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PROTEIN-BOUND OLIGOSACCHARIDES OF SEMLIKI FOREST VIRUS

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SUMMARY

Semliki Forest virus was grown in BHK cells and labeled in vivo with radioactive monosaccharides. Pronase digests of the virus chromatographed on Bio-Gel P 6 revealed glycopeptides of A-type and B-type. (For the nomenclature see Johnson, J. and Clamp, J. R. (1971) Biochem. J. 123, 739–745.) The former was labeled with [^{3}H]fucose, [^{3}H]galactose, [^{3}H]mannose and [^{14}C]glucosamine, the latter only with [^{3}H]mannose and [^{14}C]glucosamine. The three envelope glycoproteins E_1, E_2 and E_3 were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to pronase digestion. The glycoproteins E_1 and E_3 revealed glycopeptides of A-type. E_2 revealed glycopeptides of B-type. E_2 yielded additionally a glycopeptide (M_r 3100) which was heavily labeled from [^{3}H]galactose, but only marginally from [^{14}C]glucosamine, [^{3}H]fucose and [^{3}H]mannose. Whether this glycopeptide belongs to the A-type or not remains uncertain. The apparent molecular weights of the A-type units measured by gel filtration were 3400 in E_1 and 4000 in E_3; the B-type unit of E_2 had an apparent molecular weight of 2000. Combined with the findings of our earlier chemical analysis these data suggest that E_1 and E_3 contain on the average one A-type unit; E_2 probably contains one 3100 dalton unit plus one or two B-type units.

INTRODUCTION

Our group is studying the Semliki Forest virus (SF virus) membrane as an experimental model for biological membranes [1, 2]. The viral membrane is composed of a host cell-derived lipid bilayer [3–5] and three virus-specific glycoproteins: E_1 (M_r 49 000) and E_2 (M_r 52 000) and E_3 with a molecular weight of about 10 000 [6]. The viral glycoproteins form projections or spikes on the external membrane surface [7] and are necessary for the hemagglutinating ability and the infectivity of the virus particle [8]. E_1 and E_2 are attached to the membrane by a hydrophobic segment of their polypeptide chain [8], and either one or both of these glycoproteins span the lipid bilayer [9]. The molecular organization of the spikes is not known.

Chemical analysis shows that 6.3 % of total mass of SF virus consists of protein-bound carbohydrate [10]. SF virus grown in BHK cells contains the three

membrane glycoproteins E_1, E_2 and E_3 in approximately equimolar amounts [6]. The carbohydrate analysis indicated that E_1 contains about 18 mol of monosaccharides, E_2 about 28 and E_3 about 22 [6]. All three glycoproteins contain *N*-acetylglucosamine, mannose, galactose and fucose, and E_2 is particularly rich in mannose [6].

The present report describes analysis of glycopeptides obtained by pronase digestion of all three SF virus glycoproteins. The data obtained show that E_1 and E_3 glycoproteins contain one oligosaccharide labeled with radioactive glucosamine, mannose, galactose and fucose (A-type; see ref. 11). E_2 appears to contain one oligosaccharide heavily labeled with galactose; it may or may not be of the A-type. E_2 contains additionally one or two oligosaccharides labeled only with glucosamine and mannose (B-type) [11].

MATERIALS AND METHODS

Growth and radioactive labeling of the virus

A prototype strain of SF virus was grown in BHK-cells in Falcon plastic bottles of 75 cm^2 growth area (30×10^6 cells). The cells were infected with 30 plaque-forming units per cell. After 1 h adsorption at room temperature the inoculum was removed and an appropriate volume of Eagle's minimal essential medium supplemented with 0.2 % bovine serum albumin was added. Virus was grown at 37 °C for 3 h and after that the medium was changed to one containing the radioactive sugars. In most experiments this medium consisted of Eagle's minimal essential medium with 0.2 % bovine serum albumin but the glucose concentration was reduced to 0.1 of the normal concentration. The infection was then allowed to proceed for 7 h. Actinomycin D (1 μg/ml) was present during infection. The infected cells were labeled in three different ways: (1) with 1 mCi of L-[1-^{3}H]fucose (The Radiochemical Centre, Amersham, 2.6 Ci/mmol) and 250 μCi of D-[1-^{14}C]-glucosaminehydrochloride (The Radiochemical Centre, Amersham, 58 Ci/mol); (2) with 1 mCi of D-[1-^{3}H]galactose (The Radiochemical Centre, Amersham, 13 Ci/mmol) and 250 μCi of D-[1-^{14}C]-mannose (The Radiochemical Centre, Amersham, 58.7 Ci/mol); (3) with D-[1-^{3}H]-mannose (The Radiochemical Centre, Amersham, 9.1 Ci/mmol). In the third experiment the glucose content of the labeling medium was not reduced. The total volume of each labeled medium was 10 ml per bottle.

Virus purification

10 h after infection the culture fluid was harvested and the virus was purified essentially as described by Söderlund et al. [12]. The culture fluid was centrifuged at $10\,000 \times g$ for 30 min at 4 °C. The supernatant was centrifuged at 37 000 rev./min for 1.5 h in an $SW_{50.1}$ rotor of the Beckman Model L-3 ultracentrifuge. The virus pellet was resuspended in 0.1 M NaCl/0.05 M Tris (pH 7.4). The virus was further purified in a discontinuous sucrose gradient made in 0.1 M NaCl/0.05 M Tris (pH 7.4) as follows: at the bottom, 0.5 ml 50 % (w/w) sucrose, then 5 ml of linear 25–50 % (w/w) gradient and 10 ml of linear 10–20 % (w/w) gradient [13]. The gradient was centrifuged at 25 000 rev./min for 3.5 h in an SW_{27} rotor. The virus peak was collected and diluted in 0.1 M NaCl/0.05 M Tris (pH 7.4) and centrifuged for 1.5 h at 37 000 rev./min in the $SW_{50.1}$ rotor. The pelleted virus was resuspended in the buffer and stored at −70 °C.

Pronase digestion of intact SF virus

Proteolysis of SF virus was carried out essentially as described by Burge et al. [14] and Arima et al. [15]. Purified virus was disrupted with 0.1 % sodium dodecyl-sulfate and the sample was diluted ten-fold with 0.15 M Tris/acetate buffer (pH 7.8) containing 0.0015 M calcium-acetate and 0.1 % pronase (Calbiochem, B grade). One drop of toluene was added to prevent bacterial growth. The mixture was incubated at 37 °C for 4 days with daily addition of pronase to a final concentration of 0.4 %. Pronase was autodigested at 37 °C for 1 h before use to minimize possible glycosidase activity; this was carried out under the same conditions as the actual proteolysis.

Isolation of SF virus glycoproteins

SF virus glycoproteins were separated using the discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with a spacer gel [16]. Prior to electrophoresis the samples were treated with 1 % sodium dodecyl sulfate and 1 % 2-mercaptoethanol at 100 °C for 2 min .After electrophoresis the gels were sliced and each 1 mm slice was eluted for 10 h with 0.15 M Tris/acetate buffer (pH 7.8) containing 0.0015 M calcium acetate. Small aliquots from each fraction were taken to locate the radioactive glycoproteins. Each glycoprotein was digested with pronase as described above.

Gel filtration of glycopeptides

Glycopeptide samples obtained by pronase digestion were examined by gel filtration on Bio-Gel P6 (100–200 mesh, Bio-Rad) using conditions similar to those of Burge et al. [14]. The sample (300 μl) was eluted from the column (0.9 × 50 cm) with 0.15 M Tris/acetate buffer (pH 7.8) containing 0.1 % sodium dodecyl sulfate. Fractions of 0.45 ml were collected. Blue Dextran 2000 (Pharmacia) was used to determine the void volume of the column (v_0).

Estimation of the molecular weights of SF virus glycopeptides

The molecular weight estimation of SF virus glycopeptides was carried out by gel filtration using the method of Burge et al. [14]. The following radioactive standards were used to calibrate the column: lacto-*N*-difucohexaose, lacto-*N*-fucopentose and *O*-β-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucose, all three reduced with NaB^3H_4; [^{14}C]mannose and [^{14}C]acetylated [17] A-type glycopeptides of tyroglobulin, molecular weight 4100 [18], were also used. The exact position of each peak was determined by fitting a parabola through the three highest points.

RESULTS

Glycoproteins of Semliki Forest virus

Semliki Forest virus was labeled in vivo with radioactive monosaccharides in the medium of the host cells. The virus was then isolated and the viral proteins were separated with discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [16] (Fig. 1). All three glycoproteins E_1, E_2 and E_3 became labeled with radioactive mannose, glucosamine, galactose and fucose (Figs. 1A and 1B). Even the core protein, located between E_3 and E_1 in Fig. 1, contained a little radioactivity derived from the monosaccharides. Since the core protein is believed to

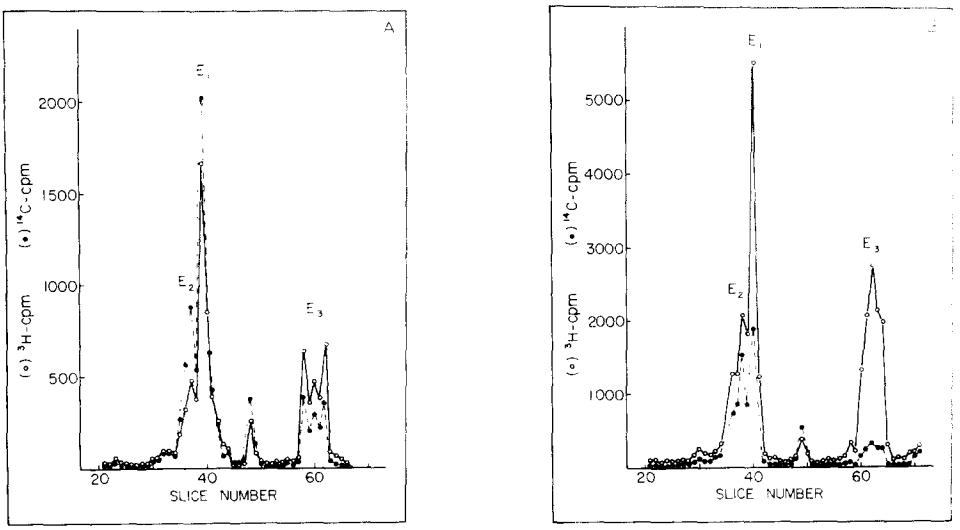

Fig. 1. Gel electrophoretic patterns of Semliki Forest virus. (a) Virus labeled with D-[1-^{14}C]-glucosamine and L-[1-^{3}H]fucose; (b) virus labeled with D-[1-^{14}C]mannose and D-[1-^{3}H]galactose. (●) ^{14}C radioactivity, (○) ^{3}H radioactivity.

be unglycosylated [19] its radioactivity indicates that some of the carbohydrate label was probably metabolized into amino acids. This conversion was probably caused by the low glucose content of the medium used in our experiments; it is not always observed in BHK cells [20]. The notion that such a conversion really took place in most of our present experiments is supported by the following observations. (1) Pronase digests of the isolated glycoproteins contained radioactive amino acids (see below; Figs. 3 and 4). (2) The amount of the radioactive amino acids in the pronase digests increased with increasing polypeptide size in the glycoproteins. For example E_1, having a polypeptide of about 45 000 daltons [6], released considerable amounts of radioactive amino acids during proteolysis (Figs. 3A and 4A), whereas E_3, having a polypeptide of only 6000 daltons, released only very small amounts of radioactive amino acids (Figs. 3C and 4C). (3) When D-[2-^{3}H]-mannose was used for the labeling of the virus, no radioactivity was found in the core protein in the gel electrophoresis, but E_1, E_2 and E_3 were labeled (data not shown). Furthermore, when the labeled virus of this experiment was subjected to pronase digestion no radioactivity was released into the amino acid fraction of the gel filtration (data not shown). The specific labeling of the oligosaccharides with [2-^{3}H]mannose [27] is believed to depend on the loss of label from mannose before it is processed through glycolysis and converted into amino acids or sugars other than fucose.

The nonspecific labeling of the viral proteins in the experiments of Fig. 1 complicates a little the interpretation of the results. As an approximation, however, E_1 and E_2 are likely to contain about 1.5 times as much amino acid radioactivity as the core protein, because their polypeptides are about 1.5 times larger than the polypeptide of the core protein [6]. Analogous considerations show that E_3 is likely to contain in its polypeptide about one fifth of the radioactivity of the core protein [6]. When the radioactivity residing in the amino acids is subtracted from the peaks it

becomes obvious that E_1, E_2 and E_3 shown in Fig. 1 contain large amounts of carbohydrate radioactivity. This confirms the results of the chemical analysis [6] which has shown that E_1, E_2 and E_3 are glycoproteins.

The E_3-band is very broad in Fig. 1. This may indicate some sort of heterogeneity in the material, but it may also indicate a rapid rate of diffusion caused by the small size of E_3. The apparent splitting of E_3 into three bands in Fig. 1A is probably fortuitous and was not observed in several other electrophoretic runs.

The small peak before E_2 (Figs. 1A and 1B) occupies the same position as the precursor protein NVP 68 found in cells infected with SF virus. This protein is believed to be essentially E_2 and E_3 linked together [6].

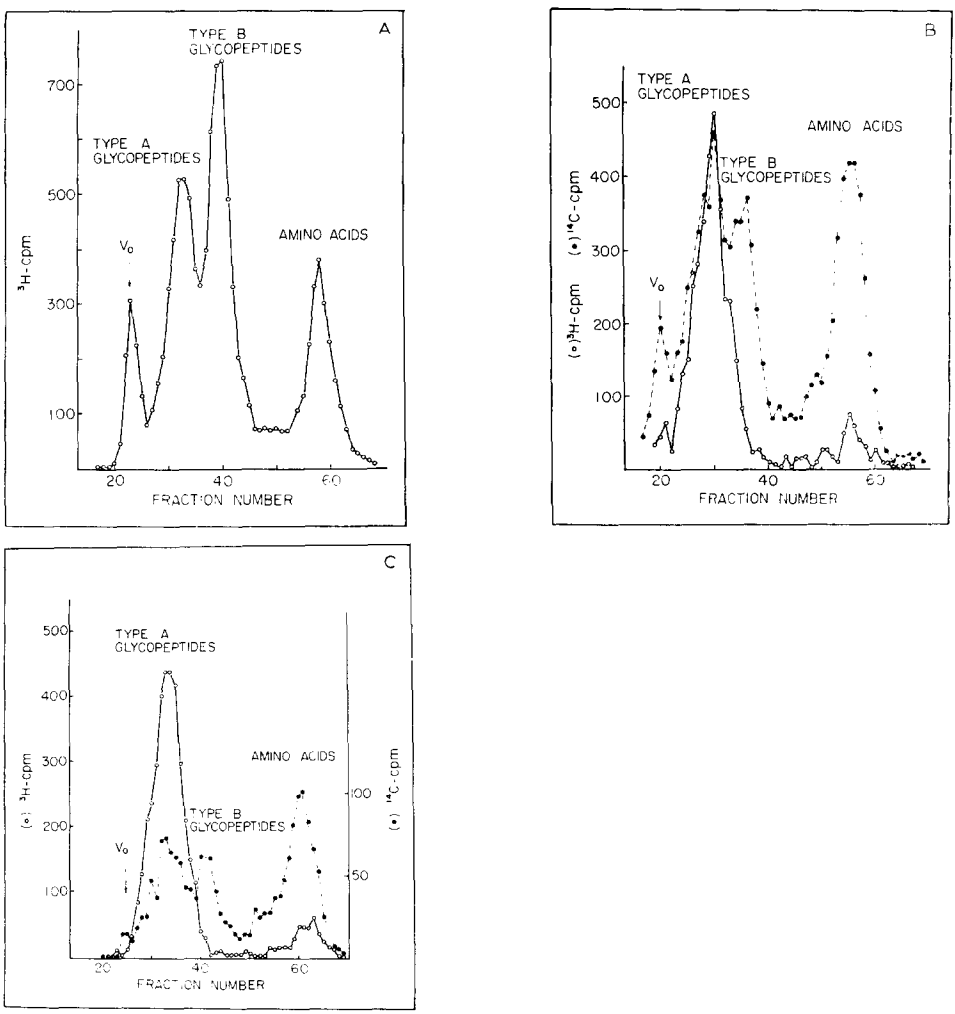

Fig. 2. Intact Semliki Forest virus labeled with different radioactive monosaccharides was disrupted with sodium dodecyl sulfate and digested with pronase. The glycopeptides were examined by gel filtration on Bio-Gel P 6. (a) Virus labeled with D-[1-^{3}H]mannose; (b) virus double labeled with D-[1-^{14}C]glucosamine and L-[1-^{3}H]fucose; (c) virus double labeled with D-[1-^{14}C]mannose and D-[1-^{3}H]galactose. (●) ^{14}C radioactivity, (○) ^{3}H radioactivity.

Glycopeptides from intact SF virus

Exhaustive digestion of glycoproteins with the protease mixture, pronase, destroys the polypeptide chains and leaves only a few amino acids attached to the oligosaccharides. These oligosaccharide structures, pronase glycopeptides, are conveniently studied by gel filtration. When pronase glycopeptides of SF virus were chromatographed on Bio-Gel P6, four major fractions were obtained (Fig. 2). The radioactivity found at or near void volume is believed to represent nondegraded glycoproteins and/or micellar glycolipids; globoside, a ceramide tetrasaccharide of M_r around 1300, eluted in this area. The second peak represents glycopeptides which contained label from [^{14}C]glucosamine, [^{3}H]fucose, [^{3}H]- and [^{14}C]mannose and [^{3}H]galactose. They are referred to as Type A glycopeptides [11]. The third peak is also a glycopeptide but it contained label only from glucosamine and mannose. It is called Type B glycopeptide [11]. The absence of fucose and galactose label from Type B glycopeptides shows that the label of these sugars was not converted into mannose and glucosamine. This is surprising; why was the label converted into amino acids of the core protein but not into the sugars of the B-glycopeptides? The explanation probably lies in the small number of monosaccharide residues in the B-glycopeptides and in the large number of amino acids in the core protein of M_r 33 000. The fourth peak of Fig. 2 represents amino acids and small peptides.

Glycopeptides of the isolated envelope glycoproteins

The individual envelope glycoproteins of SF virus were isolated with sodium dodecyl sulfate gel electrophoresis as shown in Fig. 1. This method is more rapid and more reliable than hydroxyapatite chromatography which was used earlier [6], but it gives a rather poor separation of E_1 and E_2. Satisfactory preparations were obtainable, however, by neglecting the protein fractions most likely to be mixtures of E_1 and E_2. The results described below were obtained by studying material from the early part of peak E_2 (including the peak fraction) and from the rear part of peak E_1 (including the peak fraction). The purity of isolated E_1 and E_2 could not be checked with analysis of the N-terminal amino acids because only valine is found at the N-terminus of these two proteins [19]. Fortunately, the two proteins proved to yield glycopeptides so different from each other that doubts of possible contamination were largely eliminated.

The isolated envelope glycoproteins were digested with pronase and analyzed by gel filtration on Bio-Gel P 6 (Figs. 3 and 4). No glycolipid peak was observed at the void volume in these runs. The glycoproteins E_1 and E_3 contained only glycopeptides of the A-type, but the glycoprotein E_2 contained two different glycopeptides. One of these was obviously the B-type glycopeptide, and the other resembled the A-type glycopeptides. The latter contained large amounts of galactose label (Fig. 4B), but so small amounts of label from mannose (Fig. 4B) and glucosamine, as well as from fucose (Fig. 3B), that it remains unclear whether they represent radioactivity from E_2 or from E_1 contamination.

The molecular weights of the glycopeptides were estimated from the gel filtration data of Figs. 2, 3 and 4 by the methods of John et al. [21] and Burge et al. [14]. The glycopeptide of the glycoprotein E_1 (E_1A) had an apparent molecular weight of 3400, the glycopeptide of E_3 (E_3A) 4000 and the so-called A-type glycopeptide of E_2 (E_2A) 3100. The B-type glycopeptide of E_2 (E_2B) had an apparent

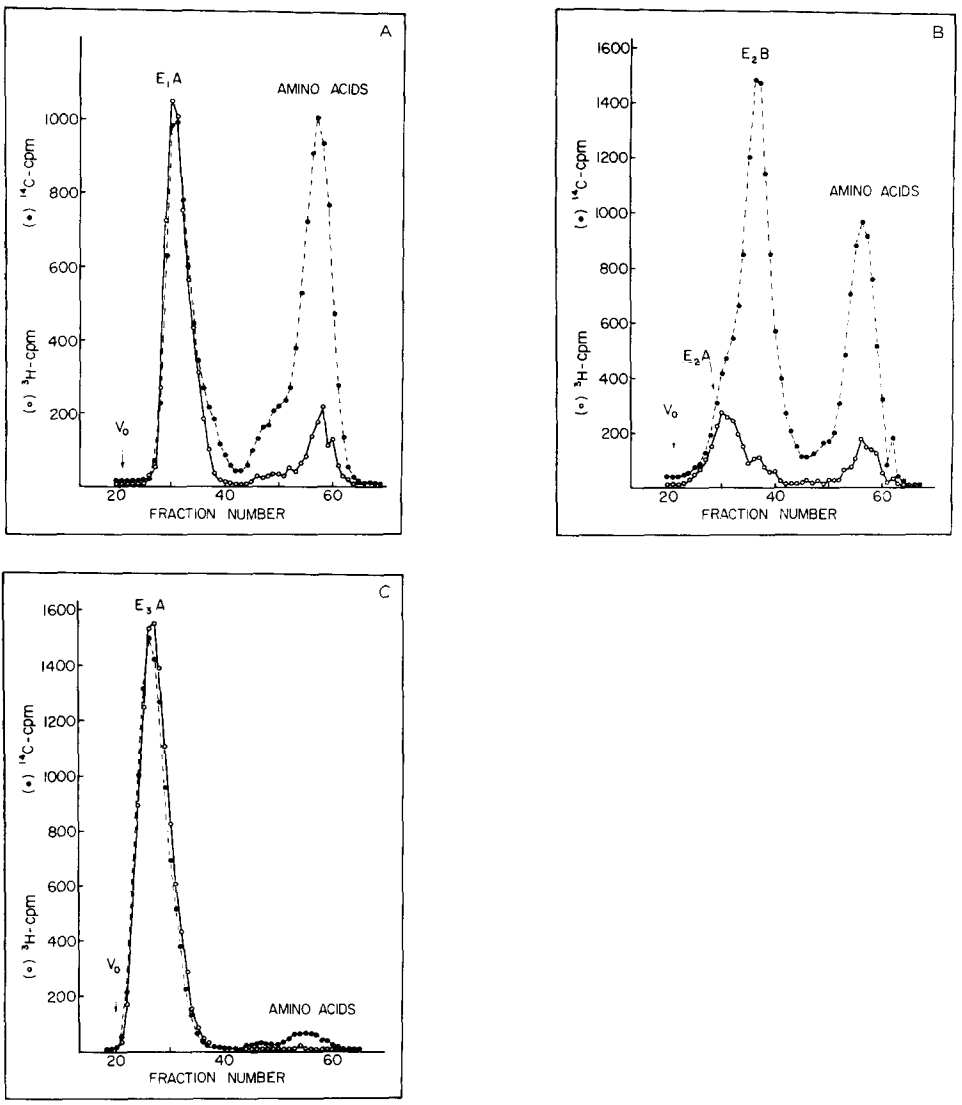

Fig. 3. Gel filtration patterns of double labeled (D-[1-^{14}C]glucosamine and L-[1-^{3}H]fucose) pronase glycopeptides on Bio-Gel P 6. (a) Glycopeptides from glycoprotein E_1; (b) glycopeptides from glycoprotein E_2; (c) glycopeptides from glycoprotein E_3. (●) ^{14}C radioactivity, (○) ^{3}H radioactivity.

molecular weight of 2000. Repeated pronase treatment of E_2B did not change its apparent molecular weight. Therefore it appears likely that the pronase digestion was exhaustive.

DISCUSSION

Using the discontinuous Neville system of gel electrophoresis in presence of sodium dodecyl sulfate, the following apparent molecular weights have been observed

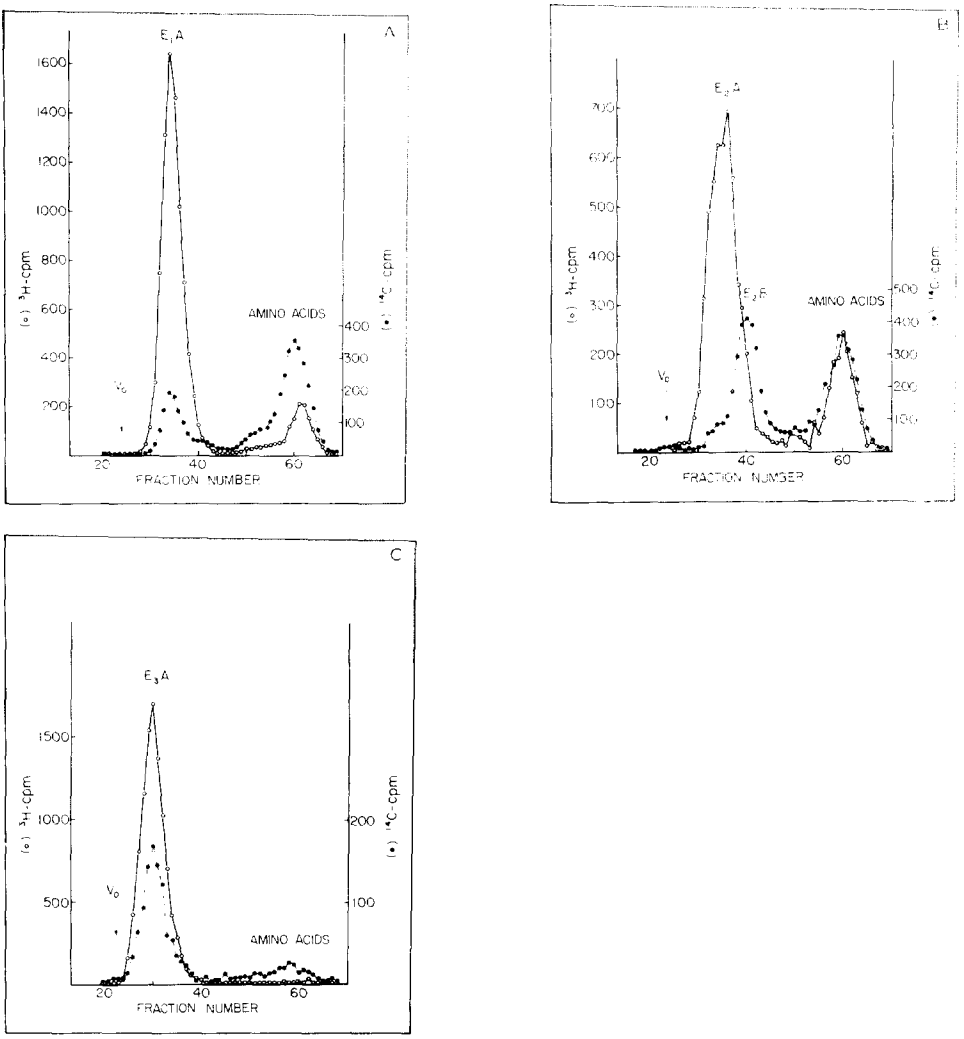

Fig. 4. Gel filtration patterns of double labeled (D-[1-^{14}C]mannose and D-[1-^{3}H]galactose) pronase glycopeptides on Bio-Gel P 6. (a) Glycopeptides from glycoprotein E_1; (b) glycopeptides from glycoprotein E_2; (c) glycopeptides from glycoprotein E_3. (●) ^{14}C radioactivity, (○) ^{3}H radioactivity.

for the SF virus envelope glycoproteins; E_1 49 000 and E_2 52 000 [6]. However, when the molecular weights of glycoproteins are determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis some uncertainty remains [22–24]. The apparent molecular weight of E_3 (M_r 10 000) was estimated using the chemical analysis data [6].

Some uncertainty remains also in the molecular weights of the pronase glycopeptides as determined by gel filtration. The structures of the molecules as well their size are important in determining the chromatographic behaviour. This is shown, for example, by the separation of pairs like maltose and isomaltose or maltotriose and isomaltotriose on Bio-Gel [21].

In some instances no extra amino acids are found in the pronase glycopeptide [15]: in others the peptide appears to be rather large [25]. The content of amino acids attached to Sindbis glycopeptides after exhaustive pronase digestion is believed to be about 10 % [14]. If this were true even here, the large oligosaccharides of SF virus would have molecular weights of about 3100 (E_1), 3600 (E_3) and 2800 (E_2), and the B-type oligosaccharide of E_2 would have a molecular weight of about 1800.

Despite all these uncertainties, we can compare the results in this paper with those of the chemical analysis [6]. The carbohydrate content of glycoprotein E_1 is 7.5 % (w/w) and the apparent molecular weight of E_1 is 49 000 [6]. This means that E_1 contains about 3700 daltons carbohydrate. As the oligosaccharide E_1A had an apparent molecular weight of 3100, it appears that E_1 contains on the average only one A-type unit. The glycoprotein E_2 (M_r 52 000) contains 11.5 % carbohydrate which corresponds to 6000 daltons [6]. Since the large oligosaccharide of E_2 has an apparent molecular weight of 2800 and the oligosaccharide E_2B of 1800, it appears that E_2 contains one of the large units and one or two B-type units. The chemical analysis has shown that E_3 contains about 4500 daltons carbohydrate [6]. Accordingly, E_3 also appears to contain on the average only one A-type oligosaccharide.

In an average SF virion there are about 290 copies of each of the three envelope proteins (calculated from the protein content of intact SF virus [10] and the molecular weights and the molar ratios of the envelope proteins [6]). Accordingly a virion contains about 290 copies of all three oligosaccharides of the large type and, in addition, 290–580 B-type oligosaccharides. Therefore the protein-bound oligosaccharides of an SF virion should add up to $3.2 \cdot 10^6$–$3.7 \cdot 10^6$ daltons, depending on the number of B-type units in the glycoprotein E_2. These values are in fairly good agreement with the data from direct carbohydrate analysis of intact SF virus, which indicated about $4 \cdot 10^6$ daltons of protein-bound carbohydrate [10].

We have previously reported the presence of galactosamine in the hydrolysates of SF virus [10]. However, we could not detect this sugar in the lipids [4] or in the hydrolysates of the isolated envelope glycoproteins which were obtained by chromatography on hydroxapatite [6]. At present we find it wise not to exclude the possibility that some of the SF virus glycoproteins may contain *N*-acetylgalactosamine.

It is possible that the oligosaccharides may not be evenly distributed among all copies of the membrane glycoproteins [26, 27]. Some molecules of the glycoprotein E_2, for instance, may contain two carbohydrate chains of the so-called A-type, whereas most E_2 molecules may contain one, and some may contain no sugar chain of this type at all. Removal of the carbohydrates leads to increased mobility of the proteins on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [28, 29]. Amino acid-labeled glycoprotein E_1 of SF virus is always observed as a sharp peak on the gel electrophoresis, but E_2 and E_3 are relatively broad bands [6, 8]. This is true also in our experiments with carbohydrate labeled proteins (Fig. 1). This suggests that E_1 is more homogeneous than E_2; the broad peak of E_3 may be caused by heterogeneity, but also by its small size and high rate of diffusion. Double labeling experiments with amino acids and sugars are needed to show whether there is any heterogeneity in E_2 and E_3 populations as regards the number of carbohydrate chains.

Sindbis virus, a close relative of SF virus, is different from SF virus in the sense that, in addition to E_2, E_1 also contains B-type oligosaccharides [27].

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