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# Glycoproteins of Sindbis Virus: Preliminary Characterization of the Oligosaccharides

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The carbohydrate content of Sindbis virus was determined by gas chromatographic analysis. The two viral glycoproteins were found to be approximately 8% carbohydrate by weight. Mannose is the sugar present in the largest amount. Smaller amounts of glucosamine, galactose, sialic acid, and fucose were also detected. Each of the two viral glycoproteins appears to contain two structurally unrelated oligosaccharides. Two of the three Sindbis-specific glycoproteins found in infected chick cells were shown to contain short, unfinished oligosaccharides.

Sindbis virus is a simple, spherical, enveloped virus which contains two glycoproteins, one nonglycosylated internal protein, a lipid bilayer, and a single-stranded RNA genome. The two glycoproteins are located on the outside of the viral membrane (2). Although the polypeptide chains of the viral glycoproteins are virally specified (8), the glycosylation of these proteins is presumably carried out in large part by normal, cellular glycosyl transferases (4).

We have determined the carbohydrate composition of the virus by gas chromatographic methods. Further, we have examined which sugars are present in the various viral oligosaccharides and which of these oligosaccharides are present on each viral glycoprotein. Finally, we have examined the biosynthesis of the viral oligosaccharides by comparison of the oligosaccharides of the virus-specific, cell-associated glycoproteins with the oligosaccharides of the mature virion.

## MATERIALS AND METHODS

**Growth and radioactive labeling of the virus.** All virus was grown in chicken embryo cells. Virus for chemical analysis was produced by two steps of growth on secondary chick cells in glass roller bottles (1,200 $cm^2$ growth surface). These cells were grown to confluence, approximately $10^8$ to $2 \times 10^8$ cells per roller bottle, at 37 C in Eagle medium supplemented with 5% calf serum and 2% heat-inactivated chicken serum.

Virus, diluted in Eagle medium containing 2% tryptose phosphate broth and 1% calf serum, was allowed to adsorb to the cells for 60 min at 30 C in a volume of 10 ml. The virus was diluted such that the multiplicity of infection was approximately 0.01 to 0.10 PFU per cell. After adsorption, 90 ml of Eagle medium containing 2% tryptose phosphate broth and 1% calf serum was added to each bottle and the infection was allowed to proceed for 24 h at 30 C.

Radioactive virus was produced by one step of growth in either primary or secondary chick cells growing on 100-mm plastic petri dishes (Falcon). Secondary cultures were used 18 to 24 h after transfer. Confluent monolayers were infected with 1.0 ml of virus (100 PFU per cell) in Eagle medium containing 2% tryptose phosphate broth and 1% calf serum. Adsorption was allowed to proceed for 60 min at either 36 C or 38.5 C with occasional rocking. Then 5 ml of Eagle medium supplemented as described above, and containing radioactive sugars, was added to each dish. The infection was allowed to proceed for 10 to 14 h at 36 C or 38.5 C. When [2-$^3$H]mannose was used as the label, actinomycin D (1 $\mu$g/ml) was present in the medium. The radioactive medium contained 5 to 25 $\mu$Ci of [6-$^3$H]glucosamine per ml (New England Nuclear Corp., 7.3 Ci/mmol), 2.5 $\mu$Ci of [1-$^{14}$C]glucosamine per ml (New England Nuclear Corp., 57.5 mCi/mmol), 8 $\mu$Ci of [2-$^3$H]mannose per ml (Amersham/Searle, 611 mCi/mmol), 25 $\mu$Ci of [1-$^3$H]-galactose (New England Nuclear Corp., 5.95 Ci/mmol) or 50 $\mu$Ci of [$^3$H]fucose per ml (New England Nuclear Corp., 4.3 Ci/mmol).

**Virus purification.** Some samples of virus were purified by the method of David (3). Others were purified by a modification of this procedure. All steps were performed at 2 to 4 C. Cell debris was removed from virus-containing medium by centrifugation at $15,000 \times g$ for 10 to 15 min. The medium was made 0.5 M in NaCl by the addition of solid NaCl. Polyethylene glycol (MCB) was added to a final concentration of 10% (wt/vol). This solution was then stirred for at least 60 min. The virus was collected by centrifugation at $15,000 \times g$ for 15 min. The pellet was dissolved in a solution of 0.2 M NaCl, 0.05 M Tris-hydrochloride, pH 7.4, and 0.001 M EDTA. This solution was sonically treated in a Raytheon sonicator at three quarters power for 30 to 45 s and then was clarified by

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centrifugation at 15,000 × *g* for 5 to 15 min. The resulting pellet was dissolved, sonically treated, and centrifuged as just described. The combined supernatants were layered onto a 15 to 30% (wt/wt) sucrose gradient in 0.15 M NaCl, 0.05 M Tris-hydrochloride, pH 7.4, and 0.001 M EDTA and centrifuged for 3 h at 24,500 rpm in a Spinco SW 27 rotor.

**Carbohydrate analysis of Sindbis virus.** Sucrose gradient-purified virus (20 to 40 mg) was precipitated by the addition of 10 volumes of ice-cold water. After at least 30 min at 0 to 2 C, the virus was collected by centrifugation at 15,000 × *g* for 15 min (12). The pellet was dissolved in 0.1 M Tris-hydrochloride, pH 7.8, and separate portions were taken for protein, sialic acid, and neutral and amino sugar analysis. Protein was determined by the method of Lowry et al. (7).

Virus for sialic acid analysis was again precipitated by fivefold dilution with water and concentrated by centrifugation. The pellet was hydrolyzed in 0.5 ml of 0.1 N $H_2SO_4$ for 1 h at 80 C. Insoluble material was removed by centrifugation and the supernatant was assayed for sialic acid by using the thiobarbituric acid method described by Warren (16). The absorbance of the cyclohexanone solution containing the chromophore was determined at both 532 and 549 nm to insure that there was not a significant amount of absorption due to nonsialic acid components.

Portions for neutral and amino sugar analysis were also concentrated by fivefold dilution with water and centrifugation. The pellets were hydrolyzed in 0.5 ml of 2 N trifluoroacetic acid for 2 h at 121 C. *Myo*-inositol (25 or 50 μg) was added as an internal standard. After hydrolysis, the samples were dried under a stream of nitrogen at 45 C. Reduction of the sugars was achieved using 5 mg of $NaBH_4$ in 0.5 ml of 0.1 N $NH_4OH$. Excess borohydride was destroyed by addition of 5 drops of glacial acetic acid. The samples were dried with a stream of nitrogen at 45 C. Boric acid was removed by five successive additions of 1 ml of methanol which was evaporated each time with a stream of nitrogen. The samples were dried overnight in vacuo over $P_2O_5$ and NaOH. The alditols were acetylated with acetic anhydride (0.5 ml) for 2 h at 100 C. The acetic anhydride was destroyed by reaction with 1.0 to 1.5 ml of a saturated $Na_2CO_3$ solution for approximately 2 h at room temperature. The acetate-buffered aqueous solution was then extracted with an equal volume of chloroform. After centrifugation in a clinical centrifuge, the chloroform layer was removed and carefully dried under a stream of nitrogen at room temperature. The residue was taken up into 30 to 50 μliters of acetone and 0.5 to 1.0 μliter was used for injection into the gas chromatograph.

The separations were performed on a Perkin-Elmer model 900 gas chromatograph with a flame ionization detector. The helium carrier gas flow was maintained at 60 ml/min. The peak areas were quantitated with the aid of an Infotronics electronic integrator, model CRS-208.

The alditol acetates were separated utilizing 4-foot stainless steel columns of either 3% ECNSS-M on Gas Chrom Q or 1% Silar 5CP on Gas Chrom Q (all from Applied Science). Samples were injected at a column temperature of 140 C and, after 4 to 6 min, the temperature was raised at 2°/min to a final temperature of 210 C. Identification of the sugar peaks was made by comparison of retention times with known sugar standards. The peak areas were corrected for detector response and hydrolysis losses using correction factors obtained by subjecting standard sugars to the same procedure.

In all virus preparations there was an unknown noncarbohydrate component, present in variable amounts, which chromatographed with galactose on ECNSS-M but was separable from the component sugars on Silar 5CP. The other sugars gave similar values on both column types.

**Pronase digestion.** Radioactive virus samples to be compared by gel filtration were combined prior to Pronase digestion. Unlabeled sucrose gradient purified virus (approximately 100 μg) was added as carrier. The virus was concentrated by dilution with water and centrifugation at 18,000 × *g* for 15 min. The pellet was dissolved in 0.5 ml of 0.1 M Tris-hydrochloride, pH 8.0, and 0.01 M $CaCl_2$. Pronase digestion was for 24 to 30 h at 60 C with toluene present to retard bacterial growth. Pronase (Calbiochem, A grade) was dissolved in 0.1 M Tris-hydrochloride, pH 8.0, and 0.01 M $CaCl_2$ at a concentration of 10 mg/ml and was predigested for 2 h at 37 C to destroy any glycosidase activity. This solution was added in three 250-μg (25 μliters) portions at 0, 6, and 20 h. Prior to gel filtration, insoluble material was removed by centrifugation at 18,000 × *g* for 5 min.

**Gel filtration.** Glycopeptide samples were chromatographed on Bio-Gel P-6 (200 to 400 mesh, Biorad). The column (1.0 by 115 cm) was equilibrated and eluted with 0.1 M Tris-hydrochloride, pH 8.0, at 25 C. Blue Dextran 2000 (Pharmacia) was used to define the void volume, Vo, but was not used with most samples since it bound tightly to the column material. Fractions (1.0 ml) were mixed with 8 to 10 ml of a triton-X100-toluene (1:2, vol/vol) scintillation fluid and counted in a Beckman scintillation counter.

**Labeling of cell-associated glycoproteins.** The procedures for infection and labeling of cells have been described in detail (11). Briefly, a monolayer of secondary chicken embryo cells was infected with a high multiplicity of Sindbis virus, approximately 100 PFU per cell, in the presence of actinomycin D (1 μg/ml). The infection was allowed to proceed for approximately 4 h at 38.5 C at which time the medium was replaced with medium containing [$^3$H]glucosamine (65 μCi/ml). Incubation was continued for approximately 4 h more. The medium was then removed, the cells were washed to remove any attached virus, and the monolayer was dissolved in a solution of 2% sodium dodecyl sulfate (SDS), 5 mM sodium phosphate buffer (pH 7.0), 15% (vol/vol) glycerol, and 2 mM phenyl methane sulfonyl flouride.

**Purification of the glycoproteins.** To separate the Sindbis glycoproteins, viral or cellular samples were subjected to SDS polyacrylamide gel electrophoresis as has been described in detail (11). The gels were frozen and fractionated with stacked razor blades, and each slice was shaken for approximately 18 h at 25 C

with 0.5 ml of 0.1% SDS. The radioactivity in a portion (25 μliters) of each fraction was determined and appropriate fractions were combined.

Samples to be compared were then mixed, lyophilized, and redissolved in 0.5 ml of water. The glycoproteins were precipitated from this solution with 5 volumes of absolute ethanol (−20 C) with bovine serum albumin (200 μg) present as carrier. After at least 1.5 h at −20 C, the precipitate was collected by centrifugation at 18,000 × *g* for 15 min. The pellet was then dissolved in 0.5 ml of 0.1 M Tris-hydrochloride, pH 8.0, and 0.01 M $CaCl_2$ and digested with Pronase as has been described.

## RESULTS

**Carbohydrate composition of Sindbis virus.** A preliminary estimation of the carbohydrate content of Sindbis virus has been reported (15). These studies used colorimetric tests which could not distinguish between the hexoses. The carbohydrate content of Sindbis virus was determined by using gas chromatographic methods to quantitate the neutral and amino sugars. The results of this analysis are shown in Table 1. The values of sialic acid are similar to those reported previously, as would be expected since the same methods were used in both cases. The variable amounts of glucose (Table 1) probably arose principally from sucrose contamination of the viral preparation during sucrose gradient centrifugation. This may account for some of the discrepancy between the hexose values reported previously and those reported here.

The glucosamine and sialic acid are probably acetylated on the nitrogen of the amino groups; however, this has not been specifically demonstrated in this case. No galactosamine was observed in any of the viral preparations.

TABLE 1. *Carbohydrate composition of Sindbis virus*[a]

| Carbohydrate | μg per mg of protein | μg per mg of glycoprotein | mol per $10^5$ g of glycoprotein |
|---|---|---|---|
| Mannose | 23.8 | 32 | 18 |
| Glucosamine | 20.4 | 27 | 13 |
| Galactose | 7.1 | 9.4 | 5.2 |
| Sialic acid | 5.1 | 6.8 | 2.3 |
| Fucose | 2.3 | 3.1 | 1.9 |
| Glucose | 4.0 | 5.3 | 2.9 |
| Total (without glucose) | 58.7 | 78.3 | 40.4 |

[a] The values represent the average of duplicate injections of six portions of one virus preparation. All values have an error of approximately 10%. Analysis of other virus preparations gave similar results except for glucose which has given values as low as 1.2 μg per mg of protein and as high as 15 μg per mg of protein. The calculations for glucosamine are based on the assumption that it is *N*-acetylated. The conversion from micrograms per milligram of protein to micrograms per milligram of glycoprotein was made using the observation that the unglycosylated viral core protein represents 25% of the total radioactivity in virus preparations labeled with a mixture of radioactive amino acids (B. Sefton, unpublished observation).

**Gel filtration of the glycopeptides from the viral glycoproteins.** Exhaustive digestion of a glycoprotein with the protease mixture, Pronase, destroys the polypeptide chain and leaves only a few amino acids attached to the oligosaccharides (14). The carbohydrate-containing structures which remain after this digestion are generally referred to as glycopeptides. This term is somewhat misleading and ambiguous since most of the mass of a Pronase-generated glycopeptide is carbohydrate, not polypeptide, and because other types of molecules, carbohydrate-containing tryptic peptides (9), and uncharacterized carbohydrate-containing polypeptides (13) are referred to as glycopeptides. When the term glycopeptide is used here, it will mean a carbohydrate-containing molecule surviving an exhaustive Pronase digestion.

It has been shown that Pronase digestion of Sindbis virus grown in hamster cells yielded glycopeptides which were separable into four peaks by gel filtration, whereas the glycopeptides of Sindbis virus grown in chick cells were separable into only three peaks, which co-chromatographed with the three smaller peaks of hamster-grown virus (1). By using radioactive sugar and colorimetric assays, a preliminary carbohydrate content of these glycopeptides was proposed (1). Through the use of [2-$^3$H]mannose, the metabolism of which does not produce other labeled sugars, and a column allowing much greater resolution than before, it has been possible to more precisely estimate the carbohydrate content and general structure of the Sindbis glycopeptides. Purified Sindbis virus, grown in chick cells and labeled with [1-$^{14}$C]glucosamine, was mixed with purified virus, also grown in chick cells, labeled with [2-$^3$H]mannose, or [1-$^3$H]galactose or [$^3$H]fucose. The mixtures were digested exhaustively with Pronase, and the resulting glycopeptides were compared by gel filtration on Bio-Gel P-6 (Fig. 1). This procedure separated the glycopeptides from whole virus grown in chick cells into four glucosamine-containing peaks, one more than previously reported. These peaks of glycopeptides are designated S1, S2, S3, and S4, the S referring to the fact that separation by gel filtration is based on the size of the glycopeptide. The largest glycopeptide, S1, co-chromatographs with the largest glycopeptide of virus grown in hamster cells (K. Keegstra and B. Sefton, manuscript in preparation), previously designated $H_A$ (1). The three smaller

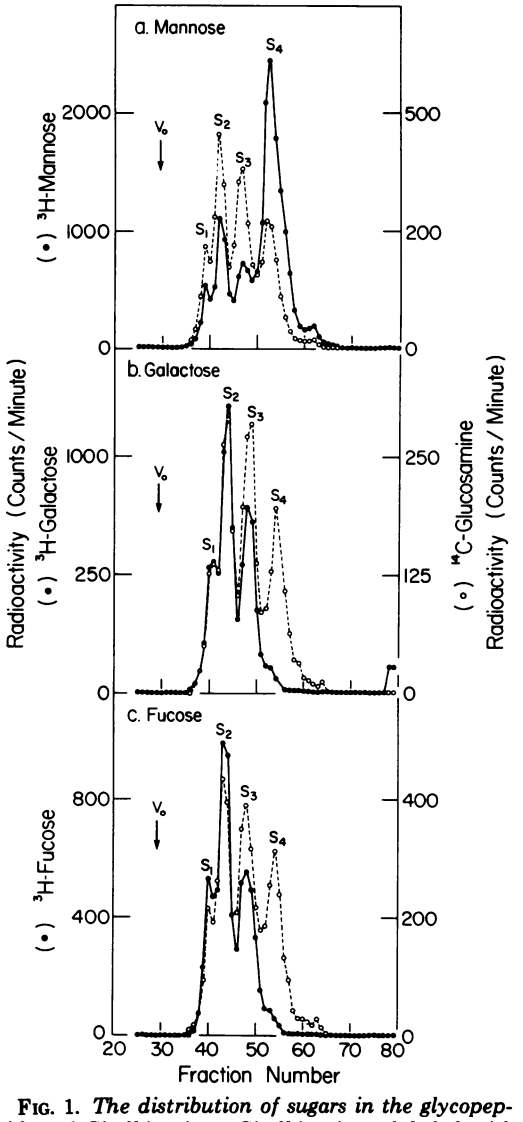


FIG. 1. *The distribution of sugars in the glycopeptides of Sindbis virus. Sindbis virus, labeled with [1-¹⁴C]glucosamine, was mixed with virus labeled with either [2-³H]mannose or [1-³H]galactose or [³H]fucose. The mixtures were digested with Pronase and chromatographed on Bio-Gel P-6. Symbols: (a) [2-³H]mannose (●) and [1-¹⁴C]glucosamine (○); (b) [1-³H]galactose (●) and [1-¹⁴C]glucosamine (○); (c) [³H]fucose (●) and [1-¹⁴C]glucosamine (○).*

peaks, S2, S3, and S4, appear to correspond to those previously designated $C_A$, $C_B$, and $C_C$, respectively. Whether glycopeptide S1 from virus grown in chick cells is identical in structure to the largest glycopeptide from virus grown in hamster cells is not known.

All four glycopeptide peaks also contained mannose in addition to glucosamine (Fig. 1). The ratio of mannose to glucosamine (counts per minute of [³H]mannose per counts per minute of [¹⁴C]glucosamine) was the same in glycopeptides S1 and S2, about 2.4, somewhat less in glycopeptide S3, 2.0, and much greater in glycopeptide S4, 9.2 (Table 2). In fact, 60% of the mannose present in all of the glycopeptides was found in glycopeptide S4. Glycopeptides S1, S2, and S3 appear to also contain galactose and fucose. Glycopeptide S4 was labeled by neither galactose nor fucose and thus appears to contain only glucosamine and mannose. If the amount of [³H]mannose, [³H]galactose or [³H]fucose is normalized to the amount of [¹⁴C]glucosamine in each glycopeptide (Table 2), glycopeptide S1 is seen to contain the same amount of mannose and more of both galactose and fucose than glycopeptide S2. Glycopeptide S2, in turn, appears to contain more mannose, galactose, and fucose than glycopeptide S3. These conclusions agree closely with preliminary results obtained by gas chromatographic analysis of the sugar compositions of the various purified glycopeptides (K. Keegstra and B. Sefton, unpublished data).

It was not known to which of the two Sindbis glycoproteins these glycopeptides were attached. Therefore, Sindbis virus, labeled with either [³H]glucosamine or [¹⁴C]glucosamine, was subjected to SDS polyacrylamide gel electrophoresis. These gels were fractionated and each glycoprotein was eluted from the gel. Glycoprotein E1, labeled with [³H]glucosamine was mixed with glycoprotein E2, labeled with [¹⁴C]glucosamine, and the mixture was digested with Pronase and examined by gel filtration (Fig. 2). Both glycoproteins contained four gly-

TABLE 2. *Apparent ratios of mannose, galactose, and fucose to glucosamine in the glycopeptides of Sindbis virus grown in chick cells*[a]

| Glycopeptide fraction | Ratio of sugars (counts per min of [³H]sugar per counts per min of [¹⁴C]glucosamine) | | |
|---|---|---|---|
| | Mannose/ glucosamine | Galactose/ glucosamine | Fucose/ glucosamine |
| $S_1$ | 2.38 | 4.11 | 2.32 |
| $S_2$ | 2.41 | 3.91 | 2.15 |
| $S_3$ | 1.99 | 2.69 | 1.42 |
| $S_4$ | 9.16 | 0 | 0 |

[a] These ratios were calculated directly from the data in Fig. 1. Fractions at the minima between peaks were divided between the two fractions in approximate proportion to the peak areas. The ratios presented here are all relative, due to the different specific activities of the sugars used. Therefore, these numbers do not correspond to the actual chemical ratios of the various sugars.

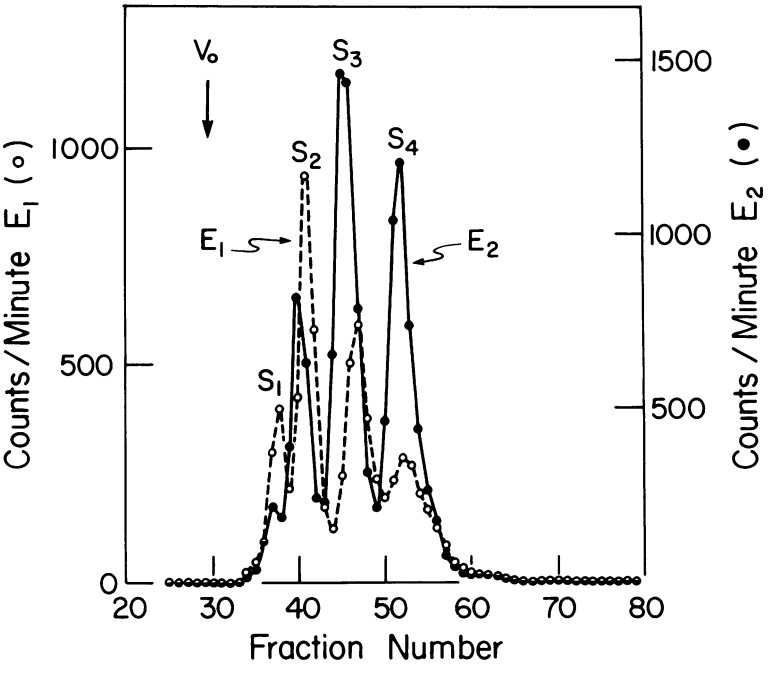


FIG. 2. *Comparison of the glycopeptides of the two Sindbis glycoproteins by gel filtration. Glycoprotein E1, labeled with [$^3$H]glucosamine, and glycoprotein E2, labeled with [$^{14}$C]glucosamine, were purified by SDS polyacrylamide gel electrophoresis. The glycoproteins were mixed, digested with Pronase, and compared by chromatography on Bio-Gel P-6. Symbols: glycoprotein E1, [$^3$H]glucosamine (○); glycoprotein E2, [$^{14}$C]glucosamine (●).*

copeptides. Thus, each of the four peaks seen with whole virus (Fig. 1) consists of a mixture of glycopeptides from the two glycoproteins. Two differences in the patterns seen with the purified glycoproteins should be noted. First, the three larger glycopeptides, S1, S2, and S3, from glycoprotein E2 reproducibly eluted from the column one fraction before the corresponding glycopeptides from glycoprotein E1. This difference is not due to the choice of isotope since it is not affected by reversal of the labels (not shown). Second, although both glycoproteins contain four glycopeptides, the percentage of protein-bound glucosamine found in the individual glycopeptides is noticeably different in the two glycoproteins.

**Gel filtration of the glycopeptides from cell-associated virus-specific glycoproteins.** Sindbis-infected chick cells contain three virus-specific glycoproteins, the two virion glycoproteins, E1 and E2, and a precursor to E2, called glycoprotein PE2 (10). It was observed that the precursor, although partially glycosylated, appears to lack fucose and to contain less galactose than its product, E2 (11). It was of interest to determine whether this apparently incomplete glycosylation of the precursor was reflected in the size of its glycopeptides. Therefore, the precursor glycoprotein, PE2, was eluted from an SDS-polyacrylamide gel of [$^3$H]glucosamine-labeled Sindbis-infected cells, and mixed with [$^{14}$C]glucosamine-labeled glycoprotein E2, which had been eluted from a gel of purified virions. The mixture was digested with Pronase and the glycopeptides were examined by gel filtration (Fig. 3).

Clearly the patterns are very different. Little of the glycopeptide material from glycoprotein PE2 was as large as virion glycopeptide S3. A majority of the glucosamine label eluted from the column between glycopeptides S3 and S4, a position which does not correspond to any glycopeptide found in mature virions. Glycopeptides the same size as virion glycopeptide S4 were present in glycoprotein PE2, but a distinct peak comigrating with glycopeptide S4 was not detected. No glycopeptides smaller than S4 were present in glycoprotein PE2.

A comparison of the glycopeptides of the cell-associated and virion forms of glycoproteins E1 and E2 was also made. These experiments were performed in the same manner as that in Fig. 3. Cell-associated glycoproteins, labeled with [$^3$H]glucosamine, were purified from SDS-

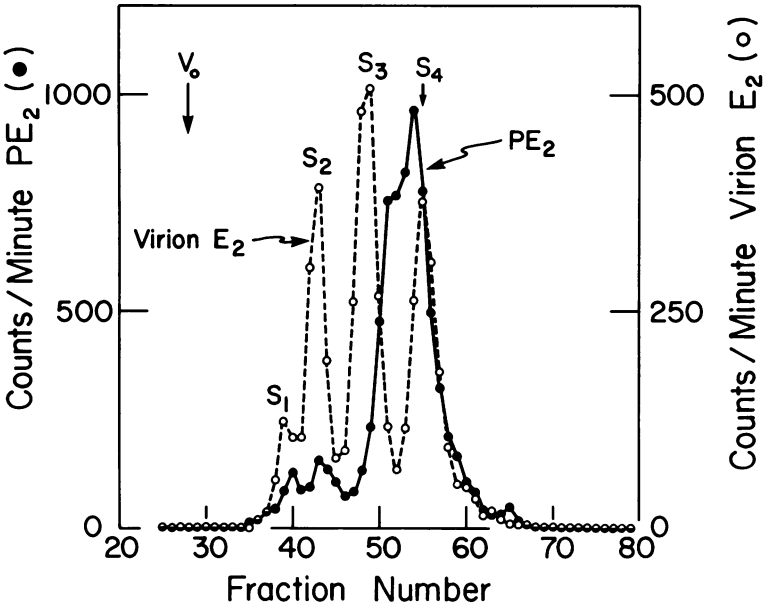


FIG. 3. *Comparison of the glycopeptides of glycoprotein PE2 with those of glycoprotein E2 from purified virions by gel filtration. Glycoprotein PE2, labeled with [$^3H$]glucosamine, and glycoprotein E2, labeled with [$^{14}C$]glucosamine, were purified by SDS polyacrylamide gel electrophoresis of infected chick cells and purified virions, respectively. The eluted proteins were mixed, digested with Pronase, and compared by chromatography on Bio-Gel P-6. Symbols: glycoprotein PE2, [$^3H$]glucosamine (●); glycoprotein E2 from purified virions, [$^{14}C$]-glucosamine* (O).

polyacrylamide gels and mixed with similarly purified [$^{14}$C]glucosamine-labeled glycoproteins from mature virions. The glycopeptides of cell-associated glycoprotein E2 were similar but not identical to those of virion glycoprotein E2 (Fig. 4). The four glycopeptides found in mature virions are also found in the cell-associated form of the glycoprotein. The nonvirion glycopeptides seen with glycoprotein PE2, those eluting between glycopeptides S3 and S4, were not present. The differences in the two patterns here were small. Glycopeptide S3 from cell-associated glycoprotein E2 eluted from the column one or two fractions later than glycopeptide S3 from virions, and the fraction of protein-bound glucosamine present in the larger glycopeptides, S1, S2, and S3, was less in cell-associated glycoprotein E2 than in virion glycoprotein E2.

When the glycopeptides of cell-associated glycoprotein E1 were compared with those of virion glycoprotein E1, a more complicated picture was seen (Fig. 5). Glycopeptides which co-chromatographed with those in virions were present in cell-associated glycoprotein E1, as was a glycopeptide which was not present in mature virions. This nonvirion glycopeptide eluted from the column between virion glycopeptides S3 and S4, an elution position very similar to that of the majority of the glycopeptides of glycoprotein PE2. As with glycoprotein PE2, no glycopeptides smaller than glycopeptide S4 were detected in cell-associated E1.

## DISCUSSION

These results allow a number of conclusions about the structure and the biosynthesis of the oligosaccharides of the Sindbis virus glycoproteins to be drawn.

**Structural features of the glycoproteins and their oligosaccharides.** The two virion glycoproteins were previously shown to be similar in molecular weight, to be present in approximately equimolar amounts, and to contain approximately equal amounts of carbohydrate (10, 11). Therefore, the value determined here for the amount of carbohydrate per milligram of viral protein, 59 $\mu$g (Table 1), when corrected for the amount of protein present in the non-glycosylated virion core protein, suggests that the glycoproteins are approximately 8% carbohydrate by weight. This figure in turn suggests that each glycoprotein contains approximately

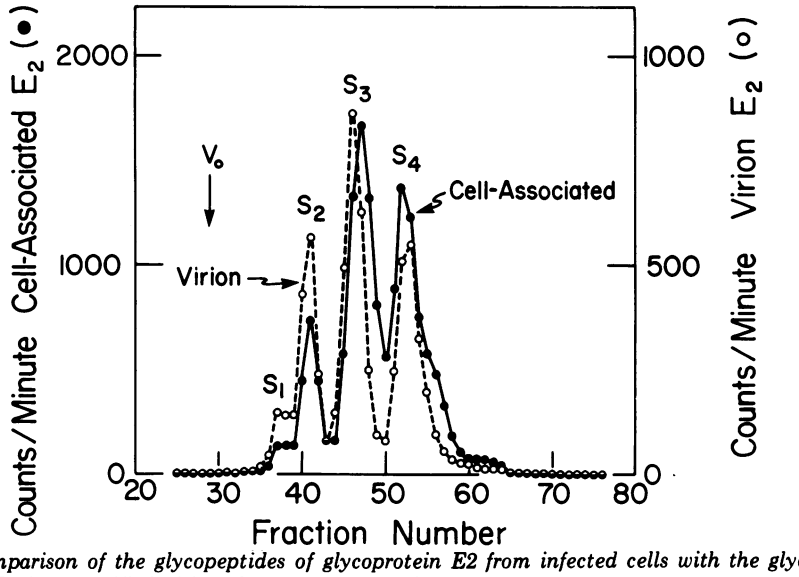


FIG. 4. *Comparison of the glycopeptides of glycoprotein E2 from infected cells with the glycopeptides of glycoprotein E2 from purified virions by gel filtration. Glycoprotein E2, labeled with [$^3H$]glucosamine, was purified by SDS polyacrylamide gel electrophoresis from infected chick cells and mixed with glycoprotein E2, labeled with [$^{14}C$]glucosamine, which had been similarly purified from purified virions. The mixture was digested with Pronase and chromatographed in Bio-Gel P-6. Symbols: cell-associated glycoprotein E2, [$^3H$]glucosamine (●); virion glycoprotein E2, [$^{14}C$]glucosamine (○).*

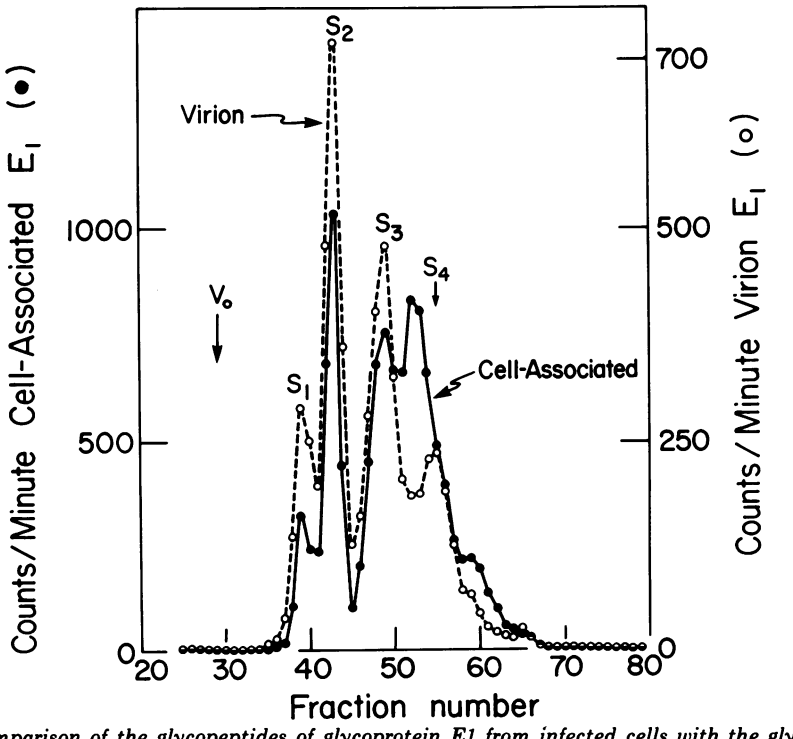


FIG. 5. *Comparison of the glycopeptides of glycoprotein E1 from infected cells with the glycopeptides of glycoprotein E1 from purified virions by gel filtration. Glycoprotein E1, labeled with [$^3H$]glucosamine, was purified by SDS polyacrylamide gel electrophoresis from infected chick cells and mixed with [$^{14}C$]glucosamine labeled glycoprotein E1, purified in a similar manner from purified virions. The mixture was digested with Pronase and chromatographed on Bio-Gel P-6. Symbols: cell-associated glycoprotein E1, [$^3H$]glucosamine (●); virion glycoprotein E1, [$^{14}C$]glucosamine (○).*

4,000 daltons of carbohydrate, although there is necessarily some uncertainty in these figures since the true polypeptide molecular weight of these glycoproteins is not rigorously known.

All of the oligosaccharides are connected to the polypeptide by an alkali-stable linkage. This was demonstrated by the absence of released oligosaccharides after treatment of the viral glycoproteins with 0.2 N NaOH and 0.25 M $NaBH_4$ for 48 h at 25 C. This stability indicates that the oligosaccharides are most probably like serum-type oligosaccharides, which are characterized by an alkali-stable, N-glycosidic linkage between glucosamine and asparagine (6). The stability in alkali of the carbohydrate to protein linkage, together with the absence of galactosamine, eliminates the possibility that the viral glycoproteins contain a mucin-type oligosaccharide characterized by an alkali-labile bond between galactosamine and serine or threonine (6).

There appear to be two different types of glycopeptides in Sindbis virus. Glycopeptides S1, S2, and S3 are examples of the A-type glycopeptides as described by Johnson and Clamp (5). They contain glucosamine, mannose, galactose, and fucose (Fig. 1) and glycopeptides S1 and S2, at least, contain sialic acid (K. Keegstra and B. Sefton, unpublished data). Glycopeptide S4 is apparently a B-type glycopeptide, again using the nomenclature of Johnson and Clamp. It contains only glucosamine and mannose. It has been suggested that glycopeptide S4 is an incomplete form of glycopeptides S2 and S3 (12). This now appears unlikely given the closeness of the apparent molecular weights of glycopeptides S3 and S4, 2,490 and 1,660, respectively (1), and the fact that the ratio of mannose to glucosamine in the smaller glycopeptide, S4, is 4.5 times greater than the ratio in glycopeptide S3. It is nearly impossible to hypothetically convert a glycopeptide with the size and sugar composition of glycopeptide S4 into a glycopeptide with the sugar composition of glycopeptide S3 without constructing a glycopeptide much larger than the observed size of S3. Indeed, preliminary gas chromatographic analysis of the actual sugar composition of glycopeptides S3 and S4 also indicates that glycopeptide S4 is not a precursor to glycopeptide S3 (K. Keegstra and B. Sefton, unpublished data).

The separated Sindbis glycoproteins each contain four glycopeptides (Fig. 2). However, this does not mean that each glycoprotein molecule contains four sites of oligosaccharide attachment. Given the estimated molecular weights of the four glycopeptides: 3,300, 2,800, 2,490 and 1,660 (1), a glycoprotein containing all four oligosaccharides would contain approximately 10,250 daltons of carbohydrate, which is much greater than the measured average weight of carbohydrate per glycoprotein, approximately 4,000. These apparently conflicting data can be reconciled if it is assumed that each glycoprotein contains, on the average, two glycopeptides, one A-type, either S1 or S2 or S3, and one B-type, S4.

A number of questions about the viral glycopeptides are as yet unanswered. First, it is not known how many points of attachment for carbohydrate there are per glycoprotein. The simplest assumption is that there is one for a B-type glycopeptide and one for an A-type glycopeptide. Second, it is not clear whether the similarly-sized glycopeptides from the two glycoproteins are identical in structure. All three large, A-type glycopeptides from glycoprotein E2 elute from a Bio-Gel P-6 column one fraction before the corresponding glycopeptide from glycoprotein E1. It is not known whether this is evidence for a difference in carbohydrate or whether it is due merely to a difference in the amino acids which remain attached to the oligosaccharides. Third, it is not clear whether the three A-type glycopeptides found on one of the glycoproteins are related structures differing only in the degree of completion of the carbohydrate chain or whether they are each structurally unique oligosaccharides. Again, the simplest assumption is that with each of the two glycoproteins, glycopeptides S2 and S3 are incomplete forms of glycopeptide S1.

**Biosynthesis of the oligosaccharides.** It was previously observed that glycoprotein PE2, the intracellular precursor to glycoprotein E2 (10), contains less galactose and fucose than its product (11). This apparent incomplete glycosylation is consistent with the observation made here that a large majority of the oligosaccharides bound to glycoprotein PE2 are smaller than those bound to either cell-associated glycoprotein E2 or to virion glycoprotein E2 (Fig. 3). Appropriately, glycoprotein PE2 is particularly deficient in those glycopeptides which contain a majority of the galactose and fucose, glycopeptides S1 and S2.

The size of most of the glycopeptides of glycoprotein PE2 (smaller than glycopeptide S3 and larger than glycopeptide S4) is unique to the cell-associated viral proteins. The virion glycoproteins do not contain glycopeptides this size. Since it was earlier shown that glycoprotein PE2 appears to already contain a majority of the glucosamine and mannose and some of the galactose found in glycoprotein E2 (11), the

nonvirion glycopeptides in glycoprotein PE2 are probably, at least in part, incomplete glucosamine, mannose, and galactose containing cores of glycopeptides S1, S2, and S3.

Further, the fact that glycoprotein PE2 contains significant amounts of glucosamine and mannose (11), coupled with the fact that no glycopeptides smaller than glycopeptide S4 were detected, suggests that glycoprotein PE2 already contains an essentially mature version of the glucosamine and mannose glycopeptide, S4.

The glycopeptides of cell-associated glycoprotein E2 are slightly different from those of virion glycoprotein E2. Specifically, a larger fraction of the protein-bound glucosamine is found in the smaller glycopeptides, S3 and S4, in cell-associated glycoprotein E2 than in virion glycoprotein E2. Since cell-associated glycoprotein E2 may be located exclusively on the cell surface (11, 12), this difference suggests that some glycosylation may occur on the cell surface and may actually accompany the release of virus from the cell.

Both virion and nonvirion glycopeptides are found in cell-associated glycoprotein E1. The nonvirion glycopeptides elute from the column in a position, between glycopeptides S3 and S4, very similar to that of the nonvirion glycopeptides of glycoprotein PE2. It has been suggested that there are two populations of glycoprotein E1 in Sindbis-infected cells, one is intracellular, under-glycosylated and in an equimolar complex with glycoprotein PE2; the other is on the cell surface, more fully glycosylated and in an equimolar complex with glycoprotein E2 (11). The glycopeptide pattern of the whole population of cell-associated glycoprotein E1 is fully consistent with this model. Some glycopeptides from glycoprotein E1 are complete, as they are in glycoprotein E2, and some are incomplete, as they are in glycoprotein PE2.

Finally, only three virus-specific glycoproteins are found in Sindbis-infected chick cells, PE2, E1, and E2 (11). In none of these glycoproteins are found glycopeptides smaller than glycopeptide S4 (Fig. 3, 4, and 5). This suggests that the carbohydrate proximal to the polypeptide chain is assembled sufficiently rapidly that incomplete, very short glycopeptides are not detected. Alternatively, the sugars proximal to the polypeptide chain could be attached to the protein as a large, presynthesized unit.

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