

Separation of Alphavirus Nucleocapsids from Envelope Fragments

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Methods for the preparation of alphavirus nucleocapsids and envelope fragments for biochemical, electron microscopical and serological investigations are described. Difficulties which have been encountered previously in attempts to isolate viral nucleocapsids for morphological studies, namely disruption of the fragile particles, have been overcome by treatment with formaldehyde.

Introduction

Members of the genus alphavirus within the family Togaviruses are spherical enveloped RNA viruses. The structure of alphaviruses has been investigated by several workers: The virions are composed of two glycoproteins in the viral membrane (E1 and E2) and of one nucleocapsid protein (C) within the range of 50 000 (E1 and E2) and 30 000 (C) daltons, respectively (for review see Mussgay *et al.* [1]; Strauss and Strauss [2]). A further glycoprotein (E3) only found in Semliki Forest virions has a molecular weight of about 10 000 daltons [3]. Chemical analysis of Semliki Forest virus yielded about 250 molecules of the C-protein in the virion; there was reported to be good evidence in both Sindbis and Semliki Forest viruses for an equimolar ratio of the envelope proteins E1 and E2 and C [4, 5]. The nucleocapsids of alphaviruses can be isolated by treatment of the virions with deoxycholate [6, 7] or with Triton X-100 [3] but in no case exact electron microscopic investigations were performed since the isolated nucleocapsids are very fragile. We have developed a simple method for separation of membrane fragments from viral nucleocapsids for electron microscopical, biochemical and serological analysis.

Materials and Methods

Viruses

The origin of Sindbis virus and Semliki Forest virus was described by Mussgay and Rott [8] and

Mussgay *et al.* [9], respectively. Both viruses were plaque-purified, adapted to BHK-cells and were propagated in roller cultures of BHK-21 cells.

Purification of viruses

The culture medium was collected about 20 h after infection and clarified by low-speed centrifugation. The supernatant fluids were spun at 19 000 rpm for 3 h in a rotor 19 of a Spinco model L3 ultracentrifuge. The pellets were resuspended in TEN-buffer as described [9]. The concentrated virus was applied to a 5–30% (w/w) linear sucrose gradient and centrifuged at 25 000 rpm in a SW 25 rotor for 90 minutes. The opalescent band was collected, diluted to twice the volume with TEN-buffer, spun at $200\,000 \times g$ for 30 minutes and resuspended in TEN-buffer. This final virus suspension contained about 10^{12} PFU per ml and was the starting material for the core-preparation. Further purification was achieved in a linear 20–60% (w/w) sucrose gradient in TEN-buffer.

Labeling of viruses

For labeling of viral proteins the infected cells were incubated with a mixture of VM3A and Eagle medium [10] containing $10 \mu\text{Ci/ml}$ [^{35}S]methionine (Buchler, Braunschweig). The labeling period was for 12–16 h. Radioactive virus was concentrated and purified as described above.

Preparation of viral nucleocapsids

Concentrated and purified Sindbis and Semliki Forest viruses were layered onto a freshly prepared density gradient in steps from 5–60% (w/w) sucrose

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in TEN-buffer. The layer of 5% sucrose contained 2% (v/v) Triton X-100. The layer of 20% sucrose contained 0.5% (v/v) formaldehyde for preparation of Sindbis virus nucleocapsids and 0.1% (v/v) formaldehyde for preparation of Semliki Forest virus nucleocapsids. The pH of the formaldehyde containing sucrose solution was adjusted to 8.5. Centrifugation was performed in a SW 41 rotor for 30

minutes at 10000 rpm and then for 18 h at 39000 rpm. Fractions were collected from the bottom of the tube. The refractive index of each fraction was determined using an Abbe 3L refractometer. Aliquots (50 μ l) of each fraction were spotted on Whatman glass fibre paper (GF/C) and precipitated with 10% trichloroacetic acid and examined for radioactivity by liquid scintillation counting.

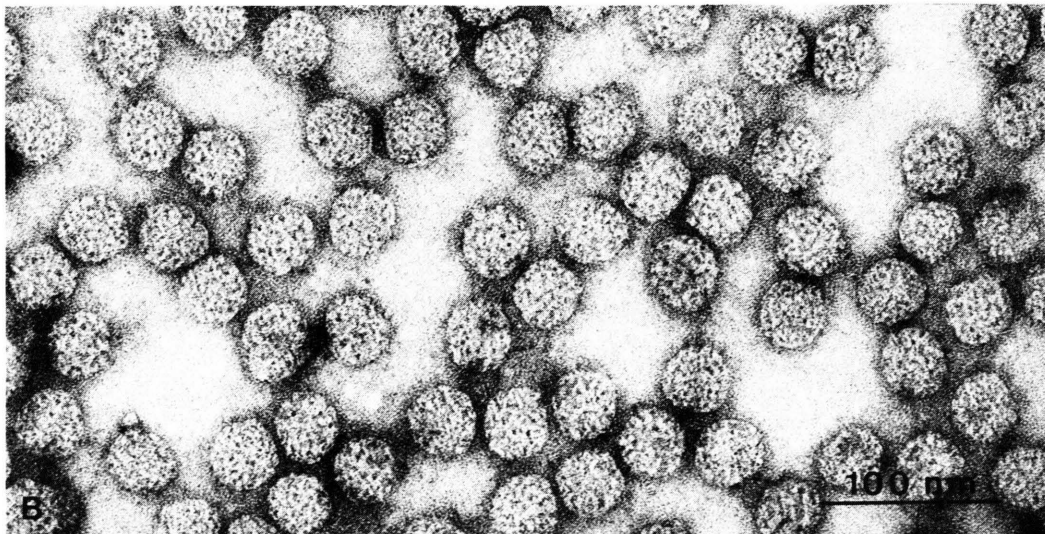
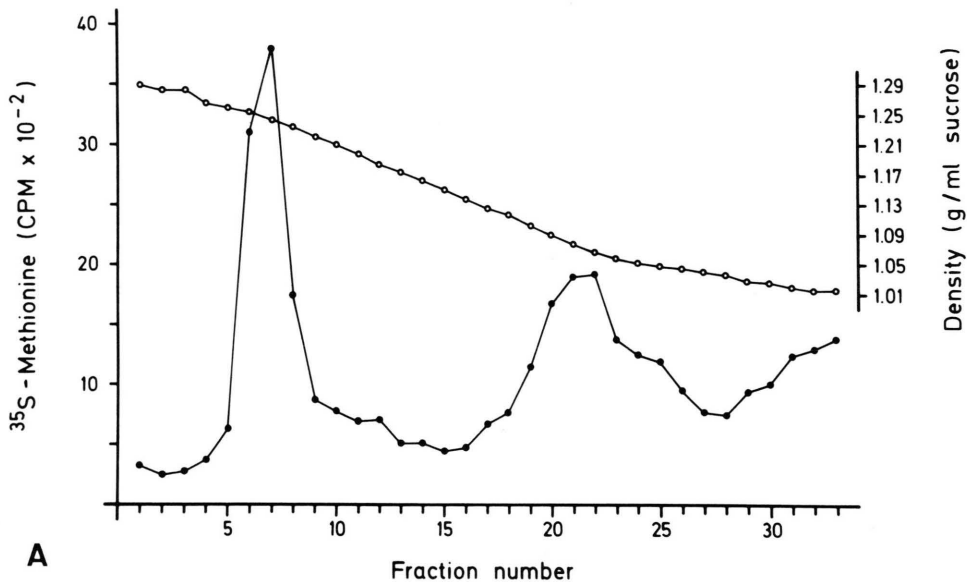


Fig. 1. Separation of viral cores and membrane fragments. A: Distribution of radioactivity after density gradient centrifugation of Semliki Forest virus treated with Triton X-100. B: Sindbis virus cores uncoated with Triton X-100 followed by density gradient centrifugation. Triton X-100 was present in the layer of 5% sucrose. Formaldehyde (0.5%) was included in the layer of 20% sucrose. The viral cores were recovered at a density of 1.24 g/cm³.

Polyacrylamide gel electrophoresis (PAGE)

PAGE of viral proteins (not treated with formaldehyde) was performed as previously described [10]. In several experiments a discontinuous system similar to that described by Laemmli [11] was used. The electrode buffer consisted of 0.05 M Tris, 0.38 M glycine (pH 8.5), 0.1% SDS and 0.1% 2-mercaptoethanol. Optimal separation of viral proteins in both electrophoretic systems was achieved by using a SDS-mixture (MCB-Pierce) described earlier [12]. Molecular weights were estimated by the procedure of Shapiro *et al.* [13]. Radioactive labeled proteins were mixed with unlabeled proteins of defined molecular weights, the position of which act as highly reproducible internal markers in the gels. After destaining gels were fractionated and counted as described earlier [14].

Electron microscopic preparation

For electron microscopic observation the visible band of viral nucleocapsids after density gradient centrifugation was removed. Sindbis virus nucleocapsids from the gradient were diluted 10 times with TEN-buffer and concentrated by sedimentation at $200\,000 \times g$ for 1 h. Semliki Forest virus nucleocapsids were diluted ten times with TEN-buffer containing 0.5% formaldehyde and were then allowed to stand for two hours at 20 °C. This material was concentrated by sedimentation through a cushion of 20% sucrose in TEN-buffer. The pellets were resuspended with TEN-buffer. Negative staining of the particles was carried out with 1% unbuffered solution of uranyl acetate or with phosphotungstic acid (2 per cent) at pH 6.8. Specimens were observed with a Siemens Elmiskop 102 at 80 kV and a nominal magnification of 60 000.

Results

Separation of virus cores and membrane fragments

Preparation of Sindbis and Semliki Forest virus cores and membrane fragments was achieved by treatment of purified virus with Triton X-100 followed by density gradient centrifugation. The pH of the Triton containing sucrose solution had to be adjusted to 8.5. Fig. 1A shows the sedimentation profile of radioactive labeled and uncoated Semliki

Forest virus as an example. A negatively stained preparation of Sindbis virus nucleocapsids is shown in Fig. 1B. The viral cores banded at a density region of 1.24 g/cm³. The membrane fractions were recovered in the region of 1.05 to 1.09 g/cm³.

Polyacrylamide gel electrophoresis

Intact virions and fractions obtained after sedimentation of uncoated virus in the described sucrose gradient but without formaldehyde were analyzed in PAGE. Fig. 2 demonstrates the results using a continuous SDS-phosphate-buffer system. Three peaks (E1, E2, C) were observed when Sindbis virus was subjected to electrophoresis (Fig. 2A), whereas only two peaks (E1 + E2, and C) were resolved if Semliki Forest virus was electrophoresed, because the two membrane proteins, E1 and E2, revealed the same relative electrophoretic mobility. After electrophoresis of fractions containing the membrane (Fig. 2B, E) and the core components (Fig. 2C, F), respectively, it was shown that the membrane proteins as well as the core proteins migrated at identical positions as the proteins of the corresponding whole viruses. Only little amounts of the core protein were detected in the membrane fraction. The viral cores obtained from the density region of 1.24 g/cm³ contained only traces of the membrane proteins. After electrophoresis of Semliki Forest virus in a discontinuous gel system both membrane proteins could be resolved (Fig. 3).

Molecular weights of viral proteins

Molecular weight estimation of the polypeptides performed for identification of the viral proteins for both viruses in the two different electrophoretic systems are tabulated in Table I. These values reflect the mean obtained from several determinations in both gel systems except for the membrane proteins of Semliki Forest virus which were only determined in the discontinuous system.

Table I. Molecular weights of Sindbis virus and Semliki Forest virus proteins.

	Sindbis virus	Semliki Forest virus
E ₁	53 000	51 000
E ₂	48 000	49 000
C	30 000	32 000

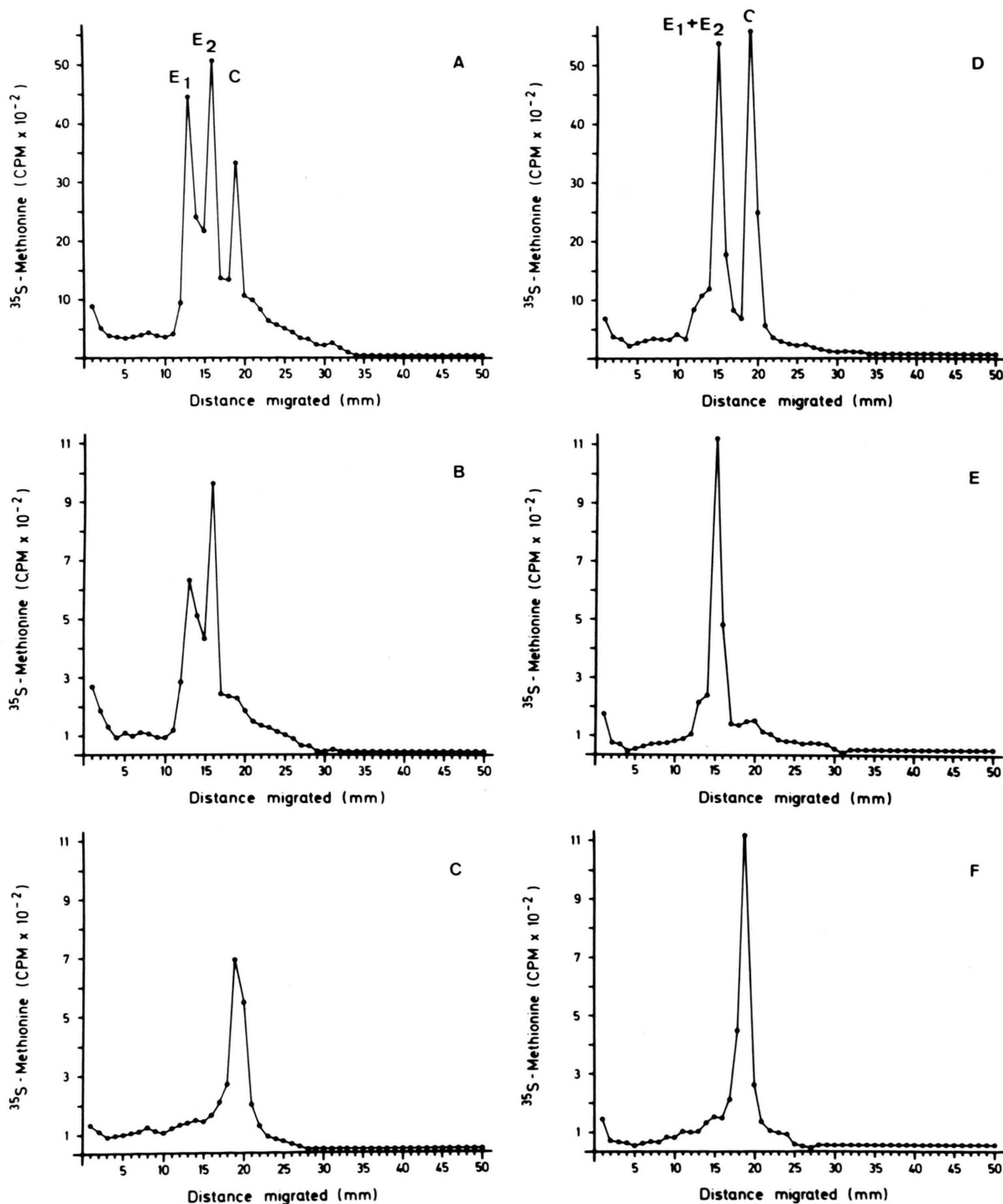


Fig. 2. PAGE of alphavirus proteins. Purified Sindbis (A) and Semliki Forest (D) viruses were disrupted with SDS and electrophoresed in polyacrylamide gels using a continuous system. Membrane proteins (E_1 and E_2) of Sindbis virus (B) and Semliki Forest Virus (E) were found in the density region 1.05 to 1.09 g/cm³ of the gradient shown for example in Fig. 1 A. Core protein of Sindbis virus (C) and Semliki Forest Virus (F) from the density region 1.24 g/cm³.

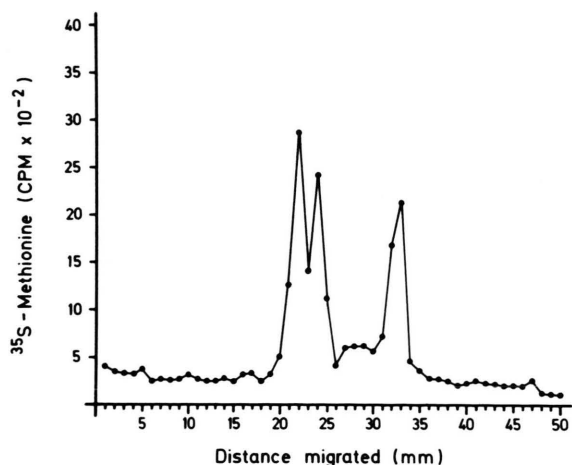


Fig. 3. PAGE of Semliki Forest virus proteins. Purified Semliki Forest virus was disrupted with SDS and electrophoresed in polyacrylamide gel using a discontinuous system. The envelope proteins E1 and E2 were resolved.

Discussion

The results presented demonstrate the preparation of subviral particles from enveloped viruses for electron microscopical, biochemical and possibly serological investigations. The structural relationship of protein complex extracted by non-ionic detergents could be correlated to the whole virus. The lipoprotein coat of the Alphaviruses Sindbis and Semliki Forest was released by treatment of purified virions with Triton X-100 followed by separation of the viral components in a 5–60% sucrose gradient. The relative low density of viral nucleocapsids (1.24 g/cm³) compared with that of real nucleoproteins (in the range of 1.3 to 1.4 g/cm³) may be explained by detergent molecules which bind to hydrophobic

regions of the nucleocapsid protein instead of lipids from the viral envelope. In order to achieve minimal destruction of nucleocapsids for electron microscopical studies formaldehyde was included into the layer of 20% sucrose in the density gradient. Optimal concentration of formaldehyde has to be tested for each virus. For example, 0.5% formaldehyde were used in the case of Sindbis virus, whereas only 0.1% were suitable for Semliki Forest virus, otherwise aggregation of nucleocapsids occurred. The distribution of viral components (not treated with formaldehyde) in the gradient could be shown by polyacrylamide gel electrophoresis. As it was shown in Fig. 2 a good separation of membrane and core components was achieved.

By using a continuous standard system of electrophoresis it was not possible to resolve the membrane proteins of Semliki Forest virus (Fig. 2D, E). The electrophoretic mobility of these proteins was different in discontinuous buffer system as it was shown in Fig. 3. The molecular weights of viral proteins were determined in both electrophoretic systems for identification (Table I). The data for molecular weights cited in literature for Sindbis virus vary considerably. Sindbis virus envelope proteins were determined to have molecular weights varying from 45 000 to 63 000 daltons (for review see refs. [1, 2, 15, 16]). These variations may be due to microheterogeneity in the carbohydrate moiety of the glycoproteins.

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