JOURNAL OF VIROLOGY, Aug. 1978, p. 436-442
0022-538X/78/0027-0436$02.00/0
Copyright © 1978 American Society for Microbiology
Vol. 27, No. 2
Printed in U.S.A.

Chromatographic Separation of the Polyoma Virus Proteins and Renaturation of the Isolated VP_1 Major Capsid Protein†

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

Received for publication 27 February 1978

Treatment of purified polyoma virions with 6 M guanidine-hydrochloride and 0.01 M β-mercaptoethanol resulted in the immediate loss of both hemagglutinating and plaque-forming ability. Gel filtration through Sepharose CL-6B beads allowed separation of the dimer, VP_1, VP_2, VP_3, and histone proteins VP_{4-7} in highly purified form. Renaturation of the purified VP_1 protein resulted in the formation of subunits that were morphologically, biophysically, and immunologically similar to native virion capsomeres.

Due to the hydrophobic nature of the polypeptides that make up the polyoma virion, the isolation and identification of these proteins has been limited to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Eight virion proteins have been resolved by gel electrophoresis (molecular weights): dimer (86,000), VP_1 (45,000 to 46,000), VP_2 (31,000 to 35,000), VP_3 (21,000 to 23,000), and four cellular histones, VP_4 (18,000), VP_5 (16,000), VP_6 (14,000), and VP_7 (11,000) (4, 6, 8, 14, 15, 17, 18). Tryptic peptide analysis of the isolated proteins indicated that the dimer and VP_1 proteins are identical (4, 9). VP_2 and VP_3 also have similar tryptic peptides, with VP_2 having additional peptides, but both polypeptides are distinct from VP_1. The histone proteins, VP_{4-7}, have been determined to be cellular histones, possibly modified following polyoma infection (16). Although SDS-polyacrylamide gel electrophoresis offers good resolution and has been a valuable tool for the identification of the virion proteins, the recovery of native protein is difficult because SDS has such a strong binding affinity for proteins.

To circumvent the SDS denaturant, we have developed an alternative method for separation of the polyoma viral proteins. This system involves gel filtration in the presence of 6 M guanidine-hydrochloride (GuHCl) and the reducing agent, β-mercaptoethanol, as described by Fish et al. (5). Dialysis of the isolated major protein, VP_1, to remove the GuHCl denaturant permits the recovery of the protein in its native state. The role that the VP_1 polypeptide plays in the virion structure will be discussed.

Virus to be used in these experiments was grown in primary mouse kidney or mouse embryo cells (3, 19) in serum-free Dulbecco-modified Eagle medium (13). Virus purification has been described previously (1, 13). ^{32}P-labeled virions were produced by maintaining the infected cell cultures in phosphate-free media with 20 μCi of radiolabeled inorganic phosphate per ml. The preparation of ^{3}H-amino acid-labeled polyoma virions and the in vitro ^{125}I virion labeling have also been described (7, 15). GuHCl (Sigma Chemical Co., practical grade) was purified by the method of Bryce and Crichton (2). Briefly, 6 M GuHCl was heated to 60°C with activated charcoal for 4 h and then filtered through Whatman no. 42 ashless filter paper. The preparation of protein samples and the details for the preparation of SDS-15% polyacrylamide gels have been described previously (1a, 15).

GuHCl was chosen over other similar protein denaturants because it caused the most rapid and complete dissociation of the polyoma virion. The virion was found to be extremely stable to 6 M urea solution, since hemagglutination and plaque-forming ability were not markedly reduced. The addition of 0.1 M β-mercaptoethanol to the urea dissociation mixture caused a 75% reduction in hemagglutinating and plaque-forming activity. However, when virions were incubated in 6 M GuHCl, with or without β-mercaptoethanol, an immediate loss of all hemagglutinating and plaque-forming activity was observed (data not shown).

Prior to chromatography of viral proteins, GuHCl-denatured ^{14}C-labeled molecular weight standards were chromatographed. It was found that the effluent volume at which each marker protein eluted in this chromatographic system was proportional to the logarithm of its molecular weight (Fig. 1). Chromatography of GuHCl-denatured polyoma virion proteins through Sepharose CL-6B beads demonstrated five major peaks of radioactivity (Fig. 1). The peak

† Contribution no. 78-265-J from the Kansas Agricultural Experiment Station, Kansas State University.

fractions from each protein species were dialyzed and analyzed by SDS-polyacrylamide slab gel electrophoresis. With SDS-disrupted polyoma virions as a reference, the column-purified proteins were identified. Peak A was determined to be the dimer; peak B, VP_1; peak C, VP_2; peak D, VP_3; and peak E contained the four histone proteins, VP_{4-7}. All the proteins isolated from the column were highly purified, with the exception of VP_2, which was slightly contaminated with VP_1. No additional proteins were detected.

To determine whether polyoma DNA was coeluting with the polyoma polypeptides, an experiment was performed in which ^{32}P-labeled polyoma virions were also disrupted and analyzed in the GuHCl chromatographic system (Fig. 1). ^{32}P radioactivity could be detected only in the void volume of the column, indicating that

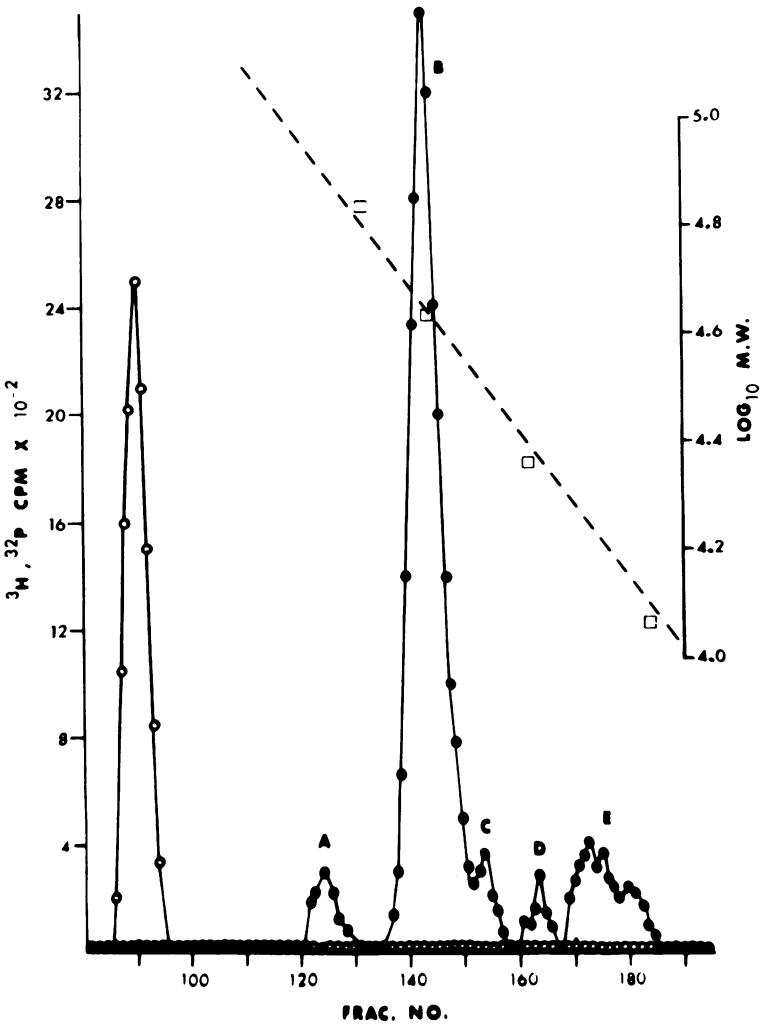

FIG. 1. *Gel filtration of radiolabeled polyoma virion proteins in GuHCl and β-mercaptoethanol. Electrophoretically pure radiolabeled polyoma virions (2 to 5 mg) were lyophilized and resuspended in 1 ml of Tris buffer (0.1 M, pH 8.5). β-Mercaptoethanol was added to a final concentration of 0.1 M, and the mixture was allowed to incubate at 4°C for 2 h. Solid GuHCl (Sigma Chemical Co., grade 1) was then added to a final concentration of 6 M, and the volume was adjusted to 2 ml with Tris buffer (0.01 M, pH 7.4). The pH of the sample was then adjusted to pH 4.0, and it was allowed to incubate at room temperature for 3 h. Chromatography was performed at a flow rate of 2 ml/h on a column (100 by 2.5 cm, Glenco column) of Sepharose CL-6B (Pharmacia) equilibrated with 6 M GuHCl, 0.1 M β-mercaptoethanol, and Tris buffer (0.01 M, pH 4.0). Samples of 1.8 ml were collected. Aliquots from each fraction were analyzed for radioactivity. Molecular weight standards from left to right are bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c. Symbols:* (○) *^{32}P,* (●) *^3H-amino acid,* (□) *molecular weight standards.*

the viral DNA was not broken down by the GuHCl. In addition, the DNA was sufficiently separated from the viral proteins such that it was not likely to influence their migration through the column matrix. In addition, no viral protein was found to be comigrating with the viral DNA, indicating that the GuHCl had disrupted any protein-DNA interactions present in the virion.

Since the polyoma viral proteins have been well characterized only in SDS-polyacrylamide gels, it was essential to compare the molecular weights and protein ratios of the GuHCl-isolated proteins with the SDS-polyacrylamide gel system. The molecular weight estimates for the viral proteins were almost identical between the two systems. The only significant difference was in VP_2, which eluted at an apparent molecular weight of 31,000 in the GuHCl column system as compared to a molecular weight of 34,000 in the SDS-polyacrylamide gel system. The polypeptide composition of the virus was also very similar in the two systems.

Gel filtration in GuHCl is not only suitable for the fractionation of viral proteins, but has the advantage that the denaturant may be removed by dialysis, allowing refolding of the protein to its native configuration. Since VP_1 makes up approximately 75% of the total viral protein, we chose to initiate our investigations of the

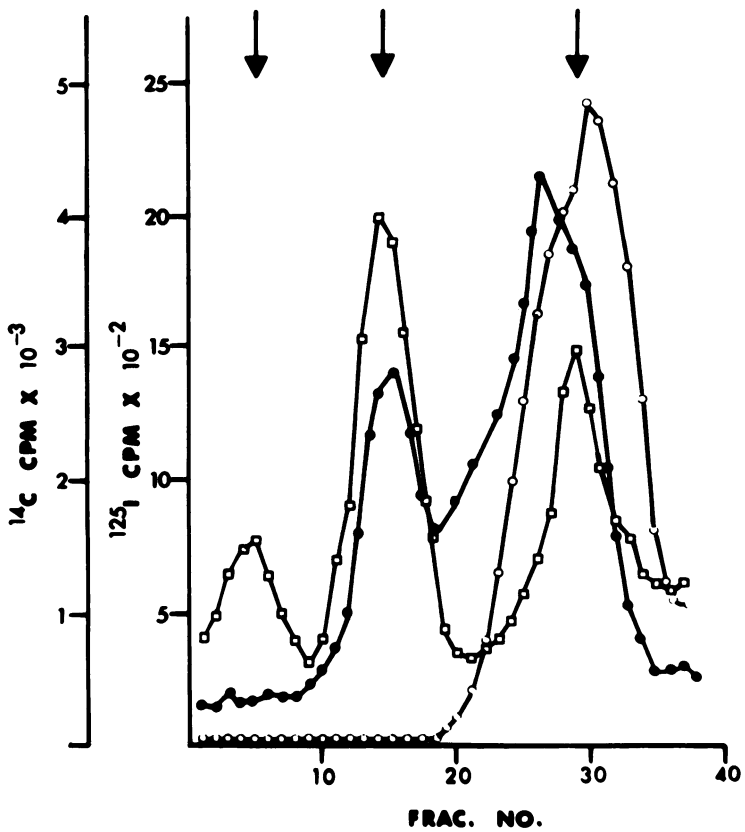

FIG. 2. *Velocity sedimentation of polyoma capsomeres, renatured GuHCl VP_1, and marker proteins in sucrose gradients. The renaturation of GuHCl VP_1 was performed by sequential dialysis against 500 volumes of cold buffer (4°C) in the following order: (i) 4 M GuHCl, 0.25% Triton X-100, 0.1 M β-mercaptoethanol, in Tris buffer (0.01 M, pH 7.4); (ii) 2 M GuHCl, 0.25% Triton X-100, 0.1 M β-mercaptoethanol, in Tris buffer (0.01 M, pH 7.4); (iii) 0.25% Triton X-100, 0.1 M β-mercaptoethanol, 0.15 M NaCl, in Tris buffer (0.01 M, pH 7.4). Each dialysis step was allowed to go for 24 h at 4°C. Dialysis against buffer (iii) was repeated twice. ^{125}I-labeled polyoma virions were exposed to 1 mM EGTA, 3 mM DTT, and 0.15 M NaCl in 0.05 M Tris buffer (pH 8.5) for 30 min at room temperature to dissociate the virions to the capsomere level. Samples of each preparation were layered onto 5 to 20% sucrose gradients (5 mM EGTA, 0.15 M NaCl, 0.25% Triton X-100, in Tris buffer, 0.01 M, pH 8.5) and centrifuged in an SW50.1 rotor at 40,000 rpm for 17 h (4°C). Arrows from left to right indicate position of 18S, 12S, and 5S capsomere subunits. Symbols: (□) polyoma capsomeres, (●) renatured GuHCl VP_1, (○) protein markers.*

GuHCl-isolated proteins with this polypeptide. Following renaturation and concentration of the VP_1 protein, the purity of the preparation was again analyzed by SDS-polyacrylamide gel electrophoresis. Only a single protein, which migrated exactly with VP_1 from intact virions, could be detected.

The renatured VP_1 was also analyzed on velocity sucrose gradients (Fig. 2). Polyoma capsomeres, prepared by ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid–dithiothreitol (EGTA-DTT) dissociation of intact virions (1), were sedimented in a parallel gradient for comparison. The EGTA-DTT capsomeres sedimented as 18S, 12S, and 5S protein species. The renatured VP_1 demonstrated two well-defined peaks of radioactivity. The heavier peak sedimented to a position in the gradient identical to that of the 12S virion capsomere species and comprised approximately 40% of the radioactivity recovered from the gradient. The lighter peak of radioactivity, which comprised approximately 60% of the total radioactivity recovered, sedimented as a very broad species of protein with a mean sedimentation value of 6S. This radioactive peak demonstrated a very definite skew on the 12S side of the peak, indicating that more than one species of protein might be present. This type of profile for the VP_1 was obtained only when the renaturation process was allowed to proceed very slowly, as described in the legend of Fig. 2. VP_1, which was subjected to a very quick dialysis against Tris buffer (0.01 M, pH 7.4), resulted in an aggregation of protein such that over 90% of the radioactivity was recovered in the pellet of the gradient (data not shown). No detectable 12S and 6 to 7S protein species were observed.

Also shown in Fig. 2 is the sedimentation pattern observed when the in vitro-labeled protein markers (bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c*) were analyzed on parallel sucrose gradients. These proteins sediment slightly slower than the VP_1 6S protein species (Fig. 2). This indicates that the VP_1 6S species might consist of a family of VP_1 monomers, dimers, and possibly trimers. The VP_1 12S species would appear to be a larger multimer of VP_1 monomers which have associated with each other to form a distinct subunit species.

Since there was a distinct species of the renatured VP_1 which cosedimented with the native 12S capsomere species, samples of the renatured VP_1 were examined in the electron microscope. A significant number of structures were observed which closely resembled virion capsomeres in both size and shape (Fig. 3). The renatured VP_1 structures measured approximately 9.0 nm in diameter, while native virion capsomeres were determined to be approximately 10 nm in diameter. The VP_1 subunits were also morphologically similar to native capsomeres, appearing to have a hollow center

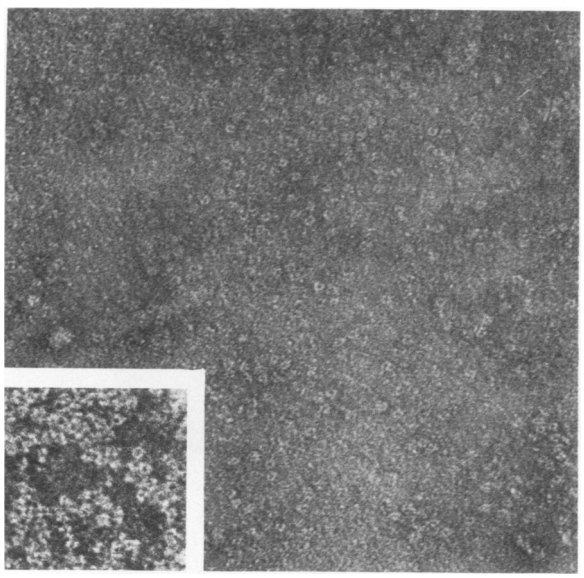

FIG. 3. *Electron microscopy of renatured GuHCl VP_1 capsomere subunits. Renatured VP_1 samples were mounted on carbon-coated grids and stained with 2% aqueous phosphotungstic acid. Specimens were examined with a Phillips 201 electron microscope operated at 60 kV. Magnification, ×180,000 (insert, ×240,000).*

around which a ring of proteins was arranged.

The sedimentation pattern and electron microscopy both indicated that at least a portion of the renatured VP_1 had assembled into capsomere subunits. Further investigations were performed with a series of well-characterized, purified immunoglobulin G (IgG) samples present in our laboratory. Following adsorption of the renatured VP_1 to mouse kidney cells, anti-capsomere IgG was tested for its reactivity to

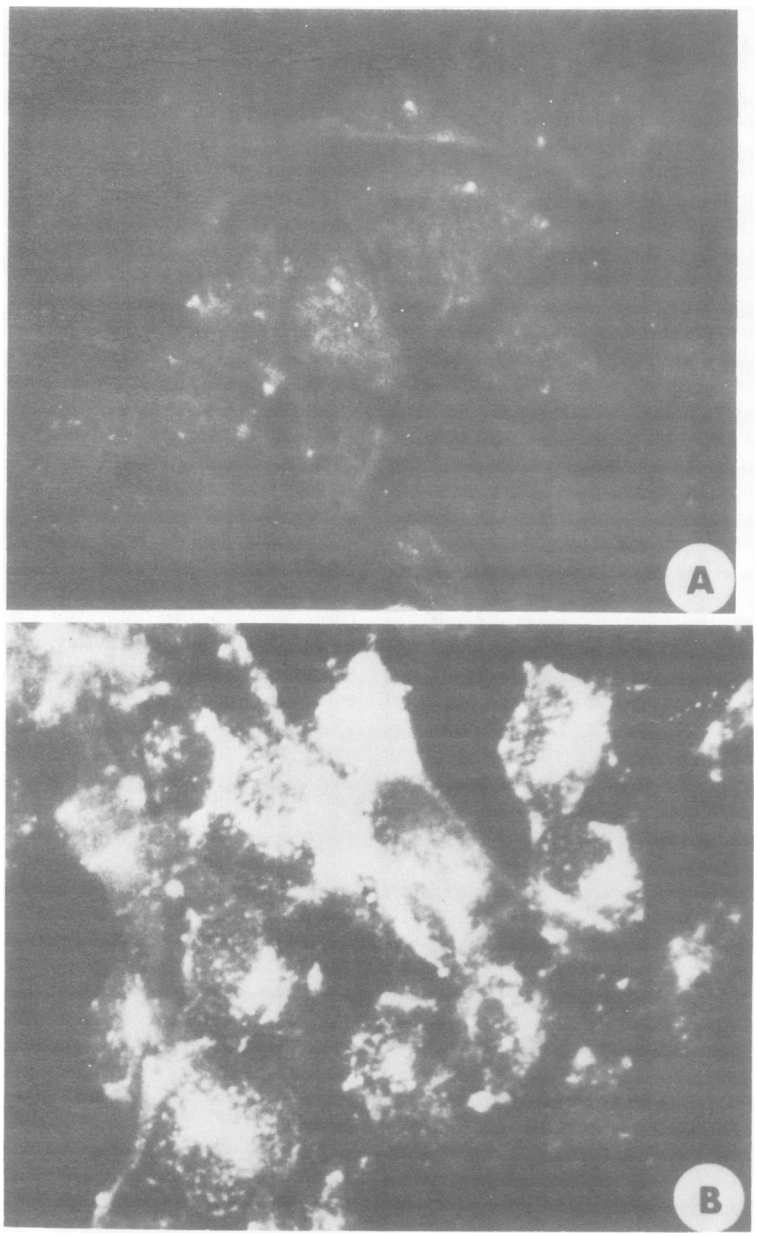

FIG. 4. *Immunofluorescence photomicrographs of renatured VP_1 capsomeres adsorbed to mouse kidney cells using normal rabbit serum and anti-polyoma capsomere serum. The preparation of monospecific antibodies has been described previously (16). IgG was purified from whole serum by triple salt extraction using sodium sulfate (10), followed by chromatography over a DEAE-Sephadex column (11). Renatured VP_1 was allowed to adsorb to mouse kidney cells for 1 h at room temperature, and the unadsorbed protein was removed by repeated washes with phosphate-buffered saline. The indirect method of fluorescent-antibody assay was used, using fluorescein-conjugated goat anti-rabbit globulin (Difco). (A) Normal rabbit serum; (B) anti-polyoma capsomere rabbit serum.*

the renatured VP_1 (Fig. 4). As can be seen, a very strong reaction was observed between the renatured VP_1 capsomeres and the anti-capsomere IgG (Fig. 4B). Some cells were observed to be virtually covered with the VP_1 capsomeres, while others demonstrated a very particulate fluorescence. Normal rabbit serum, isolated from rabbits prior to inoculation, demonstrated no fluorescence with the renatured VP_1 capsomeres (Fig. 4A).

Similar fluorescent-antibody assays were performed with IgG to intact polyoma and monospecific IgG directed against the individual virion polypeptides isolated from SDS-polyacrylamide gels (16). All these antibodies were highly purified and have been well characterized in previous investigations in this laboratory (12, 16). Only antibodies directed against capsomeres and intact polyoma virions were found to react with the renatured VP_1 capsomeres (data not shown), with anti-capsomere IgG giving the strongest reaction. No reactivity of the renatured VP_1 capsomeres with the antibodies directed against the SDS-polyacrylamide gel-isolated viral proteins was observed.

Recently, we reported that polyoma virions could be dissociated to a DNA-protein complex and individual capsomere subunits following exposure to EGTA-DTT (1, 1a). The capsomere subunits sedimented as 18S, 12S, and 5S protein species in sucrose gradients. The 18S and 12S protein species were composed of 92 and 86% VP_1, respectively. VP_2 and VP_3 made up the remaining portion of the protein associated with these two capsomere subunit species. Since the capsid shell of the virions consists of 60 hexons and 12 pentons, one can calculate that 83% of the total capsomere population are the hexon subunits. The 18S and the 12S subunit populations comprised 84% of the total capsomere population. In addition, the molecular weights of the 18S and 12S subunits are approximately 600,000 and 300,000, respectively. Based on these findings, we proposed that these two species were the hexon capsomeres of the virion (the 18S species would appear to be a hexon dimer) and that each hexon capsomere was composed of six molecules of VP_1. Obviously, the ultimate proof to this proposal was to isolate a capsomere subunit in which only VP_1 was present. Since it seemed that we could not achieve this goal by virion dissociation, the alternative was to isolate pure VP_1 and then assemble the capsomere subunit in vitro.

The results presented here indicate that polyoma capsomeres, presumably the hexons, can be formed by the association of VP_1 proteins alone. Furthermore, these VP_1 capsomeres appear to be identical to capsomeres purified from the intact virion. The VP_1 capsomeres sediment identically to the 12S capsomeres released by the EGTA-DTT dissociation of intact virions. In addition, the VP_1 capsomeres react very strongly with anti-capsomere IgG. These results provide evidence that the correct number and arrangement of VP_1 proteins have occurred in the formation of the VP_1 capsomere such that it is biophysically and antigenically very similar to native virion capsomeres. The inability of the VP_1 capsomeres to react with monospecific IgG to SDS-denatured virion proteins is also of importance. The renaturation of proteins sometimes leads to folded proteins that are only partially renatured; i.e., species are obtained which contain major portions of the original conformation, but also contain regions of altered structure due to the improper folding of the polypeptide chain. The lack of any reactivity with IgG to the SDS-polyacrylamide gel-isolated virion proteins would indicate that there are no significant denatured regions in the proteins which make up the VP_1 capsomere subunits. Together with the EGTA-DTT dissociation data, these results provide evidence that the hexons of the polyoma virion are composed of only VP_1. This would be consistent with the observations of Walter and Deppert (20).

Probably the most significant contribution of these findings is that the ability to isolate and subsequently renature the virion proteins offers an ideal experimental system for future investigation. To our knowledge, this is the first time that individual polyoma proteins have been available in their native configuration for experimentation. These renatured proteins can now be used for further investigations dealing with their structure and function in the virion.

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

We thank Joe Kendall, Kim Osborne, Viola Hill, Diane Potts, and Cheryl Brooks 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.
1a. **Brady, J. N., V. D. Winston, and R. A. Consigli.** 1978. Characterization of a DNA-protein complex and capsomere subunits derived from polyoma virus by treatment with ethyleneglycol-bis-*N,N'*-tetraacetic acid and dithiothreitol. J. Virol. **27**:193–204.
2. **Bryce, C. F. A., and R. R. Crichton.** 1971. The subunit structure of horse spleen apoferritin. I. The molecular weight of the subunit. J. Biol. Chem. **246**:4198–4205.
3. **Consigli, R. A., J. Zabielski, and R. Weil.** 1973. Plaque assay of polyoma virus on primary mouse kidney cell cultures. Appl. Microbiol. **26**:627–628.
4. **Fey, G., and B. Hirt.** 1974. Fingerprints of polyoma virus proteins and mouse histones. Cold Spring Harbor Symp. Quant. Biol. **39**:235–241.
5. **Fish, W. W., K. G. Mann, and C. Tanford.** 1969. The estimation of polypeptide chain molecular weights by

gel filtration in 6M guanidine hydrochloride. J. Biol. Chem. **244**:4989–4994.
6. **Frearson, P. M., and L. V. Crawford.** 1972. Polyoma virus basic proteins. J. Gen. Virol. **14**:141–155.
7. **Frost, E., and P. Bourgaux.** 1975. Decapsidation of polyoma virus: identification of subviral species. Virology **68**:245–255.
8. **Gibson, W.** 1974. Polyoma virus proteins: a description of the structural proteins of the virion based on polyacrylamide gel electrophoresis and peptide analysis. Virology **62**:319–336.
9. **Hewick, R. M., M. Fried, and M. D. Waterfield.** 1975. Nonhistone virion proteins of polyoma: characterization of the particle proteins by tryptic peptide analysis by use of ion exchange columns. Virology **66**:408–419.
10. **Kekwick, R.** 1940. The serum proteins in multiple myelomatosis. Biochem. J. **34**:1248–1257.
11. **Levy, H., and H. Sober.** 1960. A simple chromatographic method for preparation of gamma globulin. Proc. Soc. Exp. Biol. Med. **103**:250–252.
12. **Mackay, R. L., and R. A. Consigli.** 1976. Early events in polyoma virus infection: attachment, penetration, and uncoating. J. Virol. **19**:620–636.
13. **McMillen, J., M. S. Center, and R. A. Consigli.** 1976. Origin of polyoma virus-associated endonuclease. J. Virol. **17**:127–131.
14. **McMillen, J., and R. A. Consigli.** 1974. In vitro radioisotopic labeling of proteins associated with purified polyoma virions. J. Virol. **14**:1627–1629.
15. **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.
16. **McMillen, J., and R. A. Consigli.** 1977. Immunological reactivity of antisera to sodium dodecyl sulfate-derived polypeptides of polyoma virions. J. Virol. **21**:1113–1120.
17. **Murakami, W. T., and B. S. Schaffhausen.** 1974. The protein composition of SV40 and polyoma virions, p. 43–62. *In* Molecular studies in viral neoplasia. 25th Symposium Fundamental Cancer Research, M. D. Anderson Hospital Tumor Institute, Houston, Tex. The Williams & Wilkins Co., Baltimore.
18. **Roblin, R., E. Harle, and R. Dulbecco.** 1971. Polyoma virus proteins. I. Multiple virion components. Virology **45**:555–566.
19. **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.
20. **Walter, G., and W. Deppert.** 1974. Intermolecular disulphide bonds: an important structural feature of the polyoma virus capsid. Cold Spring Harbor Symp. Quant. Biol. **39**:255–257.