Biochimica et Biophysica Acta, 307 (1973) 287–300

BBA 76288

STEPWISE DISSOCIATION OF THE SEMLIKI FOREST VIRUS MEMBRANE WITH TRITON X-100

ARI HELENIUS and HANS SÖDERLUND

Department of Serology and Bacteriology and Department of Virology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29 (Finland)

(Received November 24th, 1972)

SUMMARY

1. The dissociation of the Semliki Forest viral membrane by the neutral detergent Triton X-100 was studied using analytical and preparative ultracentrifugation.

2. The binding of Triton to the membrane started below the critical micelle concentration of Triton and increased thereafter with increased Triton concentration.

3. Release of the nucleocapsids from the virus was observed when more than 0.2–0.4 mg of Triton was bound per mg membrane.

4. When more than 1.6 mg of Triton was bound the membranes dissociated into small protein–lipid–detergent complexes.

5. With still larger amounts of Triton present delipidation of the membrane protein took place.

6. The nucleocapsids did not bind detectable amounts of detergent.

INTRODUCTION

Neutral detergents have frequently been used to solubilize biological membranes and to isolate membrane proteins. However, little exact data are presently available on the mechanisms by which these detergents act. Of the neutral detergents Triton X-100 has proved one of the most effective solubilizers but its effect on proteins is still mild, most membrane enzymes retain their activity in its presence[1]. We have studied the effect of Triton X-100 on the membrane of Semliki Forest virus (SF virus), which is one of the simplest biological membranes known[2,3].

SF virus, a Group A arbovirus, has a spherical nucleocapsid consisting of RNA and one polypeptide species[4]. The nucleocapsid is surrounded by a lipid–protein membrane which the virus acquires as it leaves the host cell by budding through the plasma membrane[4,5]. The lipid composition of the viral membrane is very similar to that of the host cell plasma membrane[6]. Low-angle X-ray diffraction studies show that the lipids in the membrane are arranged in a bilayer structure[7]. The membrane contains only two glycoprotein species, both of which have an apparent molecular weight of about 50000[8,9]. The bulk of the membrane protein is located on the outside

Abbreviations: SF virus, Semliki Forest virus; CMC, critical micelle concentration.

of the lipid bilayer in the form of "spikes"[5]. Like most membrane proteins these viral proteins are insoluble in aqueous media (in the absence of detergents)[3].

Our results show that Triton X-100 solubilization of the SF virus membrane proceeds through certain well-defined stages beginning with detergent binding at concentrations below the critical micelle concentration (CMC). The membranes apparently lose their structural stability as detergent is bound, and rupture. At higher detergent concentrations they are fragmented into discrete protein–lipid–detergent complexes; in the presence of very high concentrations separate protein–detergent and lipid–detergent complexes are obtained.

MATERIALS AND METHODS

Virus

Semliki Forest virus (SF virus), prototype strain, grown in monolayer cultures of baby hamster kidney cells, BHK21 clone WI-2, was used in this study. Cultivation origin and passage history of cells and virus was as described by Kääriäinen *et al.*[10] Virus was purified from the cell culture fluid (8 l/batch), after low speed centrifugation and vacuum dialysis concentration, by high speed centrifugation and two successive bandings in 5–50% potassium tartrate gradients[10]. The virus was finally pelleted resuspended in 0.05 M Tris–0.1 M NaCl buffer (pH 7.4) and stored at 0 °C. This buffer was used in all subsequent experiments.

Virus, double labelled with [3H]lysine (250 Ci/mole, The Radiochemical Centre, Amersham) and $^{32}P_i$ (carrier-free orthophosphate, Institutt for Atomenergi, Kjeller, Norway), or ^{32}P labelled, was prepared as described by Kääriäinen and Söderlund[11]. When relatively more label was required in the phospholipids, cells (about $3 \cdot 10^8$) were prelabelled for 22 h with $^{32}P_i$ (20 μCi/ml) in Eagle's minimal essential medium containing one-tenth of the normal amount of phosphate and 10% calf serum. The cells were then washed with Hank's salt solution and the virus inoculum added (30–50 plaque-forming units/cell). After the 1-h absorption period at 37 °C the cells were again washed and the labelling continued with 10 μCi/ml of $^{32}P_i$ in lysine-free minimal essential medium containing 1 μg/ml of actinomycin D. [^{3}H]Lysine (μCi/ml) was added 3 h postinfection and the cell culture fluid harvested at 10 h. Labelled virus was purified as was the unlabelled, omitting the vacuum dialysis step.

The purified virus banded sharply on both isopycnic and velocity gradients and was homogeneous in the analytical ultracentrifuge. Under the electron microscope a homogeneous population of viral particles was found (Fig. 9A). After sodium dodecyl sulphate–polyacrylamide gel electrophoresis only two bands were stained by Coomassie blue, corresponding to the membrane and the nucleocapsid proteins (cf. Fig. 7). When the virus was treated with formyl methionyl [^{35}S]sulphone methyl phosphate[12] as described by Gahmberg *et al.*[13] very little label (< 3%) was found in the nucleocapsid protein, indicating that the nucleocapsids were inaccessible to the reagent. Judging by these criteria the virus was pure and the particles intact. The labelled virus, according to similar criteria, was radiochemically pure.

The chemical composition of SF virus used in the calculations was RNA, 6%; nucleocapsid protein, 12%; membrane protein, 45%; lipid, 31%; and nonlipid carbohydrate, 6% (ref. 39). The molecular weight of the virus is approximately $6.4 \cdot 10^7$.

Detergent

Triton X-100, a polyoxyethylene *p-tert*-octylphenol with an average chain length of 9–10 ethylene oxide units (mean mol. wt 638), was purchased from Rohm et Haas, Philadelphia, Pa. Thin-layer chromatography[14] showed about 10 regularly spaced spots demonstrating the great polydispersity of the polyoxyethylene chains. No major impurities were detected. The ^{3}H-ring-labelled Triton X-100 was a generous gift from Dr W. R. Lyman, Rohm et Haas.

Analytical ultracentrifugation

Analytical ultracentrifugation was performed with a Beckman Spinco Model E centrifuge equipped with a split-beam photoelectric scanner, in conjunction with a monochromator and an ultraviolet absorption optical system. The An-D rotor was operated at 14290 and 42040 rev./min, at 20 °C and double-sector cells with 12 mm optical path were used. The Triton X-100 solutions (0.19 ml) and SF virus suspensions (0.01 ml), both kept at 0 °C, were added to the cells separately. The wavelength was set to 276 nm, the absorption maximum for Triton X-100. When determined in the centrifuge the $E^{1\%}_{276\ nm}$ of Triton in 0.05 M Tris–0.1 M NaCl buffer (pH 7.4) was 21.2 ± 0.3. Because the partial specific volumes of SF virus and the subviral components are unknown, the sedimentation coefficients could not be corrected to standard conditions. Binding of Triton X-100 to SF virus was determined essentially by the sedimentation velocity method of Steinberg and Schachman[15]. The recorder displacement in the supernatant region after 30 min at 14290 rev./min and 30 min at 42040 rev./min gave the absorbance of the nonsedimenting material. As the Triton X-100 monomers and micelles did not sediment appreciably under these conditions, the absorption at 276 nm could be ascribed to free Triton in the cell, provided that no slowly sedimenting components from the virus were present. To obtain the amount of detergent bound identical runs were performed omitting the SF virus. From the difference in supernatant absorption in the presence and absence of SF virus the binding could be calculated. The binding is usually expressed as mg Triton per mg membrane protein.

Sucrose gradient centrifugations

Ribonuclease-free sucrose (Mann Research Laboratories, New York) was used in the gradients and unless otherwise indicated the gradient contained 135 μg Triton X-100 per g. The samples were prepared by adding the required amount of precooled (0 °C) detergent stock solution (5.0 mg Triton per ml Tris–NaCl buffer), Tris–NaCl buffer and the mixture of carrier virus and labelled virus, in that order, to a final volume of 0.1 ml, into a cold glass tube using precooled pipettes. After mixing, the sample was incubated at 20 °C for 10 min. The centrifugations were performed at 20 °C in a Beckman L2 centrifuge. The rotors used were SW 50.1 (at 45000 rev./min) and SW 27 (at 25000 rev./min). The 5 ml 20–50% (w/w) sucrose gradients were collected from below as 0.25-ml fractions and the 18-ml gradients (15–30%; w/w) as 0.50-ml fractions. Aliquots were taken for radioactivity assays (75 and 150 μl, respectively), lipid extraction (100 and 150 μl), haemagglutination titration and electron microscopy.

Determination of Triton X-100 binding using sucrose gradient centrifugation

Detergent binding in the gradients was measured using ^{3}H-labelled Triton X-100

(0.286 mCi/g or 0.072 mCi/g) and unlabelled or ^{32}P-labelled virus. Binding was detected as a ^{3}H-radioactivity peak. The amount of detergent bound was calculated either directly from the number of counts in the peak (Fig. 8A) or from the loss of counts observed in the upper portion of the gradient compared to a similar gradient without the virus (Fig. 5C and Fig. 6) (*cf.* the method of Hummel and Dreyer[16]). To obtain the detergent:protein ratio the protein was determined either by quantitative amino acid analysis, or estimated from the protein label ([^{3}H]lysine) present in the corresponding peak of an identical centrifugation with labelled virus and unlabelled Triton X-100. The two methods agreed within 10%.

Other methods

Radioactivity was determined using a Wallac 8100 liquid scintillation counter. The aqueous samples were added to vials containing 10 ml of Bray's[17] solution. To determine ^{32}P-labelled lipid the samples were subjected to lipid extraction (chloroform–methanol–water, 8:4:3, by vol.), and samples taken from the lipid phase and evaporated to dryness in the vial. Electron microscopy and negative staining with 1% potassium phosphotungstate was performed as described earlier[18]. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed using the method of Weber and Osborn[19] in which the two membrane proteins are not resolved. The radioactivity of the 2-mm gel slices was determined in Nuclear Chicago solubilizer–toluene as described by Caliquiri and Mosser[20]. Protein was determined by the method of Lowry *et al.*[21] with 0.1% sodium dodecyl sulphate in the reaction mixture using human albumin as standard. Quantitative amino acid analysis was performed as described[22] using norleucine as an internal standard. Haemagglutination titre was assayed according to Clarke and Casals[23] using 0.2% goose erythrocytes at pH 5.8, and is expressed as haemagglutination titre/ml. Sucrose concentrations were determined by refractometry with buffer corrections.

RESULTS

Analytical ultracentrifugation

CMC determination. Micellar solutions of Triton X-100 in 0.05 M Tris–0.1 M NaCl buffer (pH 7.4) were subjected to analytical ultracentrifugation in order to determine the CMC (Fig. 1). At 20 °C the micelles sedimented at about 1 S. After 6 h at 42040 rev./min the ultraviolet absorbance remained constant near the meniscus and the concentration of nonsedimenting monomeric detergent could be calculated. This concentration was 150 μg/ml (235 μM), which is in good agreement with recent CMC values reported by Ray and Nemethy[24].

Binding of Triton X-100 to SF virus and disruption of the virus. Centrifugations were performed with a constant amount of virus (100 μg SF virus protein) and increasing amounts of Triton (0–400 μg/ml). Representative scanner traces from these runs are shown in Figs 2A–2D. The amount of free detergent and detergent bound to the virus was determined as described in Materials and Methods, and plotted in Fig. 3 as a function of the total Triton concentration. Triton binding started below the CMC and increased thereafter with increasing detergent concentration, the concentration of free detergent did not increase beyond about 160 μg/ml. Centrifugations performed with constant Triton concentration (180 μg/ml) and variable amounts of virus (25, 37,

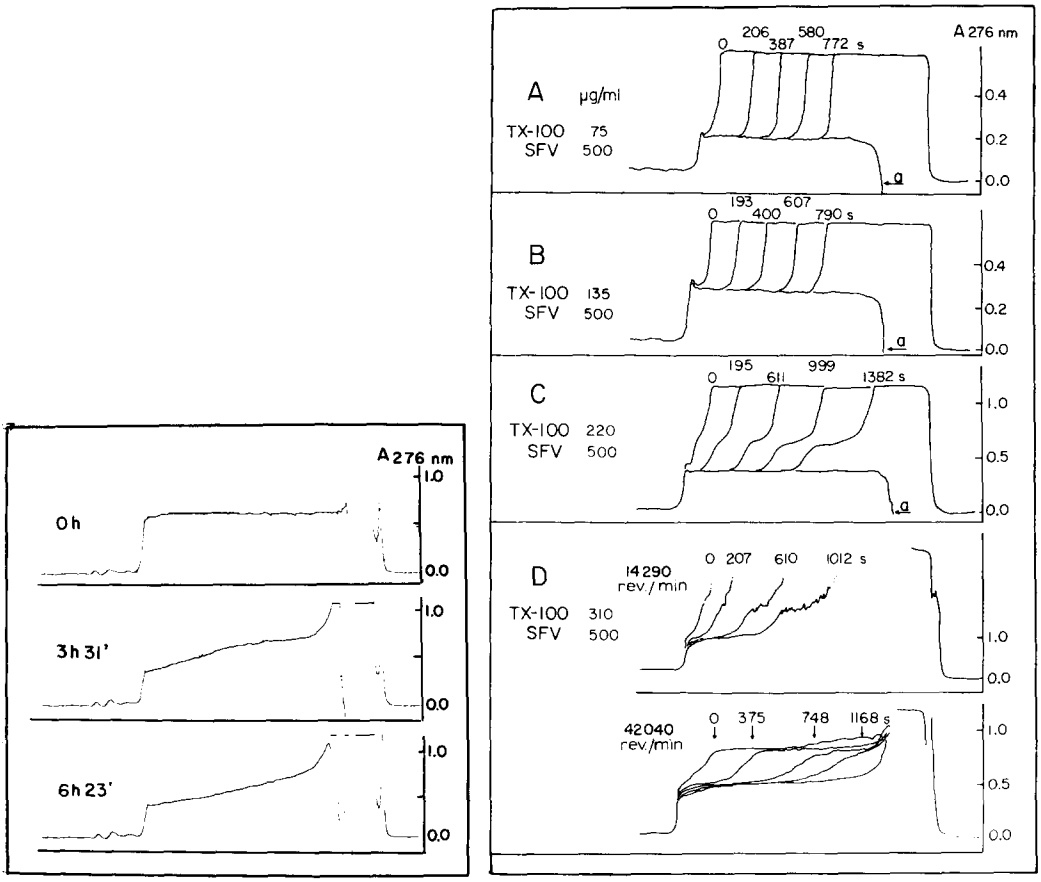

Fig. 1. Analytical ultracentrifugation of Triton X-100 solution containing 0.295 mg Triton X-100/ml Tris–NaCl buffer (pH 7.4). An An-D rotor with a double-sector, 12-mm optical path cell was used at 42040 rev./min, 20 °C. The absorbance at 276 nm was recorded at the times indicated. Zero time represents the time when the desired speed was reached.

Fig. 2. Analytical ultracentrifugation of SF virus in solutions with varying Triton X-100 concentrations. SF virus and Triton concentrations are indicated in the figure. The sample volume was 0.2 ml. Each sample was run for 30 min at 14290 rev./min (20 °C). Thereafter the speed was increased to 42040 rev./min (20 °C) for another 30 min. The traces were recorded at 276 nm and have been superimposed for clarity. A, B and C (excluding the "a" traces) were obtained during the 14290 rev./min centrifugation. The traces marked "a" show the situation at the end of the 42040 rev./min run. That these traces drop below the baseline at the bottom of the cell is probably due to the pelleted virus. In D the traces obtained during the 14290 rev./min and 42040 rev./min runs are shown separately. Zero time represents the time when the desired speed was obtained. Please note different scales of absorbance. TX-100, Triton X-100; SFV, Semliki Forest virus.

50, 65, 100 μg of viral protein in the 0.2-ml sample): all gave a free detergent concentration of 155 ± 4 μg/ml. The Triton X-100 bound in these runs was thus 25 μg/ml and independent of the virus concentration.

The intact virus sedimented at 250 S. With increasing detergent concentrations slower boundaries appeared. The first (140 S, Fig. 2C) was detected when the amount

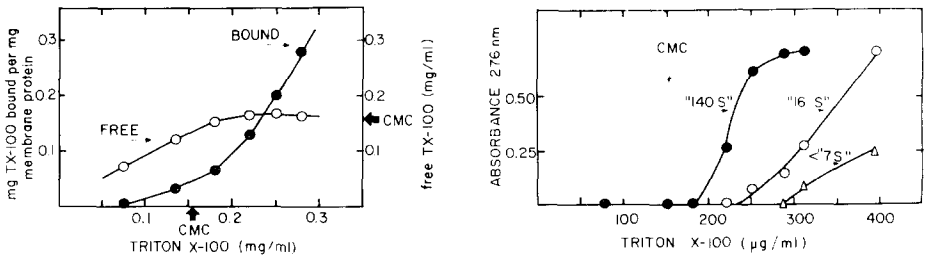

Fig. 3. Bound (●—●) and free (○—○) Triton X-100 at different concentrations of Triton X-100 in the presence of SF virus (100 μg viral protein). The concentrations were determined by analytical ultracentrifugation. Arrows indicate the CMC.

Fig. 4. Ultraviolet absorbance of the major subviral components observed in the analytical ultracentrifuge at varying concentrations of Triton X-100. 100 μg viral protein was used. 140 S (○—○), 16 S (●—●) and < 7 S (△—△).

of Triton X-100 bound had reached 0.17 mg per mg viral protein. When 0.26 mg per mg protein was bound a new major boundary (16 S) was observed (Fig. 2D, 42040 rev./min) and when the binding was above 0.37 mg per mg protein slowly moving components (< 7 S) were detected (Fig. 2D, 42040 rev./min). The ultraviolet absorbances of these boundaries are shown in Fig. 4.

The new boundaries were interpreted as being subviral components due to the action of Triton X-100. The results suggested that, although the binding of Triton X-100 to the virus increased linearly with increasing detergent concentration, the disruption occurred in discrete steps.

Preparative ultracentrifugation

In order to characterize the breakdown process and to isolate the intermediates we used preparative ultracentrifugation in sucrose gradients. The samples contained virus (100 μg viral protein unless otherwise indicated) and increasing amounts of detergent. The chemical composition of the various components obtained were studied using [^{3}H]lysine label in the viral proteins and ^{32}P label in the RNA and the phospholipids. Being soluble in chloroform–methanol, the ^{32}P activity in the lipids could be distinguished from that in the RNA. The distribution of [^{3}H]lysine label between nucleocapsid and membrane protein in any fraction was determined after sodium dodecyl sulphate–polyacrylamide electrophoresis. The haemagglutinating activity of the membrane proteins also provided a means to distinguish between the membranes and the nucleocapsids. ^{3}H-labelled Titron X-100 was used to study the detergent binding to the individual components.

Our results show four main stages in the breakdown of the virus with increasing concentrations of Triton X-100. These are: (1) binding of Triton X-100 prior to disruption, (2) disruption into nucleocapsids and membranes, (3) disintegration of membrane into protein–lipid–detergent complexes, and (4) delipidation of the membrane proteins.

Binding of Triton X-100 prior to disruption. In the presence of low Triton X-100 concentrations the virus sedimented as a sharp band, the buoyant density of which gradually decreased as detergent concentration increased (Figs 5A–5C, Table I). By using labelled Triton X-100, binding to the virus could be detected; up to 0.2 mg

Triton X-100 per mg membrane protein could be bound before large scale rupture of the virus occurred (Fig. 5C). The decrease in density of the virus may be due, at least in part, to the large partial specific volume of Triton X-100 ($\bar{v}=0.99$, ref. 25). In

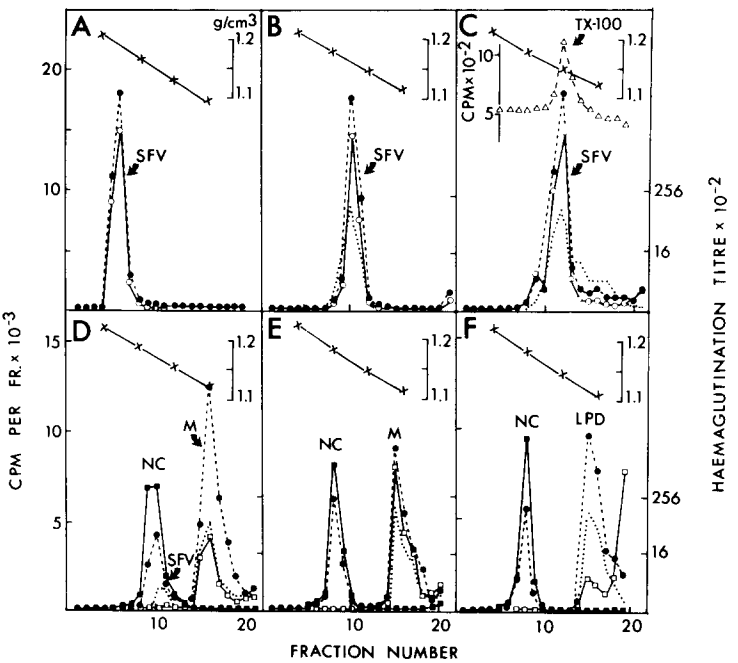

Fig. 5. Sucrose gradient centrifugation of labelled SF virus in the presence of varying amounts of Triton X-100. The 20–50% (w/w) sucrose gradients were in Tris–NaCl buffer, and all, except A contained 135 μg Triton/g. Samples were layered on the gradients and centrifuged at 45000 rev./min in a SW50.1 rotor for 80 min at 20 °C. The samples contained [^{3}H]lysine- and ^{32}P labelled SF virus (100 μg of viral protein) and Triton as follows: (A) Triton-free control; (B) 16.3 μg; (C) 26.3 μg; (D) 36.3 μg; (E) 63.3 μg; (F) 100 μg. In the [^{3}H]Triton binding experiment the sample (0.2 ml) contained 200 μg viral protein and 52 μg [^{3}H]Triton. The gradient contained 135 μg [^{3}H]Triton/g. The curve obtained is shown in C (insert). [^{3}H]Lysine (●---●), total ^{32}P activity (○—○), ^{32}P-labelled lipid (□—□), [^{32}P]RNA (■—■), [^{3}H]Triton (△---△), haemagglutination titre activity (·····) and sucrose (×—×). NC, nucleocapsid; M, membrane; and LPD, lipid–protein–detergent complex. Bottom at left.

negatively stained electron micrograms of this stage the virus differed from the untreated SF virus in the deformed membranes, some of which still contained nucleocapsids (Figs 9A and 9B). Although suggesting this the electron microscopic results may, however, do no more than that. The negative staining procedure in the presence of detergents may have introduced artifacts; the drying and dilution steps are likely to have affected the detergent binding equilibrium and ruptured the membranes.

Disruption into nucleocapsids and membranes. Centrifugations identical to those described above were performed with more detergent in the samples. Two new peaks appeared (marked NC and M in Figs 5D and 5E), traces of which were discernible in Fig. 5C. Part of the original virus peak (marked SFV in Fig. 5D) was still present when 36.3 μg Triton X-100 was added to the sample but had disappeared in the presence of 63.3 μg (Fig. 5E).

TABLE I

BUOYANT DENSITY OF SEMLIKI FOREST VIRUS IN THE PRESENCE OF INCREASING TRITON X-100

100 μg viral protein in 0.1-ml sample on 20–50% sucrose gradients in a SW50.1 rotor at 45000 rev./min, 20 °C for 80 min.

Triton in sample (μg)	*Triton in gradient* (mg/g)	*Buoyant density* (g/cm^3)
0	0	1.18*
0	0.135	1.17
16.3	0.135	1.16
26.3	0.135	1.15

* *Cf.* ref. 10.

The peak designated NC was, according to physical, chemical and morphological criteria[11], membrane-free nucleocapsids: The peak contained practically all the RNA and the nucleocapsid protein but no membrane protein, phospholipid or haemagglutination activity. During 80-min centrifugations on 20–50% sucrose gradients the peak did not reach the isopycnic position but it was pelleted in 10-h runs, which indicates high particle density. In velocity centrifugation the material sedimented as a homogeneous peak (*cf.* Fig. 6) at 150 S compared to 80 S ribosomes. Electron microscopy revealed particles of typical nucleocapsid appearance (see Fig. 9B).

Gradients containing ^{3}H-labelled Triton X-100 showed that the nucleocapsids did not bind detergent in detectable quantities (Fig. 6). Using a large amount of virus

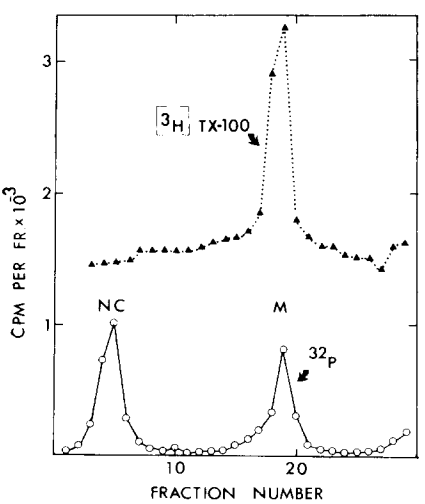

Fig. 6. Sedimentation velocity centrifugation of ^{32}P-labelled SF virus in a 15–30% sucrose gradient. The sample contained 100 μg viral protein and 71 μg [^{3}H]Triton (0.072 mCi/g) in 0.1 ml; and the gradient 135 μg [^{3}H]Triton/g. The centrifugation was at 25000 rev./min for 5 h, 20 °C, in a SW27 rotor. Identical runs with double-labelled virus and unlabelled Triton showed the peaks to be nucleocapsid (NC) and membrane (M). ^{32}P (○—○), [^{3}H]Triton (▲—▲).

(3 mg viral protein) the detection limit was lowered to 0.03 mg Triton X-100 bound per mg nucleocapsid. Even so binding was not detected.

Peak M, an opalescent layer, banded at 1.12 mg/ml (Figs 5D and 5E) in its isopycnic position. Practically all the phospholipid, membrane protein and haemagglutination activity was found in the peak, but no RNA and only trace quantities of nucleocapsid protein were present (Fig. 7). Velocity centrifugation of the membranes revealed a homogeneous peak (Fig. 6) but electron micrographs of the material showed, in addition to large vesicular or elongated structures, some smaller round particles (Fig. 9C). These small complexes may have been formed by the breakdown of some of the membranes during negative staining.

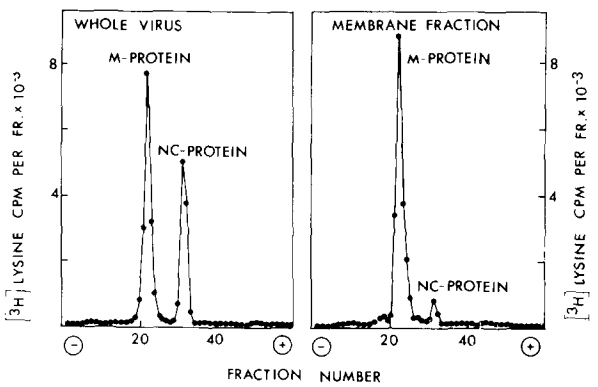

Fig. 7. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of the [^{3}H]lysine- and ^{32}P-labelled SF virus and the corresponding membrane fraction from a sedimentation velocity centrifugation (*cf.* Fraction 19 in Fig. 6). The two membrane proteins were not resolved.

The upper limit of ^{3}H-labelled Triton X-100 binding in the M fraction could be as high as 0.9 mg per membrane protein (Fig. 6) although the nucleocapsid-releasing concentration varied with experimental conditions: During analytical ultracentrifugation, disruption started at 0.14 mg Triton X-100 bound per mg membrane protein. On sucrose gradients (20–50%) during 80-min runs the nucleocapsid-releasing amount bound was 0.2 mg per mg membrane protein but during 10-h runs it was 0.1 mg.

The addition of 30–75 μg of Triton X-100 to the samples occasionally caused the formation of particles, which may represent intermediate stages in the breakdown, dependent on the experimental conditions. A buoyant density of 1.13 g/cm^3 was obtained for these particles, which contained both nucleocapsid and membrane components. They sedimented somewhat slower than the free nucleocapsids in the velocity gradients and bound considerable amounts of ^{3}H-labelled Triton X-100. In the electron micrograms distorted membrane structures were seen to which one or more nucleocapsids were attached.

Disintegration of membranes into protein–lipid–detergent complexes. When 100 μg of Triton X-100 was added to the samples some of the phospholipid, membrane protein and added detergent remained on top of the gradients (Figs 5F and 8A). The rest of the membrane material sedimented slower than the original nucleocapsid-free membranes (Fig. 8A, Table II). Electron microscopy of the peak fractions revealed homogeneous round particles with an average diameter of 25 nm (Fig. 9D). The results

summarized in Table II indicate that when more than 0.9 mg of Triton X-100 was bound per mg membrane protein the membranes were broken into homogeneous protein–lipid–detergent complexes.

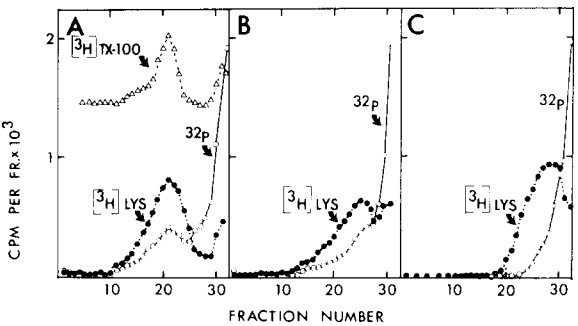

Fig. 8. Sedimentation velocity centrifugation of labelled SF virus with a high concentration of Triton X-100 in the sample. The gradients and conditions were as in Fig. 6, except that unlabelled Triton was used and the running time was 10 h. The samples contained [^{3}H]lysine- and ^{32}P-labelled SF virus (100 μg viral protein) in 0.1 ml and A, 100 μg; B, 164 μg; C, 400 μg Triton. The [^{3}H]Triton curve in A was obtained from an identical run with unlabelled virus and [^{3}H]Triton present both in the sample and the gradient. [^{3}H]Lysine (●—●), total ^{32}P (○—○), [^{3}H]Triton (△---△). The nucleocapsids were pelleted.

TABLE II

DISRUPTION OF THE SEMLIKI FOREST VIRUS MEMBRANE

100 μg viral protein in 0.1-ml samples on 15–30% sucrose gradients containing 135 μg Triton X-100 or ^{3}H-labelled Triton X-100/g in an SW27 rotor at 25000 rev./min, for 5 or 10 h at 20 °C. Fractions were 0.5 ml.

Triton in sample (μg)	*Sedimentation rate (fractions/h)*	*Phospholipid/protein (w/w)*	*Triton bound (mg/mg protein)*	*Appearance in electron micrographs*
71	2.2	0.4	0.9	Membranous
100	1.2	0.2	0.8	Globular
164	0.7	0.1	—	Globular
400	0.4	0.0*	0.2*	"Rosette"-like*

* In 0.05% Triton X-100 solution (Simons, K., Helenius, A., Garoff, H. and von Bonsdorff, C.-H., unpublished).

Delipidation of the membrane protein. As the quantity of Triton X-100 added to the sample was increased above 100 μg the sedimentation rate of the protein–lipid–detergent complexes gradually decreased, and the proportion of phospholipid was reduced (Figs 8A–8C, Table II). When 400 μg Triton X-100, or more, was added to the sample and subjected to 80 min centrifugation, all the membrane material remained on top of the gradient (20–50% sucrose). Similar methods have been used before to obtain the nucleoproteins from enveloped viruses. Prolonged centrifugation under similar conditions allows the membrane protein and lipid components of SF virus to be completely separated (Simons, K., Helenius, A., Garoff, H. and von Bonsdorff, C.-H., unpublished).

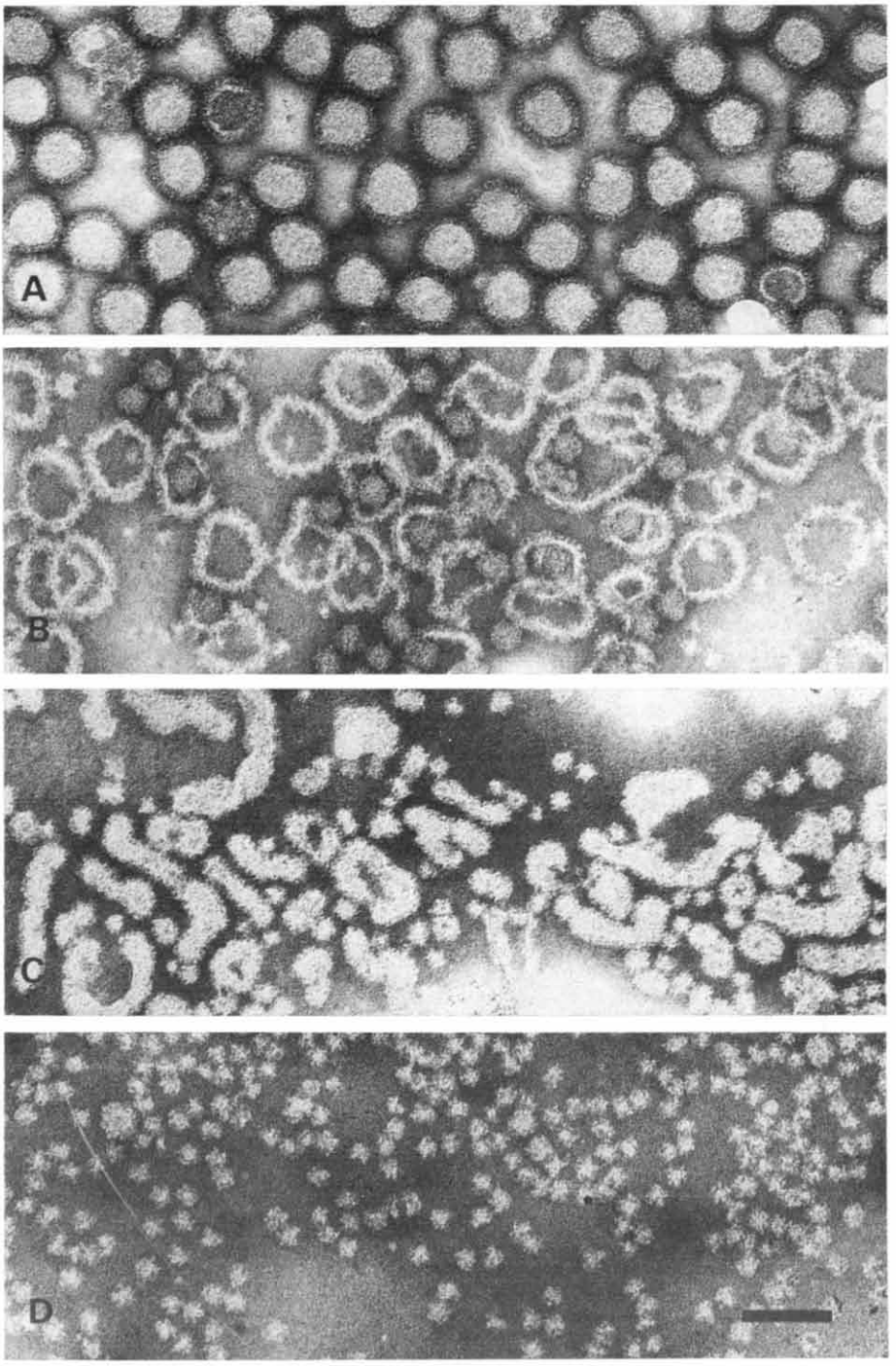

Fig. 9. Electron micrographs obtained after negative staining with potassium phosphotungstate. A, Control SF virus. B, SF virus treated with low Triton X-100 concentration (210 μg SF virus protein and 45 μg Triton X-100 in 0.1 ml). The round 40 nm particles are the nucleocapsids. C, Membrane fraction (sample taken from the membrane peak obtained by centrifugation on a sucrose gradient (Fraction 19 of Fig. 6). D, Lipid–protein–detergent complexes (sample from Fraction 21, Fig. 8A). The bar is 100 nm, all the pictures have the same magnification.

DISCUSSION

Both analytical and preparative ultracentrifugation showed that binding of Triton X-100 precedes the rupture of the SF virus membrane and its solubilization. Binding starts well below the CMC and thus it seems probable, at least in the low concentration range, that the detergent is bound in monomer form. In the presence of virus the concentration of free detergent does not rise significantly above the CMC, so it is possible that micelles do not play any important role even at higher concentrations. Furthermore, the amount of Triton X-100 bound at a given concentration was independent of the quantity of virus present. These data are in agreement with the general conclusions recently drawn by Tanford[26] from the behaviour of alkyl sulphates and sulphonates in the presence of proteins and membranes. Tanford showed that these amphipathic molecules were bound to proteins and membranes rather than forming micelles, provided that the negative standard free energy of binding was greater than that of micelle formation. The standard free energy of micelle formation is obtained from $\Delta F = RT$ ln CMC[27]. Under the conditions described here (*i.e.* 0.05 M Tris–0.1 M NaCl buffer, pH 7.4) the value for the Triton X-100 micelle formation was -7 kcal/mole; the value for binding to the SF virus membrane should be still larger.

Previous reports show that Triton acts on membranes at low concentrations: Bonsall and Hunt[28], using gel filtration and surface tension measurements, have shown that binding to human erythrocyte stroma and phospholipid bilayers starts at about 150 μg/ml. Solubilization studies with chloroplasts[29], rat liver plasma membranes[30] and erythrocyte stroma[28] indicate that Triton takes effect at 100–200 μg/ml. Furthermore activation of certain membrane enzymes by Triton takes place within the same concentration range[31,32].

It is important, in this context, to point out that Triton X-100 does not consist of a homogeneous molecular species; the length of the polyoxyethylene chains follows the Poisson normal distribution, with an average chain length of 9–10 units[33]. This introduces some uncertainty to the interpretation of the binding data. It can, however, be expected that the properties of the mixture are close to those of the homogeneous compound with a 9–10-unit chain [33]. Pure, homogeneous *p-tert*-octylphenyl polyoxyethylenes are presently difficult to obtain.

Binding of Triton reduced the buoyant density of SF virus. It also resulted in the increased susceptibility of the membrane to rupture. The nucleocapsid-releasing concentration depended on the conditions of centrifugation, but under favourable conditions the membrane could bind 0.2 mg Triton per mg membrane protein and remain intact. This corresponds to a molar detergent to lipid ratio of 1:4, or to 9000 moles of Triton per mole of virus. By using nucleocapsid-releasing Triton concentrations membranes could be isolated, which were virtually nucleocapsid free. Before disintegrating further these could bind at least 0.9 mg Triton X-100 per mg membrane protein, or 40000 moles Triton per mole viral membrane.

At higher Triton concentrations the bulk of the membrane material was found as small protein–lipid–detergent complexes. Shibuya *et al.*[29] have spectroscopic evidence for similar complexes after treatment of chloroplasts with Nonidet P40 (*p-tert*-octylphenol polyoxyethylene with an average chain length of 9 units). Due mainly to the behaviour of detergent-solubilized membranes in the analytical ultracentrifuge, it was commonly believed that detergents dissociated the membranes into their constituent

"lipoprotein subunits"[34]. The more recent work of Engelman *et al.*[35] and others[36,37] has shown that both charged and nonionic detergents and bile salts, if present in high concentrations, separate the protein moiety from the lipids. Our results indicate that before total delipidation discrete protein–lipid–detergent complexes occur. Their existence is not necessarily in conflict with the bilayer model of membrane structure: It is likely that the complexes are a result of a phase transition in the lipid bilayer caused by the rising detergent content. The transition from a lamellar to a micellar phase can be expected because the detergent molecules strongly favour the latter structure in dilute aqueous solutions. Similar phase transitions have been proposed by Lucy[38] to explain the cell-lysing effect of lysolecithin and by Bonsall and Hunt[28] for the Triton X-100 solubilization of erythrocyte membranes.

The protein–lipid–detergent complexes were gradually delipidated as the concentration of Triton was increased. Finally lipid-free membrane protein was obtained as small, homogeneous complexes binding large amounts of Triton (Simons, K., Helenius, A., Garoff, H. and von Bronsdorff, C.-H., unpublished). We have previously shown that lipophilic proteins isolated from membranes and lipoproteins bind considerable amounts of Triton whereas no binding to hydrophilic proteins could be detected[22]. The nucleocapsid, which in the virion is in close contact with the inner leaflet of the viral membrane lipid bilayer[7], does not bind Triton. This suggests, that the nature of interactions between the nucleocapsid proteins and the bilayer are different from those of the viral membrane proteins and the bilayer.

ACKNOWLEDGEMENTS

The authors wish to thank Drs Leevi Kääriäinen and Kai Simons for valuable criticism and advice. We also thank also Miss Arja Saarinen and Miss Ritva Saarlemo for skillful assistance. The investigation was supported by grants from Sigrid Jusélius Foundation, the National Research Council for Medical Sciences, and the Association of Finnish Life Assurance Companies, Helsinki, Finland.

REFERENCES

1 Tzagoloff, A. and Penefsky, H. S. (1971) in *Methods in Enzymology* (Jakoby, B., ed.), Vol. XXII, pp. 219–230, Academic Press, London
2 Stoeckenius, W. and Engelman, D. M. (1969) *J. Cell. Biol.* 42, 613–646
3 Simons, K., Kääriäinen, L., Renkonen, O., Gahmberg, C. G., Garoff, H., Helenius, A., Keränen, S., Laine, R., Ranki, M., Söderlund, H. and Uterman, G. in *Membrane Mediated Information* (Kent, P. W., ed.), Oxford, in the press
4 Joklik, W. K. and Zweerink, H. J. (1971) *Annu. Rev. Genet.* 5, 297–360
5 Acheson, N. H. and Tamm, I. (1967) *Virology* 32, 128–143
6 Renkonen, O., Gahmberg, C. G., Simons, K. and Kääriäinen, L. (1971) *Virology* 46, 318–326
7 Harrison, S. C., David, A., Jumblatt, J. and Darnell, J. E. (1971) *J. Mol. Biol.* 60, 523–528
8 Simons, K., Keränen, S. and Kääriäinen, L. (1973) *FEBS Lett.* 29, 87–91
9 Schlesinger, M. J., Schlesinger, S. and Burge, B. W. (1972) *Virology* 47, 539–541
10 Kääriäinen, L., Simons, K. and von Bonsdorff, C.-H. (1969) *Ann. Med. Exp. Fenn.* 47, 235–248
11 Kääriäinen, L. and Söderlund, H. (1971) *Virology* 43, 291–299
12 Bretscher, M. S. (1971) *J. Mol. Biol.* 58, 775–781
13 Gahmberg, C. G., Simons, K., Renkonen, O. and von Bonsdorff, C.-H. (1972) *Virology* 28, 179–182
14 Nadeau, H. G. and Waszeciak, P. H. (1967) in *Nonionic Surfactants* (Schick, M. J., ed.), pp. 893–920, Marcel Dekker, New York

15 Steinberg, I. Z. and Schachman, H. K. (1966) *Biochemistry* 5, 3728–3747
16 Hummel, J. P. and Dreyer, W. J. (1962) *Biochim. Biophys. Acta* 63, 530–532
17 Bray, G. A. (1960) *Anal. Biochem.* 1, 279–285
18 Söderlund, H., Kääriäinen, L., von Bonsdorff, C.-H. and Weckström, P. (1972) *Virology* 47, 753–760
19 Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406–4412
20 Caliquiri, L. A. and Mosser, A. G. (1971) *Virology* 46, 375–386
21 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
22 Helenius, A. and Simons, K. (1972) *J. Biol. Chem.* 247, 3656–3661
23 Clarke, D. H. and Casals, J. (1958) *Am. J. Trop. Med. Hyg.* 7, 561–573
24 Ray, A. and Nemethy, G. (1971) *J. Am. Chem. Soc.* 93, 6787–6793
25 Meunier, J. C., Olsen, R. W. and Changeux, J. P. (1972) *FEBS Lett.* 24, 63–68
26 Tanford, C. (1972) *J. Mol. Biol.* 67, 59–74
27 Hall, D. G. and Pethica, B. A. (1967) in *Nonionic Surfactants* (Schick, M. J., ed.), pp. 516–557, Marcel Dekker, New York
28 Bonsall, R. W. and Hunt, S. (1971) *Biochim. Biophys. Acta* 249, 266–280
29 Shibuya, I., Honda, H. and Maruo, B. (1968) *J. Biochem. Tokyo* 64, 571–576
30 Dunnick, J. K., Marinetti, G. V. and Greenland, P. (1972) *Biochim. Biophys. Acta* 266, 684–694
31 Greville, G. D. and Chappell, J. B. (1959) *Biochim. Biophys. Acta* 33, 267–268
32 Banerjee, S. P., Dwooh, I. L., Kharma, V. K. and Sen, A. K. (1970) *Biochim. Biophys. Acta* 211, 345–355
33 Becher, P. (1967) in *Nonionic Surfactants* (Schick, M. J., ed.), pp. 604–626, Marcel Dekker, New York
34 Korn, E. (1966) *Science* 153, 1491–1498
35 Engelman, D. M., Terry, T. M. and Morowitz, H. J. (1967) *Biochim. Biophys. Acta* 135, 381–390
36 Salton, M. R. J. and Schmitt, M. D. (1967) *Biochem. Biophys. Res. Commun.* 27, 529–534
37 Helenius, A. and Simons, K. (1971) *Biochemistry* 10, 2542–2547
38 Lucy, J. A. (1970) *Nature* 227, 815–817
39 Laine, R., Söderlund, H. and Renkonen, O. (1973) *Intervirology*, in the press