Structure and Composition of the Adenovirus Type 2 Core

DENNIS T. BROWN, MONIKA WESTPHAL, BYRON T. BURLINGHAM,[1] UTE WINTERHOFF,
AND WALTER DOERFLER*

Institute of Genetics, University of Cologne, Cologne, Germany

The structure and composition of the core of adenovirus type 2 were analyzed by electron microscopy and biochemical techniques after differential degradation of the virion by heat, by pyridine, or by sarcosyl treatment. In negatively stained preparations purified sarcosyl cores reveal spherical subunits of 21.6-nm diameter in the electron microscope. It is suggested that these subunits are organized as an icosahedron which has its axes of symmetry coincident with those of the viral capsid. The subunits are connected by the viral DNA molecule. The sarcosyl cores contain the viral DNA and predominantly the arginine/alanine-rich core polypeptide VII. When sarcosyl cores are spread on a protein film, tightly coiled particles are observed which gradually unfold giving rise to a rosette-like pattern due to the uncoiling DNA molecule. Completely unfolded DNA molecules are circular. Pyridine cores consist of the viral DNA and polypeptides V and VII. In negatively stained preparations of pyridine cores the subunit arrangement apparent in the sarcosyl cores is masked by an additional shell which is probably formed by polypeptide V. In freeze-cleaved preparations of the adenovirion two fracture planes can be recognized. One fracture plane probably passes between the outer capsid of the virion and polypeptide V exposing a subviral particle which corresponds to the pyridine core. The second fracture plane observed could be located between polypeptide V and the polypeptide VII-DNA complex, thus uncovering a subviral structure which corresponds to the sarcosyl core. In the sarcosyl core polypeptide VII is tightly bound to the viral DNA which is susceptible to digestion with DNase. The restriction endonuclease EcoRI cleaves the viral DNA in the sarcosyl cores into the six specific fragments. These fragments can be resolved on polyacrylamide-agarose gels provided the sarcosyl cores are treated with pronase after incubation with the restriction endonuclease. When pronase digestion is omitted, a complex of the terminal EcoRI fragments of adenovirus DNA and protein can be isolated. From this complex the terminal DNA fragments can be liberated after pronase treatment. The complex described is presumably responsible for the circularization of the viral DNA inside the virion. The nature of the protein(s) involved in circle formation has not yet been elucidated.

The chemical composition of the adenovirus core has been studied intensively. The core contains the viral DNA and two different proteins, polypeptides V and VII (17, 18, 22, 23, 32, 33, 34) according to the nomenclature of Maizel et al. (26). Polypeptide VII is the alanine/arginine-rich protein (17, 22, 32, 34, 38) which contains 18 to 19% of alanine and 21 to 23% of arginine residues. The core proteins have been isolated by several methods (17, 18, 22, 33, 34) and have been characterized as to molecular weight and number of polypeptide chains per virion (17, 18). Polypeptides V and VII have molecular weights of 48,500 and 18,500, respec-

tively, and it is estimated that there are 180 and 1,070 molecules per virion, respectively (17, 18, 32, 33, 34). Polypeptide VII comprises approximately 14% of the viral protein, and it has been calculated that the arginine residues of this protein are sufficient to cover approximately 60% of the phosphate charges of the viral DNA (32). Laver (22) has suggested that the alanine/arginine-rich protein molecules are evenly distributed along the DNA molecule.

As yet, the arrangement of the core inside the adenovirion and the relation between the viral DNA and the core proteins have received little attention. Robinson et al. (36) and Doerfler et al. (11) have provided evidence that the viral DNA in the virion is kept in a circular configu-

ration perhaps through an as yet unidentified "linker protein" (36). Everitt et al. (18) and Everitt and Philipson (17) have proposed that the core polypeptide V is associated both with the viral DNA and the penton bases, whereas polypeptide VII is tightly bound to the viral DNA.

Recently, Everitt et al. (16) have developed a refined model of the type 2 adenovirion. This model is based on results obtained by enzymatic iodination of intact and disrupted virions, by immunoprecipitation of intact virions with specific antisera, and by chemical cross-linkage of the structural proteins. These results suggest that polypeptides V, VI, VII, and VIII are located inside the virion. Polypeptide V is found in close proximity to the penton-bases, to hexons, and to protein IIIa. Polypeptide V can be chemically cross-linked also to polypeptide VII, hence some molecules of polypeptide V must be located close to polypeptide VII.

In the present study, the ultra structure of adenovirus cores was investigated by electron microscopy of freeze-etched preparations of virions in infected cells and by electron microscopy of negatively stained cores isolated by several procedures. The data obtained support a model of icosahedral arrangement of the DNA-polypeptide VII complex inside the virion. Sarcosyl cores of adenovirus type 2 consist of the viral DNA and polypeptide VII predominantly. In contradistinction, pyridine cores (32, 33) contain viral DNA and polypeptides V and VII. A comparison of the composition of sarcosyl and pyridine cores therefore suggests that the type of association of these two polypeptides with the core must be fundamentally different. Sarcosyl cores are tightly packed, gradually unfold on storage, and contain circular viral DNA which is sensitive both to pancreatic DNase and the EcoRI restriction endonuclease. Evidence is presented that the terminal EcoRI endonuclease fragments of adenovirus type 2 DNA, A and C, are linked by protein. A complex between the DNA termini and protein has been isolated.

MATERIALS AND METHODS

Media and solutions. Cells were grown in Eagle medium or Eagle medium modified for suspension cultures (15) supplemented with 10% calf serum. Calf serum was purchased from Flow Laboratories or from Grand Island Biological Laboratories. The composition of phosphate-buffered saline (PBS) was described previously (14); TE is 0.01 M Tris-hydrochloride, pH 7.2 to 7.5, 0.001 M EDTA; CsCl solution A consisted of 15 g of CsCl (Merck, Darmstadt) and 10 ml of TE. Buoyant CsCl solution contained n grams of CsCl and n/2 ml of 0.02 M Tris-hydrochloride, pH 8.0. For the determination of radioactivity toluene-

methanol- and toluene-based scintillators were used which consisted of 5 g of 2,5-diphenyloxazole (Merck, Darmstadt) and 0.3 g of 2,2'-p-phenylene-bis-(5-phenyloxazole) (Merck, Darmstadt) per liter of a 1:1 toluene methanol mixture or of toluene. Electrophoresis buffer (TEB buffer) consisted of 0.089 M Tris-hydrochloride, 0.089 M boric acid, 0.0025 M EDTA, 0.01 M propylamine, and 0.5% sarcosyl.

Cells and virus. KB cells (CCL17 from the American Type Culture Collection) were grown in monolayer cultures in Eagle medium (15) or in Eagle medium for suspension cultures both supplemented with 10% (vol/vol) calf serum. Human adenovirus type 2 (Ad2) was propagated in KB cells in suspension cultures and was purified as published previously (10, 11, 13). The production of Ad2 radioactively labeled with [6-^{3}H]thymidine, [^{14}C]formate-sodium or a mixture of ^{3}H-labeled amino acids described elsewhere (10, 11, 13). All radioactively labeled compounds were purchased from Amersham-Buchler, Braunschweig, Germany.

Viral DNA. Adenovirus DNA was extracted from CsCl-purified virions by methods reported previously (5, 11, 13).

Infection of cells for electron microscopy. KB cells growing in monolayer cultures on 100-mm diameter plastic dishes were infected with CsCl-purified Ad2 at a multiplicity of 50 to 100 PFU/cell as described previously (3).

Preparation of sarcosyl cores from adenovirions. (i) Equilibrium sedimentation in CsCl density gradients. Between 5 and 10 optical density units at 260 nm of CsCl-purified Ad2 (^{14}C-labeled) in 0.3 ml of buoyant CsCl solution were mixed with 0.07 ml of 10% sarcosyl (N-lauroylsarkosin sodium salt) in water and 0.63 ml of TE and incubated at ambient temperature for 30 min. In some experiments 5 μg of ^{3}H-labeled Ad2 DNA was added as density reference. Subsequently, this mixture was layered on top of 3.5 to 4.0 ml of CsCl solution A in a nitrocellulose tube of the SW56 rotor and centrifuged to equilibrium in an L2-65B Beckman ultracentrifuge at 37,000 rpm and 4 C for at least 60 h. After centrifugation, 5-drop fractions were collected. Refractive indices of every tenth fraction were determined in a Zeiss refractometer. Radioactivity was measured in 5- to 10-μl aliquots of each fraction.

(ii) Gel filtration on Sepharose 2B columns. Sarcosyl cores were also obtained by adjusting the CsCl purified Ad2 preparation to 0.5% sarcosyl in TE and passing this mixture over a Sepharose 2B column (1 cm in diameter by 10 to 15 cm in length) equilibrated with the same buffer. The column was developed at 4 C with 0.5% sarcosyl in TE at a flow rate of 2 ml/h.

Preparation of pyridine cores. Pyridine cores were prepared according to the procedure of Prage et al. (33, 34). Ad2 (^{14}C-labeled), which had been purified by equilibrium centrifugation in CsCl density gradients, was dialyzed against 0.005 M Tris, pH 8.1, and disrupted in 10% pyridine and the cores were subsequently isolated by zone sedimentation on 10 to 25% sucrose density gradients in 0.002 M Tris-hydrochloride, pH 7.5, and 0.0002 M EDTA. The samples were centrifuged in the SW41 rotor of the Spinco

L2-65B ultracentrifuge at 30,000 rpm for 125 min at 4 C.

Disintegration of adenovirus particles by heat. CsCl-purified preparations of Ad2 were dialyzed into PBS or 0.02 M Tris-hydrochloride, pH 8.1, and were heated at 56 C for 5 min (39). These virions were examined in the electron microscope directly, or after rebanding in CsCl density gradients. In some cases, the polypeptide composition of the rebanded virus preparations was also analyzed by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Enzymes and reaction conditions. DNase, electrophoretically purified, was bought from the Worthington Biochemical Corp., and was used without further pretreatment. Adenovirus cores in a volume of 0.1 ml were incubated in 0.01 M Tris-hydrochloride, pH 7.5, and 0.005 M $MgCl_2$ with 1 μg of DNase per ml at ambient temperature for 5, 10, or 20 min. In some of the experiments, DNase digestion was performed in 0.01 M Tris-hydrochloride, pH 7.2, 0.01 M $CaCl_2$, and 0.01 M $MnCl_2$ (35). After DNase treatment, some of the samples were incubated with 500 μg of pronase per ml and 1% SDS at 37 C for 30 min and subsequently were extracted with 0.5 ml of redistilled phenol saturated with TE. The phenol and aqueous phases were separated by centrifugation, and the aqueous phase was dialyzed against one-tenth strength electrophoresis buffer and analyzed by electrophoresis on polyacrylamide (1.5%)-agarose (0.8%) gels as described below.

Pronase B was obtained from CalBiochem, dissolved in 0.01 M Tris-hydrochloride, pH 7.5. at a concentration of 5 mg/ml, and preincubated at 37 C for 2 h. Each lot of Pronase B purchased was assayed after autodigestion for the absence of endo- or exonuclease activity by incubating ^3H-labeled Ad2 DNA with 250 μg of preincubated pronase in 0.01 M Tris-hydrochloride, pH 7.2, 0.1 M NaCl, and 0.002 M $MgCl_2$ for 60 min at 37 C. Subsequently, the size of the DNA was determined on neutral sucrose density gradients (6).

Restriction endonuclease RI from Escherichia coli (EcoRI). The purification of the restriction endonuclease EcoRI has been described previously (11; R. N. Yoshimori, Ph. D. thesis, Univ. of California, San Francisco, 1971). For the experiments described in this report, the DEAE-cellulose fraction of the endonuclease was dialyzed against 0.01 M potassium phosphate, pH 7.0, 0.35 M NaCl, 0.001 M EDTA, and 0.007 M β-mercaptoethanol and was chromatographed on a phosphocellulose (Whatman, P-11) column (1 by 10 cm) previously equilibrated with 0.01 M potassium phosphate, pH 7.0, 0.35 M NaCl, 0.001 M EDTA, 0.007 M β-mercaptoethanol, and 10% glycerol. The phosphocellulose column was developed with a gradient of NaCl from 0.35 to 0.70 M in the same buffer. The EcoRI endonuclease was eluted at approximately 0.5 M NaCl. The enzyme was stored in the same buffer it was eluted in, and proved stable at 0 to 4 C for several months. The reaction conditions for this enzyme as employed in this laboratory were reported elsewhere (11).

Sarcosyl cores of Ad2 to be digested with the EcoRI restriction endonuclease were dialyzed into TE and treated, in a total volume of 100 to 150 μl of 0.05 M Tris-hydrochloride (pH 7.5) and 0.01 M $MgCl_2$, with 10 to 30 μl of the phosphocellulose fraction of the enzyme at 37 C for 60 min. Subsequently, 5 μl of a 0.2 M EDTA solution, and in some of the experiments 10 μl each of a Pronase solution (5 mg/ml) and a 10% SDS solution, was added and incubation was continued for 30 min at 37 C. In other experiments, Pronase addition was omitted (for details see legend to Fig. 13). The mixture was then extracted with phenol (TE saturated), the aqueous and phenol phases were separated by centrifugation, and the aqueous phase was dialyzed against one-tenth strength electrophoresis buffer and analyzed by electrophoresis on polyacrylamide-agarose gels as described below.

Gel electrophoresis. (i) Analysis of polypeptides in SDS-polyacrylamide gels. The procedures of Maizel (25) were used with minor modifications. Samples of sarcosyl or pyridine cores or intact adenovirions were dialyzed against 0.01 M ammonium-acetate, lyophilized in a Virtis lyophilizer, resuspended in 0.1 ml of sample buffer (0.0625 M Tris-hydrochloride, pH 6.8, 2% SDS, 1% mercaptoethanol, and 10% glycerol), and heated to 100 C for 2 min. Subsequently, 5 μl of a 0.25% solution of bromophenol blue was added, and the samples were analyzed on 13% polyacrylamide gels. Cylindrical gels of 14 cm in length were used. The composition of the gels and of the electrode buffer was described previously (13, 37). Electrophoresis was performed for 5.5 to 7 h at ambient temperature and 100 V. The gels were then fixed in 20% trichloroacetic acid, stained in 0.25% Coomassie blue in 20% methanol and 7% acetic acid for periods ranging from 30 min to 16 h and were destained in 20% methanol and 7% acetic acid for several days. In some of the experiments in which radioactively labeled proteins were analyzed, the gels were cut with a slicer and each slice (usually 0.8 to 1.0 mm thick) was processed as described below. For some experiments slab gels were used of identical composition (21) and electrophoresis was performed in an apparatus as described by Studier (41).

(ii) Analysis of DNA fragments in 1.5% polyacrylamide-0.8% agarose gels. The composition of the gels and of the TEB electrophoresis buffer was described earlier (13). DNA samples (0.1 to 0.2 ml) in one-tenth strength of electrophoresis buffer were layered on cylindrical gels, and electrophoresis was carried out at 40 V at ambient temperature for 8.5 to 12 h. In most experiments the EcoRI fragments of ^{14}C-labeled Ad2 DNA were added as molecular weight markers. Immediately after electrophoresis, gels were cut into 0.8- to 1.0-mm slices and the slices were processed for counting. Individual gel slices were placed into plastic tubes or directly into scintillation vials and extracted with 0.2 to 0.5 ml of 0.1% SDS with shaking at 37 C for 18 h. The extracts were adsorbed to GF/83 glass-fiber filters, dried at 80 C, and counted in 5 ml of the toluene-based scintillator in a Packard TriCarb liquid scintillation spectrometer, model 3385 or 3330. In some experiments portions

of the extracts were counted directly in 5 ml of the toluene/methanol-based scintillator.

Electron microscopy. Monolayers of KB cells infected with Ad 2 were transferred to an ice bath and fixed with ice-cold 2% glutaraldehyde in PBS. After fixation on ice for 60 min, the cells were scraped from the plates, washed with cold PBS, and stored at 4 C until prepared for electron microscopy.

Infected cells were prepared for thin sectioning after glutaraldehyde fixation as previously described (3). Cells were postfixed for 1 h with a 1% solution of osmium tetroxide in Millonig phosphate buffer (27). The post-fixed cells were embedded in epon 812 by the procedure of Luft (24). Silver to silver-gold sections were cut on a Reichart OM U-2 ultra microtome.

Freeze etching of glutaraldehyde-fixed virus-infected cells was carried out by the procedure described by Brown et al. (4). Some specimens were fractured and etched after washing in distilled water prior to freezing.

Negative staining was carried out by the procedure of Anderson (2). Phosphotungstic acid was prepared as a 2% solution in distilled water and was adjusted to pH 7.2 by addition of NaOH.

Preparation of purified viral cores for the electron microscope was carried out by a modification of the aqueous spreading procedure of Davis et al. (9). The spread monolayers of DNA and protein were transferred to carbon-coated grids and were rotary shadowed at an angle of 10° with platinum carbon.

Specimens were photographed in a Siemens 101 electron microscope utilizing either bright or dark field optics. The magnification of the instrument was calibrated with a carbon grating replica having 2,160 lines per mm (Ernest Fullam Co., no. 1002).

RESULTS

Morphology of Ad2. It is generally accepted that the adenovirions have a mean diameter of about 80 nm (20, 43) and that the adenovirus capsid is composed of hexons which are 7 to 8 nm in diameter and pentons which are 8.0 nm in diameter. More recent studies have revealed the hexon to be a rod-like structure composed of three proteins with an overall diameter of 9 nm and a length of 11 nm and the penton base to represent a sphere of 8-nm diameter (30, 45; G. Wadell, Ph.D. thesis, Karolinska Institutet, Stockholm, 1970). We reinvestigated the dimensions of the type 2 adenovirion for the purpose of relating the observations on the internal morphology of the particle to the structure of the outer surface. We measured the size of adenovirus hexons and pentons in both the intact virion (Fig. 1a) and as free structures after release from the virion by heating (Fig. 1b). We also determined the center-to-center spacing of the hexons on the face of the intact virion and in groups of hexons released from the triangular faces of the virus icosahedron during

heating (Fig. 1b). The dimensions of the various structures are compiled in Table 1. The predicted size of the virion obtained by measuring the center-to-center spacing of the hexons was 86.16 nm using the equations $E = dT^{\prime\prime}$ and $D = E/0.618$ (40), where E is the edge of one equilateral triangular face, D the diameter of the icosahedron, d the center-to-center spacing of the hexons, and T the triangular number of the icosahedron (for adenovirus $T = 25$). The measured value for the particle was 97.5 nm. This suggests that in negatively stained preparations the virions are distorted by compression as described by Anderson (1) and by Moody (28), such that the observed cross-sectional diameter is increased by 13%. By utilizing the equation $V = 2.1817\ E^3$, where V is the volume of an icosahedron and E the edge of one of the equilateral triangular faces, we found the total volume of the virion to be 3.29×10^5 nm^3.

Morphology of the core of Ad 2. Subtracting the thickness of the hexon-penton shell from the complete virion left a subcapsomere space of volume 1.35×10^5 nm^3 and an internal diameter of 64.15 nm. The measured diameter of cores seen in thin-sectioned intact virions was 63.9 nm. The smaller size probably results from shrinkage during the embedding process (29). The volume of the space attributed to the core was therefore 41% of the total volume of the virion.

The 63.9-nm electron dense core seen in ultra-thin sections of the intact virion (Fig. 2) revealed no information regarding the organization of the components of the core. We initially attempted to study the interior of the adenovirion by freeze-cleaving of purified virions and of virions attached to the surface of cells. Such

TABLE 1. *Measured and calculated dimensions of Ad 2 and its structural components*

| Structure	Technique	Average size in nm
Hexon diameter	Negative stain	9.1
Hexon length	Freeze etching	12.0
Hexon length	Thin sectioning	10.5
Penton diameter	Negative stain	8.3
Center-to-center spacing of hexons	Negative stain	10.7
Width of virion	Negative stain	97.5
Width of virion	Thin section	85.6
Width of virion	Freeze etching	87.0
Width of virion	Calculated	86.16
Volume of virion	Calculated	3.29×10^5 nm^3
Diameter of core	Thin section	63.9
Diameter of core	Freeze etching	60.0
Diameter of core	Calculated	64.1
Volume of core	Calculated	1.35×10^5 nm^3
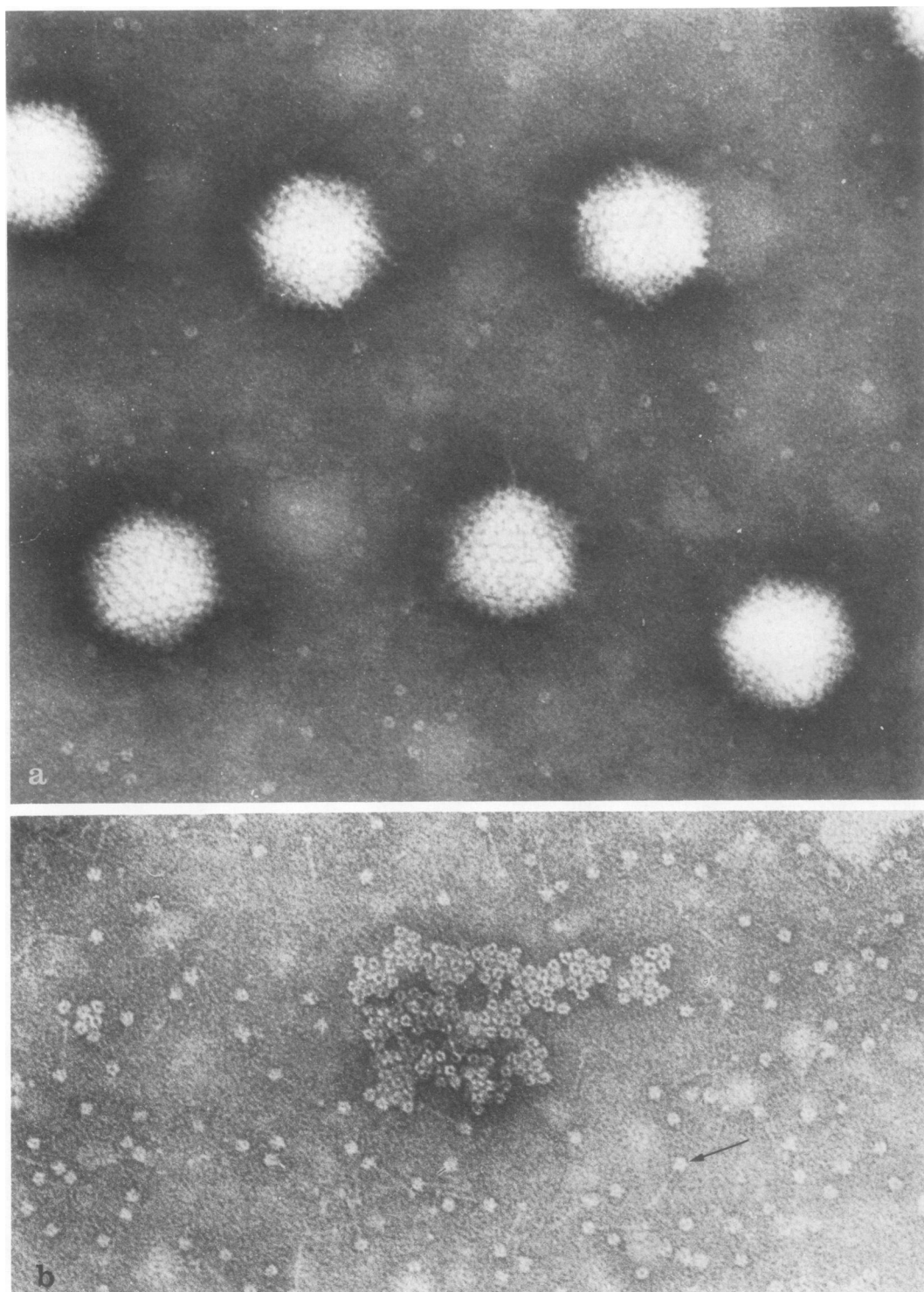
Fig. 1. *Negatively stained preparations of (a) intact type 2 adenovirions and (b) virus capsomeres released from the virion by heat treatment. The intact virions (a) show the classical arrangement of six- and fivefold organized capsomeres. It is possible on a number of the virions to measure accurately the center-to-center spacing of the capsomeres and the diameters of the subunits. The capsomere in the disrupted viral capsid are also organized in sixfold arrays (b). The hexons have the same cross-sectional diameter as those in the intact virion and in addition have a recognizable substructure. Pentons with fibers can be seen in the background (arrow). Magnification, ×226,400.*
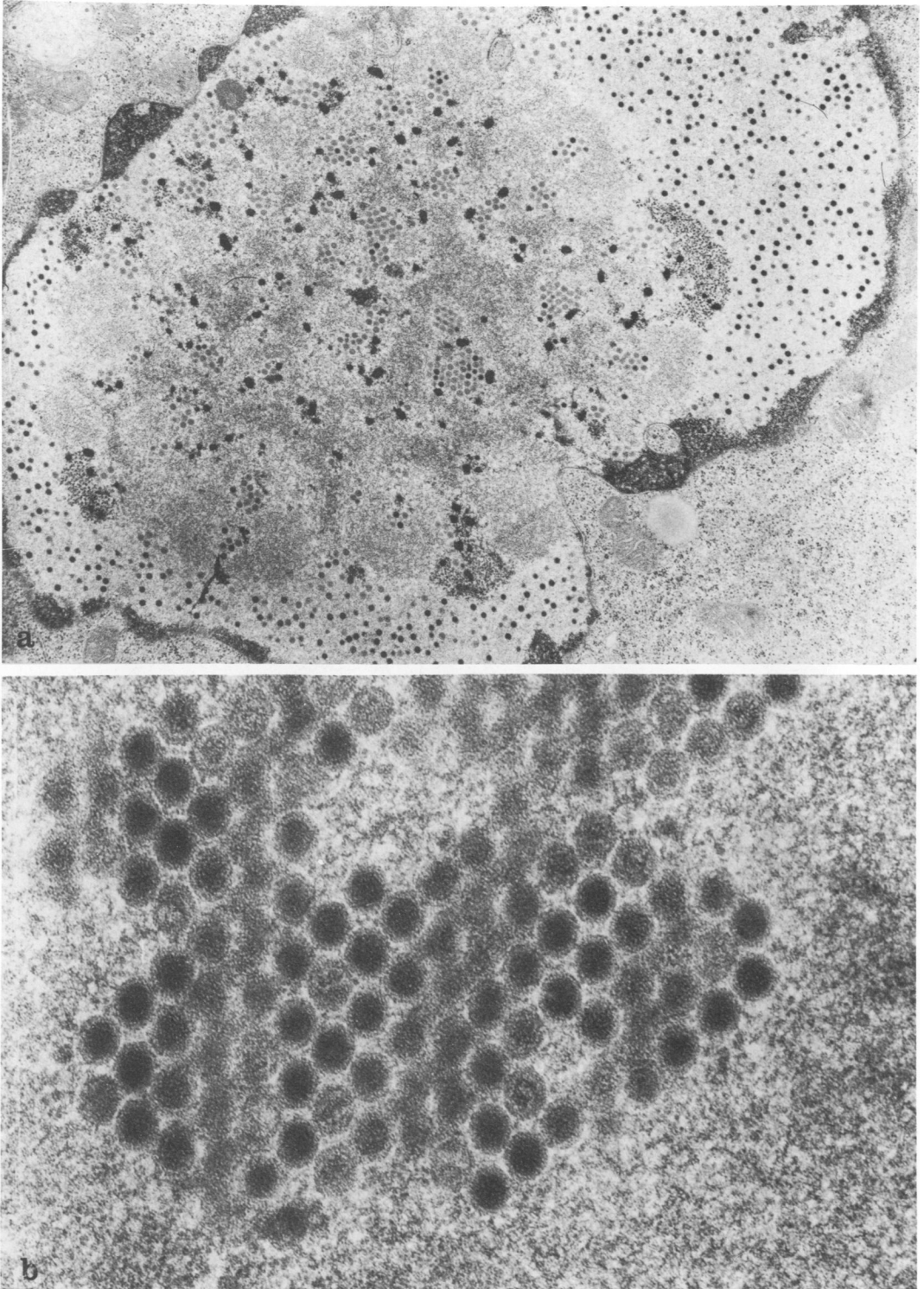
FIG. 2. *Ultrathin sections of intranuclear adenovirus paracrystalline structures. (a) Low magnification of cell at 36 h postinfection. Small crystalline structures are visible in the nucleus. (b) High magnification of a paracrystalline intranuclear inclusion. The cross-sectioned virions have a distinct electron dense center and an electron transparent layer of capsomeres. Magnification: (a) ×10,600; (b) ×80,000.*

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experiments never produced fracture planes which passed through the interior of the virions, but rather always exposed the outer surface of the particles (see ref. 3; Fig. 3). We interpret this result to indicate differences in the hydration of the interior of the isolated virion relative to the surrounding medium creating a resistance to the developing fracture plane. Altering the concentrations of cryoprotective (glycerol) did not produce any change in the image of the virion after freeze-cleaving. In a further attempt to produce fracture planes within the virion we have freeze-cleaved intact KB cells late after infection with Ad 2. At late times after infection (35 to 40 h postinfection), the nuclei of infected cells contain localized areas of very high concen-
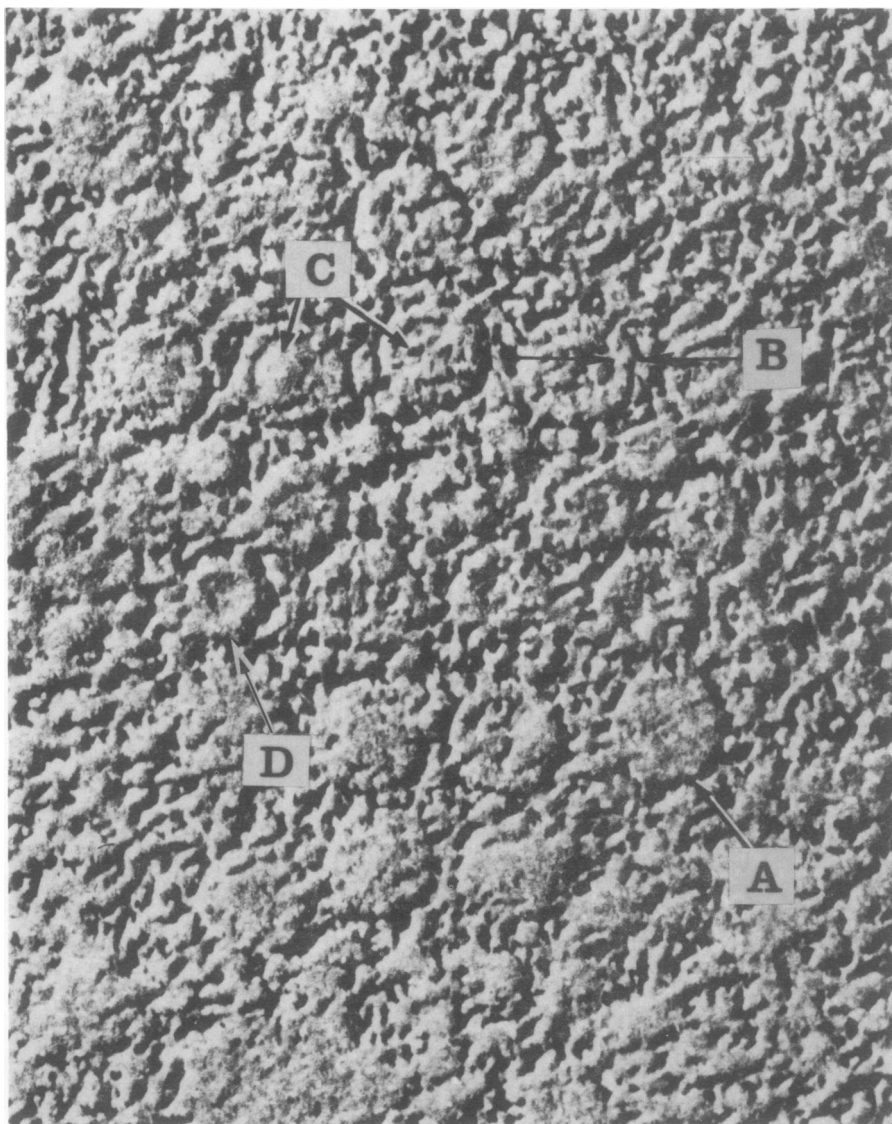
FIG. 3. A paracrystalline inclusion of the type shown in Fig. 2 after freeze-etching in 30% glycerol and shadowing with platinum carbon. Some of the virions are cross-fractured producing a relatively flat structure in which the outer ring of capsomeres is poorly resolved (A). Other virions have a more distinctly resolved outer ring of capsomeres (double arrow B). The internal core structure (C) is resolved in some virions as either a smooth spherical structure (C left) or containing a knob-like substructure (C right). Occasionally, virions have been cleaved such that the inner content is removed showing the inner smooth surface of the capsid (D). Magnification: ×162,000.
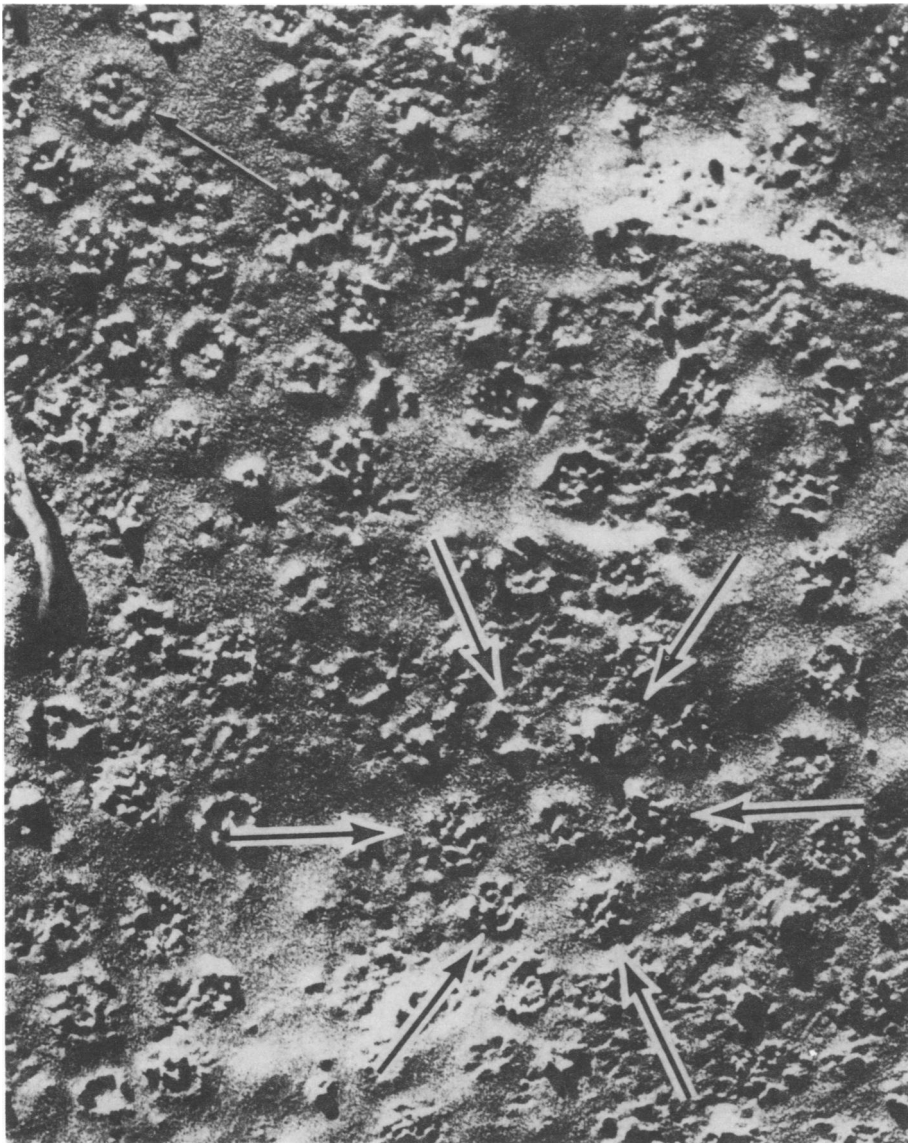
FIG. 4. *An area of a large intranuclear paracrystalline inclusion after freeze-etching in distilled water and shadowing with platinum carbon. The hexagonal arrangement of the virions is preserved in the freeze-etched paracrystals (large double white-black arrows). Many of the virions are broken showing internal substructure (single white-edged arrow). Magnification: ×140,000.*

trations of virions (paracrystals) (Fig. 2a, b).

It was hoped that the virions in the cell nucleus would be similar in their cleaving characteristics to the surrounding nucleoplasm allowing fracture planes to pass through the outer layer of capsomeres and exposing the interior of the virion. The paracrystals were readily revealed by freeze-cleaving (Fig. 3 and 4), and in contrast to extracellular virus, virions

within the nucleus could be cross-fractured. Cleavage planes passed through the outer layer of capsomeres and exposed in a varied manner the internal structure of the virions. In some instances the virions were cut so that a smooth surface was produced which barely revealed the outer ring of capsomeres (Fig. 3). Such particles were fractured close to the center and had a cross-sectional diameter slightly larger than

that found in thin-sectioned virions (Fig. 2b). The difference in the size of the freeze-etched virions compared to thin-sectioned structures can probably be accounted for by shrinkage occurring during dehydration and embedding for thin sections. The diameters of the freeze-etched virions of 87 nm agrees with the size of the virion predicted in Table 1.

In other virus particles the fracture plane passed through the outer layer of capsomeres and then over the internal contents revealing the inside of the virion as a smooth sphere or as composed of closely packed spheres with a diameter of 9 to 10 nm (Figs. 3 and 4). In virions fractured in this fashion the thickness of the outer hexon layer was 10.5 to 12 nm. Some virions were broken such that the core was removed and the inner surface of the capsids was exposed. This inner surface was smooth in appearance (Fig. 3).

Isolation of sarcosyl cores from purified virions. When CsCl-purified preparations of [^{14}C]formate-labeled Ad2 were treated with sarcosyl and subsequently centrifuged to equilibrium in CsCl density gradients as described above, the bulk of the ^{14}C-label in Ad2 banded in a density position approximately 0.2 g × cm^{-3} lighter than that of the ^{3}H-labeled marker DNA which was purified from Ad2 virions by the SDS-Pronase procedure (13; Fig. 5a). The ^{14}C-label on top of the gradient was due to viral protein. The apparent buoyant density of Ad2 DNA in this experiment was higher than the value of ρ = 1.716 g × cm^{-3} reported previously (10), perhaps due to the presence of small amounts of sarcosyl. For the analysis of the sarcosyl cores (see below) fractions 27 to 29 were used. It will be shown by electron microscopy (see Fig. 8) that these fractions contain viral cores.

The data presented in Fig. 5b and c reveal that sarcosyl cores can be readily separated from the bulk of the soluble viral proteins by gel filtration on Sepharose 2B. When [^{3}H]thymidine-labeled Ad2 was treated with sarcosyl, the cores eluted in a single sharp peak (Fig. 5b), whereas Ad2 labeled with a mixture of ^{3}H-labeled amino acids yielded two peaks of radioactivity upon treatment with sarcosyl and gel
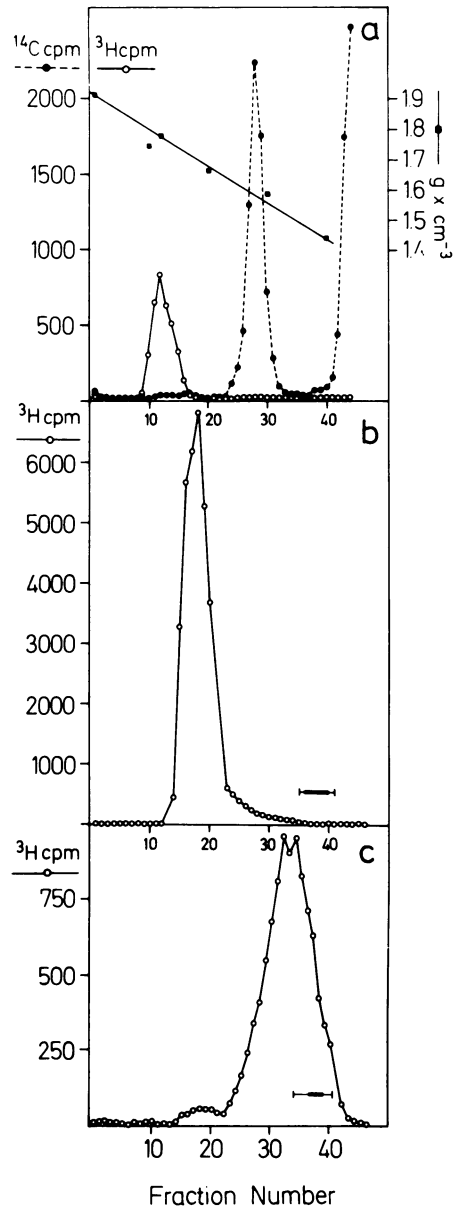

FIG. 5. *Isolation of sarcosyl cores. (a) Equilibrium sedimentation in CsCl density gradient. Approximately 1.8 optical density units at 260 nm of [^{14}C]formate-labeled Ad2 in buoyant CsCl solution were treated with sarcosyl and centrifuged to equilibrium in a CsCl density gradient together with ^{3}H-labeled Ad2 DNA (0.3 μg) as described. The refractive indexes*

were measured in every tenth fraction and the densities were calculated as described elsewhere (44). The ^{3}H and ^{14}C activities were determined in 5- to 10-μl portions of each fraction as described. (b) Gel filtration on Sepharose 2B column. Approximately 2.3 optical density units at 260 nm of [^{3}H]thymidine-labeled Ad2 in buoyant CsCl solution were treated with sarcosyl in TE and passed over a Sepharose 2B column as described. The bar indicates the position of the dinitrophenyl-alanine dye which was used as a marker. (c) In a similar experiment 2.0 optical density units at 260 nm of Ad2 labeled with a mixture of ^{3}H-labeled amino acids were used under conditions identical to those described in (b).

filtration (Fig. 5c). The minor peak represents the sarcosyl cores, the major peak the viral proteins (Fig. 5c). In this experiment, about 3.9% of the ^{3}H-amino acid label was recovered with the sarcosyl cores. This finding indicates that only part of polypeptide VII is present in the sarcosyl core, since polypeptide VII represents 14% of the viral protein (32). A part of polypeptide VII was apparently released and could be found in the main peak (Fig. 5c). At the present time, the mechanism of this release is not understood.

Sarcosyl cores prepared by gel filtration were indistinguishable in the electron microscope from sarcosyl cores obtained after equilibrium centrifugation.

A third way of preparing Ad2 cores was by the pyridine method (33) which yielded structures different from sarcosyl cores as judged by electron microscopy and the polypeptide composition (see below).

Electron microscopy of purified cores. Subviral particles produced by heat treatment (39), pyridine treatment (33), or by treatment with sarcosyl were examined in the electron microscope. It was necessary to utilize a variety of preparative procedures for the analysis of each type of subviral particle, as a procedure which produced good results with one type of subviral structure frequently produced no information when applied to another.

Electron micrographs of structures produced by heating adenovirions to 56 C for 5 min are shown in Fig. 6. Best preparative results were obtained by negative staining of these structures with neutral phosphotungstic acid. The subviral structures observed were similar to those described by Russell et al. (39). Free hexons, groups of hexons, pentons, and amorphous structures were seen (Fig. 6a). The amorphous structures were larger than the intact virions from which they were derived and had an average diameter of 167 nm, suggesting that they represent particles which have lost their structural integrity and have relaxed into a larger volume. The subviral particles produced by this procedure were found to contain most of the viral structural proteins (see below). Some substructure was recognized in the amorphous spheres (Fig. 6b). Spherical structures about 20 nm in diameter with a center-to-center spacing of about 30 nm can be distinguished. It was not possible to determine if these substructures reflect an internal organization of the virion or a topological alteration of the viral surface.

Particles produced by treatment with pyridine produced similar results whether stained positively with uranyl acetate or negatively
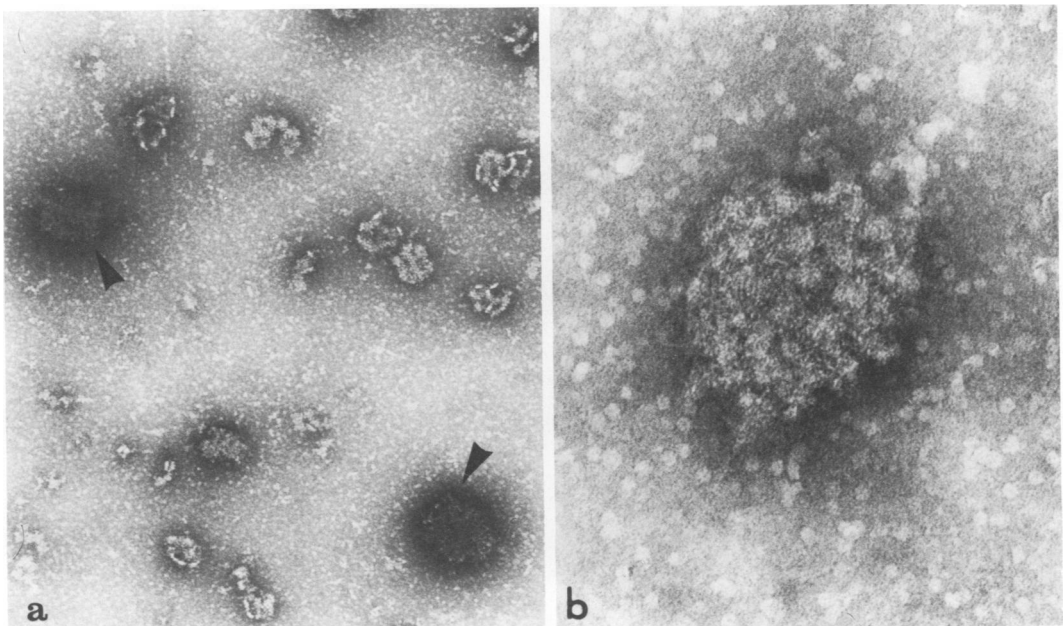

FIG. 6. *Negatively stained preparation of Ad2 after heating at 56 C for 5 min. (a) Low magnification showing viral capsid components and large amorphous cores (arrows). (b) Higher magnification of a subviral structure showing a subunit-like organization. In the background can be seen free capsomeres. Negatively stained with phosphotungstic acid. Magnification: (a) ×80,000; (b) ×200,000.*

stained with phosphotungstic acid (Fig. 7). The pyridine core structure was best preserved by diluting the preparation in 0.37% formaldehyde in PBS after gradient purification. The pyridine cores were tightly packed and had a mean diameter of 66 nm (Fig. 7a). Occasionally, partially decomposed structures were observed which seemed to contain a variable number of smaller spheres approximately 22 nm in diameter (Fig. 7b). The internal spherical structures seen in the pyridine cores had a halo of associated material which appeared to be of uniform thickness. It is possible that the sphere-associated material holds the 22-nm substructures together in the tightly packed structures (Fig. 7a) and coats the entire core preventing the visualization of substructure in the majority of particles observed.

Sarcosyl cores produced different images in the electron microscope depending upon the preparative procedure employed. The sarcosyl cores were rather fragile when examined by negative or positive staining, but could be preserved by treatment with formaldehyde as described above. Positively stained sarcosyl cores (Fig. 8b) revealed electron dense structures with a somewhat variable diameter which measured 66 to 67 nm in the best preserved structures. The structures tended to aggregate,

and frequently fibrous material, possibly DNA, radiated from the centrally located electron dense structures. The size variation described above appeared to result from the reduction in size of the centrally located dense body as more DNA was released from the particle. When the particles were negatively stained (Fig. 8a), it was no longer possible to identify the DNA fibers or to find such well defined electron dense centers as seen in positively stained preparations. The negatively stained structures, rather, appeared as a collection of smaller spheres with a constant diameter of 21.6 nm. Discrete clusters of eight to ten 21.6-nm diameter spheres were observed (Fig. 8c).

A comparison of the morphology of positively and negatively stained sarcosyl particles suggests that the structures of 67 nm diameter described above are composed of a number of spheres with a diameter of 21.6 nm. These spheres appeared very electron dense and were not distinguished in positively stained preparations. In negatively stained preparations the differential penetration of stain into the 67-nm particle and the partial exclusion of stain from the interior of the 21.6-nm substructures facilitates visualization of the internal morphology of the sarcosyl particles.

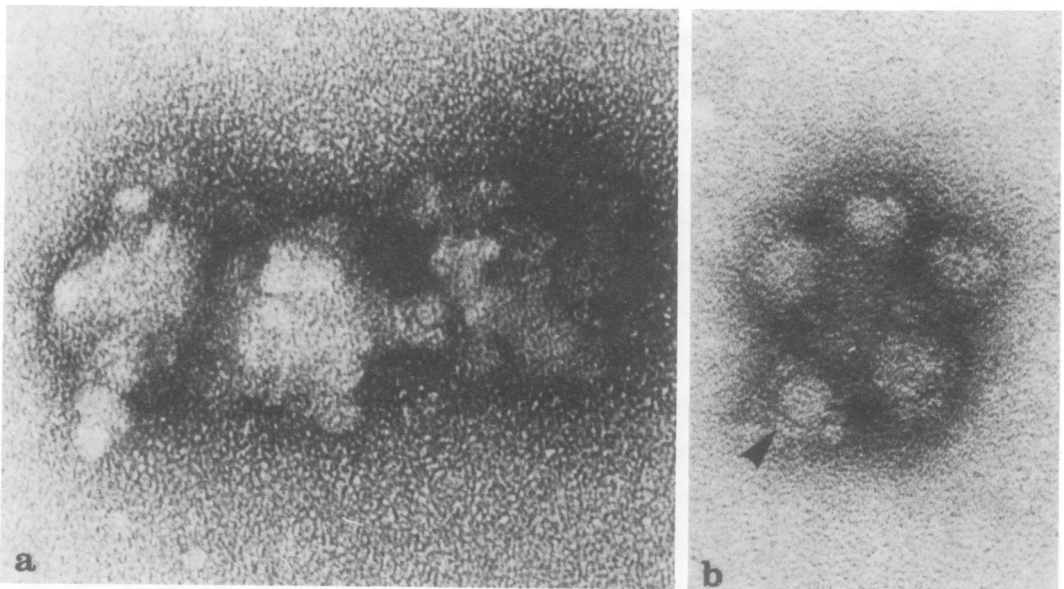We attempted to verify that the fibrous

FIG. 7. Viral cores released from adenovirions by treatment with pyridine. (a) Typical pyridine cores. The structures are hydrophobic and adhere to one another. The surface is smooth and bulges as though there were spherical structures under the surface. (b) An unusual pyridine core. The particle appears to have opened somewhat revealing the spherical structures within. The spheres are coated with some material which may have been on the outer surface of the core (arrow). Negatively stained with uranyl acetate. Magnification: (a) ×370,000; (b) ×300,000.
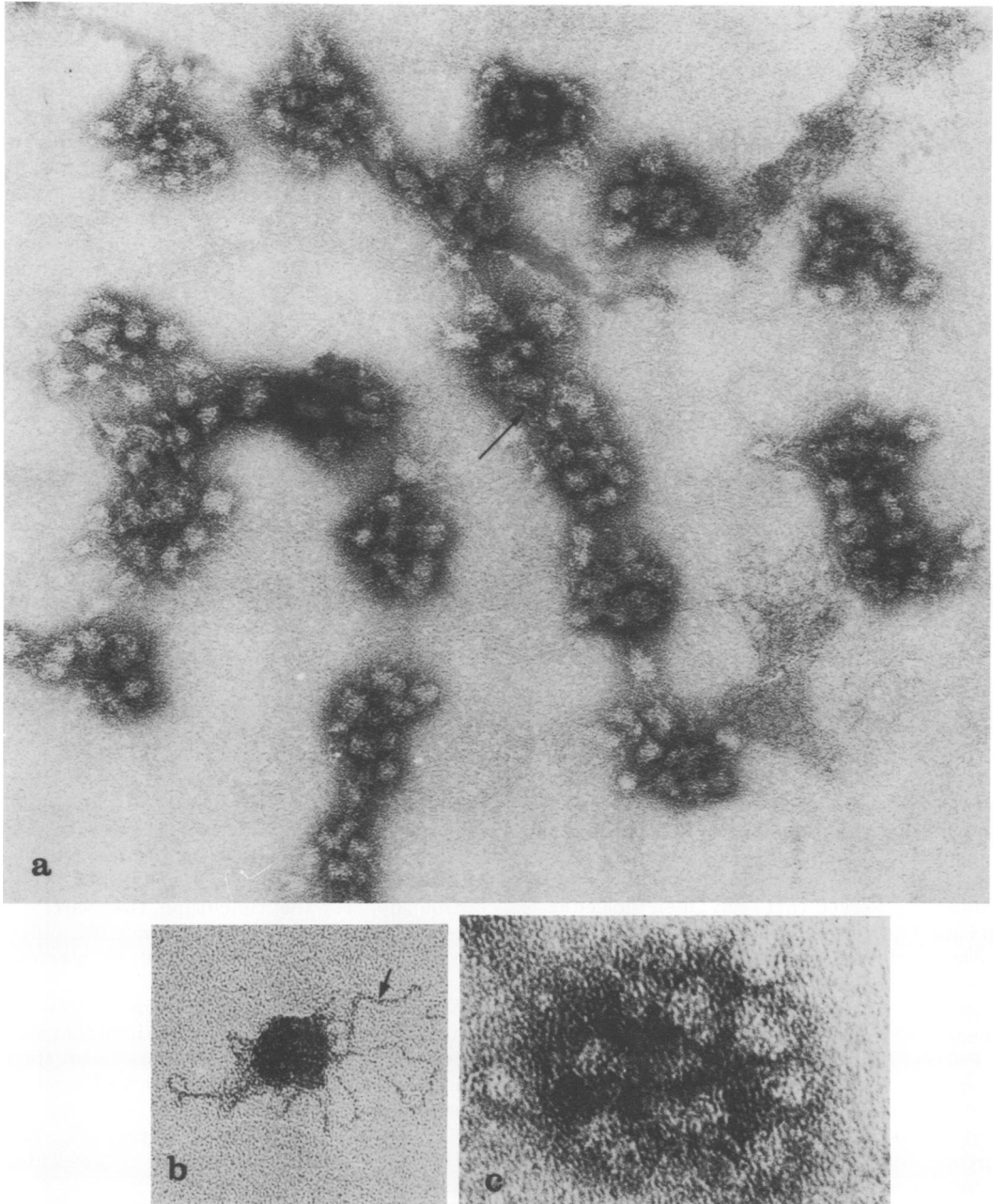
FIG. 8. *Cores released from Ad2 after treatment with sarcosyl.* (a) *Cores negatively stained with uranyl acetate. One cannot detect the free DNA or a well-defined central dense body. Rather the cores are made up of a number of smaller spherical structures. Unlike the pyridine cores these spheres have no halo of material around them. The core subunits sometimes appear to be held together by fibrous material (arrow).* (b) *A sarcosyl core positively stained with uranyl acetate. The arrow points to DNA fibers protruding from the electron dense core.* (c) *High magnification of a negatively stained sarcosyl core, showing 9 or possibly 10 subunits. Magnification:* (a) ×116,000; (b) ×156,000; (c) ×420,000.

structures seen radiating from the positively stained sarcosyl cores was DNA by spreading sarcosyl cores on a protein monolayer. To our surprise, freshly prepared particles did not spread into the random DNA structure as expected, but rather appeared as tightly packed particles of approximately the size described for the positively stained particles (Fig. 9a). Occasionally, these particles had small protrusions on their surface suggesting the association with these structures of free DNA (Fig. 9a). If the particles were allowed to stand for a few days at 4 C, the amount of free DNA protruding increased, whereas the size of the central core decreased concomitantly (Fig. 9b). Free ends of DNA were rarely seen. As the DNA was released from the dense center of the particle, it formed a rosette of closed loops around the central structure. The loops of DNA were frequently highly twisted along their length, but opened at their distal ends like hair pins (Fig. 9b, c). With increasing time of incubation at 4 C, the cores seemed to release progressively more DNA into long random structures, some of which represented one genome equivalent of DNA (10 to 11 μm in length). Even in these highly disorganized arrays free ends of DNA were only rarely seen, and the DNA was simply organized in larger loops which radiated as before from the surface of a smaller core structure (Fig. 9d).

If the preparations were allowed to stand for a period of about 1 week, the core-like structures were only rarely seen. Instead randomly arranged circles of one adenovirus genome length similar to those described by Robinson et al. (36) were observed (Fig. 10). Open molecules of one and two viral lengths could also be found in these preparations. Treatment of the purified sarcosyl cores with pronase at any stage after preparation converted the dense bodies into linear molecules of one genome length.

Polypeptide composition of sarcosyl cores from Ad2. Since the buoyant density of sarcosyl cores was found to be considerably lower than that of Ad2 DNA (Fig. 5a), it was likely that sarcosyl cores consisted of DNA and protein. It is not known whether sarcosyl can bind to the cores and alter their buoyant density. Therefore, reliable estimates of the amount of protein remaining associated with sarcosyl cores cannot be made from the buoyant density. The polypeptide composition of sarcosyl cores was determined by electrophoresis on SDS-polyacrylamide gels using both ^{3}H-amino acid-labeled (Fig. 11a–c) and unlabeled cores (Fig. 12, slot no. 2). A comparison of the polypeptide patterns presented in Fig. 11b and c demonstrates that

digestion of the sarcosyl particles with DNase prior to electrophoresis does not affect the apparent polypeptide composition of the sarcosyl cores. In the experiment shown in Fig. 12 (slot no. 2) the sarcosyl cores were analyzed after digestion with DNase which also registered in the gel pattern. The identity of polypeptide VII was demonstrated by comparing slot no. 2 with slot no. 3 which contained DNase by itself (Fig. 12). The data indicate that it is predominantly the alanine/arginine-rich polypeptide VII which was associated with the sarcosyl cores. The identity of this polypeptide was further documented by co-electrophoresis with [^{14}C]-formate-labeled polypeptides from adenovirions (Fig. 11a), and by the high proportion of arginine and alanine in the amino acid composition of sarcosyl cores. The sarcosyl cores contained also a small amount of the hexon polypeptide (II). From the data presented in Fig. 11a and b, it can be estimated that <3% of the total hexon polypeptides were still associated with sarcosyl cores. Small amounts of polypeptides VIII and IX were also present in the sarcosyl cores. This finding is not surprising, since polypeptides VIII and IX are associated with the hexons (16, 17, 18).

As a control, pyridine cores were prepared by the method of Prage et al. (33) and were analyzed by electrophoresis on SDS-polyacrylamide gels (Fig. 12, slot no. 5). It has been shown previously (17, 18, 32, 33) that pyridine cores contain exclusively polypeptides V and VII, and this finding is also documented by the results shown in Fig. 12, slot no. 5. This control supports the conclusion that sarcosyl cores are free of polypeptide V, since this polypeptide can readily be detected in electrophoretograms of core preparations.

When purified virions were heated to 56 C for 5 min (39), repurified by equilibrium centrifugation in CsCl density gradients, and then analyzed by electron microscopy and electrophoresis on SDS-polyacrylamide gels, the resulting cores had a typical structure (Fig. 6b) and contained most of the viral polypeptides (not shown).

The organization of the viral DNA in sarcosyl cores. The sarcosyl cores contain intact viral DNA. When [^{3}H]thymidine-labeled sarcosyl cores were extracted by the SDS-pronase-phenol method (10, 13), Ad2 DNA was obtained which co-sedimented at 31S with ^{14}C-labeled, intact Ad2 marker DNA in neutral sucrose density gradients. After incubation of [^{3}H]thymidine-labeled sarcosyl cores with pancreatic DNase for 5 to 20 min as described
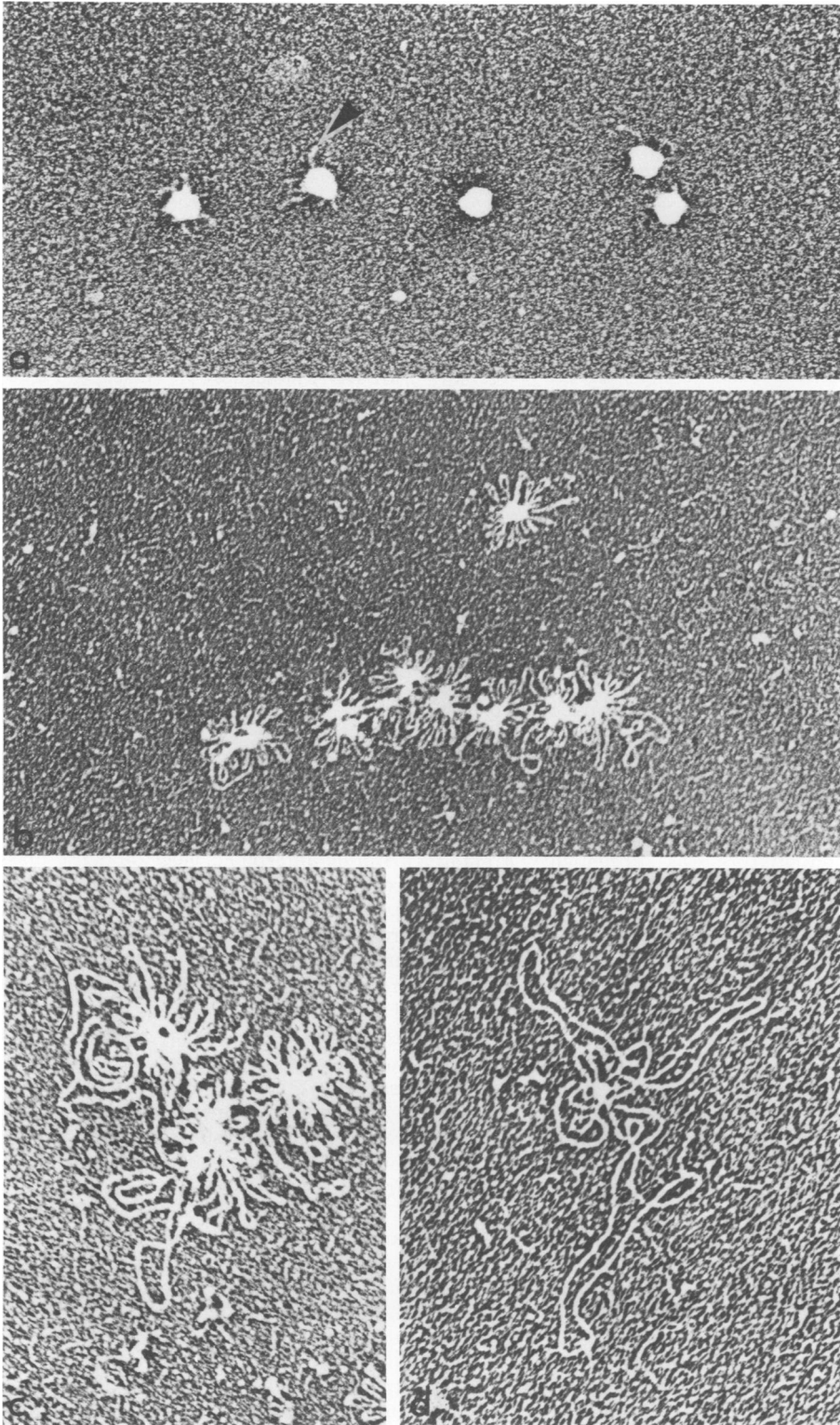
FIG. 9. *Electron micrographs of sarcosyl cores prepared by spreading on a protein monolayer at various times after isolation. (a) First day of purification: the cores are tightly packed structures from which occasionally a small amount of DNA protrudes. (b) Second day after isolation: more DNA protrudes in a radial fashion from the cores. The loops of DNA are closed structures. Free ends are not seen. (c) Third day after isolation: the central core becomes smaller and the free DNA loops larger. The loops remain closed. (d) Four to five days after isolation: the central dense body has almost disappeared and the free DNA loops represent nearly the complete genome length. Magnification: (a) ×40,000; (b) ×38,000; (c) ×60,000; (d) ×60,000.*
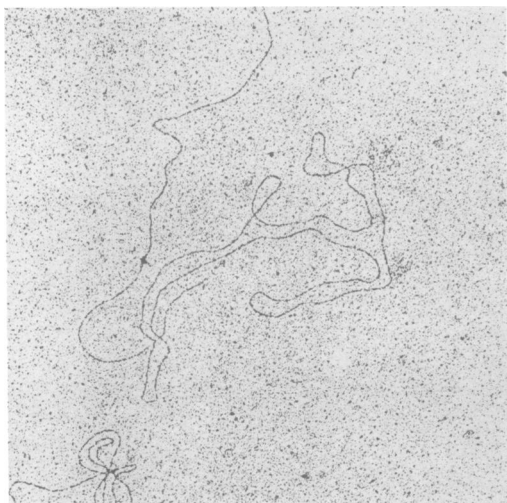
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FIG. 10. *DNA of Ad2. Sarcosyl core at approximately 1 week after isolation. The genome is a circle of viral genome unit length. The central core as seen in Fig. 9 is no longer apparent. Magnification: ×32,710.*

above, all the ³H-label was converted to low-molecular-weight material which migrated with the bromophenol blue dye front upon electrophoresis in polyacrylamide-agarose gels.

When the sarcosyl cores were incubated with EcoRI restriction endonuclease and when the incubation mixture was subsequently treated with Pronase, the [³H]thymidine-labeled Ad2 DNA in the sarcosyl cores was cleaved into the six specific fragments (31) which co-electrophoresed with the six specific EcoRI fragments from ¹⁴C-labeled marker Ad2 DNA (Fig. 13a). These results were obtained independently of the time after isolation of the cores and indicate that, although the viral DNA was closely associated with polypeptide VII in the core, the DNA remained fully susceptible to digestion with pancreatic DNase and that the six palindromic sites recognized by the restriction endonuclease EcoRI were accessible to and can be cleaved by the enzyme.

Strikingly different results were obtained when sarcosyl cores were treated with the EcoRI restriction enzyme and were subsequently not digested with pronase (Fig. 13b). In this case the amount of the ³H-labeled EcoRI A fragment was reduced in amount relative to the ¹⁴C-labeled marker DNA, and the ³H-labeled EcoRI C fragment was missing. Some of the ³H-label was retained on top of the gel (Fig. 13b); this could be due to the missing DNA fragments which are complexed with protein and thus cannot enter the gel, whereas in the experiment

illustrated in Fig. 13a this protein was removed by pronase digestion.

This interpretation was confirmed by the data presented in Fig. 13c. Upon extraction and Pronase digestion of the ³H-labeled material on top of the gel shown in Fig. 13b, the ³H-labeled EcoRI A and C fragments were released from this complex and could be identified by co-electrophoresis with the appropriate ¹⁴C-labeled marker fragments (Fig. 13c). Fragments A and C are located at either terminus of the Ad2 DNA molecule (31) (insert to Fig. 13a) and are presumably involved in the circularization of the DNA molecule inside the virion (36). The major polypeptide found associated with sarcosyl cores was polypeptide VII. It is not yet clear whether some of the polypeptide VII chains or, perhaps more likely, another as yet unidentified polypeptide present in minute quantities is responsible for complex formation with fragments A and C. This complex is interesting because it might stabilize the viral DNA in a circular configuration.

DISCUSSION

(i) Morphology of the adenovirus core. The electron microscopical observations presented here suggest that the interior of the adenovirion is structurally highly organized and not a simple amorphous electron dense mass. Upon treating the intact adenovirion with sarcosyl, spherical structures 66 to 67 nm in diameter arise. This value is slightly larger than the size of the core in the intact virion as measured in electron micrographs of thin sections of freeze-etch preparations. The slightly larger size of the free core may result from distortion due to compression of the particles which generally occurs in air-dried preparations. Alternatively, the increase in the size of the free core may result from an expansion of the structure once it is freed from the confines of the capsid shell.

The DNA of the 67-nm core is packed into a number of smaller spherical structures each having a diameter of 21.6 nm. There are 8 to 10 such spheres detectable for each core, although this number is probably too low as those spheres which are covered by others cannot be counted. Given the cross-sectional diameter of the substructures it is possible to calculate their volume, assuming they are spherical, by the formula $V = 4.189 \ r^3$ (40), where V is the volume and r the radius of the sphere. The volume of this structure would be $5.2 \times 10^3 \ nm^3$, and 25 of these spheres would be required to occupy the
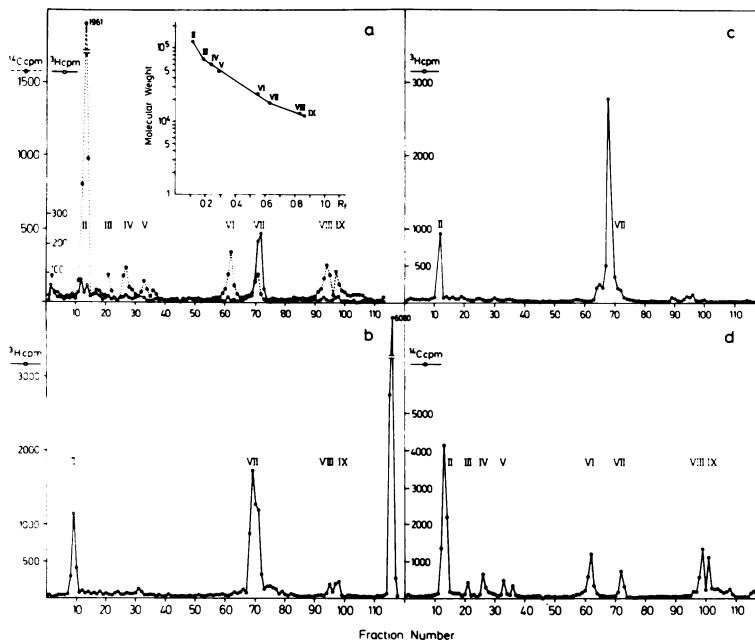
FIG. 11. *Analysis of polypeptide composition of sarcosyl cores. (a) Sarcosyl cores were prepared from ³H-labeled amino acid Ad2 by gel filtration on Sepharose 2B, dialyzed against 0.01 M ammonium-acetate, lyophilized, and resuspended in 0.1 ml of sample buffer. A small amount of ¹⁴C-labeled Ad2 marker virus was added and the sample was electrophoresed on a 13% polyacrylamide gel (21) for 7.5 h at 100 V, as described. The Roman numerals designate the viral polypeptides. In the insert the logarithms of the molecular weights of each of the viral polypeptides are plotted versus the R_f values. (b and c) Sarcosyl cores were prepared from ³H-labeled amino acid Ad2 by equilibrium centrifugation in CsCl density gradients and were dialyzed against 0.01 M NH₄-acetate buffer. One portion (b) was incubated with 50 µg of DNase under the conditions described and the reaction was stopped by the addition of EDTA. Another sample (c) was treated identically except that DNase was not added to the reaction mixture. After lyophilization and resolubilization in sample buffer, the samples were analyzed by electrophoresis on 13% polyacrylamide gels at 100 V for 7.3 h. (d) As a control the polypeptides of ¹⁴C-labeled Ad2 were analyzed by electrophoresis on 13% polyacrylamide gels.*

entire internal volume $(1.35 \times 10^5$ nm³) of the virion which we have allotted to the core (Table 1). By actual count the number of spheres in the core is lower, as would be expected, since the 21.6-nm-diameter spheres contain only the viral DNA and protein VII.

It is possible to estimate more precisely the number of spheres making up the core of the virion by calculating the volume occupied by the viral DNA and protein VII. If one assumes the viral DNA to approximate a cylinder 2.0 nm wide by 109.4 nm long (12), its volume can be calculated by the equation $V = r^2 \pi h$ (40) to be 3.4×10^4 nm³ (where V is the volume, r the radius, and h the height of the cylinder). Everitt et al. (18) have shown that each virion contains 1,070 copies of protein VII which has a molecular weight of 18,500. Assuming that this protein is globular, similar to serum albumin, it would have a volume of about 40 nm³ (42). The total volume of protein VII in the core would therefore be approximately 4.3×10^4 nm³, and the

total volume of the DNA and protein VII would be about 7.7×10^4 nm³. Thus, 14 of the 21.6-nm diameter spheres would be required to contain all of the DNA and protein VII. This number is probably too high as the above calculation does not take into account the fact that some of the DNA must run between the 21.6-nm diameter spheres connecting them together.

Another consideration in estimating the number of DNA-protein VII spheres which the core contains is that of the symmetry relationship between the icosahedral outer surface of the virion and the inner core. If the core is composed of subunits, as is implied here, and is to be packed in an orderly manner into the $T = 25$ icosahedron, that is the adenovirus capsid, a structural compatibility must exist between the morphology of the core and the capsid. This is particularly desirable if the core is to be packaged into the capsid by a mechanism of self-assembly. The conditions of compatibility are best met if the core, like the capsid, has
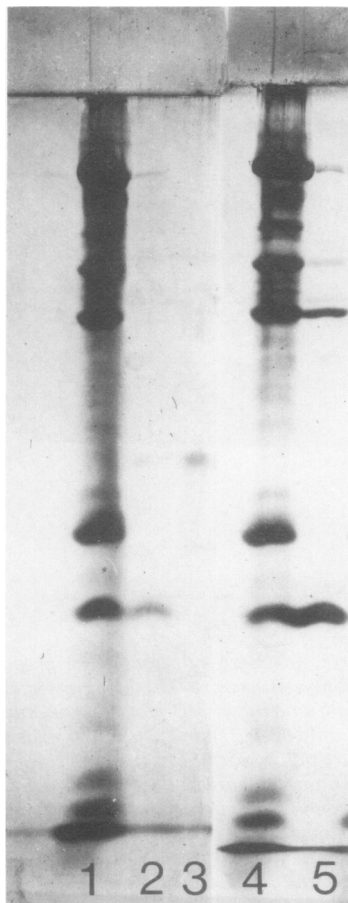
Fig. 12. *Analysis of Ad2 and subviral particles by electrophoresis on 13% polyacrylamide slab gels. Slab gel electrophoresis was performed at 40 V for 12 h under conditions as described. All samples were lyophilized and redissolved in sample buffer prior to electrophoresis. The individual slots contained the following samples: (1) Ad2; (2) sarcosyl particles prepared and treated with DNase as described in the legend to Fig. 11b; (3) DNase; (4) Ad2; (5) pyridine cores prepared according to the methods described by Prage et al. (33) and by Everitt et al. (18).*

icosahedral geometry (20, 43). Per core, 8 to 10 spheres of 21.6 nm diameter were observed in electron micrographs, and about 14 spheres were expected by the above calculations. We predict that in reality each core contains 12 of these 21.6-nm-diameter substructures. This number takes into account the possibility that because of superposition of the 21.6-nm-diameter spheres within the core they cannot all be distinguished in electron micrographs, and that not all of the viral DNA actually contributes its volume to the spherical structures. The number 12 would imply that about 19% of the viral DNA is not in

the 21.6-nm-diameter spheres, but may in fact link the 12 spheres to each other within the core. Proposing that the core contains 12 of the spheres has the added advantage that it allows an exact symmetrical relationship between the viral capsid $P = 1$, $T = 25$, and the core $P = 1$, $T = 1$, using the notation of Caspar and Klug (8). The 12 core subunits would be so oriented that the 5, 3, 2 axes of symmetry of the inner core coincide with the same axes of symmetry of the outer icosahedral capsid.

That 12 core subunits is not an unreasonable estimate for the number of 21.6-nm-diameter spheres in the virus core is also suggested by examination of an icosahedron containing 12 structural units. When such a structure is viewed from one direction, as would be the case in the electron microscope, a maximum of nine subunits can be distinguished. If the particle is slightly distorted, a maximum of 9 to 10 subunits would still be seen, the remaining subunits being obscured.

Utilizing the above calculations the DNA-protein VII complex would account for about 57% of the total volume allotted to the core of the intact virion (Table 1). Everitt et al. (16, 18) have suggested that the interior of the virus directly below the outer layer of subunits contains, in addition to protein VII and the viral DNA viral proteins V, VI, and VIII. Everitt et al. (18) have estimated the number of copies of each of the proteins per virion with the exception of protein VIII. Using the procedure described above we calculated the total volume of these other proteins, except VIII, to be 4×10^4 nm^3. When this volume is combined with that calculated for the DNA and protein VII core, it amounts to a total of about 1.2×10^5 nm^3 and accounts for all but 11% of the internal volume of the virion which should be occupied by protein VIII and water.

(ii) The protein components of the viral core. Selective procedures for the stepwise degradation of the adenovirion proved useful to elucidate its structure (16, 18, 22, 33, 39). We have used heat (56 C) (39), pyridine (33), and sarcosyl (11) treatment to disrupt the virion and get access to the core. Each of these agents produces a specific type of subviral particle. Although subviral particles prepared by heating purified virions to 56 C (39), by pyridine (33), or by sarcosyl treatment have some similarities in appearance in electron micrographs of negatively stained preparations (Fig. 6, 7, and 8), they are strikingly different in their polypeptide composition (Fig. 12). Virions heated to 56 C for 5 min and reisolated by equilibrium centrifuga-
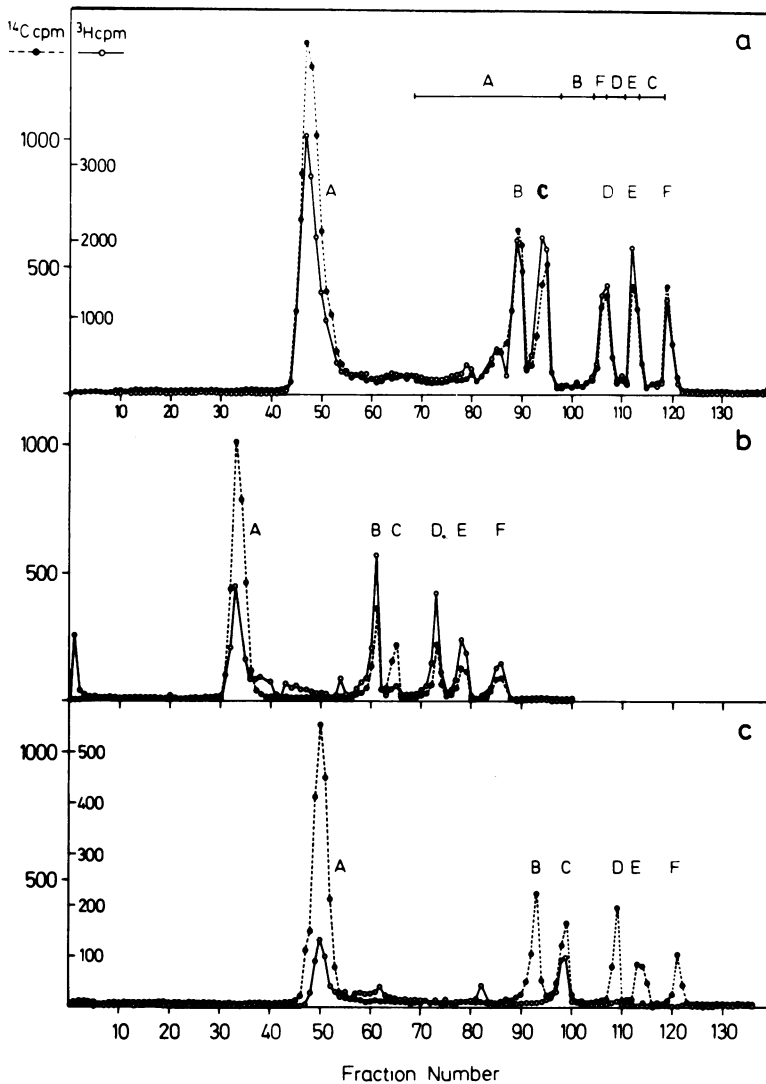
FIG. 13. *Cleavage of viral DNA in sarcosyl cores with the EcoRI restriction endonuclease. Sarcosyl cores were prepared from [³H]thymidine-labeled Ad2, dialyzed against TE, and incubated with EcoRI restriction endonuclease. All pertinent techniques have been described. Immediately prior to analysis of the cleavage products by electrophoresis on 1.5% polyacrylamide-0.8% agarose gels, 1.4 to 2.8 µg of ¹⁴C-labeled Ad2 DNA previously cleaved with the EcoRI endonuclease in a separate incubation was added as a marker. Subsequent to the incubation with EcoRI endonuclease, the marker was extracted from the incubation mixture by SDS-Pronase treatment and phenolization. Before electrophoresis all samples were dialyzed against 1/10 strength of TEB electrophoresis buffer. (a) Incubation of sarcosyl cores with EcoRI endonuclease, followed by Pronase treatment. An amount of sarcosyl particles equivalent to approximately 1.5 µg of Ad2 DNA was incubated under standard conditions with EcoRI endonuclease for 60 min at 37 C. The reaction was stopped by the addition of 5 µl of 0.2 M EDTA, 10 µl of Pronase (5 mg/ml), and 10 µl of 10% SDS and incubation at 37 C continued for 30 min. (b) Omission of Pronase incubation. Experimental conditions were essentially identical to those described under (a), except that an amount of sarcosyl particles equivalent to 0.8 µg of Ad2 DNA was used and that Pronase was not used in the incubation subsequent to cleavage by EcoRI endonuclease. The gel slice in fraction 1 was eluted in 0.2 ml of 0.1% SDS and further analyzed as described under (c). (c) Pronase treatment of the DNA-protein complex. The material remaining on top of the gel described under (b) and eluted from the gel slice (fraction 1) in 0.2 ml of 0.1% SDS was incubated at 37 C with 20 µl of Pronase (5 mg/ml) and 20 µl of 10% SDS for 45 min. The reaction mixture was chilled, and ¹⁴C-labeled Ad2 DNA fragments (EcoRI) were added prior to electrophoresis.*

tion in CsCl density gradients contain most of the viral polypeptides (results not shown), pyridine cores (18, 33) contain polypeptides V and VII (Fig. 12, slot no. 5), and sarcosyl cores contain predominantly polypeptide VII and only minor amounts of polypeptides VIII and IX and hexons (Fig. 11).

In the model of the adenovirion suggested by Everitt et al. (16), the viral polypeptide V is in close neighborhood to polypeptide VII and the penton base, and polypeptide VII is tightly bound to the viral DNA. In the sarcosyl cores described in the present report, polypeptide VII is the predominant protein component, whereas polypeptide V can be removed from the core by sarcosyl treatment. Obviously, polypeptides V and VII must be anchored in different ways in the viral core. The electron microscope data presented (Fig. 3, 4, 7, 8) are also consistent with this interpretation (see below and Fig. 14). In fact, the arginine/alanine-rich polypeptide VII can be quantitatively separated from the viral DNA by proteolytic enzymes, or alkali treatment, or by boiling in SDS. Our data, however, do not permit us to draw definite conclusions about the type of linkage between
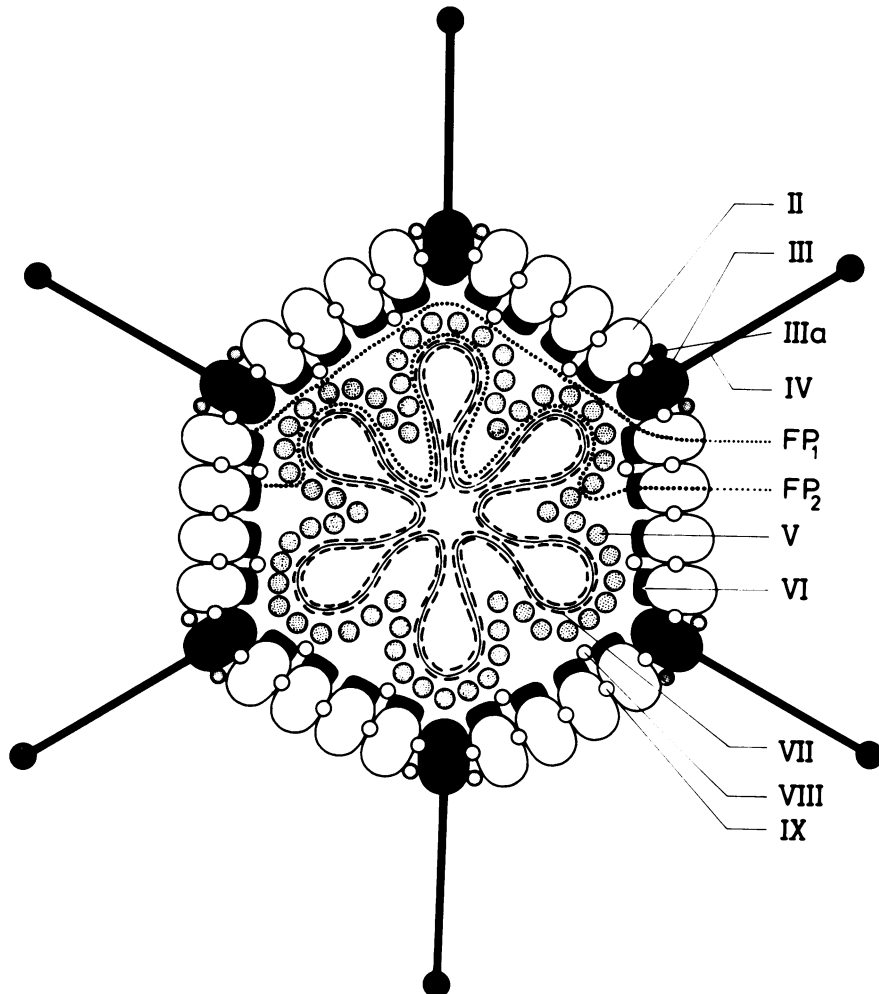

Fig. 14. *Model of the type 2 adenovirion. The Roman numerals refer to the viral polypeptides (26). The location of the fracture planes (FP_1 and FP_2) is derived from electron microscope data (see text and Fig. 3, 4, 7, and 8). The particles generated by cleavage in FP_1 is probably similar to pyridine cores, the particle that arises by cleavage in FP_2 is probably similar to sarcosyl cores. The arrangement of the viral DNA molecule as shown in the model was chosen not to presuppose any definite organization. The loops extending into each of the substructures in the model in reality represent tightly packed coils of viral DNA (see Fig. 9). The model represents a modification of the scheme proposed by Everitt et al. (16).*

polypeptide VII and the viral DNA. The polypeptide analysis of sarcosyl cores by electrophoresis on SDS-polyacrylamide gels gave very similar results, regardless of whether the sarcosyl cores were pretreated with DNase or were electrophoresed without predigestion of the DNA (Fig. 11).

Our ultrastructural studies revealed that the sarcosyl core was a tightly packed sphere which gradually unfolded upon storage at 4 C (Fig. 9). It is not understood what types of interactions, DNA-protein or protein-protein, are responsible for the packing of the DNA or whether other factors such as polyamines may play a role in the organization of the viral DNA in the core. The gradual unfolding of the sarcosyl core is perhaps a consequence of the removal of all other viral polypeptides which serve to stabilize the core structure. It is conceivable that polypeptide V is at least partly responsible for this stabilization. Differences in ionic strength and pH values in the range between 7.2 and 8.5 did not alter the morphology of the sarcosyl cores or. noticeably affect the unwinding process.

(iii) The viral DNA. The viral DNA in the sarcosyl core can be completely digested with DNase and is susceptible to cleavage by the EcoRI restriction endonuclease. The data presented in Fig. 13 demonstrate that the termini of the linear Ad2 DNA molecule are linked to each other probably by a protein, since this linkage can be disrupted by treatment with proteolytic enzymes (Fig. 13c). The finding that, upon incubation of sarcosyl cores with the EcoRI restriction endonuclease, a complex between protein and the terminal EcoRI fragments A and C can be isolated on top of the gel (Fig. 13b) further argues for the involvement of a polypeptide in this linkage. The nature of the polypeptide(s) in this complex has not yet been determined.

The viral DNA in the sarcosyl cores has been shown to be a circular molecule (11; Fig. 10) and circular adenovirus DNA has also been found after treatment of purified virions with guanidinium hydrochloride (36). These circular DNA molecules are converted to a linear form by proteolytic enzymes and the linear Ad2 DNA has a unique melting pattern (12), hence the circular molecule must be opened by proteolysis at one specific site.

We conclude that the complex between viral EcoRI DNA fragment A and C and the polypeptide(s) responsible for circularization of the viral DNA has been isolated in the experiment described in Fig. 13b. It is conceivable that polypeptide VII is involved in the circularization of the viral DNA, however, an additional,

more specialized polypeptide may be present in minute amounts and may hitherto have escaped detection.

We also consider the possibility that the adenovirus DNA is a covalently closed circular molecule and that an activated form of the virion-associated endonuclease (7) cuts the circle at the site of the specific palindrome due to the terminal repetition of the adenovirus DNA (19, 46). This endonuclease could be activated upon disruption of the structural integrity of the virion.

(iv) Correlation between morphological and biochemical findings. The presence of protein V in the pyridine cores and its absence in the sarcosyl cores correlates with the presence of the halo of material surrounding the 21.6-nm core subunits in the particles produced by pyridine, and the absence of this halo in the particles prepared by sarcosyl treatment. Protein V seems to coat the protein VII-DNA complex and to give the pyridine cores a staining characteristic which generally makes the internal structure non-distinguishable. In the freeze-cleaved virions, two fracture planes were found in the interior of the virion. One revealed the core as a smooth structure, the other showed the presence of subunits. It is conceivable that in the former case the fracture plane passed between the outer capsid and protein V, and in the latter case between protein V and the protein VII DNA subunits (Fig. 14). The smaller size of the core subunits seen in freeze-cleaved virions (Fig. 3, 4) is probably the result of the fracture plane passing over the subunits at a point somewhat distant from the true cross-section of a sphere. The most difficult aspect of fitting the viral core into the capsid is to define what types of interaction occur between the DNA-protein VII complex and the hexons and pentons of the outer shell. Everitt et al. (16) have elegantly described a number of protein-protein interactions within the virion which may explain the anchoring of the core in the viral capsid. Protein V seems to bind to both the penton and the protein VII-DNA complex (17) and could therefore attach each of the 12 core subunits to one of the 12 penton structures in the outer capsid. The strong binding of hexons to the oversize relaxed subviral structures produced by heating (Fig. 6b) suggests an attachment of the hexons to the core structure underneath. Such an interaction could be mediated by one or both of the internally located proteins VI and VIII. Our model of the adenovirion correlating ultrastructural and biochemical findings (Fig. 14) is a modification of the model proposed by Everitt et al. (16).

ACKNOWLEDGMENTS

LITERATURE CITED

1. Anderson, T. F. 1954. Preservation of structure in dried specimens, p. 122–129. In Proc. 3rd Int. Congr. Electron Microscopy, London.

2. Anderson, T. F. 1962. Negative staining and its use in the study of viruses and their serological reactions, p. 251–262. In R. J. C. Harris (ed.), The interpretation of ultrastructure, vol. 1. Academic Press Inc., New York.

3. Brown, D. T., and B. T. Burlingham. 1973. Penetration of host cell membranes by adenovirus 2. J. Virol. 12:386–396.

4. Brown, D. T., M. R. F. Waite, and E. R. Pfefferkorn. 1972. Morphology and morphogenesis of Sindbis virus as seen with freeze-etching techniques. J. Virol. 10:524–536.

5. Burger, H., and W. Doerfler. 1974. Intracellular forms of adenovirus DNA. III. Integration of the DNA of adenovirus type 2 into host DNA in productively infected cells. J. Virol. 13:975–992.

6. Burlingham, B. T., and W. Doerfler. 1971. Three size-classes of intracellular adenovirus deoxyribonucleic acid. J. Virol. 7:707–719.

7. Burlingham, B. T., and W. Doerfler. 1972. An endonuclease in cells infected with adenovirus and associated with adenovirions. Virology 48:1–13.

8. Caspar, D. L. D., and A. Klug. 1962. Physical principles in the construction of regular viruses. Cold Spring Harbor Symp. Quant. Biol. 27:1–24.

9. Davis, R. W., M. Simon, and W. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids, p. 413–428. In L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 21. Academic Press Inc., New York.

10. Doerfler, W. 1969. Nonproductive infection of baby hamster kidney cells (BHK 21) with adenovirus type 12. Virology 38:587–606.

11. Doerfler, W., H. Burger, J. Ortin, E. Fanning, D. T. Brown, M. Westphal, U. Winterhoff, B. Weiser, and J. Schick. 1974. Integration of adenovirus DNA into the cellular genome. Cold Spring Harbor Symp. Quant. Biol. 39:505–521.

12. Doerfler, W., and A. K. Kleinschmidt. 1970. Denaturation pattern of the DNA of adenovirus type 2 as determined by electron microscopy. J. Mol. Biol. 50:579–593.

13. Doerfler, W., U. Lundholm, U. Rensing, and L. Philipson. 1973. Intracellular forms of adenovirus DNA. II. Isolation in dye-buoyant density gradients of a DNA-RNA complex from KB cells infected with adenovirus type 2. J. Virol. 12:793–807.

14. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167–182.

15. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130:432–437.

16. Everitt, E., L. Lutter, and L. Philipson. 1975. Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirus type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. Virology, vol. 65.

17. Everitt, E., and L. Philipson. 1974. Structural proteins of adenoviruses. XI. Purification of three low molecular weight virion proteins of adenovirus type 2 and their synthesis during productive infection. Virology 62:253–269.

18. Everitt, E., B. Sundquist, U. Pettersson, and L. Philipson. 1973. Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. Virology 52:130–147.

19. Garon, C. F., K. Berry, and J. Rose. 1972. A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Natl. Acad. Sci. U.S.A. 69:2391–2395.

20. Horne, R. W., S. Brenner, A. P. Waterson, and P. Wildy. 1959. The icosahedral form of adenovirus. J. Mol. Biol. 1:84–86.

21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

22. Laver, W. G. 1970. Isolation of an arginine-rich protein from particles of adenovirus type 2. Virology 41:488–500.

23. Laver, W. G., H. G. Pereira, W. C. Russel, and R. C. Valentine. 1968. Isolation of an internal component from adenovirus type 5. J. Mol. Biol. 37:379–386.

24. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409–414.

25. Maizel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins, p. 179–246. In K. Maramorosh and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press Inc., New York.

26. Maizel, J. V., Jr., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology 36:115–125.

27. Millonig, G. 1961. Advantages of a phosphate buffer for OsO$_4$ solutions in fixation. J. Appl. Physiol. 32:1637.

28. Moody, M. F. 1967. Structure of the sheath of bacteriophage T4. I. Structure of the contracted sheath and polysheath. J. Mol. Biol. 25:167–200.

29. Parsons, D. F. 1969. X-ray diffraction of membrane structure changes occurring during electron microscope specimen preparation, p. 326–327. In C. A. Arceneaux (ed.), 27th Annu. Meet. Elec. Microscop. Soc. Claiton's Publishing Division, Baton Rouge.

30. Pettersson, U., and S. Höglund. 1969. Structural proteins of adenoviruses. III. Purification and characterization of the adenovirus type 2 penton antigen. Virology 39:90–106.

31. Pettersson, U., C. Mulder, H. Delius, and P. Sharp. 1973. Cleavage of adenovirus type 2 DNA into six unique fragments with endonuclease R·RI. Proc. Natl. Acad. Sci. U.S.A. 70:200–204.

32. Prage, L., and U. Pettersson. 1971. Structural proteins of adenoviruses. VII. Purification and properties of an arginine-rich core protein from adenovirus type 2 and type 3. Virology 45:364–373.

33. Prage, L., U. Pettersson, S. Höglund, K. Lonberg-Holm, and L. Philipson. 1970. Structural proteins of adenoviruses. IV. Sequential degradation of the adenovirus type 2 virion. Virology 42:341–358.

34. Prage, L., U. Pettersson, and L. Philipson. 1968. Internal basic proteins in adenovirus. Virology 36:508–511.

35. Price, P. A., W. H. Stein, and S. Moore. 1969. Effect of divalent cations on the reduction and reformation of the disulfide bonds of deoxyribonuclease. J. Biol. Chem. 244:929–932.

36. Robinson, A. J., H. B. Younghusband, and A. J. D. Bellet. 1973. A circular DNA-protein complex from adenoviruses. Virology **56:**54-69.

37. Rosenwirth, B., S. Tjia, M. Westphal, and W. Doerfler. 1974. Incomplete particles of adenovirus. II. Kinetics of formation and polypeptide composition of adenovirus type 2. Virology **60:**431-437.

38. Russell, W. C., W. G. Laver, and P. J. Sanderson. 1968. Internal components of adenovirus. Nature (London) **219:**1127-1130.

39. Russell, W. C., R. C. Valentine, and H. G. Pereira. 1967. The effect of heat on the anatomy of the adenovirus. J. Gen. Virol. **1:**509-522.

40. Selby, S. M. (ed.). 1972. Standard mathematical tables, 20th edition, p. 15-18. The Chemical Rubber Co., Cleveland, Ohio.

41. Studier, F. W. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79:**237-248.

42. Tanford, C. 1961. Physical chemistry of macromolecules. J. Wiley and Sons, Inc., New York.

43. Valentine, R. C., and H. G. Pereira. 1965. Antigens and structure of the adenoviruses. J. Mol. Biol. **13:**13-20.

44. Vinograd, J., and J. E. Hearst. 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. Fortschr. Chem. Org. Naturst. **20:**372-422.

45. Wilcox, W. C., H. S. Ginsberg, and T. F. Anderson. 1963. Structure of type 5 adenovirus. II. Fine structure of virus subunits. Morphologic relationship of structural subunits to virus-specific soluble antigens from infected cells. J. Exp. Med. **118:**307-314.

46. Wolfson, J., and D. Dressler. 1972. Adenovirus 2-DNA contains an inverted terminal repetition. Proc. Natl. Acad. Sci. U.S.A. **69:**3054-3057.