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Dissociation of Polyoma Virus by the Chelation of Calcium Ions Found Associated with Purified Virions[1]

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Analysis of polyoma virions by X-ray fluorometry demonstrated that calcium (Ca^{2+}) was associated with the purified virion. Treatment of purified virions with ethyleneglycol-bis-*N*,*N*'-tetraacetic acid (EGTA), which chelates Ca^{2+}, and the reducing agent dithiothreitol caused the virions to dissociate. Electron microscopy revealed that the virions were dissociated to the capsomere level. Incubation of polyoma virions with 150 mM NaCl, 10 mM EGTA, and 3 mM dithiothreitol was optimum for the dissociation reaction. The pH for the dissociation reaction ranged from 7.5 to 10.5. Cesium chloride density gradient centrifugation indicated that both EGTA and dithiothreitol were necessary for dissociation to occur; neither reagent alone dissociated the virus. The major protein product of the dissociated viral particles sedimented at 12S. Relationships between these experiments and the alkaline carbonate-bicarbonate dissociation of polyoma are discussed.

To understand the viral uncoating and assembly mechanisms in a host cell, it is important to understand how the intact virion is stabilized. Previous investigators have approached the problem of virion stability by studying the in vitro dissociation of polyoma virions. The dissociation of virions to the capsomere level was obtained by use of alkaline carbonate-bicarbonate buffers (17). This carbonate method was later improved by adding reducing agents that cleaved disulfide bonds in the capsid proteins (7, 10, 21). However, the mechanism by which the carbonate-bicarbonate system caused dissociation of the virion was not understood.

We report here experiments which demonstrate that Ca^{2+} ions are associated with purified polyoma and are, in part, responsible for maintaining virion integrity. Taking advantage of the binding affinity of ethyleneglycol-bis-*N*,*N*'-tetraacetic acid (EGTA) for Ca^{2+}, we studied calcium chelation, which results in the dissociation of the virion to capsomeres and a DNA-protein complex. After EGTA and dithiothreitol (DTT) treatment, more than 98% of the viral hemagglutinating (HA) activity was lost within 30 min. Concomitant with the loss of HA activity, electron microscopy revealed that the virion was dissociated to the capsomere level.

The data presented in this report demonstrate the importance of Ca^{2+} in maintaining the integrity of the virus. Furthermore, we propose that in vitro polyoma dissociation systems previously described (7, 8, 17) were dependent upon chelation of the calcium associated with the virion.

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MATERIALS AND METHODS

Cell and virus propagation. The preparation of primary cultures of mouse embryo and kidney cells has been described (5, 19). Briefly, for cell cultures, each roller bottle was seeded with 100 ml of a cell-medium mixture containing 2×10^6 cells per ml. 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 (13).

Virus purification. Virus was purified from the infected-cell lysate either as described previously for small volumes (13) or as described by Friedmann and Hass (9), by polyethyleneglycol precipitation, for large volumes. The CsCl gradients used to purify the virus were prepared as described by Brunck and Leick (2). Briefly, the concentrated virus was brought to a density of 1.20 g/cm^3 by adding 1.8-g/cm^3 stock cesium chloride. A 2.5-ml portion of this solution was then layered onto 2.5 ml of 1.35-g/cm^3 CsCl in an SW50.1 nitrocellulose centrifuge tube and centrifuged for 15 h at 35,000 rpm. Two discrete viral bands were observed after centrifugation: complete virus (buoyant density, 1.33 g/cm^3) and incomplete virus (buoyant density, 1.28 g/cm^3). The viral bands were collected separately. The respective viral preparations were then diluted with Tris buffer (0.01 M, pH 7.4) to a density of 1.20 g/cm^3 and layered onto a four-step CsCl velocity gradient consisting of 0.9 ml of 1.35-, 1.32-, 1.29-, and 1.26-g/

cm^3 CsCl layered in an SW50.1 nitrocellulose centrifuge tube. The gradients were centrifuged at 35,000 rpm for 3 h. The viral bands were collected and dialyzed for 24 h against Tris buffer (0.01 M, pH 7.4) at 4°C. After dialysis, the viral preparations were analyzed for purity by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (15). The virus was stored frozen at −20°C until used.

Preparation of ^{14}C-amino acid-labeled virions. The preparation of ^{14}C-amino acid-labeled polyoma virus has been previously described (12).

^{45}Ca-labeled polyoma virions. Four hours after the mouse kidney cells were seeded, the media and unattached cells were removed by aspiration, and Eagle medium (2× amino acids, 2× vitamins, 10% dialyzed fetal calf serum, Ca^{2+} free) containing 2 μCi of ^{45}Ca (New England Nuclear Corp., Boston, Mass.) per ml (20 Ci/g) was added to each plate. When cells reached confluency, the cultures were infected with polyoma virus and maintained in serum-free, Dulbecco-modified Eagle medium. Infected cells and media were harvested 72 h later, and the ^{45}Ca-labeled polyoma virus was purified.

Dissociation of polyoma virions. Stock solutions of each component of the dissociation buffer (Tris, EGTA, and NaCl) were prepared and stored at 4°C, with the exception of DTT, which was prepared fresh for each assay. Specific experimental conditions for polyoma dissociation are described with each experiment. All dissociation reaction mixtures were incubated at room temperature.

Quantitative assays. Cesium chloride densities were determined from the refractive index by using a refractometer (Bausch & Lomb, Inc., Rochester, N.Y.) and were calculated by using the Vinograd-Hearst equation (20). Radioactivity was quantitated in a toluene-Triton (3:1) scintillation fluid by using a Beckman LS-233 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Protein was determined by the method of Lowry et al. (11), with bovine serum albumin as the standard. The hemmagglutination assay has been described previously (4).

Velocity sedimentation. Dissociated polyoma virions were layered onto 5 to 20% sucrose gradients containing 5 mM EGTA, 0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 8.5), and 0.25% Triton X-100. Centrifugation was in an SW50.1 rotor at 40,000 rpm (4°C) for 10 h. Sedimentation coefficients were determined by comparison with a 7S immunoglobulin marker.

Electron microscopy. Samples were mounted on Formvar films and stained with 2% aqueous phosphotungstic acid. Specimens were examined with a Philips 201 electron microscope operated at 60 kV.

Inhibition studies. Experiments were designed to determine whether adding exogenous magnesium or calcium cations could prevent dissociation of the virion. The dissociation buffer contained 5 mM EGTA, 3 mM DTT, 0.15 M NaCl, and 0.01 M Tris-hydrochloride (pH 8.5) and was altered by adding Mg^{2+} (10 mM) or various concentrations of Ca^{2+} (5, 10, or 20 mM) 30 min before adding polyoma virus. Virion dissociation was measured by the hemagglutination assay.

RESULTS

Analysis of polyoma virions by X-ray fluorescence revealed that Ca^{2+} was associated with the purified virions (B. Curnutte, Jr., unpublished data). Based upon these observations, Ca^{2+} chelation studies were undertaken to determine whether Ca^{2+} played a role in maintaining the integrity of the virus. EGTA was chosen as the chelating agent in these experiments due to its high affinity for Ca^{2+} (18).

Optimization of dissociation mixture. To determine the importance of Ca^{2+} in maintaining virion structural integrity, the optimum conditions for polyoma virion dissociation were elucidated. The components of the dissociation mixture (EGTA, DTT, and NaCl) and the pH were individually optimized. We first determined the effect of various NaCl concentrations on virion dissociation. As NaCl approached physiological concentrations, dissociation of the virion was optimum (Fig. 1A). Therefore, 0.15 M NaCl was selected as the desired concentration for future experiments.

It was found that EGTA concentrations (Fig. 1B) higher than 150 mM inhibited the dissociation of the virions, whereas concentrations of ca. 0.5 to 10 mM EGTA gave the greatest degree of virion dissociation. The concentration of EGTA selected for further experiments was 10 mM.

By using 0.15 M NaCl and 10 mM EGTA, the optimum concentration of DTT was investigated. Little difference in the extent of virion dissociation was observed between concentrations of 1 to 10 mM at pH 7.5 (Fig. 1C). At a DTT concentration of 3 mM, more dissociation was observed at pH 9.5 than at 7.5. This prompted us to investigate the pH dependence of the dissociation reaction.

Tris-hydrochloride-buffered dissociation mixtures from pH 6.0 to 10.5 were tested (Fig. 1D). The alkaline pH of the buffer system was found to have maximum efficiency for virion dissociation. A pH of 9.5 was found to cause the greatest dissociation, since less than 1% of the viral HA activity remained after 30 min of incubation. It should also be noted that the dissociation mixture at pH 7.5 proceeded efficiently, although to a somewhat lesser extent, since only 12.5% of the original HA activity remained after 3 h of dissociation. A pH of 8.5 was selected for future experiments, as it afforded the highest degree of disruption under only slightly alkaline conditions. Thus, the optimum conditions found for maximum polyoma dissociation were: 0.15 M NaCl, 3 mM DTT, 10 mM EGTA, and 0.01 M Tris buffer (pH 8.5).

These optimum conditions were then used to

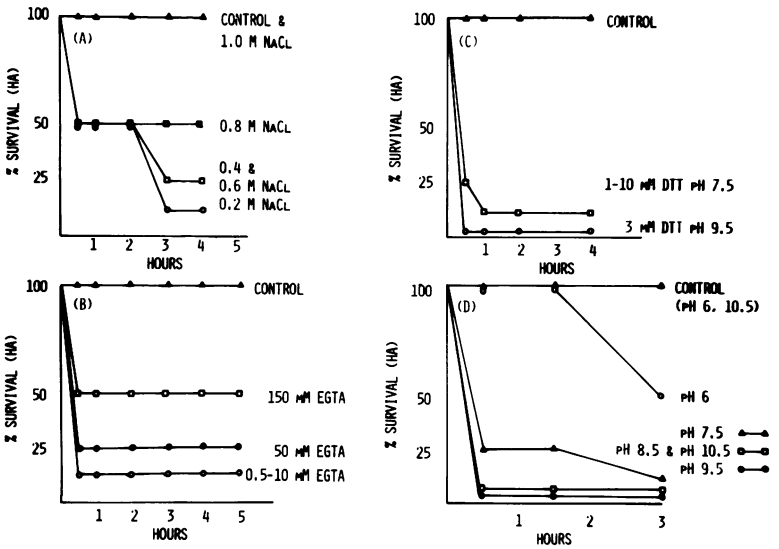

FIG. 1. *Optimal conditions for polyoma dissociation by EGTA-DTT as determined by the hemagglutination assay. (A) NaCl optimization; dissociation mix contained 50 mM EGTA, 3 mM DTT, and NaCl (as indicated) in 0.01 M Tris buffer (pH 7.5); (B) EGTA optimization; dissociation mix contained EGTA as indicated, 0.15 M NaCl, and 3 mM DTT in 0.01 M Tris buffer (pH 7.5); (C) DTT optimization; dissociation mix contained 10 mM EGTA, 0.15 M NaCl, and DTT (as indicated) in 0.01 M Tris buffer (pH 7.5 or 9.5); (D) pH optimization; dissociation mix contained 10 mM EGTA, 3 mM DTT, and 0.15 M NaCl in 0.01 M Tris buffer at indicated pH. Control preparations contained all components of the dissociation mixture except EGTA and DTT.*

determine the rate of polyoma virion dissociation (Fig. 2). More than 75% of the HA activity was lost within the first 5 min, and more than 94% of the HA activity was lost within 15 min postincubation. Within 30 min, less than 1% of the initial virion population remained as HA-positive particles.

Electron microscopy of dissociated virions. Dissociating virions were examined with an electron microscope. The inset in Fig. 3A shows untreated, purified virions. Figure 3A depicts viral particles after a 5-min exposure to the dissociation mixture. The virions have lost much of the intact structure and appear as puddles of loosely associated virions with some individual capsomeres apparent. Figure 3B shows the dissociated virions after a 15-min exposure to the EGTA-DTT dissociation buffer. Intact virions were not detected, and the capsomeres appear to be individualized across the field.

Effect of exogenous Ca^{2+} or Mg^{2+} on the dissociation of polyoma virions. Experiments were performed to determine whether adding exogenous cations could prevent the action of EGTA in virion dissociation. Adding Mg^{2+} to the dissociation mixture had little effect in preventing the dissociation of virions, but adding Ca^{2+} to the dissociation buffer completely prevented virion dissociation (Table 1).

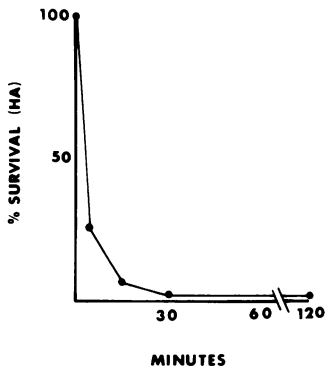

FIG. 2. *Kinetics of viral dissociation under optimal conditions. After adding virus to the dissociation mixture (10 mM EGTA, 3 mM DTT, and 0.15 M NaCl in 0.01 M Tris buffer, pH 8.5), portions were removed at indicated times and assayed by hemagglutination.*

If Mg^{2+} were the cation responsible for viral stability, then the addition of Mg^{2+} should have prevented the dissociation. The added Mg^{2+} would have competed with the virion-bound cation for the EGTA-binding sites. That Mg^{2+} did not prevent dissociation suggested that the virion-bound cation had a greater affinity for EGTA than for Mg^{2+}.

Since Ca^{2+} prevented dissociation, the Ca^{2+}

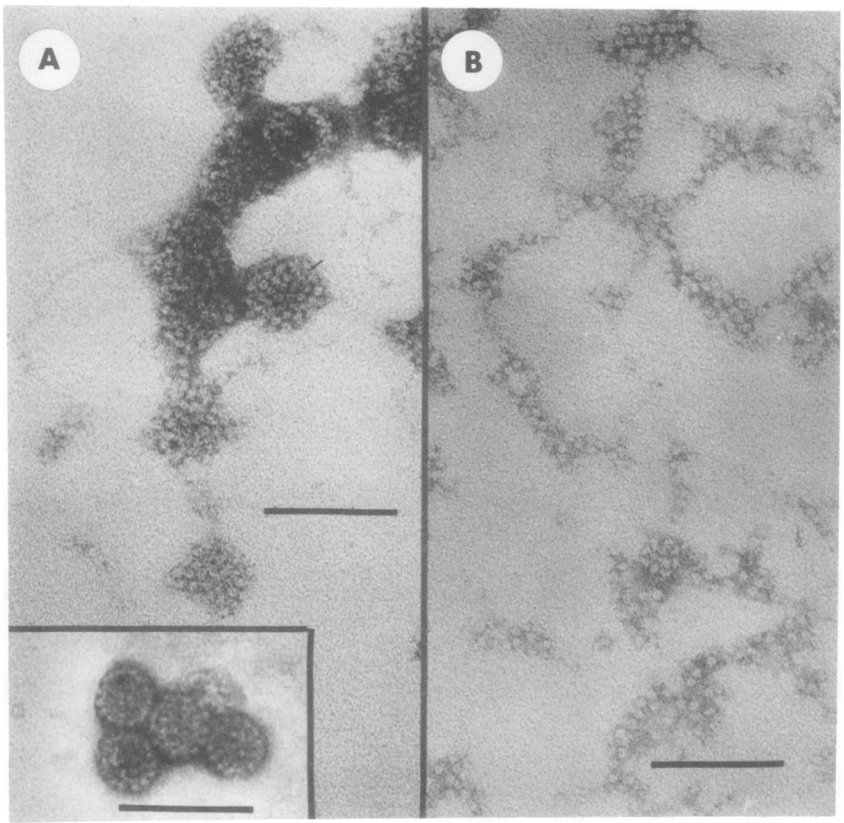

FIG. 3. *Dissociation of polyoma virions determined by electron microscopy. (A, inset) Untreated polyoma virions; (A) virions after 5-min exposure to dissociation buffer; (B) virions after 15-min exposure to dissociation buffer. Bars equal 100 nm.*

ion must compete on an equal basis for the EGTA sites available. Inhibition by exogenous Ca^{2+} can also be used to eliminate Mn^{2+}, Cd^{2+}, and Zn^{2+} as the cations responsible for viral stability, since these ions bind more strongly to EGTA than does Ca^{2+}. Thus, if these ions were involved, exogenous Ca^{2+} could not have prevented dissociation. Therefore, we conclude that Ca^{2+} plays an important role in virion stability.

Incorporation of ^{45}Ca into complete and empty virion particles. To substantiate that Ca^{2+} was, indeed, a part of the intact purified virion, experiments were conducted to label polyoma virions with the ^{45}Ca radionuclide. Since incorporating Ca^{2+} into cellular (and viral) proteins depends upon the availability of calcium-phosphate pools within the mitochondria (Peter Gray, personal communication), mouse kidney cells were grown to confluency in the presence of ^{45}Ca media and subsequently infected with polyoma. After purification, complete and empty polyoma virions were found to be ^{45}Ca associated (Fig. 4A). Radioactivity was found to be coincident with HA activity in both populations of virions. Figure 4B shows complete polyoma virions collected from a CsCl velocity gradient (Fig. 4A) and then recentrifuged in a second CsCl velocity gradient. These complete particles band as a single species that coincide with the HA activity. The small number of counts apparent at the top of the gradient most likely represent Ca^{2+} that has been stripped from the virus due to the high ionic strength of the CsCl gradient. Figure 4C shows the profile of complete virions (isolated as for Fig. 4B) that were exposed to the EGTA-DTT dissociation buffer and centrifuged in a second CsCl velocity gradient. The complete loss of any HA activity and the shift of the ^{45}Ca counts to the top of the gradient are evident. These experiments further substantiate the association of Ca^{2+} with polyoma virus and the importance of the cation in maintaining the

intact virion structure.

Requirement of EGTA and DTT for dissociation of polyoma virions. The dissociation of polyoma by EGTA and DTT was analyzed by CsCl centrifugation to determine the extent of virion dissociation. Figure 5 shows the results of velocity sedimentation of the virions in CsCl gradients before and after treatment with EGTA, DTT, or EGTA-DTT. Figure 5A illustrates the complete virion banding pattern. Figure 5B and C represent the virion profile after exposure of the virus to either DTT or EGTA, respectively. In both cases, little difference is noticed between the sedimentation pattern of untreated virions or those treated with EGTA or DTT alone. However, after a 30-min exposure to both EGTA and DTT at pH 8.5, a dramatic shift to a lighter density was observed (Fig. 5D). Thus, neither EGTA nor DTT alone is sufficient for the dissociation of the virion, but combined they demonstrate effective virion dissociation. Density determinations on cesium chloride equilibrium gradients demonstrated similar results. Untreated polyoma, polyoma-EGTA, and polyoma-DTT-treated virions banded at a density of 1.33 g/cm^3, and the polyoma-EGTA-DTT-dissociated virus banded at 1.245 g/cm^3.

TABLE 1. *Effect of exogenous Ca^{2+} or Mg^{2+} on the dissociation of polyoma virus*

Reaction mixture	HA titer	Reduction (%)
Dissociation buffer[a] (no additions)	100	97
Dissociation buffer plus 5 mM Ca^{2+}[b]	3,200	0
Dissociation buffer plus 10 mM Ca^{2+}	3,200	0
Dissociation buffer plus 20 mM Ca^{2+}	3,200	0
Dissociation buffer plus 10 mM Mg^{2+}[b]	200	94
0.01 M Tris (pH 8.5)	3,200	0

[a] 5 mM EGTA, 3 mM DTT, and 0.15 M NaCl in 0.01 M Tris buffer (pH 8.5).

[b] Exogenous Ca^{2+} or Mg^{2+} added to dissociation mixture 30 min before adding virus.

Determination of sedimentation value of virion dissociation product. Further characterization of the virion dissociation products was carried out by velocity sedimentation in sucrose gradients (Fig. 6). Three protein species were observed that migrated at 5S (peak A), 12S (peak B), and 18S (peak C) when compared to the 7S immunoglobulin marker. The S values for peaks B and C are slightly larger than those previously reported for capsomeres produced by the carbonate dissociation of polyoma virions (8). Polypeptide analysis of these dissociated virion subunits (Fig. 6) by sodium dodecyl sulfate-polyacrylamide gels and characterization of a DNA-protein complex (data not

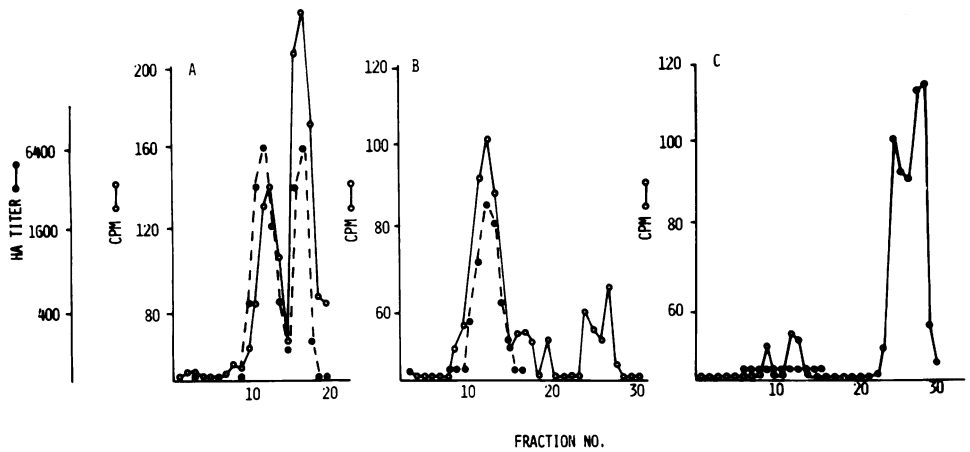

FIG. 4. *CsCl gradient analysis of ^{45}Ca-labeled complete and empty polyoma virions. Samples were layered onto shallow CsCl gradients (as described in the text) and centrifuged in an SW50.1 rotor at 35,000 rpm for 2.5 h. (A) Complete and empty polyoma virions; (B) complete virions (fractions 10 to 15) isolated from gradient A, dialyzed against 0.01 M Tris buffer (pH 7.4), and recentrifuged in a second shallow CsCl gradient; (C) complete polyoma isolated as in (B) and exposed to the dissociation buffer prior to centrifugation. Symbols: ○, ^{45}Ca; ●, HA titer.*

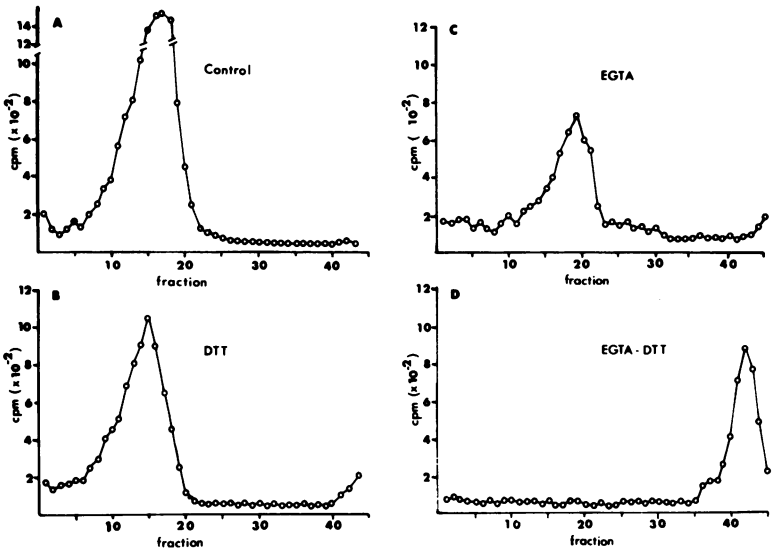

FIG. 5. *CsCl gradient analysis of polyoma virions after various chemical treatments. Samples were layered onto shallow CsCl gradients and centrifuged in an SW50.1 rotor at 35,000 rpm for 2.5 h. (A) Untreated polyoma virions; (B) polyoma virions after a 30-min exposure to DTT (3 mM); (C) polyoma virions after a 30-min exposure to EGTA (10 mM); (D) polyoma virus after a 30-min exposure to 10 mM EGTA-3 mM DTT. All incubations were conducted at pH 8.5 and contained 0.15 M NaCl. Symbol: ○, ^{14}C-amino acid-labeled virions.*

shown), which can be isolated by similar procedures, will be presented in a subsequent publication.

DISCUSSION

The data presented in this report demonstrate that the divalent cation Ca^{2+} is an integral part of the virus and plays a major role in stabilizing the intact polyoma virion structure. X-ray fluorometry, inhibition studies (Table 1), and incorporation of ^{45}Ca into complete and incomplete particles (Fig. 4) substantiate the presence of this cation in association with the intact virion. Chelation of this cation, along with the disruption of disulfide bonds, leads to the dissociation of the virion into capsomere subunits (Fig. 3) and a DNA-protein complex (unpublished data). Optimization of the virion dissociation demonstrates that the conditions necessary for the reaction are closer to physiological conditions than previously reported systems (7, 8, 17).

Previously, the most widely used system to dissociate polyoma virions involved incubating virions in a carbonate-bicarbonate buffer at pH 10.5 (7, 17). The hypothesis for the mechanism of this dissociation reaction was that the buffer merely provided the alkaline environment necessary for the disruption mechanism to proceed. Our observations that the EGTA-DTT-mediated dissociation proceeded even at near-neutral pH led us to question the pH role as an absolute requirement. Additionally, we showed that alkaline pH alone (Fig. 1D) was not sufficient in noncarbonate buffer systems to cause virion disruption. If high pH alone was not sufficient to cause dissociation, then some other property of the carbonate buffer system must be responsible. We propose that the mechanism of disruption in alkaline carbonate buffers is similar to the one responsible for disruption by the EGTA-DTT system. Both EGTA and the carbonate ion have the ability to bind Ca^{2+}. The carbonate system requires a high pH because the formation of insoluble calcium carbonate is favored under alkaline conditions. Recently, it was demonstrated that polyoma virions could be dissociated by treatment with low concentrations of sodium dodecyl sulfate (22). It would be interesting to determine whether sodium dodecyl sulfate also has the ability to chelate Ca^{2+}.

The presence of Ca^{2+} on other papovaviruses has not been determined. However, available data indicate that Ca^{2+} or some other cation might be associated with these viruses. In vitro dissociation of simian virus 40 has been accomplished by use of the alkaline carbonate system (1) and, more recently, by incubation in a Tris-glycine buffer (3). It is interesting that, after removal of a hydrogen ion from the α-amino

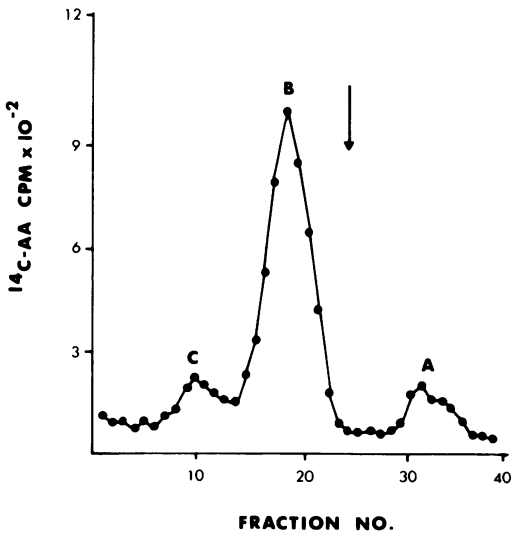

FIG. 6. *Velocity sedimentation of EGTA-DTT-dissociated polyoma virus. ^{14}C-amino acid-labeled virions were exposed to 10 mM EGTA, 3 mM DTT, and 0.15 M NaCl in 0.01 M Tris buffer (pH 8.5) for 20 min. Sample was layered onto a 5 to 20% sucrose gradient and centrifuged in an SW50.1 rotor at 40,000 rpm for 10 h (4°C). Arrow indicates position of 7S immunoglobulin marker. Symbol: ●, ^{14}C-labeled amino acid virions.*

group of glycine at pH 9.6, this amino acid is capable of binding Ca^{2+} through ionic interactions between the amino and carboxyl groups (16). The human papovaviruses that have been recently studied are also readily dissociated by the alkaline carbonate system (6). Thus, the mechanism we propose for dissociation of polyoma by EGTA-DTT might be a common property to all papovaviruses.

The EGTA-DTT virion dissociation system described here provides opportunities for further investigations. For the first time, it is possible to dissociate the virion without resorting to extremely alkaline conditions. These conditions have enabled us to isolate and characterize a nucleoprotein complex from intact virions and to compare these complexes with those obtained previously with the carbonate disruption system (14, manuscript in preparation).

Our observations of the importance of Ca^{2+} in maintaining viral structure represent an important step toward understanding the events that occur during viral uncoating in vivo. After adsorption, the polyoma virion is transported to the nuclear membrane in a monopinocytotic vesicle. Subsequently, the virion DNA and capsid proteins are visualized in the nucleus by autoradiography. However, electron microscopy revealed that the parental virus products which entered the nucleus were not recognizable as intact virions (12). In light of our present findings, we propose that the nuclear membrane may have the ability to remove Ca^{2+} from the parental virion, possibly through the action of a calcium-binding protein(s). If nuclear microenvironments exist that provide the necessary biological reducing conditions (e.g., glutathione, cysteine), the criteria for uncoating would be fulfilled. Under these conditions, the outer protein coat of the virus could be dissociated, allowing the inner DNA-protein core to be released in the nucleus to initiate the replication cycle. We are presently investigating this hypothesis.

Finally, since the presence of Ca^{2+} in mature polyoma virions has been demonstrated, we have to consider the involvement of cations in maintaining the virion integrity, uncoating, and assembly in vivo as well as in vitro. In addition, the potential involvement of cations with other viruses should be considered.

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ADDENDUM IN PROOF

Since this paper was submitted, a publication by Durham et al. (Virology 77:524–533, 1977) appeared that describes the association of Ca^{2+} with various plant viruses and the role of Ca^{2+} binding in virus disassembly.

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