Effect of Formamide on the Capsid Morphology of Adenovirus Types 4 and 7

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Treatment of adenovirus types 4 and 7 with formamide disrupted the virions, degrading the capsids into predominantly single capsomers. As shown by electron microscopic observation, disruption proceeded in the following sequence: (i) reduction of the electron density of the virions, suggesting release of an internal component; (ii) progressive cleavage of the capsid into two or more segments and the formation (type 7 only) of capsomer "sheets"; (iii) final cleavage of the capsid into single or groups of a few capsomers. The sequence appeared similar for both adenoviruses; for both types, the rate and extent of disruption were dependent on the formamide concentration, but type 7 was more easily disrupted than type 4 by short-term (5 to 10 sec) treatment at the low (10%) concentration. At 30% formamide, the intercapsomer bonds of either type were fully cleaved, and the capsids were completely degraded into predominantly single capsomers. Pretreatment with formaldehyde did not prevent this degradation. Under suitable conditions, virus-derived remnants can be observed among the breakdown products. These remnants have been shorn of capsomers and presumably represent intact internal nucleoprotein.

Degradation of the adenoviruses by physical or chemical agents has become an important means for the study of their morphology and structure-function interrelationships. Since the degradation products might retain the antigenicity of the whole virion, gentle means should be sought for degrading the capsid shells into capsomer units. Previous study at this laboratory demonstrated that the intercapsomer bonds in adenovirus types 4 and 7 virions and hemagglutinins could be cleaved by formamide treatment (2). This communication describes further the results of an electron microscopic study of the process of adenovirus degradation using various concentrations of formamide.

MATERIALS AND METHODS

Viruses. Adenovirus types 4 and 7 were prepared and purified as described previously (2).

Treatment of adenovirus type 4 suspension with 30% formamide. Purified adenovirus type 4 was treated in suspension with formamide (30% for 1 hr at 37 C) as described previously by Neurath et al. (2). The treatment was terminated by dialysis against 0.01 M phosphate buffer at 4 C for 1 hr. Both the control and formamide-treated adenovirus type 4 virions were then negatively stained for electron microscopic observation.

Kinetic studies on the disruption of adenovirus types 4 and 7 virions with formamide. The virions were treated with formamide at three concentrations (10,

20, and 30%) and for three different periods at each concentration (5 to 10 sec, 1 min, and 5 min). A single drop of the virus suspension, maintained at 4 C prior to use, was applied from a capillary pipette onto a bare carbon film previously stripped from mica and held on a specimen screen (400-mesh copper). The excess liquid was removed 1 min later with the pipette. One drop of an aqueous solution of formamide at one of the three given concentrations was then applied to the specimen on the carbon grid surface, and was allowed to remain for one of the three given treatment periods. The treatment was terminated by pipetting off the excess formamide and immediately staining for microscopy. All of the foregoing manipulations were performed at 26 C.

Treatment of formaldehyde-inactivated type 4 adenovirus with 30% formamide. Purified adenovirus type 4 virions in phosphate-buffered saline (PBS; 0.075 M phosphate and 0.072 M NaCl, pH 7.2) were treated with 0.01% formaldehyde. The mixture was incubated for 1 hr at 37 C and dialyzed against PBS overnight. The virions were treated as before for 5 min with 30% formamide. Controls consisted of completely untreated virions, and both untreated and formaldehyde-inactivated virions were treated with distilled water for 5 min before being stained.

Electron microscopy. Specimens were negatively stained with 2.0% ammonium molybdate (pH 6.0). Specimens were observed with an RCA EMU-3H electron microscope employing double-condenser illumination at 50 kv. Images were recorded at an

electron optical magnification of 43,714 times. Except where otherwise stated, the marker bar in the figures indicates a distance of 0.1 μm .

RESULTS

Treatment of adenovirus type 4 suspension with 30% formamide. Previous results, based on rate zonal centrifugation experiments (2), indicated that treatment with formamide at a relatively high concentration (33%) degrades the virions of adenovirus types 4 and 7 predominantly into single capsomers. These electron microscopic studies confirmed and extended the previous work.

The control virions (Fig. 1a) are similar to those described by Wadell et al. (7) for the same adenovirus type, except that the projections, including the distal knob, measured 22.5 to 24.0 nm instead of about 20.0 nm as described by the above authors. The fibrous extension upon which the 4.5 nm (diameter) knob is fitted is barely perceptible; however, in some cases, the width of the fiber is estimated to be 2.0 to 2.5 nm. Because of the depth of the local concentration of the stain surrounding the fiber, a precise dimension is difficult to determine.

Treatment of the virions with 30% formamide resulted in a breakdown of the capsids into single capsomers (8.5 to 9.0 nm in diameter) and into various multicapsomer patterns (Fig. 1b). Several characteristic, recurring patterns were chosen from Fig. 1b, enlarged, and assembled (Fig. 2) to demonstrate their probable points of origin within a constituent triangular face of the viral icosahedron. The corresponding arrangements are also shown diagrammatically.

One common arrangement (Fig. 2c) consists of two rows of six capsomers that probably fit into the total triangular face (Fig. 2d). This can be interpreted as two faces positioned edge to edge, with one row shifted to accommodate the other, suggesting that the edge itself has to some degree resisted the formamide treatment. The arrow in Fig. 2c indicates an intercapsomer fiber, which can be seen stretched and bent out of position. Fibers of this type are generally detected along an outside row, or along the probable connecting edge between two whole triangular faces of a virion. The fiber shown here is about 1.0 nm wide and 4.5 nm long.

Groups of six and four capsomer units arranged as parallelograms are shown in Fig. 2e and 2g, respectively. Triangular forms are seen in Fig. 2i and 2k. Various other arrangements can be observed in Fig. 2o, 2q, and 2s. Under the prescribed conditions, the action of formamide is apparently indiscriminate, since certain capsomer patterns or portions of them originate from within a given triangular face as well as from its edges.

In some of the micrographs (Fig. 2k and 2m), the intercapsomer fibers are clearly in an extended condition. The fiber in Fig. 2k measures about 1.0×6.0 nm, that in Fig. 2m about 10×40 nm.

Besides the breakdown products just described, other forms such as the virus-derived bodies shown in Fig. 1b (circle) and Fig. 3b and 3d were occasionally observed. These bodies had hexagonal outlines (presumably thus retaining the icosahedral form) and measured slightly less than the intact virions shown in Fig. 3a and 3c (67.0 versus 76.0 nm); the difference might be accounted for by the width of one nonvertex capsomer, suggesting that these bodies may represent virions "stripped" of capsomers. In some instances, individual capsomers were observable upon their surfaces, but whether these capsomers were originally attached to the bodies or had been relocated there from other capsids was not known.

Kinetic studies on the disruption of adenovirus type 4 virions with formamide. The studies were extended to determine the optimal conditions of formamide treatment. The starting material was the type 4 adenovirus preparation (Fig. 4a). Attached apical extensions were not detected by the electron microscopic examination of this control preparation.

Treatment with 10% formamide for 5 to 10 sec caused a loss of electron density, suggesting release of an internal component; the capsids being cracked open were separated into two or more fragments. Few loose capsomers were visible. Essentially identical results were obtained with adenovirus type 7 (see Fig. 7). Prolongation of treatment to 1 min appeared not to increase the fragmentation. However, when either the time was prolonged to 5 min or the concentration was increased to 20%, a greater number of capsids were completely cleaved into two or more segments, and more capsomer aggregates and single capsomers were noted (Fig. 4b). Similar results were obtained with 30% formamide after 5 to 10 sec. After 5 min treatment at the 30% concentration, however, the remnants appeared to consist solely of single capsomers and various sized aggregates (Fig. 5).

Treatment of formaldehyde-inactivated type 4 adenovirus with 30% formamide. Although formaldehyde is known to have a tanning effect on proteins and to destroy the infectivity of adenoviruses, pretreatment with 0.1% formaldehyde did not prevent the subsequent disruption of capsids by treatment with 30% formamide for 5 min (see Fig. 6 and compare with Fig. 4a, control). As in type 4 adenovirus not inactivated by formaldehyde (Fig. 5), large numbers of loose capsomers were visible, and no intact virions or empty capsid shells could be observed. Although formaldehyde

FIG. 1. (a) Purified adenovirus type 4, shown in reverse contrast. (b) Breakdown products of same adenovirus after treatment with 30% formamide for 1 hr at 37 C (also in reverse contrast). Shown in circle is one of the virus-derived bodies (similar ones are shown enlarged in Fig. 3).

FIG. 2. Fine structure (shown in reverse contrast) and accompanying model diagrams of several of the capsomer arrays resulting from treatment of adenovirus type 4 with 30% formamide for 1 hr at 37 C. The arrays illustrated here are only a few of the possible patterns that can be formed by breakdown of each of the 20 triangular faces of the viral icosahedron.

did not preserve the intercapsomer bonds, it did appear to preserve more of the virus-derived bodies, many of which remained fixed in place upon the carbon substrate (Fig. 6).

Electron microscopic observation of the sequential steps in the process of disruption of adenovirus type 7. The control virions for adenovirus type 7 were similar to those of type 4 previously shown in Fig. 4a.

The fibrous apical extensions often observed

attached to adenovirus type 4 virions could only rarely be noted in this preparation.

Treatment with 10% formamide for 5 to 10 sec gave the results shown in Fig. 7. The virions have lost their electron density (compare with the single, apparently still intact particle in Fig. 7a) and have entered various stages of cleavage. Many of the capsid shells exhibit dense, folded edges. A few single capsomers are already evident.

The enlargements in Fig. 7b through 7e illus-

FIG. 3. Comparison of purified adenovirus type 4 particles (a and c) with the virus-derived bodies (b and d) resulting from treatment with 30% formamide for 1 hr at 37 C. The bodies have retained the basic icosahedral symmetry.

trate a possible manner of sequential, stepwise disruption of a type 7 adenovirion. Figure 7b shows a narrow, straight-line cleavage for perhaps three-quarters the distance across the icosahedral capsid shell. In Fig. 7c and 7d, the cleavage has progressed and the two segments are pulling apart. In Fig. 7e, they have completely separated. Also formed are a few whole capsomer sheets (Fig. 8) having straight-line boundaries (Fig. 8c, arrow) and several virus-derived bodies that are almost as large as intact virions and are obviously not typical capsids (Fig. 8b, arrows). Although the capsid in Fig. 8c has broken along a nearly straight line, most capsomer sheets are irregular in shape. The sheets cannot be matched with the virions from which they come, since the original capsid shells have lost their structural continuity.

The type 7 virions were much more susceptible to low concentration (10%), short-term (5 sec) formamide treatment than were the type 4 virions, which under the same conditions exhibited no cleavage of the capsid shells into sheets. Instead capsids of the type 4 virions tended to retain their morphological identity until conditions were employed for their complete degradation into capsomer units.

Treatment with 10% formamide for a longer

period (1 min) produced more individual capsomers and virus-derived bodies (Fig. 9) similar to those shown for adenovirus type 4 (Fig. 3, 6). The latter structures are slightly smaller than that of intact virions (bracket).

Exposure to 20% formamide for 5 to 10 sec degraded the type 7 virions largely into capsomer sheets. Several types of capsomer groupings can be observed (Fig. 10a through 10f). The latter illustrations are accompanied by a flattened out icosahedron fitted with the corresponding model groups (Fig. 10e and 10g). Since these groups include parallelogram forms which contain up to 36 capsomer units, they can cover portions of at least four triangular faces (Fig. 10a, 10d, and 10f, shown diagrammatically in views 1 and 5 of Fig. 10e and views 2 and 3 of Fig. 10g). It cannot be readily ascertained which capsomers within the parallelogram-shaped sheets represent the rib or edge portions of the icosahedron. In Fig. 10b and 10c, however, the faces and corresponding edges can be seen. In Fig. 10b, a triangular face containing at least one complete six-capsomer unit edge is shown; in Fig. 10c a similar edge appears as a prism, the center portion of which contains six capsomer units and is flanked on either side by a row containing five capsomer units. Figures 10b

FIG. 4. (a) Purified adenovirus type 4 particles, employed as the starting material for the kinetic study of formamide degradation. (b) The same particles following treatment with 20% formamide for 1 min at 26 C.

FIG. 5. Complete degradation of adenovirus type 4 resulting from treatment with 50% formamide for 5 min at 26 C. The viral capsids have been completely broken down into capsomers or groups of capsomers.

FIG. 6. Failure of formaldehyde treatment to prevent the disruption of the adenovirus type 4 capsid by formamide. Adenovirus type 4 was pretreated with formaldehyde and subsequently treated with 30% formamide for 5 min at 26 C. No reduction in cleavage of intercapsomer bonds is evident, but the combined treatment has increased the number of virus-derived bodies or viral remnants.

and 10c are shown diagrammatically in Fig. 10e, views 3 and 4. In Fig. 10f and diagrammatically in Fig. 10g, a whole section of a virion containing portions of five triangular faces has been flattened

and separated in place on the carbon substrate. The short-term (5 to 10 sec) treatment with 20% formamide has weakened the virion enough along vulnerable stress points (presumably edges) to

FIG. 7. Steps in the disruption of the adenovirus type 7 capsid. For this preparation, adenovirus type 7 was treated with 10% formamide for 5 to 10 sec at 26 C. (a) Field view of capsids in various random stages of cleavage. One virion is still intact. (b) through (e) Enlargements of several capsids from the field, showing the probable sequence of steps in cleavage.

FIG. 8. Sheets of capsomers formed by treatment of adenovirus type 7 with 10% formamide for 5 to 10 sec at 26 C. (a) General field view; (b) and (c) enlarged portions of the field. Some of the capsomer sheets demonstrate straight edges (c, arrow) as well as irregular boundaries. Bodies which do not show typical capsomer images can also be seen (b, arrows).

FIG. 9. Increased numbers of virus-derived bodies (bracket) are found following treatment of adenovirus type 7 with 10% formamide for 1 min at 26 C. The bodies demonstrate an extensive removal of the capsomers as a result of the treatment.

cause its collapse but not its complete breakdown. As a result, not all the triangular faces are resolved into similar groupings.

Increase in the period of treatment to 5 min caused further fragmentation of the capsomer sheets into many single, free-lying capsomers, triplet forms (three capsomers in triangular form), and other small arrays. Degradation of the capsomer sheets appears to have proceeded by random cleavage of the intercapsomer bonds rather than preferentially along the edges of the triangular faces. The complete degradation of the adenovirus type 7 virions was accomplished by treatment with 30% formamide for less than 10 sec (Fig. 11). Although this treatment caused single capsomers to predominate, occasional small arrays may still be observed (*see arrow*). The more darkly stained areas still contain some images reminiscent of the virus-derived bodies (bracket), which are presumably in an advanced stage of degradation (compare with Fig. 9).

DISCUSSION

The electron micrographs show that formamide, even at low concentration (10%), causes an almost instantaneous rupture of the adenovirions, and that higher concentrations (20%, 30%) and longer periods of exposure (1 min, 5 min) cause progressive cleavage of intercapsomer bonds. The capsid shells of adenovirus types 7 and 4 are completely degraded to individual capsomers by treatment with 30% formamide at 26 C for 10 sec and 5 min, respectively. Thus, the virions of adenovirus type 7 are more susceptible to rupture than are those of type 4. Neurath et al. (*submitted for publication*) found that under similar conditions type 5 is even more resistant. Hence, adenoviruses appear to differ in their susceptibility to degradation by formamide.

Under optimal conditions, formamide indiscriminately severs all intercapsomer bonds in both adenovirus types 7 and 4. Under less severe conditions, it preferentially attacks only the most vulnerable bonds, and degradation tends to occur at the edges of points of fit of the triangular faces of the viral icosahedron, probably because the intercapsomer bonds at these points are under more stress and thus more vulnerable to attack than are the bonds located within the faces.

Pretreatment with dilute formaldehyde did not alter the ability of formamide to cleave the intercapsomer bonds. Formaldehyde did, however, appear to enhance the stability of the virus-derived bodies. Since formaldehyde pretreatment had previously been demonstrated not to reduce the antigenicity of similar preparations, it may become feasible to utilize some combination of

formaldehyde and formamide to stabilize the antigenic structure of the adenoviruses.

The electron micrographs to date have revealed no structural differences between the capsomers

before and after formamide treatment of either type of adenovirus. Admittedly, morphological changes may occur that are too subtle to be detected by present means and yet significant enough

FIG. 10. Capsomer arrays resulting from 20% formamide treatment (5 to 10 sec) of type 7 adenovirions, accompanied by models illustrating the probable origin of each geometric pattern. (a) through (d) Capsomer arrays shown enlarged; (e) models corresponding to the patterns of capsomers appearing in (a) through (d), positioned on a flattened out icosahedral template; (f) capsomer array most likely formed from collapse of the capsid about a fivefold axis of symmetry; (g) corresponding model positioned on the flattened out icosahedral template to show the probable origin of such an array.

to cause loss of biological activity; however, Neurath et al. (2) demonstrated that the complement-fixation titers of adenovirus types 4 and 7 capsomers (hexons) are unaltered by treatment

with formamide at concentrations of 30% or greater.

The characteristic clusters of nine capsomers (type A groups), found by Smith et al. (6) after

FIG. 11. Complete degradation of the adenovirus type 7 virions by treatment with 30% formamide for 5 to 10 sec at 26 C. Although single capsomers now predominate, an occasional small array can still be seen (arrow). The darkly stained areas, one of which is indicated by a bracket, contain fibrous masses which may have evolved by further degradation of the virus-derived bodies (Fig. 9).

treatment of the capsids of adenovirus type 2 with low concentrations of sodium lauryl sulfate, were observed rarely in these studies and only after treatment of type 7 with 20% formamide for 5 min. Subsequently, however, we discovered that large quantities of type A groups could be obtained if type 7 adenovirions were stored at 26 C for several days in either 0.01 M phosphate buffer (pH 7.0) or 2.0% ammonium benzoate (*unpublished data*). Russell et al. (5) also demonstrated the presence of type A groups following heat treatment of adenovirus type 5. If, as Smith et al. (6) proposed, the type A groups represent the 20 triangular facets of the adenovirus icosahedron, the relative paucity of such groups after formamide treatment may be due to the efficient and indiscriminate cleavage of intercapsomer bonds by formamide.

Evidence has been mounting for the existence of an additional, previously unrecognized adenovirus component. The internal component found by Russell et al. (5) within the capsid of adenovirus type 5 is structurally similar to the virus-derived bodies appearing in these micrographs. Moreover, the studies of Rouse and Schlesinger (4) established that the capsomer proteins and whole virion proteins differ in arginine content, thus pointing to the existence of an arginine-rich component in some part of the virion other than the capsomers. Their data, along with those of Russell et al. (5) and of others, who have studied the intracellular "uncoating" of the adenovirus and have discovered the existence of an internal component, part of which is viral deoxyribonucleic acid (DNA), suggest that the newly recognized protein is associated with viral DNA and might be concerned with the assembly of preformed viral precursors into the complete infectious virion (1, 3). The fate of this internal component in the process of breakdown of the viral capsid will require further studies.

No penton antigens were observed among the degradation products. With 30% formamide, they would have been converted to vertex capsomers and projections (fibers; 2), but at lower concentrations and after short periods of treatment some should have been present. Their absence suggests

either their degradation during the staining procedures or their failure to stain adequately in the detached state.

On occasion, a fibrous component could be detected between certain capsomers within the arrays resulting from the formamide treatment (30% for 1 hr at 37 C) of adenovirus type 4 (Fig. 2c, k, and m). The unique intercapsomer location of such a fiber implies that it functions architecturally in the connection of capsomers. A more precise definition of the possible structural and functional relationships of this component has yet to be determined.

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