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Purification and analysis of infectious virions and native non-structural antigens from cells infected with tick-borne encephalitis virus

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SUMMARY

By employing the techniques of column chromatography and membrane filtration, we have succeeded in purifying flavivirus particles with low particle to infectivity ratios and free from contamination with cellular proteins. Virus particles purified by this method have an average diameter of 53 nm, a particle to infectivity ratio of less than 10, and a K_D (partition coefficient) consistent with a molecular weight of $2.63 \cdot 10^7$. In addition it has been possible to purify the extracellular form of non-structural protein 1 (NS1), which in its native form appears to be a hexamer. It is also apparent from these studies that the slowly sedimenting haemagglutinin particle (or SHA) is an artifact of purification methods using gradient centrifugation. This technology should not only prove useful in the laboratory for studying the detailed structure of these viruses and the proteins encoded by them, but should also prove useful in industrial vaccine manufacture where large volumes of highly pathogenic material are handled.

INTRODUCTION

Flaviviruses cause some of the most important infectious diseases of man; the most widespread being yellow fever and dengue fever. In addition tick-borne encephalitis (TBE) is a significant public health problem in the Soviet Union and many eastern and central european countries. Japanese encephalitis is also widespread in China and the far east[1]. Although there are successful vaccines against some flaviviruses, research on the development of new and improved vaccines has been hampered by difficulties in purifying intact and infectious particles by conventional techniques. Moreover, research on the physical structure of these viruses and their extracellular

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antigens has also been hampered as conventional methods of purification and concentration such as gradient centrifugation and pelleting either distort or disrupt the virus particle, resulting in high particle to infectivity ratios[2].

The procedure described here involves ultrafiltration and gel exclusion chromatography to purify and concentrate viral particles and the major non-structural antigen NS1, whereby non-physiological conditions such as high sucrose concentrations, pellet formation, high salt and pH changes are avoided. These all can and do lead to irreversible changes in the integrity of virus particles and the structure of viral proteins.

A further advantage of this technology is that it is more readily adapted to industrial scale up than is ultracentrifugation. At present several vaccines for human use including that against TBE, use ultracentrifugation to purify the product[3,4]. For the best results it is necessary to handle large amounts of pathogenic material in the centrifuge and if the machine fails, containment of infectious aerosols is difficult and costly. Although some pressurisation occurs during gel chromatography and ultrafiltration these forces are much lower than those generated in a centrifuge and thus containment is easier. Apart from the problems discussed above, some flaviviruses grow to low titres and thus need extensive concentration. In addition cells infected with these viruses overproduce convoluted tubules, and as these tubules are of a similar size and density to virus particles they copurify with them when conventional procedures are used.

We report here the application of these techniques to the purification of flaviviruses which, in the past, have proved to be particularly difficult to prepare. The procedure reported here not only produces intact, pure and infectious virions and extracellular antigens, but is readily adaptable to the high containment conditions necessary for handling pathogenic viruses under laboratory and industrial conditions.

MATERIALS AND METHODS

Virus growth

Seed stocks of the Neudorfl isolate of Central European TBE was grown in suckling mouse brain as previously described[5]. For each batch of virus $3 \cdot 10^{10}$ PS cells were grown in roller culture and infected at a multiplicity of infection of 10. After 42 h in serum-free L15 medium, the supernatant was removed from the cultures and clarified by centrifugation at 2000 *g* and 4°C for 10 min and then at 10 000 *g* and 4°C for 10 min. The resultant supernatant was then made 0.01% (w/v) with respect to sodium azide; and aprotinin was added to a final concentration of 10 units per ml. All further procedures were performed at 4°C.

Virus concentration

Clarified supernatants (2-l samples) were concentrated 10-fold on Minitan PTHK OMT05 filters (molecular weight cut-off, 100 000), and diluted ten times in pH 8.0 phosphate buffer [$Na_2HPO_4 \cdot 12H_2O$ (0.0185 *M*)–K_2HPO_4 (0.0013 *M*)–NaCl (0.13 *M*)]. After a second 10-fold concentration, the virus was diluted 10 times again in phosphate buffer and finally concentrated to 30 ml for column chromatography.

Column chromatography of infected cell supernatants

Aliquots of about 20 ml of clarified virus concentrate were applied to a column (69.5 cm × 2.2 cm I.D.) of Sephacryl S300 at 4°C in phosphate buffer (as defined for virus concentration) at a flow-rate of 72 ml/h. The eluate was monitored at $O.D._{280}$ using an LKB Uvicord S UV monitor and 3-ml fractions were collected. Aliquots for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were immediately boiled for 1 min in electrophoresis sample buffer and stored at −20°C. Those for infectivity assay were stored at −20°C in the presence of 1% bovine serum albumin (BSA). On the basis of infectivity assays and SDS-PAGE, the fractions containing live virus were pooled, concentrated to 10 ml on an Amicon XM300 membrane and applied to a column (79 cm × 1.6 cm I.D.) of Sephacryl S500 at a flow-rate of 46 ml/h. The void volume of the S300 column was determined using Blue Dextran; and that of the S500 column was determined using a formalin-fixed, trypsin treated suspension of *Escherichia coli.*

Purification of SCF antigen

Fractions from the first S300 column which contained the SCF antigen (as determined by SDS-PAGE) were pooled, concentrated to 15 ml on an Amicon XM50 membrane and applied to a second S300 column.

Electron microscopy

A drop of virus suspension was placed on a 400-mesh copper specimen grid coated with a formvar–carbon film. After approximately 30 s the suspension was removed by touching the edge of the grid with moist filter paper. A drop of negative stain (1%, w/v, sodium silicotungstate, pH 6.8, or 0.5% uranyl acetate, pH 4.3) was then placed on the grid and similarly removed after approxamately 10 s. Grids were allowed to air dry before being examined in a Philips EM400T electron microscope operated on a accelerating voltage of 80 kV. Micrographs were recorded on an Ilford EM technical film at 60 000 × magnification.

SDS-PAGE

Column fractions were analysed by SDS-PAGE under reducing conditions, as described by Stephenson *et al.*[6]. The gels were fixed in 7% acetic acid and stained using Coomassie blue and/or Bio-Rad silver stain.

Infectivity assays

Infectious viral particles were assayed in monolayers of PS cells as described previously[7].

Sucrose gradient centifugation

Virus particles and extracellular antigens, labelled with ^{35}S methionine were prepared as described previously[8].

Reagents

Sephacryl was obtained from Pharmacia (Milton Keynes, U.K.); Minitan membranes from Millipore (Watford, U.K.) and membranes were obtained from Amicon (Stonehouse, U.K.). [^{35}S]Methionine was from Amersham International (Amersham, U.K.).

RESULTS

Purification of virus particles

Chromatography of infected cell supernatants on Sephacryl S300 resulted in three species of particle being detected by monitoring the column eluate at O.D. $_{280}$ (Fig. 1). Virtually all infectious virus particles were present in the void volume (peak 1) and when this material was analysed by PAGE (Fig. 2) the major virion envelope protein E was clearly seen. Staining of the virion capsid protein C and the minor envelope protein M is very poor and is only uneqivocally seen when column fractions are concentrated 50-fold or more. As peak 1 represents the void volume of the column other particles are found apart from virions. PAGE analysis however (Fig. 2) indicates that very little protein, apart from virus proteins, is present in peak 1. Electron microscopy indicates that these non-virion particles are similar to the heterogenous smooth membranes reported from cells infected with alphaviruses[9]. PAGE analysis also reveals that the major protein in peak 2 is the extracellular form of the non-structural virus specific protein (NS1), previously referred to as the "soluble complement fixing antigen"[10]. Electron microscopy and PAGE analysis both confirm that the material in peak 3 is the amorphous granular cell debris seen in similar samples from cells infected with alphaviruses[9].

Virus particles were further purified by pooling fractions 40–55 and concentrating the preparation with XM300 membranes in stirred cells. Subsequent chromatography on Sephacryl S500 columns (Fig. 3) enabled infectious virus particles to be resolved by the column matrix, eluting with a K_D (partition coefficient) of 0.66. This figure is consistant with a particle of molecular weight of $2.38 \cdot 10^7$, as determined by conventional methods. PAGE analysis shows that fractions 43–55 from this column contain pure virus particles (Fig. 4).

It is clear from the data described above that only two virus-specific particles are released from the cell, but when similar material is analysed by gradient centrifu-

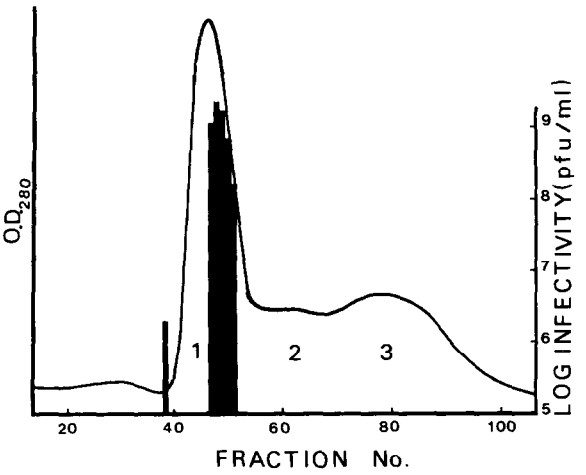

Fig. 1. Purification of TBE extracellular antigens by chromatography on S300. Clarified, concentrated infected cell supernatants were applied to the column and eluted in phosphate buffer as described in methods. —— = O.D.$_{280}$ profile; ■ = infectivity in plaque forming units (pfu)/ml, assayed in PS cells.

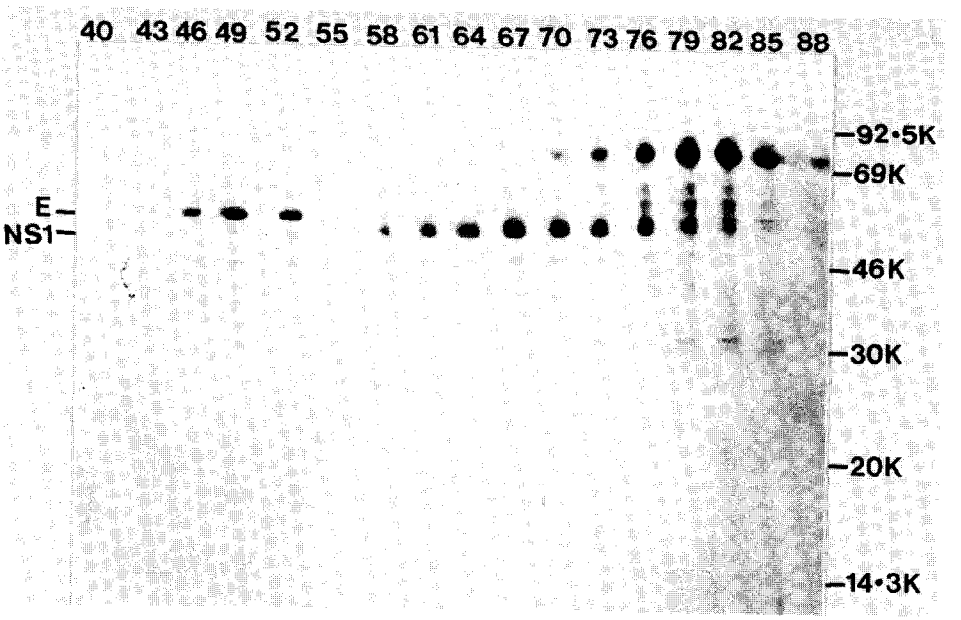

Fig. 2. PAGE of selected fractions from Fig. 1. Aliquots of 20 μl were boiled in sample buffer and analysed as described in Materials and methods. Gels were stained with Coomasie blue. Gel tracks are indicated by the fraction numbers of the samples and the positions of molecular weight markers are indicated on the right hand side. K = Kilodalton.

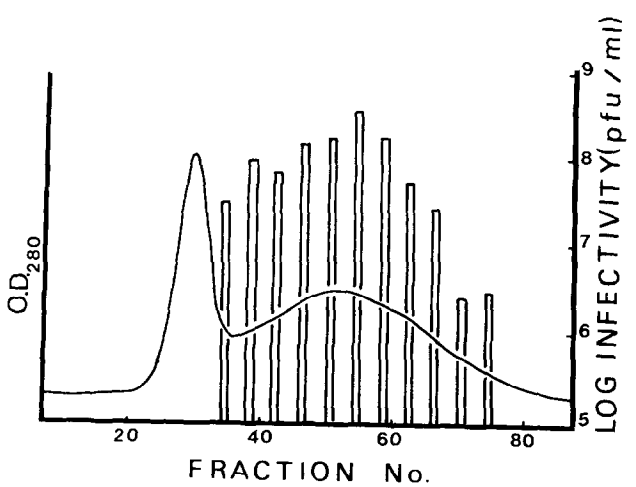

Fig. 3. Purification of TBE virions on S500. Fractions from Fig. 1, containing virus particles, were concentrated and applied to the column as described in Materials and methods. — = $O.D._{280}$ profile; bars indicate infectivity.

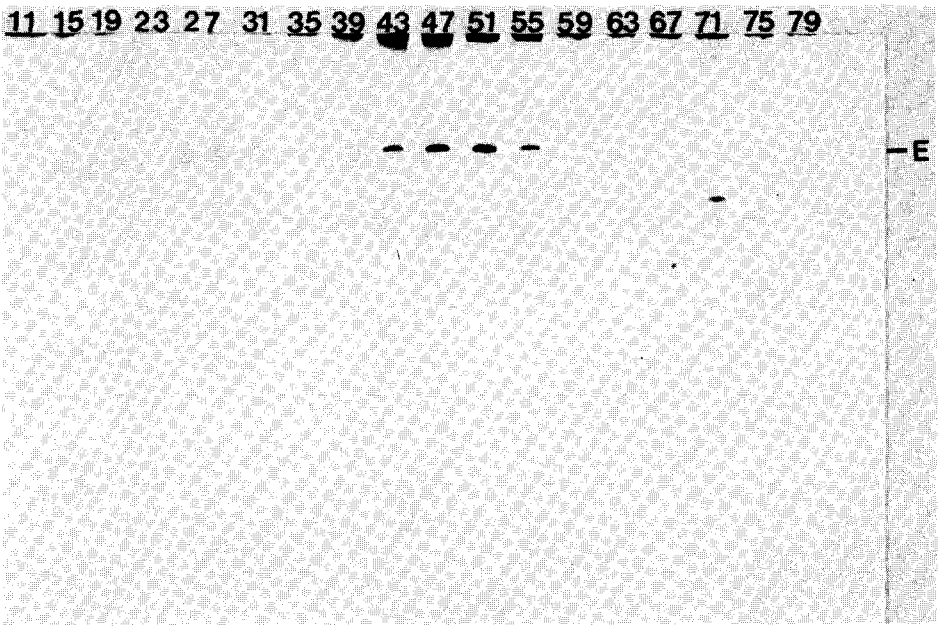

Fig. 4. PAGE of selected fractions from Fig. 3. Samples were prepared and analysed as in Fig. 2. Track designations are as for Fig. 2.

gation (Fig. 5) three species are seen. In addition to infectious virions and NS1 a third moiety, the SHA (slowly sedimenting haemagglutinin) can be observed. This particle contains only the E polypeptide and has been reported for several mosquitoborne flaviviruses as well as TBE[10,11].

Recovery of biological activity

The recovery of infectious virus particles was monitored throughout the purification procedure by assaying infectivity in PS cells, as described above (Table I). Some virus was lost during the initial concentration step and on each chromatography, but the overall recovery rate of 32% is significantly better than that achieved by conventional means and is similar to that reported for alphaviruses[9].

Physical integrity of the virus particles

The physical integrity of the virus particles was examined by electron microscopy. Fig. 6 shows a typical field of virus particles with an average diameter of 53 nm, a figure similar to that reported for other flaviviruses. Although some of the particles appeared damaged most virions were intact. The damaged particles are almost certainly a result of the sample preparation as it has been known for some time that flavivirus particles are very sensitive to disruption by techniques used to prepare samples for electron microscopy.

The particle to infectivity ratio was calculated from similar preparations by the method described in ref. 9 and these calculations gave a value of 6.8, similar to that reported for alphaviruses.

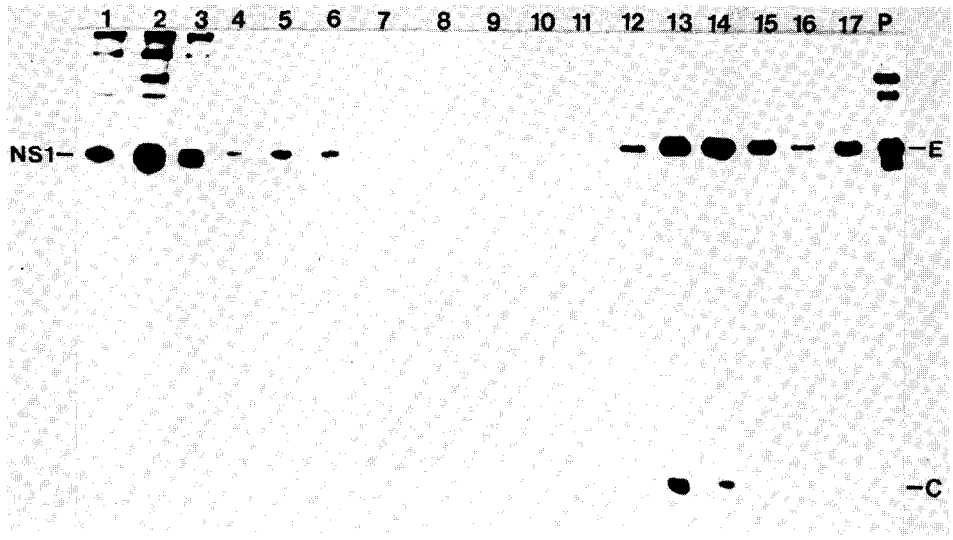

Fig. 5. Sucrose gradient analysis of extracellular antigens from cells infected with TBE virus. Gradient sedimentation and analysis of gradient fractions by autoradiography and PAGE were performed as in Materials and methods with sedimentation being from left to right. Gel tracks are designated by the fraction numbers from the sucrose gradient. P = Pellet from the gradient.

Purification of the extracellular form of NS1

Extracellular NS1 was further purified by pooling fractions 61–70 from peak 2 (Fig. 1) and concentrating the preparation on XM50 membranes. This preparation was reapplied to an S300 column and purified as described in Materials and methods. As can be seen in Fig. 7, the extracellular NS1 elutes with a K_D of 0.22 and is clearly resolved from virus particles, cellular proteins and serum. A typical PAGE analysis of a chromatography run (Fig. 8) shows no evidence of virus particles and thus the $O.D._{280}$ peak 1 in Fig. 7 must represent membranous particles as described above. PAGE analysis also shows some contamination of the NS1 peak fractions by serum albumin; but this can be completely removed if required by further chromatography on S300 (data not shown). By calibrating the S300 column with aldolase, thyroglobulin, ferri-

TABLE I

EFFICIENCY OF RECOVERY OF INFECTIOUS VIRUS DURING PURIFICATION BY ULTRAFILTRATION AND CHROMATOGRAPHY

Sample	*Plaque forming units (total)*	*Recovery (%)*[a]
Clarified supernatant	$2.0 \cdot 10^{11}$	–
Concentrated supernatant	$1.5 \cdot 10^{11}$	75
Pooled peaks from S300 column	$9.5 \cdot 10^{10}$	63
Pooled peaks from S500 column	$6.35 \cdot 10^{10}$	67
Overall recovery	–	32

[a] Apart from the overall recovery these figures represent the yield at each step.

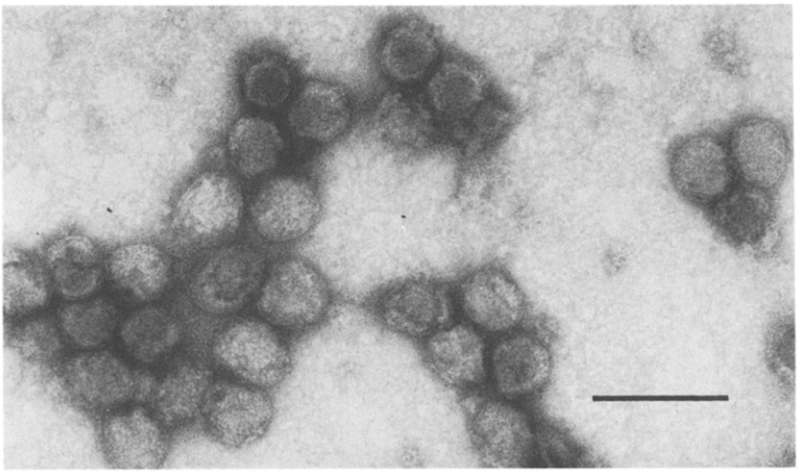

Fig. 6. Electron microscopy of purified TBE virus from Fig. 3; negatively stained with uranyl acetate. Bar marker equals 100 nm.

tin and catalase, the extracellular NS1 protein elutes with an apparant molecular weight of 331 000, consistent with it being in form of a hexamer.

DISCUSSION

Flaviviruses have been responsible for several severe diseases of mankind for over three centuries[12]. Although some successful vaccines have been developed against these viruses, protection against many flavivirus diseases is either poor or non-existant[13]. Research in this area has been hampered by several factors, the pathogenic nature of the agents, poor growth in tissue culture for many isolates and difficulty in purifying virus particles and extracellular antigens.

Conventional methods of virus purification such as sucrose gradient centrifugation, pelleting or ion-exchange chromatography frequently result in loss of infectivity and distortion of viral particles. The techniques reported here do not employ any harsh chemical or physical environments and therefore we have succeeded in purifying TBE virus with high yields and low particle to infectivity ratios.

TBE virus particles prepared by this methodology elute with a K_D of 0.66 which

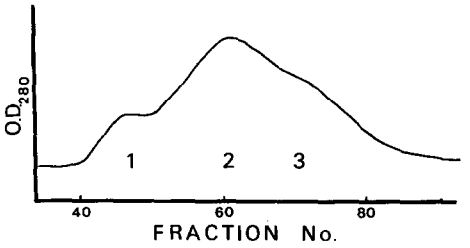

Fig. 7. Purification of extracellular NS1 by chromatography on S300, as described in Materials and methods.

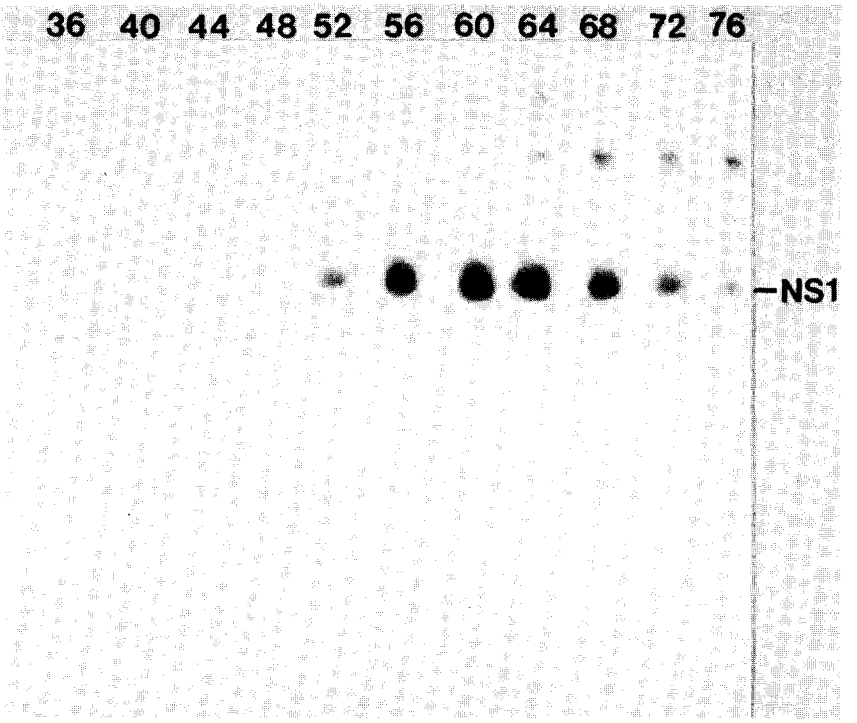

Fig. 8. PAGE analysis of selected fractions from Fig. 7. Samples were prepared and analysed as in Fig. 2. Track designations are as for Fig. 2.

is consistent with the particle diameter of 53 nm observed by electron microscopy and with a molecular weight of $2.63 \cdot 10^7$. As determined by PAGE the virus preparations are clear of any contaminating cell protein and by electron microscopy they also appear to be clear of cell membranes. The material stained with silver, but not coomassie blue, appearing at the top of the gels is thought to be viral nucleic acids.

It is surprising to note that the SHA particles observed when extrallular material is purified on sucrose gradients is not detected during column chromatography. It is possible therefore that the SHA does not represent a true extracellular product from infected cells but is a by-product of the breakdown of virus particles during gradient purification.

Although the extracellular NS1 is not as clearly resolved from cellular proteins as are virus particles, it is still possible to obtain preparations of this protein which are free from detectable contaminants. It is difficult to estimate the yield of this protein as it has no biological property which is not shared by either virion or cellular components. The elution profile of NS1 is consistent with it being present as a hexamer in the extracellular environment. Studies with other flaviviruses however have indicated that NS1 is a dimer[14]. This apparent discrepancy can be resolved if it is remembered that the studies with dengue virus involved exclusively SDS-PAGE analysis of NS1 and thus the protein was at least partially denatured. In addition it has been shown in our laboratory that the extracellular form of NS1 is more heavily glycosylated than

its intracellular counterpart[8] and therefore it is possible that NS1 is indeed a dimer in the infected cell, but the additional glycosylation associated with its export enables three dimers to associate into a hexamer. This hexamer may well be sensitive to low concentrations of SDS as sequence analysis reveals several hydrophobic domains in this protein. Multimeric proteins are much better immunogens than their monomeric counterparts and the observation that NS1 is a hexamer is consistent with it being a good immunogen in animals infected or vaccinated with TBE virus[15].

In conclusion we have demonstrated a method for purifying TBE virus that gives preparations of high titre and free from contaminating proteins. In addition the virus is highly infectious and maintains a native conformation. The extracellular form of NS1 can also be purified by this method and this protein appears as a hexamer in its native state.

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REFERENCES

1 E. G. Westaway, *Adv. Virus Res.*, 33 (1987) 45–90.
2 G. Wengler and G. Wengler, *J. Virol.*, 63 (1989) 2521–2526.
3 F. X. Heinz, C. Kunz and H. Fauma, *J. Med. Virol.*, 6 (1980) 213–221.
4 L. B. El'bert, A. V. Gorgorina, M. K. Khanina, G. L. Krutyanskaya, V. P. Grachev, S. G. Drozdov, N. M. Ral'f, E. V. Finogenove, I. M. Rodin, A. V. Tyutanov, Y. V. Pervikov, V. M. Borisov, N. L. Kondrat'eva and M. E. Gurevich, *Vopr. Virusol.*, 3 (1980) 341–345.
5 J. R. Stephenson, A. J. Crooks and J. M. Lee, *J. Gen. Virol.*, 68 (1987) 1307–1316.
6 J. R. Stephenson, A. J. Hay and J. J. Skehel, *J. Gen. Virol.*, 36 (1977) 237–248.
7 J. R. Stephenson, J. M. Lee and P. D. Wilton-Smith, *J. Gen. Virol.*, 65 (1984) 81–89.
8 J. M. Lee, A. J. Crooks and J. R. Stephenson, *J. Gen. Virol.*, 70 (1989) 335–343.
9 A. J. Crooks, J. M. Lee and J. R. Stephenson, *Anal. Biochem.*, 152 (1986) 295–303.
10 W. E. Brandt, R. D. Cardiff and P. K. Russell, *J. Virol.*, 6 (1970) 500–506.
11 R. D. Cardiff, T. G. McCloud, W. E. Brandt and P. K. Russel, *Virology*, 41 (1970) 569–572.
12 R. E. Shope, in R. W. Schlesinger (Editor), *The Togaviruses*. Academic Press, New York, 1980, pp. 47–82.
13 J. R. Stephenson, *Vaccine*, 6 (1988) 471–480.
14 G. Winkler, V. B. Randolph, G. R. Cleaves, T. E. Ryan and V. Stollar, *Virology*, 162 (1988) 187–196.
15 P. Hambleton, J. R. Stephenson, A. Baskerville and C. N. Wiblin, *Infect. Immun.*, 40 (1983) 995–1003.