JOURNAL OF VIROLOGY, July 1978, p. 193-204
0022-538X/78/0027-0193$02.00/0

Vol. 27, No. 1
Printed in U.S.A.

Characterization of a DNA-Protein Complex and Capsomere Subunits Derived from Polyoma Virus by Treatment with Ethyleneglycol-Bis-*N,N'*-Tetraacetic Acid and Dithiothreitol†

JOHN N. BRADY, VERN D. WINSTON, AND RICHARD A. CONSIGLI*
Division of Biology, Kansas State University, Manhattan, Kansas 66506

Received for publication 26 January 1978

Treatment of polyoma virions with ethyleneglycol-bis-*N,N'*-tetraacetic acid (EGTA) and dithiothreitol (DTT) at pH 8.5 resulted in the dissociation of the virions into a DNA-protein complex and individual structural capsomere subunits. The sedimentation value of the DNA-protein complex in sucrose gradients was approximately 48S, and it had a density of 1.45 g/cm^3 in equilibrium CsCl gradients. Alkaline sucrose analysis of the DNA within this DNA-protein complex demonstrated that approximately 75% of the DNA is component 1. The proteins associated with the DNA were dissociated by treatment with either NaCl or the anionic detergent Sarkosyl. VP_1 and the histone proteins VP_{4-7} were the major proteins associated with the DNA. Treatment of the DNA-protein complex with alkaline pH resulted in the specific removal of VP_1. Electron microscopy of the 48S DNA-protein complex demonstrated that it is a very tightly coiled structure that is slightly larger than the intact virion. Treatment of the complex with either NaCl or with pH 10.5 buffer resulted in the loss of protein and subsequent loosening of the DNA-protein complex such that the DNA could be visualized. The capsomere subunits released as a result of the EGTA-DTT treatment sedimented as 18S, 12S, and 5S subunits in sucrose gradients. Electrophoretic analysis of the isolated capsomeres demonstrated that VP_1, VP_2, and VP_3 were present in each species, although the ratios of the proteins varied. In addition to the structural proteins, histones VP_{4-7} were found to be predominantly associated with the 5S capsomere subunit.

The structure of polyoma virus was described in detail in a recent review by Finch and Crawford (6). The virus particles have icosahedral symmetry, and the virion surface is composed of 72 capsomeres, 12 pentons at the vertices of the icosahedron and 60 hexons on the faces. However, at the present time there is a great deal of uncertainty as to how the different polypeptides fit into the virion structure. The histones are closely associated with the internal viral DNA of the particle (9, 18), but are also believed to be external on the capsid structure (11, 12, 16, 20). The involvement of proteins VP_1, VP_2, and VP_3 in the capsid structure and their relationship to the hexons and pentons is unknown. In addition, it is not clear whether the virus-specific structural proteins are in any way associated with the viral DNA.

To investigate these questions, we have examined the dissociation products of a new in vitro dissociation method which we have recently developed for polyoma virions (1). This dissociation procedure is dependent upon the chelation of Ca^{2+} ions that are found associated with the virion and the disruption of disulfide bonds. This dissociation procedure may be carried out under more physiological conditions than could previously described systems (8, 9, 23) and has proven to be advantageous in dissecting the structure of the virion. The purpose of this paper is to describe the biophysical and biochemical properties of the DNA-protein complex and capsomere subunits which are released by this dissociation technique. Furthermore, the data from these investigations allow us to predict a clearer picture of the virion structure.

MATERIALS AND METHODS

Cell and virus propagation. The preparation of primary cultures of mouse embryo (MEC) and mouse kidney (MKC) cells has been described (5, 27). For roller-bottle cell cultures, each bottle was seeded with 100 ml of a cell-media mixture containing 2×10^6 cells per ml prepared as described above. Wild-type polyoma virus was used to infect cells at a multiplicity of infection of 10. Infected cultures were maintained in serum-free Dulbecco-modified Eagle medium (17).

† Contribution no. 78-202-J from the Kansas Agricultural Experiment Station, Kansas State University.

Virus purification. Virus was purified from the infected-cell lysate either as described previously for small volumes (17) or as described by Friedmann and Haas (10) by polyethyleneglycol precipitation for large volumes. The CsCl gradients used to purify the virus were prepared as described by Brunck and Leick (2) and described in greater detail previously (1).

Preparation of radioactively labeled polyoma virions. The preparation of [^{3}H]deoxyribosylthymine ([^{3}H]dT)-labeled and in vitro ^{14}C-protein-labeled polyoma virions has been previously described (18, 19). Purified polyoma virions were labeled in vitro with ^{125}I by the method of Frost and Bourgaux (12).

Dissociation of polyoma virions. Stock solutions of each component of the dissociation buffer (Tris, ethyleneglycol-bis-*N,N'*-tetraacetic acid [EGTA], and NaCl) were prepared and stored at 4°C, with the exception of dithiothreitol (DTT), which was prepared fresh for each assay. Specific experimental conditions for polyoma dissociation are described in each experiment. All dissociation reaction mixtures were incubated at room temperature.

Quantitative assays. Cesium chloride densities were determined from the refractive index with a Bausch and Lomb refractometer and calculated by using the equation of Vinograd and Hearst (28). Radioactivity was quantitated in a toluene-Triton (3:1) scintillation fluid with a Beckman LS-233 liquid scintillation counter.

Velocity sedimentation. Polyoma virions or products derived from dissociated virions were layered onto either 5 to 20% or 10 to 30% (wt/vol) sucrose gradients containing 5 mM EGTA, 0.15 M NaCl, 0.01 M Tris-hydrochloride (pH 8.5), and 0.25% Triton X-100 (Rutger Chemical Co.). Centrifugation was in an SW50.1 rotor at 40,000 rpm (4°C) for 40 min, 2 h, or 12 h as described in each experiment, or in an SW41 rotor at 34,000 rpm for 3 h. Sedimentation coefficients were estimated by comparison with 7S immunoglobulin marker for proteins or with 64S T_4 DNA and 21S polyoma DNA markers for the DNA-protein complexes.

Formaldehyde fixation of DNA-protein complex. The [^{3}H]dT-labeled 48S DNA-protein complex was isolated from a 5 to 20% sucrose gradient (SW50.1, 40,000 rpm for 2 h) and dialyzed against 0.2 mM EDTA–0.15 M NaCl (pH 7.1) for 36 h at 4°C. After dialysis, samples were mixed with phosphate-buffered formaldehyde (6% final concentration) by the method of Hancock (14). The samples were then dialyzed against several changes of 0.2 mM EDTA (pH 7.1) to remove excess formaldehyde.

Buoyant density determinations of DNA-protein complex. Each of the formaldehyde-fixed DNA samples, prepared as described above, was mixed with an appropriate amount of CsCl to give a final density of 1.70 g/cm^3 for Hirt DNA and 1.45 g/cm^3 for the 48S DNA-protein complex. The samples were centrifuged to equilibrium at 35,000 rpm (SW50.1 rotor) for 48 h (10°C). Fractions were collected and assayed for radioactivity and density determinations.

Electrophoresis. Purified virions, DNA-protein complexes, and capsomeres were precipitated by the addition of cold trichloroacetic acid (25% final concentration). After 2 h at 0°C, the precipitates were collected by centrifugation at 10,000 rpm (Sorvall HB-4 rotor) for 30 min. Each pellet was washed with 1 volume of 25% trichloroacetic acid and subsequently with 2 volumes of ice-cold acetone and air dried. Solubilization of the precipitates has been described (18). The sodium dodecyl sulfate (SDS)-polyacrylamide slab gel consisted of a 15% running gel and a 5% stacking gel. Samples were loaded and subjected to electrophoresis at 15 mA until the samples were concentrated at the interface, at which time the current was increased to 30 mA for the duration of the run. Electrophoresis was stopped when the tracking dye eluted from the running gel. The running buffer consisted of 0.1% SDS–0.38 M glycine in Tris buffer (0.05 M, pH 8.4). After electrophoresis, the gels were fixed and stained by allowing the gel to soak overnight in 50% methanol–7.5% acetic acid–0.1% Coomassie brilliant blue. The gel was destained by repeated washes with 5% methanol–7.5% acetic acid.

Autoradiography of slab gels. The dried radiolabeled gels were exposed to Kodak no-screen medical X-ray film (NS-2T). The film was developed with D-19 Kodak developer for 5 min, washed in 1% acetic acid for 3 min, and then transferred to Kodak fixer for 10 min.

Quantitation of protein bands from autoradiograms. The autoradiograms were scanned with a Joyce-Loebl double-beam recording microdensitometer, model MK III C. Subsequently, the area under each peak was determined, and the percentage of each polypeptide was calculated by comparison with the total area of all the proteins within a given sample.

Electron microscopy. Nucleoprotein complexes were prepared essentially as described by Christiansen and Griffith (3). Fractions from sucrose gradients were fixed by adding 0.1 volume of 10% formaldehyde. After 15 min of incubation on ice, 0.1 volume of 8% glutaraldehyde was added, and the mixture was incubated for another 15 min on ice. A drop of fixed material was then applied to Formvar-covered grids and then dehydrated in a graded series of ethanol. Dehydrated grids were stained with freshly prepared 1% uranyl acetate. The preparation of capsomeres for electron microscopy has been described previously (1). Observations were made with a Phillips 201 electron microscope operated at 60 kV.

RESULTS

Sedimentation analysis of EGTA-DTT-dissociated polyoma. After dissociation of both [^{3}H]dT- and in vitro ^{14}C-protein-labeled virions, the dissociation products, as well as intact virions, were compared on parallel sucrose gradients (Fig. 1). Intact polyoma virions sediment very rapidly to the bottom portion of the gradient and have a sedimentation coefficient of 240S. However, after treatment of the virions with EGTA-DTT, there was a dramatic decrease in the sedimentation rates of both [^{3}H]-dT- and ^{14}C-protein-labeled virions. No radioactivity could be detected in the region of intact polyoma virions after dissociation. When the dissociated [^{3}H]dT-labeled virions were exam-

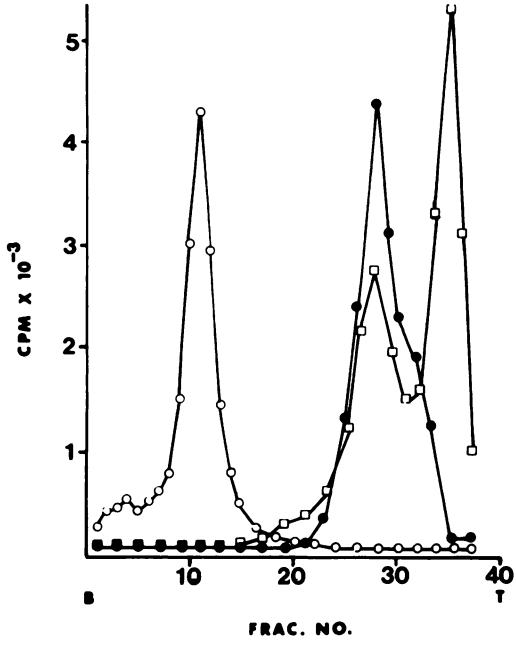

FIG. 1. *Sedimentation of polyoma virions and EGTA-DTT-dissociated virions in sucrose gradients. [³H]dT- and ¹⁴C-protein-labeled virions were exposed to 1 mM EGTA-3 mM DTT-0.15 M NaCl in 0.05 M Tris buffer (pH 8.5) for 30 min at room temperature. Samples were layered onto a 10 to 30% sucrose gradient and centrifuged in a SW50.1 rotor at 40,000 rpm for 40 min (4°C). Symbols: ○, [³H]dT polyoma virions; ●, [³H]dT-dissociated virions; □, [¹⁴C]-HCOH-dissociated virions.*

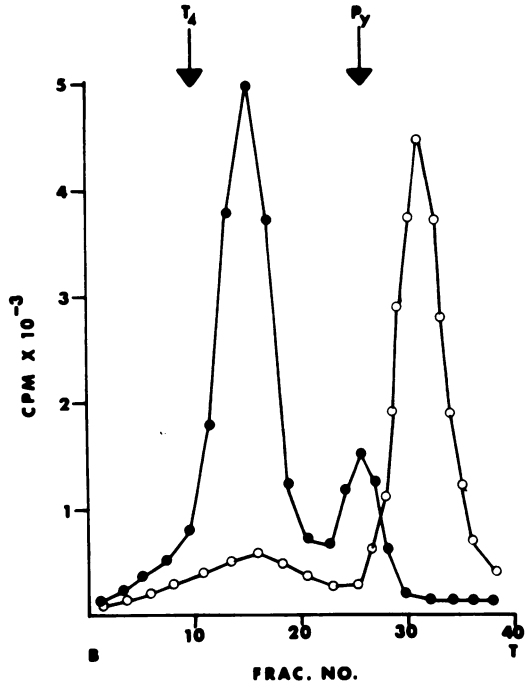

FIG. 2. *Sedimentation of EGTA-DTT-dissociated virions in sucrose gradients. ¹²⁵I- and [³H]dT-labeled virions were dissociated as in Fig. 1. Samples were layered onto a 5 to 20% sucrose gradient and centrifuged in a SW50.1 rotor at 40,000 rpm for 2 h. Arrows indicate position of 64S T4 DNA and 21S polyoma DNA markers analyzed in parallel gradients. Symbols: ●, [³H]dT; ○, ¹²⁵I-amino acid.*

ined, one main peak of radioactivity which sedimented at approximately 50S was apparent. When ^{14}C-protein-labeled virions were examined, two peaks of radioactivity were resolved. The heavier peak of radioactivity sedimented coincident with the 50S DNA peak described above. The lighter protein peak, which contained most of the radioactivity, sedimented at approximately 15S and contained the individual capsomere subunits of the virus (see below).

Sedimentation analysis of the DNA-protein complex. To examine the DNA-protein complex released by the EGTA-DTT dissociation more closely, [^{3}H]dT-labeled virions were dissociated and centrifuged for 2 h to better resolve the DNA-protein complex from the capsomeres (Fig. 2). T_4 DNA and polyoma DNA were used as sedimentation markers in a parallel gradient. Two distinct bands of DNA are reproducibly seen on these gradients. The lighter peak migrates at approximately 21 to 24S. This species of DNA was a minor component in fresh preparations of purified virions, but was observed to increase after prolonged storage at 4°C or when the virions were frozen. The heavier DNA peak sedimented at approximately 48S when compared with the T_4 and polyoma DNA markers. In fresh preparations of polyoma virions, this peak contained approximately 95 to 97% of the total DNA released. The complex appears to be very stable, because no shift in sedimentation value was observed even when the dissociation mix was allowed to incubate at room temperature for 2 h (data not shown). Alkaline sucrose analysis of the DNA within the complex, after removal of associated proteins with 0.25% Sarkosyl (Fig. 3A) demonstrated that 75% of the DNA is component 1 (data not shown).

The sedimentation pattern of protein-labeled virions after dissociation is also seen in Fig. 2. Two peaks of radioactivity were observed, one which sedimented coincident with the 48S viral DNA as well as a large amount of slow-sedimenting protein near the top of the gradient. The presence of protein-labeled material coincident with the DNA peak provided evidence for the association of protein with the DNA.

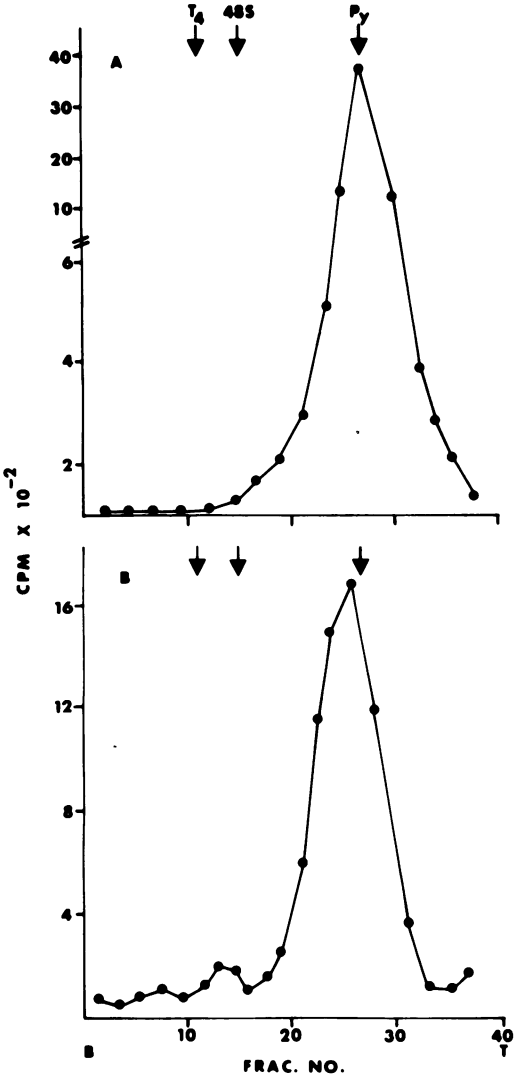

FIG. 3. *Sedimentation analysis of 48S DNA-protein complex after various chemical treatments. 48S DNA-protein complex was isolated from gradients as shown in Fig. 2 and dialyzed against 0.15 M NaCl-0.25% Triton X-100 in 0.01 M Tris buffer (pH 8.5). The complex was then treated with 1.0 M NaCl or 0.25% Sarkosyl for 15 min at room temperature and were analyzed on sucrose gradients as described in the legend to Fig. 2. Arrows indicate position of T_4 DNA, dialyzed 48S complex, and polyoma DNA which were used for markers. Symbol: ●, [^{3}H]dT.*

Chemical properties of the 48S complex. Due to the high sedimentation value of the 48S DNA complex recovered from dissociated virions and the presence of labeled virion protein cosedimenting with the DNA complex, it was essential to investigate the association of these proteins with the DNA. Treatment of the 48S complex isolated from gradients as shown in Fig. 2 with 0.25% Sarkosyl resulted in the conversion of the 48S complex to a peak which cosedimented with 21S polyoma DNA (Fig. 3A). After treatment of the complex with 1.0 M NaCl, the sedimentation coefficient was reduced to a value intermediate between that of the intact complex and naked 21S polyoma DNA (Fig. 3B). The sedimentation value of the NaCl-treated complex was approximately 23 to 24S, indicating that not all of the proteins had been removed by the high-salt treatment. When NaCl concentrations less than 1.0 M (0.8 M) were used, a very broad heterogeneous DNA peak was observed that ranged from the position of the 48S complex marker to the naked 21S polyoma DNA marker (data not shown).

Buoyant density of formaldehyde-fixed 48S DNA-protein complex. To estimate the amount of protein associated with the DNA of the 48S DNA-protein complex, the buoyant density of the complex was determined. The 48S complex was isolated from gradients identical to those previously described (Fig. 2). After fixation of the protein to the DNA, the complex was centrifuged in an isopycnic CsCl gradient. Hirt-extracted 21S viral DNA, which had been phenol extracted, was centrifuged in parallel gradients before and after fixation with formaldehyde. Polyoma DNA which had been subjected to formaldehyde fixation had a buoyant density of 1.68 g/cm^3 (Fig. 4B), whereas the unfixed DNA had a buoyant density of 1.70 g/cm^3 (Fig. 4A). These values are in agreement with previously reported values (18, 31). The 48S DNA-protein complex banded at a density of 1.45 g/cm^3 for the major peak and 1.47 g/cm^3 for the minor peak (Fig. 4C). The 1.47-g/cm^3 species likely represents a subclass of the 1.45-cm^3 density species that lost some protein during the various manipulations before fixation. Assuming the density contributions of the DNA and protein are additive, one can calculate the percentage of protein present in the 48S complex. Using a viral DNA density of 1.70 g/cm^3 and the average density of 1.28 g/cm^3 (polyoma empty capsids) for the protein, we have calculated that the 48S DNA-protein complex consists of 60% protein and 40% DNA. This corresponds to a molecular weight of approximately 7.5×10^6. This value, in turn, corresponds to approximately 33% of the total molecular weight of the intact polyoma virion.

Determination of proteins associated with the 48S DNA-protein complex. The proteins found associated with the 48S DNA-protein complex were analyzed by SDS-polyacrylamide slab gel electrophoresis. To minimize the possibility that contaminating capsomere subunits sedimented with the complex, the com-

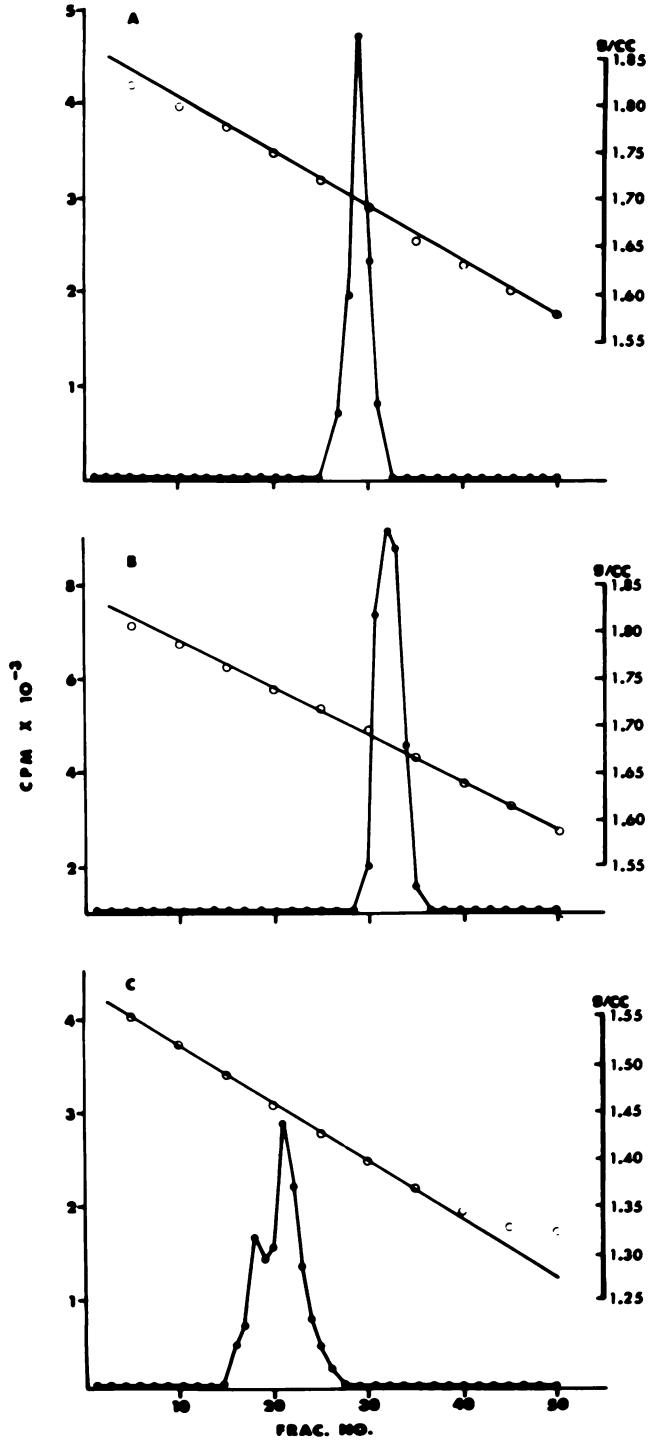

FIG. 4. *Buoyant density determination of polyoma DNA, HCOH-fixed polyoma DNA, and HCOH-fixed 48S DNA-protein complex. The complex, along with the polyoma DNA, was prepared as described in the text, mixed with CsCl, and centrifuged to equilibrium at 35,000 rpm for 48 h. Radioactivity and density determinations were made on the fractions. (A) Polyoma DNA; (B) HCOH-fixed polyoma DNA; (C) HCOH-fixed 48S complex. Symbol: ●, [^{3}H]dT.*

plex was isolated after centrifugation at 34,000 rpm (SW41 rotor) for 3 h. The increased length of the centrifuge tube allows for better separation between the complex and the capsomere subunits. The centrifugation procedure yielded a profile of DNA-protein complex and capsomeres similar to the one shown in Fig. 2.

Figure 5A is the autoradiogram of intact ^{125}I-labeled polyoma virions which shows the three structural proteins (VP_1, VP_2, and VP_3) and the histone proteins (VP_{4-7}). The gel pattern for the 48S DNA-protein complex shows that VP_1 and the histone proteins VP_{4-7} are the major proteins associated with the DNA (Fig. 5C). However, when the gels are overloaded with large quantities of the complex, VP_2 and VP_3 can also be detected, although in very small quantities with respect to VP_1 and the histones (Fig. 6; see below). The distribution of proteins in intact virions and the 48S DNA-protein complex are shown in Table 1.

To further investigate the presence of large amounts of VP_1 in the 48S DNA-protein complex, two additional experiments were performed. In the first experiment, after isolation of the complex from the sucrose gradient, the complex was recentrifuged through a second sucrose gradient. Electrophoretic analysis of this complex revealed a pattern identical to that shown in Fig. 5C. No significant change in the amount of VP_1 or histones was detected (data not shown). In the second series of experiments, the 48S complex was isolated on a sucrose gradient, and the pH of the isolated complex was adjusted to pH 9.0, 9.5, 10.0, and 10.5 and then analyzed on a second series of sucrose gradients (Fig. 7). The following results were obtained: treatment of the 48S complex with pH 9.0 and 9.5 buffers induced no significant change in the sedimenta-

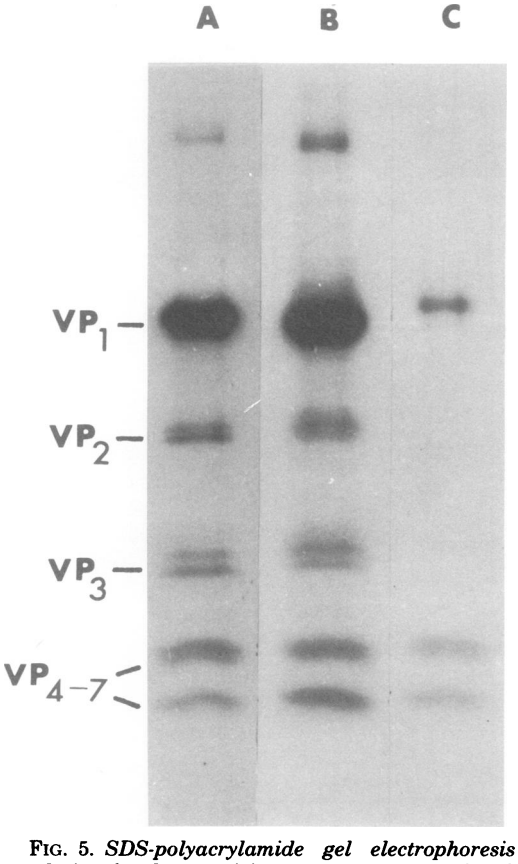

FIG. 5. *SDS-polyacrylamide gel electrophoresis analysis of polyoma virions, capsomeres, and 48S DNA-protein complex. Samples were isolated and prepared for electrophoresis as described. (A) Polyoma virions; (B) polyoma capsomeres; (C) 48S DNA-protein complex.*

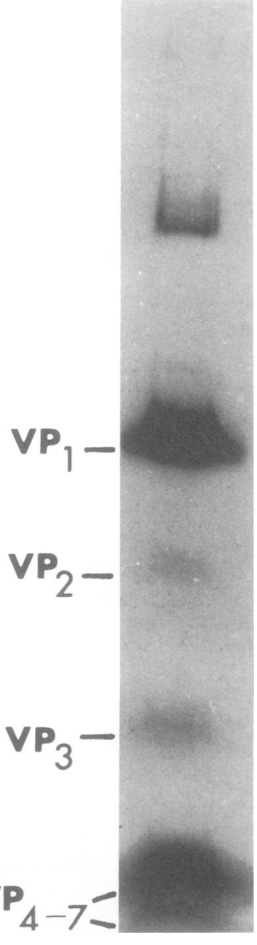

FIG. 6. *SDS-polyacrylamide gel electrophoresis of 48S DNA-protein complex. Samples were isolated and prepared in same manner as in Fig. 5, except that a large quantity of the complex was analyzed.*

TABLE 1. *Distribution of proteins in purified virions, 48S DNA-protein complex, and capsomere subunits*

Sample	Protein (%) present in:[a]			
	VP_1	VP_2	VP_3	VP_{4-7}
Virions	68.7	6.4	5.3	19.6
Capsomeres	84.8	4.9	4.5	5.8
48S DNA-protein complex	52.7	—[b]	—[b]	47.3

[a] The area of the peak for each protein is expressed as the percentage of the total area occupied by the proteins detected in the sample. Each value is the average of a number of separate determinations.

[b] VP_2 and VP_3 were detected in the 48S complex, but only when the VP_1 and histone proteins were present in such large amounts that protein estimation by densitometry was particularly subject to error.

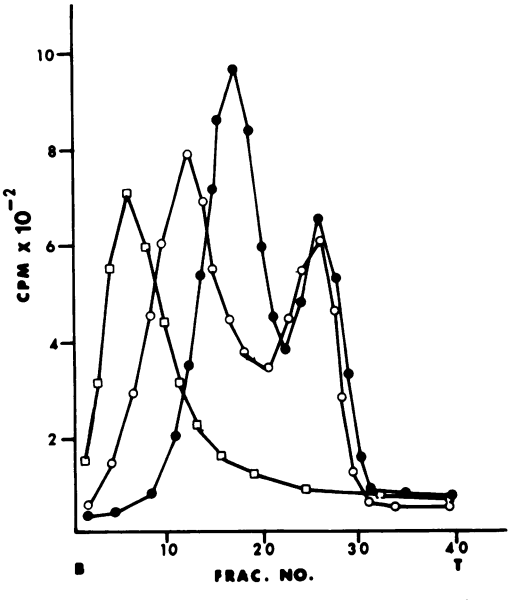

FIG. 7. *Effect of pH treatment on sedimentation of 48S DNA-protein complex. The 48S DNA-protein complex was isolated from 5 to 20% sucrose gradients centrifuged in an SW41 rotor at 34,000 rpm for 3 h at 4°C and dialyzed as described in Fig. 3. After dialysis, the pH of the complex was adjusted as indicated by the addition of glycine buffer to a final concentration of 0.2 M. Samples were then centrifuged through a second SW41 gradient as above except the centrifugation time was increased to 3.5 h. Radioactivity determinations were made on the fractions and then stored at 4°C for electrophoretic analysis. Symbols: □, pH 8.5; ○, pH 10.0; ●, pH 10.5. All samples were labeled with [^{3}H]dT.*

tion properties of the complex (data not shown). However, after treatment at pH 10.0, the 48S complex decreased in sedimentation value to approximately 38 to 40S (Fig. 7). At pH 10.5, the 48S complex sedimented even slower, with a mean sedimentation value of 28 to 30S (Fig. 7). To determine which proteins were being dissociated from the DNA as the pH increased, the pH-altered complexes isolated on the gradients shown in Fig. 7 were analyzed on SDS-polyacrylamide gels. At pH 10.0, less VP_1 was found associated with the DNA. At pH 10.5, the histone proteins were essentially the only species of protein associated with the DNA. Only trace amounts of VP_1, if any, could be found associated with the pH 10.5-altered complex (data not shown).

Experiments were also performed to determine whether 21S polyoma DNA, after incubation with dissociated virions, could be converted to a 48S complex. No conversion of the 21S DNA to 48S complex was observed; instead, the DNA sedimented identical to the 21S marker DNA (data not shown). This indicates that the complex is not an aggregation of viral DNA and proteins that arises during the dissociation procedure, and furthermore suggests that the complex structure is stable and does not turn over.

Electron microscopy of 48S DNA-protein complex. The DNA-protein complex recovered from the 48S peak of sucrose gradients (Fig. 2) was also examined in the electron microscope. The DNA-protein complex was visibly different from intact virions, incomplete virions, and individual capsomeres (Fig. 8a, b, and c). Under the mild conditions of the dissociation and isolation, relatively compact structures were usually observed (Fig. 8d, e, and f). These DNA-protein complex structures had an average diameter of approximately 50 nm, as compared with intact virion particles which measured 43 nm in diameter. In some preparations, the DNA-protein particles were observed to assume a more relaxed structure (Fig. 8g, h, and i). After treatment of the 48S DNA-protein complex with 0.8 M NaCl, a wide variety of structures that ranged from compact structures, like those observed under lower salt concentrations, to structures where the DNA-protein complex appears to have been disrupted considerably were observed (Fig. 8j, k, and l).

Incubating the 48S complex with pH 10.0 buffer caused a drastic change in morphology. No compact structures were visible. These pH-altered structures were generally amorphous, with gobular areas alternating with regions that appeared to be naked DNA (Fig. 8m, n, and o). Increasing the pH of the prefixing step to pH 10.5 caused further dissociation of DNA and protein, resulting in structures which consisted of predominantly naked DNA (Fig. 8p, q, and r).

Determination of proteins associated with capsomere subunits. Electrophoretic

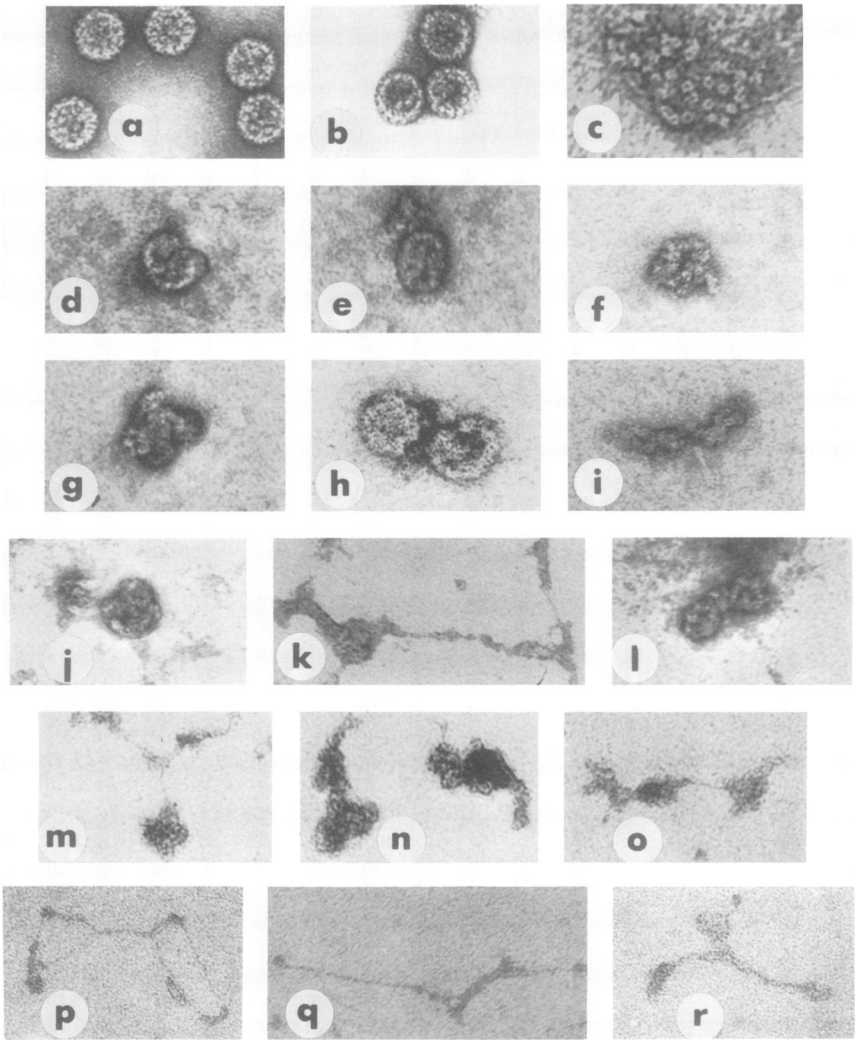

FIG. 8. *Polyoma dissociation products visualized by electron microscopy. (a) Control: intact virions; (b) control: incomplete virions; (c) polyoma capsomeres isolated from gradient as in Fig. 2; (d) through (r) products of polyoma virus dissociation recovered from the 48S peak of gradients as shown in Fig. 2. Portions of the pooled fractions were adjusted to the desired NaCl concentration or pH, incubated for 30 min on ice, fixed, mounted on specimen grids, stained with uranyl acetate, and examined. (d) through (i), pH 8.5 control; (j) through (l), adjusted to 0.8 M NaCl, pH 8.5; (m) through (o), adjusted to pH 10.0 with 0.1 M glycine buffer; (p) through (r), adjusted to pH 10.5 with 0.1 M glycine buffer. Magnification, ×188,000.*

analysis of capsomeres isolated from gradients as shown in Fig. 2 were performed. The results of the autoradiogram of this gel is shown in Fig. 5B. The major protein of the virion, VP_1, makes up approximately 85% of the protein associated with the capsomeres (Table 1), whereas VP_2 and VP_3 contribute about 4 to 5% each. Surprisingly, histones VP_{4-7} were also found with the capsomere subunits, although the concentration (6%) was significantly lower than in complete virions (20%).

To determine the protein composition of the individual capsomere species, the capsomeres were separated into 18S, 12S, and 5S subunits as described previously (1) (Fig. 9). The 18S subunit species comprised approximately 14% of the total capsomere population (Table 2). Of the total protein associated with this species, 92% was VP_1, whereas VP_2 and VP_3 each contributed about 4% and histones VP_{4-7} were not detected. The 12S subunit species constituted 63% of the total subunit population. A total of 85% of the protein in the 12S subunit was determined to be VP_1; VP_2 and VP_3 were found to comprise 8%

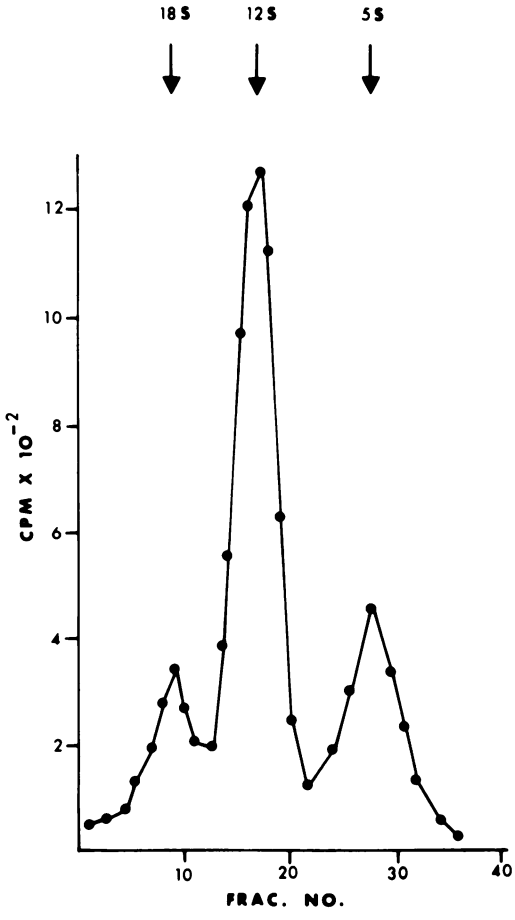

FIG. 9. *Velocity sedimentation of EGTA-DTT-dissociated polyoma virus. ^{125}I-radiolabeled virions were exposed to 1 mM EGTA, 3 mM DTT, and 0.15 M NaCl in 0.01 M Tris buffer (pH 8.5) for 30 min. Sample was layered onto a 5 to 20% sucrose gradient and centrifuged in an SW50.1 rotor at 40,000 rpm for 12 h (4°C). Symbol: ●, ^{125}I.*

TABLE 2. *Distribution of proteins in purified virions and capsomere subunits*[a]

Sample	% Total[b]	Protein (%) present in:[c]			
		VP_1	VP_2	VP_3	VP_{4-7}
Virions	—	68.7	6.4	5.3	19.6
5S	23.7	21.3	31.2	40.1	7.4
12S	62.7	85.8	8.1	6.1	—
18S	13.6	92.1	3.7	4.2	—

[a] Capsomere subunits were isolated from a sucrose gradient as described in the legend to Fig. 9.

[b] The amount of radioactivity in each capsomere species is expressed as the percentage of the total radioactivity recovered from the gradient.

[c] The area of the peak for each protein is expressed as the percentage of the total area occupied by the proteins detected in the sample. Each value is the average of a number of separate determinations.

and 6% of the total protein, respectively, and histones VP_{4-7} were not detected. The 5S capsomere species constituted approximately 24% of the total capsomere population. VP_3 was the dominant protein in this species and comprised 40% of the total protein. VP_2 comprised 31% of the total protein, whereas VP_1 was found to contain 21% of the protein. The 5S species was found to be the major depot of the histone proteins VP_{4-7} which made up approximately 7% of the total protein contained in the 5S species.

Looking at the protein distribution in a somewhat different manner, of the total VP_1 protein found in the isolated capsomere subunits, 70% was found in the 12S species, 21% was in the 18S species, and only about 9% was in the 5S species. For the VP_2 protein, 60% of this protein was located in the 5S species, with 37 and 3% located in the 12S and 18S capsomere species, respectively. Approximately 70% of the total VP_3 was found associated with the 5S species, 27% was in the 12S, and 3% was in the 18S capsomere species. The external histones were found to be associated with only the 5S species (data not shown).

DISCUSSION

The data reported in this paper demonstrate that the in vitro dissociation of polyoma virions by EGTA-DTT at pH 8.5 results in the release of a DNA-protein complex and the structural capsomere subunits (Fig. 1, 2, and 8). Both the DNA-protein complex and the capsomeres are substantially different than those previously described using other in vitro dissociation systems (4, 9). The experimental evidence presented indicates that the polyoma EGTA-DTT dissociation products are not artifacts of the dissociation procedure. Rather, it seems more likely that they represent components of the virion that were not observed previously because of the harshness of the alkaline dissociation systems.

Evidence is presented in this report which substantiates the association of specific polyoma virion proteins within the 48S complex. The 48S sedimentation value is significantly larger than that of 21S naked polyoma DNA or the 25 to 30S DNA-protein complex isolated from carbonate-treated virions (9). The 48S complex is not formed by the nonspecific association of capsomere proteins with 21S viral DNA (data not shown). Radioactively labeled virion proteins were found to cosediment with the complex (Fig. 1 and 2). The complex was extremely sensitive to either NaCl or Sarkosyl, reagents which are used commonly to disrupt DNA-protein interactions (Fig. 3). Electrophoretic analysis of the 48S DNA-protein complex demonstrated the presence of substantial quantities of VP_1 and the

histone proteins VP_{4-7} (Fig. 5C) Treatment of the complex with alkaline pH resulted in a decrease in the sedimentation value of the complex (Fig. 7). Electrophoretic analysis of the pH-altered complex revealed that the sedimentation value decreased due to the loss of the VP_1 associated with the DNA. These experiments clarify the absence of DNA-associated VP_1 in the pH 10.5 carbonate DNA-protein complexes. The pH of this dissociation system was simply too harsh to allow VP_1 to remain associated with the DNA-histone complex. Finally, electron microscopy of the 48S DNA-protein complex demonstrated that it was not naked DNA, but rather a tightly coiled structure that is extensively associated with proteins (Fig. 8). These electron microscopic observations of the polyoma 48S DNA-protein complex are consistent with other studies of intact eukaryotic chromatin stained with uranyl acetate. Striking similarities exist between negatively stained preparations of rat liver chromatin (7), calf lymphocyte chromatin (15), and the 48S DNA-protein complex described above. Metal-shadowed preparations of DNA-protein complexes recovered from simian virus 40-infected cells by Christiansen et al. (4) are also similar in size and shape. The 48S complex is also in agreement with the theoretical model for the morphology of the internal viral DNA-protein core of simian virus 40 by Martin (21). Both our electron microscopic observations and the mathematical considerations of Martin indicate that the "core" of the papovaviruses is a very compactly folded structure.

It is interesting to compare the similarities between the EGTA-DTT DNA-protein complex with the nucleoprotein complexes isolated from infected cell nuclei (18). The nucleoprotein complex isolated from infected cell nuclei by the Triton extraction procedure measures 50 to 60S (13, 18, 25, 26), whereas our virion-isolated complex sediments at 48S. Isopycnic centrifugation of the complexes in CsCl reveals identical densities of 1.45 g/cm^3 for both complexes, indicating similar DNA-to-protein ratios. Electrophoretic analysis of the protein associated with the complex isolated from infected-cell nuclei (18) indicated that it contained VP_1 (44%), VP_2 (3.5%), VP_3 (4.5%), and the histones VP_{4-7} (48%). Our data indicate that approximately 53% of the 48S complex-associated protein was VP_1, whereas the histones VP_{4-7} comprised approximately 47% of the total protein (Table 1). VP_2 and VP_3 were detected in the 48S complex (Fig. 6), but only when the histone and VP_1 proteins were present in such large quantities that protein estimation by Joyce-Loebl densitometry was particularly difficult and subject to error. From the appearance of the gels, it could be estimated that the VP_2 and VP_3 might represent <1% of the total protein present in the 48S complex. Certainly, the two complexes demonstrate certain similarities, considering that the two were isolated under quite different conditions. What is of particular interest is that the complex isolated from infected-cell nuclei may be chased into mature virions in vivo, indicating that it might be an important precursor to viral assembly (18). These results indicate that the EGTA-DTT dissociation of polyoma virions possibly reverses the assembly process that occurs in vivo.

Previously, the virion capsomere subunits obtained by alkaline carbonate dissociation of polyoma virions were repored to sediment as 7S and 10S subunits and contain the structural proteins VP_1, VP_2, and VP_3 (9). There was approximately five to six copies of VP_1, one copy of VP_2, and one copy of VP_3 in both species of capsomeres. These protein ratios made it difficult to determine whether the VP_2 and VP_3 were actually integral structural components or contaminants which were tightly bound to the true capsomere subunits even after dissociation. If we consider just the hexons, the above ratios do not eliminate the possibility that a hexon is composed of five copies of VP_1 and one copy of VP_2 or VP_3. In addition, two distinct classes of capsomere subunits, corresponding to hexons and pentons, were not observed. The capsomeres obtained with the EGTA-DTT dissociation, however, are substantially different and allow us to reconsider these questions.

The protein composition of the capsomere subunits obtained by EGTA-DTT dissociation of polyoma virions revealed two distinct capsomere species. The first capsomere species is made up of 18S and 12S capsomere subunits which are composed predominantly of VP_1 (Table 2). The second capsomere species is made up of the 5S capsomeres which are enriched in VP_2 and VP_3 and contain the external histones VP_{4-7}. The molecular weight estimates of the VP_1-enriched 18S and 12S capsomeres were calculated to be 600,000 and 300,000, respectively, by the method of Martin and Ames (22). Because the protein compositions of the 18S and 12S capsomeres are so similar, we conclude that the 18S peak is likely made up of 12S capsomere dimers in which the linking disulfide bonds have not been reduced sufficiently to allow the capsomeres to be found as individual subunits. In addition, it seems likely that the 18S and 12S capsomeres are the hexon capsomeres. Because there are 60 hexons and 12 pentons per virion, the hexons should comprise approximately 83% of the total capsomere population. When ^{3}H-amino acid-labeled virions were used for analysis, the 18S and 12S species totaled 84% of the

total capsomere population. In the case of the 12S subunit, we calculate that this capsomere species would contain 10 to 11 copies of VP_1 per 1 copy of VP_2, and 1 copy of VP_3. Again, considering the hexon structure of six protomers comprising a single capsomere, both the molecular weight estimate and the protein ratios would seem to indicate that the hexon subunit is composed of six protomers of VP_1. In a separate set of experiments conducted in our laboratory, we have purified the virion VP_1 protein to homogeneity by guanidine-hydrochloride column chromatography and subsequently renatured the VP_1. The renatured VP_1 protein has been shown to be morphologically, biophysically, and antigenically similar to native virion capsomeres and sediments as a 12S capsomere species in sucrose gradients (J. N. Brady and R. A. Consigli, submitted for publication). These data indicate that in all probability the polyoma hexon capsomere is indeed made up of only VP_1. This structure is in agreement with previously published observations (24, 29, 30).

The second species of structural capsomere subunits sediments as a 5S species of protein. As mentioned previously, this species is greatly enhanced in VP_2, VP_3, and external histones VP_{4-7} when compared with the 12S and 18S species (Table 2). All of the histones detectable in the total capsomere population are present in the 5S species, whereas about 70% of the VP_2 and 60% of the VP_3 are associated with this species. Only 9% of the total VP_1 is present in the 5S species. Certainly, the protein composition of the 5S species is quite different from the 18S and 12S species. Could this capsomere subset represent the penton component of the virion?

Walter and Etchison (29, 30) have shown that the disposition of VP_2 and VP_3 in the virus particle is clearly different from that of VP_1. By treating purified virions with low concentrations of SDS in the absence of reducing agents, intact virion particles were converted to empty shells with the release of DNA and its associated proteins. A concomitant loss of VP_2 and VP_3 accompanied the conversion of full particles to empty shells. It was not clear, however, whether the loss of VP_2 and VP_3 was due to their association with DNA or to the removal of pentons from the capsid. Although there have been reports that show a low level of association between VP_3 and DNA (4), the results that we obtained for the 48S complex would argue that the true viral DNA core has virtually no VP_2 and VP_3 associated with it compared with the 5S capsomere species.

It is also interesting that the 5S capsomere species contains the external histone proteins found associated with the capsomere population as a whole. Frost and Bourgaux (12) have hypothesized the existence of external histones on the polyoma virion. Subsequently, Mackay and Consigli (16) suggested that these external histone proteins might serve as the nuclear transport recognition factor(s) that determines the fate of the virus particle once it initiated attachment to a host cell receptor. Because Mackay and Consigli have also shown that viral attachment to the cell membrane involves the interaction of penton(s) with the membrane, it would seem plausible that the histones be located in close association with the penton subunits. Evidence for this close association between histones and pentons was reported recently by McMillen and Consigli (20). They demonstrated that immunoglobulin G directed against SDS-polyacrylamide gel-derived histones was capable of neutralizing both the hemagglutinating and plaque-forming ability of the virion. Because monovalent F(ab') fragments were also able to neutralize the viral infectivity, it is unlikely that antibody-mediated aggregation of the virus resulted in the neutralization. These observations would certainly be consistent with the theory that the 5S species constitutes the penton subunits of the virion.

In conclusion, the experiments presented in this manuscript allow us to predict the following model for the structure of the polyoma virion. It appears that the outer protein shell of the virion has a composition similar to adenovirus in that the hexon and penton capsomeres are made up of different proteins. The polyoma hexons, which are the major capsomere species and no doubt provide the main structural framework of the virion, are composed of six protomers of VP_1. Hexon-hexon linkages are most likely due to disulfide bridges as proposed by Walter and Etchison (29, 30). The penton capsomeres appear to be made up of VP_2 and VP_3. In close association with the viral penton are a compliment of external histones VP_{4-7}. Because the adsorption of both complete and incomplete virions (incomplete virions lack DNA and histones) to the host cell membrane has been shown to involve the penton subunit (16), it would appear that viral proteins VP_2 and VP_3 serve the virion as membrane receptor proteins. The external histones, which are closely associated with the penton subunit, enable the host cell to differentiate between complete and incomplete virions and ultimately determine the fate of the virion. Additionally, it seems likely that the Ca^{2+} ion which we have shown to be important in the maintenance of viral structure is located within the penton capsomere subunit. Inside the virion protein shell, the viral DNA is associated with a particular subset of the histones and VP_1 poly-

peptide. This DNA-protein complex is a very compact core which would be required to package this size of DNA and associated proteins inside the small icosahedron virion.

This model not only confirms previous observations, but also represents the first definitive model in which the virion proteins can be placed precisely within the virion structure. This not only helps to understand the polyoma structure, but also helps to assign specific functions for the virion proteins. In addition, the model presents three very interesting observations. First, the DNA core of the virion contains significant quantities of VP_1 in addition to histones VP_{4-7}. Second, there appears to be a specific set of histones VP_{4-7} which are located with the external viral penton. Third, Ca^{2+} ions play a significant role in maintaining virion integrity. It would be extremely interesting to investigate these observations further to determine the exact function of the DNA-associated VP_1 and the external histones.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant CA-07139 from the National Cancer Institute.

We would like to express our appreciation to Joe Kendall, Kim Osborne, Cheryl Brooks, Viola Hill, and Diane Potts for their excellent technical assistance.

LITERATURE CITED

1. **Brady, J. N., V. D. Winston, and R. A. Consigli.** 1977. Dissociation of polyoma virus by the chelation of calcium ions found associated with purified virions. J. Virol. **23**:717-724.
2. **Brunck, C. F., and V. Leick.** 1969. Rapid equilibrium isopycnic CsCl gradients. Biochim. Biophys. Acta **179**:136-144.
3. **Christiansen, G., and J. Griffith.** 1977. Salt and divalent cations affect the flexible nature of the natural beaded chromatin structure. Nucleic Acids Res. **4**:1837-1851.
4. **Christiansen, G., T. Landers, J. Griffith, and P. Berg.** 1977. Characterization of components released by alkali disruption of simian virus 40. J. Virol. **21**:1079-1084.
5. **Consigli, R. A., J. Zabielski, and R. Weil.** 1973. Plaque assay for polyoma virus on primary mouse kidney cell cultures. Appl. Microbiol. **26**:627-628.
6. **Finch, J. T., and L. V. Crawford.** 1975. Structure of small DNA containing animal viruses. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comprehensive virology, vol. 5. Plenum Publishing Corp., New York.
7. **Finch, J. T., M. Noll, and R. D. Kornberg.** 1975. Electron microscopy of defined lengths of chromatin. Proc. Natl. Acad. Sci. U.S.A. **72**:3320-3322.
8. **Friedmann, T.** 1971. *In vitro* reassembly of shell-like particles from disrupted polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **68**:2574-2578.
9. **Friedmann, T., and D. David.** 1972. Structural roles of polyoma virus proteins. J. Virol. **10**:776-782.
10. **Friedmann, T., and M. Haas.** 1970. Rapid concentration and purification of polyoma and SV40 with polyethylene glycol. Virology **42**:248-250.
11. **Frost, E., and P. Bourgaux.** 1975. Electrophoretic analysis of the structural polypeptides of polyoma virus mutants. Virology **65**:286-288.
12. **Frost, E., and P. Bourgaux.** 1975. Decapsidation of polyoma virus: identification of subviral species. Virology **68**:245-255.
13. **Goldstein, D. A., M. R. Hall, and W. Meinke.** 1973. Properties of nucleoprotein complexes containing replicating polyoma DNA. J. Virol. **12**:887-900.
14. **Hancock, R.** 1970. Separation by equilibrium centrifugation in CsCl gradients of density-labeled and normal deoxynucleoprotein from chromatin. J. Mol. Biol. **48**:357-360.
15. **Hozier, J., M. Renz, and P. Nehls.** 1977. The chromosome fiber: evidence for an ordered superstructure of nucleosomes. Chromosoma **62**:301-317.
16. **Mackay, R. L., and R. A. Consigli.** 1976. Early events in polyoma virus infection: attachment, penetration, and nuclear entry. J. Virol. **19**:620-636.
17. **McMillen, J., M. S. Center, and R. A. Consigli.** 1976. Origin of polyoma virus-associated endonuclease. J. Virol. **17**:127-131.
18. **McMillen, J., and R. A. Consigli.** 1974. Characterization of polyoma DNA protein complexes. I. Electrophoretic identification of the proteins in a nucleoprotein complex isolated from polyoma-infected cells. J. Virol. **14**:1326-1336.
19. **McMillen, J., and R. A. Consigli.** 1974. In vitro radioisotopic labeling of proteins associated with purified polyoma virions. J. Virol. **14**:1627-1629.
20. **McMillen, J., and R. A. Consigli.** 1977. Immunological reactivity of antisera to sodium dodecyl sulfate-derived polypeptides of polyoma virion. J. Virol. **21**:1113-1120.
21. **Martin, R. G.** 1977. On the nucleoprotein core of Simian Virus 40. Virology **83**:433-437.
22. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372-1379.
23. **Perry, J. L., C. M. To, and R. A. Consigli.** 1969. Alkaline degradation of polyoma virions. J. Gen. Virol. **4**:403-411.
24. **Ponder, B. A. J., A. K. Robbins, and L. V. Crawford.** 1977. Phosphorylation of polyoma and SV_{40} virus proteins. J. Gen. Virol. **37**:75-84.
25. **Qureshi, A. A., and P. Bourgaux.** 1976. Polypeptides of a viral DNA-protein complex from polyoma virus infected cells. Virology **74**:377-385.
26. **Seebeck, T., and R. Weil.** 1974. Polyoma viral DNA replicated as a nucleoprotein complex in close association with host cell chromatin. J. Virol. **13**:567-576.
27. **Smith, G. L., and R. A. Consigli.** 1972. Transient inhibition of polyoma virus synthesis by Sendai virus (parainfluenza I). I. Demonstration and nature of the inhibition by inactivated virus. J. Virol. **10**:1091-1097.
28. **Vinograd, J., and J. E. Hearst.** 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. Prog. Chem. Org. Nat. Prod. **20**:372-422.
29. **Walter, G.** 1974. Inter-molecular disulfide bonds an important structural feature of polyoma virus capsid. Cold Spring Harbor Symp. Quant. Biol. **39**:255-257.
30. **Walter, G., and D. Etchison.** 1977. Subunit interactions in polyoma virus structure. Virology **77**:783-797.
31. **Weil, R.** 1963. The denaturation and renaturation of the DNA of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **49**:480-487.