Glycosidase Susceptibility: a Probe for the Distribution of Glycoprotein Oligosaccharides in Sindbis Virus

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Intact Sindbis virus and Triton-solubilized viral glycoprotein were treated with α-mannosidase and with a preparation of mixed glycosidases from *Diplococcus pneumoniae* to probe the accessibility of carbohydrate units on the viral surface. The products of glycosidase attack on Triton-solubilized virus showed that most carbohydrate units of the glycoproteins are good substrates for these enzymes. The relative resistance of most of the viral oligosaccharides in intact virus particles showed that much of the carbohydrate is not accessible to glycosidases, probably because it is not exposed at the viral surface. The only completely accessible carbohydrate units on Sindbis glycoproteins were the type A oligosaccharides of E2. This differential accessibility of Sindbis oligosaccharides is discussed in relation to the organization of the viral surface.

Sindbis virus is structurally among the simplest of the lipid-containing animal viruses that mature by budding through the plasma membrane of an infected cell (1, 3, 17). The Sindbis particle has proved to be sufficiently regular and stable in structure to permit studies of its lipid-protein organization (8) and its surface organization (37). It has a spherical nucleocapsid, assembled from subunits of a single protein species (C; molecular weight, 30,000) and viral RNA (34). The nucleocapsid is enveloped by a membrane composed of a lipid bilayer and two distinct glycoprotein species (E1 and E2; both of molecular weight ~50,000). Each glycoprotein has both complex "type A" and simple "type B" *N*-glycosidically linked carbohydrate units, which have been characterized by radioactive labeling and gel filtration of the four viral glycopeptide fractions (30). Electron micrographs of negatively stained Sindbis virus particles show that the glycoproteins are organized with trimer clustering in a T = 4 icosahedral surface lattice (37).

There is, as yet, little information on the fine structure of the viral surface--e.g., details of the location of carbohydrate units or the relative exposure of the two glycoproteins. There is some suggestion from the iodination data of Sefton et al. (31) that E1 is less exposed at the viral surface than E2. The objective of the present study was to probe the surface arrangement of glycoprotein carbohydrate units with glycosidases. We digested intact virus and viral glyco-

protein solubilized by Triton X-100 with α-mannosidase and with a preparation of mixed glycosidases from *Diplococcus pneumoniae* to probe the distribution of carbohydrate units on the viral surface. The mixed glycosidases contain three exoglycosidases and an endoglycosidase, endo-β-*N*-acetylglucosaminidase D. The endoglycosidase is specific for oligosaccharides having mannosyl di-*N*-acetylchitobiose core sequences, which occur frequently on *N*-glycosidically linked carbohydrate units (15). The enzyme hydrolyzes the di-*N*-acetylchitobiose sequence, thus cleaving the oligosaccharide at a glycosidic linkage near the carbohydrate-peptide linkage site and releasing the mannose-containing portion of the glycan core.

MATERIALS AND METHODS

Virus growth and purification. Unlabeled Sindbis virus was grown and purified as described by von Bonsdorff and Harrison (37). Radioactive virus was produced by a single growth step in primary chicken embryo fibroblasts growing on 100-mm plastic tissue culture dishes. Confluent monolayers were infected with 1.0 ml of virus per plate diluted into Eagle minimal essentials medium supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1 μg of actinomycin D per ml. Virus labeled with a mixture of radioactive amino acids was prepared with Eagle minimal essential medium containing one-tenth the normal concentration of all amino acids except glutamine. The multiplicity of infection was 125 PFU/cell. Adsorption proceeded for 1 h at 37°C with occasional rocking of the plates, and 4.5 ml of Eagle medium supplemented as above was then added to each plate. Radioactive sugars were added at 1 h postinfection; radioactive amino acids were added at

3 h postinfection. The infection proceeded for 10 to 14 h at 37°C.

Radioactive virus was purified essentially as described by Sefton and Keegstra (30). However, the virus was centrifuged through a discontinuous sucrose gradient, which functions as a combined velocity and density gradient (37). The gradient consisted of a 0.5-ml cushion of 50% sucrose in TNE buffer (0.15 M NaCl, 0.05 M Tris-hydrochloride, pH 7.4, 1 mM EDTA) onto which a 6.0-ml, 25 to 50% linear sucrose-TNE gradient was layered. Over this, a 10.0-ml, 10 to 20% sucrose-TNE gradient was layered. A 1.5-ml virus sample was centrifuged at 24,500 rpm (80,000 × g) for 5 h at 4°C in an SW27 rotor.

Radioactive sugars used for viral growth included (per plate) 62.5 μCi of [2-^{3}H]mannose (Amersham/Searle, 2 Ci/mmol), 62.5 μCi of [6-^{3}H]glucosamine (New England Nuclear Corp., 10 Ci/mmol), 12.5 μCi of [1-^{14}C]glucosamine (New England Nuclear Corp., 56.6 mCi/mmol), 125 μCi of [1-^{3}H]galactose (New England Nuclear Corp., 6 Ci/mmol), and 125 μCi of [1,5,6-^{3}H]fucose (New England Nuclear Corp., 2.4 Ci/mmol). Amino acid-labeled virus was prepared with (per plate) 12.5 μCi of a ^{14}C-labeled L-amino acid mixture (New England Nuclear Corp.) or with 62.5 μCi of a ^{3}H-labeled L-amino acid mixture (New England Nuclear Corp.).

Infectivity was titrated by plaque assay on confluent monolayers of secondary chicken embryo fibroblasts (25). Hemagglutination titer was assayed by the method of Clarke and Casals (4) with 0.2% goose erythrocytes (GIBCO Diagnostics) at pH 5.8.

Preparation of glycosidases. α-Mannosidase (*Canavalia ensiformis*) was obtained from Boehringer Mannheim Corp. One hundred microliters of crystalline enzyme suspension was dissolved in an equal volume of TNE buffer (0.15 M NaCl, 0.05 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA) and dialyzed overnight against the same buffer. When assayed with p-nitrophenyl-α-D-mannoside (Sigma Chemical Co.) in TNE at pH 7.4, the enzyme had an activity of 0.12 U/ml. In addition, 0.01 U of β-N-acetylglucosaminidase activity per ml was present.

Neuraminidase (*Clostridium perfringens*) was obtained from Boehringer Mannheim. The enzyme was dissolved in 0.15 M NaCl, 0.05 M Tris-hydrochloride, pH 7.4, 2 mM CaCl$_2$ at a concentration of 1 mg/ml and assayed on fetuin (GIBCO Diagnostics). Free sialic acid was determined by the thiobarbituric acid assay of Warren (38).

An enzyme preparation containing β-galactosidase, β-N-acetylglucosaminidase, neuraminidase, and endo-β-N-acetylglucosaminidase D activities was prepared essentially as described by Muramatsu and co-workers (14, 20, 21). Crude enzyme was obtained by ammonium sulfate fractionation of the culture medium of *D. pneumoniae* Type I and further purified by gel filtration on a Sephadex G-150 column (3 by 95 cm). Fractions were assayed for glycosidase activities as described by Muramatsu and Egami (22), with the appropriate p-nitrophenyl pyranosides (Sigma Chemical Co.) as substrates.

Neuraminidase present in the mixed glycosidases was assayed on fetuin as described above. Active

fractions were pooled and concentrated by dialysis against polyethylene glycol 20,000 (Fisher Scientific), and the preparation then dialyzed against 0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.5.

The mixed glycosidase preparation contained 0.025 U of β-N-acetylglucosaminidase per ml, 0.015 U of β-galactosidase per ml, and 0.45 U of neuraminidase per ml when assayed in TNE buffer at pH 7.4. Activities were unchanged in the presence of 0.5% (wt/vol) Triton X-100. Neither α-fucosidase nor α-mannosidase activity was detected. The enzyme mixture was free of proteolytic activity in that it did not release radioactive label from ^{14}C-amino acid-labeled virus, nor did it alter the quantitative distribution of label in polyacrylamide gel electrophoresis patterns of amino acid-labeled virus. The mixed glycosidases were used for digestion of Sindbis virus and Sindbis glycoproteins since endo-β-N-acetylglucosaminidase D acts on intact carbohydrate units only in the presence of the three exoglycosidases (14, 20, 21).

Solubilization of Sindbis membrane proteins with Triton X-100. Triton X-100 was added to Sindbis virus to dissociate the viral membrane into soluble glycoprotein-detergent complexes, using a membrane solubilization scheme originally devised by Helenius and co-workers for Semliki Forest virus (SFV) (10, 32). Sindbis virus (50 to 100 μg of protein) was suspended in 1% Triton X-100 in TNE to a final concentration of 1 μg of virus protein per μl and incubated for 20 min or more at room temperature. At this Triton/viral protein ratio, soluble glycoprotein-detergent complexes form containing approximately 2 polypeptide chains and 75 molecules of Triton (32).

Glycosidase treatment of Sindbis virus. A portion of labeled virus was combined with 50 to 100 μg of protein of unlabeled carrier virus. The virus was precipitated from a solution of low ionic strength (24), and the pellet was resuspended in TNE or in 4 volumes TNE and 1 volume of 5% Triton X-100 to solubilize the membrane proteins. The volume of buffer added was adjusted to give a solution of 1 μg of virus protein per μl.

To establish the time course of mixed glycosidase digestions, the release of glucosamine from intact virus and from Triton-solubilized glycoprotein was measured as a function of incubation time. In this particular case, glucosamine-labeled protein was precipitated from solution with trichloroacetic acid to terminate the reaction. The percent precipitable glucosamine decreases with time for 12 h; release of carbohydrate has essentially ceased by 16 h. Thus, in all subsequent digestions, a given volume of radioactive virus solution prepared as above was mixed with an equal volume of the mixed glycosidases or α-mannosidase and incubated for 16 h at 37°C. Controls consisted of the same volume of radioactive virus solution mixed with an equal volume of TNE and similarly incubated. Incubations were terminated by placing the incubation mixtures on ice.

Sindbis virus was digested with neuraminidase (*Clostridium perfringens*) for 16 h at 37°C. The virus, in TNE at a concentration of 1 μg/μl, was

incubated with 0.015 U of neuraminidase per 50 μg of viral protein.

Intact, glycosidase-treated virus particles were recovered as follows: up to 100 μl of reaction mixture (intact virus plus glycosidases) was diluted with an equal volume of TNE, and the solution was layered over 400 to 500 μl of 10% sucrose in TNE in a 3/16-inch (ca. 0.5-cm) cellulose nitrate tube (Beckman). The treated virus was pelleted by centrifugation for 1 h at 35,000 rpm (114,000 $\times$ g) in the SW50.1 rotor; the supernatant contained material released from the virus by the glycosidases. The released material was carbohydrate labeled but not amino acid labeled.

The combined viral proteins were recovered from Triton-containing incubation mixtures as follows: 200 μl of 0.2% bovine serum albumin in TNE was added to the incubation mixture, and the protein was precipitated with ice-cold n-butanol (26). The protein pellet could then be resuspended in 0.5 ml of 0.1 M Tris-hydrochloride, pH 8.0, 0.01 M CaCl$_2$ without addition of detergent. Finally, solutions were dialyzed for 2 days against three changes, 1 liter each, of the same buffer.

Gel electrophoresis. Polyacrylamide discontinuous gel electrophoresis was performed essentially as described by Laemmli (16). The concentrations of Tris-hydrochloride in the stacking and resolving gels were 0.062 and 0.188 M, respectively, a modification used by Sefton et al. (31). Stacking gels contained 3% (wt/vol) acrylamide and were 1.0 to 2.0 cm in length. Resolving gels contained 7.5% (wt/vol) acrylamide and were 10.5 cm in length. All gels were prepared with acrylamide recrystallized from chloroform. Before electrophoresis, samples were dissolved or diluted into "sample buffer" containing 5 mM sodium phosphate buffer, pH 7.5, 1% sodium dodecyl sulfate (SDS), 10% glycerol, 0.001% bromophenol blue, and 5% β-mercaptoethanol. Samples were then boiled for 2 to 3 min to completely denature the proteins. Electrophoresis proceeded at a constant current of 1.5 mA per gel until the bromophenol blue reached the bottom of the gel, about 5 h. After electrophoresis, gels were removed from the glass tubes, frozen at $-70°$C, and then cut into 1-mm slices.

Measuring the release of carbohydrate from viral glycoprotein. Since the glycosidases do not contain protease activity, release of carbohydrate from the viral glycoprotein corresponds directly to a decrease in the sugar/protein ratio. By using a mixed-label incubation mixture, it is possible to monitor the change in sugar/protein ratio. This procedure was used to measure release of carbohydrate label from Triton-solubilized membrane glycoprotein as well as from intact virus particles.

Portions of ^{14}C-amino acid-labeled virus and ^{3}H-sugar-labeled virus were mixed with 50 μg of unlabeled carrier virus. The virus was precipitated from a solution of low ionic strength, resuspended, and then incubated with or without glycosidases. The incubation was terminated by adding to a given volume of incubation mixture 3 volumes of electrophoresis sample buffer. Samples were reduced with β-mercaptoethanol and subjected to SDS-polyacryl-

amide gel electrophoresis (Fig. 1). Gels were then processed for counting as described below. From each gel pattern, the ^{3}H/^{14}C ratios of E1, E2, and total viral protein (i.e., ratio of total ^{3}H counts per minute to total ^{14}C counts per minute) were calculated. Assuming that radioactive label accurately reflects the chemical distribution of carbohydrate or amino acid, the sugar released by glycosidases can be expressed as a percentage of the initial amount:

$$\left[1 - \frac{(^3\text{H}/^{14}\text{C})\text{glycosidase-treated}}{(^3\text{H}/^{14}\text{C})\text{control}}\right] \times 100.$$

By using the appropriate ^{3}H/^{14}C ratios, it is possible to determine the release of carbohydrate from the individual glycoproteins as well as from the whole virus.

Two systems were used interchangeably to count gel slices. Slices were either shaken with 0.1% SDS (30) or solubilized with Protosol (New England Nuclear Corp.). Slices shaken with SDS were counted in 9 ml each of ScintiVerse (Fisher Scientific) containing 8% (vol/vol) water and 5% (vol/vol) glacial acetic acid. Slices solubilized with Protosol were counted in 5 ml each of toluene containing 5% (vol/vol) Protosol and 4% (vol/vol) Liquifluor (New England Nuclear Corp.). The same counting efficiency was obtained with each system. All liquid scintillation counting was performed on a Beckman liquid scintillation counter.

Isolation of Sindbis glycoproteins. Glycosidase-treated glycoproteins were isolated after SDS-polyacrylamide gel electrophoresis of the incubation mixtures or pelleted virus. Gels were cut into 1-mm slices, and each slice was shaken with 0.5 ml of 0.1% SDS (30) to elute the glycoprotein. Approximate fractions were then pooled and lyophilized.

Preparation of glycopeptides. Glycopeptides were prepared from labeled viral glycoproteins or from labeled virus by exhaustive digestion with a protease preparation from *Streptomyces griseus* (Sigma Chemical Co.). The procedures followed were essentially those of Sefton and Keegstra (12, 28, 30), with digestion for 48 h at 60°C.

Gel filtration. Glycopeptides were chromatographed on Bio-Gel P-4 (200 to 400 mesh, Bio-Rad). The column (0.9 by 115 cm) was equilibrated and eluted with 0.1 M Tris-hydrochloride, pH 8.0 (12, 30). One-milliliter fractions were collected and counted in 9 ml each of ScintiVerse (Fisher Scientific) containing 8% (vol/vol) water and 5% (vol/vol) glacial acetic acid. The column was calibrated with ovomucoid glycopeptide (23), ovalbumin glycopeptide (39), and stachyose. The excluded volume (V_o) was determined from the elution position of blue dextran (Pharmacia). The total volume (V_t) was determined from the elution position of glucose.

When Sindbis glycopeptides are chromatographed on Bio-Gel P-4, the two sialic acid-containing glycopeptides, S1 and S2, do not separate but elute together in a single peak, and glycopeptides S3 and S4 are resolved. S1 and S2 may be resolved on Bio-Gel P-6 (30), but P-4 was chosen here to match optimal fractionation with the expected size of glycopeptides from glycosidase-digested glycoprotein.
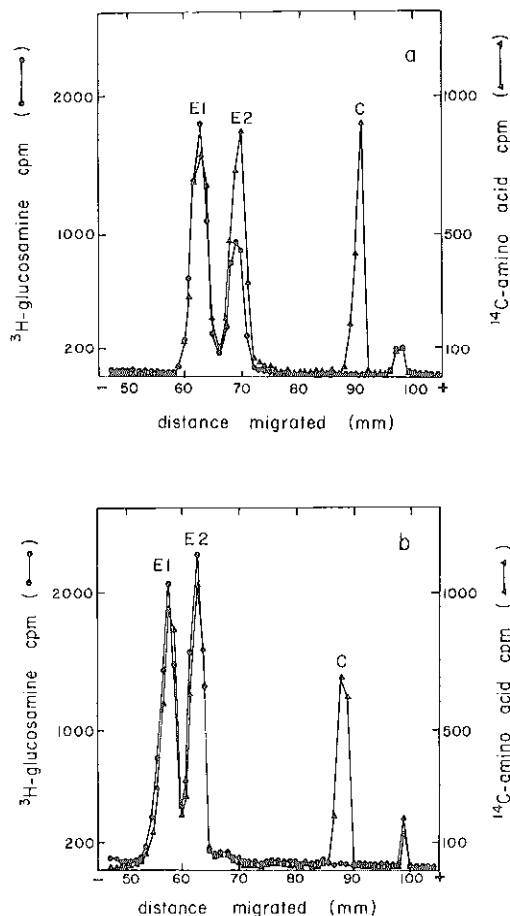
FIG. 1. *Polyacrylamide gel electrophoresis of [3H]glucosamine and 14C-amino acid-labeled Sindbis virus. Intact virus was incubated for 16 h at 37°C with (a) or without (b) mixed glycosidases and then subjected to electrophoresis as described in Materials and Methods. Symbols: (⊚) [3H]glucosamine label; (▲) 14C-amino acid label.*

On each glycopeptide pattern, the elution positions of glycopeptides from untreated viral glycoprotein are indicated.

Oligo- and monosaccharides released by glycosidase digestion were chromatographed on Bio-Gel P-2 (200 to 400 mesh, Bio-Rad) or Sephadex G-15 (Pharmacia). The P-2 column (0.9 by 100 cm) was equilibrated and eluted with 10 mM NH_4HCO_3, pH 7.4. One-milliliter fractions were collected and counted in 9 ml each of ScintiVerse (Fisher Scientific) containing 8% (vol/vol) water and 5% (vol/vol) glacial acetic acid. The Sephadex G-15 column was used for all Triton X-100-containing samples. The column (0.9 by 115 cm) was equilibrated and eluted with 10 mM NH_4HCO_3, pH 7.4, 0.5% Triton X-100. One-milliliter fractions were collected and counted as above. These columns were calibrated with stachyose or raffinose. Column parameters V_o and V_t

were determined from the elution positions of blue dextran (Pharmacia) and glucose, respectively. To determine the elution positions of all standards used for column calibration, 100-μl portions from each fraction were analyzed for hexose by the anthrone reaction (33).

RESULTS

Labeled carbohydrate released from glycosidase-treated Sindbis glycoproteins. Intact, carbohydrate-labeled Sindbis virus retains more than half its carbohydrate label after digestion with mixed glycosidases (Table 1). Glycoprotein E2 appears to lose significantly more of its carbohydrate label than does E1 (Fig. 1). Although E2 loses all its galactose label and a significant amount of its glucosamine and mannose label, E1 loses virtually no mannose label and only about 10% of its glucosamine label. This difference cannot be due to a difference in the amount of labeled carbohydrate in the two glycoproteins. Gel electrophoresis patterns of all preparations of sugar-labeled virus indicated that untreated E1 and E2 have approximately equal amounts of labeled glucosamine, mannose, or galactose. The larger glycoprotein E1 contains approximately four times as much fucose label as E2. Very similar distributions of labeled carbohydrate were previously reported by Sefton and Burge (29). The α-mannosidase apparently did not remove mannose from intact virus; i.e., no loss of mannose label was detected after α-mannosidase digestion of intact virus (Table 2).

After Triton solubilization of the viral membrane, mixed glycosidases release approximately half the total viral mannose and glucosamine label (Table 3). Glycoprotein E1 loses more than half its mannose or glucosamine label and almost all its galactose label. However, mixed glycosidases release about the same percentage of the glucosamine or man-

TABLE 1. *Carbohydrate label released from intact Sindbis virus by mixed glycosidase digestion*

Labeled sugar	Amt of label released from:		
	E1[a]	E2[a]	Total viral protein[b]
Glucosamine	9 ± 3	44 ± 2	25 ± 1
Mannose	4 ± 2	26 ± 4	14 ± 1
Fucose	2 ± 1	0	1 ± 1
Galactose	21 ± 8	96 ± 5	52 ± 1

[a] Released label is expressed as a percentage of total label incorporated into E1 or E2. Values given are the mean values of three determinations ± standard deviations.

[b] Released label is expressed as a percentage of total viral label. Values given are the mean values of three determinations ± standard deviations.

nose label of E2 whether or not E2 has been solubilized with Triton. The α-mannosidase releases approximately one-fourth the total mannose label from Triton-solubilized glycoprotein, whereas the enzyme fails to release any mannose label from intact Sindbis virus (Table 2).

Characterization of carbohydrate-labeled material released from viral glycoproteins by mixed glycosidases. Mixed glycosidases release from intact virus a single mannose-labeled oligosaccharide with a molecular weight of 700, as determined by gel filtration of the released material on Bio-Gel P-2 (data not shown) or Sephadex G-15 (Fig. 2). This oligosaccharide also contains about 7% of the total viral glucosamine label (data not shown). Additional released glucosamine elutes near glucose on the P-2 column, as does galactose (data not shown), indicating that glucosamine and galactose are released as monosaccharides.

Mixed glycosidases release two oligosaccharides from Triton-solubilized glycoprotein. When Triton-containing reaction mixtures are chromatographed on Sephadex G-15, all amino acid label elutes in the column void volume

TABLE 2. *Mannose label released from Sindbis glycoproteins by α-mannosidase digestion*

Substrate	Amt of label released from:		
	E1[a]	E2[a]	Total viral protein[b]
Intact virus	0	0	0
Triton-solubilized glycoprotein	20	34	25

[a] Released label is expressed as a percentage of total mannose label in E1 or E2.

[b] Released label is expressed as a percentage of total viral label.

TABLE 3. *Carbohydrate label released from Triton-solubilized Sindbis glycoproteins by mixed glycosidase digestion*

Labeled sugar	Amt of label released from:		
	E1[a]	E2[a]	Total viral protein[b]
Glucosamine	56 ± 2	47 ± 2	51 ± 2
Mannose	56 ± 3	29 ± 4	44 ± 2
Fucose	0	0	1
Galactose	96 ± 4	96 ± 4	85 ± 2

[a] Released label is expressed as a percentage of total label incorporated into E1 or E2. Values given are the mean values ± standard deviations from the mean of three determinations.

[b] Released label is expressed as a percentage of total viral label. Values given are the mean values ± standard deviations from the mean of three determinations.
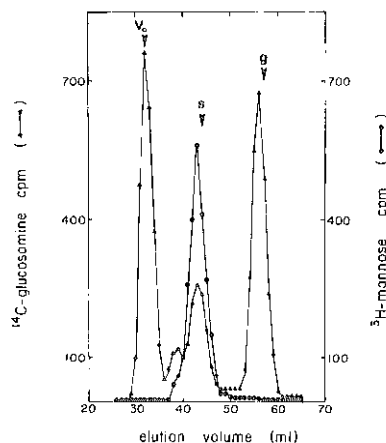
(data not shown). Glucosamine label elutes in the column void volume and in three included peaks with molecular weights of approximately 1,100, 700, and 240 (Fig. 2). The 1,100-dalton peak and the 700-dalton peak are also labeled by mannose; these are probably oligosaccharides. The 240-dalton peak corresponds in size to free N-acetylglucosamine. The smaller oligosaccharide coelutes on Sephadex G-15 with the single oligosaccharide released by mixed glycosidase digestion of intact Sindbis (Fig. 2).

Neuraminidase activity in mixed glycosidases. The neuraminidase activity of the mixed glycosidases was examined by preparing [³H]glucosamine-labeled glycopeptides from glycosidase-treated virus and comparing them by cochromatography with [¹⁴C]glucosamine-labeled glycopeptides from neuraminidase-treated virus (Fig. 3). Neuraminidase converts the two largest glycopeptides, S1 and S2, to species that comigrate with S3 (12; confirmed here by the ¹⁴C-labeled pattern in Fig. 3). Glycopeptides from mixed glycosidase-treated virus are likewise never larger than S3, indicating that the neuraminidase in mixed glycosidases removes all sialic acid from viral glycoprotein-carbohydrate units.

Glycopeptides from mixed glycosidase-treated viral glycoprotein. To determine directly the extent to which glycosidases degrade carbohydrate units of Sindbis glycoproteins,

FIG. 2. *Sephadex G-15 chromatography of [³H]mannose-labeled material released from intact [³H]mannose-labeled Sindbis virus by mixed glycosidase digestion, cochromatographed with the incubation mixture containing Triton-solubilized, [¹⁴C]-glucosamine-labeled Sindbis virus plus mixed glycosidases. Symbols: (●) [³H]mannose label; (▲) [¹⁴C]glucosamine label. Arrows indicate the positions of stachyose and glucose markers detected by the anthrone test.*

carbohydrate-labeled glycopeptides were prepared from glycosidase-digested material and chromatographed on Bio-Gel P-4. Differential glycosidase attack on E1 and E2 was examined after digestion of intact or Triton-solubilized virus by subjecting the incubation mixtures to SDS-gel electrophoresis to separate and recover the two glycoproteins. Glycopeptides prepared from these fractions were examined by gel filtration. Glucosamine-labeled glycopeptide patterns are shown in Fig. 4 and 5, whereas fucose- and mannose-labeled patterns are not shown. Instead, the distribution of label in these patterns is summarized in Tables 4 and 5.
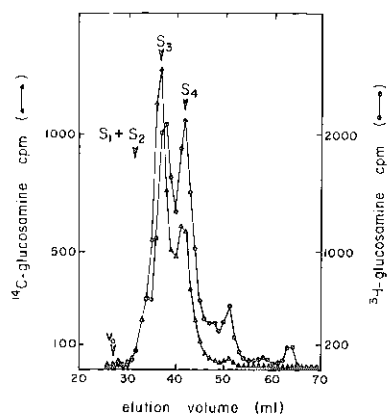

Fig. 3. *Bio-Gel P-4 chromatography of [³H]glucosamine-labeled glycopeptides prepared from intact virus digested with mixed glycosidases plus [¹⁴C]glucosamine-labeled glycopeptides prepared from neuraminidase-treated virus. Symbols: (●) [³H]glucosamine label; (▲) [¹⁴C]glucosamine label.*

After mixed glycosidase digestion of intact virus, [³H]glucosamine-labeled glycopeptides from E1 elute in two distinct peaks (Fig. 4). The larger peak is asymmetric and has a modal molecular weight of about 2,000. This value is slightly less than that of the S3 glycopeptide from untreated E1 which, on the Bio-Gel P-4 column, has a molecular weight of 2,100. The asymmetric peak is also labeled by mannose and fucose (Table 4). The second peak of label corresponds in size to the smallest Sindbis glycopeptide, S4, with a molecular weight of 1,600; it is also labeled by mannose. Fucose sometimes labels a 1,200-dalton glycopeptide, but a glycopeptide of the same size and containing the same percentage of fucose label also tends to appear among glycopeptides from untreated E1 (M. McCarthy, Ph.D. thesis, Harvard University, Cambridge, Mass., 1976). It is, therefore, unlikely that the glycopeptide is a product of glycosidase digestion.

After mixed glycosidase digestion of intact virus, [³H]glucosamine-labeled glycopeptides from E2 elute in a broad peak with a leading shoulder and in several low-molecular-weight species smaller than S4 (Fig. 4). Mannose and fucose elute with a similar profile, but mannose does not label low-molecular-weight glycopeptides (Table 4). The shoulder has an elution position corresponding to a molecular weight of about 1,800. This is smaller than S3 from untreated E2, which has a molecular weight of about 2,300 on the Bio-Gel P-4 column. The main peak corresponds in elution position to S4, with a molecular weight of 1,600. The low-molecular-weight glycopeptides range in molecular weight from 700 to 1,100 and contain a large
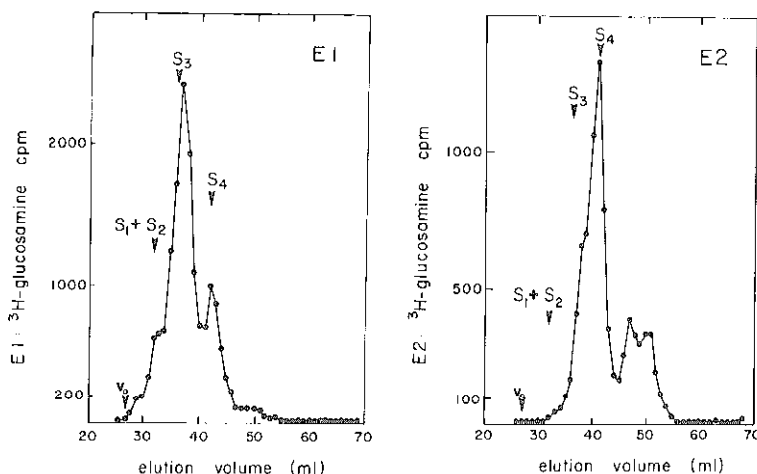

Fig. 4. *Bio-Gel P-4 chromatography of glycopeptides prepared from [³H]glucosamine-labeled E1 or E2 after mixed glycosidase digestion of intact Sindbis.*
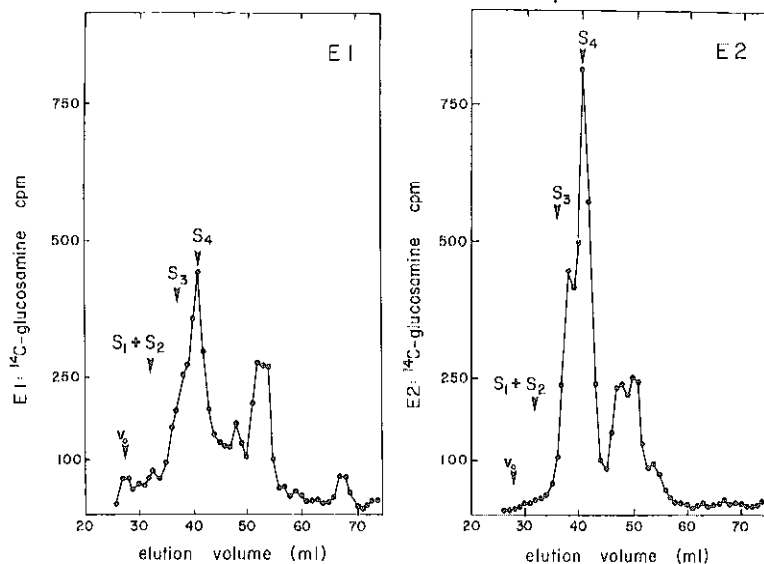
FIG. 5. *Bio-Gel P-4 chromatography of glycopeptides prepared from [^{14}C]glucosamine-labeled E1 or E2 after mixed glycosidase digestion of Triton-solubilized Sindbis.*

TABLE 4. *Distribution of carbohydrate label among glycopeptides of E1 and E2 after mixed glycosidase digestion of intact Sindbis*

Labeled sugar	% Label released		% Total amt of E1 or E2 label in glycopeptide of modal mol wt: [a]					
			E1			E2		
	E1	E2	2,000 (~S3)	1,600 (S4)	700-1,100	1,800 (~S3)	1,600 (~S4)	700-1,100
Glucosamine	9 ± 3	44 ± 2	68 ± 1	23 ± 2	0	12 ± 5	30 ± 2	14 ± 3
Fucose	0	0	83 ± 3	0	17 ± 1	35 ± 4	18 ± 1	48 ± 2
Mannose	4 ± 2	26 ± 4	55 ± 2	41 ± 2	0	6 ± 4	70 ± 4	0

[a] Values are the mean ± standard deviation from the mean of two determinations.

TABLE 5. *Distribution of label among the glycopeptides of E1 and E2 after mixed glycosidase digestion of the Triton-solubilized glycoprotein*

Label	% Released		% Total amt of label originally incorporated into E1 or E2 in glycopeptide of mol wt:							
			E1				E2			
	E1	E2	2,000 (S3 size)	1,600 (S4 size)	700-1,100	<700	1,800 (S3 size)	1,600 (S4 size)	700-1,100	<700
[^{14}C]glucosamine	56	47	5	18	19	2	11	23	17	2
[^{3}H]fucose	0	0	4	14	70	12	23	8	63	7
[^{3}H]mannose	56	29	7	26	11		7	64		

portion of the fucose label of E2.

After mixed glycosidase digestion of Triton-solubilized Sindbis virus, [^{14}C]glucosamine-labeled glycopeptides from E1 elute in both high- and low-molecular-weight positions on the Bio-Gel P-4 column (Fig. 5). The high-molecular-weight glycopeptide material elutes in a broad peak with a leading shoulder. The shoulder has an elution position corresponding to a molecular-weight range of 1,800 to 2,000. The main

peak of label corresponds in elution position to S4. The peak and its shoulder account for approximately 25% of the total glucosamine label of E1, 20% of the total fucose label, and 35% of the total mannose label, taking into account the carbohydrate label released from E1 (Table 5). The low-molecular-weight material is distributed among three glycopeptides eluting at about 1,050, 760, and 350. (The latter peak elutes outside the reliable fractionation range

of Bio-Gel P-4.) Of these three glycopeptides, the larger two are labeled by mannose, glucosamine, and fucose; the smallest is labeled only by glucosamine and fucose.

After mixed glycosidase digestion of Triton-solubilized Sindbis virus, the gel filtration patterns of glycopeptides from E2 are comparable to the patterns of glycopeptides prepared from E2 after mixed glycosidase digestion of intact virus (Fig. 5; Table 5). Slightly more fucose label elutes with the low-molecular-weight glycopeptides; but, in general, the two sets of glycopeptide patterns have similar distributions of fucose, mannose, and glucosamine label.

Glycoprotein E2 loses approximately 45% of its glucosamine label upon mixed glycosidase digestion of intact or Triton-solubilized Sindbis (Tables 1 and 2). The S4 glycopeptide of E2 accounts for approximately 27% of the label in glucosamine-labeled glycopeptides prepared from the untreated glycoprotein (M. McCarthy, Ph.D. thesis). Thus, the S4-sized glycopeptide accounts for approximately the same percentage of the E2 glucosamine label before and after digestion (Tables 4 and 5). Similarly, the S4-sized glycopeptide accounts for approximately the same percentage of the total mannose label of E2 before and after digestion. As judged by the distribution of carbohydrate label among glycopeptides, the type B (S4) carbohydrate units of E2 appear to be relatively unaffected by the mixed glycosidases. Similarly, the simple glycopeptides of thyroglobulin, resembling those of Sindbis in size and composition, are resistant to the mixed glycosidases (14). This suggests that low-molecular-weight glycopeptides probably derive from type A oligosaccharides on E2 that have been extensively degraded by the mixed glycosidases. On E1 there is some indication that the mixed glycosidases, acting on Triton-solubilized virus, degrade some of the type B oligosaccharides. The amount of E1 mannose label eluting in the position of S4 decreases from 35% (McCarthy, Ph.D. thesis) to 26% (Table 5), taking into account the percent mannose label released from E1.

Glycopeptides from α-mannosidase-treated viral glycoproteins. α-Mannosidase does not release mannose label from intact Sindbis, but it will release mannose label from Triton-solubilized virus (Table 2). Glycopeptides were prepared from the combined [^{3}H]mannose-labeled viral glycoproteins after α-mannosidase digestion of Triton-solubilized Sindbis. The glycopeptides were then chromatographed on Bio-Gel P-4 (Fig. 6). The glycopeptide mannose label elutes in three peaks. The first peak corre-
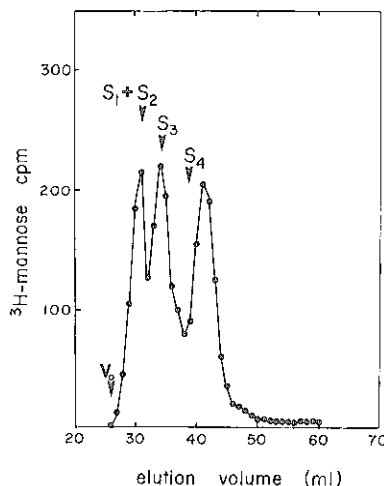

FIG. 6. *Bio-Gel P-4 chromatography of [^{3}H]-mannose-labeled glycopeptides prepared from Triton-solubilized Sindbis virus digested with α-mannosidase.*

sponds to glycopeptides S1 and S2, which do not resolve on Bio-Gel P-4; the second peak corresponds in elution position to glycopeptide S3; the third peak elutes with a molecular weight of about 1,300, making it smaller than S4. Since α-mannosidase removes 25% of the total mannose label from the combined Triton-solubilized viral glycoproteins (Table 2), the two larger glycopeptides contain 50% of the original total label. Correspondingly, 50% of the total mannose label is found in the type A glycopeptides from untreated, mannose-labeled Sindbis as determined from P-4 gel filtration patterns (data not shown). The third glycopeptide contains 30% of the total mannose label; S4 from untreated virus would contain 50%. The glycopeptide pattern (Fig. 6), therefore, indicates that α-mannosidase degrades only the type B oligosaccharides of Triton-solubilized glycoprotein.

Effects of glycosidase digestion on infectivity and hemagglutination. The infectivity of Sindbis virus is not altered by digestion of intact virus with the mixed glycosidases (Table 6), but the incubation conditions do cause some heat inactivation. The infectivity titer of both control and glycosidase-treated virus decreases approximately 10-fold after incubation for 16 h at 37°C. This may be due to aggregation of incubated virus, although mixed glycosidase-treated virus appears to sediment normally when centrifuged through a sucrose gradient (see below). The observed heat inactivation may obscure differences in the infectivity of control and glycosidase-treated virus. With

TABLE 6. *Infectivity and hemagglutination of chicken embryo fibroblast-grown Sindbis virus*

Added glycosi-dases	Infectivity[a] after h at 37°C:		Hemagglutination[b] after h at 37°C:	
	0	16	0	16
None	1×10^{11}	1.1×10^{10}	12,800	4,900
E	1×10^{11}	7×10^{9}		5,500

[a] Infectivity is expressed as PFU per milliliter. The viral protein concentration is 0.5 mg/ml. E, Mixed glycosidases. Values given are the means of four determinations.

[b] Hemagglutination units equal the reciprocal of the end point dilution factor. Titers are expressed as hemagglutination units per milliliter and are the means of three to six determinations. Virus (0.5 mg of protein/ml) was incubated at 37°C for the indicated time period prior to assay.

shorter incubation periods, heat inactivation of virus might not be a factor in determining infectivity titers. But, unlike the case with Triton-solubilized glycoprotein (see Fig. 7), the mixed glycosidases release carbohydrate from intact virus very slowly. No more than 5% of the glucosamine label is released after 2 h of incubation at 37°C (data not shown). Thus, it may not be possible to determine the infectivity of mixed glycosidase-treated virus under conditions where digestion is complete and no heat inactivation of virus occurs.

Antibodies to glycoprotein E2 will neutralize infectious Sindbis, suggesting that E2 mediates virus-cell interaction during infection (5). Since mixed glycosidases release much of the E2 carbohydrate, and since the infectivity of digested virus remains the same as that of control, intact E2 carbohydrate units do not appear to be necessary for virus-cell interaction. A similar conclusion was reached by Schlesinger et al. (27), who showed that Sindbis virus containing incomplete type A oligosaccharides has the same infectivity as virus with normal oligosaccharides. The apparent insensitivity of infectious titer to removal of exposed oligosaccharides suggests strongly that carbohydrate has little role in infectivity. Oligosaccharides inaccessible to glycosidases are probably inaccessible to cellular receptors as well, and a major change in surface configuration would be required to involve them in virus-cell interaction.

Like infectivity, the hemagglutinating activity of intact Sindbis is not altered by digestion with mixed glycosidases (Table 6). Although the hemagglutination titer of treated virus is slightly higher than that of control virus, this difference in titer is not significant. Sindbis virus hemagglutination is very sensitive to pH, and titers are variable. The hemagglutination titer is also heat sensitive. After 16 h at 37°C, the titer of a 0.5-mg/ml solution of Sindbis virus decreased from 12,800 U/ml to 4,900 U/ml.

Triton-glycoprotein micelles prepared from SFV have been shown to hemagglutinate gander erythrocytes (32). Likewise, Triton-solubilized Sindbis virus has the same titer as intact virus at equal concentrations of virus protein. Since the mixed glycosidases will remove carbohydrate from both Triton-solubilized Sindbis glycoproteins, it is possible to assay the effect of extensive carbohydrate removal on hemagglutinating activity. Solubilized, unlabeled Sindbis virus was assayed for hemagglutination titer after various lengths of incubation time (Table 7) and, in a parallel experiment, Triton-solubilized, [³H]glucosamine-labeled virus was assayed by trichloroacetic acid precipitation for release of carbohydrate (Fig. 7). At no time was the hemagglutination titer of any glycosidase-treated sample significantly lower than that of the control, even though glucosamine was rapidly released from solubilized viral glycoproteins. The hemagglutination titer of all samples did decrease with longer incubation times. This result suggests that the Triton-glycoprotein micelles are not stable for long periods of time at

TABLE 7. *Hemagglutination of Triton-solubilized Sindbis virus[a]*

Added glycosidases	Hemagglutination after h at 37°C:				
	0	1	2	3	8
None	6,400	1,600	3,200	3,200	1,000
Mixed glycosidases	6,400	1,600	1,600	1,600	1,000
Mixed glycosidases + α-mannosidase	3,200	1,600	1,600	1,600	500

[a] Hemagglutination units equal the reciprocal of the end point dilution factor; titers are expressed as hemagglutination units per milliliter. Sindbis virus was solubilized with Triton X-100 as described in Materials and Methods. Virus (0.33 mg of protein/ml) was incubated at 37°C for the indicated time period before assay.
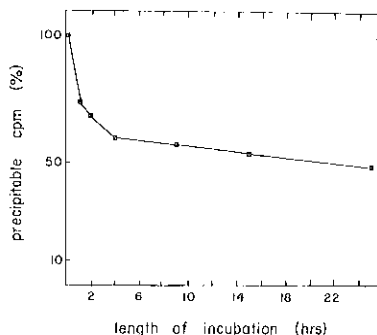

FIG. 7. *Time course of mixed glycosidase digestion of Triton-solubilized, [³H]glucosamine-labeled Sindbis virus. Viral protein was precipitated from the reaction mixture with trichloroacetic acid; then the percent precipitable [³H]glucosamine label was measured as a function of time.*

37°C, perhaps due to aggregation into larger complexes. Nevertheless, glycosidase-treated glycoprotein hemagglutinates like untreated glycoprotein, implying that the complete carbohydrate structures are not necessary for Sindbis virus-erythrocyte recognition. Since the glycosidases do not remove 100% of the carbohydrate, it is, of course, always possible that hemagglutinating activity depends on internal carbohydrate residues not removed by the glycosidases.

Kennedy (13) has reported that when intact SFV is treated with a mixture of glycosidases, both infectivity and hemagglutinating activity are lost. The commercial glycosidases used by Kennedy removed greater than 95% of the labeled carbohydrate from the intact SFV. Assays were performed at pH 7.0 in salt solutions of more than 0.1 M. In contrast, the mixed glycosidases from *D. pneumoniae* removed at most 50% of any labeled carbohydrate from intact Sindbis. Treated SFV apparently tends to aggregate, and this aggregation may have contributed to loss of biological activities. Treated Sindbis does not aggregate as long as the pH of the reaction mixture is maintained at 7.2 to 7.6. At lower pH values, both treated and untreated Sindbis will aggregate. Provided the commercial glycosidases used by Kennedy were free of protease activity, his results suggest that carbohydrate units on SFV might be fully exposed. Nevertheless, the aggregation of SFV makes it impossible to establish from glycosidase digestions whether or not the carbohydrate units are essential for biological function.

DISCUSSION

The results can be summarized as follows:

(i) When intact Sindbis is treated with mixed glycosidases, all glycopeptides from E1 are approximately the size of S3 or S4; no endoglycosidase cleavage has occurred. Glycopeptides from E2 include S4, some residual S3, and new low-molecular-weight species ranging from 700 to 1,100 and labeled only by glucosamine and fucose.

(ii) When Triton-solubilized virus is similarly treated, glycopeptides from E1 now include both high- and low-molecular-weight species labeled by glucosamine, fucose, and mannose. Glycopeptides from E2 are very similar to those obtained from E2 after mixed glycosidase treatment of intact virus.

Endo-β-N-acetylglucosaminidase D susceptibility of Sindbis oligosaccharides. Endo-β-N-acetylglucosaminidase D hydrolyzes the di-N-acetylchitobiose sequence found in N-glycosidically linked oligosaccharides with branched oligomannosyl di-N-acetylchitobiose core sequences. The enzyme requires an unsubstituted α-mannosyl residue linked at the C3 position of the branch mannose; hence, external sugars in the oligosaccharide must be released by exoglycosidase degradation (35). The action of the endoglycosidase then releases a mannose-containing oligosaccharide with N-acetylglucosamine at its free reducing terminus. Typically, these oligomannosyl cores contain one glucosamine residue and three to five mannose residues. The free oligosaccharides generated by mixed glycosidase digestion of intact or Triton-solubilized Sindbis are typical of oligomannosyl cores released by endo-β-N-acetylglucosaminidase D from other glycoproteins with N-glycosidically linked oligosaccharides. On the basis of its molecular weight, the single 700-dalton oligosaccharide released from intact Sindbis may contain three mannose residues and one glucosamine residue per mole. The larger oligosaccharide released only from Triton-solubilized Sindbis may contain four to five mannose residues per glucosamine. Since the mixed glycosidases release about the same amount of carbohydrate label from E2 whether or not Triton is present, the larger oligosaccharide must be derived from E1 carbohydrate units.

The low-molecular-weight glycopeptides obtained from E2 or E1 after mixed glycosidase digestion of Triton-solubilized virus are similar in size and composition to the glycopeptide products generated by endo-β-N-acetylglucosaminidase D acting on glycopeptides or intact glycoproteins with asparagine-linked oligosaccharides (2, 14, 18, 19, 21). These "inner-core" glycopeptides typically contain a single residue of glucosamine and often one of fucose. The low-molecular-weight glycopeptides of E1 appear to contain mannose as well (Table 5). Given the approximate size of these glycopeptides, it is unlikely that mannose is present in a complete oligomannosyl di-N-acetylchitobiose sequence. Instead, a single mannose residue may be linked to the innermost or proximal core N-acetylglucosamine residue on some E1 type A oligosaccharides. Muramatsu (20) isolated such inner-core glycopeptides after endo-β-N-acetylglucosaminidase D digestion of mouse myeloma γ-globulin glycopeptides.

A subpopulation of the Sindbis type A oligosaccharides is resistant to endo-β-N-acetylglucosaminidase D. The outer chains of these oligosaccharides are partially degraded by the exoglycosidases present in the mixed glycosidases, since none of the glycopeptides from glycosidase-treated glycoprotein is larger than S3.

Resistant oligosaccharides remain after glycosidase digestion of isolated type A glycopeptides (M. McCarthy, Ph.D. thesis). Thus, resistance to the endoglycosidase is probably due to oligosaccharide structure, not to interference by the polypeptide chain. Glycopeptides prepared from the G protein of vesicular stomatitis virus grown in polyoma virus-transformed BHK cells also contain a subpopulation of endoglycosidase-resistant structures (18). Moyer and Summers have suggested that endoglycosidase-resistant oligosaccharides have different carbohydrate sequences near the carbohydrate-peptide linkage region. However, resistant oligosaccharides may differ from susceptible structures in the carbohydrate sequence and/or anomeric configuration of one or more external glycosidic linkages. For example, externally linked α-fucosyl or α-N-acetylglucosaminyl residues are not sensitive to any of the exoglycosidases used. Their presence in an oligosaccharide chain would prevent sequential release of external carbohydrate residues, blocking the unsubstituted mannose necessary for endoglycosidase activity. It is, therefore, not possible to determine from mixed glycosidase digestion alone whether oligosaccharides that are resistant to endo-β-N-acetylglucosaminidase D do have different core carbohydrate sequences.

Distribution of glycoprotein carbohydrate on the surface of Sindbis virus. The products of glycosidase attack on Triton-solubilized Sindbis virus affirm that most carbohydrate units of the glycoproteins are good substrates either for mixed glycosidases or for α-mannosidase. The relative resistance of most of these units in intact virus particles, therefore, shows that much of the carbohydrate is not accessible to glycosidases, probably because it is not exposed at the viral surface. Sindbis glycoproteins cannot be represented as spikes with carbohydrate chains attached only at the periphery: most oligosaccharide chains are exposed only at their nonreducing termini, or not at all.

The only completely accessible carbohydrate units on Sindbis glycoproteins are the type A oligosaccharides of E2. When intact virus is digested with the mixed glycosidases, most of these oligosaccharides are degraded by exoglycosidases and by endo-β-N-acetylglucosaminidase D. Thus, residues near the carbohydrate-peptide linkage, as well as external sugars, are exposed on the viral surface. External regions of some E1 type A oligosaccharides are also accessible to enzymes. There is complete removal of sialic acid and some loss of external galactose and glucosamine from these oligosaccharides, although the amount released is not

enough to suggest that all type A oligosaccharides on E1 have accessible terminal carbohydrate residues. None of the E1 oligosaccharides of intact virus appears to be degraded by endoglycosidase: the carbohydrate-peptide linkage sites of E1 type A oligosaccharides are not exposed. Type B oligosaccharides of E1 and E2 are both inaccessible on intact Sindbis: only after Triton disruption are these oligosaccharides degraded by α-mannosidase.

These interpretations depend on the assumption that the surface structure of Sindbis virus is not altered or disrupted by glycosidase incubation. We examined virus by X-ray diffraction after prolonged incubation at 37°C (no enzyme was present, since very large quantities of virus were required for the experiment, but conditions were otherwise identical); no disruption had occurred and the usual, very clear diffraction pattern was observed (8). In addition, mixed glycosidase-treated virus was examined by two methods—velocity gradient centrifugation and electron microscopy—to determine whether or not the mixed glycosidase digestion alters viral size or shape. In no instance are the properties or appearance of incubated virus different from those of the control. Thus, glycosidase treatment does not alter the overall regularity and rigidity of the viral particle, nor does it disturb the regular surface organization of the glycoproteins described by von Bonsdorff and Harrison (37).

Iodination of intact Sindbis virus has provided another probe for protein accessibility. Low levels of iodination of E1 and extensive iodination of E2 on intact virus contrast with essentially equivalent levels in SDS-disrupted virus (31). These results indicate a differential reactivity of tyrosine residues that parallels oligosaccharide exposure. At least one significant portion of E1 appears to be exposed on the surface, however, since antibodies to E1 will inhibit Sindbis hemagglutination (5).

Can we relate the differential accessibility of Sindbis oligosaccharides to the organization of the viral surface? X-ray scattering experiments have shown that the outer polar groups of the lipid bilayer in Sindbis lie at about 25.5 nm from the particle center, and that glycoprotein subunits project 8.0 to 9.0 nm outward from this bilayer (8). Probably a small, hydrophobic "tail" on each subunit penetrates the lipid, as has been demonstrated in SFV (6, 7, 36). The tightest inter-subunit contacts occur in the region between 26.0 to 30.0 nm from the particle center; beyond 30.0 nm, glycoprotein subunits must taper considerably, since the spherically averaged density is rather low (8). Electron

micrographs show that the glycoproteins are tightly clustered as trimers and that these trimer clusters are regularly arranged in a T = 4 icosahedral lattice (37). There must also be some association between subunits in adjacent trimers to maintain the overall organization, but micrographs show these intertrimer contacts to be much less extensive than those within one cluster. That is, deep grooves between trimers form a triangular network in the surface, the grooves extending inward almost to the phospholipid bilayer (Fig. 8a; cf. reference 37). To a first approximation, then, we might expect the accessible region of the virus surface to correspond to the tapered outer part of the glycoprotein structural units ($r = 30.0$ to 34.0 nm), as well as to those parts of the subunit surfaces between radii of 26.0 and 30.0 nm that face the surface lattice grooves (Fig. 8b).

In this description of the viral surface, where might oligosaccharides of Sindbis glycoproteins be located? Certainly they must be on subunit surfaces, since sugar residues are unlikely to be buried in the hydrophobic interior of a protein, and since they are exposed to glycosidases by Triton disruption of the surface lattice. Triton is generally assumed to dissociate subunits at the surface, but not to unfold them radically (32). Type A oligosaccharides of E2 are accessible to endo-β-N-acetylglucosaminidase D even on intact Sindbis. The carbohydrate may, therefore, be linked either near the tip of the glycoprotein subunit or to a lateral surface facing outward from the tightly clustered trimer (Fig. 8b). The other oligosaccharides are largely buried: those of type B are completely so, and those of type A of E1 are accessible only at the outermost sugars. We imagine the carbohydrate-peptide linkage of these oligosaccharides to lie at closely apposed interfaces of adjacent subunits, inaccessible to endo-β-N-acetylglucosaminidase D. The oligosaccharide chain could then extend along the subunit interface towards the exterior, where terminal carbohydrate residues on some oligosaccharides would be accessible to exoglycosidases (Fig. 8b). This description is consistent with the arrangement of carbohydrate in the Fc fragment of human immunoglobulin G—the only glycoprotein structure that has been solved by X-ray diffraction. In Fc, extended oligosaccharide chains lie facing each other at the interface between two C_H2 domains (11). Most of the sugars are packed against polypeptide surfaces on the inside of the Fc dimer, with terminal carbohydrate residues appearing to project beyond this "protected" contact region. Such a picture suggests that carbohydrate can mediate
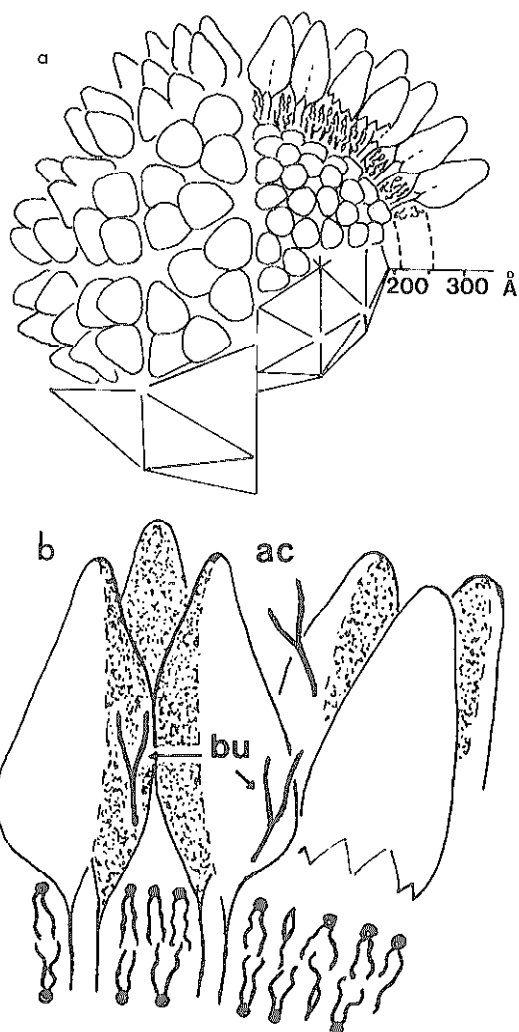

FIG. 8. (a) Cross-sectional diagram of Sindbis virus, with features of surface organization described in the text. Each pear-shaped structure unit on the surface represents two polypeptide chains; the units are trimer clustered, with rather marked grooves between the trimers (4). (b) Enlarged schematic view of glycoprotein subunits, showing two trimers and possible locations for oligosaccharides. The shaded surfaces might represent E1, with relatively inaccessible tyrosines and carbohydrate-peptide linkages. Oligosaccharides designated ac represent accessible locations; those designated bu represent sites largely or completely buried in the intact structure. Triton disruption of the surface lattice would expose these residues, even without unfolding of the polypeptide chains.

protein-protein contacts and that glycosylation may therefore play a role in regulating assembly of viral membranes.

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