HA_{02}.

To test this hypothesis, proteases have been blocked by the use of three compounds: the trypsin inhibitor TLCK, the chymotrypsin inhibitor TPCK, and PHMS which inhibits both enzymes. The effect of these substances on the synthesis of the HA_0 proteins is shown in Fig. 2. Infected cells were kept in the presence of glucosamine and labeled by a short pulse with [^{3}H]amino acids followed by a short chase period with cold amino acids. Only HA_0 can be detected under these conditions, HA_{01} and HA_{02} are absent. If the chase period is increased to 1 h, HA_0 decreases with a concomitant increase of HA_{01} and HA_{02}. However, if the protease inhibitors are added during the chase period, HA_{01} and HA_{02} do not appear. This indicates that HA_0 is converted into HA_{01} and HA_{02} by proteolytic cleavage.

Effect of glucosamine and deoxyglucose on the carbohydrate content of the hemagglutinin glycoproteins. Previously we have obtained indirect evidence that the inhibitory effect of glucosamine and deoxyglucose on the biosynthesis of the functional hemagglutinin is due to a block of glycosylation (20). In a direct approach towards this problem we determined now the extent of glycosylation by a series of experiments using radioactive sugars.

First we examined the effect of deoxyglucose on the uptake, activation, and incorporation into glycoproteins of glucosamine which is one of the major constituent sugars of the viral hemagglutinin (23).

Four hours after infection cells were labeled for 3 h with [^{14}C]glucosamine, and the amount of radioactivity was determined in the acid-soluble and in the acid-insoluble fraction. A small proportion of the radioactivity in the latter fraction could be extracted with chloroform-methanol, whereas the bulk of the label was bound to protein. Over 90% of the protein-bound label was present in its original form as detected by acid hydrolysis followed by paper electrophoresis. As will be shown later, most of this radioactive glucosamine is incorporated into hemagglutinin glycoprotein. The incorporation of glucosamine into protein is inhibited by deoxyglucose (Fig. 3). Ten millimolar deoxyglucose drastically reduces the radioactivity to about 3% of the uninhibited control.

Figure 3 also shows that deoxyglucose has no effect on the amount of radioactivity which is found in the acid-soluble fraction. This fraction contains most of the metabolic intermediates of glucosamine involved in glycosylation. The metabolic intermediates have been analyzed by paper chromatography. Figure 4 shows that

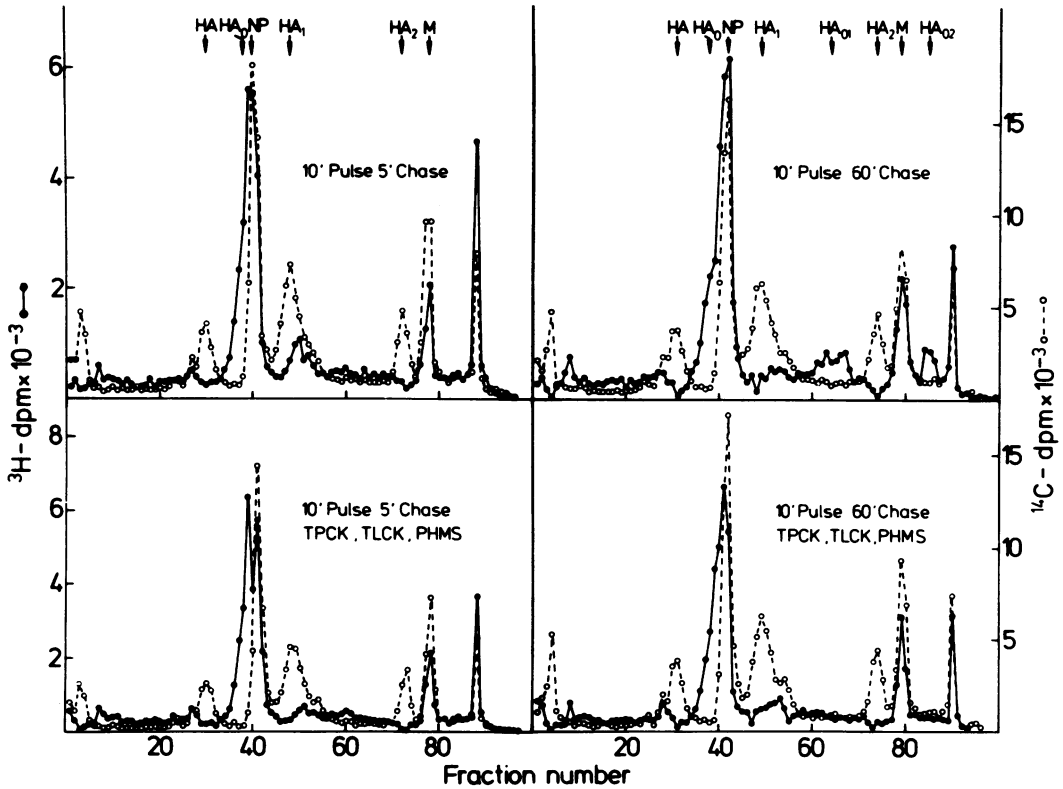

FIG. 2. *Effect of protease inhibitors on the cleavage of polypeptide HA_0. Infected cells were incubated in the presence of 20 mM glucosamine and labeled with a mixture of [^3H]leucine, [^3H]tyrosine, and [^3H]valine for 10 min. The pulse was followed by 5- and 60-min chase periods which were carried out without and with 0.1 mM TLCK, 0.1 mM TPCK, and 0.1 mM PHMS. The proteins were analyzed by polyacrylamide gel electrophoresis. The virus-specific proteins of infected cells grown in the absence of all inhibitors and labeled for 1 h with [^{14}C]amino acids were included as internal markers.*

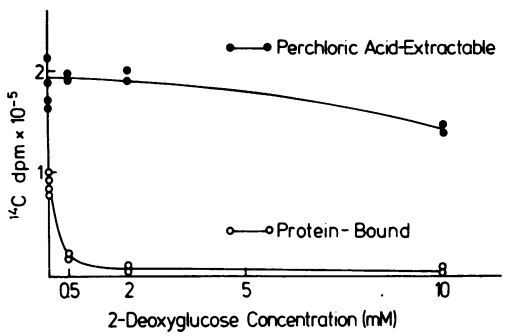

FIG. 3. *Effect of deoxyglucose on the uptake and incorporation of radioactive glucosamine into infected cells. Infected cells were incubated in the absence and the presence of various doses of deoxyglucose. Four hours after infection cells were labeled for 3 h with [^{14}C]glucosamine. The medium was removed and the cells were extracted with perchloric acid. The radioactivity in the acid-soluble and in the lipid-extracted insoluble fraction was determined.*

deoxyglucose slightly changes the distribution of the radioactivity among these compounds. At the highest concentration of the inhibitor the fraction containing both hexosamine and *N*-acetylhexosamine increases from 13 to 30%, whereas the UDP-derivative of *N*-acetylhexosamine decreases from 78 to 62%. This slight decrease of radioactivity in the activated amino sugars (ca. 20%) cannot account for the drastic reduction of glucosamine incorporation into the glycoprotein portion (ca. 97%) (Fig. 3).

The reduced incorporation into protein could be caused, however, by a drastic increase of the UDP-*N*-acetyl-glucosamine pool. It was, therefore, necessary to determine the effect of the inhibitor on the size of this pool. The total amount of UDP-*N*-acetylhexosamine has been measured by an enzymatic assay, and it was found that in the presence of deoxyglucose the pool of the activated amino sugars is elevated (Fig. 5). However, the two to threefold increase

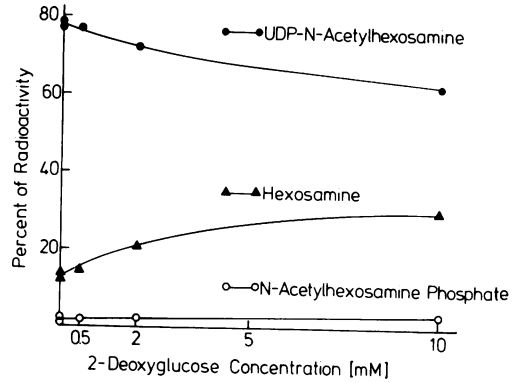

FIG. 4. *Effect of deoxyglucose on the distribution of radioactive glucosamine in the perchloric acid extract. Infected cells were incubated in the absence and the presence of various doses of deoxyglucose and were pulse-labeled with [^{14}C]glucosamine. The perchloric acid extract of the cells was analyzed by paper chromatography as described in Materials and Methods. The relative amount of radioactivity in free hexosamine and its derivatives was determined.*

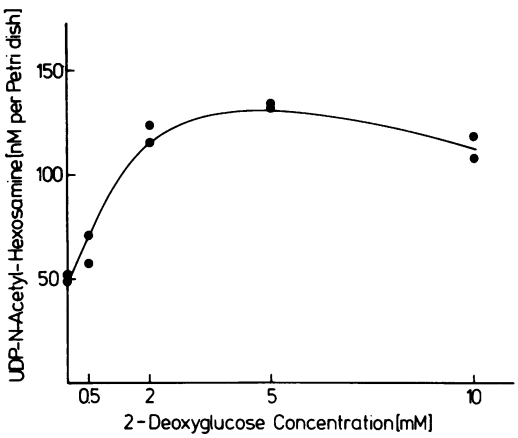

FIG. 5. *Effect of deoxyglucose on the UDP-N-acetylhexosamine pool. Infected cells were incubated in the absence and presence of increasing doses of deoxyglucose. Seven hours after infection the cells were extracted with perchloric acid and the absolute amount of activated hexosamine was determined as described in Materials and Methods.*

of the pool at maximal inhibitor concentration was by far too small to explain the 30-fold reduction of [^{14}C]glucosamine incorporation into glycoprotein (Fig. 3).

Thus, the labeling studies with radioactive glucosamine described so far indicate that deoxyglucose blocks the incorporation into glycoprotein, whereas it has no or only slight effects on the uptake and the activation of the amino sugar.

To examine the extent of glycosylation of the individual hemagglutinin proteins, influenza virus-infected chicken embryo cells were labeled with both a radioactive sugar and a mixture of radioactive amino acids. These experiments were carried out in the absence and presence of inhibiting doses of glucosamine or deoxyglucose. The cells were labeled for 3 h to guarantee that the profile obtained represented a steady-state condition. Such a long pulse was also necessary for sufficient incorporation of the carbohydrate label.

When the cells were labeled with radioactive glucosamine, the patterns shown in Fig. 6 were obtained. Because of the long pulse, most of the hemagglutinin protein is present in the cleaved form. HA, HA_1 and HA_2 are labeled by radioactive glucosamine, most of which is still present in these glycoproteins in its original form as has been shown above. At a deoxyglucose concentration of 1 mM, HA has obtained a higher electrophoretic mobility. HA_1 is broadened and migrates faster, too. In both peaks the ratio of ^{3}H to ^{14}C is significantly lower than in the absence of the inhibitor, indicating a decrease of the glucosamine content. At 5 mM deoxyglucose, glucosamine incorporation is further decreased. HA_1 can no longer be discriminated as a distinct peak. The data indicate that the shift of HA and HA_1 to higher electrophoretic mobilities is paralleled by a decrease of the glucosamine content.

When mannose was used as carbohydrate label, the patterns seen in Fig. 7 were obtained. There seems to be some conversion of the mannose label into amino acids, as suggested by the tritium incorporation into the carbohydrate-free NP protein. When 5 mM glucosamine is added, the incorporation of radioactive mannose into the hemagglutinin proteins is drastically reduced. Similar results have been obtained, when deoxyglucose was used instead of glucosamine (data not shown).

When radioactive fucose was used, again only the hemagglutinin proteins were labeled (Fig. 8). In the presence of glucosamine or deoxyglucose (data not shown), there is a dose-dependent reduction of fucose incorporation. The available evidence suggests that radioactive fucose is not converted into other sugars if it is incorporated into glycoproteins (18, 35). Previously we have reported that the total amount of fucose in HA_1 and HA_2 is higher than in HA (*In* R. D. Barry and B. M. J. Mahy, (ed.), *Negative Strand Viruses*, in press). Figure 8 shows similar results: even in the presence of inhibiting doses of glucosamine, HA_1 contains significantly more fucose than HA. These data suggest that the precursor molecule HA is less complete, with

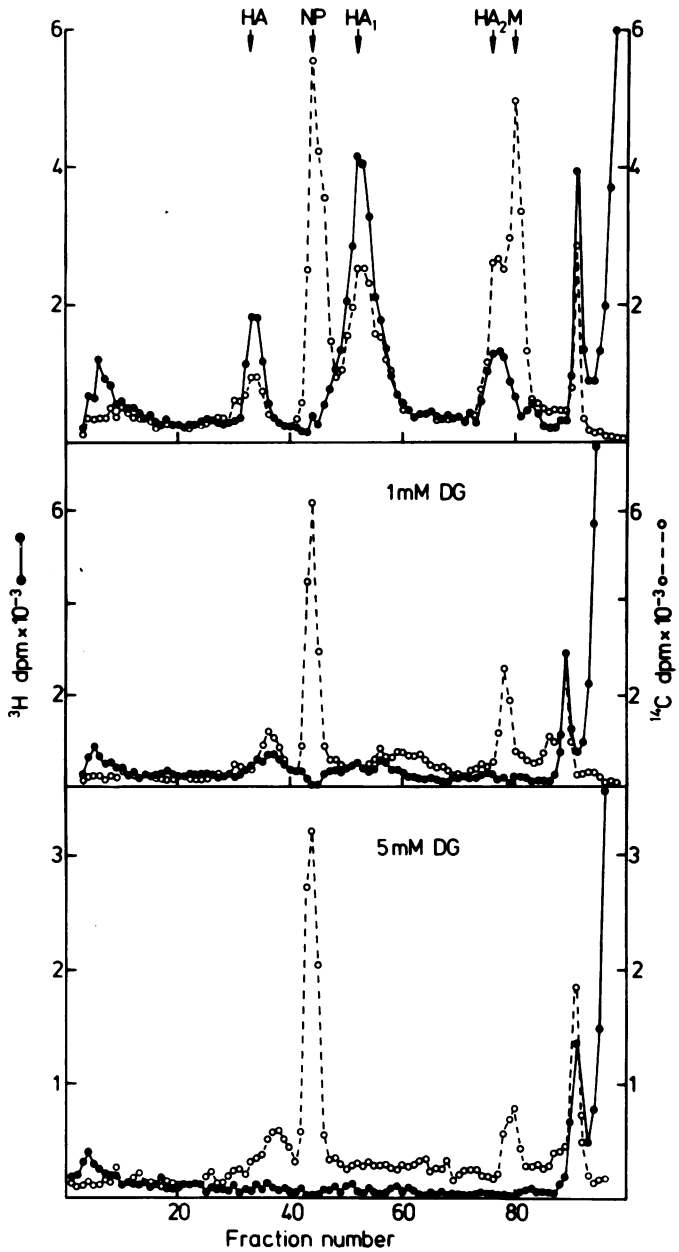

FIG. 6. *Effect of deoxyglucose on the incorporation of glucosamine into the hemagglutinin proteins. Infected cells were incubated in the absence (top panel) and the presence of 1 mM (middle panel), and 5 mM (lower panel deoxyglucose, respectively. The cells were labeled by a 3-h pulse with [^{14}C]amino acids and [^{3}H]glucosamine. The proteins were analyzed by polyacrylamide gel electrophoresis.*

regard to fucose, than the cleavage products.

Radioactive galactose is incorporated into viral glycoproteins predominantly in its original form (18, 35). When it was used as a label for the hemagglutinin glycoproteins, the pattern in Fig. 9 was obtained. As has been found in the case of fucose, the total amount of galactose in HA_1 and HA_2 is significantly (ca. 3-fold) higher than in HA. This finding suggests that HA is less complete, also with regard to galactose, than the cleavage products. In the presence of deoxyglucose the incorporation of galactose is reduced similarly to that of the other sugars analyzed (data not shown).

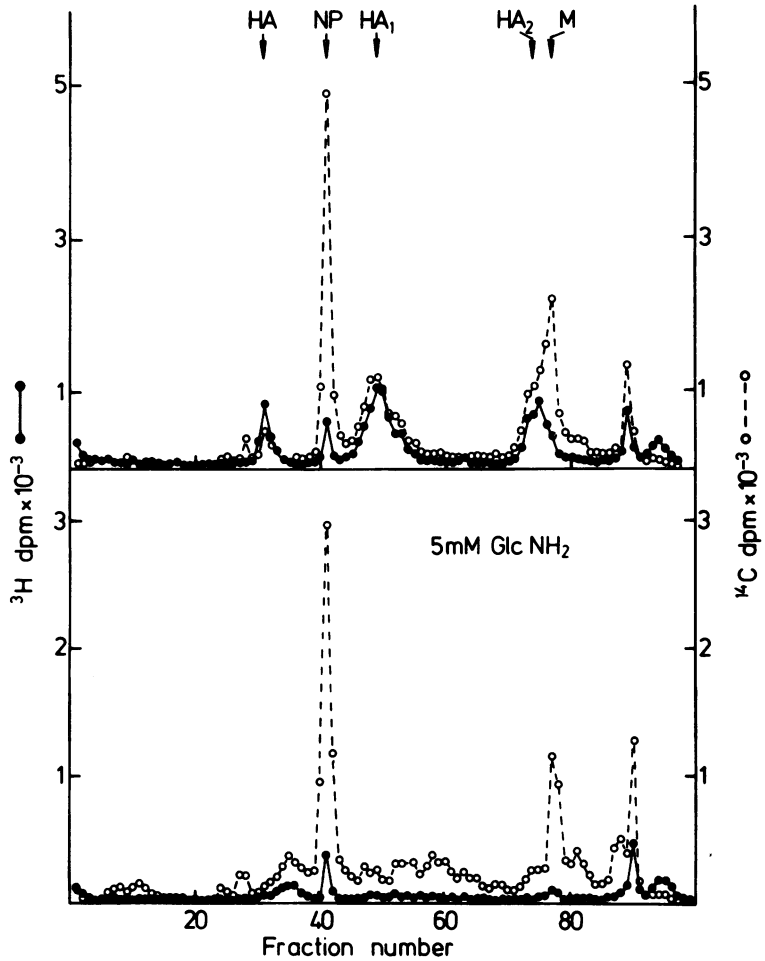

FIG. 7. *Effect of glucosamine on the incorporation of mannose into the hemagglutinin proteins. Infected cells, were incubated in the absence (top panel) and the presence of 5 mM glucosamine (lower panel). They were labeled by a 3-h pulse with [^{14}C]amino acids and [^{3}H]mannose.*

The double-label experiments indicate that increasing doses of glucosamine and deoxyglucose cause an increase of the electrophoretic mobility of the hemagglutinin glycoproteins with a concomitant reduction of carbohydrate incorporation. It has been pointed out above that the incorporation of carbohydrate label approximates the relative carbohydrate content, and that under the conditions used the apparent molecular weight as indicated by the electrophoretic mobility approximates the actual molecular weight. The relationship between carbohydrate content and molecular weight of HA at various stages of inhibition is shown in Fig. 10. Double-label experiments as described above have been carried out with [^{3}H]fucose, [^{3}H]galactose, [^{3}H]mannose, and [^{3}H]glucosamine as carbohydrate label. The experiments were performed in the absence and the presence of increasing doses of glucosamine and deoxyglucose (legend, Fig. 10).

At the various stages of inhibition, the molecular weight of HA has been determined by gel electrophoresis. On the basis of the ratio of ^{3}H to ^{14}C the carbohydrate content of each HA peak was calculated relative to the uninhibited control. Figure 10 clearly shows that the decrease of the molecular weight of HA (76,000) is paralleled by a reduction of the carbohydrate content. When the molecular weight is reduced to that of HA_0 (64,000), if any, only a small fraction of carbohydrate is left.

The incorporation of radioactive glucosamine is reduced more drastically than that of the

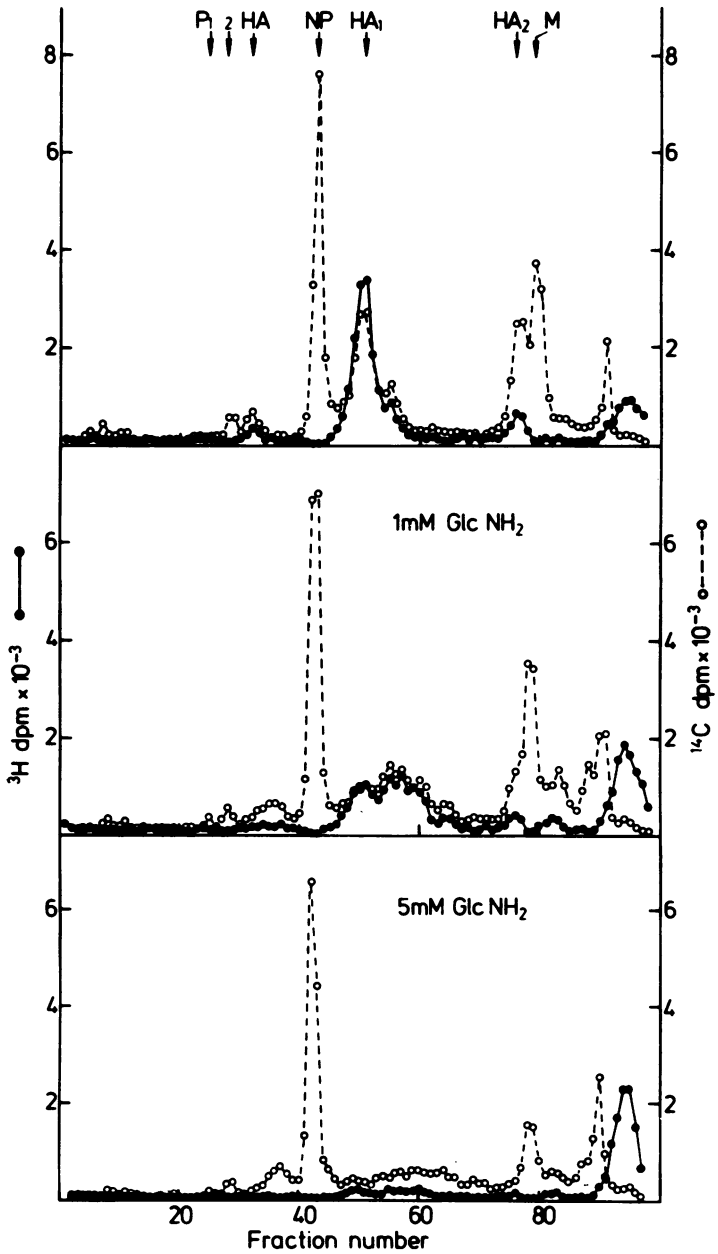

FIG. 8. *Effect of glucosamine on the incorporation of fucose into the hemagglutinin proteins. Infected cells were incubated in the absence (top panel), and the presence of 1 mM (middle panel), and 5 mM (lower panel) glucosamine, respectively. They were labeled by a 3-h pulse with [^{14}C]amino acids and [^{3}H]fucose.*

other sugars. This finding might suggest a comparatively higher decrease of the glucosamine content. More likely, however, in the presence of deoxyglucose the [^{3}H]glucosamine label has been diluted out by the elevation of the UDP-hexosamine pool (Fig. 5).

DISCUSSION

In the presence of deoxyglucose and glucosamine the size of the hemagglutinin glycoproteins decreases. The precursor as well as the cleavage products are affected. The extent of

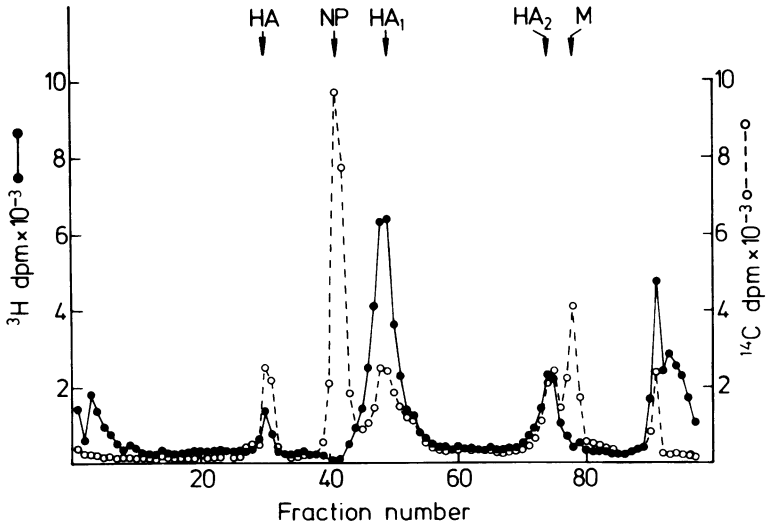

FIG. 9. *Incorporation of galactose into the hemagglutinin glycoproteins. Infected cells were labeled by a 3-h pulse with [^{14}C]amino acids and [^{3}H]galactose.*

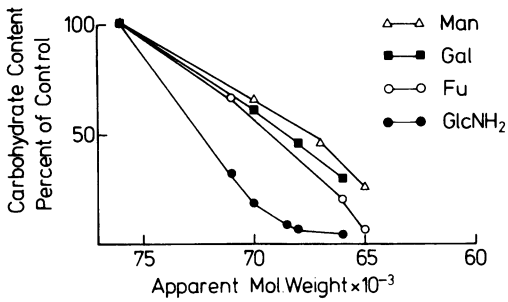

FIG. 10. *Relationship between carbohydrate content and molecular weight of glycoprotein HA at various stages of inhibition. Infected cells were labeled by 3-h pulses with [^{14}C]amino acids and [^{3}H]sugars. Experiments were carried out with [^{3}H]fucose and [^{3}H]mannose at 0, 1, 5, and 20 mM glucosamine, respectively, with [^{3}H]glucosamine at 0, 0.5, 1, 2, 5, and 10 mM deoxyglucose; and with [^{3}H]galactose at 0, 1, 5, and 10 mM deoxyglucose. The cells were subjected to polyacrylamide gel electrophoresis. At the various stages of inhibition the molecular weight of HA has been determined. On the basis of the ratio of ^{3}H to ^{14}C the carbohydrate content of each HA peak was calculated relative to the uninhibited control.*

the decrease is dose dependent. Thus, as the sugar concentration increases glycoprotein HA with a molecular weight of 76,000 is gradually converted into a compound of the molecular weight of 64,000 which has been designated HA_0 (20). Similar effects of deoxyglucose on the glycoproteins of Semliki Forest virus (13) and herpes simplex virus (5) have been reported recently.

When the interference of deoxyglucose and glucosamine with the biosynthesis of the hemagglutinin glycoproteins was detected first, it had already been suggested that this interference might be caused by an impairment of glycosylation and that the difference in molecular weight between HA and HA_0 might be due to a lack of carbohydrate in the latter (20). To prove this hypothesis directly it was necessary to determine the extent of glycosylation of the HA glycoproteins in the absence and the presence of the inhibitors. This has been accomplished by incorporation studies with radioactive sugars. We have not been able to detect a significant interference of the inhibitor with the uptake of the sugar label or with the pool size of the metabolic intermediates. Therefore, the amount of radioactivity incorporated into the hemagglutinin proteins can be taken as a measure for the carbohydrate content. The experiments show that there is a progressive decrease of the carbohydrate content which parallels the change in size of HA and its cleavage products. The decrease comprises all constituent sugars: glucosamine, mannose, galactose, and fucose. HA_0 synthesized under conditions of maximal inhibition appears to be almost free of carbohydrate. This suggests that the difference in molecular weight between HA_0 and HA approximates the carbohydrate complement of the latter (20). The difference amounts to 12,000 daltons. This value agrees well with the carbohydrate content determined by chemical analysis (23). These data taken together provide firm evidence that the inhibitory effect of

deoxyglucose and glucosamine is, indeed, due to an impairment of glycosylation.

Our knowledge on the mechanism by which these sugars inhibit glycosylation is still incomplete. The glycoproteins of Semliki Forest virus (13) and of influenza virus (Wöllert and Klenk, unpublished data) can be labeled by radioactive deoxyglucose. Kaluza et al. (13) have also obtained suggestive evidence that deoxyglucose is incorporated into the glycoproteins as an analogue for mannose thereby terminating further elongation of the carbohydrate side chains. It is, therefore, conceivable that at least in the case of deoxyglucose, HA_0 still contains some carbohydrate. Quite definitely, however, this can be only a small proportion of the carbohydrate complement of the fully glycosylated glycoprotein as has been pointed out above. The mechanism by which glucosamine inhibits glycosylation is at present not understood.

The presence of carbohydrates appears to be necessary for the integrity of the hemagglutinin glycoprotein. This concept is based on the following observations. The extent of glycosylation does not affect the rate of synthesis of the precursor and at the various stages of inhibition, the precursor is synthesized as a rather homogeneous molecular species. The cleavage products, however, exhibit a progressive heterogeneity in size which parallels the loss of carbohydrate. These findings suggest that only a small area of the fully glycosylated polypeptide is susceptible to proteolytic enzymes. Under these conditions, cleavage yields two well defined glycoproteins. If less carbohydrate is attached to the polypeptide the enzymes find more cleavage sites and generate a larger variety of cleavage products. Similar observations have also been made in the case of Semliki Forest virus (13). They are compatible with the concept that the carbohydrates protect the glycoprotein from proteolytic degradation.

The carbohydrate composition of the precursor HA and its cleavage products appears to be different. Precursor and products are equally well labeled with glucosamine and mannose, but there is a considerably higher incorporation of fucose and galactose into the cleavage products. These observations confirm previous ones (*In* R. D. Barry and B. M. J. Mahy, (ed.), *Negative Strand Viruses*, in press). They support the concept that the sugars are attached to the polypeptide in a stepwise fashion: first glucosamine and mannose as the core and then galactose and fucose as the terminal sugars of the carbohydrate side chains. This concept is also supported by the findings of Compans (4) and Stanley et al. (34) who came to similar conclusions by different approaches.

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