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Sindbis Virus Glycoproteins: Effect of the Host Cell on the Oligosaccharides

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Sindbis virus was grown in four different host cells and the carbohydrate portions of the glycoproteins were analyzed. Sindbis virus grown in BHK-21 cells has more sialic acid and galactose than Sindbis virus grown in chicken embryo cells. In other respects the carbohydrates from virus grown in these two hosts are very similar. Sindbis virus grown either in chick cells transfromed by Rous sarcoma virus or in BHK cells transformed by polyoma virus was also examined. In comparisons of virus from normal and transformed cells, differences in the amount of sialic acid were observed; but otherwise the carbohydrate structures appeared basically similar. The growth conditions used for the host cell also affected the degree of completion of the carbohydrate chains of the viral glycoproteins.

Sindbis virus is an enveloped animal virus in which the virion is comprised of a ribonucleoprotein complex which is surrounded by a lipid bilayer from which glycoprotein spikes protrude (5, 10). The virion contains two glycoproteins, designated E1 and E2 (15). Each has a molecular weight of approximately 50,000. In the virus produced by chicken embryo fibroblasts, each glycoprotein molecule has two types of oligosaccharide structures (17). One type is a rather complex oligosaccharide containing glucosamine, mannose, fucose, galactose, and usually sialic acid; the other type of oligosaccharide structure contains only glucosamine and mannose (17). In keeping with previous reports (11, 17), the more complex oligosaccharides will be referred to as A-type glycopeptides and the less complex molecules as B-type glycopeptides.

The Sindbis glycoproteins are probably glycosylated in large part, if not entirely, by host cell transferases (3, 4, 9). The host cell specificity of these enzymes has been used to explain the apparent differences in viral oligosaccharide structure which are observed when the glycopeptides of Sindbis virus, grown in different host cells, are compared (3, 4). This hypothesis suggests that Sindbis virus might be useful as a probe for investigating the mechanisms by which various host cells glycosylate proteins. For instance, it has been suggested that transformed cells glycosylate proteins differently than normal cells, and that this results in altered cell surface carbohydrates in transformed cells (1, 2, 8, 22, 23). If the glycosylation of Sindbis virus does reflect the mechanisms present in the host cell, it should be possible to use Sindbis virus to analyze differences in glycosylation mechanisms between normal and transformed cells.

This paper presents data on the extent to which the host cell can affect the glycosylation of the Sindbis virus glycoproteins. A number of variables have been studied. The oligosaccharides from virus grown in avian cells are compared with those from virus grown in mammalian cells. The effect of viral transformation of the host cell is examined by comparing the oligosaccharides of Sindbis virus grown in normal and transformed cells. The effects of cell density and of growth temperature are also examined.

MATERIALS AND METHODS

Growth of Sindbis virus in BHK-21/13 cells and polyoma-transformed BHK cells. BHK-21/13 and polyoma virus-transformed BHK cells were the gift of P. W. Robbins. Cells were passaged either in roller bottles or on Falcon plates, but were never passaged more than 10 times consecutively. Both cell lines were grown at 37 C in Eagle minimal essential medium (F-11, GIBCO), supplemented with 5% fetal calf serum. For growth of nonradioactive virus, cells were grown to confluence in roller bottles (1,200-cm^2 growth surface) and infected at a multiplicity of 0.01 to 0.1 PFU/cell. Virus was harvested after 24 h at 37 C and purified as described previously (6, 17). For growth of glucosamine-labeled virus, cells were grown to confluence at 37 C on 100-mm Falcon plates with the appropriate medium (see above) and were infected with 100 PFU/cell in 1.0 ml of medium. After absorption for 1 h, 5.0 ml of medium containing radioactive sugar was added to each plate. The infection pro-

ceeded for 12 to 16 h at 37 C. Virus was then harvested and purified as described previously (6, 17). The radioactive medium contained either 2.5 μCi of [6-^{3}H]glucosamine-hydrochloride (New England Nuclear Corp., 2.41 or 10.13 Ci/mM) or 1.0 μCi of [1-^{14}C]glucosamine-hydrochloride (New England Nuclear Corp., 56.5 mCi/mM) per ml.

Growth of Sindbis virus in RSV-transformed chick cells. Primary cultures from C/O, COFAL-negative chicken embryos (SPAFAS, Norwich, Conn.) were infected with Schmidt-Ruppin Rous sarcoma virus (RSV), subgroup A, and incubated at 38.5 C for 3 days in medium 199 supplemented with 2% tryptose phosphate broth, 1% calf serum and 1% heat-inactivated chick serum. The cells were then transferred and grown as secondary cultures in medium 199 supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated chick serum for 58 h. The fully transformed cells were then infected with Sindbis virus at a multiplicity of 50 PFU/cell and incubated for 12 h at 36 C in medium 199 supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated chick serum, and containing 2.5 μCi of [6-H^3]glucosamine (New England Nuclear, 7.3 Ci/mM) per ml. The virus was purified as described previously (6, 17).

Carbohydrate analysis of Sindbis virus grown in BHK-21/13 cells. Sucrose gradient-purified virus was precipitated by addition of 10 volumes of ice-cold water and was collected by centrifugation at 15,000 × *g* for 15 min. This virus was resuspended with sonication twice more in cold water, each time collecting the pellet by centrifugation. The pellet was again resuspended in cold water and aliquots were taken for protein, sialic acid, and neutral and amino sugar determinations. Samples were then extracted with chloroform-methanol (1:1 vol/vol) to remove lipid. The multiple water washings were performed to remove both sucrose and Tris, since both of these compounds give rise to gas-chromatographic peaks overlapping with the neutral sugars we wished to analyze. Tris, when acetylated, gives rise to a variable gas chromatographic peak described previously (17).

The quantitation of sialic acid was performed using the procedure of Warren (20). Protein determinations were performed by using the procedure of Lowry et al. (13). Neutral and amino sugars were analyzed by gas chromatography of the alditol acetate derivatives. Alditol acetates were prepared as described previously (17), with the following exceptions: after acetylation with acetic anhydride, 1 ml of toluene was added with mixing and the solution was evaporated under a stream of dry air at 25 to 35 C. The residue was taken up in $CHCl_3$ and injected into the gas chromatograph. The separations were performed on a Hewlett-Packard 5710A gas chromatograph and the peaks were electronically integrated with a Hewlett-Packard 3370B integrator. The alditol acetates were separated on a 6-foot (ca. 182.88 cm) glass column packed with 1% OV-275 on Gas Chrom Q (Applied Science) using a nitrogen carrier gas flow rate of 20 ml/min. Samples were injected at a column temperature of 170 C, and after 16 min the temperature was raised at 2 C/min to a final temperature of 220 C.

Protease digestion of viral glycoproteins and Bio Gel P-6 column chromatography of the resultant glycopeptides. Treatment of Sindbis glycoproteins with a protease preparation from *Streptomyces griseus* (Sigma Chemical Co.) degrades the protein chains leaving oligosaccharides with only a few amino acids attached (18). In experiments comparing [^{14}C]- and [^{3}H]glucosamine-labeled glycopeptides, aliquots of virus containing the two labels were first combined and unlabeled carrier virus (100 μg) was added. Virus was then precipitated by adding 10 volumes of cold water and collected by centrifugation at 15,000 × *g* for 15 min. The pellet was resuspended in 0.3 ml of 0.1 M Tris-hydrochloride, pH 8.0, containing 0.01 M $CaCl_2$ and digested for 24 to 30 h at 60 C. Protease was first dissolved in 0.1 M Tris-hydrochloride, pH 8.0, at a concentration of 10 mg/ml and then predigested (to digest any endogenous glycosidases) at 37 C for 2 h before use. This solution was added to the virus preparation in 100-μl aliquots at 1 and 12 h. Samples were centrifuged at 18,000 × *g* for 15 min to remove any insoluble material.

Glycopeptide samples were chromatographed on Bio Gel P-6 (200 to 400 mesh, Bio-Rad). The column (1.0 by 115 cm) was equilibrated and eluted with 0.1 M Tris-hydrochloride, pH 8.0. Fractions (1.0 ml) were collected, combined with 10 ml of triton-toluene (1:1 vol/vol) counting solution [6.0 g of 2,5 diphenyloxazole and 0.25 g of 1,4-bis-(5-phenyloxazolyl)benzene per liter] and counted in either a Beckman or in a Nuclear-Chicago scintillation counter. In some experiments, fractions were counted in a cocktail containing 2.67 g of 2,5 diphenyloxazole and 0.67 g of 1,4-bis-(5-phenyloxazolyl)benzene per liter of triton-toluene (2:1 vol/vol). This cocktail dissolved fractions slightly less efficiently, but gave essentially the same results. Samples containing both ^{14}C and ^{3}H were corrected for crossover counts before plotting the data.

Neuraminidase treatment of Sindbis virus glycopeptides. When the glycopeptides were to be treated with neuraminidase after protease digestion, the samples to be compared were digested with protease separately as described above, except that 0.01 M potassium phosphate buffer, pH 7.8, was used in place of the 0.1 M Tris-hydrochloride buffer. After the protease digestion, each sample was placed in a boiling water bath for 2 min. Then each sample was adjusted to pH 5.2 by adding 0.5 M KH_2PO_4. Neuraminidase (0.02 units of neuraminidase from *Clostridium perfringens* obtained from Sigma Chemical Co.) was added to one sample and the reaction was allowed to proceed for 1 h at 37 C. The control sample was similarly incubated, but in the absence of neuraminidase. After this incubation, both tubes were placed in a boiling water bath for 2 min to inactivate the neuraminidase. The samples were then mixed and placed on a Bio Gel P-6 column and eluted as described above. For this experiment, 0.5-ml fractions were collected and the radioactivity present in each fraction was determined as described above. A control experiment showed that the patterns of glycopeptides were identical when neither glycopeptide preparation was incubated with neuraminidase.

RESULTS

Comparison of the oligosaccharides of Sindbis virus grown in chick or BHK cells. To determine the effect of the species of the host cell on the glycosylation of Sindbis glycoproteins, we have examined the viral oligosaccharides by two different approaches: (i) comparison by Bio Gel P-6 chromatography of the glycopeptides derived by protease digestion of the viral glycoproteins; (ii) analysis of the carbohydrate compositions of the viral glycoproteins.

Figure 1 shows a Bio Gel P-6 elution profile of the glycopeptides from [6-^{3}H]glucosamine-labeled virus grown in chick cells and [1-^{14}C]glucosamine-labeled virus grown in BHK cells. Several features are noteworthy. The virus grown in BHK cells contained glycopeptides separable by gel filtration into four peaks. These four peaks co-chromatographed with the four glycopeptides derived from virus grown in chick cells. However, the glycopeptides from the two virus preparations differ in the relative proportion of glucosamine label present in each glycopeptide. Significantly more of the glucosamine label in the virus from BHK cells was present in the larger glycopeptides S1 and S2 than was present in these glycopeptides from virus grown in chick cells.

Table 1 shows a comparison of the sialic acid, neutral sugar, and amino sugar composition of the Sindbis grown in BHK cells with previously published data (17) for virus grown in chick cells. Virions isolated from these two hosts contain approximately the same amount of carbohydrate and have similar though nonidentical sugar compositions. Sindbis grown in BHK cells contains more galactose and sialic acid than virus grown in chick cells. In addition, virus from BHK cells contains significantly less mannose than virus from chicken cells. It should be noted that the virus from chick cells was grown at 30 C (17), whereas the virus from BHK cells was grown at 37 C. Sindbis grown in chick cells at 37 C have a carbohydrate composition similar to that grown at 30 C, except there is less sialic acid in the virus grown at 37 C (see below).

TABLE 1. *Carbohydrate compositions of Sindbis grown in two different host cells*[a]

Monosaccharide	μg of sugar/mg of virion protein	
	Chick cells	BHK cells
Mannose	23.8	17.4
Glucosamine	20.4	18.2
Galactose	7.1	10.7
Neuraminic acid	5.1	9.6
Fucose	2.3	1.4
Glucose	4.0	5.2

[a] All values have an error of approximately 10%, except for fucose, where the error is ±0.5 μg/mg of protein. The values for virus grown in chick cells were taken from previously published data (17). The values for virus grown in BHK cells were based on triplicate aliquots of one virus preparation, but similar results have been obtained with other preparations. The values for glucosamine are based on the assumption that it is *N*-acetylated.

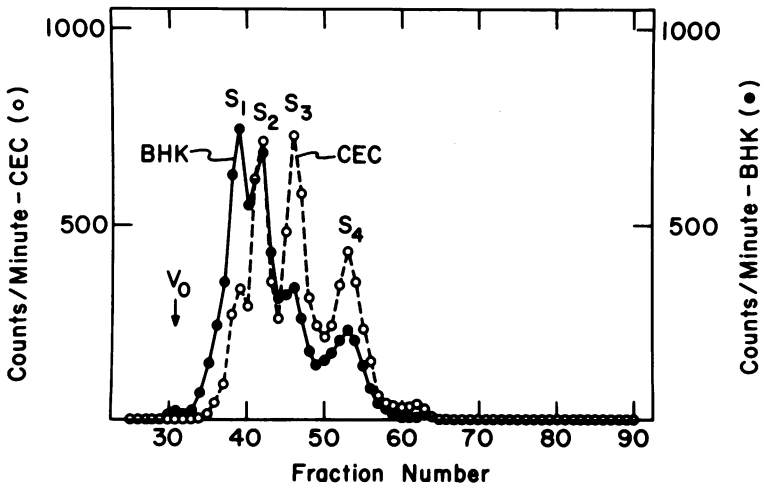

FIG. 1. *Comparison of glucosamine-labeled glycopeptides from Sindbis virus grown in BHK-21 cells* (●, [C^{14}]*glucosamine*) *with glucosamine-labeled glycopeptides from Sindbis virus grown in chick cells* (○, [H^3]*glucosamine*). *Aliquots of each purified Sindbis virus preparation were combined, digested exhaustively with proteases, and chromatographed on Bio Gel P-6.*

Effect of neuraminidase treatment. It was suggested previously (17) that glycopeptides S1 and S2 are more complete forms of glycopeptide S3, in that they probably contain additional residues of sialic acid. To examine this hypothesis, glycopeptides from Sindbis virus were treated with neuraminidase and then compared by Bio Gel P-6 chromatography with untreated glycopeptides. The results of this experiment are shown in Fig. 2. It is clear that neuraminidase treatment caused glycopeptides S1 and S2 to be converted to species which comigrated with glycopeptide S3. The new peak at fractions 145–150 is most probably free sialic acid, which is labeled when virus is labeled with radioactive glucosamine (21). If either glycopeptide S1 or S2 was first isolated by gel filtration and then treated with neuraminidase, the product of such treatment comigrated with glycopeptide S3 (data not shown). Results similar to those in Fig. 2 have been obtained with virus grown in either BHK cells or polyoma virus-transformed BHK cells.

Comparison of Sindbis glycopeptides from virus grown in normal and virus-transformed BHK and chick cells. There is considerable evidence that alterations in the activity of cellular glycosyl transferases and alterations of surface oligosaccharides accompany viral transformation of cells (1, 8, 22, 23). To examine whether transformation of the host would affect the glycosylation of the Sindbis virus glycoproteins, the virus was grown in RSV-transformed chicken embryo cells and in a line of polyoma virus-transformed BHK cells.

The glucosamine-labeled glycopeptides of virus grown in RSV-transformed chick cells were compared by gel filtration with those of virus from normal chick cells (Fig. 3). Both preparations contained glycopeptides of four sizes. The material present at the void volume of the column (fractions 28 and 29) is probably hyaluronic acid and not viral glycopeptide (12). The virus from RSV-transformed chick cells contained glycopeptides which co-chromatograph with glycopeptides S1, S2, and S3 of virus from normal cells. Relatively, a larger fraction of total glucosamine label was present in glyco-

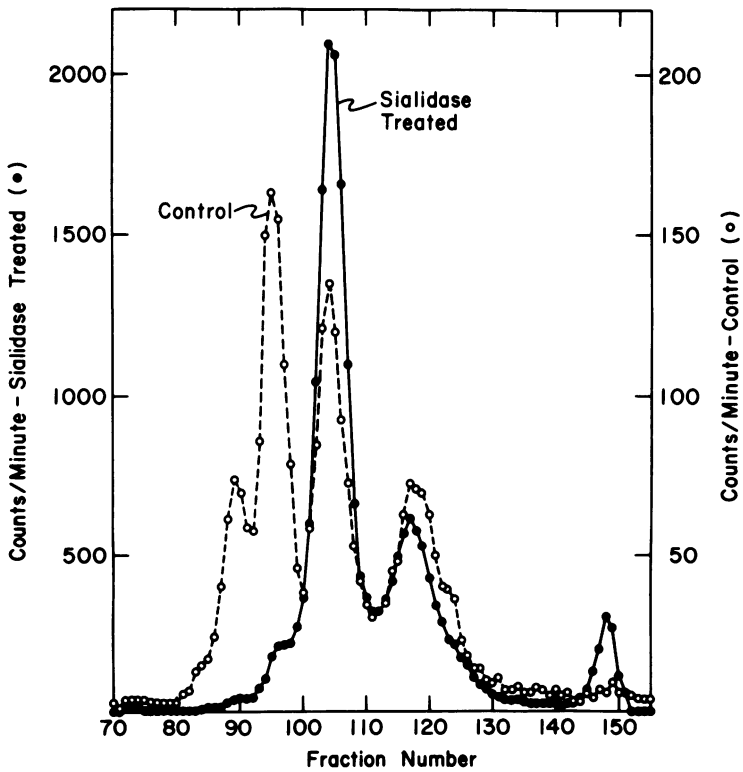

FIG. 2. *Neuraminidase treatment of Sindbis virus glycopeptides. Aliquots of purified Sindbis virus, one labeled with [^3H]glucosamine and one labeled with [^{14}C]glucosamine, were digested with proteases separately. The glycopeptides labeled with ^3H glucosamine (●) were subsequently treated with neuraminidase. These were combined with untreated [^{14}C]glucosamine-labeled glycopeptides (○) and chromatographed on Bio Gel P-6.*

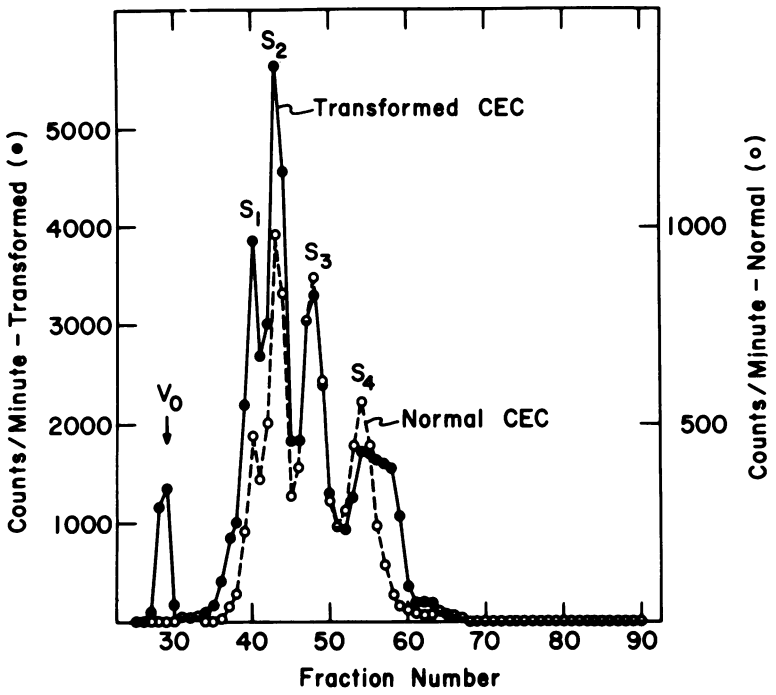

FIG. 3. *Comparison of glucosamine-labeled glycopeptides from Sindbis virus grown in RSV-transformed chick cells* (●, [H^3]*glucosamine*) *with glucosamine-labeled glycopeptides from Sindbis virus grown in normal chick cells* (○, [C^{14}]*glucosamine*). *Aliquots of each purified Sindbis virus preparation were combined, digested exhaustively with proteases, and chromatographed on Bio Gel P-6.*

peptide S1 of virus from transformed cells than in glycopeptide S1 from normal cells. Glycopeptide S4 of virus from RSV-transformed cells appeared slightly smaller and more heterogeneous than glycopeptide S4 of virus from normal chick cells.

Similarly, the glucosamine-labeled glycopeptides of Sindbis virus grown in a polyoma virus-transformed BHK cell line were compared with those of virus grown of normal BHK cells (Fig. 4). Again, glycopeptides of four sizes were present in both virus preparations. However, as in the above comparisons, the fraction of total glucosamine label in two various glycopeptides was different in the two samples. The virus from the polyoma virus-transformed cells contained relatively less of glycopeptide S1 and relatively more of glycopeptide S3 than virus from normal BHK cells.

Effect of growth temperature upon the glycosylation of viral glycoproteins. The glucosamine-labeled glycopeptides of Sindbis virus grown at 30 C were compared by gel filtration with those from virus grown at 38.5 C (Fig. 5). The growth temperature had a significant effect on the relative ratio of the various glycopeptides. The virus grown at 30 C contained a larger amount of glycopeptide S1 and a smaller amount of glycopeptide S3 than the virus grown at 38.5 C.

Effect of host cell density upon the glycosylation of viral glycoproteins. It has been reported that cell density has an effect upon the glycosylation of membrane glycoproteins (2, 22). To examine if this effect is reflected in the viral glycoproteins, Sindbis virus was grown in BHK cells at low density (10^6 cells/100-mm plate) and high density (7.8×10^6 cells/100-mm plate). Comparison by Bio Gel P-6 chromatography of the glycopeptides of the virus preparations revealed a slight increase in the relative amount of glycopeptide S1 in virus grown at lower cell densities. The extent of the increase was small but reproducible in three different experiments. In similar experiments using polyoma virus-transformed BHK cells, the opposite effect was observed, revealing a slight decrease in the amount of glycopeptide S1 in virus grown at low cell density. With chick cells as the host, there was no difference in the glycopeptides of virus grown at high cell density or low cell density.

DISCUSSION

The envelope glycoproteins of Sindbis virus contain glycopeptides separable by gel filtration

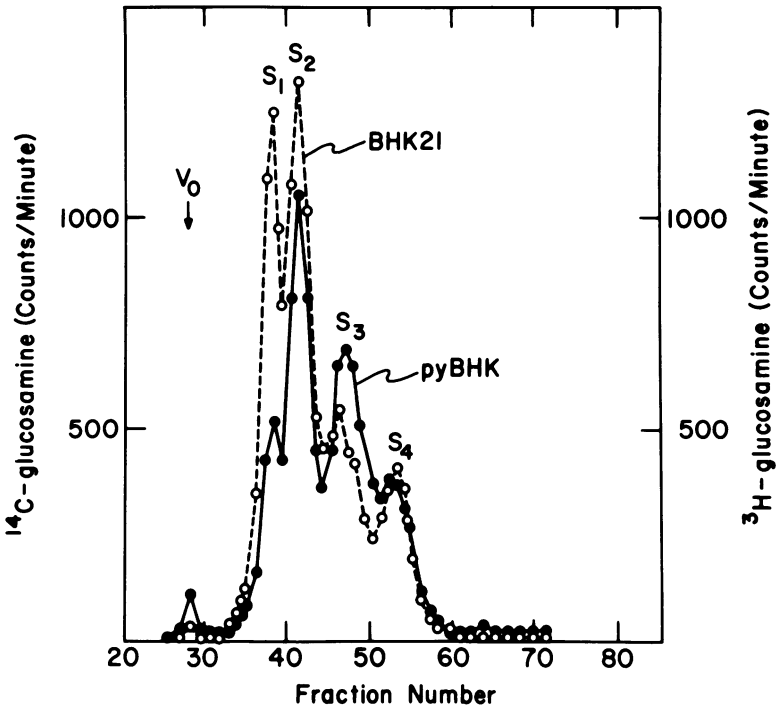

FIG. 4. *Comparison of glucosamine-labeled glycopeptides from Sindbis virus grown in polyoma virus-transformed BHK cells (●, [^{14}C]glucosamine) glucosamine-labeled glycopeptides from Sindbis virus grown in BHK-21 cells, (○, [^{3}H]glucosamine). Aliquots of each purified Sindbis virus preparation were combined, digested exhaustively with proteases, and chromatographed on Bio Gel P-6.*

into four peaks (labeled S1–S4 in Fig. 1–4) in each of four different host cells. It has been shown that glycopeptides S1, S2, and S3 contain mannose, glucosamine, galactose, and fucose, whereas glycopeptide S4 contains only glucosamine and mannose (17). Based on this and on other data, it was suggested that glycopeptides S1 and S2 represent more complete forms of glycopeptide S3 (17). This is supported by the data in Fig. 2, which show that glycopeptides S1 and S2 can be converted by neuraminidase digestion to a form which co-chromatographs with glycopeptide S3 on a Bio Gel P-6 column. Figure 2 shows that the elution position of glycopeptide S3 is unaffected by neuraminidase treatment, indicating that this glycopeptide contains no sialic acid. The carbohydrate compositions of purified glycopeptides support this conclusion and demonstrate that glycopeptide S1 contains two residues of sialic acid, whereas glycopeptide S1 has one residue of sialic acid (Keegstra and Burke, unpublished data). Based on these data and the total carbohydrate composition of the Sindbis virus glycoproteins, it has been suggested that each Sindbis glycoprotein contains two sites of carbohydrate attachment, one giving rise to glycopeptide S4 and the second giving rise to either glycopeptide S1, S2, or S3. The data presented here suggest that this is also true for Sindbis grown in BHK cells. The increase in galactose and sialic acid content of virus grown in BHK cells (Table 1) is consistent with the observation that this virus contains more of glycopeptide S1, the most complete form of the A-type glycopeptide.

The data presented here do not indicate whether or not glycosylation occurs at the same sites on the viral polypeptide in each host. It is possible that there are changes in the linkages of the core structure of the glycopeptides which are not detected in these experiments. More detailed studies are currently underway to examine these possibilities. Despite these reservations, it appears that the glycosylation of Sindbis virus glycoproteins by several different hosts is quite similar. Etchison and Holland (7) concluded that the glycosylation of the glycoprotein of vesicular stomatitis virus by four mammalian cell lines was also basically similar. This result with vesicular stomatitis virus and these results with Sindbis virus suggest that the glycosylation of a particular viral glycoprotein might possibly vary only as a function of the ability of the host to assemble specific viral glycopeptides. An extreme example of this is the

observation of Schloemer and Wagner (16) that vesicular stomatitis virus grown in mosquito cells lacks, as do mosquito cells, sialic acid.

Although the glycosylation of the Sindbis glycoproteins appears similar in the hosts examined, there are obvious host-dependent variations in the extent to which the A-type glycopeptides are completed. Sindbis virus grown in BHK cells contains A-type glycopeptides with a greater degree of completion than the A-type glycopeptides from virus grown in chick cells. This results both in a larger proportion of glycopeptide S1 (Fig. 1) and in a larger amount of sialic acid and galactose (Table 1) in the virus grown in BHK cells. Sindbis virus grown in RSV-transformed chick cells also contains A-type glycopeptides which are completed to a greater degree than virus grown in normal chick cells (Fig. 4). However, it is not possible to generalize about the effect of transformation on the completion of the A-type oligosaccharide. When virus from polyoma virus-transformed BHK cells was compared with that from BHK cells, it was seen that there was a decrease, rather than an increase, in the degree of completion of the A-type glycopeptide (Fig. 3). Finally, both the temperature of growth and cell density of the host cells can affect the degree of completion of the A-type glycopeptide.

Transformation of chick cells by RSV and of BHK by polyoma virus also produced slight alterations in the B-type oligosaccharides in peak S4. In both cases, the S4 peak eluted at a slightly different retention volume relative to the normal counterpart. In the case of RSV transformation of chick cells, the Sindbis S4 peak was also much more heterogeneous in size. Whether such changes represent differences in completion of these oligosaccharides or some other structural modification is unknown and is currently under investigation.

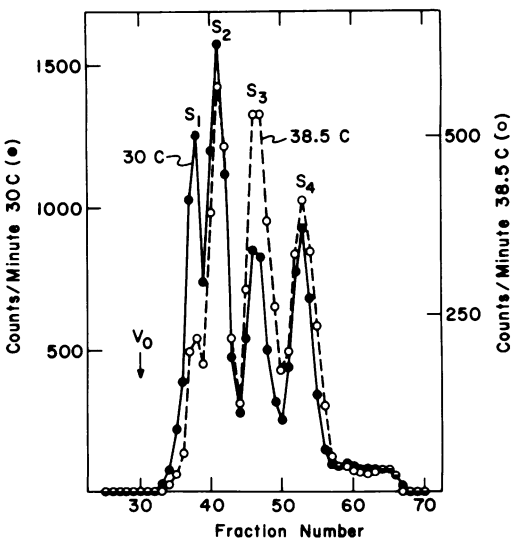

FIG. 5. *Comparison of glucosamine-labeled glycopeptides from Sindbis virus grown in chick cells at 30 C (●, [^{3}H]glucosamine) with glycopeptides from Sindbis virus grown in chick cells at 38.5 C (○, [^{14}C]-glucosamine). Aliquots of each purified Sindbis virus preparation were combined, digested exhaustively with proteases, and chromatographed on Bio Gel P-6.*

There are several possible explanations for the changes in glycopeptide patterns which have been observed. (i) The changes in the relative levels of glycopeptides S1, S2, and S3 might reflect parallel changes in the levels of synthetic glycosyl transferase activities (in particular sialyl transferase) present in the host cell. (ii) The changes in the degree of completion might reflect changes in the levels of degradative glycosidase activities (either in the cell membrane or free in the culture medium). (iii) The glucosamine- and mannose-containing portion of the A-type glycopeptides might be structurally altered in some hosts in such a way as to change the efficiency with which these chains are completed. (iv) The rates of virus maturation and envelope protein glycosylation in these cells may vary in such a way as to alter the extent to which the oligosaccharides are completed. This latter mechanism seems the most likely explanation for the differences observed as a function of growth temperature (Fig. 5). It is certainly possible that more than one of these mechanisms may contribute to the observed differences in degree of completion of the viral A-type glycopeptides in the different host cells.

The effects of transformation of the host cell on the degree to which the Sindbis virus A-type glycopeptides are completed are consistent with several other reports. There is evidence for both increased sialyl transferase activity and larger cell surface glycopeptides following RSV transformation of chick cells (22, 23). Lai and Duesberg (12) observed that the glycopeptides of a number of avian tumor viruses were larger in virus grown in RSV-transformed chick cells than in virus grown in phenotypically normal cells. Warren et al. (22, 23) observed that the size of fucose-containing chick cell surface glycopeptides was larger after RSV transformation and that this increase in size was due to the presence of sialic acid. These two results are similar to the observed increased completion of the A-type Sindbis virus glycopeptides by transformed chick cells.

On the other hand, Grimes (8) has observed that polyoma virus-transformed BHK cells have significantly less sialyl transferase activity

than do BHK cells. This is consistent with the observed decreased completion of the A-type viral glycopeptides in polyoma virus-transformed BHK cells.

Moyer and Summers (14) have reported that the glycopeptides isolated from VSV grown in polyoma-transformed BHK cells are similar in size to the glycopeptides isolated from virus grown in BHK-21 cells. However, they reported that the glycopeptides from virus grown in these two hosts yielded different products when degraded with a mixture of glycosidases, indicating that there was some undefined structural modification. The experiments reported here neither confirm nor rule out such differences in the glycopeptides of Sindbis virus grown in these two hosts.

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