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SOLUBILIZATION OF THE SEMLIKI FOREST VIRUS MEMBRANE WITH SODIUM DEOXYCHOLATE

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SUMMARY

The effects of increasing concentrations of sodium deoxycholate on Semliki Forest virus have been studied. Sodium deoxycholate begins to bind to the virus at less than 0.1 mM free equilibrium concentration and causes lysis of the viral membrane at 0.9 ± 0.1 mM free equilibrium concentration when $2.2 \pm 0.2 \cdot 10^3$ mol of sodium deoxycholate are bound per mol of virus. Liberation of proteins from the membrane begins at 1.5 ± 0.1 mM sodium deoxycholate and the proteins released are virtually free from phospholipid above 2.0 mM sodium deoxycholate. The overall mechanism of sodium deoxycholate solubilization of the viral membrane resembles that of Triton X-100 and sodium dodecyl sulphate except that with sodium deoxycholate the various stages of membrane disruption occur at about 10-fold higher equilibrium free detergent concentrations. At sodium deoxycholate concentrations higher than 2.3 mM the viral spike glycoproteins can be separated by sucrose gradient centrifugation or gel filtration into constituent polypeptides E1, E2 and E3. E1 carries the haemagglutinating activity of the virus.

INTRODUCTION

The bile salts are common working tools in the solubilization of membranes and the isolation of integral membrane proteins. Deoxycholate and cholate dissolve membranes effectively and they do this frequently without inactivation of enzymes or dissociation of functional protein complexes (see refs. 1–4). In the presence of excess deoxycholate, the membrane proteins become separated from the lipids [5, 6] and remain soluble by virtue of bound bile salt [4]. The properties of bile salts and bile salt-lipid mixtures in aqueous solution have been well studied (for reviews see refs. 7–11). They differ from the other commonly used detergents with aliphatic and aryl-aliphatic chains in that they form smaller micelles, the micelles have a different struc-

Abbreviation: SF virus, Semliki Forest virus.

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ture, the critical micellar concentration is generally higher and the capacity to solubilize phospholipids is greater. The different properties are due to the molecular structure of bile salts, they have a rigid hydrophobic steroid ring structure, and instead of a clear-cut polar head moiety the polar groups are distributed along one face of the bean-shaped molecule.

We have previously studied the effects of Triton X-100 (arylaliphatic) and sodium dodecyl sulphate (aliphatic) on a simple membrane virus, Semliki Forest virus (SF virus). We observed the following successive stages in the dissociation of its membrane with increasing detergent concentration: (1) binding of detergent to the membrane, (2) lysis of the membrane, (3) solubilization into lipoprotein-detergent complexes, and (4) delipidation of membrane proteins [12–14]. These general features seem to apply to the interaction of these and similar detergents with more complex biological membranes as well (see ref. 4). Whether the bile salts act on membranes by a similar mechanism has not previously been studied in detail. In this study we have analyzed the effect of increasing concentrations of the bile salt, sodium deoxycholate on SF virus.

SF virus is composed of a nucleocapsid surrounded by a membrane. The nucleocapsid contains one RNA molecule (about $4.0 \cdot 10^6$ daltons) and a lysine-rich protein species ($3.4 \cdot 10^4$ daltons) [15]. The nucleocapsids are assembled in the cytoplasm of the host cell and acquire the surrounding membrane from the plasma membrane when budding out of the cell [16]. The lipid composition of the viral membrane is very similar to that of the host cell plasma membrane [17], the lipids being present in a bilayer arrangement [18]. The polypeptides of the membrane, E1 and E2 (both $5 \cdot 10^4$ daltons) and E3 ($1 \cdot 10^4$ daltons), are virus specified, contain carbohydrate and form spikes on the surface of the membrane [19]. The membrane proteins are insoluble in aqueous solution and can only be extracted by detergents [13]. E1 and E2 have been shown to contain hydrophobic peptide segments that anchor them to the lipid bilayer [20, 21] and one or both of these segments extend through the bilayer into close contact with the nucleocapsid surface [22].

MATERIALS AND METHODS

Materials. Sodium deoxycholate (lot number S4197) was purchased from Mann Research Laboratories (U.S.A.). It gave one spot on thin-layer chromatography and contained less than 2 % of other bile salts when analyzed by gas-liquid chromatography [6]. ^{14}C-labelled deoxycholic acid was from Mallinckrodt (U.S.A.). [^{35}S]methionine was from Radiochemical Centre, England, [^{3}H]leucine, [^{3}H]-isoleucine and [^{3}H]valine were from New England Nuclear (U.S.A.), and ^{32}P-labelled carrier-free orthophosphate from the Institutt for Atomenergi (Norway). The RNAase-free sucrose used for gradients was from Mann (U.S.A.). Thyroglobulin (porcine) and pancreatic RNAase were from Sigma (U.S.A) and human γ-globulin from Kabi (Sweden). Dimethylsuberimidiate was synthesized from suberonitrile (Suchard, Switzerland) [23].

Virus. A prototype strain of SF virus was grown in monolayer cell cultures of BHK-21 cells. The culture procedure, virus purification and purity controls were as described by Kääriäinen et al. [24]. For calculations we used the chemical composition of SF virus as given by Laine et al. [15]: 6 % RNA, 12 % nucleocapsid protein, 44 %

membrane protein, 31 % lipid and 6 % protein-bound carbohydrate, and a molecular weight for SF virus of $64 \cdot 10^6$ [15]. Radioactive labelling of SF virus with [^{3}H]-isoleucine, [^{3}H]leucine, and [^{3}H]valine, [^{35}S]methionine and with [^{32}P]orthophosphate was carried out as described previously [12, 24].

Determination of sodium deoxycholate binding to SF virus. The binding determinations and all other experiments, unless otherwise indicated, were performed in 50 mM Na_2HPO_4 (Baker, the Netherlands), 100 mM NaCl (Fluka, Switzerland) titrated to pH 8.0 with 50 mM NaH_2PO_4 (Merck, W. Germany). We have previously used a sucrose gradient centrifugation method to determine the binding isotherms for sodium dodecyl suphate and Triton X-100 (see Fig. 9). Preliminary experiments indicated, however, that much higher free equilibrium sodium deoxycholate concentrations were needed to obtain a comparable degree of binding. To be able to determine the bound sodium deoxycholate above the high "background" of free sodium deoxycholate, higher concentrations of virus were required than those obtained by the sucrose gradient method. We therefore had to use another technique. An aliquot of ^{3}H-labelled virus was pelleted by centrifugation and the pellet was resuspended in a small volume of buffer containing a certain concentration of ^{14}C-labelled sodium deoxycholate. After equilibration an aliquot was taken from the suspension, and the protein and sodium deoxycholate concentrations were determined from the radioactivity. The virus was then repelleted and the remaining sodium deoxycholate and protein concentrations in the supernatant were determined as above. The free sodium deoxycholate concentration was calculated from the supernatant ^{14}C activity after centrifugation and the ratio of bound sodium deoxycholate to protein was calculated from the differences in ^{14}C and ^{3}H activities before and after centrifugation.

The detailed procedure was as follows: 100-μl samples of ^{3}H-labelled SF virus (specific activity $1.5 \cdot 10^9$–$2.0 \cdot 10^9$ cpm/g protein) containing 50 μg protein in 50 mM Tris · HCl, pH 7.2, and 100 mM NaCl were added to 500 μl phosphate buffer in 600-μl centrifuge tubes. The tubes were centrifuged at 20 000 rev./min for 2 h at 4 °C in a SW 50.1 Beckman rotor equipped with adapters. The supernatant was removed and 50 μl of sodium deoxy[^{14}C]cholate (0.1–3.0 mM sodium deoxycholate with specific activities $0.1 \cdot 10^{12}$–$5.0 \cdot 10^{12}$ cpm/mol) was added at 0 °C. The virus pellet was left to resuspend overnight at 4 °C and further resuspended with a Pasteur pipette. The tubes were then incubated at 5 ± 1 or 23 ± 1 °C for 2 h, and aliquots (two times 10 μl) were taken for ^{3}H and ^{14}C determination. The remaining suspension was centrifuged at 12 500 rev./min for 1h in the SW 50.1 rotor at 20 ± 1 °C to pellet the virus with the bound sodium deoxycholate. After centrifugation, two 10-μl aliquots were taken from the supernatant and the ^{3}H and ^{14}C determined.

Analytical ultracentrifugation. Analytical ultracentrifugation was performed in a Beckman Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. Double sector cells, with a 20 mm optical path and the AnE rotor were used. Sodium deoxycholate solutions (700 μl containing 0–3 mM sodium deoxycholate in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl) were added to the cells followed by 10 μl SF virus suspension (100 μg protein). The samples were mixed and centrifuged immediately at 20 °C. The sedimentation of intact virus and large subviral particles was usually followed at 15 000 rev./min and the sedimentation of smaller dissociation products were observed after acceleration to 30 000 or 40 000 rev./min. The cells were scanned at both 260 and 280 nm so that RNA : protein ratio of the boundaries

could be estimated. From a centrifugation in 35 mM sodium dodecyl sulphate, a concentration at which the virus dissociates into free RNA and protein [14], the individual absorption coefficients of these components were estimated.

RNAase digestion of SF virus at different sodium deoxycholate concentrations. To buffer solutions (130 μl) containing 0–1.5 mM sodium deoxycholate, ^{32}P-labelled virus (5 μl) containing less than 1 μg of virus protein was added and the mixture incubated at 23$\pm$1 °C for 10 min. With these low concentrations of virus the amount of sodium deoxycholate bound could be neglected and the free equilibrium sodium deoxycholate concentration could be taken as being equal to the total sodium deoxycholate concentration. After the incubation 0.25 μg of pancreatic RNAase in 1 μl buffer was added and the mixture incubated for 3 min at 23$\pm$1 °C, cooled to 0 °C and immediately layered on pre-cooled 10–50 % sucrose gradients. After centrifugation in a SW 50.1 rotor for 80 min at 45 000 rev./min and 4 °C, fractions were collected from the bottom and the ^{32}P radioactivity determined. The intact virus banded at the isopycnic density (1.185 g/cm^3) and digested virus was found at the top of the gradient.

Negative staining and electron microscopy. SF virus in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl was mixed at 4 °C with an equal volume of sodium deoxycholate solution in the same buffer to give a final concentration of 0.10 mg protein/ml and the appropriate sodium deoxycholate concentration (0–5 mM). A drop was applied to carbon-coated Formvar grids and stained with 1 % potassium phosphotungstate, pH 7.0. The specimens were examined in a Siemens Elmiskop 1A electron microscope at an initial magnification $4 \cdot 10^4$.

Sucrose gradient centrifugation in the presence of sodium deoxycholate. 13 ml sucrose gradients (10–50 % w/w sucrose in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl) were prepared with different concentrations of sodium deoxycholate (0–7.5 mM). SF virus (50 μg viral protein in 200 μl buffer) containing added ^{32}P- and ^{3}H-labelled virus, was layered on to the gradients. No sodium deoxycholate was added to the virus samples. The gradients were centrifuged at 20 °C in a MSE 6 $\times$ 14 ml rotor or the International 488 titanium swing-out rotor at 195 000 $\times$ g for 16 or 24 h. Fractions (0.5 ml) were collected from the bottom and the pellets were dissolved in 0.5 ml 35 mM sodium dodecyl sulphate buffer. Aliquots were taken for radioactivity determination, lipid extraction and polyacrylamide gel electrophoresis.

By extracting the lipid it was possible to distinguish between ^{32}P-labelled phospholipid and [^{32}P]RNA: aliquots (150 μl) were diluted to 300 μl with buffer, 50 μl RNAase solution (30 μg RNAase/ml in buffer containing 30 g Triton X-100/l) was added and the samples were left at 23$\pm$1 °C for 30 min. Then 1.4 ml of chloroform/methanol (2 : 1, v/v) was added and the solution was mixed and left to equilibrate for 2 h. Thereafter 250-μl samples were taken from both the lower organic solvent phase and the upper aqueous phase and these were placed in glass scintillation vials. After incubation overnight at 50 °C the solvents had evaporated and scintillation fluid (10 ml) was added.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Gel electrophoresis in glass tubes was performed using either the continuous buffer system described by Weber and Osborn [25] or that described by Davis and Stark [26]. Slab gel electrophoresis was done using the discontinuous buffer system described by Neville [27]. The radioactivity in cylindrical gels was determined by slicing the gels into 2-mm

segments, treating the slices with Nuclear Chicago Scintillator fluid and counting [28]. The slab gels were impregnated with 2,5-diphenyloxazole (PPO), dried and developed by the fluorographic film detection method described by Bonner and Laskey [29].

Cross-linking of the viral proteins obtained after dissociation with sodium deoxycholate. 5–20 μg of ^{3}H-labelled protein in 50–100 μl buffer containing sodium deoxycholate were mixed with an equal volume of 200 mM triethanolamine containing 10 mg/ml dimethylsuberimidate and the same sodium deoxycholate concentration as in the protein sample. After incubation for 2 h at 23±1 °C the cross-linking reaction was terminated by the addition of sodium dodecyl sulphate (final concentration 35 mM) and 2-mercaptoethanol (final concentration 300 mM) and heating for 15 min at 70 °C. The cross-linked products were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate-according to Davis and Stark [26].

Other methods. Protein was determined by the method of Lowry et al. [30] with 35 mM sodium dodecyl sulphate in the reaction mixture. For the binding isotherm experiments quantitative amino acid analysis with norleucine as an internal standard was used [19]. Radioactivity was measured in a Wallac 8100 or a Nuclear Chicago Mark III liquid scintillation counter in vials containing 10 ml Tritosol [31]. Haemagglutination titres were assayed according to Clarke and Casals [32] using 0.2 % goose erythrocytes. Sucrose concentrations were determined by refractometry with appropriate corrections for the buffer. The critical micellar concentration of sodium deoxycholate was determined from the solubilization of methyl orange [33].

RESULTS

Properties of sodium deoxycholate. The critical micellar concentration of sodium deoxycholate in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl was determined to be 2.6±0.3 mM at 23±1 °C. The average number of sodium deoxycholate molecules per micelle in these conditions is about 16 [34]. In 50 mM sodium phosphate, pH 8.0, 500 mM NaCl the critical micellar concentration was 1.1 mM. Since solutions containing more than 2 mM sodium deoxycholate tended to form gels at 4 °C, most of our experiments were performed at 20–25 °C.

Sodium deoxycholate binding to SF virus. Fig. 1A shows the sodium deoxycholate binding isotherm at 20±2 °C in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl. When the number of mol of bound sodium deoxycholate per mol SF virus, $\bar{\nu}$, was plotted against the free equilibrium sodium deoxycholate concentration, c, the points with c smaller than 0.9 mM fitted a straight line. This suggested that binding in the low sodium deoxycholate concentration region represented partitioning of sodium deoxycholate into the viral membrane rather than binding to a set of saturable binding sites. The partition coefficient calculated from the slope of the line was $0.12 \cdot 10^3$ mol sodium deoxycholate/kg lipid (M). A plot of $1/\bar{\nu}$ against $1/c$ indicated that the increase in binding above 0.9 mM sodium deoxycholate was cooperative [35]. The binding values have not been corrected for binding to the broken non-sedimented virus. Therefore the values above 0.9 mM represent minimal values and the true binding isotherm above 0.9 mM sodium deoxycholate may be still steeper.

Only a small number of experiments were done to study the effects of ionic strength and temperature on the binding isotherm. The results show that increased

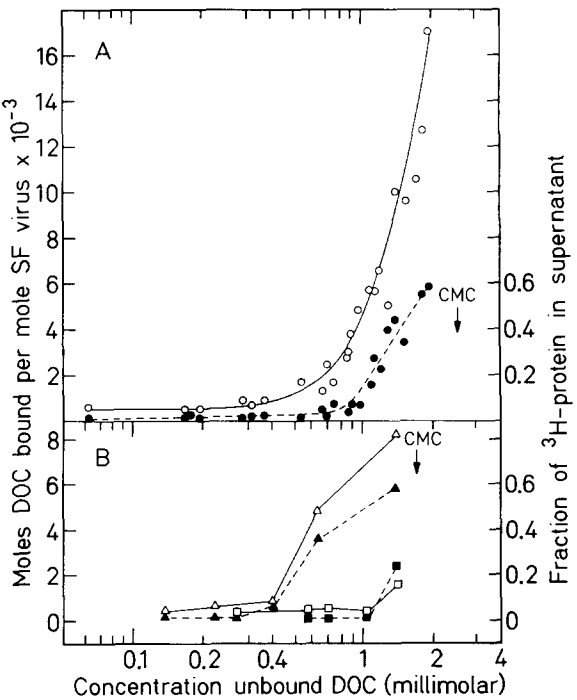

Fig. 1. The binding of sodium deoxycholate (DOC) to SF virus determined from the supernatant protein and sodium deoxycholate concentrations before and after centrifugation. (A) ○—○, binding at 20 °C in 50 mM sodium phosphate buffer, pH 8.0, containing 100 mM NaCl; ●—●, the fraction of ^{3}H-labelled protein remaining in the supernatant. (B) □—□, binding at 4 °C, buffer same as above; ■—■, the fraction of ^{3}H-labelled protein in the supernatant at 4 °C; △—△, binding at 20 °C in 50 mM sodium phosphate, pH 8.0, 500 mM NaCl; ▲—▲, the respective fraction of ^{3}H-labelled protein in the supernatant. The binding values are uncorrected for sodium deoxycholate bound to the unsedimented virus. The arrows show the critical micellar concentration at 24 °C in the buffer of low (A) and high (B) ionic strength.

salt concentration (500 mM NaCl) shifted the start of the cooperative phase of binding (and the start of viral disruption) from 0.9 to 0.4 mM free equilibrium sodium deoxycholate (cf. Figs. 1A and 1B). A decrease in temperature from 20 to 4 °C, on the other hand, shifted these processes to higher free sodium deoxycholate concentrations (from 0.9 to about 1.1 mM) (see Fig. 1B).

Sensitivity to RNAase in increasing sodium deoxycholate. In the intact virus particle the RNA is protected by the viral membrane against digestion by external RNAase, whereas the RNA in the exposed nucleocapsid is sensitive to RNAase. Fig. 2A shows the relative amount of intact virus after a brief treatment with pancreatic RNAase at 20 °C in the presence of increasing concentrations of sodium deoxycholate. By this criterion, the lysis of the membranes started at 0.9 ± 0.1 mM sodium deoxycholate. At 1.2 mM sodium deoxycholate all the virus was lyzed.

Sedimentation velocity in increasing sodium deoxycholate. The effects of increasing sodium deoxycholate on the integrity of the virus were further studied by analytical ultracentrifugation using dilute suspensions of virus (0.1 mg viral protein

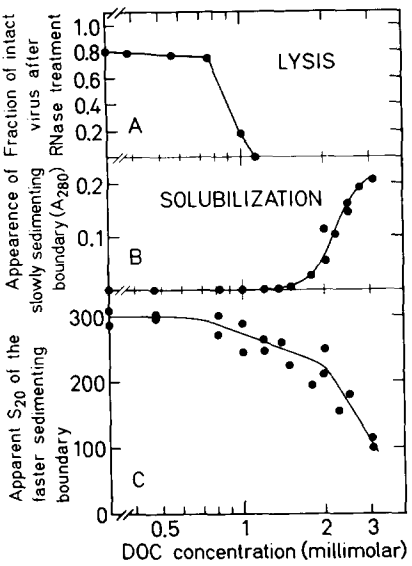

Fig. 2. Lysis, solubilization and sedimentation behaviour of SF virus studied by RNAase treatment and analytical ultracentrifugation. For detailed procedures see text. DOC, sodium deoxycholate.

per ml). Between 0 and 0.8 mM sodium deoxycholate, the change in the sedimentation coefficient of the virus was less than 3 % (Fig. 2C). Between 0.8 and 1.4 mM sodium deoxycholate, there was a gradual decrease in the apparent sedimentation coefficient from 290 S to 210±20 S. The scan of the virus boundary indicated homogeneity (Fig. 3) and there was no sign of more slowly sedimenting ultraviolet-absorbing material, which would have indicated release of nucleocapsids or solubilization of the membranes. At 1.4 mM sodium deoxycholate slowly sedimenting material with an apparent sedimentation coefficient of less than 25 S began to appear. Scanning of the cell at two wavelengths (260 and 280 nm) showed that this material contained protein and less than 1 % RNA. At 3.0 mM sodium deoxycholate, more than 90 % of the viral protein was released into this form (Fig. 2B). Concomitant with the solubilization of the viral membrane, partial dissociation of the nucleocapsids also seemed to take place: The apparent sedimentation coefficient of the

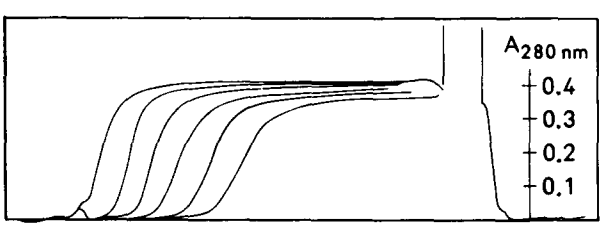

Fig. 3. Analytical ultracentrifugation of SF virus (100 μg protein per ml) in 1.2 mM sodium deoxycholate in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl, 20 °C, 14 290 rev./min. Recorded at 4-min intervals.

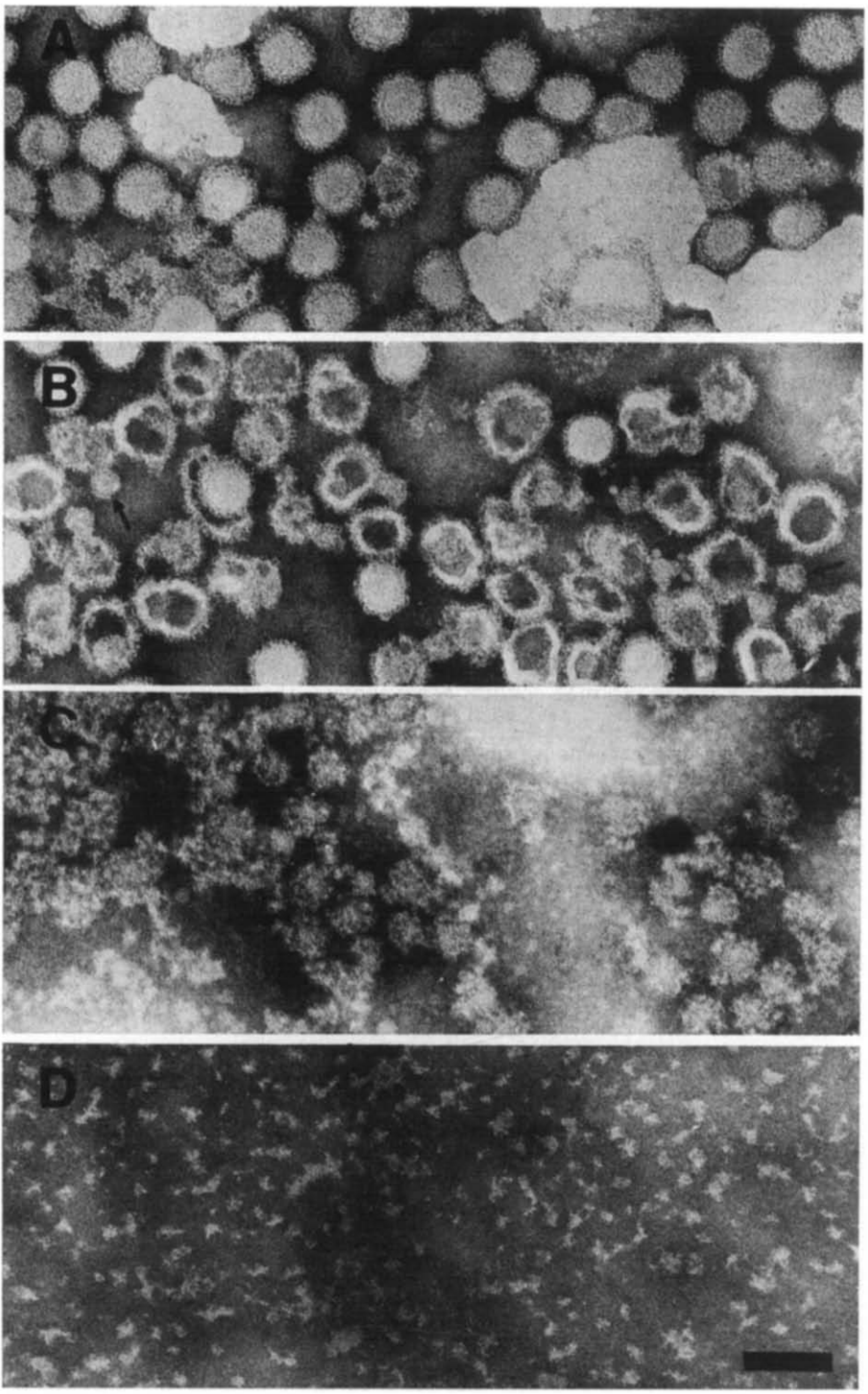

Fig. 4. Electron micrographs of SF virus obtained after negative staining with potassium phosphotungstate. A, control SF virus; B, after treatment with 1.0 mM sodium deoxycholate; C, after treatment with 2.25 mM sodium deoxycholate, and D, after treatment with 2.75 mM sodium deoxycholate. The bar is 100 nm and all the pictures have the same magnification.

faster sedimenting particles decreased from 210±20 S at 1.7 mM sodium deoxycholate to 105±5 S at 3.0 mM sodium deoxycholate (Fig. 2C). At 2.5 mM sodium deoxycholate the protein : RNA ratio of these particles (obtained by scanning at 260 and 280 nm) was about 2 mg protein per mg RNA, which is the ratio observed in isolated intact nucleocapsids. At 3.0 mM sodium deoxycholate, however, there was only 0.2 mg protein per mg RNA.

Electron microscopy of SF virus after sodium deoxycholate treatment. The dissociation of SF virus with increasing concentrations of sodium deoxycholate was also studied by electron microscopy (Figs. 4A–4D). For these studies a low concentration (0.1 mg/ml) of virus was used so that the free sodium deoxycholate concentration would be approximately equal to the total sodium deoxycholate concentration. After treatment with 1.0 mM sodium deoxycholate, some of the virus particles were lyzed (Fig. 4B) and the nucleocapsids (see arrows) either remained in contact with the lysed membrane or were detached. At 2.25 mM sodium deoxycholate the morphology of the membrane was grossly altered (Fig. 4C) and at 2.75 mM sodium deoxycholate and higher concentrations (Fig. 4D) the virus dissociated into small particles of heterogeneous size and shape.

Sucrose gradient centrifugation at increasing sodium deoxycholate concentrations. Sucrose gradients were prepared containing various concentrations of sodium deoxycholate. The virus samples (usually 200 μl containing 50 μg viral protein and added traces of ^{3}H- and ^{32}P- labelled virus) were layered on to the gradients. With such small quantities of virus equilibrium with the gradient sodium deoxycholate concentration was probably attained in the first millimeters of migration in the gradient. The use of virus labelled in its components, allowed the separate quantitation of protein, phospholipid and RNA. The relative amounts of membrane protein and nucleocapsid protein in some of the fractions was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulphate [25].

Figs. 5A–5F shows the results obtained in 10–50 % (w/w) sucrose gradients with 0, 1.0, 1.5, 2.0, 2.25 and 5.0 mM sodium deoxycholate. In the sodium deoxycholate-free control (Fig. 5A) the bulk of the virus banded at its isopycnic position (1.18 g/cm^3). Analysis in sodium dodecyl sulphate gels showed that 84 % of the ^{3}H counts were in the membrane proteins (as shown by the boxed-in number in the figure), and the remaining 16 % in the nucleocapsid protein. With 1.0 mM sodium deoxycholate in the gradient, two bands were obtained; one at the position of the control virus and the other at 1.16 g/cm^3 (Fig. 5B). Electron microscopy of the material from the latter band showed lysed virus, some free nucleocapsids and nucleocapsid-free membrane sacs (cf. Fig. 4B), but no intact virus particles. As compared to intact virus, the particles in this band contained less RNA relative to phospholipid and less nucleocapsid protein relative to membrane protein. This was because part of the nucleocapsids had dissociated from the lysed virus and sedimented to the bottom.

In 1.5 mM sodium deoxycholate (Fig. 5C) there was still virus banding at 1.18 g/cm^3. The second band (fractions 10–13) appeared clearly heterogeneous and contained no detectable RNA. This band probably consisted of a mixture of lysed membranes, membrane fragments and lipoprotein complexes of varying size and lipid content. In addition, there was a minor band (fractions 18–20) consisting of protein and phospholipid, suggesting the presence of smaller lipoprotein complexes. No dis-

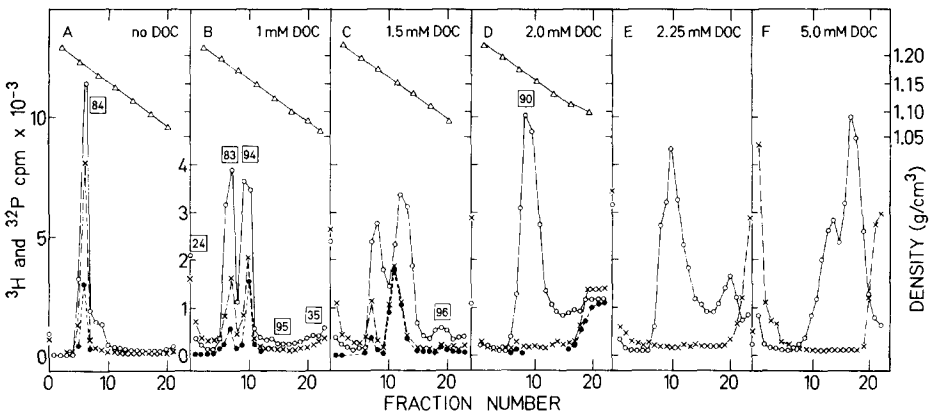

Fig. 5. Sucrose gradient centrifugation of SF virus in the presence of sodium deoxycholate (DOC). Gradients: 13 ml 10–50 % sucrose in 50 mM sodium phosphate, pH 8, 100 mM NaCl and the concentration of sodium deoxycholate indicated. Centrifugation at 195000 × *g* for 16 h at 20 °C. ○ — ○, ^{3}H-labelled protein; × — ×, total ^{32}P; ● — ●, lipid ^{32}P, and △ — △, density. The boxed in numbers in A, B, C, and D indicate the percentage of membrane protein counts of total protein counts in the respective peaks. Sedimentation was to the left.

sociation of lipid and protein could be detected, all the ^{32}P-labelled phospholipid activity remained associated with the ^{3}H-labelled protein peaks. With 2.0 mM sodium deoxycholate in the gradient (Fig. 5D) almost all the phospholipid was recovered in the five uppermost fractions of the gradient. It was separate from the major protein band which had a sedimentation coefficient of 25–30 S. Although free from RNA, this band still contained 10 % nucleocapsid protein. At 2.25 mM sodium deoxycholate (Fig. 5E) all phospholipid remained at the top of the gradient. At this sodium deoxycholate concentration a major protein band occurred in fractions 19–21. It sedimented with a rate of 3–5 S using IgG as reference. The band became more prominent when the sodium deoxycholate concentration was increased until it at about 5 mM sodium deoxycholate comprised 55 % of the membrane protein in the gradient. Further increase in gradient sodium deoxycholate concentration did not change the sedimentation pattern any more, but when the centrifugation time was increased from 16 to 24 h an additional slowly sedimenting band (band III in Fig. 6) could be resolved.

The polypeptide composition of the three bands (I, II and III) and the pellet (Fig. 6) was analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (Fig. 7). The pellet contained the nucleocapsid protein, band I contained all the E2 and some residual E1, band II contained E1, and the small peak III contained the E3. Separation of the four polypeptides could thus be obtained in a single step. Haemagglutination activity was detected in peak II only.

A similar separation of the viral polypeptides could be obtained by gel filtration of SF virus at 22±1 °C in a Sepharose 6B column (0.8 × 50 cm) equilibrated with 5 mM sodium deoxycholate in 50 mM sodium phosphate, pH 8, 100 mM NaCl (not shown). The virus (0.4 mg viral protein in 0.3 ml) was applied to the column without added sodium deoxycholate. The nucleocapsid protein emerged at the void volume, the E2 (and some E1) with $K_{av} = 0.35$, the E1 with $K_{av} = 0.62$, and the E3

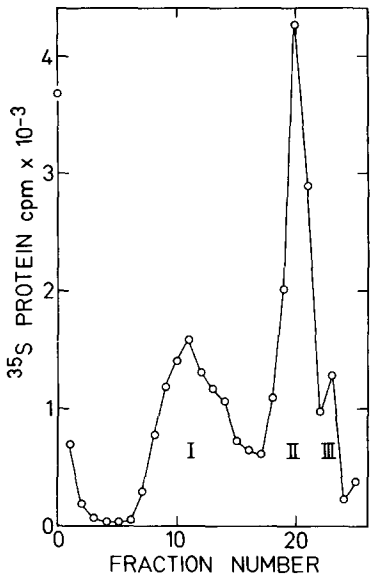

Fig. 6. Sucrose gradient centrifugation of [^{35}S]methionine-labelled SF virus in 7.5 mM sodium deoxycholate for 24 h. Except for the higher sodium deoxycholate concentration and longer centrifugation time, conditions were as in Fig. 5. Sedimentation was to the left.

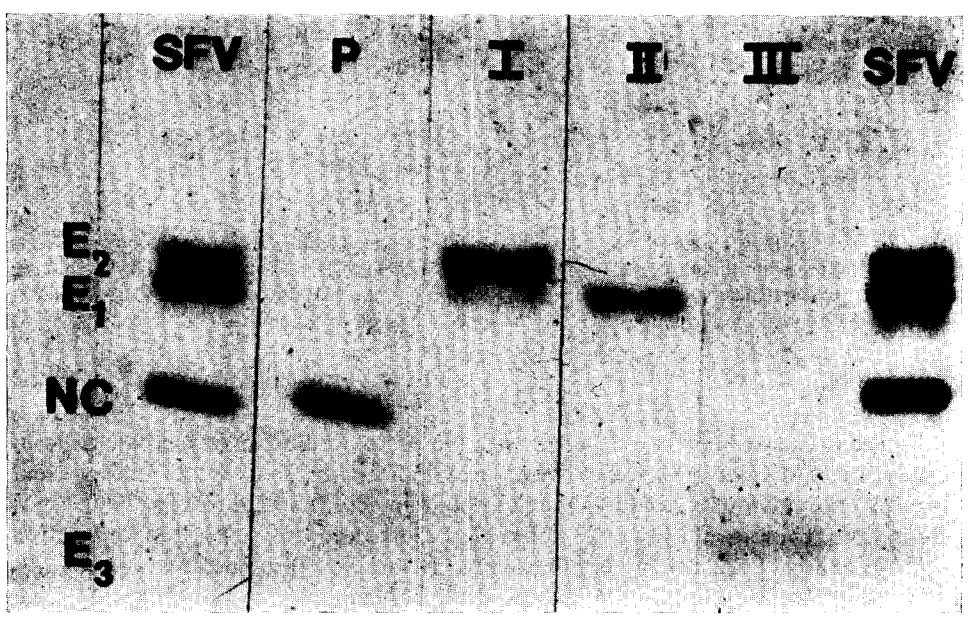

Fig. 7. Polyacrylamide slab gel electrophoresis in sodium dodecyl sulphate of fractions from the 7.5 mM sodium deoxycholate gradient in Fig. 6 and of [^{35}S]methionine-labelled control virus. The bands were developed by a fluorographic film detection method [29]. SFV, control virus; P, pellet.

with $K_{av} = 0.88$. The RNA eluted in the void volume and the phospholipids close to the full volume of the column.

The E1 and E2 glycopolypeptides have apparent molecular weights of $49 \cdot 10^3$ and $52 \cdot 10^3$ [19]. The fact that they can be separated by sucrose gradient centrifugation and gel filtration should thus be due to a difference in their states of aggregation. To study this question fractions from peaks I and II (Fig. 6) were treated with dimethylsuberimidate (a bifunctional cross-linking agent) and the products analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. With material from peak I (not shown) 60 % of the ^{3}H-labelled protein was in the upper part of the gel and the remaining proteins occurred as smaller oligomers with only 16 % of the protein in monomer form. With peak II material on the other hand, 48 % of the material was found in the monomer band (Fig. 8). These patterns suggest that E2 occurs as an aggregate and that E1 occurs as a monomer in sodium deoxycholate concentrations above 5 mM. That E1 is a monomer in sodium deoxycholate is further supported by the pattern obtained from spike protein extracted by Triton X-100. The Triton X-100 complexes are known to contain dimers of E1 and E2 [13] and they show a predominance of dimers after cross-linking (Fig. 8).

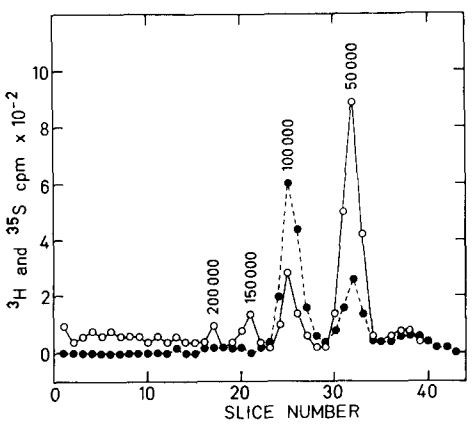

Fig. 8. Polyacrylamide slab gel electrophoresis in sodium dodecyl sulphate of isolated spike proteins after cross-linking with dimethylsuberimidate. ○—○, ^{3}H-labelled E1 in 5 mM sodium deoxycholate; ●—●, ^{35}S-labelled spike protein extracted with Triton X-100 [13]. The cross-linked ^{3}H- and ^{35}S-labelled proteins were electrophoresed in the same gel. Cathode to the left.

DISCUSSION

The dissociation of the SF virus with increasing sodium deoxycholate concentration can be divided into four stages depending on the free equilibrium concentration of sodium deoxycholate; (1) prelytic sodium deoxycholate binding, 0–0.7 mM sodium deoxycholate, (2) lysis, 0.8–1.1 mM sodium deoxycholate, (3) solubilization and delipidation, 1.5–2.3 mM sodium deoxycholate and (4) dissociation of the spike subunit structure, above 2.3 mM sodium deoxycholate.

Prelytic sodium deoxycholate binding. At concentrations below 0.9 mM, sodium deoxycholate binds to the virus and our data seem to suggest that this binding represents partitioning of sodium deoxycholate molecules between the membrane and

the aqueous phase with a partition coefficient of 0.120 mol sodium deoxycholate/kg lipid (M). A small set of discrete saturable binding sites could, however, go undetected. Recent sodium deoxycholate binding studies with sonicated egg lecithin and egg lecithin-cholesterol liposomes (molar ratio 1 : 1) performed by N. M. Green (personal communication) have yielded binding isotherms similar to that obtained for the virus. The partition coefficients measured by Green were $0.260 \cdot 10^3$ mol sodium deoxycholate/kg lipid (M) for the egg lecithin liposomes and $0.024 \cdot 10^3$ mol sodium deoxycholate/kg lipid (M) for the egg lecithin-cholesterol liposomes. SF virus contains phospholipids and cholesterol in a ratio of 1.2 to 1.

Detergents of the aliphatic and arylaliphatic type are generally assumed to become intercalated between the lipid molecules of the bilayer membrane when bound. It is more difficult to picture the localization of sodium deoxycholate and other bile salts in lipid bilayers. They are bulky molecules and have the polar groups spread over the length of the molecule. Small [36] has postulated that the bile salts may be present as dimers within the bilayer with the hydrophilic surfaces of the molecules turned towards each other.

Lysis. Lysis of the viral membranes began at 0.9 mM free equilibrium sodium deoxycholate. At this sodium deoxycholate concentration $2.2 \pm 0.2 \cdot 10^3$ mol of sodium deoxycholate are bound per mol of SF virus. There are about $35 \cdot 10^3$ molecules of lipid and $7 \cdot 10^2$ molecules of protein in each viral membrane. Thus the mol fraction of sodium deoxycholate to lipid required for lysis is 0.06. The equivalent mol fractions for sodium dodecyl sulphate and Triton X-100 are 0.15 and 0.14, respectively (ref. 14 and our unpublished results). There is another significant difference between sodium deoxycholate and the other two detergents: the equilibrium concentration needed to cause lysis is 10-fold higher for sodium deoxycholate (Fig. 9). The relatively low affinity of sodium deoxycholate has been observed also with other membranes; red blood cells and microsomal vesicles lyse above 0.6–0.7 mM sodium deoxycholate, whereas 0.02 mM Triton X-100 and 0.01 mM sodium dodecyl sulphate suffice [37–39].

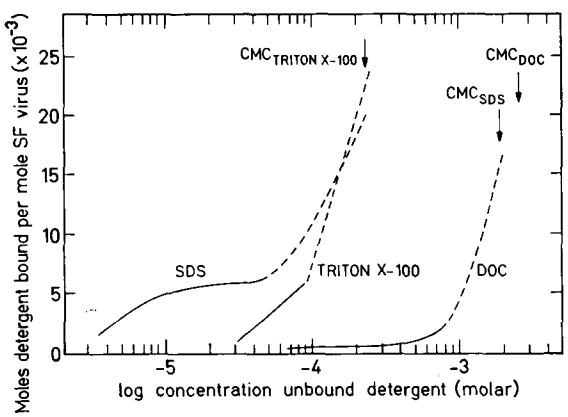

Fig. 9. Isotherms for binding of sodium dodecyl sulphate (SDS), Triton X-100 and sodium deoxycholate (DOC) to SF virus. The sodium dodecyl sulphate isotherm (4 °C) is from Becker et al. [14] and the Triton X-100 isotherm (4 °C) is our unpublished data. The sodium deoxycholate isotherm was determined at 20 °C. The detergent concentrations at which viral lysis occurs are indicated by the broken lines. CMC, critical micelle concentration.

The lysis of the viral membrane with sodium deoxycholate coincided with a cooperative increase in binding. This binding is probably due to the sudden accessibility of the internal membrane surface and perhaps also to the loss of restraints on the membrane upon lysis. Similar cooperative binding increases were also observed with sodium dodecyl sulphate and Triton X-100 (ref. 14, our unpublished results) and with the binding of the surface active local anaesthetic chlorpromazine to red blood cell membranes [40]. The data of Green indicate that egg lecithin liposomes begin to lyse at 0.9 ± 0.1 mM equilibrium sodium deoxycholate at conditions similar to ours, and that there is a concomitant cooperative increase in binding.

All the virus particles were not lysed at a single free sodium deoxycholate concentration. Sometimes occasional intact viruses could be observed even at 2 mM sodium deoxycholate. Whether the nucleocapsid dissociated from the membrane when it lysed or not depended on the conditions. In sucrose gradients containing 1.5 mM sodium deoxycholate, the nucleocapsids were effectively released, but not during the analytical ultracentrifugations. Sodium deoxycholate has previously been used to release nucleocapsids from SF virus [24] and the closely related Sindbis virus [41]. The nucleocapsids we obtained using sodium deoxycholate were inferior to those obtained with Triton X-100. Usually they contained some bound envelope protein and at sodium deoxycholate concentrations above about 2 mM they became unstable.

Solubilization and delipidation. The solubilization of the viral membrane (defined as the release of slowly sedimenting material) began at about 1.5 mM sodium deoxycholate. As in the case of sodium dodecyl sulphate and Triton X-100 the initial products of solubilization included lipo-protein complexes. These probably also contained bound sodium deoxycholate. With increasing sodium deoxycholate concentration the lipid was removed from the protein and at 2.25 mM sodium deoxycholate the membrane proteins were free from phospholipid. The sequence of events with increasing sodium deoxycholate resembles that observed with both sodium dodecyl sulphate and Triton X-100 with the important difference that with sodium deoxycholate the free equilibrium concentrations required for solubilization and delipidation are about 10-fold higher. Our data indicate that the solubilization of the SF virus membrane occurs at a free equilibrium concentration below or close to the critical micellar concentration of sodium deoxycholate. The critical micellar concentration of bile salts, however, is not as well defined a concentration interval as that of aliphatic and arylaliphatic detergents, nor is it easy to measure unequivocally.

Comparison of our data with the results of previous studies on membrane solubilization by sodium deoxycholate is complicated by the fact that we have correlated the effects with the free equilibrium sodium deoxycholate concentration (which below the critical micellar concentration essentially gives a measure for the chemical potential of sodium deoxycholate in the system) and not with the sodium deoxycholate to membrane ratio, as has been done by most other investigators. Differences in experimental conditions also make direct comparison difficult, since the properties of sodium deoxycholate are very sensitive to the nature and concentration of counter ions, the pH and also, to some extent, to temperature (see ref. 34). Taken together the work on a variety of different membranes [5, 37, 42–52] indicates, however, that the overall scheme of binding, lysis, solubilization and delipidation as described

above for SF virus membranes may be generally operative with sodium deoxycholate. Meissner and Fleischer [51] and Garland and Cori [52] have shown that soluble lipoprotein complexes occur as intermediates in the solubilization of sarcoplasmic and microsomal membranes.

Sodium deoxycholate has been widely used in membrane reconstitution studies based on the spontaneous reassembly of solubilized lipid and protein upon removal of detergent (see ref. 2). The reason why sodium deoxycholate (and cholate) have proved more useful than other detergents can partly be understood on the basis of our present results. The fact that the free equilibrium detergent concentration at all stages of membranes dissociation are 10-fold higher with sodium deoxycholate than with Triton X-100 and sodium dodecyl sulphate facilitates the removal of this detergent by dialysis or gel filtration. These methods remove the unbound detergent and thereby indirectly also, by equilibration, the bound detergent. When reconstitution is performed by dilution of solubilized membranes, the extent of dilution needed to reduce the amount of sodium deoxycholate bound and reverse the solubilization effect is much smaller than with Triton X-100 and sodium dodecyl sulphate. The overall amount of detergent required for effective membrane solubilization seems to be similar for the three detergents [4, 45].

Dissociation of the membrane spike glycoproteins. The viral membrane spikes have a molecular weight of $1 \cdot 10^5$ [19], and contain, most likely, one copy of E1, E2 and E3. We have not been able to separate the constituent spike polypeptides in Triton X-100 solution as has been achieved with the proteins of the related Sindbis virus (J. Dalrymple, personal communication; K. Keegstra, personal communication). In sodium dodecyl sulphate this can be done, but the proteins are denatured [19]. Sodium deoxycholate can, as shown here, be used for the separation under conditions which are essentially non-denaturing. This has now made it possible to assign the haemagglutinating activity of SF virus to the E1 polypeptide. A more detailed study of the properties of the membrane glycoproteins in sodium deoxycholate will be presented elsewhere.

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