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STRUCTURE OF THE LIPID-CONTAINING BACTERIOPHAGE ϕ6

DISRUPTION BY TRITON X-100 TREATMENT

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Summary

The structure of the lipid-containing bacteriophage ϕ6 was studied by means of controlled Triton X-100 disruption and subsequent isolation of subviral particles. Rate-zonal centrifugation yielded two fractions, a nucleocapsid fraction with RNA, proteins P1, P2, P4, P7, P8, and about half of the protein P5 and a membrane fraction with associated proteins P3, P6, P9, P10, and the rest of the protein P5. Following isopycnic sucrose gradient centrifugation, an empty capsid fraction was obtained which lacked RNA but contained a protein composition similar to the nucleocapsid except for the absence of P5. The membrane fraction isolated after isopycnic centrifugation was morphologically indistinguishable from that isolated after rate-zonal centrifugation but contained only proteins P3, P6, P9 and P10. By treating ϕ6 with Triton X-100 prior to isopycnic sucrose gradient centrifugation the viral membrane was further separated into submembrane structures and the attachment protein, P3, could be isolated in rather pure form.

Introduction

The *Pseudomonas phaseolicola* bacteriophage ϕ6 [1] has two unique characteristics among bacterial viruses. The viral double-stranded RNA genome is divided into three polycistronic segments [2] and the polyhedral nucleocapsid is surrounded by a membrane [1,3]. The nucleocapsid also contains a virion-associated RNA polymerase activity [4—6]. This phage therefore provides an excellent model system for the elucidation of the replication process in the

double-stranded RNA viruses and for the study of the structure and assembly of biological membranes.

The virion contains an electron-dense core, obviously the location of the viral genome [3,7]. The core is surrounded by a capsid structure, around the outside of which is a membranous outer layer [3,8]. This membrane envelope can be fractured into two leaflets, indicating that this is a typical bilamellar biological membrane [9]. The virus is effectively inactivated by organic solvents and detergents [1], thus exposing the capsid [3,8]. The virus codes for ten structural polypeptides (P1—P10), one precursor polypeptide (P11) and one non-structural polypeptide (P12) [10]. P1, P2, P4 and P7 are early proteins and, together with the late protein P8, form the nucleocapsid [11,12]. Proteins P3, P5, P6, P9 and P10 can be extracted from the virion with Triton X-100 [11,12] and are therefore considered as components of the viral membrane [10]. Protein P8 is located on the surface of the nucleocapsid and protein P3 is on the surface of the whole virion [12]. The protein P3 together with P6 are needed for host penetration [13]. At least, proteins P1 and P2 are required for RNA synthesis [10] and P5 is responsible for the lytic activity found early and late in the infection [14—16].

We have studied the structure of bacteriophage $\phi 6$ using controlled disruption of the virus with the non-ionic detergent, Triton X-100. This technique was developed by Helenius and Söderlund [17] for the study of Semliki Forest virus structure. The same approach has been used later to study the structure of other enveloped viruses (see, for example, Refs. 18—20). We describe here the isolation of $\phi 6$ subviral particles and their characterization using electron microscopy and sodium dodecyl sulphate-polyacrylamide gel electrophoresis and discuss the structure of this virus based on these and previous results.

Materials and Methods

Chemicals. The labeled chemicals, [5-^{3}H]uridine (27 Ci/mmol), [U-^{14}C]protein hydrolysate (57 mCi/mgatom carbon) and [^{32}P]orthophosphate (10 mCi/ml) were all obtained from The Radiochemical Centre, Amersham, U.K. Triton X-100 was of scintillation grade from Koch-Light Laboratories. All other chemicals were of analytical grade.

Growth, labeling and purification of the phage. Bacteriophage $\phi 6$ and its host *P. phaseolicola* HB10Y were originally provided by Dr. Anne K. Vidaver [1]. The NBY * medium [21] was used for unlabeled-phage production and measuring of the infectivity. The host was grown at 26°C with aeration to $6 \cdot 10^8$ cells/ml and phage added to a final phage : bacterium ratio of 30 : 1. The culture was incubated with aeration until lysis occurred.

RNA labeling. A modified MSC * medium [22] was used. The Mn^{2+}, Zn^{2+} and Fe^{3+} salts were replaced by adding 100 ml of city water per l of medium. At the time of infection, 4 μCi/ml of [5-^{3}H]uridine was added. Otherwise the procedure was the same as for unlabeled-phage production.

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* NBY: contains (per l) 5.0 g nutrient broth (Difco), 2.0 g yeast extract (Difco), 2.0 g K_2HPO_4, 0.5 g KH_2PO_4, 5.0 g glucose, 0.25 g $MgSO_4 \cdot 7H_2O$. MSC: contains (per l) 5.9 g K_2HPO_4, 2.2 g KH_2PO_4, 1.32 g $(NH_4)_2SO_4$, 0.13 g $MgSO_4 \cdot 7H_2O$, 10.0 g sucrose, 1.0 g casamino acids (vitamin free, Difco).

^{32}P-labeling of RNA and phospholipids. TG9 medium [23] was used. In place of the specific amino acids used by Sands [23], our medium contained 1 g Difco casamino acids per l. The bacteria were grown to $3 \cdot 10^8$ cells/ml and infected with a phage : bacterium ratio of 30 : 1. At the time of infection 15 μCi/ml of [^{32}P]orthophosphate was added.

Protein labeling. M9 medium [24] was used except that the added amino acids were replaced by adding 20 mg Difco casamino acids per l. The host was grown to $6 \cdot 10^8$ cells/ml and [U-^{14}C]protein hydrolysate (2 μCi/ml) was added 6 min prior to infection. Otherwise the procedure was repeated as with unlabeled phage.

Purification. After lysis the cell debris was removed by low-speed centrifugation and the virus in the supernatant was concentrated with polyethylene glycol 6000 using the method of Yamamoto et al. [25]. The polyethylene glycol-treated phage was allowed to stand on ice overnight and was then pelleted by centrifugation at 10 000 rev./min, (Sorvall GSA rotor) for 15 min at 4° C. The pellet was carefully resuspended into buffer A [1] to 1/100 of the original lysate volume. This phage sample was purified by centrifugation through a linear 10—40% (w/w) sucrose gradient in buffer A in a Spinco SW27 rotor at 24 000 rev./min for 80 min at 4° C. After centrifugation the infectious band was removed, diluted 1 : 3 into buffer A and pelleted using the same rotor at 24 000 rev./min for 2 h. The pellet was resuspended for at least 3 h in a desired volume of buffer A and layered on a linear 30—60% (w/w) sucrose gradient in buffer A. The gradients were then centrifuged in an SW27.1 rotor at 24 000 rev./min for 18 h at 4° C. The infectious band was collected, diluted 1 : 5 into buffer A and pelleted as before. The pellet was resuspended into buffer A to obtain a final protein concentration of 1 mg/ml (determined by using the method of Lowry et al. [32]) for unlabeled phage.

Disruption of the phage with Triton X-100. The unlabeled purified virus preparation was separately mixed with the different labeled purified viruses to obtain about 1 mg viral protein/ml and this mixture was used in the different centrifugation procedures.

Rate-zonal centrifugation. 150-μl samples were layered on linear 20—50% (w/w) sucrose gradients in buffer A containing 0, 100, 130 or 150 μg of Triton X-100/ml gradient. Centrifugation in an SW50.1 rotor was performed at 40 000 rev./min for 100 min at 4° C, after which the positions of the visual bands were determined. 150-μl fractions were collected from the bottom and the infectivity as well as the radioactivity of the fractions were measured.

Isopycnic sucrose gradient centrifugation of the whole virus. 150-μl samples, as in the rate-zonal centrifugation, were layered on linear 30—60% (w/w) sucrose gradients in buffer A containing 0, 100, 120 or 140 μg Triton X-100/ml gradient and centrifuged at 35 000 rev./min for 20 h at 4° C (SW50.1 rotor). The fractions were collected and measured as above. In addition, the refractive index was determined.

Isopycnic sucrose gradient centrifugation of the viral membrane. ^{32}P- or [^{3}H]uridine-labeled virus preparations were separately mixed with purified unlabeled virus. This virus preparation containing 1 mg protein/ml was treated with 150 μg Triton X-100/100 μg of virus protein for 3 min at room temperature. 150-μl samples were layered on linear 10—40% (w/w) sucrose gradients

in buffer A containing 150 μg Triton X-100/ml gradient. After centifugation at 35 000 rev./min for 20 h at 4°C (SW50.1 rotor), the positions of the visible bands were measured, the tubes were punctured from the bottom and the gradients pumped through a Uvicord S ultraviolet monitor operating at A_{280} and fractionated. Also the radioactivity of the fractions was measured.

Electron microscopy. The samples for electron microscopy were taken for unlabeled peak fractions, dialysed overnight against buffer A and stained on grids either with 1% (w/v) potassium phosphotungstate, pH 6.5, or with 0.5% (w/v) uranyl oxalate, pH 5.5. All micrographs were taken with a JEM 100 B electron microscope operating at 80 kV.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The discontinuous gel system used was essentially that described by Laemmli [26] with the modifications described previously [27]. The separation gel was 15% (w/v) acrylamide and the gels 1 mm thick. The staining of the gels was performed by using the method of Fairbanks et al. [28] but omitting solution 3.

Results

Disruption of φ6 to subunits rate-zonal centrifugation in the presence of Triton X-100

The effect of Triton X-100 on the radioactivity labeled ([^{14}C]protein, [^{3}H]-uridine or [^{32}P]) φ6 was studied using rate-zonal sucrose gradient centrifugation in the presence of increasing concentrations of Triton X-100. The virus sedimented as one sharp band if Triton X-100 was absent. Infectivity of about $2 \cdot 10^{12}$ plaque-forming units (p.f.u.)/ml was found in the corresponding peak fraction. Most of the input infectivity (75%) and radioactivity (85%) were recovered in this peak (Fig. 1A). The ten structural proteins of the virion [11,12] were found in this peak (Fig. 3, slot φ6). As long as the Triton X-100 concentration in the gradient was below 130 μg/ml the virus sedimented as a single band (Fig. 1B) but the infectivity was almost completely lost. The sedimentation rate of the virus was reduced with increasing Triton X-100 concentration, probably due to the detergent binding to the viral membrane. These uninfective φ6 particles had the same protein composition as the intact viruses (Fig. 3, slot TX). Both intact and uninfective particles described above are morphologically similar to intact viruses previously studied by using electron microscopy [3].

When the Triton X-100 concentration in the gradient was increased to 130 μg/ml, virus disruption began (Fig. 1C) and at a concentration of 150 μg/ml two separate classes of subviral particles were obtained (Fig. 1D). These particles were identified as the faster sedimenting nucleocapsid and the slower sedimenting membrane based on electron microscopy (Fig. 2) and label distribution (Fig. 1). All RNA label and slightly less than half of the protein and phosphorus labels were found in the nucleocapsid fraction. Electron-microscopic examination of this material revealed polyhedral capsids with projections (Fig. 2B, arrows). The capsid alone had a diameter of about 45 nm but with projections about 50 nm. The projections are more clearly seen at higher magnification in Fig. 2B. These capsids formed large aggregates (not shown) and only rarely were individual capsids seen in electron-microscopic prepara-

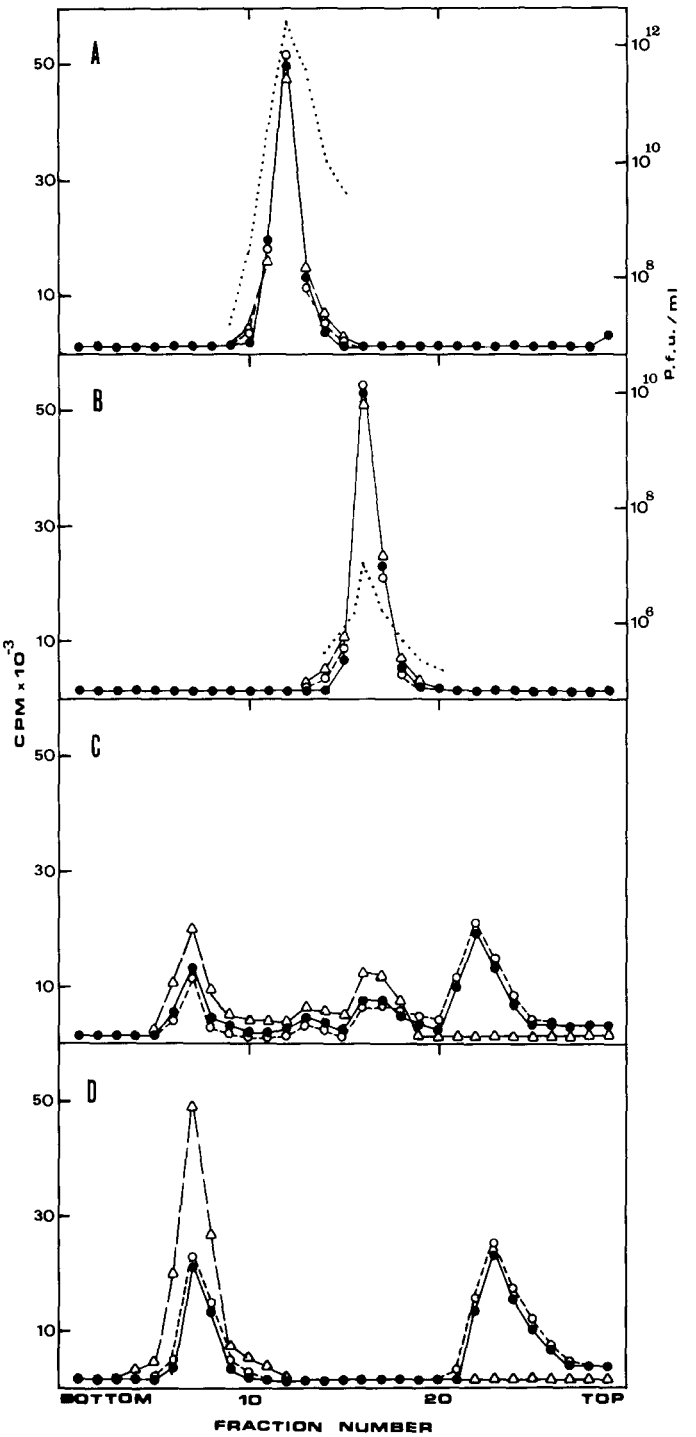


Fig. 1. Disruption of ϕ6 by rate-zonal sucrose gradient centrifugation in the presence of Triton X-100. ϕ6 was sedimented through a 20—50% (w/w) linear sucrose gradient in buffer A using an SW50.1 rotor (40 000 rev./min) for 100 min at 4°C with 150 μl sample volume (1 mg protein/ml). (A) No detergent, (B) 100, (C) 130 and (D) 150 μg Triton X-100 per ml gradient. . . . . , infectivity (p.f.u./ml); ●———●, [^{32}P]; △———△, [^{3}H]uridine; ○———○, ^{14}C-labeled amino acids.

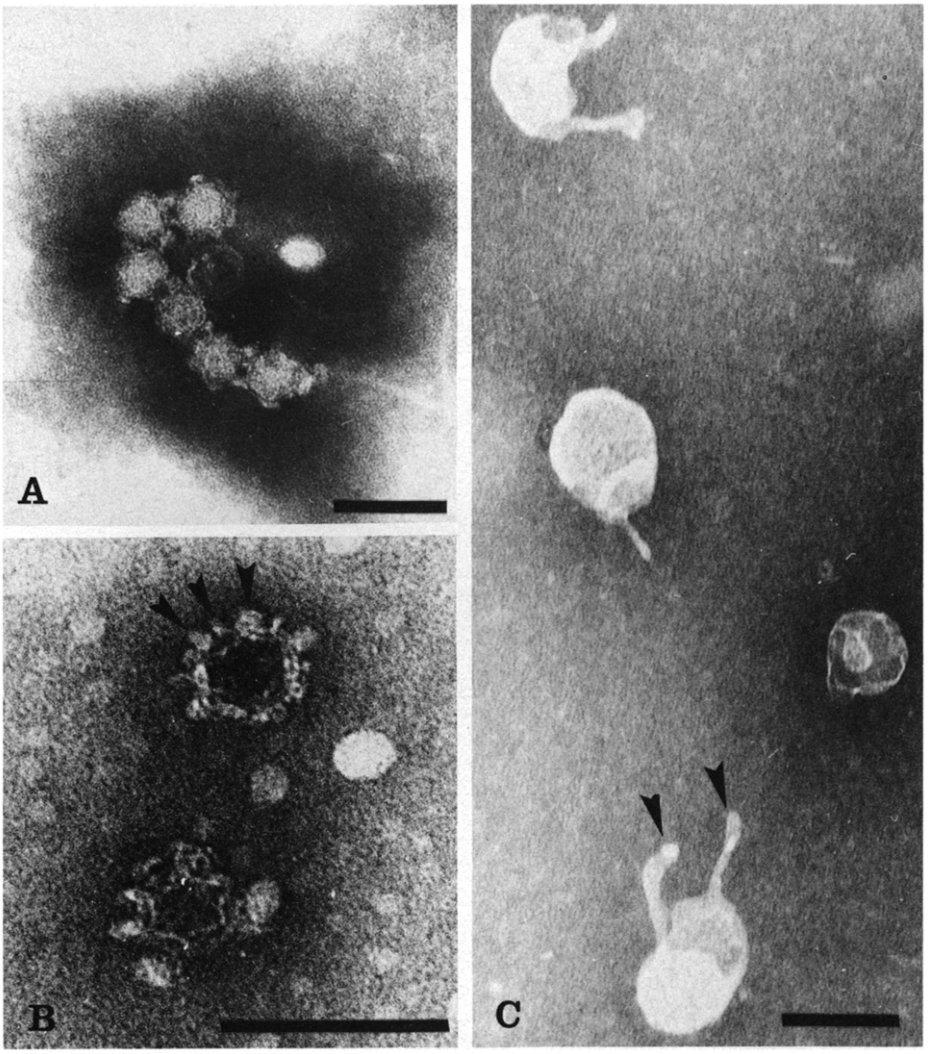


Fig. 2. (A) and (B) potassium phosphotungstate staining of the nucleocapsid and (C) the membrane fractions shown in Fig. 1D. The bar represents 100 nm.

tions. This nucleocapsid fraction contained the proteins P1, P2, P4, P7, P8 and some of proteins P5 (Fig. 3, slot NC).

The slower sedimenting envelope peak (Fig. 1D) had the rest of the protein and phosphorus labels. Fig. 2C shows the image of this fraction. The material contained disrupted membrane vesicles with an outer diameter between 80 and 180 nm. These vesicles had often one or two long projections (arrow in Fig. 2C) and an opening. The envelope had a protein composition of P3, P6, P9, P10, and some of protein P5 (Fig. 3, slot M). Thus, the viral proteins could be clearly separated to those of the nucleocapsid or the membrane except for protein P5 which under these conditions was about equally divided between the nucleocapsid and the membrane.

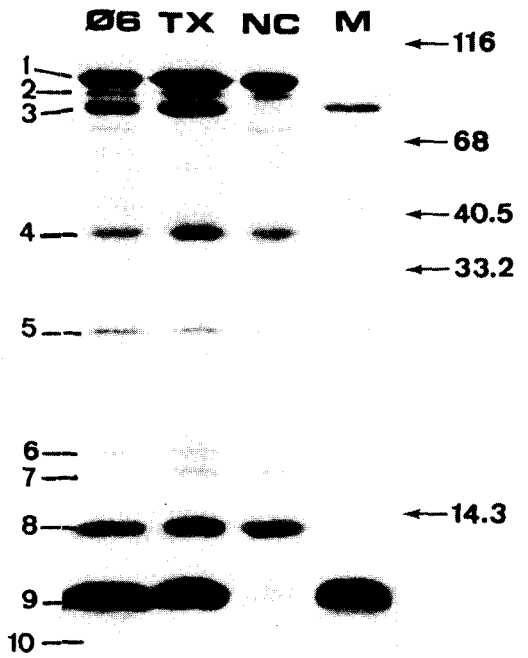


Fig. 3. Sodium dodecyl sulphate-polyacrylamide (15%) gel elctrophoresis of $\phi 6$ and isolated subviral particles (after Triton X-100 treatment in rate-zonal centrifugation). TX, material from the uninfective peak in Fig. 1B. NC, nucleocapsid material from the faster sedimenting peak in Fig. 1D. M, membrane material from the slower sedimenting peak in Fig. 1D. Standards: 116, β-galactosidase (M_r 116 000); 68, bovine serum albumin (M_r 68 000); 40.5, creatine kinase (M_r 40 500); 33.2, arabinose-binding protein (M_r 33 200); 14.3, lysozyme (M_r 14 300).

Viral subunits obtained by isopycnic sucrose gradient centrifugation in the presence of Triton X-100

The Triton X-100 disruption of $\phi 6$ was further studied using 30—60% (w/w) isopycnic sucrose gradients containing increasing concentrations of Triton X-100. In the absence of the detergent, the virus showed a density of 1.230—1.240 g/cm^3 at equilibrium. The infectivity of this peak was about $2 \cdot 10^{12}$ p.f.u./ml and the protein, RNA and phosphorus radioactive labels were all recovered in this peak (Fig. 4A). Addition of Triton X-100 to the gradient (up to 120 μg/ml) again caused the loss of infectivity (Fig. 4B) but no change in the label distribution (Fig. 4B), morphology (not shown) or protein composition (Fig. 6, slot TX) was detected. The only change in addition to the loss of infectivity was the decrease in particle density (1.200 g/cm^3) when the gradient contained 100 μg Triton X-100/ml (Fig. 4B), obviously reflecting Triton X-100 binding to virus particles.

The virus disruption started at a slightly lower concentration (120 μg/ml) of Triton X-100 than in the rate-zonal centrifugation and was complete at a concentration of 140 μg/ml (Fig. 4C and D). The nucleocapsids pelleted under these conditions (not shown) whereas membrane vesicles were found as before in the less dense part of the gradient. This membrane fraction had a very similar morphology (Fig. 5B) and protein composition, except for the lack of

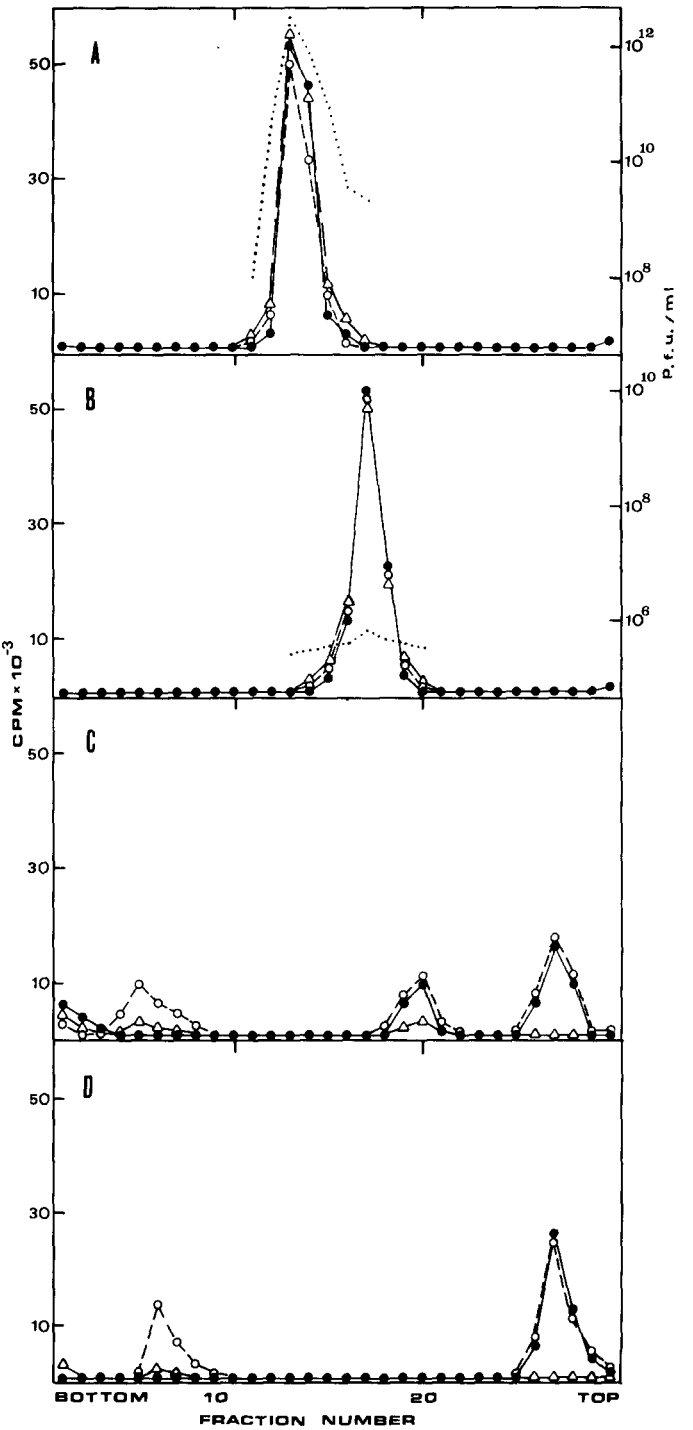


Fig. 4. Disruption of ϕ6 by isopycnic sucrose gradient centrifugation in the presence of Triton X-100. The material was centrifuged into equilibrium in a 30—60% (w/w) linear sucrose gradient in buffer A: rotor and samples as in Fig. 1. (A) No detergent, (B) 100, (C) 120 and (D) 140 μg Triton X-100 per ml gradient. Symbols are the same as in Fig. 1.

protein P5 (Fig. 6, slot M), to that from rate-zonal centrifugation (Figs. 2C and 3, slot M, respectively). P5 was found together with some of P3 on the top of the gradient (not shown).

In addition to the membrane vesicles already obtained by rate-zonal centrifugation, a new class of subviral particles was observed in the more dense part of the gradient. This peak contained only protein label and when examined under the electron microscope (Fig. 5A) only empty capsid structures could be detected; a polyhedral one with an outer diameter between 40 and 46 nm (arrow A), a spherical one with an outer diameter of about 43 nm (arrow B), and a compact spherical structure with a diameter of about 37 nm (arrow C). Some of the spherical particles had a round projection connected to the capsid (arrow D). These projections varied in size and were resistant to the water-soluble stain used. The number of these structures varied between preparations and was roughly estimated to occur with 5—15% of the round particles and in the micrograph shown they are more numerous than normal. The empty capsids did not form aggregates in electron-microscopic preparations as did the nucleocapsid. The proteins found in this fractions were P1, P2, P4, P7 and P8 (Fig. 6, slot C).

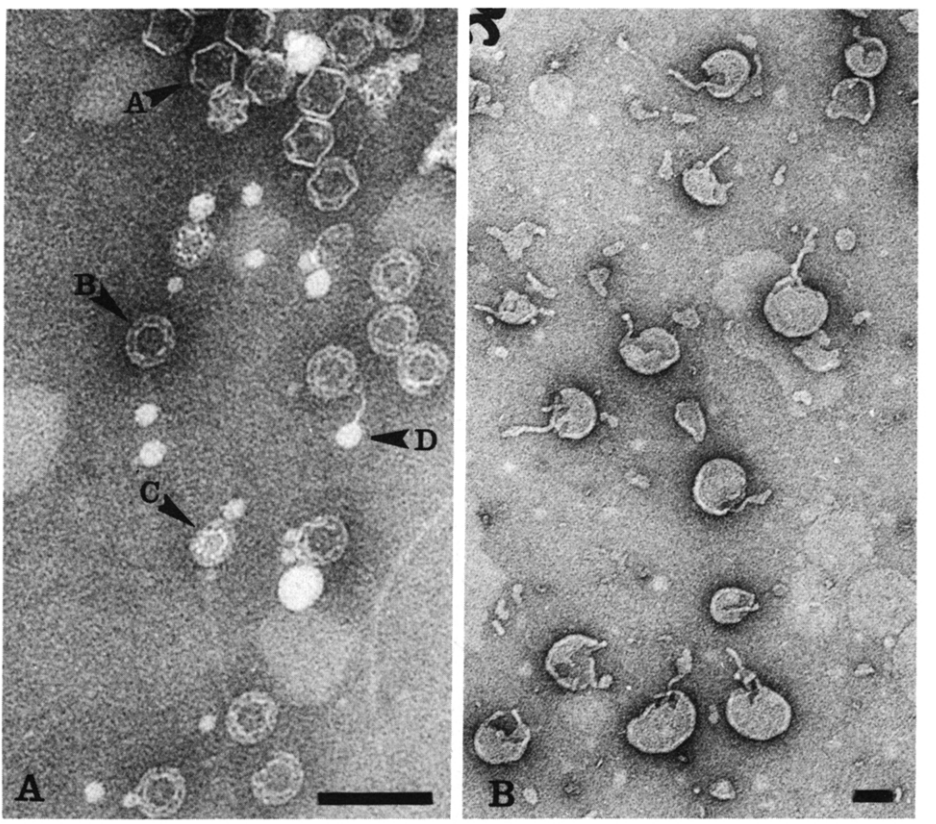


Fig. 5. (A) Negative staining of the capsid fraction from isopycnic centrifugation in the presence of Triton X-100 (Fig. 4D). (B) Membrane fraction from the same gradient but stained with uranyl oxalate. The bar respresents 100 nm.

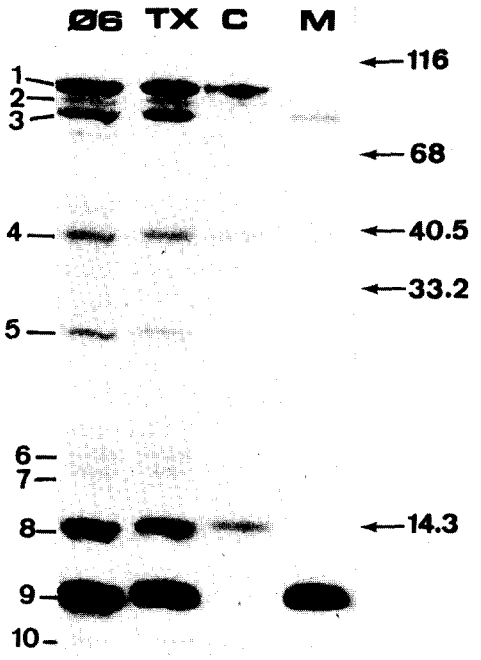


Fig. 6. Sodium dodecyl sulphate-polyacrylamide (15%) gel electrophoresis of ϕ6 and isolated subviral particles (after Triton X-100 treatment in isopycnic centrifugation). TX, material from the uninfective peak in Fig. 4B. C, capsid material from the heavy peak in Fig. 4D. M, membrane material from the light peak in Fig. 4D. The standards are the same as in Fig. 3.

Membrane fractions obtained after Triton X-100 treatment and isopycnic sucrose gradient centrifugation in the presence of Triton X-100

The membrane components released from ϕ6 with Triton X-100 were further separated by pretreatment of the virus for 3 min with Triton X-100 prior to applying the virions onto the gradient which also contained the detergent. Under these conditions, the nucleocapsids and empty protein capsids were pelleted but the released membrane material formed three separate peaks (designated I, II and III) detected by the absorbance at 280 nm (Fig. 7). The large absorbance at the top of the gradient was due to the Triton X-100 added to the sample. No uridine label was detected in the gradient (except in the pellet). The amount of ^{32}P label in the peaks corresponded to the ^{32}P label found in the membrane fractions both in rate-zonal and isopycnic centrifugations. Thus, the ^{32}P label in the gradient was due to the phospholipids of the virus. Peaks I and II contained nearly all the phospholipids whereas only trace amounts were found in peak III (Fig. 7).

Electron micrographs of fraction I (Fig. 8A and B) showed membrane vesicles of the same size as those isolated in previous experiments (Figs. 2C and 5B). However, these vesicles lack the long projections described earlier. Sometimes tiny projections were seen connected to either side of the vesicles (Fig. 8A and B, arrows). This fraction contained proteins P9 and P10 (Fig. 9, slot I). Fraction II contained small vesicles with diameters between 30 and 40 nm (Fig. 8C and D) and proteins P6, P9 and P10 (Fig. 9, slot II). Peak III

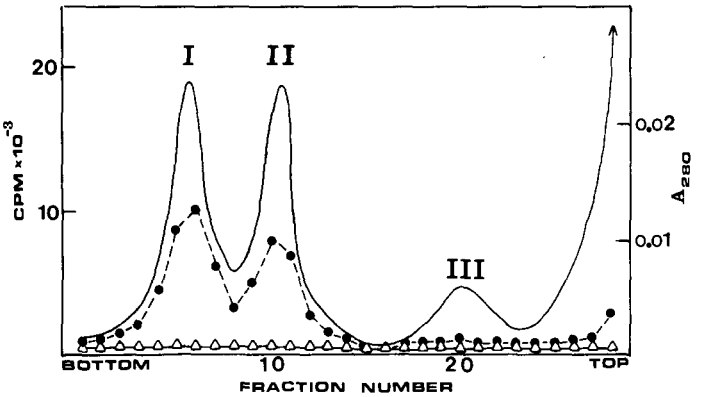


Fig. 7. Isopycnic centrifugation of the membrane fraction in a 10—40% (w/w) sucrose gradient in buffer A containing 150 μg Triton X-100 per ml gradient. The virus was treated with 100 μg of Triton X-100/100 μg of virus protein prior to centrifugation. ———, A_{280}; △———△, [^{3}H]uridine; ●———●, ^{32}P.

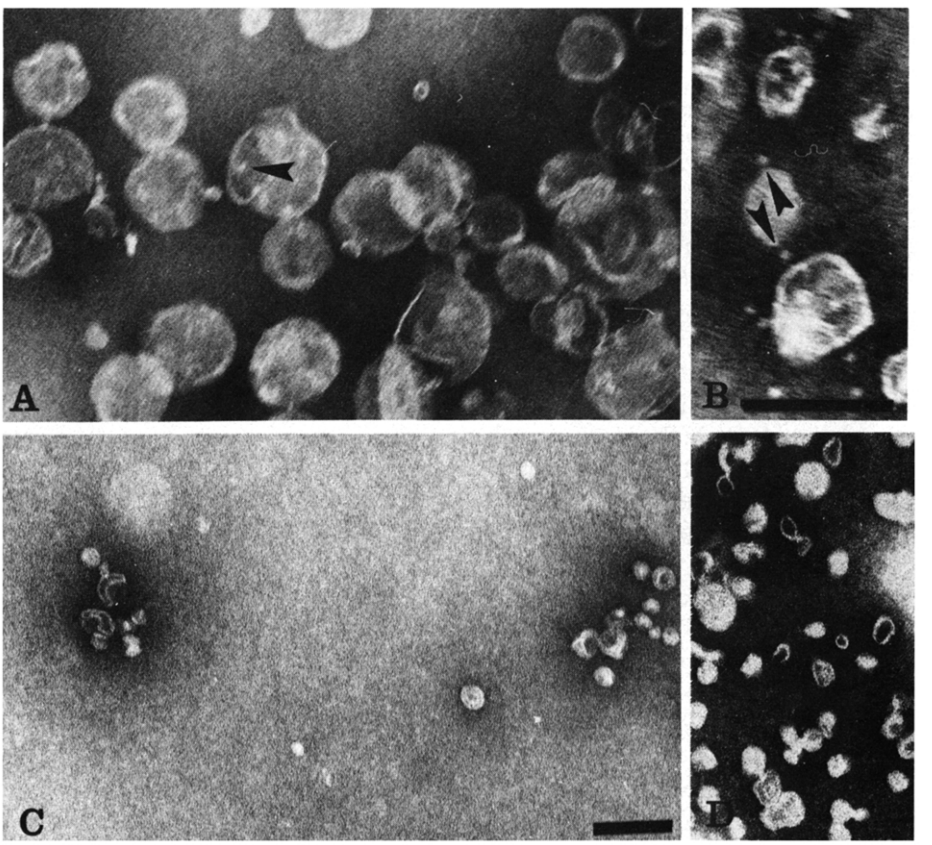


Fig. 8. Negative staining of the membrane fractions shown in Fig. 7. (A) Potassium phosphotungstate staining of the heaviest fraction (I). (B) Larger magnification of vesicles shown in A. (C) Fraction II stained with uranyl oxalate and (D) with potassium phosphotungstate. The bar represents 100 nm.

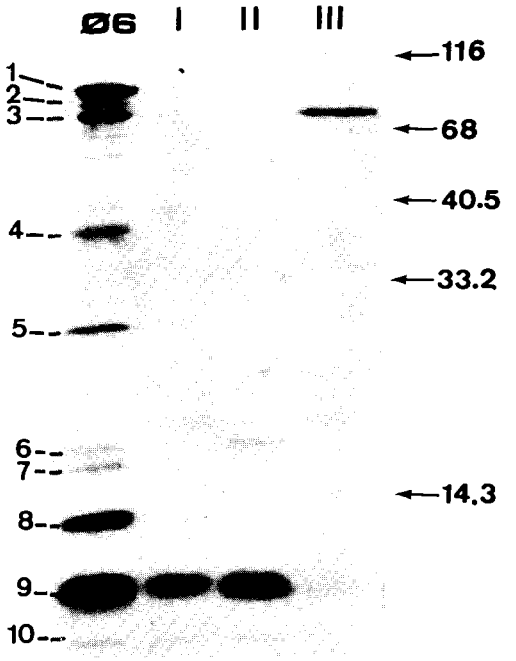


Fig. 9. Sodium dodecyl sulphate-polyacrylamide (15%) gel electrophoresis of isolated membrane fractions I, II and III (after Triton X-100 pretreatment of the virus and subsequent isopycnic centrifugation in the presence of the detergent). The standards are the same as in Fig. 3.

was almost solely composed of protein P3 and some P9 (Fig. 9, slot III) probably as a contamination due to membrane vesicles detected in this fraction by electron microscopy (not shown).

Discussion

Both rate-zonal and isopycnic centrifugations resulted in the release of the phage envelope as a single large membrane vesicle with a single rupture site through which the nucleocapsid had probably escaped. This rupture site was often surrounded by one or two long membranous projections. These projections round the rupture site could be remnants of the phage tail structure. It is reasonable to assume that the rupture takes place at the site where the blunt membranous tail is attached and where the membrane fusion during the infection takes place [3,9], thus representing a weak area in the phage membrane.

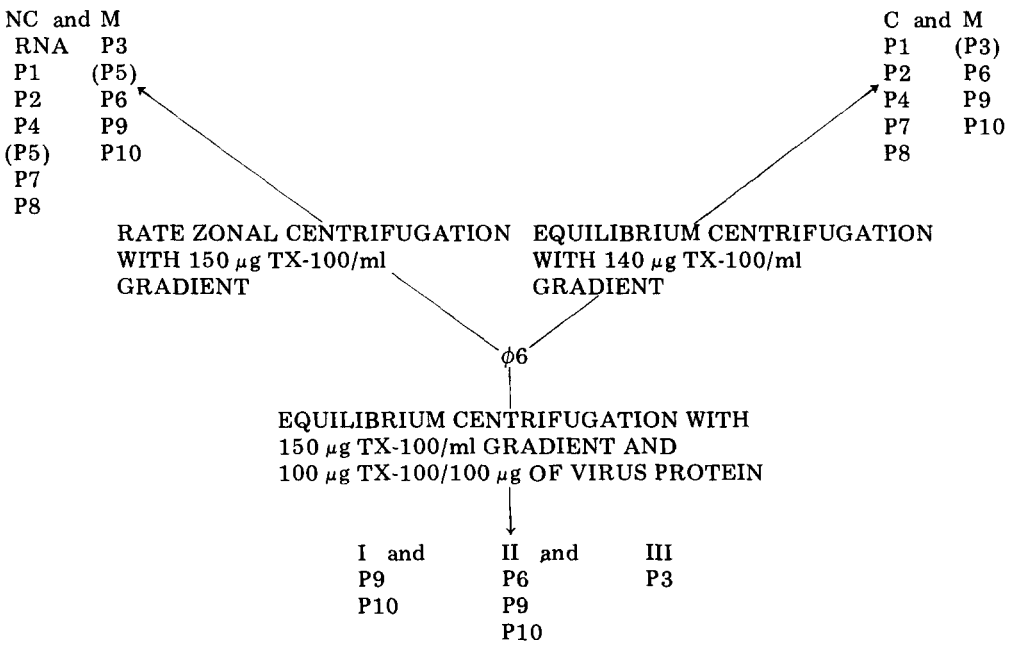
The isolated envelopes contain the viral proteins P3, P6, P9, P10 and part of P5. This protein composition is in agreement with previous data where these proteins could be extracted from the virion with Triton X-100 [11,12].

The viral envelope could be further separated into subfractions after pretreatment of the virus with Triton X-100 and subsequent isopycnic centrifugation in the presence of the detergent (Fig. 7). The results obtained suggest that P9 and P10 together with lipids form the bulk membrane. Protein P6 can be extracted from the membrane more easily than P9 and P10. The recognizer protein P3 [29] appears more loosely attached to the envelope as part of it is

released when the nucleocapsid and the membrane are separated from each other by rate-zonal centrifugation and as it can be separated from lipids with higher Triton X-100 concentrations (Fig. 7). Protein P3 can also be specifically removed from intact virions by butylated-hydroxytoluene treatment with simultaneous loss of infectivity (unpublished results). Protein P3 is the only protein localized on the outer surface of the virion [12] and it has been suggested that it is not particularly hydrophobic and is attached to the viral envelope through protein P6 [30].

Nucleocapsids isolated after rate-zonal centrifugation contained the viral RNA and proteins P1, P2, P4, P7, P8, and some of P5. These capsids had short projections on the outer surface which may arise from attachment of residual viral phospholipids or Triton X-100 onto hydrophobic proteins (probably P5) still present on the nucleocapsid surface. The heavy aggregation of nucleocapsids after removal of the detergent by dialysis supports the idea of a hydrophobic surface.

After isopycnic centrifugation it was possible to obtain small amounts of capsids which did not contain the viral RNA. The protein pattern of these empty capsids was similar to that of the nucleocapsids except that no protein P5 was detected. Neither did these empty capsids contain the projections observed on the surface of nucleocapsids. This fraction contained several types of particle. It is difficult to give the reason for the appearance of different types of empty capsids but the staining procedure may affect the structure of empty capsids, resulting in stepwise disruption. The image of these capsids is similar to the procapsids reported to contain only proteins P1, P2, P4 and P7 [31]



Scheme 1. Summary of the ϕ6 disruption results obtained in this study using different types of centrifugation in the presence of the non-ionic detergent, Triton X-100 (TX-100). C, capsid; NC, nucleocapsid; M, membrane material.

except that some round capsids isolated by us showed tail-like stain-resistant projections. It is possible that the stain removes P8 from empty capsids as discussed in Ref. 31 and that this is the reason for the similar morphology of these two types of particle. However, it is probable that the nucleocapsids shown in Fig. 3A contain also P8.

Scheme 1 gives a summary of the different subviral particles isolated after centrifugation of the virus in the presence of Triton X-100. Based on these results, we interprete the ϕ6 structure as follows. The viral RNA with proteins P1, P2, P4, P7 and P8 make up the nucleocapsid of the virus. It is possible that the inner core alone, without protein P8, is responsible for the polyhedral shape of the nucleocapsid. Protein P5 is probably located in between the nucleocapsid and the envelope. The proposed location and behaviour of P5 are in accordance both with the observed lytic activity of this protein [14—16] and with the penetration model where the nucleocapsids have to pass the peptidoglycan layer after the viral envelope and the host outer membrane have fused [3,9,29]. The envelope surrounding the nucleocapsid is basically composed of proteins P9 and P10 in addition to phospholipids. The recognizer protein, P3, is only loosely attached to the membrane. This attachment could be brought about through protein P6 as suggested previously [30].

P5 seems to have affinity to both the membrane and the nucleocapsid. This model is principally in accordance with the structure of ϕ6 suggested by Mindich et al. [13] based on biosynthetic data using different types of ϕ6 mutants.

In conclusion, we have isolated nucleocapsid, empty protein capsid and membrane of bacteriophage ϕ6. The membrane was further separated into three submembrane components; large and small vesicles with typical protein composition as well as a single protein species.

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