Virus-Dependent Glycosylation

BARTHOLOMEW M. SEFTON

Tumor Virology Laboratory, The Salk Institute, San Diego, California 92112

The oligosaccharides of the membrane glycoproteins of Sindbis virus, vesicular stomatitis virus, and Rous sarcoma virus were compared on the basis of apparent size and sugar composition. It appears that each virus acquires a different set of oligosaccharides during growth in a single type of cell.

The external proteins of all lipid-enveloped animal viruses are glycosylated. The viral glycoproteins which have been examined have been found to have attached to them a number of oligosaccharide side chains, each consisting of 10 to 20 sugars (2, 3, 6, 11, 19). It is unclear how the sites on the polypeptide for attachment of carbohydrate are selected and how the structure of the oligosaccharide to be assembled is specified. Because of the small size of the genomes of those viruses whose glycoproteins have been studied, it is highly unlikely that these viruses carry the structural genes for most of the enzymes necessary for oligosaccharide synthesis (2, 3, 6, 7). Indeed, since there is as yet no evidence for virus-specific glycosyltransferases, it is quite possible that normal cellular enzymes carry out the entire glycosylation of these virus-specific polypeptides.

The question therefore arises as to whether the virus influences the glycosylation of its membrane glycoproteins. Several studies have examined the effect that the host cell can have on the oligosaccharide side chains of a viral glycoprotein. It has been shown that the carbohydrate compositions of both Sindbis virus and vesicular stomatitis virus (VSV) show some dependence on the cell in which the virus is grown (3, 6, 10, 21), the greatest differences being found in the content of sialic acid. However, the observed differences in composition are small and, while it is obviously correct to conclude that the host cell does affect viral glycosylation, the overall similarities of the glycosylation of either Sindbis virus or VSV in various hosts is more striking than the dissimilarity.

The sizes of the glycopeptides of Sindbis virus and VSV, grown in the same cells, have been directly compared (2). It was concluded that the oligosaccharides of the two viruses were fundamentally similar. This suggests either that viral glycosylation is a function solely of the host cell or that it is virus specified and that both Sindbis virus and VSV specify the same oligosaccharides. However, more recent results indicate that the glycoproteins of Sindbis virus produced by chicken cells contain two structurally dissimilar oligosaccharides (19) whereas the glycoproteins of VSV, produced by a variety of mammalian cells, contain two structurally similar oligosaccharides (5, 6). To interpret these observations unequivocally, the comparison of the oligosaccharides of Sindbis virus and VSV, grown in the same cells, has been repeated, and, in addition, a comparison of the oligosaccharides of Sindbis virus and the avian tumor viruses, both grown in chicken cells, has been made.

MATERIALS AND METHODS

Chicken embryo primary cell cultures were prepared essentially as described by Rein and Rubin (16). Cells for the growth of either Sindbis virus or VSV were from fertilized eggs obtained from Hen-X, Lakeside, Calif. Cells for the growth of the Prague avian sarcoma virus were from COFAL-negative chicken embryos obtained from SPAFAS, Norwich, Conn. Cells for the growth of the nontransforming B77 virus (NT-B77) were from C/O, gs-negative embryos obtained from Heinsdorf and Nelson, Redmond, Wash. Uninfected primary cultures were grown in enriched Eagle medium supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1% chicken serum. Avian tumor virus-infected primary cells were grown in medium similar to the above except that it contained heat-inactivated chicken serum and, in some experiments, polybrene (2 µg/ml). Morphologically transformed secondary cultures were grown in enriched Eagle medium supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated chicken serum and, in some experiments, 1% dimethyl sulfoxide (Me$_2$SO). This medium was changed frequently. Secondary and tertiary cultures of NT-B77-infected cells were grown in medium similar to that used for the primary cells except that it contained Me$_2$SO (1%).

BHK cells were obtained from John Holland, University of California, San Diego, and were grown

in enriched Eagle medium supplemented with 5% calf serum. The chicken cells used for the growth of the Prague virus were incubated at 38.5 C. All other cells were incubated at 37 C. The nontransforming B77 virus was obtained from Peter Vogt, University of Southern California. The VSV (Indiana serotype) was obtained from John Holland, University of California, San Diego.

Growth of radioactive virus. In some experiments, cells to be used for the growth of either Sindbis virus or VSV were incubated for 24 h prior to infection in glucose-free Eagle medium supplemented with either 2% tryptose phosphate broth and 1% calf serum (chicken cells) or 5% calf serum (BHK cells). This was done to induce the accelerated transport of exogenous sugars (14). The procedures for the growth of Sindbis virus and VSV were essentially the same. Infection of chicken cells was done in complete Eagle medium supplemented with 2% tryptose phosphate broth and 1% calf serum. Infections of BHK cells were done in complete Eagle medium supplemented with 5% calf serum. The virus, either 100 Sindbis PFU per cell or 30 VSV PFU per cell, was allowed to adsorb in a small volume of medium for 60 to 90 min at 37 C. Medium containing the appropriate radioactive sugar was then added to each dish and the infection was allowed to proceed for 13 to 16 h at 37 C. The labeling medium contained either [^{14}C]glucosamine (New England Nuclear, 222 mCi/mmol) at a concentration of 2.5 μCi/ml, [6-^{3}H]glucosamine (New England Nuclear, 10.12 Ci/mmol) at a concentration of 6 to 12.5 μCi/ml, or [2-^{3}H]mannose (Amersham/Searle, 2 Ci/mmol) at a concentration of 6 to 25 μCi/ml.

This procedure was altered slightly for the growth of Sindbis virus in B77-transformed cells. In this case, morphologically well-transformed secondary cultures of B77-infected chicken cells were infected with Sindbis virus at a multiplicity of 10 PFU per cell. After adsorption, Eagle medium supplemented with 10% tryptose phosphate broth, 4% calf serum, 1% heat-inactivated chicken serum, and 1% Me$_2$SO and containing [^{3}H]glucosamine (3 μCi/ml) was added to the cells and the infection was allowed to proceed for 15 h.

The avian tumor viruses were labeled differently.

(i) Labeling of Prague virus. Medium 199, supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated chicken serum and containing [^{14}C]glucosamine (New England Nuclear, 52 mCi/mmol, 2 μCi/ml), was added to well-transformed Prague virus-infected secondary chicken cells. This medium was removed after 24 h and replaced with similar, unlabeled medium. Cell debris was removed from the radioactive medium by centrifugation and the medium was stored at 4 C. After another 8 h, the unlabeled medium was harvested, cleared of cell debris, and combined with the radioactive medium.

(ii) Labeling of nontransforming B77 virus. Eagle medium, supplemented with 2% tryptose phosphate broth, 1% calf serum, 1% heat-inactivated chicken serum, and 1% Me$_2$SO and containing [^{3}H]glucosamine (15 μCi/ml), was added to NT-B77-infected tertiary chicken cells. This medium was removed after 24 h, cleared of cell debris by cen-

trifugation, and stored at 4 C. Fresh medium containing [^{3}H]glucosamine (10 μCi/ml) was then added to the cells, and incubation was continued for another 24 h. This medium was then removed, treated as just described, and replaced with fresh medium containing 8 μCi of [^{3}H]glucosamine per ml, and incubation was continued for another 40 h. This radioactive medium was in turn removed, cleared of cell debris, combined with the previously harvested labeled media at 4 C, and replaced with fresh unlabeled medium. This medium was removed from the cells 18 h later, treated as described above, and combined with the radioactive media. The virus-containing media were not frozen prior to purification.

Virus purification: Sindbis virus. Sindbis virus was purified as was described in detail previously (19). Essentially, after the removal of cellular debris, the virus was concentrated by precipitation with polyethylene glycol and then subjected to sucrose gradient velocity sedimentation.

Virus purification: VSV. Virus-containing medium was centrifuged (5,000 × g for 5 min) to remove cellular debris, and the virus was then concentrated by centrifugation at 130,000 × g for 60 min. The viral pellet was dissolved in a small volume of 0.2 M NaCl, 0.05 M Tris-hydrochloride (pH 7.4), and 0.001 M EDTA by sonic extraction and then sedimented through a 15 to 30% (wt/wt) sucrose gradient containing 0.15 M NaCl, 0.05 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA, and 0.5% chicken serum. Centrifugation was for 45 to 60 min at 110,000 × g.

Virus purification: avian tumor viruses. Prague avian sarcoma virus was purified essentially as described by Duesberg et al. (4). The virus-containing medium was freed of cellular debris by centrifugation, and the virus was precipitated with ammonium sulfate. The precipitate was collected by centrifugation and redissolved in a small volume of 0.2 M NaCl, 0.05 M Tris-hydrochloride (pH 7.4), and 0.001 M EDTA, with the use of sonic extraction. The virus was then sedimented through 20% sucrose and collected on a cushion of 65% sucrose. After dilution, the virus was centrifuged to equilibrium in a 20 to 55% sucrose gradient containing 0.15 M NaCl, 0.05 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA, and 0.3% fetal calf serum. The virus-containing fractions were diluted and centrifuged for 90 min at 230,000 × g, and the resulting viral pellet was stored at −70 C. Nontransforming B77 virus was purified as above, except that the virus was removed from the growth medium by centrifugation at 177,000 × g for 1 h rather than by precipitation with ammonium sulfate. This modification appears to yield virus with greater radiochemical purity.

Glycoprotein purification. Viral glycoproteins were purified by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis in 7.5% gels, as was described previously (19). The gels were sliced with stacked razor blades, and the proteins of interest were eluted into 0.1% SDS, lyophilized, redissolved in water, and precipitated with ethanol.

Gel filtration. Gel filtration on Bio-Gel P-6 was as described previously (19). Samples to be compared

were mixed and then concentrated. Purified glyco-proteins were concentrated by precipitation with ethanol and centrifugation at 15,000 × g for 15 min, whole VSV virions were pelleted by centrifugation at 200,000 × g for 60 min, and whole Sindbis virions were precipitated by dilution to low ionic strength and collected by centrifugation at 15,000 × g for at least 5 min (20). The mixed samples were then dissolved in a small volume (0.25 to 0.50 ml) of 0.1 M Tris-hydro-chloride (pH 8.0), containing 0.01 M $CaCl_2$, and 0.5 mg (50 μl) of Pronase (Calbiochem, type CB, B grade) was added. Digestion was carried out for 30 to 72 h at 60 C, and additional Pronase (250 μg, 25 μl) was added approximately twice a day. The Pronase had been incubated in 0.1 M Tris-hydrochloride (pH 8.0), containing 0.01 M $CaCl_2$, for 120 min at 37 C prior to use. The samples were then put directly onto a column of Bio-Gel P-6 (200/400 mesh, 0.9 by 115 cm), equilibrated with 0.1 M Tris-hydrochloride (pH 8.0), and 1.0-ml fractions were collected. The flow rate was approximately 6 ml/h. Radioactivity was determined in a Beckman scintillation counter using a scintilla-tion fluid consisting of fluors dissolved in a 2:1 (vol/vol) mixture of toluene and Triton X-100.

Mild acid hydrolysis. Glycopeptides, dissolved in 0.1 M Tris-hydrochloride (pH 8.0), containing 0.01 M $CaCl_2$, were acidified with 2 N HCl to pH 1. The mixture was then incubated for 30 min at 80 C, cooled, neutralized with either 2 N NaOH or 2 N Tris base, and put directly on the column.

Strong base hydrolysis. Whole Sindbis virus and the purified major glycoprotein of B77 virus, dissolved in 0.1% SDS, were mixed and precipitated with ethanol. The pellet was dissolved in 0.3 ml of 1 M NaOH, 1 M $NaBH_4$, and incubated for 4 h at 100 C (13). After the sample was cooled, the excess $NaBH_4$ was destroyed with concentrated HCl and the com-plete reaction mixture was put directly on the col-umn. No effort was made to remove the insoluble SDS.

RESULTS

The oligosaccharides of the envelope glyco-proteins of Sindbis virus, VSV, and two avian tumor viruses were compared. The oligosaccha-rides of these viruses, labeled biosynthetically with either glucosamine or mannose, were freed from the polypeptide by exhaustive Pronase digestion, and the resulting carbohydrate-con-taining molecules were compared by gel filtra-tion. The labeled molecules studied are referred to as glycopeptides. In composition, they are principally carbohydrate, but they contain, in addition, a small and unknown number of amino acids. Separation by gel filtration is on the basis of size, but is also affected by molecu-lar shape. Glycoprotein oligosaccharides are, in general, highly branched structures (1) and it is not clear how each of the molecular dimensions contributes to the chromatographic behavior. Further, it is probable that oligosaccharides of

similar size and shape, but of different composi-tion, would co-chromatograph during gel filtra-tion. Thus, although observed chromatographic differences are most probably real, observed chromatographic similarities may be merely fortuitous.

The glycopeptides of whole Sindbis virus and of whole VSV were compared on a column of Bio-Gel P-6 (Fig. 1). Radioactive glucosamine was used as the label and both viruses were grown in chicken embryo cells. Some similari-ties between the two viruses are obvious, as was previously reported (2). A majority of the gluco-samine label in VSV co-chromatographed with the two large Sindbis virus glycopeptides, S1 and S2. A small amount of material from VSV eluted as a shoulder, larger even than the largest Sindbis virus glycopeptide. Some VSV material (less than 12%) eluted as a small peak approximately coincident with the Sindbis glycopeptide S3. Only very little gluco-samine label from VSV (4%) eluted from the column at the position of the smallest Sindbis virus glycopeptide, S4. The glucosamine label from VSV which appeared at the void volume varied from preparation to preparation and was probably nonviral material since it was absent from purified glycoproteins.

The relative proportions of the four Sindbis virus glycopeptides seen in gel filtration is somewhat different in virus produced by ham-ster cells (3, 10). Therefore, the comparison of the glucosamine-labeled Sindbis virus and VSV glycopeptides was repeated, this time using
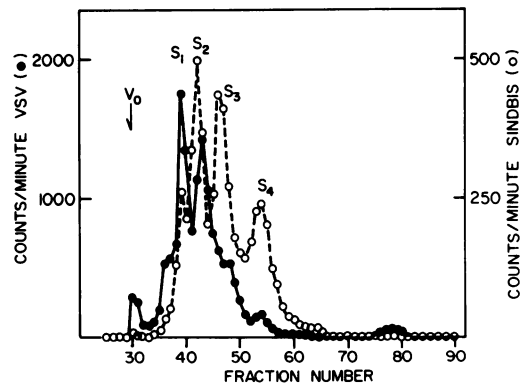

FIG. 1. *Glycopeptides of Sindbis virus and VSV, both produced by chicken cells. Whole Sindbis virus, labeled with [¹⁴C]glucosamine and grown in chicken cells, was mixed with whole VSV, labeled with [³H]glucosamine and grown in chicken cells, and the mixture was digested with Pronase and applied to a column of Bio-Gel P-6. Symbols: ●, VSV, labeled with [³H]glucosamine; ○, Sindbis virus, labeled with [¹⁴C]glucosamine.*

viruses grown in hamster cells (Fig. 2). The two patterns seen here were generally similar to those seen with virus produced from chicken cells. A majority of the VSV glycopeptides were the same size as ths Sindbis virus glycopeptides S1 and S2, some were a little larger than any Sindbis virus glycopeptide, some were approximately the same size as Sindbis glycopeptide S3, and few were the same size as the small Sindbis glycopeptide, S4.

These two experiments (Fig. 1 and 2) were done using whole virus. Possible problems with the use of whole virus, rather than purified glycoproteins, are contamination with labeled cellular glycoproteins and the presence of radioactive glycolipids. However, the glycopeptides of whole Sindbis virus purified by the methods used here are essentially identical with those of the purified viral glycoproteins (19). Further, less than 7% of the labeled glucosamine in VSV grown in chicken cells was present in material other than the viral glycoprotein, as determined by SDS polyacrylamide gel electrophoresis.

The smallest glycopeptide of Sindbis virus grown in chicken cells, S4, contains only glucosamine and mannose (3, 19) and is probably a B-type (9) or high-mannose glycopeptide (1). As such, it is structurally unlike the larger Sindbis virus glycopeptides, S1, S2, and S3. These glycopeptides are most probably A-type or complex glycopeptides. The structural dissimilarity of the two types of Sindbis virus glycopeptides is seen by comparison of the apparent ratio of mannose to glucosamine in the various glyco-

peptides. In virus grown in chicken cells, the ratio of mannose to glucosamine is four times greater in the high-mannose glycopeptide, S4, than in the larger complex glycopeptides (19).

Thus, even though VSV contained little glucosamine label which co-chromatographed with the high-mannose glycopeptide of Sindbis virus, the question of whether VSV contained such a structure was examined directly. The ratio of mannose to glucosamine in the various VSV glycopeptides was determined by comparison of the glycopeptides of mannose-labeled and glucosamine-labeled VSV by gel filtration (Fig. 3). Further, although the relative amounts of mannose and glucosamine in glycopeptide S4 of Sindbis virus grown in chicken cells have been determined (19), that glycopeptide S4 of Sindbis virus grown in hamster cells is also relatively rich in mannose had not been shown directly. Therefore, the ratio of radioactive mannose to radioactive glucosamine in the glycopeptides of Sindbis virus grown in BHK cells was similarly examined (Fig. 3). Two points seem clear: the ratio of mannose label to glucosamine label in the various VSV glycopeptides was remarkably constant, whereas the ratio of mannose label to glucosamine label in glycopeptide S4 of Sindbis virus was much greater than in the other larger Sindbis virus glycopeptides. Since less than 20% of either label is present as material other than the original sugar (3, 19), these ratios of radioactivity probably approximate the chemical ratios in the various glycopeptides. Therefore, no glycopeptide of VSV grown in BHK cells was especially mannose-rich. In contrast, as is true for Sindbis virus grown in chicken cells, Sindbis virus from BHK cells contained a very mannose-rich glycopeptide. This experiment was done using purified, mannose-labeled VSV glycoprotein, rather than whole virus. This was done to reduce the amount of uncharacterized ^{3}H-labeled material eluting at the column volume (fractions 79 to 81). With whole virus, more of this material was seen.

It appears likely that glycopeptides S1, S2, and S3 of Sindbis virus are related structures which differ largely in their content of sialic acid. This is suggested in part by the fact that these three glycopeptides co-chromatograph on Bio-Gel P-6 after the enzymatic removal of sialic acid (10). To examine whether the various VSV glycopeptides might be similarly related, the VSV glycopeptides labeled with glucosamine were subjected to mild acid hydrolysis (0.1 N HCl, 80 C, for 30 min) (2) to remove sialic acid and then examined by gel filtration (Fig. 4). For comparison, Sindbis virus glycopep-
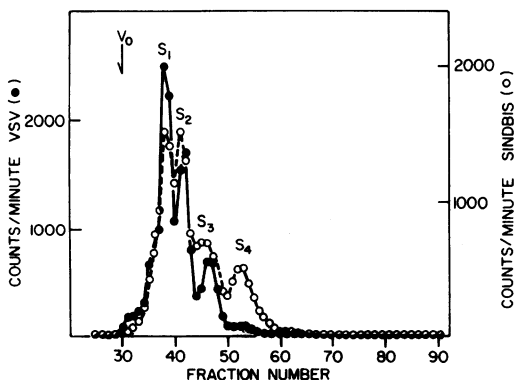

FIG. 2. Glycopeptides of VSV and Sindbis virus, both produced by BHK cells. Whole VSV, labeled with [^{14}C]glucosamine and grown in BHK cells, was mixed with whole Sindbis virus, labeled with [^{3}H]glucosamine and grown in BHK cells, and the mixture was digested with Pronase and applied to a column of Bio-Gel P-6. Symbols: ●, VSV, labeled with [^{14}C]glucosamine; ○, Sindbis virus, labeled with [^{3}H]glucosamine.
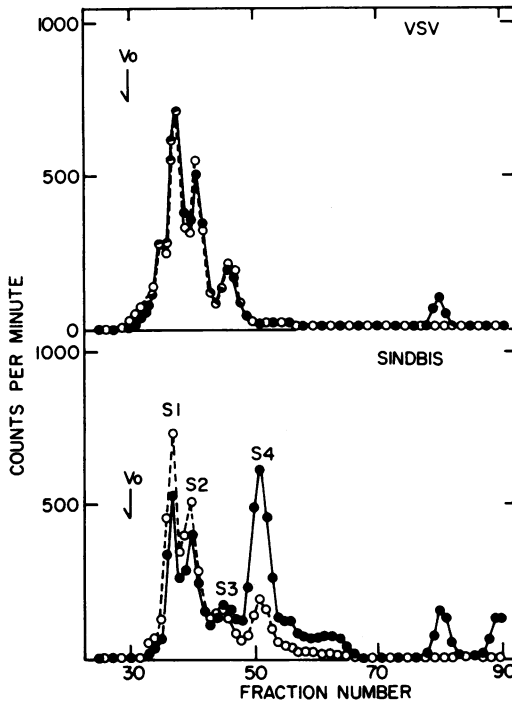
FIG. 3. Comparison of the mannose-labeled glyco-peptides with the glucosamine-labeled glycopeptides of VSV and of Sindbis virus. (Top) The VSV glycopro-tein, labeled with [2-³H]mannose, was mixed with whole VSV, labeled with [¹⁴C]glucosamine, and the mixture was digested with Pronase and applied to a column of Bio-Gel P-6. (Bottom) Whole Sindbis virus, labeled with [2-³H]mannose, was mixed with whole virus, labeled with [¹⁴C]glucosamine, and the mixture was concentrated by dilution to low ionic strength and collection of the precipitate by centrifugation (20). The pellet was dissolved in 2% Nonidet P-40 and precipitated with ethanol. This pellet was then di-gested with Pronase and applied to a column of Bio-Gel P-6. All samples were from BHK cells. Symbols: ●, labeled with [2-³H]mannose; ○, labeled with [¹⁴C]glucosamine.

FIG. 4. Comparison of the glycopeptides of VSV and Sindbis virus after the removal of sialic acid. Whole Sindbis virus, labeled with [¹⁴C]glucosamine, and whole VSV, labeled with [³H]glucosamine, were digested with Pronase separately. The resulting glyco-peptides were then mixed, subjected to mild acid hydrolysis, neutralized, and applied to a column of Bio-Gel P-6. Both samples were grown in chicken cells. Symbols: ●, Sindbis virus, labeled with [¹⁴C]glucosamine; ○, VSV, labeled with [³H]glucosa-mine.

tides, also labeled with glucosamine, were mixed with the VSV glycopeptides prior to hydrolysis. Chemical removal of sialic acid gave results with both viruses indistinguishable from those obtained with neuraminidase (data not shown, but see reference 10, Fig. 2 for compari-son). After the removal of sialic acid, the majority of the VSV glycopeptides co-chromatographed with glycopeptide S3 of Sind-bis virus. This result is consistent with a major-ity of the VSV glycopeptides being related structures which differ in their content of sialic acid and is similar to what is observed with the Sindbis virus glycopeptides. However, it can also be seen that some glucosamine-labeled
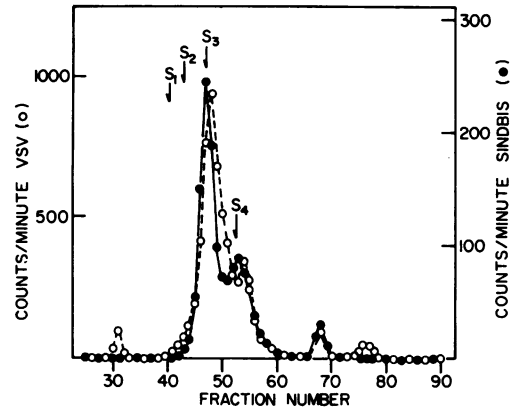
material from VSV behaved as material slightly smaller than the smallest Sindbis virus glyco-peptide, S4, after the removal of sialic acid (Fig. 4). This material is not seen prior to the removal of sialic acid (see Fig. 1 and 2) but is seen in all VSV glycopeptides from which the sialic acid has been removed, regardless of the host cell, the isotope of the label, or the method of removal of the sialic acid. The labeled material at fractions 67 to 69 in Fig. 4 is almost certainly free sialic acid which was released from the glycopeptides during hydrolysis and which is labeled with glucosamine.

The glycopeptides of Sindbis virus were also compared with those of the major glycoprotein, gp 85, of the avian tumor viruses. However, this comparison is complicated by the observation that the apparent average size of the oligosac-charides acquired by the avian tumor viruses is greater during growth in morphologically trans-formed cells than during growth in infected but morphologically normal cells (11). The possibil-ity existed that the glycopeptides of Sindbis virus would be similarly affected by growth of the virus in transformed cells. Therefore, for comparison with a transforming avian tumor virus, Sindbis virus was grown in chicken cells infected with, and morphologically transformed by, the avian sarcoma virus, B77. Sindbis grew well and was purified without detectable con-tamination with either B77 virions or B77 glyco-

proteins. The glycopeptides from this preparation of Sindbis virus were compared, by gel filtration, with those of the major glycoprotein of Prague avian sarcoma virus of subgroup C, a virus which transforms chicken fibroblasts. The purified major glycoprotein of the Prague virus, rather than whole virus, was used for this comparison to eliminate contaminating cellular glycoproteins and to avoid confusion due to glycopeptides from the minor avian tumor virus glycoprotein, gp 37. The glucosamine-containing glycopeptides of Prague virus were much larger than those of Sindbis virus grown in transformed chicken cells (Fig. 5). Similar results were also obtained with the glycopeptides of Schmidt-Ruppin Rous sarcoma virus of subgroup A, Prague sarcoma virus of subgroup B, and B77 virus of subgroup C (data not shown).

Nontransforming derivatives of avian sarcoma viruses have been isolated and characterized (22). These viruses are apparently produced by spontaneous deletion of that part of the sarcoma virus genome which is required for the transformation of fibroblasts in culture (12). The glucosamine-containing glycopeptides of the major glycoprotein of one of these viruses,

NT-B77, were compared with those of Sindbis virus by gel filtration. Since this avian tumor virus does not transform fibroblasts, Sindbis virus grown in normal chicken cells was used for the comparison. Again, the glycopeptides of the nontransforming avian tumor virus were much larger than those of Sindbis virus (Fig. 6). A similar result was obtained when the glycopeptides of a transformation-defective derivative of Prague virus of subgroup B were examined (data not shown).

It is possible that the difference in size of the glycopeptides of the avian tumor viruses and those of Sindbis virus was due to differences in the content of sialic acid. Therefore, glycopeptides from Prague avian sarcoma virus and from Sindbis virus grown in transformed chicken cells were mixed, subjected to mild acid hydrolysis, and compared by gel filtration (Fig. 7). The glycopeptides from the Prague virus were larger than those from Sindbis virus after the removal of sialic acid from both sets of glycopeptides. Thus, the difference in apparent size of glycopeptides from these two viruses cannot be exclusively the result of differences in the content of sialic acid.

A problem in the comparison of the sizes of glycopeptides is that the contribution of the remaining amino acids to the apparent size of the glycopeptides is unknown. It is even possible that the large glycopeptides found after Pronase digestion of the avian tumor viruses consisted of two small oligosaccharides connected by a polypeptide resistant to digestion
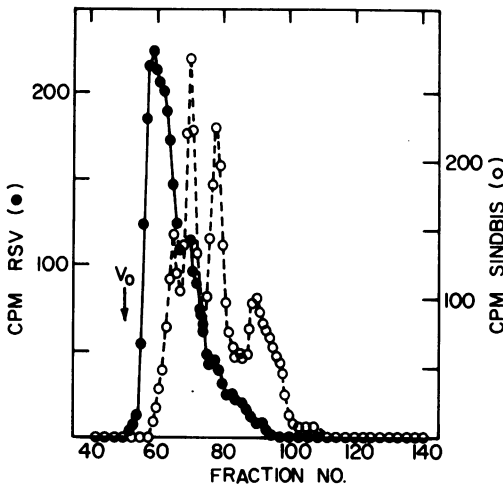
FIG. 5. *Comparison of the glycopeptides of the major glycoprotein of Prague avian sarcoma virus with the glycopeptides of Sindbis virus grown in transformed chicken cells. Whole Sindbis virus, labeled with [³H]glucosamine and grown in B77-transformed chicken cells, was mixed with the purified major glycoprotein of Prague avian sarcoma virus (subgroup C), labeled with [¹⁴C]glucosamine, and the mixture was digested with Pronase and applied to a column of Bio-Gel P-6. In contrast to other experiments, 10-drop fractions (approximately 0.6 ml) were collected. Symbols: ●, Prague avian sarcoma virus, labeled with [¹⁴C]glucosamine; ○, Sindbis virus, labeled with [³H]glucosamine.*
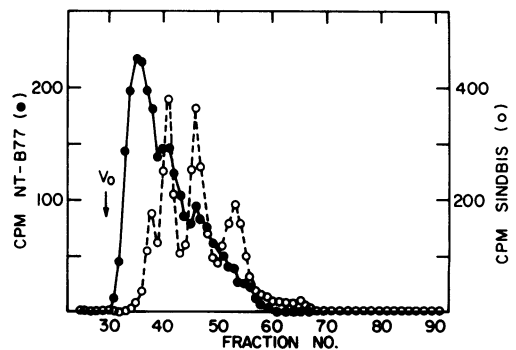
FIG. 6. *Comparison of the glycopeptides of a nontransforming derivative of B77 avian sarcoma virus with the glycopeptides of Sindbis virus. Whole Sindbis virus, labeled with [¹⁴C]glucosamine, was mixed with the purified major glycoprotein of NT-B77 virus (subgroup C), labeled with [³H]glucosamine, and the mixture was digested with Pronase and applied to a column of Bio-Gel P-6. Symbols: ●, NT-B77, labeled with [³H]glucosamine; ○, Sindbis virus, labeled with [¹⁴C]glucosamine.*
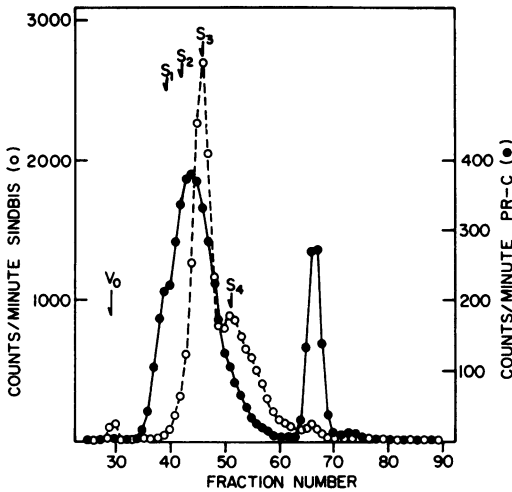
FIG. 7. *Comparison, after the removal of sialic acid, of the glycopeptides of the major glycoprotein of Prague avian sarcoma virus with the glycopeptides of Sindbis virus grown in transformed chicken cells. Whole Sindbis virus, grown in B77-transformed chicken cells and labeled with [³H]glucosamine, was mixed with the purified major glycoprotein of Prague avian sarcoma virus, labeled with [¹⁴C]glucosamine. The mixture was digested with Pronase, hydrolyzed in mild acid, neutralized, and applied to a column of Bio-Gel P-6. Symbols:* ●, *Prague avian sarcoma virus, labeled with [¹⁴C]glucosamine;* O, *Sindbis virus, labeled with [³H]glucosamine.*

with Pronase, rather than a single oligosaccharide attached to a few amino acids. Glycopeptides containing more than one oligosaccharide have been observed in the IgA₁ immunoglobulin (1). Therefore, the major glycoprotein of B77 avian sarcoma virus and whole Sindbis virus grown in transformed chicken cells were mixed and hydrolyzed in strong base (1 N NaOH, 1 M NaBH₄, for 4 h at 100 C), a procedure which frees intact oligosaccharides from a number of glycoproteins (13). The sizes of the resulting oligosaccharides were then compared by gel filtration. The pattern seen was qualitatively similar to that in Fig. 5. The oligosaccharides of the avian sarcoma virus were larger than those of Sindbis virus after hydrolysis of both samples in base (data not shown). This result suggests that the amino acids resistant to Pronase digestion are not responsible for the apparent difference in size between the glycopeptides of Sindbis virus and the avian tumor viruses. However, this procedure is unsatisfactory for the quantitative production of oligosaccharides from Sindbis virus since some destruction of the oligosaccharides was apparent. No destruction of the oligosaccharides from B77 was observed.

DISCUSSION

The purpose of these comparisons was to examine the extent to which the enveloped viruses influence the glycosylation of their membrane glycoproteins. That the host cell is not solely responsible for viral oligosaccharide structure seems clear from three results reported here. During growth in both avian and mammalian cells, the Sindbis virus glycoproteins acquire two structurally distinguishable oligosaccharides (Fig. 3) (10, 19). One is an A-type oligosaccharide which is acidic and complex in sugar composition. The other is a B-type, high-mannose oligosaccharide which consists only of glucosamine and mannose. In contrast, during growth in these same avian and mammalian cells, the VSV glycoprotein appears to acquire A-type or acidic oligosaccharides but not B-type, high-mannose oligosaccharides (Fig. 3 and 4) (5, 6). Thirdly, during growth in chicken cells, the avian tumor viruses appear to acquire A-type oligosaccharides which are noticeably larger than those acquired by Sindbis virus. This dissimilarity in oligosaccharide size is not due to the fact that the avian tumor viruses transform chicken cells whereas Sindbis virus does not, since the glycopeptides of the viruses are different in size, both when the two viruses are grown in transformed chicken cells (Fig. 5) and when they are both grown in phenotypically normal chicken cells (Fig. 6).

Since small, host-dependent differences in the sugar composition of both Sindbis virus and VSV have been observed (3, 6, 21), it is clear that the host can also affect the glycosylation of viral proteins. However, as has been discussed elsewhere (10), it is possible to explain much of this host-dependent variation as being due to differences in the ability of different hosts to complete the synthesis of specific oligosaccharides. This interpretation is based partly on the fact that the greatest variability in the sugar composition of Sindbis virus is seen in galactose and sialic acid, two sugars which are found predominantly at the termini of A-type oligosaccharides, and in part on the fact that, although the molar ratios of the various viral glycopeptides are clearly host dependent, the size of the glycopeptides characteristic of Sindbis virus and VSV is not host dependent (6, 10; B. Sefton, unpublished data).

The most clear-cut example of the host dependence of viral glycosylation is seen in a comparison of VSV grown in mammalian cells with VSV grown in mosquito cells. The oligosaccharides of the VSV glycoprotein acquire sialic

acid during growth of the virus in both avian and mammalian cells. In sharp contrast, VSV grown in mosquito cells contains no sialic acid (17). Since the glycoprotein oligosaccharides of VSV grown in mosquito cells have not been characterized, it is not clear whether the VSV glycoprotein is merely incompletely glycosylated by mosquito cells or whether it is glycosylated in a totally novel manner. However, the fact that a sialyl transferase from BHK cells can, in vitro, add sialic acid to the glycoproteins of VSV grown in mosquito cells (17) suggests that the glycosylation of VSV in mosquito cells might be similar to that in avian and mammalian cells, except that the viral oligosaccharides all remain incomplete in mosquito cells.

It is of interest to know how these different viruses control the glycosylation of their glycoproteins. As has been discussed by others (2, 3, 6, 7), it is impossible that the genomes of the small enveloped viruses code for all the enzymes involved in oligosaccharide synthesis. In fact, the observation of Schloemer and Wagner that VSV does not acquire sialic acid in cells which lack sialyl transferase (mosquito cells) (17) demonstrates directly that VSV does not encode a sialyl transferase. Perhaps the most reasonable hypothesis is that glycosylation is carried out solely by normal cellular enzymes and that the amino acid sequence of the viral membrane polypeptides specifies both the site of attachment for carbohydrate and the structure of the assembled oligosaccharide. The observed differences between the oligosaccharides of these viral glycoproteins would therefore have as its basis differences in the nature and possibly the number of amino acid sequences recognizable as sites of carbohydrate attachment. In the absence of a virus-specific enzyme, it might be expected that viral oligosaccharides would be a limited subset of the oligosaccharides normally synthesized in the host cell. It is possible, however, that a virus could specify a single enzyme which, in concert with normal cellular enzymes, would cause the synthesis of unique oligosaccharides. There is no evidence for or against this idea.

It has been argued that each Sindbis glycoprotein acquires two oligosaccharides during growth in chicken cells (10, 19). This conclusion is based on the known mass of carbohydrate per glycoprotein and the apparent size of the viral glycopeptide oligosaccharides relative to the glycopeptides relative to the glycopeptides of fetuin and thyroglobulin (2). However, each Sindbis virus glycoprotein yields four glycopeptides after Pronase digestion. This apparent

anomaly can be explained if three of these glycopeptides come from a single site of carbohydrate attachment and represent alternative structures present at this site on the polypeptide. The evidence is consistent with three oligosaccharides at one site of attachment being structurally related but differing in the extent to which they are completed.

Similarly, based on measurements of the mass of carbohydrate per glycoprotein and the apparent size of the viral glycopeptides relative to those of fetuin, it has been argued by Etchison and Holland that each VSV glycoprotein contains two oligosaccharides (5, 6). However, Grubman et al. (8) have reported that the VSV glycoprotein contains as many as six fucose-containing tryptic peptides. It is not impossible to reconcile these apparently conflicting data. Although four VSV glycopeptides can be resolved by gel filtration (Fig. 1 and 2), only one major peak and one minor peak are seen after the removal of sialic acid from the glycopeptides. This is consistent with a large majority of the VSV glycopeptides being similar in structure but differing in their content of terminal sialic acid. Therefore, one could postulate that only two sites on the polypeptide are glycosylated but that a family of structurally related oligosaccharides, differing in their content of sialic acid and therefore also in their charge, is present at one or both sites. Were this true, there would exist more carbohydrate-containing tryptic peptides than sites of carbohydrate attachment, since tryptic peptides which are identical except for their content of sialic acid would be charged differently.

It has been reported that the glycopeptides of VSV grown in polyoma-transformed BHK cells are more resistant to digestion with a mixture of glycosidases than the glycopeptides of VSV grown in BHK cells (15). Moyer and Summers have interpreted this to mean that the structure of the oligosaccharides acquired by VSV in these two hosts is different (15). If this interpretation is correct, the generalization made here that the effects of the host cell on viral glycosylation are minor is wrong.

In conclusion, it should be remembered that these structural arguments are based only on comparisons of the apparent size of viral glycopeptides and inferences drawn from the carbohydrate composition of whole virions and the relative carbohydrate compositions of glycopeptides. The approach is obviously indirect and the detailed conclusions are necessarily preliminary. Proof must await more rigorous chemical data. These reservations, however, should not

negate the primary conclusion that the oligosaccharides of three different viruses appear to be clearly different, even in the absence of actual linkage and sequence data.

LITERATURE CITED

1. Baenziger, J., and S. Kornfeld. 1974. Structure of the carbohydrate units of IgA$_1$ immunoglobulin. II. Structure of the O-glycosidically linked oligosaccharide units. J. Biol. Chem. 249:7270–7281.
2. Burge, B. W., and A. S. Huang. 1970. Comparison of membrane protein glycopeptides of Sindbis virus and vesicular stomatitis virus. J. Virol. 6:176–182.
3. Burge, B. W., and J. H. Strauss. 1970. Glycopeptides of the membrane glycoprotein of Sindbis virus. J. Mol. Biol. 47:449–466.
4. Duesberg, P. H., H. L. Robinson, W. S. Robinson, R. J. Huebner, and H. C. Turner. 1968. Proteins of Rous sarcoma virus. Virology 36:73–86.
5. Etchison, J. R., and J. J. Holland. 1974. Carbohydrate composition of the membrane glycoprotein of vesicular stomatitis virus. Virology 60:217–229.
6. Etchison, J. R., and J. J. Holland. 1974. Carbohydrate composition of the membrane glycoprotein of vesicular stomatitis virus grown in four mammalian cell lines. Proc. Natl. Acad. Sci. U.S.A. 71:4011–4014.
7. Grimes, W. J., and B. W. Burge. 1971. Modification of Sindbis virus glycoprotein by host-specified glycosyl transferase. J. Virol. 7:309–313.
8. Grubman, M. J., E. Ehrenfeld, and D. F. Summers. 1974. In vitro synthesis of proteins by membrane-bound polyribosomes from vesicular stomatitis virus-infected HeLa cells. J. Virol. 14:560–571.
9. Johnson, I., and J. R. Clamp. 1971. The oligosaccharide units of a human type L immunoglobulin M (macroglobulin). Biochem. J. 123:739–745.
10. Keegstra, K., B. M. Sefton, and D. Burke. 1975. Sindbis virus glycoproteins: effect of the host cell on the oligosaccharides. J. Virol. 16:613–620.
11. Lai, M. M. C., and P. H. Duesberg. 1972. Differences between the envelope glycoproteins and glycopeptides of avian tumor viruses released from transformed and from non-transformed cells. Virology 50:359–372.
12. Lai, M. M. C., P. H. Duesberg, J. Horst, and P. K. Vogt. 1973. Avian tumor virus RNA: a comparison of three sarcoma viruses and their transformation-defective derivatives by oligonucleotide finger-printing and DNA-RNA hybridization. Proc. Natl. Acad. Sci. U.S.A. 70:2266–2270.
13. Lee, Y. C., and J. R. Scocca. 1972. A common structural unit in asparagine-oligosaccharides of several glycoproteins from different sources. J. Biol. Chem. 247:5753–5758.
14. Martineau, R., M. Kohlbacher, S. N. Shaw, and H. Amos. 1972. Enhancement of hexose entry into chick fibroblasts by starvation: differential effect of galactose and glucose. Proc. Natl. Acad. Sci. U.S.A. 69:3407–3411.
15. Moyer, S. A., and D. F. Summers. 1974. Vesicular stomatitis virus envelope glycoprotein alterations induced by host cell transformation. Cell 2:63–70.
16. Rein, A., and H. Rubin. 1968. Effects of local cell concentration upon the growth of chick embryo cells in tissue culture. Exp. Cell Res. 49:666–678.
17. Schloemer, R. H., and R. R. Wagner. 1975. Mosquito cells infected with vesicular stomatitis virus yield unsialylated virions of low infectivity. J. Virol. 15:1029–1032.
18. Sefton, B. M., and B. W. Burge. 1973. Biosynthesis of the Sindbis virus carbohydrates. J. Virol. 12:1366–1374.
19. Sefton, B. M., and K. Keegstra. 1974. Glycoproteins of Sindbis virus: preliminary characterization of the oligosaccharides. J. Virol. 14:522–530.
20. Sefton, B. M., G. G. Wickus, and B. W. Burge. 1973. Enzymatic iodination of Sindbis virus proteins. J. Virol. 11:730–735.
21. Strauss, J. H., B. W. Burge, and J. E. Darnell. 1970. Carbohydrate content of the membrane protein of Sindbis virus. J. Mol. Biol. 47:437–488.
22. Toyoshima, K., R. R. Friis, and P. K. Vogt. 1970. The reproductive and cell-transforming capacities of avian sarcoma virus B77: inactivation with UV light. Virology 42:163