The Effect of Enzymes on Structural and Biological Properties of Semliki Forest Virus

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SUMMARY

Treatment of purified Semliki Forest virus with neuraminidase released sialic acid, raised the isoelectric point of the virus by 0.35 pH units but had no effect on the infectivity, haemagglutinating (HA) activity or surface antigenic properties and did not alter the electrophoretic mobility of the envelope glycoproteins on polyacrylamide gels. Treatment of virus with a mixture of sugar hydrolases lowered infectivity and HA activity and slightly increased the electrophoretic mobility of the envelope glycoproteins, but had no measurable effect on surface antigenic properties. Treatment with bromelain digested the envelope glycoproteins, destroyed infectivity, HA activity and surface antigenicity and yielded a sub-viral particle containing lipid. Treatment of this particle with phospholipase C produced a core particle indistinguishable by sedimentation analysis from the nucleocapsids found in infected cells.

INTRODUCTION

Semliki Forest virus (SFV), an alphavirus, consists of a spherical nucleocapsid containing the virus RNA, surrounded by an outer lipoprotein envelope (Osterrieth & Calberg-Bacq, 1966). This envelope is derived from the plasma membrane of the host cell and is acquired as the nucleocapsid buds through the plasma membrane during virus maturation (Acheson & Tamm, 1967; Waite, Brown & Pfefferkorn, 1972). Functionally, this envelope is required for infection since isolated nucleocapsids are not infectious (Friedman & Berezesky, 1967). Two structural proteins have been found in a number of alphaviruses including SFV (Hay, Skehel & Burke, 1968; Kennedy & Burke, 1972), Sindbis virus (Strauss et al. 1968), western equine encephalomyelitis virus (Sreevalsan & Allen, 1968) and Chikungunya virus (Igarashi et al. 1970). One of these proteins, the core protein, is a component of the virus nucleocapsid, the other, a glycoprotein, forms part of the envelope. Recently a second envelope protein has been found in Sindbis virus (Schlesinger, Schlesinger & Burge, 1972) and SFV (Simons, Kera"nan & Kääriäinen, 1973). Both of these envelope proteins are reported to carry carbohydrate residues.

Spike-like projections have been observed on the surface of SFV (Osterrieth & Calberg-Bacq, 1966). Oram et al. (1971) identified these spikes as envelope glycoprotein and demonstrated that the carbohydrate moiety of the protein is exposed on the surface of the spikes. The role of the spikes in the infectivity and HA activity of alphaviruses is unclear. Osterrieth (1966) reported that treatment of SFV with the proteolytic enzyme caseinase C removes the spikes and destroys HA activity, but has little effect on infectivity. By contrast, removal of Sindbis virus spikes with the enzyme bromelain destroys infectivity, indicating that the spikes are essential for infection of the host cell (Comphans, 1971).
In the present study, purified SFV was treated with a number of degradative enzymes in an attempt to gain insight into relationships between structural and biological properties of the virus. In particular, attention was focused on the envelope glycoprotein and its component carbohydrate.

**METHODS**

**Materials.** Bromelain (5-7 units/mg), α-galactosidase (7-8 units/mg), β-galactosidase (27 units/mg), α-mannosidase (1.4 units/mg), N-acetyl-β-D-glucosaminidase (3.7 units/mg), neuraminidase (0.4 units/mg) and α-L-fucosidase (1.4 units/mg) were all obtained from Boehringer Mannheim GmbH, Germany. Sugar hydrolases from the slime mould Dictyostelium discoideum were a gift from Dr J. Ashworth, School of Biological Sciences, University of Leicester. Phospholipase C (type 1; 2-7 units/mg protein), iodoacetamide and dansyl chloride were supplied by Sigma Chemical Co., Ltd., London. Concanavalin A (B grade) was supplied by Calbiochem, Ltd., London. [4, 5-3H]-L-lysine HCl (6.7 Ci/mmol), [14C]-L-lysine HCl (310 Ci/mmol), [2, 3-3H]-L-valine (12 Ci/mmoll), [4, 5-3H]-L-leucine (41 Ci/mmol), [85S]-L-methionine (22 Ci/mmol), [1-3H]-D-glucosamine HCl (2.6 Ci/mmol), [2-14C]-uridine (60 Ci/ml), [1-3H]-l-fucose (1.7 Ci/mmol), [2-3H]-D-mannose (1.4 Ci/mmol) and [methyl-3H]-choline chloride (5.7 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks. Caseinase C was a gift from Dr Paul M. Osterrieth, Laboratory of General and Medical Microbiology, University of Liège, Belgium. Acrylamide (purum; from Fluka AG, Buchs, Switzerland) and N,N′-methylenebisacrylamide (from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) were recrystallized from chloroform and acetone, respectively (Loening, 1967). SDS (especially pure grade) was obtained from British Drug Houses, Ltd., Poole, Dorset. All other chemicals were the best grade commercially available.

**Virus growth.** The growth of the ts+ wild-type strain of SFV in suspensions of chick embryo fibroblasts has been described (Kennedy & Burke, 1972). On occasions Sindbis virus (obtained from Dr J. Porterfield, National Institute for Medical Research, Mill Hill, London) was used. The growth and purification of this virus was exactly as described for SFV. In order to radioactively label the virus, isotope was added to the growth medium to 10 µCi/ml, except for [3H]-choline and [14C]-lysine, which were added to 20 µCi/ml and 2.5 µCi/ml, respectively. Isotope was added immediately after adsorption.

**Virus purification.** In order to avoid sedimenting the virus – a procedure likely to cause damage to virus particles – the following method of purification was developed. All manipulations were performed between 3 and 6 °C. Up to 2 l of clarified virus suspension from freshly grown virus (Kennedy & Burke, 1972) was concentrated to less than 30 ml by ultrafiltration (ultrafilter model 6/HFU-1 from Bio-Rad Laboratories, Richmond, California, U.S.A.) and layered on to a 30 ml preformed 20 to 55 ~ (w/v) linear sucrose gradient prepared in 50 mM-tris containing 100 mM-NaCl and 1 mM-EDTA, pH 7-2 (TNE). The gradient was centrifuged at 65000 g from 14 to 16 h, the virus band collected, diluted to about 10 ml with TNE and layered on to a second 35 ml preformed 20 to 55 ~ (w/v) linear sucrose gradient which was centrifuged as before. The virus was collected, dialysed against TNE for 12 to 16 h, concentrated by vacuum dialysis to less than 1 ml and stored at 4 °C. Suspensions of purified virus were used within 4 days of preparation. During the purification of virus for injection into rabbits, pre-immune rabbit serum was added to both sucrose gradients to 0.1%.

**Infectivity and haemagglutination assays.** These assays were performed as described by Kennedy & Burke (1972).
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Purification of virus nucleocapsids from infected cells. Virus nucleocapsids were purified from infected chick embryo cells as described by Acheson & Tamm (1970).

Treatment with enzymes. Suspensions of virus or sub-viral particles were incubated with various enzymes as follows:

**Bromelain.** Virus at a protein concentration of 1 to 2 mg/ml in 0.1 M-tris containing 1 mM-EDTA, 50 mM-NaCl and 50 mM-dithiothreitol (pH 7.2) was incubated at 37 °C for 30 min with 1.5 units/ml of bromelain.

**Phospholipase C.** Virus or bromelain-treated sub-viral particles at a protein concentration of 1 to 2 mg/ml or 450 to 700 µg/ml respectively, in 50 mM-tris containing 1 mM-CaCl₂ (pH 7.8) was incubated at 37 °C for 4 h with 0.16 units/ml of phospholipase C.

**Neuraminidase.** Virus suspension at a protein concentration of 2 to 5 mg/ml in 50 mM-tris containing 100 mM-NaCl and 2 mM-CaCl₂ (pH 6.8) was incubated at 37 °C for 45 min with 0.6 units/ml of neuraminidase.

**Sugar hydrolases.** Neuraminidase-treated virus particles at a protein concentration of 0.5 to 2.5 mg/ml in 50 mM-tris containing 250 mM-NaCl, 1 mM-MgCl₂ and 1 mM-CaCl₂ (pH 7.0) was incubated at 37 °C for 15 min with each of the sugar hydrolases in turn in the following order: α-L-fucosidase (0.8 units/ml), α- and β-galactosidases (both 1.7 units/ml), N-acetyl-β-D-glucosaminidase (1.0 units/ml), α-mannosidase (2.4 units/ml) and finally N-acetyl-β-D-glucosaminidase (1.0 unit/ml).

In all cases controls were submitted to the same treatment in the absence of the appropriate enzyme(s).

**Buoyant density analysis.** Samples for analysis were layered on to 15 ml preformed 25 to 60% (w/v) linear sucrose gradients prepared in 50 mM-tris containing 250 mM-NaCl (pH 7.5). Sedimentation was at 75,000 g for 14 to 16 h at 4 °C in a 6 × 15 ml swing-out rotor. Fractions were collected by upward displacement and a sample of each fraction was taken for radioactivity measurement. The density of alternate samples was determined by refractometry with the aid of a calibration graph.

**Preparation of antisera.** Purified suspensions of either SFV or Sindbis virus (each containing approx. 10¹² p.f.u.) were dialysed for 16 h at 4 °C against 0.05 M-borate containing 0.12 M-NaCl and 0.02% formaldehyde (pH 9.0). Residual formaldehyde was removed by dialysis for 36 h at 4 °C against the same buffer minus formaldehyde and each preparation was concentrated by vacuum dialysis to approx. 1.4 ml. Each preparation was emulsified with an equal vol. of Freund’s complete adjuvant and injected subcutaneously into two male New Zealand rabbits. After approx. 10 weeks each rabbit was injected intravenously with a freshly prepared unemulsified antigen preparation and after a further three days each rabbit was bled. Antisera, together with pre-immune sera prepared from a bleed taken two weeks prior to the initial injection, were adsorbed with kaolin as described by Clarke & Casals (1958), reconstituted by vacuum dialysis to the original serum vol. and stored at -20 °C.

**Gel diffusion.** The agar double-diffusion technique was used; either as described by Crowle (1958), or in 46 mm plastic Petri dishes. Antisera were diluted to 1:8 before use and concanavalin A was used at a concentration of 1 mg/ml in phosphate-buffered saline. Before diffusion all antigen preparations were made 1% in Triton N-101.

**Isoelectric focusing of virus preparations.** Apparatus constructed in this Department was used for isoelectric focusing. The apparatus consisted of six vertically mounted 450 mm × 10 mm precision bore glass tubes arranged concentrically within a 12 cm diam. cylindrical Perspex cooling jacket. In order to minimize ‘wall effects’ the tubes were siliconized (using Siliclad from Becton, Dickinson and Company, New Jersey, U.S.A.). The upper and lower ends of the tubes extended outside the cooling jacket to a distance of 50 mm. The top of
each tube, which was open, passed through a rubber grommet into the upper buffer reservoir. The lower end of each tube was fitted with a side arm and the bottom was threaded to take a Quickfit and Quartz ST8/2T seal. In operation, dialysis membrane was placed over the bottom of each tube and held in place with the seal. The bottom 20 mm of the tubes was then immersed in 50 % (w/v) sucrose plus 2 % (v/v) glycerol and 1 % (v/v) sulphuric acid contained in the lower buffer reservoir. The same sucrose solution was used to fill the tubes up to the level of the side arm. Through the side arm of each tube a sucrose gradient was pumped containing 2 % (v/v) glycerol and 2 % (v/v) ampholines pH 3 to 10 (from LKB Instruments Ltd., Croydon, Surrey) consisting of three parts; firstly a 0 to 20 % (w/v) linear sucrose gradient (17 ml), then the sample in 25 % (w/v) sucrose (3 ml) and finally a 30 to 50 % (w/v) linear sucrose gradient (17 ml). In order to free the sample of salts, the virus preparation was collected by sedimentation for 2 h at 100,000 g, rinsed with 5 ml of distilled water and resuspended in 25 % (w/v) sucrose by gentle ultrasonic agitation. After the tubes had been filled, 1 % (v/v) ethylene diamine was placed in the top reservoir and plastic pipes (4 mm bore) were connected to the side arms and strapped to the apparatus to ensure hydrostatic balance. Focusing was performed at 1000 V for 18 to 24 h during which time the current fell from 1.7 mA/tube to 0.3 mA/tube. After focusing the ethylene diamine was siphoned off and the tubes unloaded by upward displacement. Fractions of 0.7 ml were collected and analysed with respect to pH and radioactivity.

Blocking of virus and sub-viral particle polypeptide sulphydryl groups. Iodoacetamide was used as described by Schlesinger et al. (1972). After blocking samples were dialysed against either 0.04 M-boric acid containing 0.04 M-tris and 0.1 % SDS (pH 8.64) or 0.05 M-phosphate buffer (pH 8.2) containing 0.1 % SDS.

Dansylation of virus polypeptides. Virus polypeptides (0.1 to 2.0 mg protein) either before or after sulphydryl blocking and in 0.05 M-phosphate buffer (pH 8.2) were dansylated as described by Talbot & Yphantis (1970). Unreacted dansyl chloride and other fluorescent side products were removed by chromatography through a 10 × 0.9 cm column of Sephadex G-25 which was eluted with 0.04 M-boric acid containing 0.04 M-tris and 0.1 % SDS (pH 8.64). Dansyl polypeptides were stored for up to 4 weeks in the dark at room temperature.

Polyacrylamide gel electrophoresis. Immediately before electrophoresis samples in 0.04 M-boric acid containing 0.04 M-tris (pH 8.64) were incubated at 100 °C for 2 min in 1 % (w/v) SDS and, if not treated with iodoacetamide, in 1 % (v/v) 2-mercaptoethanol. Samples were made 5 % (w/v) in sucrose, a drop of bromophenol blue solution (0.01 %) was added and samples were layered on to the gels. The sample vol. was 40 to 150 μl containing 10 to 100 μg of protein. A number of gel systems was used, including those of Laemmli (1970), Hay et al. (1968), Neville (1971) and Morser, Kennedy & Burke (1973). Routinely, however, a discontinuous tris-SDS system was used consisting of 10 % (w/v) acrylamide 0.27 % bisacrylamide prepared in 0.375 M-tris-HCl containing 0.05 % SDS (pH 8.4). A stacker gel was not used. The electrophoresis buffer was 0.01 M-tris containing 0.075 M-glycine and 0.01 % SDS (pH 8.4). Gels were cast to a height of 10, 12 or 19 cm in 6 mm internal diam. Perspex tubes and electrophoresis was done for the first hour at ½ mA/gel and thereafter at 1½ mA/gel for up to 14 h. After electrophoresis polypeptides were either eluted from the gel (see below) or the gel was sliced into 0.6 mm segments and each segment was dissolved and counted in a toluene: Triton X-100 scintillator as previously described (Kennedy & Burke, 1972). The same scintillator was used for an aqueous sample counting. All radioactivity values are corrected for background.

Elution of polypeptides from polyacrylamide gel. Virus polypeptides were eluted from
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Effect of enzymes on SFV crushed segments of polyacrylamide gel by continuous mixing at 37 °C for 16 to 18 h in an equal vol. of 0.1 M-tris containing 0.1 % SDS (pH 7.5). Crushed segments of 8 to 10 gels were processed together. Large gel fragments were removed by centrifuging at 2500 g for 15 min and incubated at 37 °C for 4 h in a half vol. of fresh buffer. After centrifuging the supernatant fluid was pooled with that of the first sedimentation and the preparation was further clarified by centrifuging at 12000 g for 30 min followed by filtration through a 0.45 μm pore size cellulose acetate membrane. The filtrate was made 0.05 % in Triton N-101 and dialysed for 5 days against 10 changes of 500 vol. of 50 mM-tris containing 100 mM-NaCl and 0.05 % Triton N-101 (pH 7.4) and then concentrated by vacuum dialysis to less than 0.5 ml. Overall recovery, on a radioactivity basis, ranged from 58 to 70 %.

Protein determination. Protein concentration was determined by the method of Lowry et al. (1951) as modified by Kennedy & Burke (1972).

Sialic acid determination. Sialic acid was measured by the resorcinol reaction (Svennerholm, 1957).

RESULTS

Before attempting to examine the effects of enzymes on SFV it was essential to establish polyacrylamide gel electrophoresis conditions which would resolve the two glycoproteins of our strain of virus. This study was undertaken using dansylated proteins in order to permit continuous monitoring of the polypeptides. A variety of polyacrylamide gel systems were tried, but none gave satisfactory results. However, by combining aspects of several systems, notably (i) blocking of the sulphydryl groups of the virus polypeptides using iodoacetamide, (ii) having a two stage buffer discontinuity between the gels (tris-HCl), the sample (boric acid-tris) and the electrophoresis buffer (tris-glycine) from the start of electrophoresis and (iii) lowering the SDS concentration in the gel to 0.01 %, the separation shown in Fig. 1 was achieved. Analysis of virus labelled with [3H]-glucosamine showed that the resolved polypeptides were both glycoproteins (Fig. 2). For comparison, Fig. 1 also shows the separation of the virus polypeptides obtained using the previous system (Morser et al. 1973). It should be noted that the electrophoretic mobility of the two glycoproteins relative to the core polypeptide was not the same in the two gel systems. The reason for this is unclear but it was a consistent finding and has implications for mol. wt. determinations. In order to confirm that the two resolved polypeptide bands were two distinct glycoproteins, virus was labelled with [14C]-lysine together with one of a number of [3H]-labelled amino acids in turn and the [3H]/[14C] ratio of the two bands was determined. The results, shown in Table 1, confirm that the two resolved polypeptides are discrete species.

Antigenic properties of the glycoproteins. Using the elution procedure described in Methods, the two glycoproteins were recovered from acrylamide gel, checked for purity by re-running on acrylamide gels and their antigenicity tested by gel diffusion against antiserum raised to purified formaldehyde-fixed SFV. The result is shown in Fig. 3 A. Both glycoproteins reacted with the antiserum, forming a continuous precipitin line which joined that produced against Triton-disrupted virus. This result demonstrates that the glycoproteins (i) can be recovered from acrylamide gel in antigenically active form, (ii) are the principal immune determinants of the virus, and (iii) appear to have a common antigenic determinant.

Since both proteins are glycosylated, the possibility that the sugar moiety acts as common determinant was investigated. SFV and Sindbis virus were separately grown in the same host cell system, chick embryo fibroblasts, and purified. Since the host cell is believed to specify the sugar moiety of the glycoproteins (Burge & Strauss, 1970; Strauss, Burge & Darnell, 1970) it was assumed (but not proven) that the two virus preparations would carry the same carbohydrate
Fig. 1. SDS-polyacrylamide gel electrophoresis of the dansyl-labelled polypeptides of purified SFV. Gel A, separation using the extraction procedure and gel system of Morser, Kennedy & Burke (1973). Gel B, separation following sulphhydryl 'blocking' and using the gel system described in Methods. In both gel systems migration is downwards.

Fig. 2. SDS-polyacrylamide gel electrophoresis of the polypeptides of purified SFV. Virus polypeptides labelled with [¹⁴C]-lysine (—) and [³H]-glucosamine (-----) were treated with iodoacetamide and SDS and electrophoresed on a 10% polyacrylamide gel as described in Methods. In this and in all subsequent electrophoretograms migration is towards the right.
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Table 1. The proteins of purified SFV, labelled with $^{14}$C-lysine and another $^3$H-labelled amino acid, were separated by polyacrylamide gel electrophoresis and the radioactivity ratio in the glycoproteins (EP1 and EP2) was determined.

<table>
<thead>
<tr>
<th>Double label $x$: $[^{14}$C$]$-lysine</th>
<th>Radioactivity ratio $[^{3}$H$/[^{14}$C$]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{3}$H$]$-valine</td>
<td>EP1: 1.4</td>
</tr>
<tr>
<td></td>
<td>EP2: 0.8</td>
</tr>
<tr>
<td>$[^{3}$H$]$-leucine</td>
<td>EP1: 0.9</td>
</tr>
<tr>
<td></td>
<td>EP2: 1.5</td>
</tr>
<tr>
<td>$[^{3}$H$]$-methionine</td>
<td>EP1: 0.6</td>
</tr>
<tr>
<td></td>
<td>EP2: 1.1</td>
</tr>
</tbody>
</table>

Fig. 3. Agar double-diffusion of, in A purified SFV and its two glycoproteins, EP1 and EP2 diffused against SFV antiserum (A-SFV), and in B, purified SFV and purified Sindbis virus diffused against SFV antiserum and Sindbis antiserum (A-Si). All antigens were made 1% in Triton N-101 before diffusion. Unlettered wells contained 1% Triton N-101 in phosphate-buffered saline.

groupings. When antisera prepared against these two virus preparations were diffused against disrupted SFV and Sindbis virus, precipitation only occurred between homologous antigen antibody combinations (Fig. 3 B). This result suggests that the carbohydrate moiety of alphavirus glycoproteins is not the major antigenic determinant and, therefore, is unlikely to be the common determinant of the two isolated glycoproteins of SFV. The nature of the common determinant thus remains unclear. Indeed, it should be pointed out that since both glycoproteins have very similar mol. wt. and, therefore, presumably similar diffusion coefficients that the continuous nature of precipitin line shown in Fig. 3 A may be accidental and have no biological significance. Experiments are in hand to further elucidate this point.

Treatment of virus with neuraminidase

The importance of sialic acids in virus-cell interactions has long been recognized (Pepper, 1968; Laver & Valentine, 1969; Hughes, 1973). It would seem possible therefore that the presence of approx. 2000 molecules of sialic acid per SFV particle (Laine, Söderlund & Renkonen, 1973) would have some significance in virus-cell recognition. To test this hypothesis purified SFV was incubated with neuraminidase and the treated particles recovered by
sedimentation through a pad of 40 % (w/v) sucrose, [chemical analysis showed no detectable sialic acid in the treated preparation (less than 0.05 % of the control)]. No significant change in the infectivity or HA activity of the treated particles was observed (control; infectivity of \(5 \times 10^{11}\) p.f.u./ml, HA/ml of \(8 \times 10^6\); treated; infectivity of \(4.8 \times 10^{11}\) p.f.u./ml, HA/ml of \(8 \times 10^6\)) and the treated preparation gave a precipitin line which was continuous with that obtained with the control. These results indicate that sialic acid plays little or no part in the infectivity, antigenicity or HA activity of SFV.

Next, the contribution made by sialic acid to the isoelectric point of the virus was investigated. Treatment with neuraminidase increased the pI of the virus by 0.35 pH units (Fig. 4). The result is in accordance with the removal of the exposed carboxylate ion charge of the sialic acid residues.

Finally, the electrophoretic mobility of the virus glycoproteins before and after neuraminidase treatment was determined. The result (Fig. 4 inset) clearly shows that the mobility of both glycoproteins was unchanged by neuraminidase treatment and indicated that the negative charge of the sialic acid residues does not measurably contribute to the mobility of the glycoproteins in SDS gels.

**Treatment of virus with sugar hydrolases**

In order to further investigate the role of the carbohydrate moieties, particles treated with neuraminidase were incubated with a mixture of sugar hydrolases as described in Methods and the particles recovered by equilibrium banding on sucrose gradients (buoyant
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I.18 g/ml). To check that the bulk of the sugar groups (with the possible exception of the monosaccharide directly linked to the polypeptide chain), had been removed, two approaches were used. Firstly, virus labelled with a mixture of [3H]-glucosamine, [3H]-fucose and [3H]-mannose was treated with the enzymes and the distribution of radioactivity throughout the sucrose gradient was determined. More than 95% of the radioactivity was found in the top three fractions of the gradient and none was present in the region of the gradient containing the treated particles (fractions 20 to 22). Secondly, treated and untreated preparations were diffused through agar against concanavalin A, a phytohaemagglutinin, which has been shown to react with the sugar moiety of the glycoprotein of SFV (Oram et al. 1971). In contrast with the untreated preparation, no precipitin line was observed with the treated sample, confirming that the bulk of the sugar moieties had been removed. Similar results were obtained when virus was treated with the enzymes from the slime mould.

The infectivity of the treated preparation was ~5% of that of the control and the HA activity was less than 1%. These results were complicated, however, by the observation that the treated sample underwent irreversible aggregation in salt solutions of less than about 0.1 M and it was not clear to what extent this aggregation contributed to the decreased infectivity and HA activity of the sample even though the assays were performed in salt solutions of greater than 0.1 M. By contrast to the above properties the treated preparation (in 1% Triton N-101) retained its ability to give a precipitin reaction with SFV antiserum. This finding, together with the antigenic properties of the glycoproteins which have already been described, clearly indicates that it is the polypeptide portions rather than the carbohydrate moieties of the envelope proteins that are the antigenic determinants of SFV.

Since polyacrylamide gel electrophoresis has been used to determine the mol. wt. of SFV glycoprotein(s) (Ranki, 1972; Morser et al. 1973) and since certain glycoproteins behave anomalously on acrylamide gels, it was of interest to determine to what extent the mobilities of the proteins of the treated preparation differed from those of the control. Accordingly, treated particles labelled with [3H]-lysine were mixed with untreated virus labelled with [14C]-lysine and subjected to electrophoresis. The result (Fig. 5) shows that, relative to the core polypeptide, both proteins of the envelope of the treated preparation migrated more rapidly than the glycoproteins of the control sample. This increase in mobility was equivalent to a decrease in mol. wt. of approx. 6000, or 11 to 15% of the reported mol. wt. of the envelope polypeptide(s) (Morser et al. 1973). This decrease is in close agreement with a value of (6.3/44.4) x 100 or 14.2% for the percentage by weight of the protein-bound-carbohydrate of the envelope glycoprotein reported by Laine et al. (1973). This result also suggests that the electrophoretic mobility of SFV glycoproteins is determined by their total mol. wt. and not spuriously affected by the carbohydrate residues.

Treatment of virus with bromelain

A time course of the effects of bromelain on purified SFV is shown in Fig. 6. Infectivity was rapidly destroyed, decreasing to less than 0.5% of the control by 12 min. The HA activity of the virus was also rapidly destroyed. Experiments with caseinase C gave essentially identical results. Indeed it was not possible to define proteolytic conditions (of pH, ionic strength, virus concentration or temperature) which destroyed HA activity without a concomitant loss of infectivity. These results contrast with those of Osterrieth (1966) who demonstrated a loss of HA activity with little or no loss of infectivity. No explanation can be offered to explain these differing results.

Sedimentation of bromelain-treated virus on sucrose equilibrium gradients showed (Fig. 7a) that the treated particles had a buoyant density of 1.13 g/ml compared to a value of
Fig. 5. SDS-polyacrylamide gel electrophoresis of the [\textsuperscript{3}H]-lysine-labelled polypeptides of purified SFV after treatment of virus with neuraminidase and subsequently with a mixture of sugar hydrolases (----). The polypeptides of [\textsuperscript{14}C]-lysine-labelled virus (---) were co-electrophoresed as internal markers.

Fig. 6. Effects of bromelain on purified SFV. Virus was treated with 1·5 units/ml of bromelain and at intervals samples were removed for infectivity (○—○) and HA activity (△—△) assays. An identical sample of virus was incubated in the absence of bromelain and also assayed for infectivity (●—●) and HA activity (▲—▲).
Fig. 7. Buoyant density analysis and polyacrylamide gel electrophoresis of SFV treated with bromelain. [3H]-lysine-labelled purified virus was treated with 1.5 units/ml bromelain and, together with a [14C]-lysine-labelled sample of untreated virus, was analysed, in (a), by centrifuging to equilibrium on a 20 to 65% (w/v) sucrose gradient (untreated, ○; treated, ●) and in (b), by electrophoresis on a 10% polyacrylamide gel (untreated, ---; treated, --). The arrow in (b) indicates the position of the tracker dye.

1.8 g/ml for intact virus. This decrease in buoyant density was investigated by analysing the polypeptide composition of the treated particles. Fig. 7b shows that bromelain treatment completely digests the envelope glycoproteins whilst leaving the core protein intact. Moreover, by terminating electrophoresis before the tracker dye reached the bottom of the gel, an upper mol. wt. limit of 3000 can be placed on any polypeptide fragment derived from the envelope polypeptides which escaped complete digestion.

Since Harrison et al. (1971) showed that the lipid of Sindbis virus completely separates the core protein from the envelope polypeptides, the presence of lipid in the particles treated with bromelain was investigated. Virus labelled with [3H]-choline and [14C]-lysine was treated with bromelain and analysed in toto on sucrose gradients. The result (Fig. 8) demonstrates that all of the [3H]-choline radioactivity was present in the particles treated with bromelain particles, indicating that these particles contained their full complement of lipid. The presence of the lipid layer presumably explains the failure of bromelain to destroy the core
Fig. 8. Buoyant density analysis of SFV treated with bromelain. Purified SFV, labelled with [\(^{3}H\)]-choline and [\(^{14}C\)]-lysine was treated with 1.5 units/ml of bromelain and centrifuged to equilibrium on a 20 to 65 % (w/v) sucrose gradient (\(\blacktriangle\)\(\blacktriangle\), choline and \(\blacktriangle\)\(\blacktriangle\), lysine radioactivity). A sample of untreated virus was analysed on a second gradient (\(\bullet\)\(\bullet\), choline and \(\circ\)\(\circ\), lysine radioactivity).

Fig. 9. Buoyant density analysis of whole virus treated with phospholipase C and sub-viral particles treated with bromelain. Whole virus labelled with [\(^{3}H\)]-choline and [\(^{14}C\)]-lysine (a) and sub-viral particles treated with bromelain (b) were treated with 0.16 units/ml of phospholipase C and centrifuged to equilibrium on 20 to 65 % (w/v) sucrose gradients (\(\blacktriangle\)\(\blacktriangle\), choline, and \(\blacktriangle\)\(\blacktriangle\), lysine radioactivity). Control samples, not treated with phospholipase C, were centrifuged on separate gradients (\(\bullet\)\(\bullet\), choline, and \(\circ\)\(\circ\), lysine radioactivity). The arrow in (b) indicates the position of marker nucleocapsids.
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polypeptide (Fig. 7b) and also explains the decrease in buoyant density of the treated particles compared with both intact virus and isolated nucleocapsids [buoyant density of 1.33 (Kääriäinen, Simons & Von Bonsdorff, 1969; Acheson & Tamm, 1970)]. When particles treated with bromelain were recovered from sucrose not only were they found to have no measurable infectivity or HA activity as anticipated from the results already obtained, but they also completely failed to react with SFV antibody.

Treatment with phospholipase C

Since particles treated with bromelain appeared to contain all the virus lipid, even after prolonged sedimentation, the effect of phospholipase C on both particles treated with bromelain and whole virus was investigated. Treatment of whole virus (labelled with [3H]-choline and [14C]-lysine) had no measurable effect on infectivity or HA activity. However, when analysed on sucrose gradients (Fig. 9a) virus treated with phospholipase C gave a broad, rather diffuse band which, although giving a peak coincident with untreated virus, clearly indicated that the treated particles were more fragile than whole virus. This result suggests a role for the virus lipid in strengthening the virus particle as previously suggested by Friedman & Pastan (1969). When particles treated with bromelain were exposed to the same phospholipase C treatment the result shown in Fig. 9b was obtained. All of the [3H]-choline was found at the top of the gradient and the [14C]-lysine radio activity sedimented to a position coincident with that of nucleocapsids isolated from infected cells.

DISCUSSION

From the results presented in this paper it is clear that the envelope glycoproteins of alphaviruses are the principal determinants of virus infectivity, HA activity and surface antigenicity. However, with the exception of surface antigenicity, which appears to involve both proteins, it is not possible to attribute specific biological properties to one, or other, or both of the glycoproteins. Indeed, it is not clear whether the spike projections of the virus consist of a combination of both glycoproteins or, alternatively, whether there are two types of spike each composed of a single glycoprotein. Attempts in this laboratory to resolve the two glycoproteins in biologically active form (rather than merely antigenically active form) as has been achieved for paramyxoviruses (Scheid & Choppin, 1973) and myxoviruses (Laver & Valentine, 1969) has so far been unsuccessful.

Of the two components of the glycoproteins, it seems that it is the polypeptide portion rather than the sugar residues that determines surface antigenicity and possibly also HA activity and infectivity. Indeed, it appears from the present work that the chief function of the carbohydrate residues is to provide a hydrophilic exterior for the virus particle. However, it should also be considered that the carbohydrate residues may play a role in the intracellular migration of the glycoproteins from the endoplasmic reticulum to the plasma membrane. This idea will be described in more detail elsewhere (R. W. Bingham & S. I. T. Kennedy, unpublished observations).

The results obtained with bromelain are in agreement with those described by Compans (1971) for Sindbis virus and confirm that the integrity of the polypeptide portion of the glycoproteins is essential for the infectivity, HA activity and antigenic properties of alphaviruses. In addition, the experiments with bromelain also indicate that in SFV, as in Sindbis virus, the envelope glycoproteins are located outside the lipid layer and do not pass through it. However, this conclusion should be considered tentative since the digestive action of bromelain may withdraw the glycoprotein from the lipid.
The experiments with phospholipase C demonstrate that although the enzyme can completely digest the lipid layer of bromelain-treated particles, the presence of the glycoproteins appears to largely protect the lipid from digestion. This, in turn, suggests that the glycoproteins are closely juxtaposed on the surface of the virus particle.

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REFERENCES


Effect of enzymes on SFV


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