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Adenovirus Type 5 virions asymmetrically lose icosahedral structure at low pHs of endosomes

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Adenovirus Type 5 Virions Asymmetrically Lose Icosahedral Structure at Low pHs of Endosomes.

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ABSTRACT

After receptor mediated endocytosis into an endosome, human Adenovirus Type 5 (Ad5) undergoes structural changes that allow the virus to release a viral protein, pVI, that lyses the endosome and releases the virus core into the cytosol, where it travels to the nucleus to insert its genome. Structural studies of Ad5 at a range of pHs typical of endosomes (7.5-4.5) using a transmission electron microscope show that Ad5 undergoes asymmetrical loss of proteins from vertex regions at the pH of the late endosome (pH5.15). Following further acidification, Ad5 ejects core material preferentially through one vertex—indicating an asymmetry at one vertex of the capsid. Further studies done using SDS-PAGE show that as pH is decreased, the virus capsid releases pVI and pVII, and later pV from the virus capsid. This correlates with an increase in infectivity of pH6.2 virus and a decrease in infectivity of the virus at pH 5.2 and 4.6; indicating that at those two pH values, essential proteins were lost from the capsid. We propose a revision of the process of Ad5 disassembly as the virus enters the cell via acidifying endosomes.

INTRODUCTION

Adenovirus is a nonenveloped[1] virus composed of an icosahedral shaped protein coat (capsid)[1,2] and a double stranded DNA (dsDNA) genome[1]. The genus *Mastadenovirus* contains 7 species of adenovirus and 57 unique serotypes with the ability to infect humans[3]. These serotypes cause a wide range of infections—including respiratory tract infections, gastroenteritis, conjunctivitis, and, in rare cases, cardiovascular or neurological infection[3]. Adenoviruses range in size from 80-100nm[3]; because they are relatively large viruses, they are ideal candidates for structural study.

Adenovirus type 5 (Ad5) is a strain of adenovirus that causes upper respiratory tract infections[4] and is the best studied strain. It is approximately 90nm in diameter[4]. The virus has a T=25 icosahedrally symmetric protein coat[5,6] with 20 triangular faces that have 3 fold rotational symmetry and 12 vertices

that have 5 fold rotational symmetry[6]. Each triangular face is comprised of 12 hexon trimers (for a total of 720 hexon monomers per virus)[6,7] (Figure 1A). At each of the vertices is a penton base protein, from which protrudes a homotrimeric fiber protein[5,9]. Surrounding and associated with the penton at each of the five-fold axes of symmetry is a peripentonal hexon trimers[5,6]. Because these peripentonal hexons are found surrounding each five-fold axes, each face of the virus contains 3 peripentonal hexons (one at each corner), in addition to the 9 other hexon proteins. All of these proteins together make up the virus capsid—a "hard shell" responsible for protecting the core of the virus. The core of the virus contains a linear, dsDNA genome[1] of 35935 base pairs[10], as well as minor capsid proteins (IIIa, V, VI, VII, VIII, IX, a viral protease, and several other proteins)[11]. Each of these minor proteins has a role in the Ad5 life cycle, some of which have yet to be determined. Protein VI is the most extensively studied of these minor capsid proteins and has a major role in viral entry[12].

Infection of a cell by adenovirus begins when the fiber protein of a mature adenovirus particle binds its cell surface receptor—the coxsackie virus-adenovirus receptor (CAR)[13,14,15]; after binding, the penton base of the adenovirus interacts with integrins[16] to instigate formation of a clathrin coated pit and subsequent internalization of the virus particle[17]. The clathrin coated pit then forms a transport vesicle (endosome) that carries the adenovirus deeper within the cell, en route to the nucleus[18]. The internalization of the virus begins the process of adenovirus disassembly to allow it to release its genome from the capsid and complete its infection of the host cell[19,20]. Over time, protons are pumped into the endosome; and the pH decreases from the neutral pH of ~7.5 (found in the extracellular environment)[21,22]. Because the virus capsid is metastable (sturdy for protection of the core when outside the cell, but able to undergo conformational changes that expose proteins when the capsid chemical environment changes[23,24,25])as the endosome moves further into the cell and the pH continues to decrease, conformational changes occur in the virus capsid that enable the release of certain proteins into the endosome[12,23,26,27,28]. Chief among these proteins released from the capsid is protein VI, which

has been shown to possess membrane lytic capabilities[12]. When the capsid disassembles and releases protein VI, the protein VI inserts itself into the endosomal membrane and lyses it[12,28,29], thereby releasing the adenovirus capsid into the cytosol of the cell. At this point, adenovirus attaches to a microtubule and is carried to the cell nucleus, where it attaches to a nuclear pore complex and inserts its dsDNA genome[18,30] into the host cell nucleus. The host cell enzymes transcribe this viral DNA to make viral RNA, which is exported into the cytosol and translated to make viral proteins by host ribosomes and using host amino acids[18,19,23]. These new viral proteins are assembled into new progeny adenovirus particles; these particles mature when the viral protease cleaves protein precursors into viral proteins[32]. Mature adenovirus particles are released from the host cell by puncturing the cell membrane[31]. These steps, from initial attachment at the cell surface to final release from the host cell, make up the infectious cycle of the adenovirus[18] (the steps from attachment to genome injection into the nucleus are pictured in figure 1B).

The existing model of adenovirus disassembly holds that the proteins are released from the capsid when all the pentons and peripentonal hexons are released from the virus, resulting in a “swiss-cheese” virus with no proteins at the vertices (Figure 1C)[33,34]. Several experiments have indicated that this penton release occurs within the endosome. WC Russell et. al. found that when the virus was heated for 10min at 50-56˚C , the virus ruptured at all of its 12 vertices—losing its pentons and peripentonal hexons and exposing the core of the virus (Figure 2)[33,35]. Wiethoff et. al. found that when the virus was treated at low pH and at biological (37˚C) to high (60˚C) temperatures, there was a dramatic increase in accessibility of the viral core to fluorescence measurement—indicating the removal of large numbers of capsid proteins to expose the core to solution. They also observed marked decrease (from ~80% remaining in capsid to ~10% remaining in capsid) in the amount of penton, fiber, and protein VI remaining in the capsid at temperatures from 40 ˚C to 44 ˚C and pH7.4. Lower temperatures were required for protein dissociation when the pH was lower (pH 5)—thus indicating that at high

temperatures and low pH, adenovirus releases fiber proteins, protein VI, and most of its penton proteins[12]. Both of these experiments helped to shed light on the events of adenovirus disassembly in the cell and helped to develop the "swiss cheese" virus structure that has come to be well accepted in the field.

We wanted to determine exactly what structural changes occur in the adenovirus that enable it to release protein VI and lyse the endosome; to do so, it was necessary to replicate as closely as possible the *in vivo* endosomal conditions that the adenovirus is likely to experience as it travels from the cell surface until it lyses the endosome. To do this, we needed to study the virus at biological temperatures (37 ˚C) over a range of appropriate pH values (from extracellular pH 7.5 to lysosomal pH 4.5) at biological ion concentrations[37]. Each pH treated virus sample would then be viewed using a transmission electron microscope to examine how the surface morphology of the virus changes in different chemical environments; further, it is necessary to quantify what effect the pH change has on the virus—what proteins are being removed from the capsid and how does that change affect the virus function. Our research centered on recreating a range of endosomal environments so we could pinpoint exactly what changes were occurring in the virus capsid.

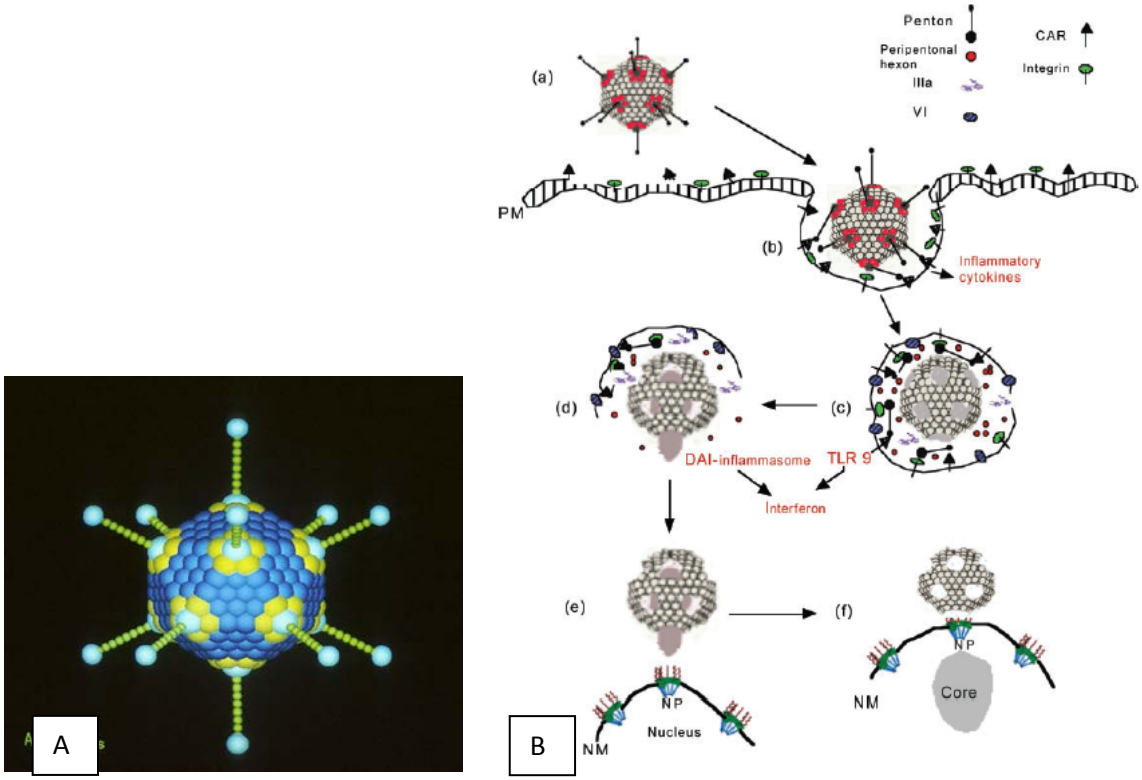

Figure 1. A- Adenovirus capsid structure. Blue proteins are hexons, yellow are peripentonal hexons, light blue are pentons. B- Infectious entry pathway of Adenovirus[36].

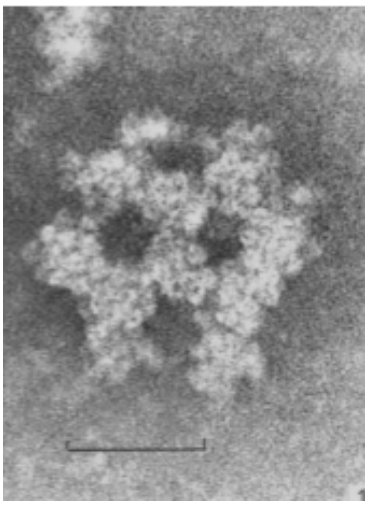

Figure 2. Micrograph of Ad5 after being heated to 50-56˚C for 10min. All vertex regions have been lost from the virus[33].

METHODS

Cell culture conditions.

Adenovirus transformed HEK 293 cells (CRC 1573, ATCC, Rockville, MD) were grown up and maintained in Dulbecco's complete modified Eagle's medium (DMEM) (Gibco); the 500 mL of medium was supplemented with 15 mL fetal bovine serum (Atlanta Biochemicals), 5mL of 120X Glutamax, and 5 mL of 100X Antibacterial/antimycotic solution. Cells were maintained in a 5% CO_2 incubator kept at 37˚C. All steps of the procedure were performed in a laminar flow fume hood using sterile technique.

Adenovirus propagation and purification.

Cells were grown to 80% confluence in each of 4- T225 cell culture flasks; to each of these flasks was added 25µl of purified recombinant Adenovirus type 5 (Ad-CMV-eGFP, containing the gene for enhanced Green Fluorescent Protein behind a strong cytomegalovirus promotor in place of the Adenovirus E1 gene). Cells were incubated and observed for appearance of cytopathic effect (cells becoming spherical in shape and detaching from the flask surface, as opposed to being irregular and flat in shape as part of the monolayer). Once cytopathic effect was nearly complete (after ~48 hours), cells were scraped from the flask surface and the cell-containing medium from each flask was pooled together and centrifuged in an Eppendorf desktop microcentrifuge at 500 rcf for 10 minutes to pellet the cells. Medium was removed and the pellet was resuspended in 12 mL total of phosphate buffered saline (PBS). Cell suspension was frozen in liquid nitrogen and subsequently thawed in a 37˚C water bath; this freeze-thaw process was repeated three times to lyse cell membranes and release virus into solution. The solution was centrifuged at 1000 rcf for 10 minutes to pellet cell debris; virus-containing supernatant was removed and virus was purified using density gradient centrifugation.

To purify virus from the viral supernatant, Optiprep Iodixanol gradients were set up in two UltraClear ultracentrifuge tubes. Each tube contained 1 mL 54% iodixanol, 4.5 mL 40% iodixanol, 4.5 mL 25%

iodixanol, and 1.5 mL 15% iodixanol. 6 mL of viral supernatant was added to the top of each density gradient. Each tube was placed in a Beckman ultracentrifuge hanging bucket, which was placed on a Beckman ultracentrifuge swinging bucket rotor and centrifuged at 27,000 rpm (~100,000xg) for 18 hours in a Beckman ultracentrifuge. Ad5 sedimented to form an opalescent band at the 25% and 40% iodixanol interface; the purified virus was removed using a syringe and needle.

pH Treatment

A range of biological endosomal pHs were replicated by dialyzing the purified virus in buffer of designated pH values. Purified Ad5 was injected into a 30K dialysis cassette (Slide-alyzer, Piecres), which was then placed in an appropriate pH buffer (20mM sodium citrate/citric acid buffer, 10% glycerol, 100mM NaCl, ultrapure H_2O) (pH values ranged from 6.9 to 4.5). If virus was to be kept at a neutral pH 7.5, it was dialyzed in tris-buffer (20mM tris, 10% glycerol, 100mM NaCl, ultrapure water). Initial dialysis was performed at 4˚C with 1L buffer being stirred for 6 hours; buffer was then replaced with 1L new buffer and stirred at 4˚C for 6 more hours. pH was confirmed both before and after dialysis using a pH meter. After dialysis, virus was removed from the cassette with a syringe and either analyzed immediately or flash frozen in liquid nitrogen and stored in a -80˚C freezer for later use.

Transmission Electron Microscopy

Purified virus samples were incubated at 37˚C for 30 minutes. In the meantime, Formvar grids were primed by irradiating them under UV light for 10 minutes. A 12 µl droplet of heated virus was placed on the dull surface of the grid and allowed to adhere for 30 min, after which time another 12 µl droplet of heated virus was placed on the grid and allowed to adhere for 30 min. The droplet was then wicked away on a piece of filter paper. A 10 µl droplet of 4% sodium silicotungstate stain was then placed on the grid for 1 minute 5 seconds and immediately wicked off the grid. The grid was then allowed to dry for 30 minutes in the laminar flow hood. Grids were then placed in a storage box for transport.

Grids were visualized using a JEOL 1010 transmission electron microscope (TEM). Micrographs were taken using AVT photography software.

Spin Concentration and SDS-PAGE

A sample of purified, dialyzed, pH7.5 Ad5 was placed in a Millipore 10K spin molecular weight cutoff (MWCO) concentrator and centrifuged in a desktop microcentrifuge at 4000 rcf for 20 min. A 200 µl sample was then collected for use in future infections and for SDS-PAGE. The remaining virus was divided into 3 equivalent volumes. One volume was then placed in a 50K spin concentrator and to the concentrator was added 4 mL of 37˚C pH 6.2 buffer; the sample was centrifuged at 4000 rcf for 20 minutes at 37˚C. A sample of the pH 6.2 flow through was collected and concentrated in a 10K concentrator. This concentration step was repeated with the addition of 4 mL more of 37˚C pH 6.2 buffer; no sample of flow through was collected after this concentration. The concentrated virus, now pH 6.2, was collected for SDS-PAGE analysis. This process was repeated for the two remaining of the original viral volumes at pH 5.2 and at pH 4.6. All steps were performed at 37˚C to replicate biological conditions. After this process, the following samples were prepared for SDS-PAGE: pH 7.5 virus (10K spin concentrated), pH 6.2 virus (50K spin concentrated), pH 6.2 viral flow through (10K spin concentrated), pH 5.2 virus (50K spin concentrated), pH 5.2 viral flow through (10K spin concentrated), pH 4.6 virus (50K spin concentrated), pH 4.6 viral flow through (10K spin concentrated).

Samples placed in 1X Laemli sample buffer and were then denatured by heating to 95˚C for 5 minutes. Samples were then loaded onto a BioRad Mini-Protean 4-15% gradient precast gel and run at 175V and 50mA for 45 minutes. The gel was then stained using a BioRad SilverStain Plus silver staining kit according to the manufacturer instructions. The gel was photographed using a Gel Logic 200 imaging system.

Flow Cytometry

12-well plates were seeded overnight with 300,000 cells/well of HEK293 cells. Medium was replaced with 1mL/well of virus dialyzed at pH 7.5, 6.5, 5.2, and 4.6 at a dilution of either 10^{-3} or 10^{-4} – all in triplicate, one well was left uninfected; plates were then incubated overnight. Medium was removed and wells were washed with .5mL of PBS. Cells were detached using .5mL of trypsin per well for 15 seconds. Cells were resuspended in 1 mL PBS and placed in a 5 mL polystyrene round bottom tube.

Samples were analyzed in an FACS flow cytometer using CellQuestPro imaging software. The instrument was calibrated using the uninfected cells. 4000 cells in the designated range were scanned for each sample and analyzed by the machine for fluorescence. Cells registering a fluorescence under 100 FL1-H were designated “uninfected” or M1; cells registering a fluorescence above 100 FL1-H were designated “infected” or M2. From this data, a chart could be made that compared the infectivity of different viral dilutions and pH values.

RESULTS

As pH decreases, Ad5 exhibits asymmetrical loss of vertex regions from the capsid followed by core ejection. To determine exactly what structural changes occur in Ad5 as pH was decreased from extracellular pH of 7.5 to lysosomal pH 4.5, samples of virus at several different pHs in that range with intracellular ion concentrations and at biological temperature were viewed using a transmission electron microscope (TEM). At extracellular pH 7.5 (Figure 3A and 3B), no distinct morphological changes were seen in the virus capsid; the shape was icosahedral, and all hexon trimers and pentons appeared to be appropriately located on the virus capsid. As pH was decreased to pH6.2 (Figure 4A and 4B), that of the early endosome, there appeared to be no significant deviations in structure from that of the pH7.5 virus; the capsid was still complete—its shape remained icosahedral and all pentons and hexon trimers appeared appropriately located. As the pH was further decreased to pH 5.15 (Figure 5A and 5B), that of

a late endosome, spots began to appear on the virus capsids. These spots were likely areas on the virus capsid where stain seeped in and formed a dark spot or pool of stain in the virus. These spots corresponded with vertex regions of the virus (Figure 5C). Spots were seen on many (but not all) capsids in the field of view at this pH. Most capsids only had one spot, if they had any at all. As pH decreased to 4.5 (Figure 6), that of the lysosome (a pH which Ad5 likely does not reach), there appeared to be an ejection of core material from the capsid. This core material formed a trail that appeared localized from one area on the virus; this area corresponded to a vertex region. Most capsids at this pH exhibited an ejection of core material from no more than one vertex region.

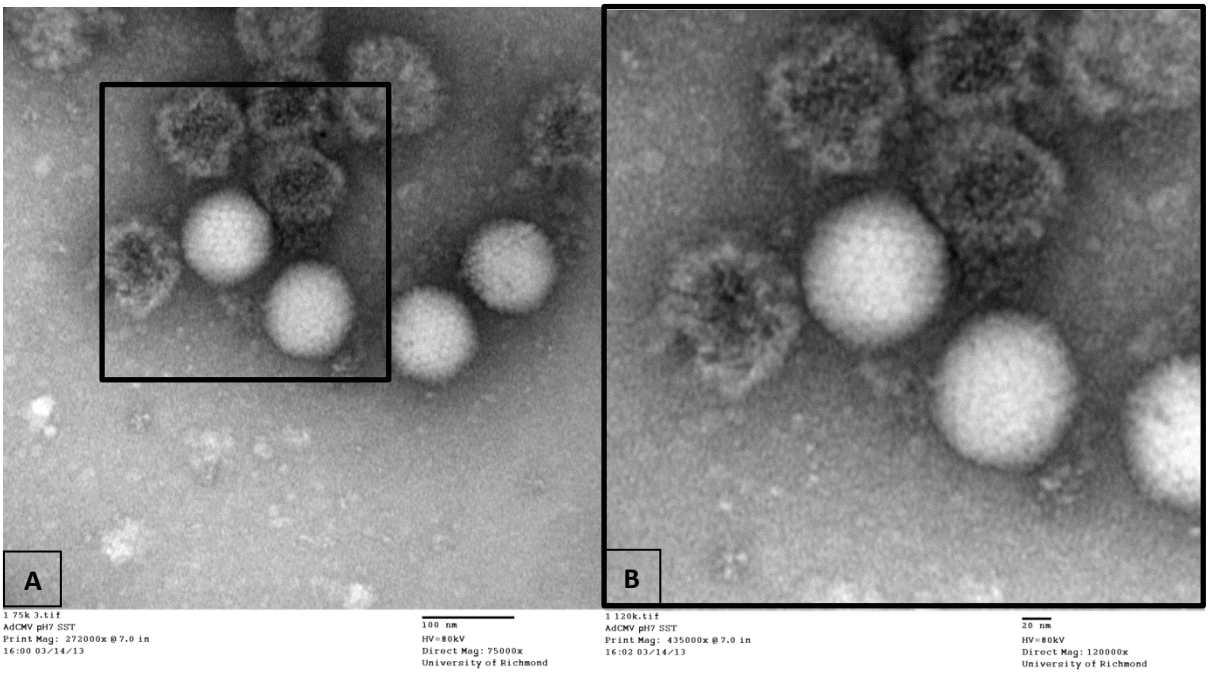

Figure 3. Transmission electron micrographs of Ad5 dialyzed at pH 7.5 and heated for 30 min at 37˚C; stained with sodium silicotungstate for 1 minute 5 seconds. A- 75000X magnification; B- 120000X magnification.

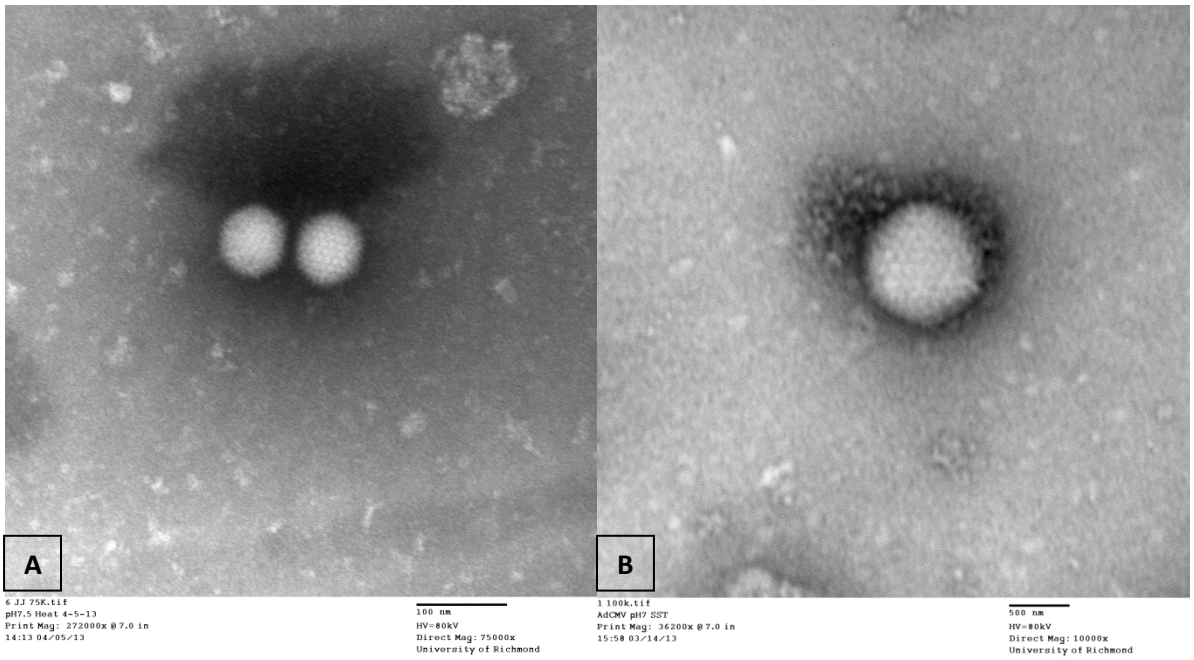

Figure 4. Transmission electron micrographs of Ad5 dialyzed at pH 6.5 and heated for 30 min at 37˚C; stained with sodium silicotungstate for 1 minute 5 seconds. A- 75000X magnification; B- 100000X magnification.

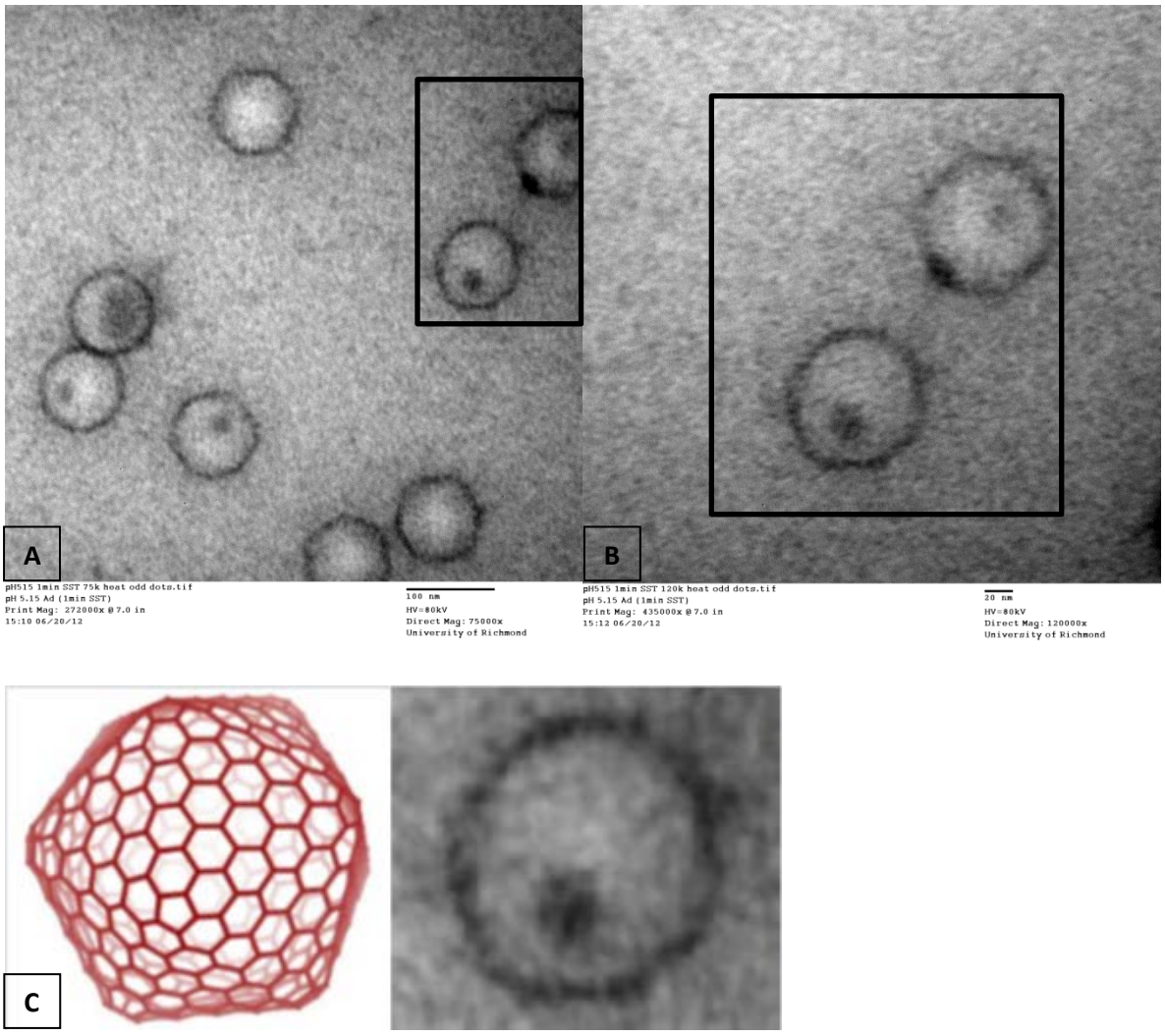

Figure 5. Transmission electron micrographs of Ad5 dialyzed at pH 5.15 and heated for 30 min at 37˚C; stained with sodium silicotungstate for 1 minute 5 seconds. A- 75000X magnification; B- 120000X magnification; C- comparison of 120000X micrograph (right) to an icosahedron generated using the Johnson Lab Icosahedron generator (left) and rotated to align with the viral triangular face in the micrograph.

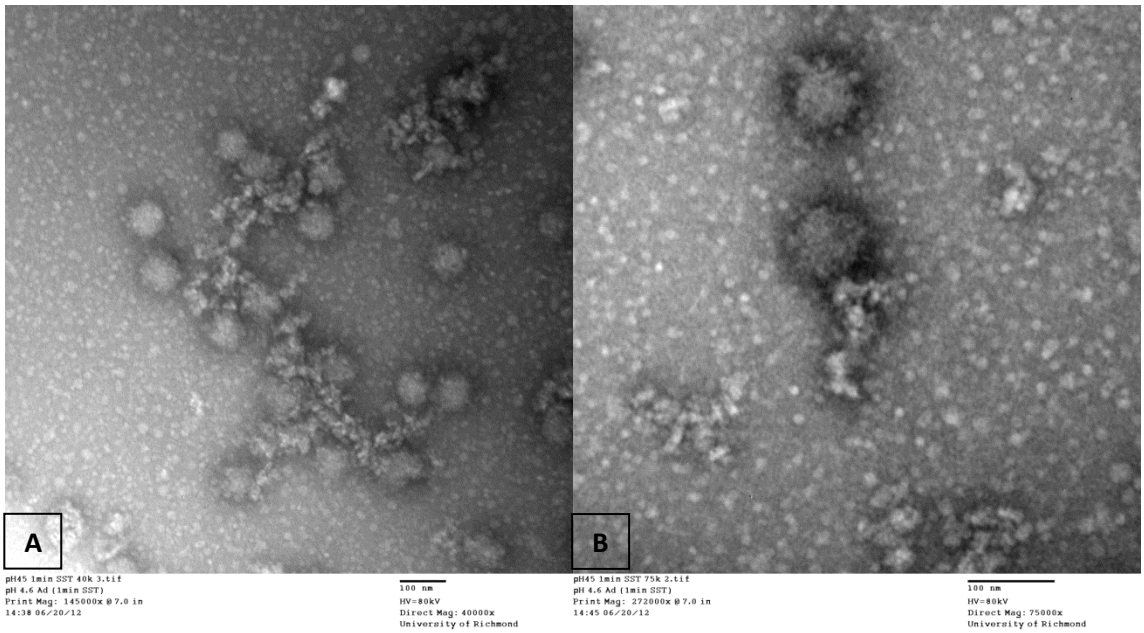

Figure 6. Transmission electron micrographs of Ad5 dialyzed at pH 4.5 and heated for 30 min at 37˚C; stained with sodium silicotungstate for 1 minute 5 seconds. A- 40000X magnification; B- 75000X magnification.

As pH is decreased, proteins V, VI, VII dissociate from the virus capsid. Virus was pH treated and spin concentrated in a spin concentrator that would allow small (<50kD) viral proteins to pass through; samples of both concentrated virus and concentrated flow through were denatured and separated by SDS-PAGE (Figure 7) to determine which proteins (that were small enough to pass through the concentrator) had dissociated from the virus capsid and were passed into solution. Virus at pH 7. 5 was centrifuged in 10K spin concentrator; 7 proteins (II, III, IIIa, IV, V, VI, and VII) were easily identifiable on the gel; this lane contained the bands that made up the whole virus. Virus at pH 6.2 was centrifuged in 50K spin concentrator. Few differences existed between this lane and that of virus at pH 7.5, indicating that few proteins have come off the virus. Viral flow through at pH 6.2 contained 4 bands—two of which can be found at approx. 21 kD and correspond with the molecular weights of proteins VI and VII; another band was visible at approx. 37 kD and another at approx. 50kD, the identities of these bands did not correspond with any known molecular weights (Table 1). Virus at pH 5.2 was centrifuged in a 50K concentrator and exhibited fewer bands than virus at both pH 7.5 and 6.2; this indicates that several

proteins likely came off the virus capsid. Viral flow through at pH 5.2 contained several more bands than did the pH 6.2 flow through. This flow through contained 8 bands, 4 of which (II, V, VI, and VII) corresponded to known viral proteins; the other 4 bands do not correspond to known viral proteins. Surprisingly, the flow through contained hexon (which should not have gone through the spin concentration step, given that a 108kD and a 50kD concentrator was used). Virus at pH4.6 was concentrated in a 50kD concentrator and had far fainter bands than the viruses at any of the other pHs, though most bands are still present in the lane. Flow through at pH4.6 showed only a faint band of what appears to be protein VI.

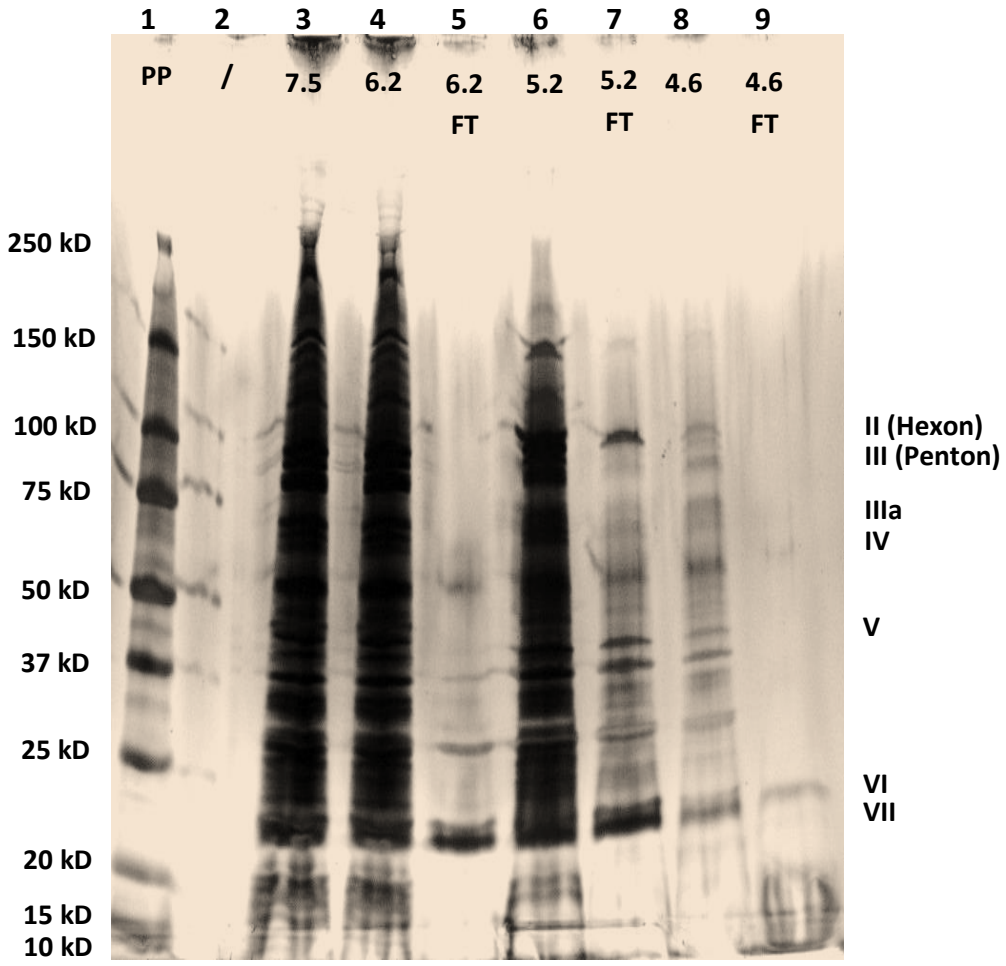

Figure 7. Silver stained 4-15% SDS-PAGE gel. Lanes from left to right are: Precision Plus protein standards, empty, pH7.5 virus spun in 10K concentrator, pH 6.2 virus spun in 50K concentrator, pH 6.2 flow through spun in 10K concentrator, pH 5.2 virus spun in 50K concentrator, pH 5.2 flow through spun in a 10K concentrator, pH 4.5 virus spun in a 50K concentrator, pH 4.5 flow through spun in a 10K concentrator. Samples were heated in Laemli sample buffer at 95˚C for 5 minutes. Gel was run at 175V and 50 mA for 45 minutes. Protein assignments were made using protein molecular weights as determined using the NCBI protein database GenBank (Table 1).

Table 1| Major and Minor Proteins of Ad5

Protein	Molecular Mass (kDa)	No. of Copies	Location	Function
II	108007	720	Hexon (trimer)	Formation of capsid shell
III	63293	60	Penton base (pentamer)	Formation of capsid shell, entry
IV	61585	36	Fiber (trimer)	Attachment to host cell
IIIa	65253	74 +/- 1	Inner capsid surface below penton base	Stabilization of capsid, entry
VI	26996	342 +/- 4	Hexon -associated, inner capsid surface	Stabilization of capsid, entry
VIII	24687	211 +/-2	Hexon-associated, inner capsid surface	Stabilization of capsid
IX	14458	247 +/-2	Outer surface of groups of nine hexons, edges of icosahedral faces	Stabilization of capsid
V	41447	157 +/-1	Core	Packaging of DNA genome
VII	21992	835 +/-20	Core, bound to DNA	Packaging of DNA genome

Infectivity decreases for virus treated at pH of late endosome and lysosome and increases for virus treated at pH of early endosome. To determine how infective virus particles were at various stages of pH-mediated dissociation, HEK 293 cells were treated with different dilutions and pHs of virus; cells were collected and mean fluorescence was measured using flow cytometry (Figure 8). Because Ad5-CMV-eGFP contains the eGFP gene, cells penetrated by the virus will express the GFP protein and fluoresce strongly. Cells with a fluorescence value of greater than 100 FL1-H were determined to be infected, cells with a fluorescence value of less than 100 FL1-H were determined to be uninfected. Mean fluorescence measured the average fluorescence of all the cells sampled—the higher the mean fluorescence, the more infected the cell sample (the greater number of cells infected or superinfected with Ad5). Compared to virus treated at pH7.5, virus treated at pH6.2 exhibited a steep increase in mean fluorescence (and thus, infectivity) at both viral dilutions. Virus treated at pH5.2 exhibited a slight decrease in mean fluorescence from pH7.5 at both dilutions. Virus treated at pH4.5 was rendered practically uninfective—it infected far fewer cells than any other pH treated virus sample. The trends for mean fluorescence were similar for both dilutions—pH 6.2 virus increased mean fluorescence

significantly from pH 7.5; pH5.2 virus decreased mean fluorescence slightly from pH7.5; and pH4.5 virus almost eliminated viral infectivity.

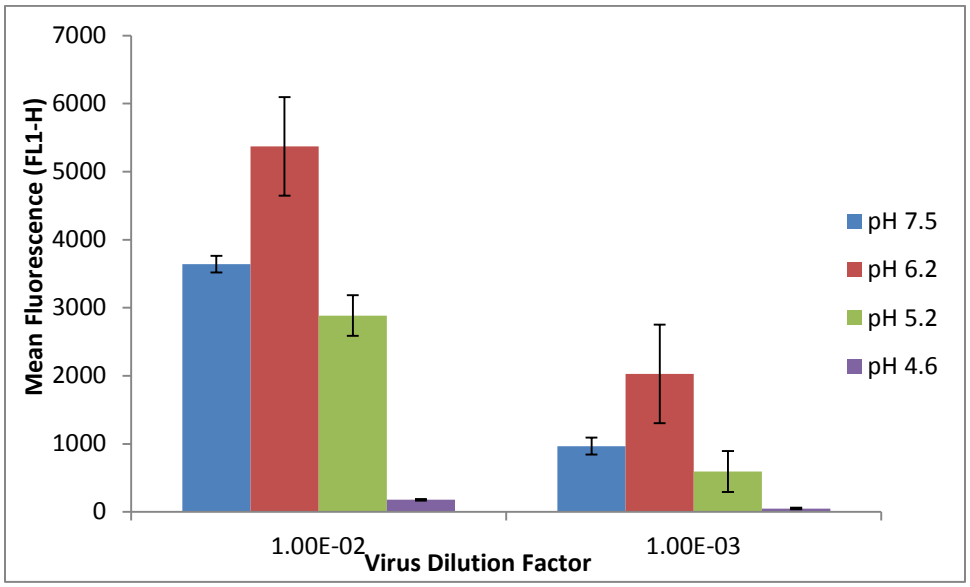

Figure 8. Flow cytometry of HEK293 cells infected with endosomal pH pretreated Ad5. Cells were incubated overnight with purified Ad5 (at either a 10^{-3} or 10^{-4} dilution) that had been dialyzed in sodium citrate/citric acid buffers of pH 6.2, 5.2, or 4.6 or a pH7.5 Tris buffer. Each treatment was done in triplicate. Cells were detached with trypsin and resuspended in PBS. Fluorescence was read with a FACS flow cytometer and analyzed with CellQuestPro software; mean fluorescence of the detached cell sample was calculated. Infected cells fluoresce more than uninfected cells. Error bars represent the standard deviation of 3 trials per dilution and per pH. The machine was calibrated using a sample of uninfected HEK293 cells.

DISCUSSION

Our studies shed some light onto the process of Ad5 disassembly as it would occur within the cell. Prior studies have indicated that partial disassembly is necessary for the virus to infect the cell[12] and that this disassembly likely involves a loss of vertex regions from the capsid[12,33,34]. It was unknown exactly what structural changes occur in the capsid under biological conditions that allow this dissociation and subsequent infection to take place.

Our studies indicated that Ad5 undergoes disassembly at the pH of late endosomes <pH5.2; this was evident by the appearance of spots on the TEM images of the virus at pH values in this region (Figure 5).

These spots were areas in which stain seeped into the virus capsid due to a missing vertex region. Further studies are needed to determine just what proteins of the vertex were removed at late endosomal pH (pentons, peripentonal hexons, etc.). It has previously been thought that Ad5 undergoes vertex dissociation at pH <5.5 and 37˚C[12]. Our results confirm that finding and also indicate something surprising—that the disassembly that occurs at the late endosomal pH values is asymmetrical; capsids appear to lose only one vertex region as they disassemble. Most viruses that were visualized at late endosomal pH showed "spots" where stain had penetrated a region where the vertex was missing. This contrasts with previous assumptions that the virus loses all of its vertex regions as it disassembles[33]. There could be many possible reasons for this asymmetrical disassembly—it is possible that there is some inherent asymmetry in the virus structure that has not before been characterized. Most structural determinations of Ad5 protein placement involve X-Ray crystallography or cryoelectron microscopy; these methods can average out asymmetries in the virus structure and make it so the capsid and its underside appear uniform in protein arrangement when this is not necessarily the case. Ahi et al have seen a similar asymmetry in Ad5 assembly; they hypothesize that IVa2, 33K and DBP localized at a single vertex; it is possible that this vertex has the capability to come off the virus capsid before the others, due to its being the last vertex added to the capsid during the assembly process and that there is something unique about this vertex that enables it to be used for genome packaging[38]. This finding of asymmetry is very interesting because it shows a similarity between adenoviruses and herpesviruses, which also have unique vertex used for both disassembly and packaging[39].

Also interestingly, as pH of the virus is decreased to lysosomal levels of approx. pH 4.5, we observed ejection of core material from one area of the virus (Figure 6). This area corresponded to a vertex region and we hypothesize that this vertex is likely the area through which capsid contents and core material exit the capsid (during disassembly or genome ejection). Further, it seems likely that this vertex is the vertex that disassembled from the capsid first (the spot in Figure 5). It is possible that decreasing the pH

to this level that the virus would likely never experience in vivo has accelerated the disassembly process and destabilized the virus to the point that it has released its core, as it would usually do after docking at the nuclear pore complex[19]. This indicates to us a progression of capsid disassembly that goes as follows: virus enters the cell via an endosome; the endosome acidifies to pH 5.2 (late endosome) and the capsid loses a vertex, through that hole is released pVI, which lyses the endosome to release the virus into the cytosol where it travels to the nucleus to eject its genome through the same hole that formed first in the virus in the endosome.

Studies of the disassembly using SDS-PAGE show us what proteins come off the virus at various pHs at biological temperatures. In the early endosome at pH6.2, few proteins come off the virus capsid and into the flow through; we see pVI and pVII and two unidentifiable proteins. Finding pVI coming off the capsid so early was surprising—this could be due to some disruption at the hexons (underneath each is hypothesized to be some pVI[40,41]) that liberates pVI into the flow through. We see this band of pVI in every lane of the gel and in every flow through; this indicates to us that something is occurring in the virus capsid that enables pVI to be released at every stage in virus entry via and endosome. This is important because pVI is responsible for lysing the endosome[12] and this shows that endosome lysis is theoretically possible at any stage in the virus entry process—even right at the very beginning, which we would not have expected seeing as how our micrographs of pH 6.2 virus indicate no capsid disruption (Figure 4), yet somehow, pVI is being released. pVII is a core protein bound to viral DNA (Table 2), finding it in the pH 6.2 flow through was surprising because it indicates exposure of the core at early endsomal pH; this is not what we would have predicted seeing as how no obvious changes have occurred to the capsid structure at pH 6.2 (Figure 4). At pH 5.2, we see far more proteins coming off the capsid. We see hexon, indicating that some of it may have slipped through the concentrator membrane, but which indicates that there has been some major shift in capsid structure enough to release some of the major capsid protein from the capsid. At this pH, we also see the elution of pV, which is not present

in the pH6.2 flow through. pV is a core protein, indicating exposure of the core proteins to solution only when the virus is at late endosomal pH—this hints that at pH5.2, major capsid disassembly has occurred, enough to release a core protein from the capsid. At pH 4.5, the virus bands are much fainter, indicating that much of the virus has broken up into its smaller component proteins that have migrated further down into (or off) the gel; this shows that the virus has undergone more disassembly compared to its disassembly at the other pH values. Finally, the only protein visible in the pH 4.6 flow through is pVI, which indicates that this protein is present as the capsid continues to disassemble.

Also interesting are the flow cytometry results, which lead us to believe that something happens to the virus between pH 7.5 and pH 6.2 that renders it more infective. It is possible that this was just a more concentrated virus sample that happened to have more viable virus particles than the pH 7.5 sample, but it is also possible that one of the proteins that is released from the capsid at pH 6.2 enhances the virus's infectivity. At pH 5.2, we saw a drop in infectivity that was likely due to the loss of the many proteins that can be seen in the in the flow through (Figure 7). These proteins are likely necessary for successful infection and since the capsid had already lost these proteins before being placed on the cells, the virus could not cause an infection. Treating the virus at pH 4.5 virtually eliminates its infectivity; this is likely due to the fact that so few proteins remain intact and with the capsid, the core is ejected from the capsid at this pH and, it is likely that the capsid is destroyed completely or it is so empty at this point of its genetic material and essential proteins that it cannot infect the cells at all.

This asymmetry we witnessed in Ad5 disassembly causes us to ask more questions about the nature of Ad5 infection. Since we know pVI is necessary to lyse endosomal membranes and release the virus into the cytosol, how much pVI is released when one vertex region is released from the virus? Is this amount of pVI enough to lyse the endosome and carry out an infection? We further question the location of pVI, which has been hypothesized to be located underneath all each hexon; is it free flowing in the virus

capsid so it can be released when a vertex is removed? Or is the amount of pVI directly underneath the vertex sufficient for membrane lysis? These questions can be answered by quantifying pVI and studying the virus with cryoelectron microscopy (and analyzing images in a way that does not average out the asymmetry in the virus structure) to determine the exact positions of proteins within an asymmetrical capsid.

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