Characteristics of Vesicular Stomatitis Virus
Envelopes Released with Saponin

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SUMMARY

The treatment of vesicular stomatitis virus with 1 % saponin induced the release of envelopes from inner virus structures. The density of treated virus particles in CsCl increased from 1.22 to 1.23 g/ml, probably owing to loss of lipids. The envelopes could be removed from the nucleoprotein helix and further purified by repeatedly ultracentrifuging in sucrose gradients. The haemagglutinating activity of the envelopes was retained. The purified envelopes had sedimentation coefficients of 380 to 400S and a density of 1.20 g/ml. Two proteins, glycoprotein G and large protein L, were dominant in polyacrylamide gel electrophoresis of purified envelopes. In electron microscopy small amounts of ribonucleoprotein N were also seen as free, unwound ribbons, close to the envelopes.

INTRODUCTION

There is general agreement (Wagner et al. 1972) on the main structural features of vesicular stomatitis virus (VSV). The purified virus particles contain three major protein components, G (glycoprotein), N (nucleoprotein) and M (matrix protein), with mol. wt. of 69,000, 50,000 and 29,000, respectively. One minor component, L (large protein), is found constantly in VSV preparations. Because of its great mol. wt. of 100,000 to 300,000, it has been suggested that it is an aggregate (Wagner & Schnaitman, 1970). However, it does not dissociate even after extraction with 8 M-urea. Recently, however, the L component has been shown to be a real protein peculiar to most animal viruses (Stampfer & Baltimore, 1973). It differs greatly from the other proteins of VSV in tryptic peptides.

Since the first demonstration of haemagglutination by VSV (Halonen et al. 1968) we have studied the relationship of haemagglutinin to virus morphology (Arstila, 1972, 1973). Haemagglutination is a property of the surface projections of the virus envelope, which are formed from the only glycoprotein found in VSV (Cartwright, Talbot & Brown, 1970). The surface projections can be removed with Nonidet P40 without loss of haemagglutinating activity. The same effect can be produced in another rhabdovirus, rabies (Schneider, Horzinek & Novicky, 1971), with the surface-active plant glycoside, saponin. This report describes a different effect of saponin on VSV, in which saponin removes whole envelopes. The envelopes were analysed and found to consist of the large protein component L in addition to the glycoprotein G forming the surface projections.
METHODS

The methods used have been described in earlier reports (Arstila, Halonen & Salmi, 1969; Arstila, 1972, 1973) and only a brief outline will be given here.

**Purification of vesicular stomatitis virus.** VSV, Indiana serotype, was grown in a suspension culture of BHK 21/13S cells, with an input multiplicity of 0.001 p.f.u./cell. The cells were maintained in BHK 21 medium with 0.4% bovine albumin, 10% tryptose phosphate broth, and no serum (Arstila et al. 1969). The extracellular virus was harvested after 40 h and then precipitated with polyethylene glycol 6000 (Arstila 1972). The precipitate, rinsed and dissolved in 0.05 M-tris-HCl buffer (TES), pH 7.4, containing 1 mM-EDTA and 0.15 M-NaCl, was further centrifuged at 20000 rev/min for 60 min in the Spinco 30 rotor, and the pellet was resuspended in TES. After sonic treatment, the virus was centrifuged in a 15 to 45% (w/w) sucrose gradient for 2 h at 20000 rev/min in the Spinco SW 25 rotor. The fractions containing virus particles, as measured by haemagglutination, were collected, diluted to contain 5% sucrose and finally pelleted by ultracentrifugation. The pellets were suspended in TES, to 1/100 of the original vol., and stored at 4 °C after sonic treatment.

**Titration of infectivity, haemagglutinating and complement-fixing activity.** Haemagglutination was measured by a microtitre technique with 0.4% bovine albumin borate saline as diluent, and a 0.25% suspension of goose erythrocytes, the final pH was 5.8, and incubation was at ice-water temperature (Halonen et al. 1968). The titres are expressed as the reciprocals of the highest virus dilutions per 0.05 ml showing complete or almost complete haemagglutination.

In complement fixation tests, antigens were titrated with optimal dilutions of anti-VSV rabbit immune serum (Arstila et al. 1969) and with two haemolytic units of complement. The amount of antibody was determined by box titration with the seed virus preparation of VSV (Arstila, 1972). The reciprocal of highest antigen dilution per 0.025 ml causing complete or almost complete fixation with immune serum was taken as the complement fixation titre.

The infective virus was measured by plaque titration in agarose suspensions of BHK 21/13S cells with BME diploid medium containing 5% calf serum (Arstila, 1972).

**Electron microscopy.** A standard negative staining method was used with 2% phosphotungstic acid (PTA) at pH 7.0. Samples on formvar-coated grids were examined in a Siemens Elmskope IA, at an instrumental magnification of 40000 and at 60 kV.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis was performed according to Maizel (1969). The samples in 1% SDS and 2-mercaptoethanol (2-ME) were heated at 100 °C for 2 min, and dialysed against a 0.01 M-phosphate buffer, pH 7.0, with 0.1% SDS and 2-ME. The acrylamide gel columns contained 5% acrylamide and 0.1% SDS. The electrode buffer was 0.01 M phosphate buffer, pH 7.0, with 0.1% SDS. Before the sample was added, the gels were run for 2 h with the electrode buffer alone for stabilization. The samples (0.2 ml with 0.05 ml of 60% sucrose and 0.05 ml of 0.01% bromphenol blue) were run for about 150 min at 8 mA/gel. Polypeptide bands were fixed overnight with 20% sulphosalicylic acid and stained with Coomassie brilliant blue.

A preparation of whole VSV, and bovine serum albumin, fraction V (Armour Pharmaceutical Company, Kankakee, Illinois) were used as reference substances. All the samples in each electrophoretic run were adjusted to contain the same concentration of protein, determined according to Lowry’s method (Lowry et al. 1951).

**Estimation of sedimentation coefficients.** Approximate S values were estimated by sedimentation in a linear 5 to 20% (w/w) sucrose gradient for 60 min at 15000 rev/min.
Vesicular stomatitis virus envelopes

in the Spinco SW 27·1 rotor, with poliovirus as reference. For this, poliovirus type 1 (160S) was grown in LLC-MK2 cells and labelled with 5 μCi/ml of [5-3H]-uridine (Radiochemical Centre, Amersham, England). The polio virus was purified in a CsCl density gradient and mixed with VSV before sedimentation. The fractions were titrated for VSV haemagglutination and counted in a liquid scintillation counter (LKB Wallac 81,000, Turku, Finland) for 3H radioactivity of the poliovirus.

Chemical treatments. Saponin ('white, pure'. E. Merck AG., Darmstadt, Germany) was diluted in a TES buffer, pH 8·2, to a 1% solution and stored at 4 °C for not more than 1 week. The standard treatment was as follows: 2 parts of virus were mixed with 1 part of 1% (w/v) saponin in a TES buffer, pH 8·2, and shaken occasionally at room temperature for 20 min.

For ribonuclease (RNase) treatments the enzyme (ribonuclease, 5 x crystallized, bovine pancreas, salt-free, A grade, 48 Kunitz units/mg, Calbiochem, Los Angeles) was diluted in TES, pH 7·4, to 1 mg/ml. For treatment, the samples were mixed with an equal vol. of ribonuclease solution and incubated for 1 h at 37 °C.

RESULTS

The effect of saponin treatment on VSV haemagglutination

Purified and concentrated preparations of the virus were mixed with saponin in different concentrations and conditions, the aim being to find the treatment giving highest haemagglutinating activity for VSV, as reported for rabies virus (Schneider et al. 1970). Saponin was diluted serially twofold from 2% (w/v) to about 0·03% and incubated with an equal vol. of VSV for 20 min at 22 °C or for 60 min at 4 °C, 22 °C or 37 °C.

Haemagglutinating activity remained equal to or slightly lower than those of controls in all conditions tested with saponin concentrations under 0·5%. Higher concentrations of saponin haemolysed erythrocytes.

The effect of saponin treatment on VSV haemagglutinins

Although saponin seemed to have little influence on VSV haemagglutination, rate-zonal sedimentation of the treated virus showed a remarkable change in the VSV bands (Fig. 1). After sedimentation for 2 h at 20000 rev/min in the Spinco SW 25 rotor, there were two close bands in a 15 to 45% (w/w) sucrose gradient, and closer to the bottom of the tube than the bands for untreated control virus. Treatment of the virus with 3% (w/v) 1% or 0·5% saponin for 20 min at room temperature or 60 min at 4 °C all produced the same effect on the light-scattering bands. An increase of the pH of treatment from 7·4 to 8·2 did not affect the HA patterns in the gradient.

In titrations of haemagglutination and complement fixation the two bands could not be differentiated (Fig. 2), but the haemagglutinating activity of VSV was spread over the dense region of the gradient. Saponin also enhanced the release of a small haemagglutinin of the type released spontaneously (Arstila, 1973). In addition, saponin removed to the top of the gradient some material with complement-fixing activity but no haemagglutinating activity.

In electron microscopy the striking effect of saponin treatment was to release VSV envelopes from the nucleoprotein helix. The denser band from the gradient contained particles with partly detached envelopes, but also many particles of normal appearance.

In polyacrylamide gel electrophoresis of the saponin-treated virus, pelleted from the denser band in the sucrose gradient, all four polypeptides of VSV were found.

On repetition of the treatment on the saponin-treated virus pelleted from a sucrose
Fig. 1. Light-scattering bands of saponin-treated (a) and control (b) preparations of vesicular stomatitis virus in 15 to 45% sucrose gradient. 1.5 ml of purified virus was mixed with 0.75 ml of 1% saponin, pH 8.2, and incubated for 20 min at room temperature, then centrifuged for 2 h at 20000 rev/min in the Spinco SW 25 rotor.

Fig. 2. Sedimentation analysis of saponin-treated and control vesicular stomatitis virus in 15 to 45% sucrose gradient after sedimentation for 2 h at 20000 rev/min in the Spinco SW 25 rotor. Prior to sedimentation purified vesicular stomatitis virus was treated with saponin (---) or as control with TES-buffer alone (- - - - - - -). Fractions were titrated for haemagglutination (HA) (a) and complement fixation (CF) (b), and measured for extinction (E) (c). The bottom of the tube is to the left.

gradient, no difference in the distribution of haemagglutinins was found after the second sucrose gradient sedimentation.

The sedimentation used after saponin treatment of VSV (15 to 45% (w/w) sucrose gradient, 2 h, 20000 rev/min and SW 25) is known to separate particles of the size of VSV partly according to density (Sokol, Clark & György, 1972). The S values of the particles were estimated therefore in a 5 to 20% (w/w) sucrose gradient with radioactively-labelled
poliovirus as reference. A sedimentation coefficient of 560 to 580S was calculated for the peak of haemagglutinating activity.

In a CsCl gradient, haemagglutinating activity after saponin treatment banded at a density of 1.23 g/ml, whereas the control virus banded at a density of 1.22 g/ml. The sedimentation in the Spinco SW 50·1 rotor was at 40000 rev/min for 18 h in a preformed linear CsCl gradient of 1.15 to 1.30 g/ml.

The infectivity of the saponin-treated VSV decreased greatly to $2.5 \times 10^6$ p.f.u./mg of protein, as compared with the control value of $2.0 \times 10^9$ p.f.u./mg, however, all infectivity was not lost.

**Purification of VSV envelopes isolated with saponin**

Because different conditions of saponin treatment had no significant effect, the simplest procedure was adopted for further experiments (2 parts of 100× concentrated, purified VSV, mixed with 1 part of 1% saponin in TES, pH 8.2, shaken for 20 min at 22 °C).

Envelopes of VSV sufficiently pure for further analysis were isolated from the saponin-treated material in the denser light-scattering band of sucrose gradient (Fig. 1) as follows. The material was first pelleted at 20000 rev/min for 60 min in the Spinco 30 rotor, then recentrifuged in a 10 to 30% (w/w) sucrose gradient for 60 min at 20000 rev/min in the Spinco SW 27 rotor and again pelleted. Each pellet was sonically disforced, then frozen
and thawed three times. This material no longer showed intact ribonucleoprotein helices, called 'skeletons' by Cartwright et al. (1970), but free, unwound ribbons of ribonucleoprotein. The free ribonucleic acid was removed by treating the pellet with 500 µg/ml of ribonuclease for 60 min at 37 °C and then centrifuged in a 10 to 30 % sucrose gradient. The final, pelleted material containing purified envelopes was examined in gradient sedimentations, electron microscopy and polyacrylamide gel electrophoresis.

Analyses of purified VSV envelopes isolated with saponin

In a 5 to 20 % (w/w) sucrose gradient the purified envelopes showed sedimentation coefficients of about 380 to 400S for the peak of haemagglutinating activity. In a CsCl density gradient the peak was found at a density of 1:20 g/ml after 45 h of sedimentation at 35000 rev/min in the Spinco SW 50-1 rotor.

In electron microscopy the purified preparation consisted entirely of envelopes with some free unwound ribonucleoprotein ribbons (Fig. 3). Virus particles or skeletons were not seen. The envelopes were rounded, swollen particles formed of one electron-translucent layer with surface projections.

Polyacrylamide gel electrophoresis of the envelope preparation seen in Fig. 3 showed total loss of the matrix protein M (Fig. 4) which maintain the form of the skeletons (Cartwright et al. 1970). Polypeptides corresponding to proteins L and G were dominant. The preparation still contained N protein, but the proportion was diminished.

No infectivity was found in the purified envelope preparation.

Fig. 4. Polyacrylamide gel electrophoresis of the same purified saponin-treated haemagglutinin of vesicular stomatitis virus as seen in Fig. 3. Preparations were dissociated with 1 % SDS and 1 % 2-mercapto-ethanol at 100 °C for 2 min and subjected to electrophoresis in 5 % polyacrylamide gel at a current of 8 mA/gel. The identity of the polypeptides in each preparation was demonstrated by mixing the envelope preparation (a) with the control virus preparation (b) and the electrophoretic pattern of the mixture is seen in the third column (c). L, large protein; G, glycoprotein; N, nucleoprotein; M, matrix protein.
**DISCUSSION**

In the present experiments saponin treatment was found to cause release of VSV envelopes from inner structures. The effect was exerted over rather wide ranges of pH, time, temperature and concentration. A distinct difference between rabies and VSV was that no coiled filament, such as Schneider *et al.* (1971) described in rabies preparations, could be found after treating VSV with saponin. Saponin enhanced the release of the small haemagglutinins of VSV, but they resembled those seen after disintegration of virus particles, consisting of fragments of virus particles with surface structures (Arstila, 1973). In a CsCl gradient no haemagglutinin was found at the approximate density of proteins, 1.29 g/ml, where the haemagglutinin of rabies was found after release with saponin.

Saponin-treated VSV was found to be slightly denser in sucrose or CsCl than untreated particles. Sokol *et al.* (1972) recently reported that during normal laboratory processing, the morphology of rabies is changed in a way similar to that described here. This was due to loss of phospholipids from the virus envelope. Electrophoretic analysis of crude saponin-treated VSV particles, in polyacrylamide gels showed that no virus polypeptides were lost. Therefore, it seems probable that the release of VSV envelopes is based on the same phenomenon as was described by Sokol *et al.* (1972) with rabies: phospholipids are removed from VSV by the lipophilic action of saponin.

The purified envelope preparation was found to contain only three virus-specific proteins, L, G and N, the proportion of N being decreased. Small amounts of N were seen in electron micrographs in the form of free, unwound nucleoprotein ribbons. Attempts to separate ribonucleoprotein from the virus envelopes by density gradient sedimentation in CsCl were unsuccessful.

The suggestion of Cartwright *et al.* (1970) that M keeps the skeletons fixed is supported by our finding of total loss of M in these preparations. The presence of G, the protein forming the surface projections, is in accordance with the findings in electron microscopy.

The same problem of contaminating N protein was encountered earlier (Arstila, 1973). It may partly be due to the high proportion of N in the virus particle (Cartwright *et al.* 1972). Cartwright, Smale & Brown (1969) have suggested that the arrangement of the surface spikes is determined by the nucleoprotein helix, to which the spikes might even be attached.

Cartwright *et al.* (1970) have found only one protein component, N from nucleoprotein ribbons. Also from the ultrastructure of the envelopes isolated with saponin it may be assumed that the protein N found in gel electrophoresis is derived from ribonucleoprotein ribbons and the protein G from the surface projections. If this is true, it leaves only the large protein L to form the possible framework of the envelope, to which lipids and surface projections are attached. A constant finding of L protein in VSV preparations and its presence in saponin-isolated envelopes even after several laboratory treatments support the idea of the real existence of L protein (Stampfer & Baltimore, 1973). Biologically it may act as a RNA transcriptase (Emerson & Wagner, 1973), but hitherto, its morphological role in VSV has not been known. These results seem to suggest that it forms at least part of the electron-translucent envelope of the virus.

Saponin treatment of VSV seems to offer a method for removing the envelopes, which after purification retain their haemagglutinating activity while losing their infectivity.
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REFERENCES


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