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Purification and Partial Characterization of a New Enveloped RNA Virus (Berne Virus)

By MARIANNE WEISS,¹ FRANZ STECK^{1†} AND
MARIAN C. HORZINEK^{2*}

¹*Institute of Bacteriology, Veterinary Faculty, University of Berne, Switzerland and* ²*Institute of Virology, Veterinary Faculty, State University, Utrecht, Yalelaan 1, 3508 TD Utrecht, The Netherlands*

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SUMMARY

In Berne (Switzerland), a virus was isolated from a horse which was found to be serologically unrelated to known equine viruses. Its growth was unaffected by iododeoxyuridine and it was inactivated by organic solvents. A purification procedure involving ammonium sulphate precipitation and sucrose gradient equilibrium centrifugation was developed and viral activities were monitored using infectivity and enzyme-linked immunosorbent assays. Purified virions of density 1.16 g/ml were shown by negative staining electron microscopy to be roughly spherical and to measure 120 to 140 nm in diameter; projections (peplomers, about 20 nm long) were identified on the virion surface. In thin sections, an envelope and an elongated core structure could be distinguished. The core, measuring about 23 nm across and 104 nm in length, appears to assume a rod-, crescent- or open ring-shape within the envelope. It has a tubular structure and shows a transverse striation (periodicity 4.5 nm). Budding at the plasma membrane was observed. Berne virus is considered as a representative of a hitherto undefined family of widespread animal viruses serologically related to recent bovine isolates in Ames, Iowa (U.S.A.) and Lyon (France).

INTRODUCTION

During routine diagnostic work, a cytopathic agent was isolated in 1972 from a rectal swab taken from a horse under observation at the Surgery Clinic in Berne, Switzerland. The animal died about 1 week after the sample had been collected showing pseudomembranous enteritis and miliary granulomas and necrosis in the liver upon post mortem examination. *Salmonella lilla* (O 6,7, z₃₈) was considered the causative agent. The Berne isolate (laboratory designation P138/72) was not neutralized by any diagnostic antiserum tested, including sera directed against known equine viruses. In this paper we describe some properties of the virus which show its unique taxonomic position.

METHODS

Cell culture. Original isolation of the P138/72 virus was made on secondary horse kidney cells. Most experiments reported here, however, were performed in EMS (embryonic mule skin) cells propagated in Eagle's minimal essential medium with Earle's salts (MEM), supplemented with 2 to 10% foetal calf serum, non-essential amino acids (1%), L-glutamine (200 mM), sodium bicarbonate and minocyclin (1%). The calf sera used had been pre-tested for absence of antibody against P138/72 virus by neutralization assay.

Virus purification. Virus purification experiments were performed with material from the 23rd to 27th passage in cell culture after two cycles of plaque cloning. Supernatants harvested from EMS cell cultures showing cytopathic effect (c.p.e.) 1 to 2 days after infection were pooled (100 ml) and used for purification. Ammonium sulphate

† This article is dedicated to the memory of Professor Franz Steck, who, after initiating these studies, died in an accident in October 1982.

precipitation (50% saturation) was allowed to proceed overnight at 4 °C; after low-speed centrifugation, the pellet was resuspended in 5 ml TES buffer (0.01 M-Tris-HCl, 0.001 M-EDTA, 0.15 M-NaCl, pH 7.4) and clarified again. This material was layered on top of a linear 15 to 50% (w/w) sucrose gradient in TES buffer and centrifuged for 5 h at 25000 rev/min in a Spinco SW27 rotor. After fractionation by unloading from the top, the infectivity and antigen concentration were determined for each fraction.

Infectivity assay. Infectious doses (ID_{50}) were routinely determined in flat-bottom microtitre trays (Greiner und Söhne, Nürtingen, F.R.G.) by adding 100 µl volumes of serial tenfold virus dilutions in MEM into wells containing a suspension of 3×10^4 EMS cells. The Spearman-Kärber formula was applied for the calculation of infectivity titres after reading the plates at 5 days after infection.

Enzyme-linked immunosorbent assay (ELISA). An indirect ELISA procedure was used for monitoring antigen in gradient fractions. Antigen was adsorbed to polystyrene microtitre plates (Dynatech Immulon flat-bottom microelisa plates) in the presence of a 0.1 M-carbonate/bicarbonate buffer pH 9.6. Horse sera with and without neutralizing antibodies to Berne virus and an anti-horse total IgG preparation conjugated to alkaline phosphatase (grade VII, Sigma) by the method of Engvall & Perlmann (1972) were employed. 4-Nitrophenyl phosphate (disodium salt hexahydrate; Merck) was used as a substrate at 1.0 mg/ml in 0.05 M-carbonate/bicarbonate buffer pH 9.8, with 1 mM-MgCl₂. Absorption measurements were made at 405 nm using a Titertek Multiscan photometer (Flow Laboratories).

Electron microscopy. Virus particles were identified in gradient fractions by negative staining using sodium phosphotungstate (2 to 5%) in water, adjusted to pH 7.2 with NaOH. Alternatively, resuspended ammonium sulphate precipitates were layered on top of a 15% sucrose layer with a 50% sucrose cushion. After centrifugation for 5 h at 25000 rev/min in a Spinco SW27 rotor, a light-scattering band had become visible at the sucrose interphase which was collected by aspiration. In some experiments, fixation was achieved by incorporating 0.5% (v/v) formaldehyde into the 50% sucrose cushion and subsequent interphase centrifugation. For thin-section electron microscopy, cells were fixed with 2.5% glutaraldehyde in 0.125 M-cacodylate buffer pH 7.2 and post-fixed in 2% OsO₄. After prestaining in a solution of 5% uranyl acetate in 70% acetone, cells were dehydrated in graded acetone concentrations and embedded in Spurr's low viscosity medium (Polaron Equipment, Watford, U.K.). Sections were stained with lead citrate (Venable & Coggeshall, 1965) and examined in a Philips EM300 electron microscope operating at 80 kV. The magnification of the viral structures presented was determined using a line grid (spacings 0.463 µm, Balzers Union, Liechtenstein) and its photographically enlarged image as a standard.

All size measurements were made in suitably enlarged electron micrographs using an IBAS interactive image analysis system (Kontron, Munich, F.R.G.) equipped with cursor, digitizer tablet, and linked to a Videoplan Computer Unit. In thin sections, linear measurements were made; in negatively stained preparations, integral values were determined for the two-dimensional projections of the virion, which were then converted into diameter values.

Organic solvents. Indications for the presence of essential lipids in the virion were obtained by testing its ether- and chloroform-sensitivity (Andrewes & Horstmann, 1949; Bögel & Mayr, 1961).

Nucleic acid determination. For a preliminary determination of the type of viral nucleic acid, the effect of 5-iodo-2'-deoxyuridine (IUdR) on virus multiplication in EMS cells was tested (Tamm & Eggers, 1963). IUdR (puriss. grade; Fluka, Buchs, Switzerland) was employed at a final concentration of 40 µg/ml cell culture medium. At this concentration no cytotoxic effect was seen during the observation period. Serial tenfold dilutions of a virus suspension in medium with or without the drug were inoculated into EMS cell cultures in microtitre plates which were observed for c.p.e. An equine rhinopneumonitis virus strain (57 F 94) and the Bucyrus strain of equine arteritis virus were adapted to the EMS cell line and used as controls in both the organic solvent and the inhibitor experiments.

Serology. Experiments to ascertain the identity of the Berne P138/72 isolate included virus neutralization, indirect immunofluorescence and ELISA. Immune sera directed against arthropod-borne and non-arthropod-borne members of the Togaviridae [Semliki Forest, Sindbis, rubella, bovine diarrhoea, equine arteritis, *Aedes albopictus* cell-fusing virus (Stollar & Thomas, 1975)], Coronaviridae (mouse hepatitis virus strain A59, porcine transmissible gastroenteritis and avian infectious bronchitis virus), Orthomyxoviridae (influenza C virus) and Herpesviridae (equine herpesvirus type 1) families were employed. In addition, sera from gnotobiotic calves after infection with two serotypes of the recently described Breda virus (Woode *et al.*, 1982) as well as a non-immune gnotobiotic calf serum were tested; from Dr A. Moussa, Laboratoire National de Pathologie Bovine, Lyon, France, immune sera against another new virus isolate (Lyon-4 virus) were obtained and included in the experiments.

Neutralization indices were determined after titration of Berne virus in the presence of immune or non-immune sera at a 1:20 dilution. Neutralization titres were calculated after serial twofold serum dilutions had been reacted with 100 ID_{50} units of Berne virus and inoculated into EMS cell monolayers in Terasaki plates (Greiner und Söhne, Nürtingen, F.R.G.). Indirect immunofluorescence tests were performed using either infected cells that had been suspended by trypsinization, dried and acetone-fixed onto microscope slides (for Lyon-4 and

transmissible gastroenteritis virus) or infected monolayers on coverslips (mouse hepatitis and infectious bronchitis viruses). Antiglobulin-fluorescein isothiocyanate conjugates were purchased from Miles-Yeda (Rehovot, Israel). For ELISA serology, supernatants from uninfected and Berne virus-infected EMS cell cultures were concentrated and purified as described (ammonium sulphate precipitation, sucrose interphase centrifugation) and bound to polystyrene microtitre plates; details of the procedure have been described elsewhere (Horzinek *et al.*, 1982).

RESULTS

Virus concentration and purification

Preliminary experiments had shown that peak infectivity titres were obtained in the supernatants of EMS cells 24 h after infection at a calculated multiplicity of 1 to 3 ID₅₀ units/cell. Since infectivity titres usually did not exceed 10^{6.5} ID₅₀ units/ml, the virus had to be concentrated and purified for further structural studies. The result of a representative experiment is shown in Table 1. Precipitation using ammonium sulphate had no detrimental effect on infectivity, and resulted in a reduction of contaminating protein in the resuspended sediment by about 90%. The protein content had decreased below detection level in the density gradient fraction which contained the maximum of infectivity. From electron microscopic observations, we assume that the virus loses projections during sucrose gradient centrifugation, which may account for the low recovery of infectivity in the peak fraction; the peak integral in Fig. 1 contains 7% of the infectivity present in the starting material. From the data presented it can be calculated that the protein content per ID₅₀ unit was reduced by a factor of >40 in the purified preparation.

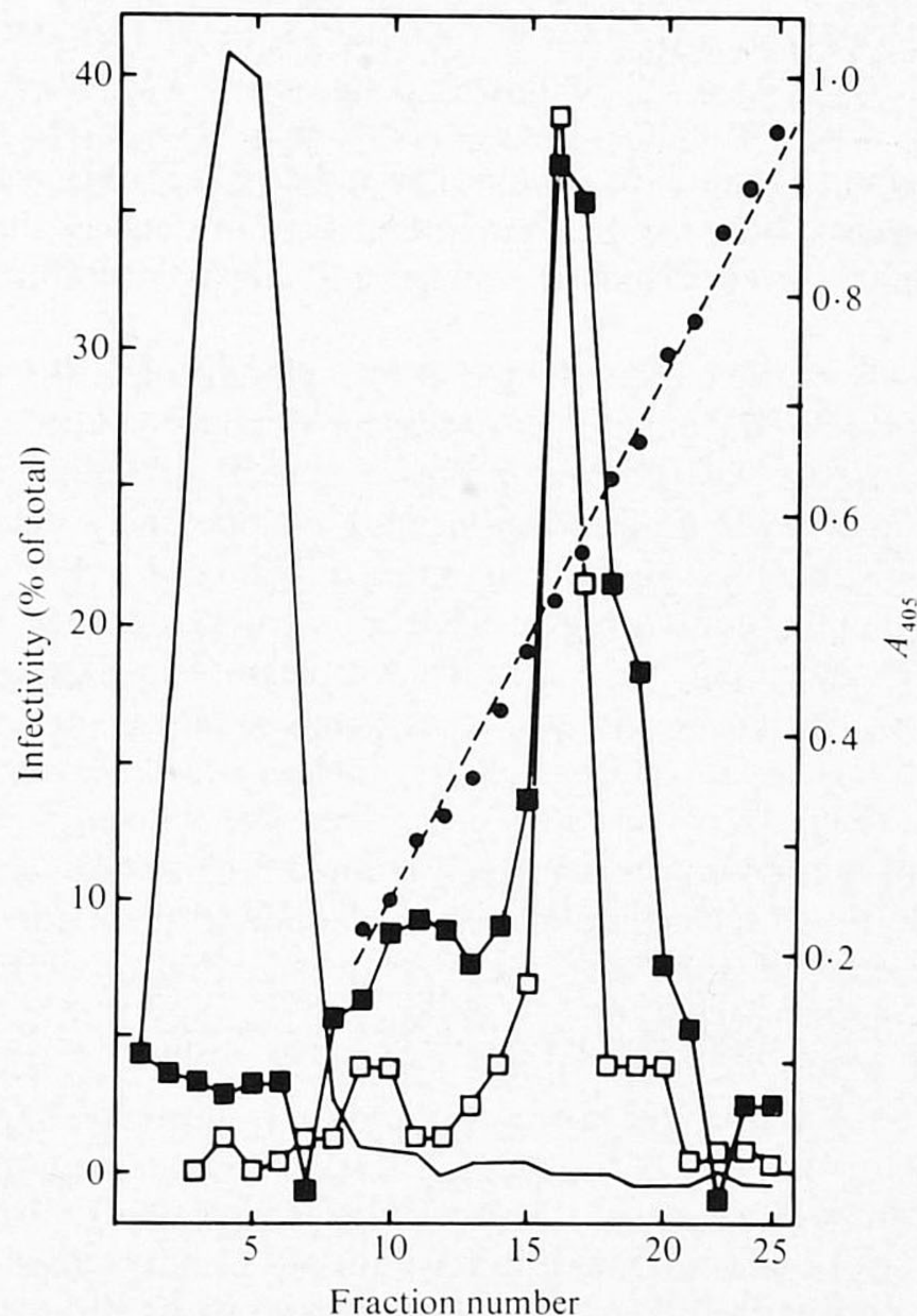


Fig. 1. Distribution of infectivity (□) and ELISA activity (■) of a Berne virus concentrate in a linear 15 to 50% sucrose gradient (●); the continuous line without symbols indicates the total protein concentration. Centrifugation was performed for 5 h at 25000 rev/min in a Spinco SW27 rotor.

Table 1. *Protein and infectivity values in a representative purification of Berne virus*

Material	Vol. (ml)	Protein (mg/ml)	%	Infectivity (ID ₅₀ /ml)	%
Culture supernatant	100	1.25	100	5.6×10^5	100
(NH ₄) ₂ SO ₄ precipitation					
Supernatant	200	0.38	61*	5.6×10^3	2
Sediment	5	2.16	9*	1.8×10^7	159
Gradient fraction ($\rho = 1.16$ g/ml)	1	<0.1	<0.1	1.8×10^6	3†

* After resuspension of the sediment, a further low-speed centrifugation had to be done in order to remove insoluble precipitate; this explains the loss of protein during the procedure.

† Viral infectivity was obviously affected by sucrose gradient centrifugation; total recovery from all fractions amounted to about 8% of the starting material.

Viral activities in the gradient were monitored by infectivity tests and ELISA; both assays indicated peak values at a sucrose density of 1.16 g/ml as calculated from the refractive indices measured. A second, minor infectivity peak was reproducibly found at an apparent density of 1.10 g/ml; it was paralleled by a shoulder in the profile of ELISA activities (Fig. 1).

Virion structure

Electron microscopy of negatively stained material from density gradients or sucrose/formaldehyde interphase centrifugations showed aggregations of predominantly smooth-surfaced spherical particles (Fig. 2*a*); some of them had a distinct C-shape (Fig. 2*b*). Virions in which the membrane was obviously damaged (Fig. 3*a*) nevertheless did not release substructures indicative of the symmetry of the nucleocapsid. However, a sausage-like internal structure with transverse striations (estimated periodicity 4.5 nm) was observed (Fig. 3*b*). Depending upon the preparation, virions were either bald or densely studded with projections (Fig. 4*a*); these peplomers have a drumstick shape, and consist of a thin stalk carrying a distal spherule (Fig. 4*b*).

Thin sections through Berne virus-infected horse kidney or EMS cells revealed densely staining spherical, elliptical and elongated particles accumulating at the cytoplasmic membrane (Fig. 5); budding at this site was observed (Fig. 5, inset). At higher magnification, a clear distinction can be made between an electron-lucent envelope and a dense core. The core may appear of uniformly high opacity (Fig. 6*a*) or contain a light centre (Fig. 6*b*). Rod-like (Fig. 7*a*) and crescent-shaped structures containing an electron-lucent centre (Fig. 7*b*) were prevalent and appeared either in the extracellular space or in the cytoplasm (Fig. 8). Fig. 7(*c*) shows enveloped twin circular structures which we interpret as cross-sections through a hollow, tubular nucleocapsid core (see Discussion). Occasionally, intracytoplasmic accumulations of twisted strands were observed (Fig. 9).

Size measurements of Berne virus in negatively stained preparations are given in Table 2. For a detailed analysis, additional measurements were made in thin-section electron micrographs: (group 1) of core diameters in cross-sections (as shown in Fig. 6*b* and 7*c*); (group 2) of core diameters in longitudinal projections (Fig. 7*a, b* and 8); (group 3) of core length, whether the structures appeared in a straight (Fig. 7*a* and 8) or bent form (Fig. 7*b*); and (group 4) of all remaining opaque internal virion structures of circular or elliptical shape (Fig. 5, 6*a* and 7*b*).

A value of 23 nm [standard error of the mean (S.E.M.) 3 nm, coefficient of variation 13%] was calculated for the core diameter (group 1). Its mean length (group 3) was 104 nm ($n = 90$, S.E.M. 16 nm, coefficient of variation 15%); maximum values of 171 nm were measured. It can be seen from Fig. 10 that the core within the virion (group 4) in all its projections shows significant deviations from a spherical outline; a mean value of 41 nm ($n = 196$, S.E.M. 11 nm, coefficient of variation 27%) was calculated. These data are compatible with an overall discoidal shape of the viral core and hence of the virion itself.

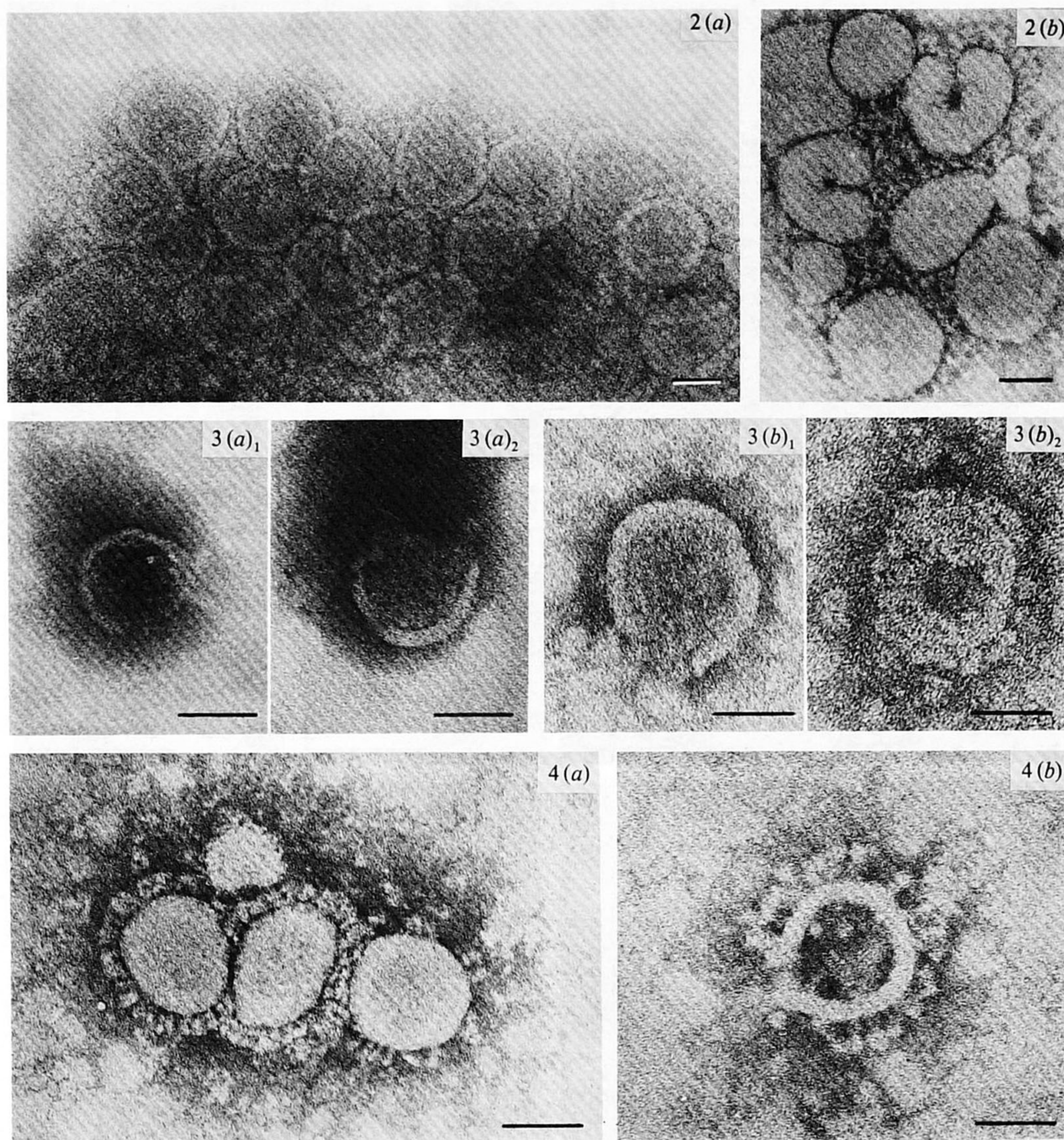
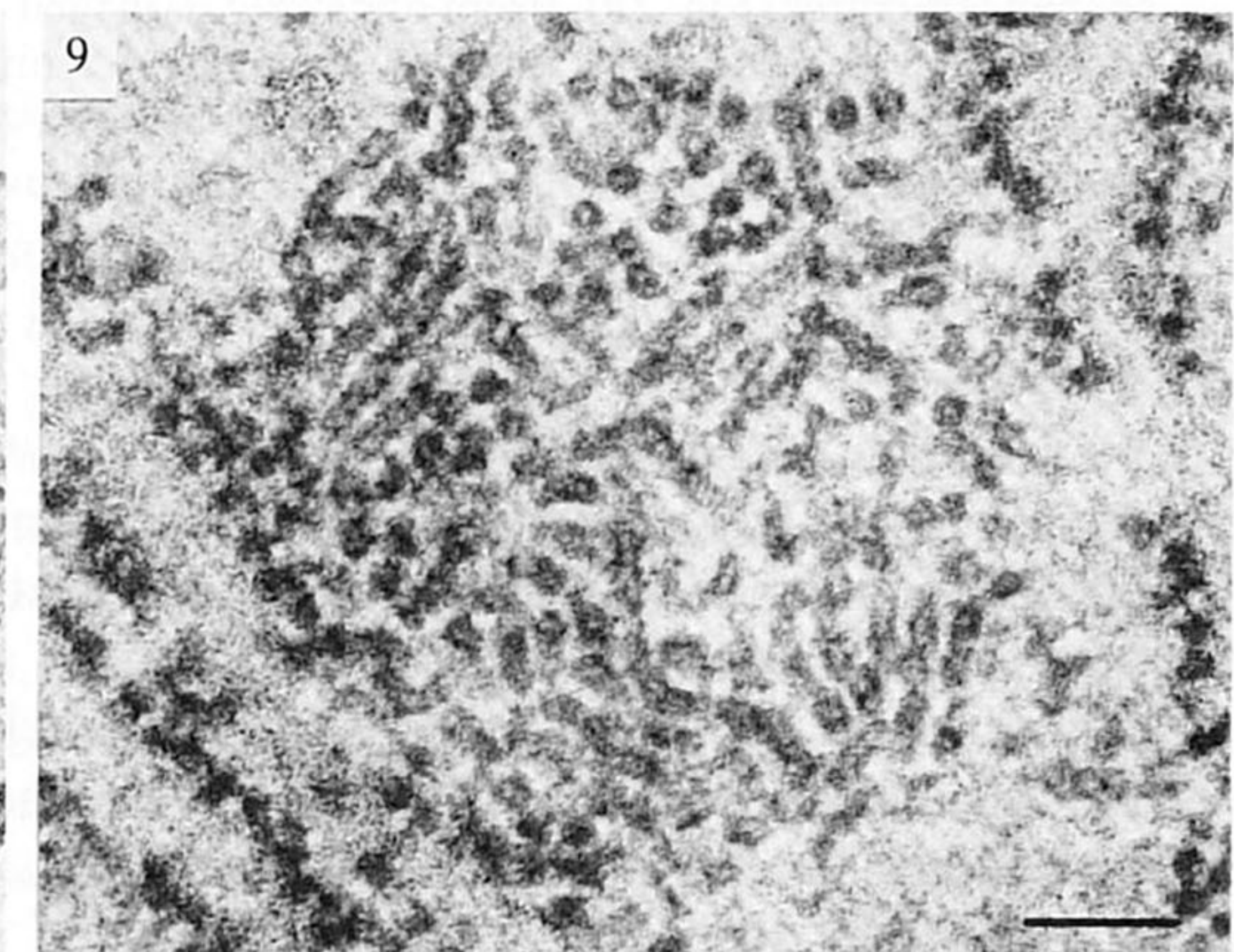
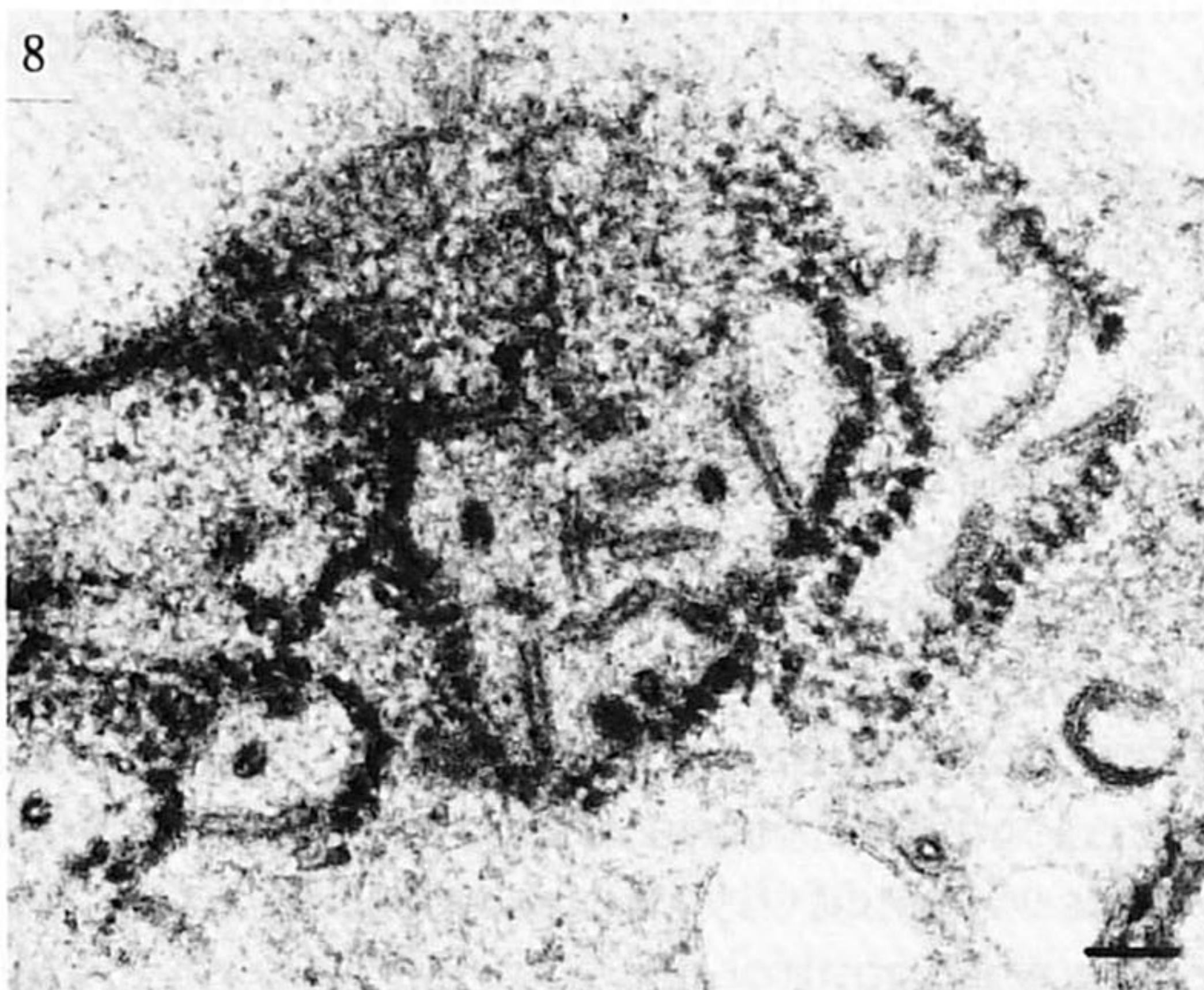
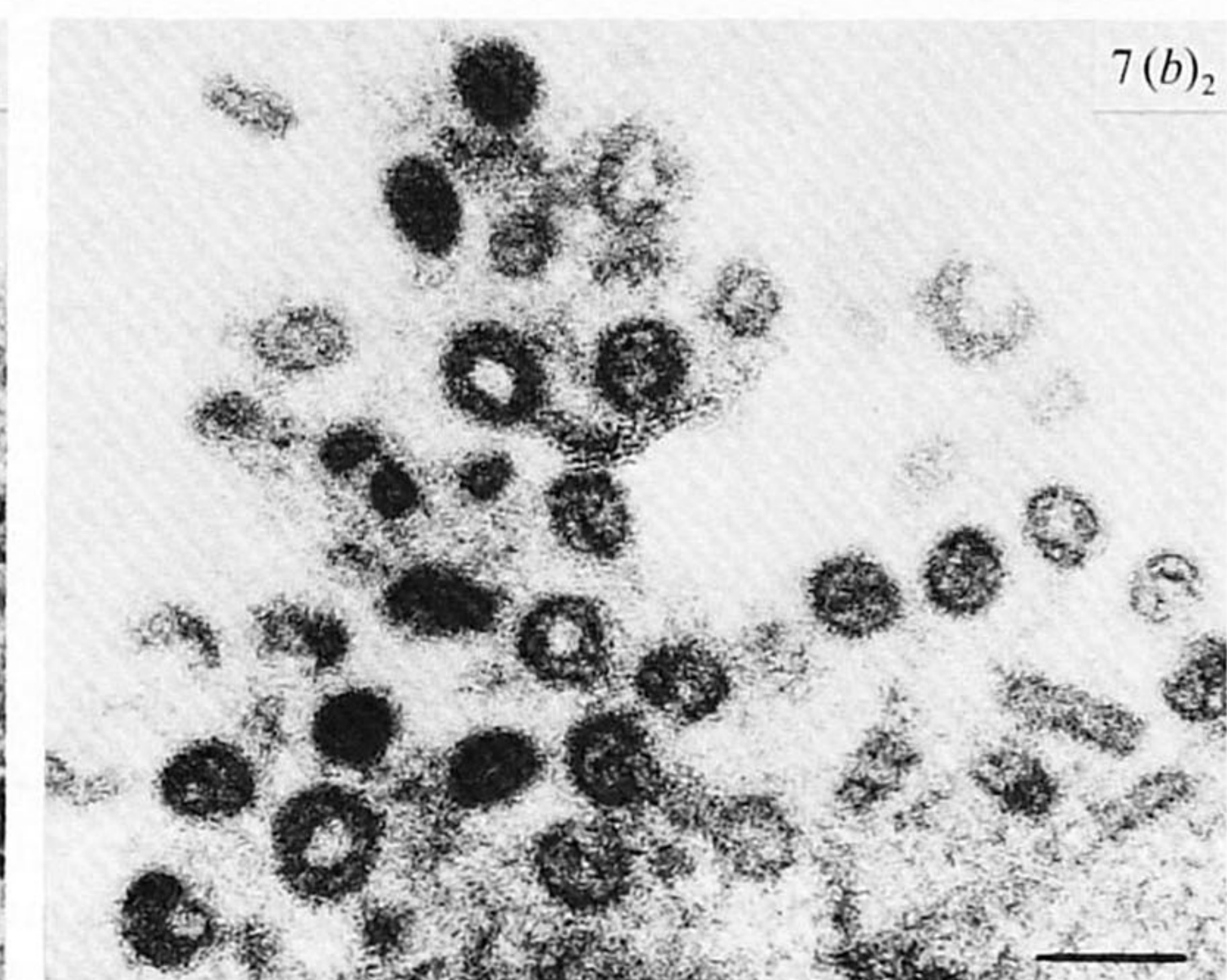
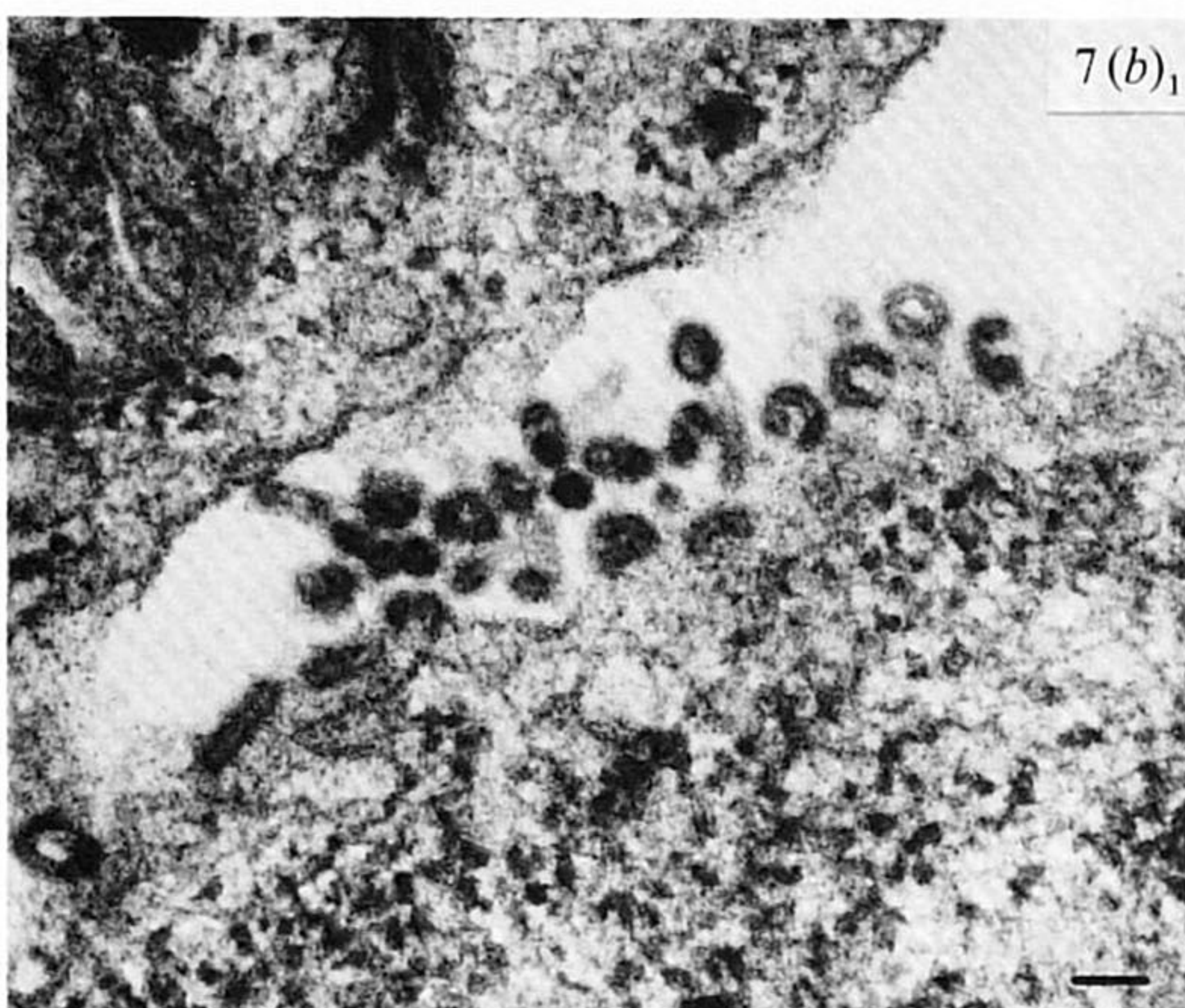
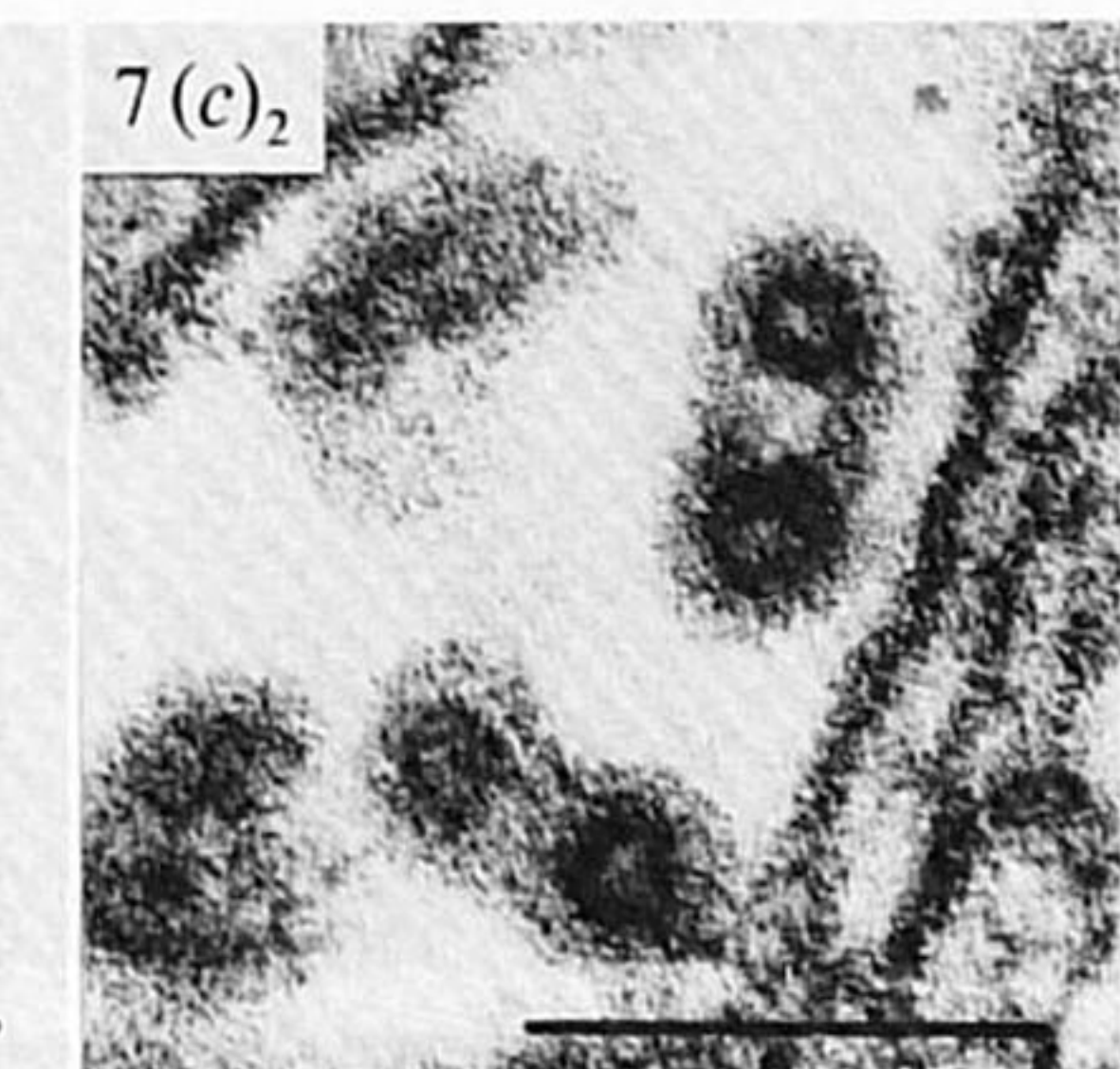
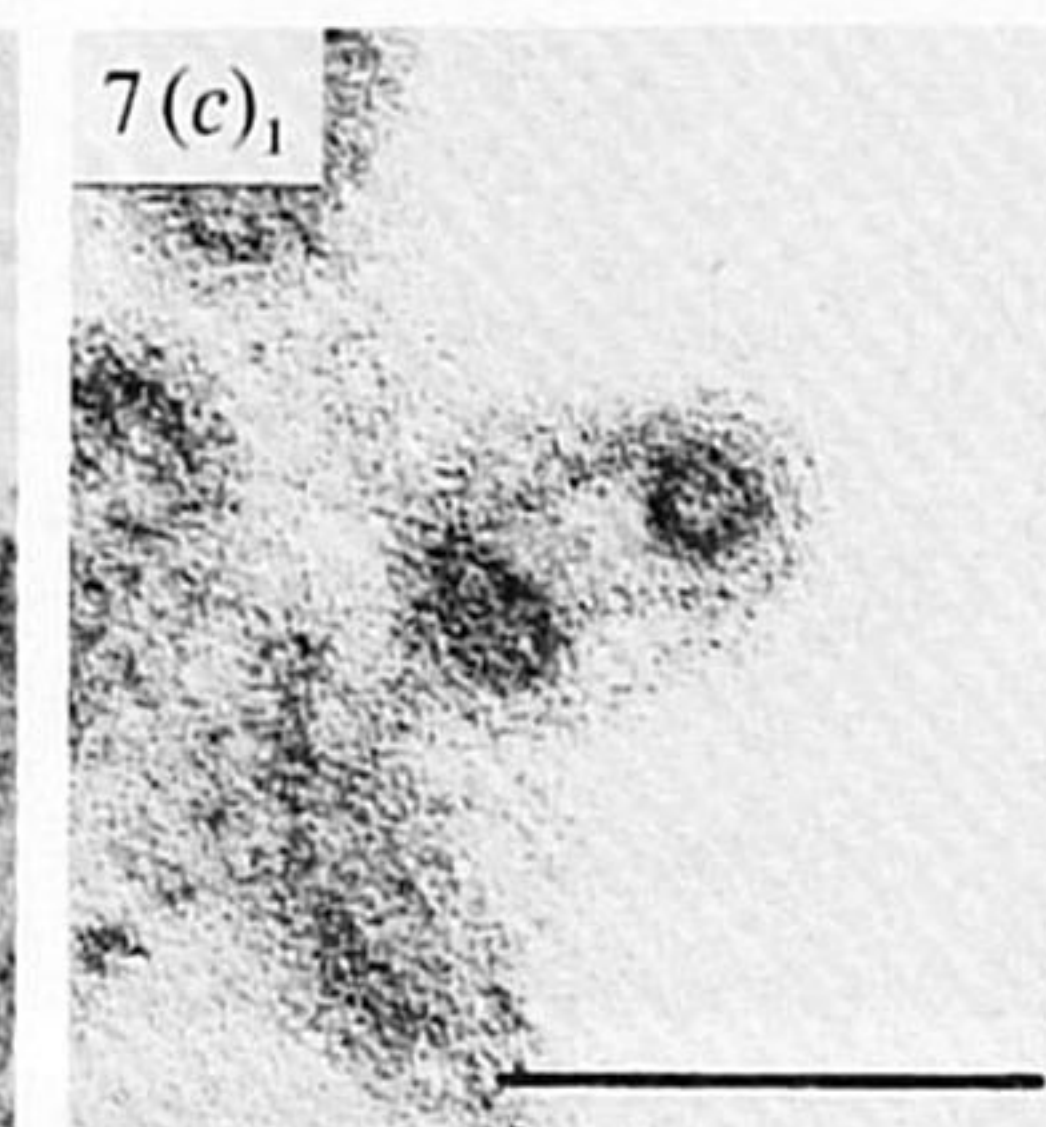
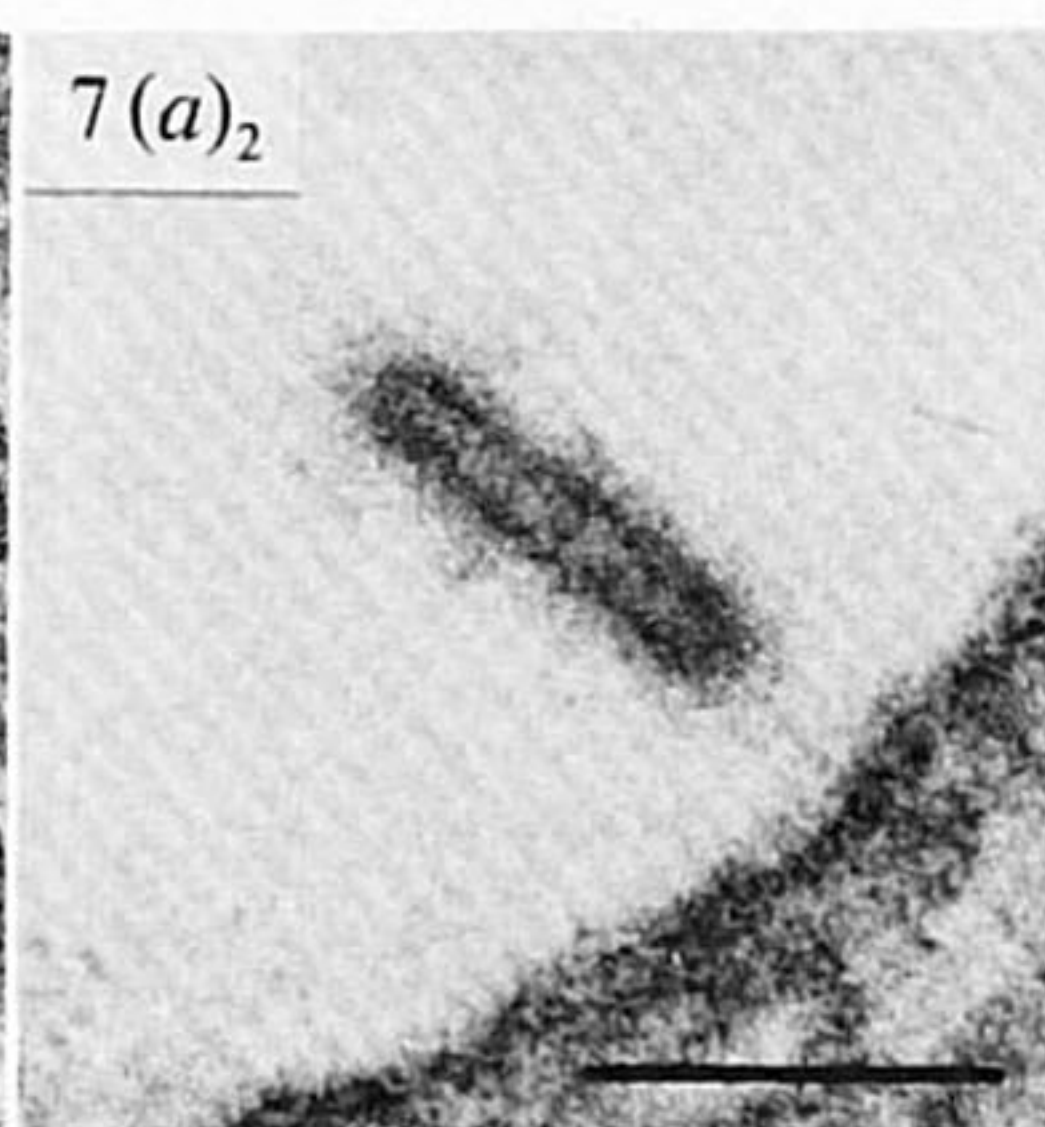
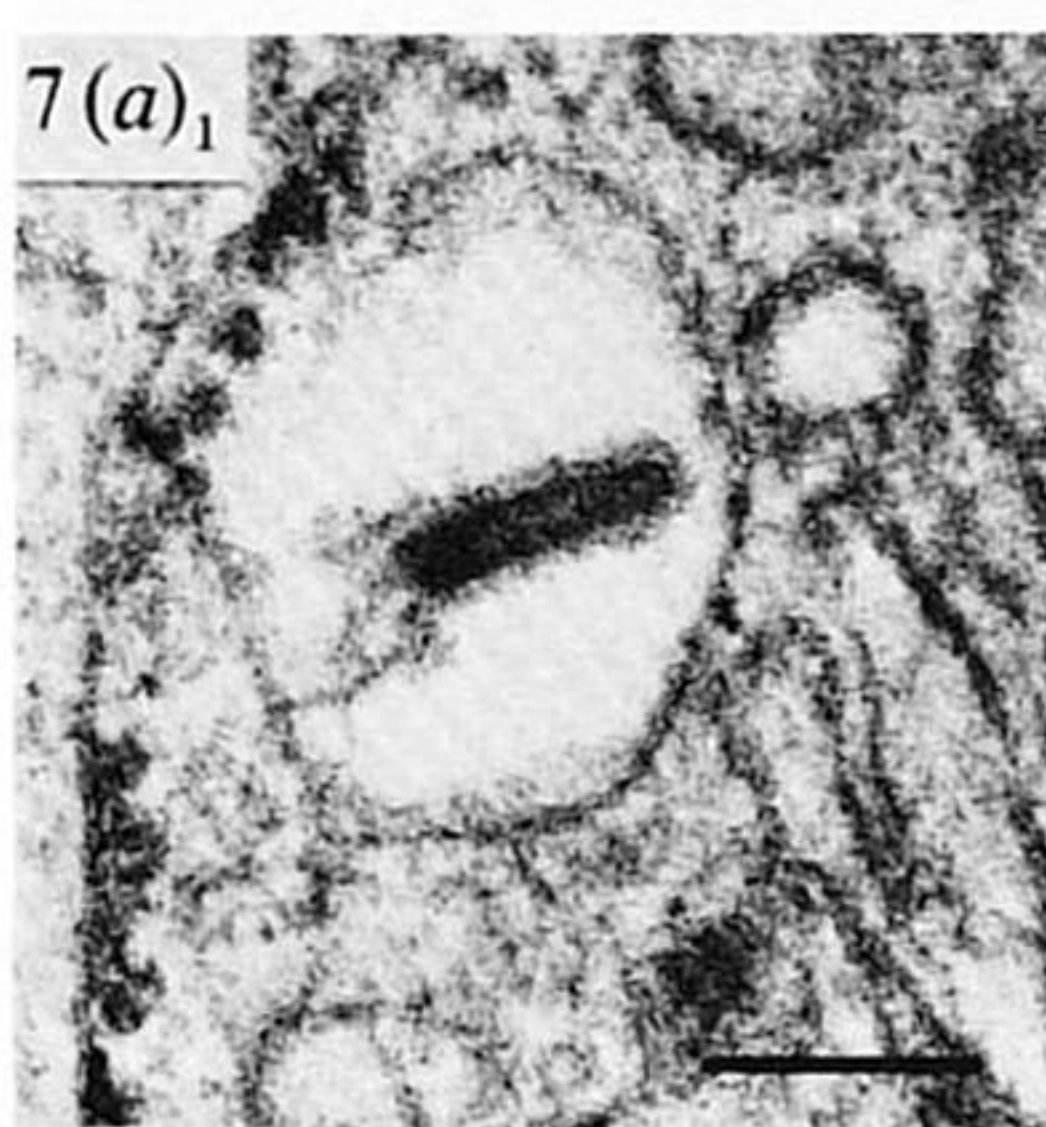
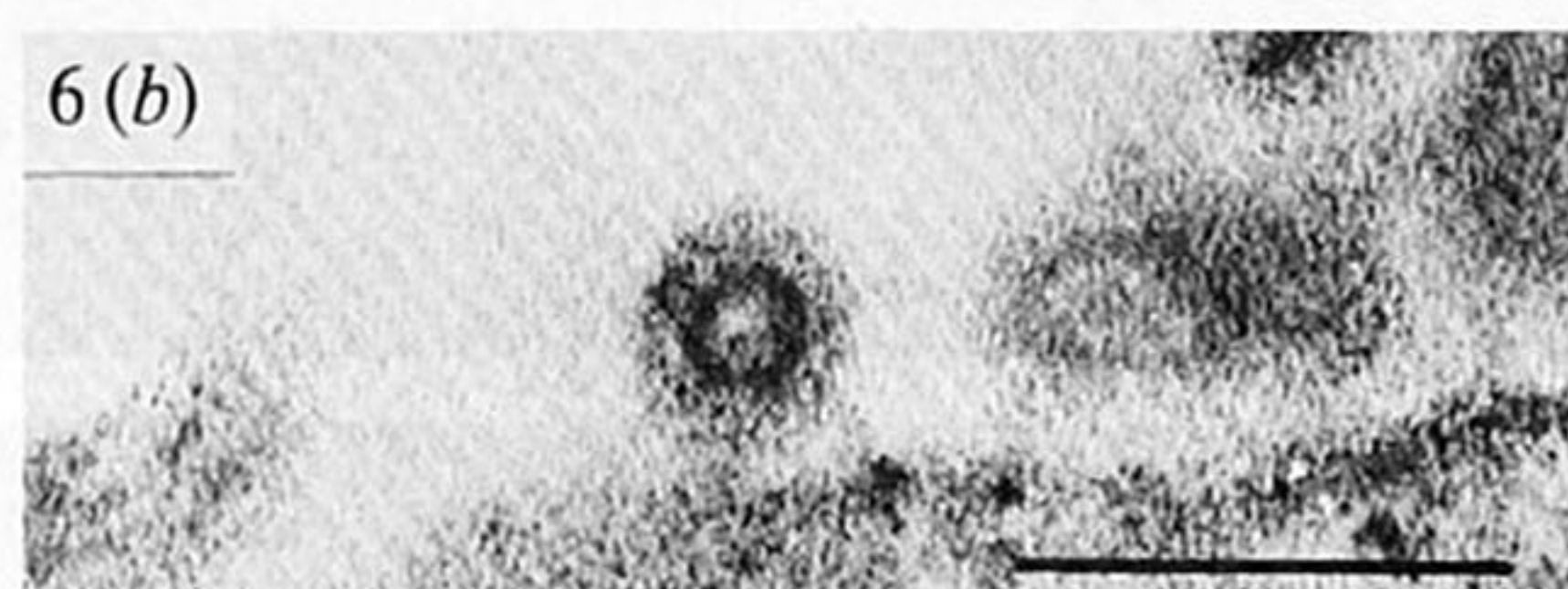
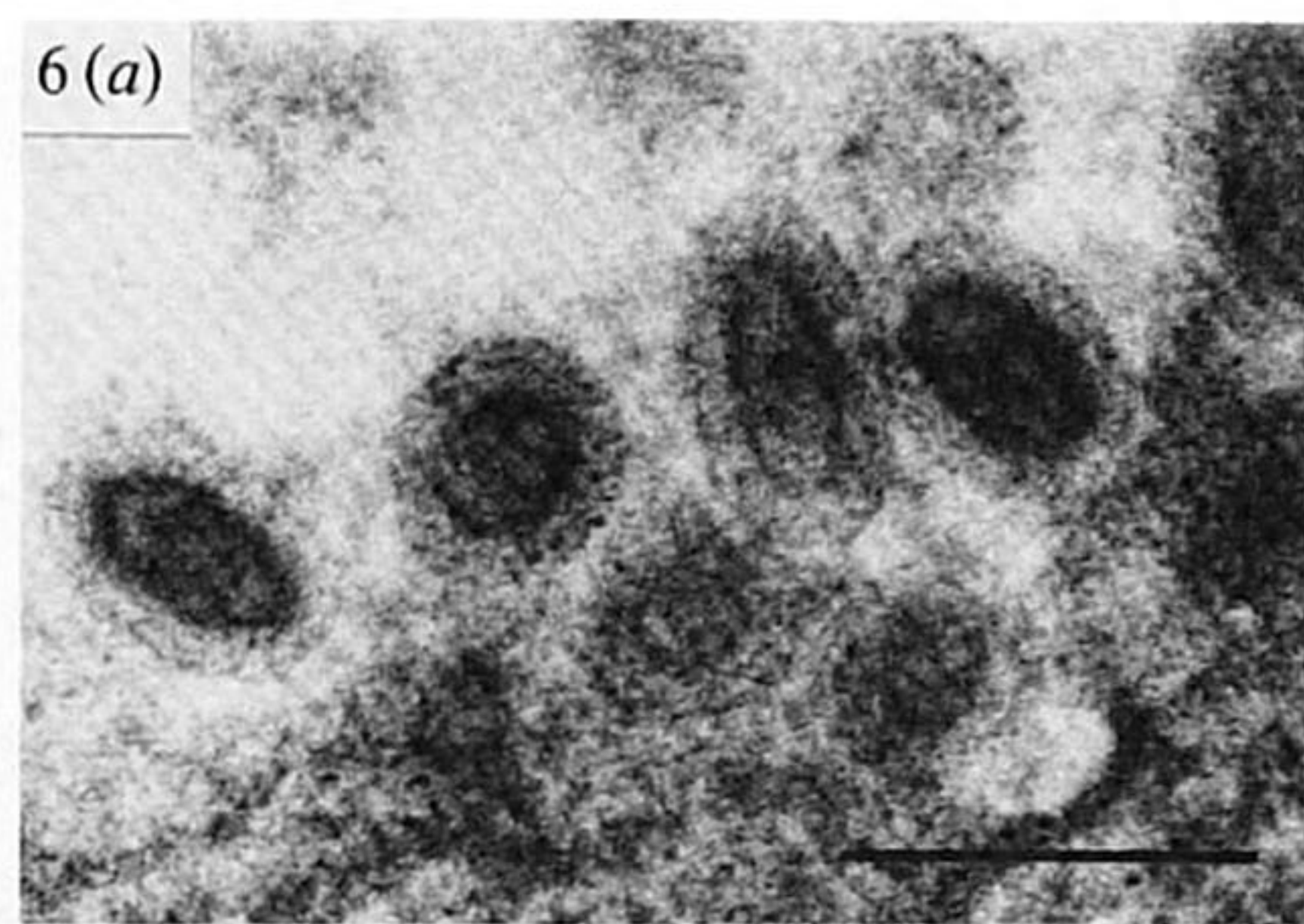
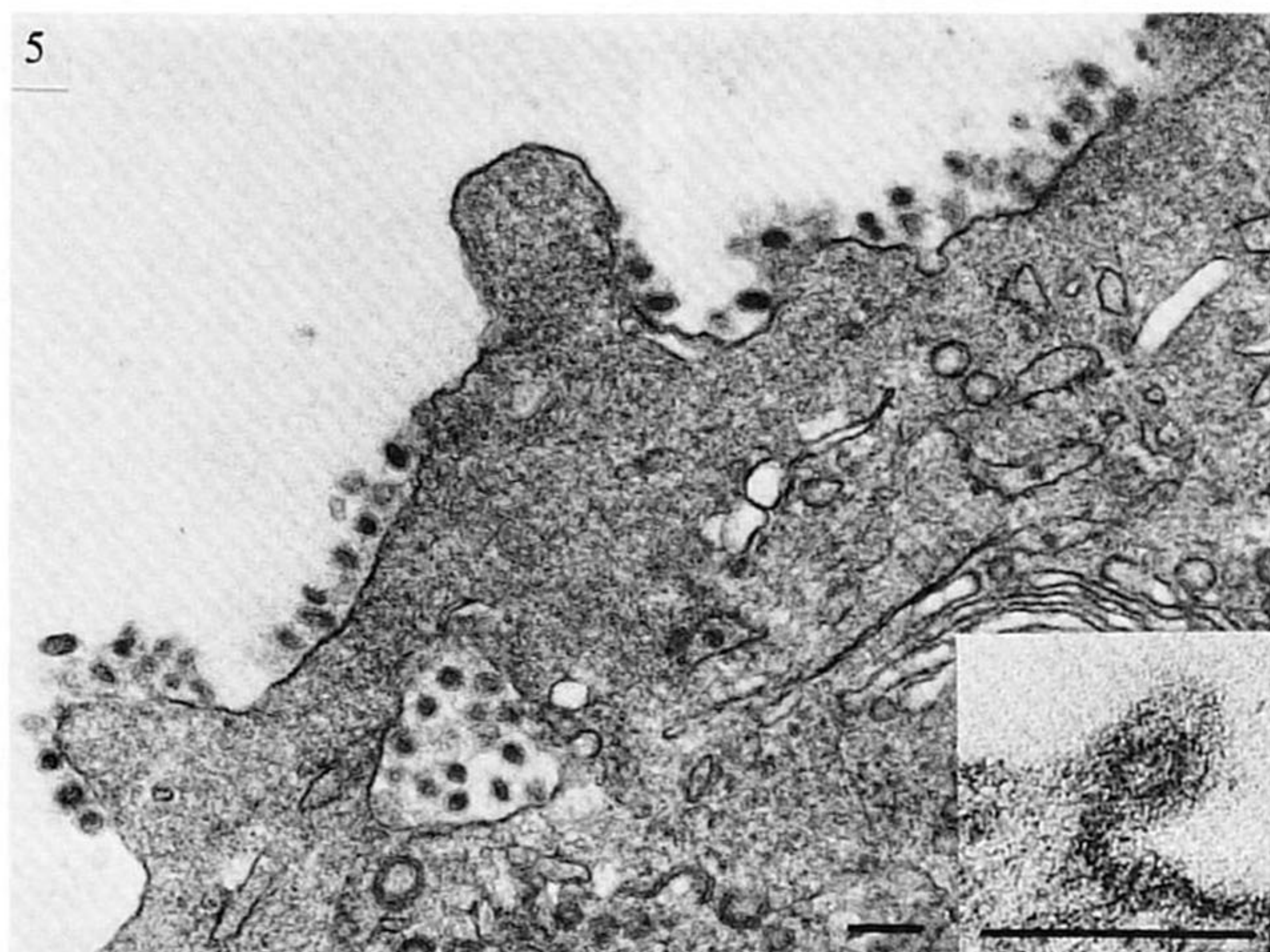


Fig. 2 to 4. Purified Berne virus from a sucrose interphase (15%–50%) centrifugation, negatively stained with phosphotungstic acid. Smooth-surfaced spherical virions (Fig. 2*a*) are shown as well as C-shaped structures (Fig. 2*b*). From obviously damaged virions the core is not released (Fig. 3*a*); it may show transverse striations (periodicity about 4.5 nm, Fig. 3*b*). Few virions carry 20 nm surface projections (Fig. 4*a*) which appear to consist of a thin stalk and a distal spherule (Fig. 4*b*). Bar markers represent 50 nm.

The presence of essential lipids in the virion was shown by treatment of a virus suspension containing $10^{5.5}$ ID₅₀ units/ml with chloroform or diethyl ether, respectively. No residual infectivity could be detected in the undiluted material after chloroform treatment and less than 0.1% in the ether-treated sample.

From the data presented in Table 3 it is concluded that the Berne virus isolate is an RNA virus, since its growth is unaffected by IUdR, whereas the DNA virus (equine herpesvirus type 1) is inhibited in replication by more than 3 log₁₀ units of infectivity; equine arteritis virus, an RNA virus (van der Zeijst *et al.*, 1975), served as another control.



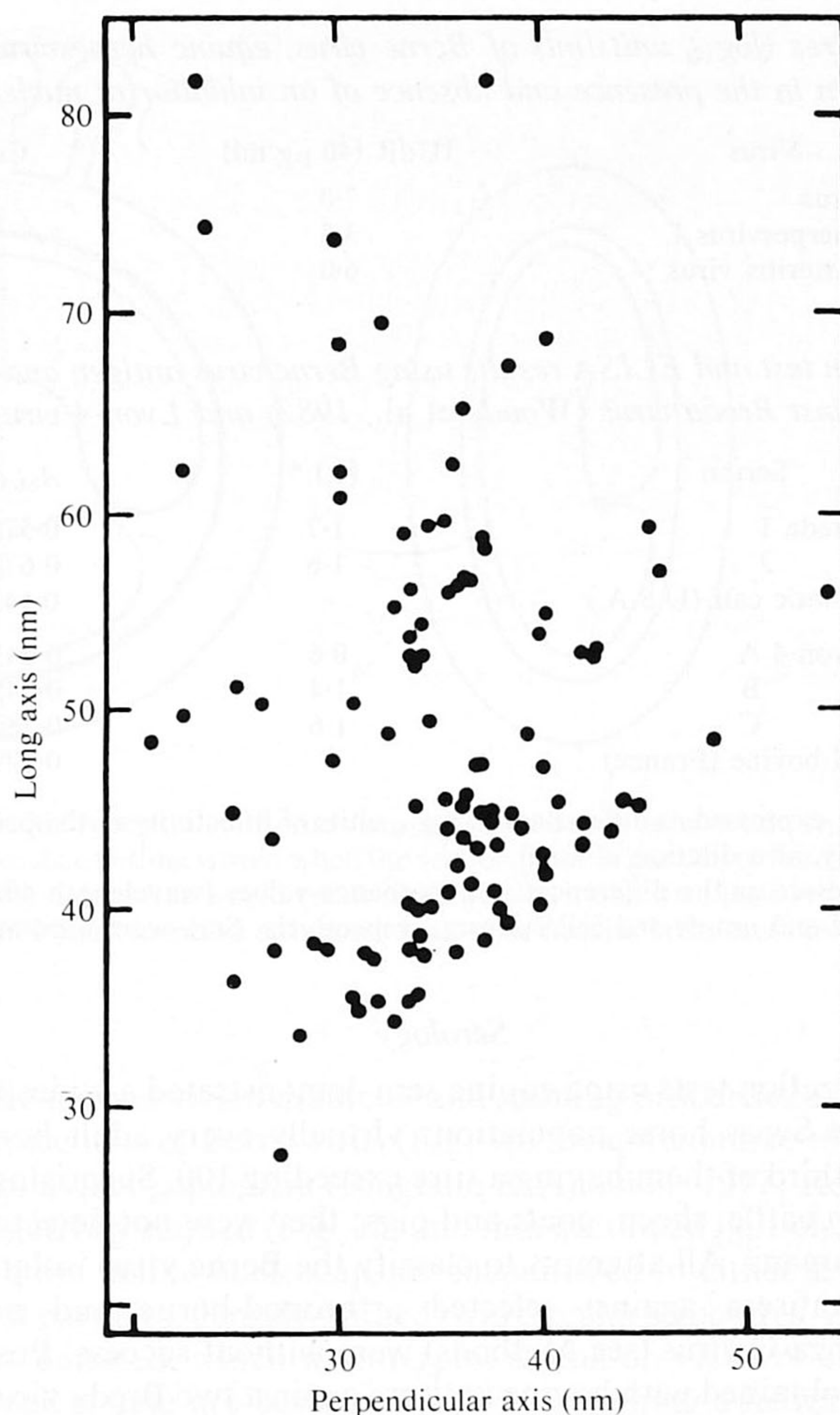


Fig. 10. Dimensions of cores ($n = 196$) of Berne virus in thin-section electron micrographs (group 4, see Results). The coordinates of each point are represented by two measurements per particle, one of its longest axis and the other perpendicular to it. The shape of the cloud is indicative of deviations of the core from the spherical form. Application of the sign test to the data confirmed that the sample did not belong to a symmetrically distributed population ($P < 0.05$).

Table 2. *Size measurements (nanometres) of Berne virus*

	Negative staining					
	Unfixed			Formaldehyde-fixed		
	Mean \pm S.E.M.	Coeff. of variation	n	Mean \pm S.E.M.	Coeff. of variation	n
Virion without projections	99 ± 14	14%	100	82 ± 12	15%	125
Surface projections	20 ± 1	5%	52	—	—	—
Membrane thickness	11 ± 1	10%	75	10 ± 1	15%	51

Fig. 5 to 9. Thin-section electron micrographs of Berne virus in horse kidney (Fig. 5 and 6*a*) and embryonic mule skin cells. Virions are shown adsorbed to the cytoplasmic membrane (Fig. 5) from which budding was observed (Fig. 5, inset). Round and elliptical core structures with opaque (Fig. 6*a*) or electron-lucent (Fig. 6*b*) centres surrounded by a tightly fitting envelope are visible. Straight rod-shaped (Fig. 7*a*), crescent- and torus-shaped cores (Fig. 7*b*) and cross-sections through the latter structures (Fig. 7*c*) are presented. Rod-like particles are found within dilated cisternae of the rough endoplasmic reticulum (Fig. 8) and accumulations of similar strands are seen in the cytoplasm of infected cells (Fig. 9). Bar markers represent 100 nm.

Table 3. *Infectivity titres (\log_{10} units/ml) of Berne virus, equine herpesvirus type 1 and equine arteritis virus grown in the presence and absence of an inhibitor of nucleic acid synthesis*

Virus	IUdR (40 μ g/ml)	Control
Berne virus	7.0	6.5
Equine herpesvirus 1	3.5	6.7
Equine arteritis virus	6.0	5.5

Table 4. *Neutralization test and ELISA results using Berne virus antigen and bovine sera with and without antibodies against Breda virus (Woode et al., 1982) and Lyon-4 virus (A. Moussa, Lyon)*

Serum	N.I.*	$A_{405}\dagger$
Anti-Breda 1	1.7	0.579
2	1.6	0.672
Gnotobiotic calf (U.S.A.)	—	0.145
Anti-Lyon-4 A	0.6	0.281
B	1.4	0.745
C	1.6	0.780
Normal bovine (France)	—	0.129

* Neutralization indices, expressed as differences in \log_{10} units of infectivity in the presence of control serum and antiserum, respectively, at a dilution of 1:20.

† ELISA readings, expressed as the differences in absorbance values (wavelength 405 nm) between antigen preparations from infected and uninfected cell cultures, respectively. Sera were used at a 1:80 dilution.

Serology

Preliminary neutralization tests using equine sera demonstrated a widespread distribution of the Berne virus in the Swiss horse population; virtually every adult horse tested possessed antibodies, about one-third of them having a titre exceeding 100. Surprisingly, antibodies were also found in sera from cattle, sheep, goats and pigs; they were not detected, however, in sera from dogs, cats and humans. All attempts to classify the Berne virus isolate by showing cross-neutralization with antisera against selected arthropod-borne and non-arthropod-borne togaviruses and influenza C virus (see Methods) were without success. Positive neutralization results, however, were obtained with bovine antisera against two Breda virus serotypes (Woode *et al.*, 1982) and Lyon-4 virus (Table 4).

In addition, indirect immunofluorescence tests were performed using antisera against members of three coronavirus antigenic clusters (mouse hepatitis, transmissible gastroenteritis and avian infectious bronchitis viruses) as well as the respective antigen preparations. No antigenic relationship could be detected between Berne virus and the coronaviruses studied. Pronounced intracytoplasmic fluorescence was observed when calf testicle cells infected with Lyon-4 virus were reacted with a horse serum known to contain neutralizing antibody against Berne virus.

Distinct reactions were observed in ELISA using cattle sera with antibodies against Breda and Lyon-4 virus, respectively. When the A_{405} values (see footnote to Table 4) were plotted against the corresponding serum dilutions, titres >640 were determined.

DISCUSSION

We have characterized the Berne virus isolate as a lipid-containing RNA virus ranging in size between 120 nm and 140 nm (Table 2). These data are based on negatively stained preparations. The infectious virion has a buoyant density of 1.16 g/ml in sucrose and consists of a projection-bearing envelope and a core of unique morphology.

In thin sections, Berne virus resembles rubella virus (Murphy *et al.*, 1968), a member of the togaviridae family (Horzinek, 1981). Oval or elongated virions are not uncommon in both viruses. The alphaviruses Chikungunya virus (Higashi *et al.*, 1967) and Semliki Forest virus (Erlandson *et al.*, 1967; Tan, 1970) and also rubella virus have been shown to produce bacilliform structures occasionally reaching lengths of more than 200 nm (Payment *et al.*, 1975).

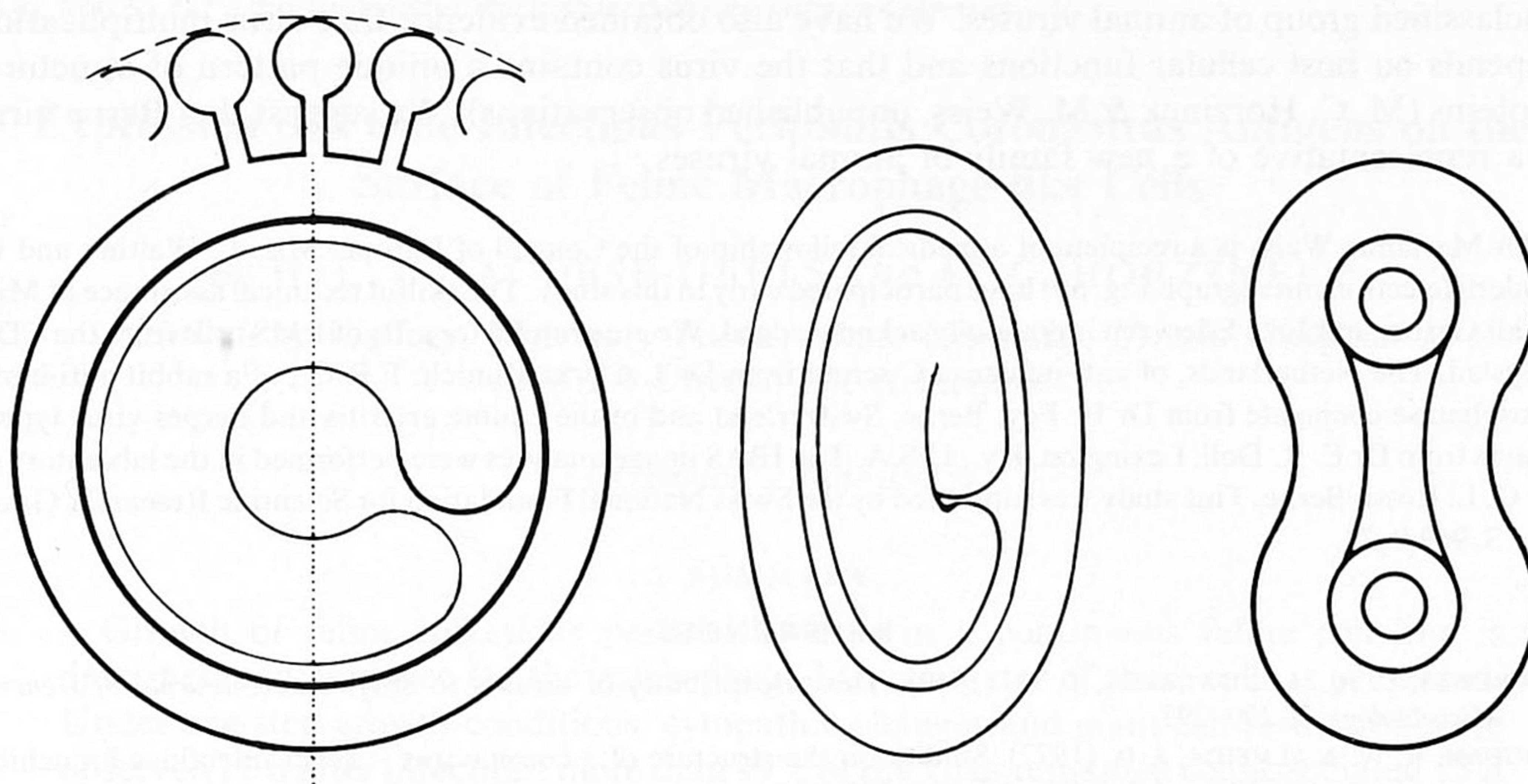


Fig. 11. Schematic interpretation of Berne virus substructures as seen in thin sections. The open torus within a circular particle outline is seen when the section plane is parallel to the discoidal particle (left). Rotation around the dotted line would result in elliptical virions with little resolution of the interior (middle, tilt about 70°) or biconcave structures with twin circular cross-sections of the core (right, tilt 90°).

while maintaining the normal virion diameter and staining properties of the core. On the other hand, the surface projections of Berne virus (Fig. 4*b*) are quite unlike those in togaviruses and rather resemble coronavirus peplomers (Bingham & Almeida, 1977; Roseto *et al.*, 1982). The cores observed in negatively stained (Fig. 3*b*) and thin-sectioned particles of Berne virus (Fig. 6 to 8), however, are quite unlike nucleocapsids encountered in either the toga- or coronavirus families. In some oval particles, densely stained twin circular structures with a diameter of about 23 nm (Fig. 7*c*) were detected, which we interpret as end-on views of cross-sectioned toroidal cores. The morphological data are best explained by assuming a helical nucleocapsid, tightly coiled into a hollow tube which is bent into an open torus. Fig. 11 represents a synthesis of the electron microscopic images presented. A tightly fitting envelope surrounds the core. It is our impression that its curved shape is maintained by the membrane; with this assumption the virion would in most instances have the morphology of a (biconcave) disc as visualized in cross-sections (Fig. 7*c*). Further support for an elongated tubular nucleocapsid, probably of helical symmetry (Fig. 3*b*), comes from the observation of extracytoplasmic enveloped rod-shaped particles (Fig. 7*a*) and of intracytoplasmic straight rods (Fig. 8) and convoluted strand structures (Fig. 9). The distribution of measurements (Fig. 10) is in agreement with our model (Fig. 11).

A disc-shaped virion is a novel structure in animal virology; the brick shape (Poxviridae), bullet shape (Rhabdoviridae) and rod shape (Baculoviridae, Marburg-like 'filo'viruses) have been the only exceptions to the rule of spherical virion morphology. Preparation artefacts must be taken into consideration; yet it is difficult to imagine how shrinkage during sample preparation could cause the observed structures in both negatively stained virions from suspensions and thin-sectioned intracellular particles. It cannot be excluded, however, that the observed morphology is the consequence of the *in vitro* passage history (as, e.g., in orthomyxoviruses) and that the C-shape (Fig. 2*b*) or open kidney-shape observed for Breda virus by Woode *et al.* (1982) in calf faecal specimens predominate under 'natural' conditions.

The serological relatedness of Berne virus with the Breda and Lyon-4 viruses demonstrated in cattle in the U.S.A. and France, respectively (Woode *et al.*, 1982; A. Moussa, unpublished results, 1982), and their lack of relatedness with selected enveloped RNA viruses have been reported. Prevalence of antibody against the Berne isolate in Swiss horse, cattle (Steck *et al.*, 1980), sheep, goat and pig sera indicate the world-wide occurrence in ungulates of a hitherto

unclassified group of animal viruses. We have also obtained evidence that virus multiplication depends on host cellular functions and that the virus contains a unique pattern of structural proteins (M. C. Horzinek & M. Weiss, unpublished observations). We suggest that Berne virus is a representative of a new family of animal viruses.

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