Carbohydrates of Influenza Virus

I. Glycopeptides Derived from Viral Glycoproteins After Labeling with Radioactive Sugars

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The carbohydrate moiety of the influenza glycoproteins NA, HA₁, and HA₂ were analyzed by labeling with radioactive sugars. Analysis of glycopeptides obtained after digestion with Pronase indicated that there are at least two different types of carbohydrate side chains. The side chain of type I is composed of glucosamine, mannose, galactose, and fucose. It is found on NA, HA₁, and HA₂. The side chain of type II contains a high amount of mannose and is found only on NA and HA₂. The molecular weights of the corresponding glycopeptides obtained from virus grown in chicken embryo cells are 2,600 for type I and 2,000 for type II. The glycoproteins of virus grown in MDBK cells have a higher molecular weight than those of virus grown in chicken embryo cells, and there is a corresponding difference in the molecular weights of the glycopeptides. Under conditions of partial inhibition of glycosylation, virus particles were isolated that contained hemagglutinin with reduced carbohydrate content. Glycopeptide analysis indicated that this reduction is due to the lack of whole carbohydrate side chains and not to the incorporation of incomplete ones. This observation suggests that glycosylation of the viral glycoproteins involves en bloc transfer of the core sugars to the polypeptide chains.

The envelope of influenza virus contains two glycoproteins, hemagglutinin and neuraminidase. The hemagglutinin glycoprotein may be present as one large molecule, HA (molecular weight, ca. 76,000), or as two smaller fragments, HA₁ (molecular weight, ca. 50,000) and HA₂ (molecular weight, ca. 30,000), which are derived by proteolytic cleavage from HA. The molecular weight of the neuraminidase glycoprotein (NA) varies over a relatively wide range, depending on the virus strain analyzed (see 6, 26). Structural analyses have concentrated on the polypeptide chain of hemagglutinin, of which partial sequences have been elucidated (1, 33). Less is known about the carbohydrate moiety of the glycoproteins. The only isolated glycoprotein on which a carbohydrate analysis has been carried out is HA₁. It was found to contain glucosamine, mannose, galactose, and fucose at molar ratios of 6, 4, 1, and 1, respectively (19).

In infected cells, the glycoproteins are synthesized at the rough endoplasmic reticulum. From there they migrate to smooth internal membranes and, presumably, further on to the plasma membrane, where they are incorporated into mature envelopes. Glycosylation is initiated in rough membranes by the attachment of glucosamine and mannose and com-

pleted in smooth membranes by the attachment of galactose and fucose (see 6, 26).

Valuable, even though indirect, information on the biological function of the carbohydrate moiety of hemagglutinin stems from experiments with glycosylation inhibitors such as 2-deoxyglucose, glucosamine, and 2-fluorohexoses. Under these conditions, the unglycosylated or partially glycosylated polypeptide chain of the glycoprotein is formed (11, 17, 27, 30). Such studies have shown that glycosylation is important for the expression of hemagglutinating activity (12) and for the resistance of these proteins to proteolytic degradation (30), but that it is not necessary for the membrane affinity of these molecules (18).

This study is focused on the carbohydrate side chains of the individual glycoproteins of influenza virus. We will report on size and constituent sugars of these oligosaccharides, on the influence of the host cell and the virus strain used, and, finally, on the oligosaccharides synthesized under conditions of partial inhibition of glycosylation.

MATERIALS AND METHODS

Virus strains. Two influenza A strains were used in this study: A/FPV/Dutch (Hav1Neq1) (referred to as FPV) and A/chick/Germany/49 (Hav2Neq1) (re-

ferred to as virus N). Seed stocks were grown in the allantoic cavity of 11-day-old embryonated eggs and stored as infected allantoic fluids at -80°C .

Cells. The viruses were grown in primary cultures of chicken embryo fibroblasts (39) and in MDBK cells (3).

Growth and radioactive labeling of virus. Confluent monolayers in plastic petri dishes (14 cm in diameter) were inoculated at a multiplicity of infection of 10 to 50 PFU per cell. After a 30-min adsorption period the cells were washed twice with phosphate-buffered saline and incubated with 12 ml of Eagle medium. ^3H -labeled sugars were added to the culture medium at a concentration of $5\text{ }\mu\text{Ci/ml}$, and ^{14}C -labeled sugars were added at $1\text{ }\mu\text{Ci/ml}$. Virus was harvested 20 h after infection.

Plaque assays and hemagglutination titrations. Plaque assays and hemagglutination titrations were carried out according to standard procedures (8, 39).

Purification of virus. Virus was purified by equilibrium centrifugation on sucrose gradients (15). Tartrate gradients (7) were used when virus was grown in the presence of inhibitors of glycosylation.

Polyacrylamide gel electrophoresis. Previously described procedures were used for gel electrophoresis in analysis (30) and preparation (25).

Pronase digestion of whole virus or isolated viral glycoproteins. Glycopeptides of viral glycoproteins were prepared by a modification of the method given by Spiro (34). Labeled virus samples (ca. 10^4 hemagglutination units) were suspended in 1 to 2 ml of 0.15 M Tris-hydrochloride, pH 7.8, containing 1.5 mM CaCl_2 , and 5 mg of Pronase in 100 μl of the same buffer was added. The enzyme was predigested for 1 h at 37°C to destroy possibly present glycosidases. After addition of a drop of toluene to prevent bacterial growth, the tubes were sealed and incubated at 37°C . After 24 h the pH value was checked and readjusted with 1 M Tris. A 2-mg amount of predigested Pronase was added, and the tubes were incubated for an additional 24 h. Thereafter, the same procedure was repeated. The incubation was stopped after a total incubation of 100 h. Radioactive virus samples to be compared by gel filtration were combined before Pronase digestion. After Pronase digestion the samples were lyophilized.

Gel chromatography. The freeze-dried samples were taken up in 1 ml of elution buffer containing blue dextran and phenol red as markers. After centrifugation, the samples were applied to Bio-Gel columns with diameters of 1 cm and lengths of 100 cm. Columns were eluted with 0.1 M pyridine-acetate buffer, pH 5.0. Fractions of 0.5 ml were collected. Radioactivity was counted, using toluene scintillation fluid containing Triton X-100 (29). Unlabeled carbohydrate was monitored by the phenol-sulfuric acid test (23).

For molecular weight determination the following marker substances were used: two glycopeptides obtained from thyroglobulin by Pronase digestion, molecular weights of 4,100 and 1,250, respectively (34); a glycopeptide derived from milk immunoglobulin, molecular weight of 2,630, which was a generous gift of A. Chéron and J. Montreuil, Lille, France (2); stachyose, molecular weight of 666.

When gel chromatography was carried out with these unlabeled markers, hemoglobin was used instead of blue dextran to eliminate interference in the phenol-sulfuric acid assay.

Determination of metabolic interconversion of radioactive marker sugars. To determine the extent of interconversion of sugar labels the virus particles were hydrolyzed for 2 h with 2 N HCl at 100°C under nitrogen in sealed tubes. The hydrolysates were dried over NaOH and P_2O_5 and taken up in water. The hydrolysates were then passed through ion-exchange columns as described by Montreuil and Spik (23). The effluent containing neutral sugars was chromatographed on Schleicher & Schuell SS2040b chromatographic paper in ethyl acetate-pyridine-water (18:5:5.75) (4). The 2 N HCl eluates containing amino sugars and amino acids were dried, taken up in water, and subjected to paper electrophoresis on Whatman no. 3 paper after reacylation (23). Essentially the same procedures were applied to analyze the radioactivity in the eluates of the Bio-Gel columns containing the fractions after Pronase digestion.

Chemicals and isotopes. L-[1- ^{14}C]fucose, 50 mCi/mmol; D-[1- ^{14}C]galactose, 40 mCi/mmol; D-[1- ^{14}C]glucosamine hydrochloride, 50 mCi/mmol; D-[1- ^{14}C]mannose, 30 mCi/mmol; L-[1- ^3H]fucose, 1 Ci/mmol; D-[1- ^3H]galactose, 5 Ci/mmol; D-[6- ^3H]glucosamine hydrochloride, 10 Ci/mmol; and D-[2- ^3H]mannose, 2 Ci/mmol, were obtained from Amersham Buchler, Braunschweig, W. Germany. Bio-Gel P10, P6, and P4 were from Bio-Rad, Munich, W. Germany. Pronase and reagents for polyacrylamide gel electrophoresis were obtained from Serva, Heidelberg, W. Germany. Chromatographic paper was from Whatman, England, and Schleicher & Schuell, Dassel, W. Germany. All other chemicals were purchased from Merck, Darmstadt, W. Germany.

RESULTS

Metabolism of marker sugars. To estimate possible conversion of sugar isotopes during the labeling period, preparations of purified virus were subjected to acid hydrolysis, and the constituent sugars were analyzed by ion-exchange chromatography, paper chromatography, and paper electrophoresis as described in Materials and Methods. The results summarized in Table 1 demonstrate that, with the exception of [1- ^{14}C]mannose, all radioactive

TABLE 1. *Metabolic stability of radioactive marker sugars*

Radioactive sugar used for labeling	Radioactivity recovered in original form (%) ^a
[2- ^3H]mannose	80
[1- ^3H]galactose	77
[1- ^{14}C]mannose	45
[1- ^{14}C]glucosamine	90

^a Percentage of radioactivity present in purified virus. For experimental details, see text.

sugars analyzed could be recovered from the virus largely in their original form. Thus, [2-³H]mannose, [1-³H]galactose, and [1-¹⁴C]glucosamine can be used as quite specific marker sugars. [1-³H]fucose and [6-³H]glucosamine can be added to this list, since they have been shown before to undergo little or no metabolic interconversion (14, 35).

Incorporation of radioactive sugars into viral glycoproteins. FPV was labeled with radioactive sugars and purified and analyzed by polyacrylamide gel electrophoresis. Radioactivity was found only in the viral glycoproteins, and the amounts present in NA, HA₁, and HA₂ were calculated (Table 2). The data show that the labeling pattern obtained with [2-³H]mannose differs from those of the other sugars by a comparatively high amount of radioactivity incorporated into HA₂. Thus, HA₂ appears to be relatively rich in mannose. This observation was confirmed by the analysis of the glycopeptides described below.

Preparation and analysis of glycopeptides. Whole labeled virus particles were digested with Pronase, and the resulting glycopeptides were analyzed on Bio-Gel P10 columns. Only one peak of radioactivity was observed when the label was glucosamine, fucose, or [2-³H]mannose. After labeling with either [1-³H]galactose or [1-¹⁴C]galactose, in addition, a minor peak of lower molecular weight could be seen, which, after labeling with [1-¹⁴C]mannose or [1-³H]mannose, rose to as much as 50% of the total amount of radioactivity (Fig. 1).

To test whether both or only one of the peaks obtained after labeling with [1-¹⁴C]mannose represented glycopeptide, analyses for the identification of carbohydrate, as described in Materials and Methods, were performed. The material present in the individual peaks was hydrolyzed with acid and subjected to ion-exchange and paper chromatography. The result can be seen in Table 3. Only the peak of higher molecular weight contained carbohydrate, most of

FIG. 1. Products obtained after Pronase digestion of influenza virions labeled with radioactive sugars. Purified FPV particles labeled with [1-³H]galactose (A) and [1-¹⁴C]mannose (B) were digested with Pronase and chromatographed on Bio-Gel P10 columns as described in the text.

TABLE 3. Radioactive constituents of material eluted from Bio-Gel P10 columns after labeling with [1-¹⁴C]mannose

Radioactivity recovered as:	% Radioactivity in:	
	High-mol-wt peak	Low-mol-wt peak
Glucosamine	25	ND ^a
Mannose	63	ND
Galactose	5	ND
Fucose	7	ND
Noncarbohydrate	ND	95

^a ND, Not detectable.

TABLE 2. Incorporation of different sugars into glycoproteins^a

Radioactive sugar	Amount of label [dpm (%)] incorporated into:		
	NA	HA ₁	HA ₂
[6- ³ H]glucosamine	3,040 (16)	10,800 (61)	4,300 (23)
[2- ³ H]mannose	850 (18)	1,810 (40)	1,950 (42)
[1- ³ H]galactose	10,360 (18)	32,800 (58)	12,500 (24)
[1- ³ H]fucose	800 (12)	4,910 (70)	1,200 (18)

^a The data are derived from polyacrylamide gels of purified virus labeled with the respective sugars.

which is mannose. The other peak did not give rise to neutral material after acid hydrolysis. This material remained almost totally on Dowex 50 ion-exchange columns. The effluents after elution with HCl were reacylated for hexosamine analysis and subjected to paper electrophoresis in borate buffer. Several peaks of radioactivity could be detected, none of which, however, comigrated with the hexosamine markers. When virus labeled with ¹⁴C-amino acids was subjected to Pronase digestion,

only the peak of lower molecular weight was obtained after gel filtration (data not shown). Thus, it appears that this material consists of amino acids or small peptides and that the glycopeptides are present only in the high-molecular-weight peak. Confirming the observations described above, these data show also that, of the different mannose labels used here, only [2-³H]mannose is a suitable carbohydrate marker.

In some experiments, radioactivity was found to elute with the void volume. As shown elsewhere, this material probably represents mucopolysaccharide (R. T. Schwarz, B. Fournet, J. Montreuil, R. Rott, and H.-D. Klenk, submitted for publication).

The experiments described so far, carried out on Bio-Gel P10, the material used in the beginning of this study, showed only one glycopeptide peak. In subsequent analyses on Bio-Gel P6 it became clear, however, that there are several types of glycopeptide. This is shown in Fig. 2, where the glycopeptides derived from whole virus labeled with various sugars have been analyzed. There are two major glycopeptide peaks. Type I glycopeptide eluted with a maximum around fraction 90 and was readily labeled by fucose, galactose, glucosamine, and mannose. Type II eluted around fraction 100 and could be discriminated as a distinct peak only after labeling with mannose; it appears to be free of fucose and galactose. After labeling with glucosamine, a shoulder on the back slope of the type I glycopeptide peak suggests that this sugar might be present also in type II. However, further experimental evidence is needed to prove this point. There appears to be a third minor glycopeptide peak eluting around fraction 113, which could be detected only after labeling with mannose.

With the Dutch strain of FPV used in most experiments in this study, all three glycoproteins separate well on polyacrylamide gels (16) and could, therefore, be easily isolated by preparative gel electrophoresis (Fig. 3). The individual glycoproteins were digested with Pronase and analyzed on Bio-Gel P6 columns. Figure 4 shows a distinct glycopeptide pattern from each glycoprotein. NA exhibited type I, type II, and the low-molecular-weight glycopeptide. HA₂ also contained the complete set of glycopeptides, but type II was present in relatively larger amounts than in NA. In glycoprotein HA₁, however, only type I glycopeptide was found.

Sizes of the glycoproteins and glycopeptides as depending on host cells and on virus strains. It is a common concept that host cell transferases are involved in glycosylation of

Fig. 2. Glycopeptides obtained from whole FPV (Dutch) particles. Virus was grown in chicken embryo cells in the presence of [³H]fucose, [³H]galactose, [6-³H]glucosamine, or [2-³H]mannose. Purified virus was subjected to Pronase digestion and chromatography on Bio-Gel P6. The rectangle around fraction 60 indicates the elution of blue dextran.

FIG. 3. Isolation of the individual glycoproteins of FPV. Virus (5×10^4 hemagglutination units) labeled with [2- ^3H]mannose was purified and subjected to preparative polyacrylamide gel electrophoresis. Aliquots of whole virus (A) and of the isolated glycopro-

viral glycoproteins, and evidence has been obtained with a togavirus that the oligosaccharide side chains may be the site of host-specific modifications (13). It was, therefore, of interest to test this hypothesis in the case of influenza virus. FPV was grown in chicken embryo cells and MDBK cells. Virus derived from chicken embryo cells was labeled with [$1\text{-}^{14}\text{C}$]glucosamine, and virus from MDBK cells was labeled with [$6\text{-}^3\text{H}$]glucosamine. Co-electrophoresis of both preparations showed a significant difference in the electrophoretic mobilities of the glycoproteins (Fig. 5A). The molecular weights of the different glycoproteins synthesized in either MDBK or chicken embryo cells have been calculated and are listed in Table 4. The viral glycoproteins formed in MDBK cells had higher molecular weights than those derived from chicken embryo cells. It was now of interest to find out whether an increase in size could also be observed with the oligosaccharide side chains. Preparations of labeled virus grown in both cell types were mixed and digested with Pronase, and the resulting glycopeptides were analyzed by chromatography on Bio-Gel P6. The results show that the molecular weight of the glycopeptide formed in MDBK cells was significantly higher than that formed in chicken embryo cells (Fig. 5B).

For determination of molecular weight the following marker substances were used: two glycopeptides obtained by Pronase digestion of thyroglobulin (molecular weights, 4,100 and 1,250, respectively), a glycopeptide derived from milk globulin (molecular weight, 2,630) (2), and stachyose (molecular weight, 666). By co-chromatography with these marker substances, the molecular weight of the glycopeptide obtained after labeling with glucosamine, i.e., the molecular weight of type I glycopeptide, was found to be 2,600 if the virus was grown in chicken embryo cells and 3,400 if grown in MDBK cells. In similar experiments, type II glycopeptide obtained from virus grown in chicken embryo cells was found to have a molecular weight of about 2,000.

We also compared the glycopeptides obtained from FPV and virus N, both grown in chicken embryo cells. There were only minor differences in the molecular weights of the glycopeptides (data not shown).

Glycoproteins and glycopeptides synthesized in the presence of inhibitors of glycosylation. 2-Deoxyglucose and glucosamine inhibit glycosylation of influenza virus glycoproteins (17). With increasing doses of inhibitor there is

teins (B, C, D) were analyzed by analytical polyacrylamide gel electrophoresis.

FIG. 4. Glycopeptides obtained from individual glycoproteins of FPV. Glycoproteins of virus labeled with $[2\text{-}^3\text{H}]\text{mannose}$ were isolated as described in Fig. 3 and digested with Pronase. The glycopeptides were analyzed by chromatography on Bio-Gel P6 (open columns). Glycopeptide of type I obtained by digestion of FPV labeled with $[1\text{-}^{14}\text{C}]\text{galactose}$ was cochromatographed as a marker (solid columns). The rectangle around fraction 55 indicates the elution of blue dextran.

a concomitant decrease of the carbohydrate content of the viral glycoproteins. Thus, it is possible to obtain viral hemagglutinin in a form that is only partially glycosylated (30). To throw

light on the mechanism of inhibition, it was of interest to find out whether the reduced carbohydrate content was due to the incorporation of incomplete oligosaccharides into the hemagglu-

FIG. 5. (A) Glycoproteins of FPV grown in MDBK cells and in chicken embryo cells. Virus grown in MDBK cells was labeled with [6-³H]glucosamine, and virus grown in chicken embryo cells was labeled with [1-¹⁴C]glucosamine. Purified preparations of each virus were mixed and co-electrophoresed on polyacrylamide gels. (B) Glycopeptides obtained from FPV grown in MDBK cells and in chicken embryo cells. The virus preparations described in (A) were mixed, digested with Pronase, and subjected to chromatography on Bio-Gel P6.

TABLE 4. Comparison of molecular weight estimates of the glycoproteins of FPV (Dutch strain) grown in different cell types

Cell type	Glycoprotein		
	NA	HA ₁	HA ₂
MDBK	82,000	59,000	31,000
CEF ^a	75,000	51,000	28,000

^a CEF, Chicken embryo fibroblasts.

tin molecule or to the lack of entire side chains.

Such an analysis was performed on purified virus N, which was grown in the presence of 2-deoxyglucose and contained only partially glycosylated hemagglutinin. Figure 6 shows that the molecular weight of the hemagglutinin of this virus (68,000) is reduced, compared to that of uninhibited control virus (72,000). Since the

shift in molecular weight reflects the loss of carbohydrate (30) and since the unglycosylated polypeptide chain of the influenza virus hemagglutinin (HA₀) has a molecular weight of about 60,000 to 64,000, one can calculate that the incompletely glycosylated hemagglutinin obtained in this experiment had lost at least 30% of its carbohydrate complement. Figure 7 shows gel chromatography of the glycopeptide obtained from the same virus preparation and from a preparation where glycosylation was inhibited by glucosamine. The analysis was performed on Bio-Gel P4 columns to detect minor differences in molecular weight. The radioactivity in the void volume was significantly increased in the case of 2-deoxyglucose-mediated inhibition. As has been pointed out above, this material most probably represents mucopolysaccharide, the synthesis of which appears to be less inhibited than that of the carbohydrate side chains of the glycoproteins. The data show that the glycopeptide peak was not shifted towards a lower molecular weight in the presence of the inhibitors. This experiment was performed with glucosamine and galactose as radioactive markers. Thus, only type I glycopeptide was labeled. However, preliminary studies with [2-³H]mannose indicate that the molecular weight of type II glycopeptide was not reduced, either, under these conditions. It can therefore be concluded that the loss of carbohydrate is due to the loss of complete oligosaccharide side chains, rather than to a decrease in their size.

DISCUSSION

This study was undertaken to throw light on the carbohydrate moiety of the glycoproteins of influenza virus by labeling with radioactive sugars. [6-³H]glucosamine, [1-¹⁴C]glucosamine, [2-³H]mannose, [1-³H]galactose, and [1-³H]fucose showed little or no conversion into other sugars or into amino acids and, thus, proved to be suitable markers. Our data show that all viral glycoproteins (NA, HA₁, and HA₂) contain glucosamine, mannose, galactose, and fucose. Similar carbohydrate compositions have been found with togaviruses (22, 32), rhabdoviruses (24), and paramyxoviruses (14), the only difference being neuraminic acid, which is absent in myxoviruses.

Analysis of the glycopeptides obtained after Pronase digestion demonstrates that these sugars are present in the form of carbohydrate side chains, of which several types exist that differ from each other in size and composition. The two major species of side chains are type I, consisting of glucosamine, mannose, galactose, and fucose, and type II, containing only man-

FIG. 6. Glycoproteins of virus *N* under conditions of partial inhibition of glycosylation. Virus *N* was grown in chicken embryo cells in the presence of [6- ^3H]glucosamine and 2-deoxy-D-glucose (2 mM) and purified. Co-electrophoresis was carried out with uninhibited control virus labeled with [1- ^{14}C]glucosamine. Virus *N* exhibits only one glycoprotein peak, because HA and NA have similar migration rates (16).

nose and, possibly, glucosamine. The HA₁ subunit of hemagglutinin has side chains of type I exclusively, whereas a large proportion of the side chains of HA₂ appears to be of type II. Neuraminidase contains both types of oligosaccharide. There is evidence that side chains similar to those of type I and II found with influenza virus are present in a variety of other glycoproteins, such as the envelope proteins of togaviruses (22, 32) and immunoglobulins (10). Our data do not exclude the possibility that a structural or metabolic relationship exists between both types, e.g., that the less complex type II might represent the core of the fully glycosylated type I oligosaccharide. However, the observation that each glycoprotein has a distinct pattern of side chains suggests that type I and type II are structurally unrelated. Whether the third minor glycopeptide, which resembles type II in localization and sugar composition, is an incomplete form of this side chain remains to be seen.

The molecular weights of the type I and II glycopeptides obtained from virus grown in chicken embryo cells were found to be 2,600 and 2,000, respectively. Since it can be assumed that the glycopeptides contain at least one and not more than two or three amino acid residues (34), one can calculate that the oligosaccharide side chains of types I and II consist of a maximum of 12 and 9 monosaccharide units each, respectively. The molecular weights of the glycopeptides allow also a rough estimate on the number of side chains present in the uncleaved

hemagglutinin glycoprotein of FPV. Since the total carbohydrate complement of HA was found to be 12,000 daltons (30), there appear to be at least five to six side chains in the molecule. At least two of these are located on HA₂, since this subunit contains type I as well as type II oligosaccharides.

It is a common concept that the carbohydrate side chains in viral glycoproteins are synthesized by host-specific sugar transferases and, therefore, are susceptible to host modifications. Thus, the glycoproteins of influenza virus grown in MDBK cells have higher molecular weights than those of virus grown in BHK cells (7) or, as shown here, in chicken embryo cells. Our data show that the difference in the molecular weights of the glycoproteins is paralleled by a difference of the molecular weights of the glycopeptides. This is clearly the case for type I side chains. Whether it holds also for type II remains to be seen. This indicates that the differences observed with the glycoproteins are due to variations in the size of the side chains rather than variations in their number. In contrast to these findings, two different influenza virus strains, virus *N* and FPV, when grown in the same host cell give rise to glycopeptides of similar molecular weights, which is again in favor of the assumption that host-cell sugar transferases are specifying to some extent the carbohydrate moiety of viral glycoproteins.

There is growing evidence that the cores of the carbohydrate side chains of glycoproteins are synthesized in covalent linkage to polyiso-

FIG. 7. Glycopeptides obtained from virus *N* grown under conditions of partial inhibition of glycosylation. Purified preparations of virus *N* grown in chicken embryo cells in the presence of [6-³H]glucosamine and 2-deoxy-D-glucose (2 mM) (A) and of virus grown in the presence of [1-³H]galactose and D-glucosamine (5 mM) (B) were analyzed. Purified virus grown in the presence of [1-¹⁴C]-glucosamine without an inhibitor of glycosylation was added as a control. Both samples were digested with Pronase and chromatographed on Bio-Gel P4.

prenyl derivatives and that they are transferred en bloc from the lipid to the polypeptide chains (for review, see 9). Formation of the sugar-lipid conjugates can be inhibited by tunicamycin (21, 36, 38). The observation that this antibiotic interferes with the formation of the glycoproteins of influenza and other viruses suggests that such lipid intermediates are also involved in the biosynthesis of viral glycoproteins (31, 37). The concept of en bloc glycosyla-

tion is supported by the present finding that the reduction of the carbohydrate content of influenza virus hemagglutinin synthesized in the presence of 2-deoxyglucose and glucosamine is due to the lack of whole carbohydrate side chains and not to the incorporation of incomplete ones. Our data suggest that these inhibitors interfere either with the formation of the oligosaccharide on the lipid matrix or with its transfer to the polypeptide. Previous studies on 2-deoxyglucose-mediated inhibition have shown that this sugar analog is activated to UDP-2-deoxyglucose and GDP-2-deoxyglucose (28) and that it is only the latter form that is involved in the interference reaction (20, 29).

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LITERATURE CITED

1. Bucher, D. J., S. S.-L. Li, J. M. Kehoe, and E. D. Kilbourne. 1976. Chromatographic isolation of the hemagglutinin polypeptide from influenza virus vaccine and determination of their amino-terminal sequences. *Proc. Natl. Acad. Sci. U.S.A.* 73:238-242.
2. Chéron, A., B. Fournet, G. Spik, and J. Montreuil. 1976. Structure complète de glycopeptides isolés des immunoglobulines IGI du colostrum de vache. *Biochimie* 58:927-942.
3. Choppin, P. W. 1969. Replication of influenza virus in a continuous cell line: high yield of infective virus from cells inoculated at high multiplicity. *Virology* 39:130-134.
4. Colombo, P., D. Corbetta, A. Pirota, G. Ruffini, and A. Sartori. 1960. A solvent for qualitative and quantitative determination of sugars using paper chromatography. *J. Chromatogr.* 3:343-350.
5. Compans, R. W. 1973. Influenza virus proteins. II. Association with components of the cytoplasm. *Virology* 51:56-70.
6. Compans, R. W., and P. W. Choppin. 1975. Reproduction of myxoviruses, p. 179-252. In H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 4. Plenum Publishing Corp., New York.
7. Compans, R. W., H.-D. Klenk, L. A. Caligiuri, and P. W. Choppin. 1970. Influenza virus proteins. I. Analysis of polypeptides of the virion and identification of spike glycoproteins. *Virology* 42:880-889.
8. Davenport, R. M., R. Rott, and W. Schäfer. 1960. Physical and biological properties of influenza virus components obtained after ether treatment. *J. Exp. Med.* 112:765-782.
9. Hemming, F. W. 1974. Lipids in glycan biosynthesis, p. 39-97. In T. W. Goodwin (ed.), *Biochemistry of lipids*. Butterworths, London.
10. Johnson, I., and J. R. Clamp. 1971. The oligosaccharide units of a human immunoglobulin M (macroglobulin). *Biochem. J.* 123:739-745.
11. Kaluza, G., M. F. G. Schmidt, and C. Scholtissek. 1973. Effect of 2-deoxy-D-glucose on the multiplication of Semliki Forest virus and the reversal of the block by mannose. *Virology* 54:179-189.
12. Kaluza, G., C. Scholtissek, and R. Rott. 1972. Inhibi-

- tion of the multiplication of enveloped RNA-viruses by glucosamine and 2-deoxy-D-glucose. *J. Gen. Virol.* 14:251-259.
13. Keegstra, K., B. Sefton, and D. Burke. 1975. Sindbis virus glycoproteins: effect of the host cell on the oligosaccharides. *J. Virol.* 16:613-620.
  14. Klenk, H.-D., L. A. Caligui, and P. W. Choppin. 1970. The proteins of parainfluenza virus SV5. II. The carbohydrate content and glycoproteins of the virion. *Virology* 42:473-481.
  15. Klenk, H.-D., R. Rott, and H. Becht. 1972. On the structure of the influenza virus envelope. *Virology* 47:579-591.
  16. Klenk, H.-D., R. Rott, M. Orlich, and J. Blödorn. 1975. Activation of influenza A viruses by trypsin treatment. *Virology* 68:426-439.
  17. Klenk, H.-D., C. Scholtissek, and R. Rott. 1972. Inhibition of glycoprotein biosynthesis of influenza virus by D-glucosamine and 2-deoxy-D-glucose. *Virology* 49:723-734.
  18. Klenk, H.-D., W. Wöllert, R. Rott, and C. Scholtissek. 1974. Association of influenza virus proteins with cytoplasmic fractions. *Virology* 57:28-41.
  19. Laver, W. G. 1971. Separation of two polypeptide chains from the hemagglutinin subunit of influenza virus. *Virology* 45:275-288.
  20. Lehle, L., and R. T. Schwarz. 1976. Formation of dolichol monophosphate 2-deoxy-D-glucose and its interference with the glycosylation of mannoprotein in yeast. *Eur. J. Biochem.* 67:239-245.
  21. Lehle, L., and W. Tanner. 1976. The specific site of tunicamycin inhibition in the formation of dolichol-bound N-acetylglucosamine derivatives. *FEBS Lett.* 71:167-170.
  22. Mattila, K., A. Luukonen, and O. Renkonen. 1976. Protein bound oligosaccharides of Semliki Forest virus. *Biochim. Biophys. Acta* 419:435-444.
  23. Montreuil, J., and G. Spik. 1968. Microdosage des glucides. Monograph, Laboratory of Biological Chemistry of the Faculty of Sciences of Lille, France.
  24. Moyer, S. A., J. M. Tsang, P. H. Atkinson, and D. F. Summers. 1976. Oligosaccharide moieties of the glycoprotein of vesicular stomatitis virus. *J. Virol.* 18:167-175.
  25. Nagai, Y., and H.-D. Klenk. 1977. Activation of precursors to both glycoproteins of Newcastle disease virus by proteolytic cleavage. *Virology* 77:125-134.
  26. Rott, R., and H.-D. Klenk. 1977. Structure and assembly of viral envelopes, p. 47-81. In G. Poste and G. L. Nicolson (ed.), *Virus infection and the cell surface*. Elsevier Press, Amsterdam.
  27. Schmidt, M. F. G., R. T. Schwarz, and H. Ludwig. 1976. Fluorosugars inhibit biological properties of different enveloped viruses. *J. Virol.* 18:819-823.
  28. Schmidt, M. F. G., R. T. Schwarz, and C. Scholtissek. 1974. Nucleoside diphosphate derivatives of 2-deoxy-D-glucose in animal cells. *Eur. J. Biochem.* 49:237-247.
  29. Schmidt, M. F. G., R. T. Schwarz, and C. Scholtissek. 1976. Interference of nucleoside diphosphate derivatives of 2-deoxy-D-glucose with the glycosylation of virus-specific glycoproteins in vivo. *Eur. J. Biochem.* 70:55-62.
  30. Schwarz, R. T., and H.-D. Klenk. 1974. Inhibition of glycosylation of the influenza virus hemagglutinin. *J. Virol.* 14:1023-1034.
  31. Schwarz, R. T., J. M. Rohrschneider, and M. F. G. Schmidt. 1976. Suppression of glycoprotein formation of Semliki forest, influenza, and avian sarcoma virus by tunicamycin. *J. Virol.* 19:782-791.
  32. Sefton, B. M., and K. Keegstra. 1974. Glycoproteins of Sindbis virus: preliminary characterization of oligosaccharides. *J. Virol.* 14:522-530.
  33. Skehel, J. J., and M. D. Waterfield. 1975. Studies on the primary structure of the influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. U.S.A.* 72:93-97.
  34. Spiro, R. G. 1965. The carbohydrate units of thyroglobulin. *J. Biol. Chem.* 240:1603-1610.
  35. Strauss, J. H., B. W. Burge, and J. E. Darnell. 1970. Carbohydrate content of the membrane protein of Sindbis virus. *J. Mol. Biol.* 47:437-448.
  36. Takatsuki, A., K. Khono, and G. Tamura. 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. *Agric. Biol. Chem.* 39:2089-2091.
  37. Takatsuki, A., and G. Tamura. 1971. Tunicamycin a new antibiotic. II. Some biological properties of the antiviral activity of tunicamycin. *J. Antibiot.* 24:224-231.
  38. Tkacz, J. S., and J. O. Lampen. 1975. Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf liver microsomes. *Biochem. Biophys. Res. Commun.* 65:248-257.
  39. Zimmermann, T., and W. Schäfer. 1960. Effect of p-fluoro-phenylalanine on fowl plaque virus multiplication. *Virology* 11:676-698.