Studies on the Assembly of Adenovirus in vitro

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SUMMARY

In vitro assembly of human adenovirus type 5 was studied using virus precursor materials from extracts of HeLa cells infected in Eagle’s complete and arginine-free medium. Radioisotope studies suggested that factors having assembly activities in vitro were present only in extracts of cells infected in complete medium. Extracts containing these assembly factors were added to extracts of cells infected in arginine-free medium and the mixtures, after incubation in the presence of stabilizing buffer solutions, contained particles of the same density as complete virus particles formed in vivo. The newly assembled products were DNase-resistant and had 1.5 to 2 log. greater infectivity in plaque assays than either of the two extracts alone or in unassembled mixtures. The factor(s) responsible for the assembly activity were found to be associated predominantly with the nuclear fraction of the infected cells and they were produced in greatest amounts in extracts taken at 12 to 16 hr after infection. Assembly activity of the extracts was lost when they were subjected to temperatures above 32°, trypsin treatment and fluorocarbon extraction before adding them to experimental assembly mixtures.

INTRODUCTION

In the absence of arginine in the tissue culture medium, cells infected with adenovirus apparently produce both structural and non-structural antigens and virus DNA, although these are not assembled into infectious virus (Rouse & Schlesinger, 1967). It was suggested that protein(s) synthesized in response to an exogenous arginine supply could function in some way in the assembly of preformed virus precursors into particles. Further insight into the factors involved was obtained by the demonstration that a component of the P antigen (Russell et al. 1967a; Russell & Knight, 1967; Hayashi & Russell, 1968) was not produced under conditions of arginine deprivation (Russell & Becker, 1968) and that this component could possibly be related to the arginine-rich proteins found within the virus particle (Russell, Laver & Sanderson, 1968). Thus it was suggested that this arginine-rich protein, whose production was arginine-dependent, could function as a limiting maturation factor in the assembly of the particle.

In this communication we describe a system for studying the assembly of adenovirus type 5 in vitro, utilizing extracts of cells infected with the virus in the presence and absence of arginine in the tissue culture medium.

METHODS

Media and cells. Eagle’s medium, supplemented with 10% tryptose phosphate broth (TPB), 10% calf serum, and antibiotics (ETC medium), was used for routine HeLa cell culture. Experimental media consisted of serum and TPB-free Eagle’s medium with arginine or without arginine, designated E + A medium and E − A, respectively.
Virus. Human adenovirus type 5, strain AD 75 (Ad. 5) was propagated in HeLa cells which had been cultured in ETC medium. After infection the cells were maintained in Eagle's complete (E + A) medium. Virus used as seed was extracted from cells by homogenization in fluorocarbon and then partially purified by caesium chloride density centrifugation (Russell, Valentine & Pereira, 1967b).

Buffers. CMF-PBS is phosphate-buffered saline (Dulbecco & Vogt, 1954) without CaCl₂ and MgCl₂; KTB is 0.1 M-KCl, 0.05 M-tris/HCl, 0.0001 M-mercaptoethanol, pH 7.8; TM is 0.01 M-tris and 0.005 M-MgSO₄, pH 7.4; TMDK is TM plus 0.002 M-dithiothreitol and 0.0001 M-EDTA, pH 7.0. When each solution contained a final concentration of 0.3 M-sucrose, it was designated +S, i.e. KTB + S.

Separation of nuclear and cytoplasmic fractions of cells. Cells for fractionation were washed once in CMF-PBS and then resuspended in KTB + S in a plastic centrifuge tube and disrupted for 3 min. using an Ultra-turrax 18/2 tissue disintegrator (Jauke & Kemkel, K. G., Stanfen, W. Germany) run at 50 v. The tube was then centrifuged at 3200 g for 30 min. at 4°C. The cytoplasmic extract was removed from the nuclear pellet which could be resuspended in a suitable volume of KTB + S. Microscopic examination revealed apparently intact nuclei and relatively little cytoplasmic contamination. This method obviated the need for a detergent to separate the nuclei. By using infected cells labelled with [³H]thymidine 10 hr after infection (when only the virus DNA is labelled – Mäntyjärvi & Russell, 1969), it could be shown that 98% of the radioactivity was located in the nuclear fraction.

Cellular, nuclear and cytoplasmic extracts were obtained by sonic treatment of a suspension of cells, nuclei, or cytoplasmic fractions in thin-walled glass vials for timed intervals in an ultrasonic bath (Burndept Ultrasonic Cleaner).

Radioisotopes. [¹⁴C]Thymidine (60.5 mc/mmole) and [³H]thymidine (25.3 c/mmole) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Radioactivity was assayed by spotting samples on filter paper squares, drying, placing in toluene-scintillating fluid, and counting in a Packard TriCarb liquid scintillation counter.

Infectivity titrations. Virus seeds, infected cell extracts and mixtures of extracts were titrated by a plaque assay involving infection of HEK cells in monolayers or in suspension followed by overlaying or plating out the cells in the presence of carboxy-methyl cellulose (Russell, 1962). Plaques were counted after 10 to 14 days incubation in CO₂ (5% + 95% air) atmosphere at 37°C.

Enzymes. Trypsin treatment of cell extracts consisted of digestion at 37°C for 30 min. followed by the addition of trypsin inhibitor. Trypsin (crystalline grade, Armour Chemicals) was used at concentration of 0.1% and soybean trypsin inhibitor (Worthington Biochemical Corporation) was used at concentration of 1%. Deoxyribonuclease sensitivity of assembly products before and after purification on caesium chloride density gradients was determined using the method described by Russell et al. (1967b). Controls using ³H-labelled infected cell DNA (Mäntyjärvi & Russell, 1969) monitored the efficiency of the enzyme under the same conditions used in the experiments.

Preparation of cells in arginine-free medium for use as extracts in assembly experiments. Monolayer cultures of HeLa cells in 20 oz bottles (containing about 2 to 3 x 10⁸ cells/bottle) were washed once with E-A medium and incubated at 37°C for 2 hr in fresh E-A medium. The medium was again changed and approximately 200 p.f.u./cell of Ad. 5 was added to the cultures. The virus inoculum was from a stock pool of purified virus which had been dialysed against E-A medium. At 10 hr after infection at 37°C the medium was removed and [³H]thymidine was added (2 μc/ml) in fresh E-A medium. Incubation at 37°C was continued for 24 to 34 hr. Uninfected radioactively labelled cells and unlabelled infected and uninfected
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Cells were treated in the same manner. Unlabelled cells infected in E-A medium were designated infected E-A cells and similar cells which had been labelled with [3H]thymidine were designated infected [3HT] E-A cells. During the preparation of each lot of cells in E-A medium, a portion of the infected cell cultures was changed to E+A medium at the time of addition of the radioisotope so that the extent of virus maturation after the restoration of arginine could be ascertained.

Preparation of cells in complete medium for use as extracts in assembly experiments. HeLa cells were infected in E+A medium in suspension by shaking for 30 min. at 37° with purified virus seed (approximate multiplicity of 200 p.f.u./cell) which had been previously dialysed against E-A medium. After infection cells were sedimented by low-speed centrifugation (3000 g, 10 min.) and resuspended in E+A medium in 8 oz. bottles (about 30 million cells/bottle) and incubated at 37°. In some experiments incubation at 37° was interrupted briefly at 10 hr after infection when [14C]thymidine was added to the medium. Cells infected in E+A medium were designated infected E+A cells; similarly infected cells labelled with [14C]thymidine were designated infected [14CT] E+A cells. Uninfected labelled cells and unlabelled infected and uninfected cells were treated in the same manner as that described above.

Collection and storage of experimental cells. Cells in E+A medium or in E-A medium were harvested at various intervals by scraping off the cells, washing once in Liebovitz medium (Liebovitz, 1963) with antibiotics, and then resuspending in a small volume of KTB+S and Liebovitz medium at a ratio of 2:1 and stored at -70°.

General plan of assembly experiments. Extracts of cells to be used in the mixtures were prepared by disruption by sonic treatment of measured amounts in thin-walled glass vials followed by centrifugation at 700 g for 10 min. to remove undisintegrated cells and cellular debris. Extracts to be tested were then mixed immediately, or were pre-treated then mixed, in KTB+S, and the mixtures were then shaken at 32° in a water bath. At intervals the incubation was stopped by placing the mixture samples into an ice bath followed immediately by fluorocarbon extraction. This was carried out by mixing equal volumes of Arklole P (I.C.I. Ltd) in the small (5 ml.) Waring Blender and running at full speed at 4° for 60 sec. The aqueous phase of the fluorocarbon extract was carefully layered on the top of two layers of caesium chloride (0.8 ml. of density 1.45 and 3.2 ml. of density 1.33) prepared in 0.005 M-tris HCl + 0.0001 M-EDTA, pH 7.8, and centrifuged in the MSE 65 ultracentrifuge at 90,000 g for 90 min. using a 3 x 5 ml. swing-out rotor or for 4 hr at 75,000 g using a 6 x 15 ml. swing-out rotor – in the latter case the tubes were filled to the top with liquid paraffin. Five-drop fractions were collected by puncturing the bottom of the centrifuge tube and the gradient of density determined using an Abbé refractometer, as described previously (Russell et al. 1967b). Fractions of the gradients within the approximate range of refractive index 1.364 to 1.368 (densities 1.33 to 1.37) were collected and pooled, diluted with buffer, and placed on top of another preformed double layer of CsCl. This consisted of 1.0 ml. of density 1.45 g./cm.³ and 3.0 ml. of density 1.33 g./cm.³ and centrifuged to equilibrium using either the 3 x 5 ml. swing-out rotor (90,000 g 18 to 24 hr) or the 6 x 15 ml. swing-out rotor (75,000 g, 36 to 48 hr). Fractions of the equilibrium gradients were collected from the bottom of the centrifuge tube and tested for refractive index and radioactivity.
RESULTS

General system of assembly in vitro

The first objective of our experiments was to test the hypothesis that virus particles could be assembled *in vitro* from virus materials which had previously been synthesized in infected human cells. Accordingly, sources of the precursor materials were selected on the basis of results of previous studies on the synthesis of cellular and virus DNA, structural and non-structural virus antigens and infectious virus (Russell *et al.* 1967a; Hayashi & Russell, 1968; Russell & Becker, 1968; Mäntyniemi & Russell, 1969). Mixtures of extracts were assayed for assembly activity, as described in the methods section. One of the extracts added to the experimental mixture was always from cells infected in E−A medium and collected 30 or more hr after infection. Under these conditions virus DNA and capsid components are pro-

![Radioactivity profiles of caesium chloride density gradients of assembly mixtures.](image)

**Fig. 1.** Radioactivity profiles of caesium chloride density gradients of assembly mixtures. Each mixture consisted of 0.5 ml. of sonicated, centrifuged extracts, or KTB+S buffer, incubated at 32° for 30 min., then extracted with fluorocarbon, as described in Methods. Times shown in parenthesis refer to time after infection when cells used in extracts were collected. ○—○, experimental mixture of infected [HT] E−A cell extract (42 hr) plus infected E+A cell extract (14 hr); ●—●, control mixture of infected [HT] E−A cell extract (42 hr) plus uninfected E+A cell extract (14 hr); □—□, control mixture of infected [HT] E−A cells extract (42 hr) plus KTB+S buffer; △, refractive indices.

duced, but not complete progeny virus nor components of the late, arginine-dependent P antigen. The other component of the assembly mixture was derived from cells infected in E+A medium taken at various intervals of time after-infection when virus DNA, capsid antigens and non-structural antigens were being synthesized. It was anticipated that the maturation factors, obviously depleted in the E−A extracts, could perhaps be obtained from the E+A extracts and made to function in the *in vitro* system.

The primary criterion for detecting the *in vitro* assembly of virus was the characteristic
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density of adenovirus in a caesium chloride density gradient. Thus, by labelling the infected
cells with [3H]thymidine, the extracted virus could be characterized as a sharp band of
radioactivity at a buoyant density of 1.345 (Russell et al. 1967b). By using two successive
gradients (a ‘velocity’ followed by an equilibrium gradient) the background of radioactivity
was reduced to very low levels and the production of very small amounts of radioactive
virus was readily detected.

![Graph showing time of appearance of assembly activity in extracts of infected E + A cells.](image1)

![Graph showing time of in vitro assembly.](image2)

Fig. 2. Time of appearance of assembly activity in extracts of infected E + A cells. Radioactive pro-
files of mixtures, as in Fig. 1, were used to calculate assembly activity for each test extract of
infected E + A cells mixed with the same extract of infected [3HT] E-A cells. Histograms show
assembly activity (% counts/min.) of the mixtures. % Counts/min. = sum of radioactivity in virus
peak fractions (counts/min. vp) divided by counts/min. in total gradient (tg) × 100 of the control
mixture subtracted from value of counts/min. vp × 100, of experimental test mixture.

Fig. 3. Time of in vitro assembly. Expts. 1 (O—O) and 2 (●—●) represent assays using two
different extracts of infected [3HT] E-A cells (40 hr) mixed with the same extract of infected or un-
infected E + A cells (14 hr). % Counts/min. at each interval of time was calculated as described in
Fig. 2.

In the preliminary experiments a number of the variables of the in vitro assay system were
investigated and it was found that, under the correct conditions, in vitro assembly of virus
could be detected. One such experiment is shown in Fig. 1 where it can be clearly seen that
there was significantly more radioactivity banding at the characteristic density of aden-
virus in the experimental mixture than in the control mixtures. Some of the parameters
involved in this in vitro assembly and of the characteristics of the assembled product are
described below.

Time of appearance of assembly-rich materials in infected cells

The ability of extracts of cells harvested at different times after infection in E + A medium
to assemble virus was examined in the in vitro incubation system. The extracts were mixed
with equal portions of an extract of infected \(^{3} HT\) E-A cells that had been collected 42 hr after infection. Significant assembly activity was detected only in extracts of infected E+A cells taken during the 12 to 18 hr after infection (Fig. 2). No assembly activity was detected when uninfected cells in E-A and E+A medium, or infected cells in E-A medium at similar intervals were used as sources of assembly materials.

In succeeding experiments, described in detail below, assays for assembly activity used extracts of cells infected in E+A medium taken at 14 hr as a source of assembly-rich materials.

**Influence of time of incubation**

Equal quantities of each of the extracts, i.e. infected E+A cells and infected \(^{3} HT\) E-A cells, were mixed in KTB+S and incubated on a shaker in a 32° water bath for various times. The results of two experiments using the same infected E+A cell extract, but two different extracts of infected \(^{3} HT\) E-A cells, indicated that assembly occurred within 15 min. of incubation (Fig. 3), since continued incubation did not appreciably change the amount of assembled virus. When equal quantities of extracts of uninfected E+A cells were mixed with infected \(^{3} HT\) E-A extracts, no assembly could be detected.

**Influence of temperature of incubation**

The optimum temperature of incubation was 32° under the experimental conditions used (Table 1). At 4°, 56° and 70°, no significant assembly could be detected, and at 22° and 37°,
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although some assembly was detected, it was significantly less than that resulting at 32°C. This indicated that assembly activity of the mixtures was temperature-dependent over a narrow range.

Influence of incubation buffers

Infected and uninfected E+A cells taken at 14 hr after infection, and after mock-infection were extracted in Liebovitz medium + KTB buffer (ratio of 1:2) and then mixed with similarly treated extracts of infected [²HT] E−A cells. Different buffer solutions were added to the mixtures before incubation and they were then incubated for 30 min. while gently shaking in a 32°C water bath. The result of two experiments, (Table 2) suggested that the incubation buffer of choice was KTB+S, followed by TM+S, TMDK+S, KTB and then PBS+S, respectively. Thus, the increased amount of Mg²⁺ in the TM and TMDK solutions did not appear to increase the yields of assembled virus in vitro, while the 0.3 M-sucrose apparently

Fig. 4. Radioactivity profiles of caesium chloride density gradients of two assembly mixtures, one of which contained extract of infected [²HT] E−A cells (42 hr) plus extract of infected [¹⁴CT] E+A cells (14 hr); ○—○, [²H]; - - - , [¹⁴C]. The other mixture contained extract of infected [²HT] E−A cells (42 hr) plus extract of uninfected E+A cells (14 hr); - - - , [²H]; △, refractive indices.
exerted a stabilizing effect on the incubation mixture. Thereafter, cells were routinely stored in a mixture of KTB+S (2 parts) + Liebovitz medium (1 part).

**Contribution of virus DNA to assembled virus particles**

Our initial experiments showed that, on the basis of radioactivity profiles, complete virus was assembled in vitro. However, these experiments used extracts of cells labelled with [H]thymidine and infected in E−A medium. To determine if the virus DNA formed in cells infected in E+A medium (and rich in assembly activity) was also incorporated into the in vitro assembled virus, cells infected in E+A medium were labelled at 8 hr after infection with [14C]thymidine and collected at 14 hr after infection. (Extracts of unlabelled

![Graph](attachment:image.png)

**Fig. 5. Influence of quantity of assembly-rich extract in mixtures on assembly activity.** Values represented by symbols (O, •) are sums of radioactivity at virus density using two different lots of extracts of infected [3HT] E−A cells mixed with same extract of infected (experimental) E+A cells after correction for uninfected controls. O, Expt. 1; •, Expt. 2.

cells infected in E+A medium prepared at the same time as the labelled cells were found to have high assembly activity.) The infected [14CT] E+A cell extracts were mixed with infected [3HT] E−A cell extracts and were incubated under optimal assembly conditions. Control mixtures were included with each assay. The results (Fig. 4) indicated that the DNA in the in vitro assembled particles was derived only from the extracts of infected [3HT] E−A cells. In all subsequent experiments, extracts of infected [3HT] E−A cells were used as the source of virus DNA and therefore the amount of radioactivity in the in vitro assembled particles was a reflection of the amount of virus DNA incorporated from these extracts. Experiments are now being done to determine from which extracts the capsid and internal components of the in vitro assembled virus are derived.
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Some characteristics of the assembly factors

Since the above experiments had demonstrated that the assembled virus DNA was apparently derived from only one of the extracts (infected E-A cells), it became possible to assay the potency of the other extract (from the infected E+A cells) in terms of its assembly activity. In these investigations, increasing amounts of these extracts were mixed with constant amounts of extracts of infected [3HT] E-A cells and the incubated mixtures assayed for assembly activity in the standard manner. In two experiments (Fig. 5) the amount of in vitro assembled virus was found to be directly related to the quantity of assembly-rich extracts in the experimental mixture. In the first experiment, the limiting factor in the observed dose-response phenomenon was apparently present in the infected [3HT] E-A cell extract, since a saturation plateau was reached. In the second experiment where a different [3HT] E-A cell extract was used with the same E+A cell extract, it is clear that a saturation level was not reached, suggesting that these different lots of infected [3HT] E-A cell extracts differed in the amounts of virus components available for assembly and also probably in the specific activity of the labelled virus DNA. With this type of assay it was thus possible to quantify various assembly-rich extracts relative to a given, pre-tested, infected [3HT] E-A cell extract, and also to test other sources thought to be potentially rich in assembly factors.

Effects of pre-treatment of extracts of infected E+A cells

Temperature effect on assembly activity. Equal portions of the assembly-rich extract were distributed into small glass vials, KTB+S was added, and the vials were treated sonically and centrifuged as previously described. The cell-free fluids in fresh vials were then placed in an ice bath and in water baths at 22°, 37°, 56° and 70° respectively for 30 min. At the end of this time equal portions of an extract of infected [3HT] E-A cells was added to each vial and the mixtures were incubated and assayed for in vitro assembly. In a similar manner, cell-free extracts of assembly rich materials frozen at -70° and -20° were assayed. Assembly ability was unaffected by temperatures of 4° or below but was reduced or lost at the temperatures above 4° (Fig. 6). This result showed that the assembly factors were relatively
thermolabile, perhaps because of enzymes which would undoubtedly be present in the various extracts.

**Effect of trypsin and fluorocarbon treatment on assembly activity.** Pre-incubation of assembly-rich extracts of cells with trypsin at 37° gave a 95 % reduction in assembly activity, close to the levels observed when uninfected cells were used as the source of assembly extracts (Table 3). In contrast, pre-incubation of extracts at 37°, both without trypsin and with trypsin + trypsin inhibitor, gave a 30 to 40 % reduction in assembly activity, suggesting that the trypsin effect was specific and that the assembly factors were probably protein in nature or perhaps that the site of assembly was trypsin-sensitive. Also, similar pretreatment with fluorocarbon eliminates the activity of the extracts, suggesting that perhaps a lipid or membrane-containing structure was also essential for the in vitro activity.

**Table 3. Effect of trypsin and fluorocarbon treatment on extracts with assembly activity**

<table>
<thead>
<tr>
<th>Pre-incubation treatment</th>
<th>Temperature of pre-incubation (°)</th>
<th>Source of pre-treated* extract</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Counts/min.†</td>
</tr>
<tr>
<td>Trypsin, 30 min. then TI + KTB + S, 5 min.</td>
<td>37</td>
<td>Infected E + A cells</td>
<td>4,500</td>
</tr>
<tr>
<td>Trypsin + TI + KTB + S for 35 min.</td>
<td>37</td>
<td>Infected E + A cells</td>
<td>10,250</td>
</tr>
<tr>
<td>KTB + S, 30 min.</td>
<td>37</td>
<td>Infected E + A cells</td>
<td>11,000</td>
</tr>
<tr>
<td>KTB + S, 30 min.</td>
<td>4</td>
<td>Uninfected E + A cells</td>
<td>14,150</td>
</tr>
<tr>
<td>Fluorocarbon, then 30 min. in KTB + S</td>
<td>4</td>
<td>Infected E + A cells</td>
<td>4,720</td>
</tr>
</tbody>
</table>

* All samples 14 hr after infection.
† Counts/min. = Sum of radioactivity ³H in virus fractions after assembly assay when extract of infected [³HT] E − A cells were added to pretreated extracts and incubated for 20 min. at 32°. TI = Trypsin inhibitor.
‡ Counts/min. corrected = radioactivity (counts/min.) of test mixtures minus counts/min. of negative control mixture (b).
§ % Assembly = Corrected counts/min. of test mixture divided by corrected counts/min. of positive control mixture (a).

**Effect of nuclear and cytoplasmic fractionation of assembly-rich cells**

Since the location of the assembly factor in infected cells rich in assembly activity was not known, equal portions of such cells were placed in two plastic centrifuge tubes and the cells in one tube were separated into their nuclear and cytoplasmic fractions, as described in the Methods section. Cellular, nuclear and cytoplasmic extracts were then mixed with equal portions of infected [³HT] E − A cells, incubated under optimal conditions for assembly in vitro and then assayed. The assembly factor rich materials were located predominantly in the separated nuclear and not in the cytoplasmic fraction (Table 4). This information was of interest since the location of the majority of virus precursor materials, including the presumed internal maturation factor antigens in cells infected in vivo, is nuclear (Russell & Becker, 1968).

The results of preliminary experiments using Nonidet P-40 (Boron, Scharff & Robbins, 1967) indicated that, although nuclear-cytoplasmic separation was apparently achieved, this detergent affected the nuclei in some way deleterious to assembly activity, which confirms the previous impression that lipid or membrane-containing structures might be of some importance in the in vitro assembly activity.
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Table 4. Assembly activity in vitro of whole cell, nuclear and cytoplasmic extracts

<table>
<thead>
<tr>
<th>Extract of E+A cells taken 14 hr after infection</th>
<th>Extract of infected [HT] E-A cells taken 42 hr after infection</th>
<th>Radioactivity in virus density fractions</th>
<th>Assembly (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected, whole cells</td>
<td>Whole cells</td>
<td>4300†</td>
<td>100</td>
</tr>
<tr>
<td>Infected, nuclear fraction</td>
<td>Whole cells</td>
<td>3300</td>
<td>78</td>
</tr>
<tr>
<td>Infected, cytoplasmic fraction</td>
<td>Whole cells</td>
<td>600</td>
<td>14</td>
</tr>
<tr>
<td>Uninfected, whole cells</td>
<td>Whole cells</td>
<td>130</td>
<td>3</td>
</tr>
<tr>
<td>Uninfected, nuclear fraction</td>
<td>Whole cells</td>
<td>88</td>
<td>2</td>
</tr>
<tr>
<td>Uninfected, cytoplasmic fraction</td>
<td>Whole cells</td>
<td>100</td>
<td>2</td>
</tr>
</tbody>
</table>

* Values represent average counts from three experiments determined after mixtures were incubated for 30 min. at 32°.
† This mixture was the positive control used to calculate % assembly.

Table 5. Infectivity titrations of mixtures used in in vitro assembly assays

Source of extracts (time of collection after infection) | p.f.u./ml. *
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected E+A cells (48 hr) plus uninfected E+A cells (14 hr)</td>
<td>8 × 10⁹ to 2.1 × 10¹⁰</td>
</tr>
<tr>
<td>Infected E+A cells (14 hr) plus uninfected E+A cells (14 hr)†</td>
<td>1.2 to 3.0 × 10⁶</td>
</tr>
<tr>
<td>Infected E-A cells (42 hr) plus uninfected E+A cells (14 hr)†</td>
<td>2.8 to 6.0 × 10⁶</td>
</tr>
<tr>
<td>Infected E-A cells (42 hr) plus infected E+A cells (14 hr)†</td>
<td>8.0 × 10⁷ to 1.2 × 10⁹</td>
</tr>
<tr>
<td>Infected E-A cells (42 hr) plus infected E+A cells (14 hr)‡</td>
<td>1.8 to 9.0 × 10⁶</td>
</tr>
</tbody>
</table>

* Values represent range of p.f.u./ml. of three separate experiments.
† Incubated for 20 min. at 32°.
‡ Incubated for 20 min. at 4°.

Some properties of the material assembled in vitro

Of crucial importance in this in vitro assembly system is the question of the nature of the assembled product. Is this merely an aggregate of virus and cellular materials with the characteristic density of adenovirus, or does the system actually assemble virions? Infective virus could, of course, be detected in both of the extracts used in the system. However, when these mixtures were incubated together under the optimum conditions, there was a significant increase in the virus infectivity (Table 5). As might be expected, moreover, the efficiency of the in vitro assembly was very much less (less than 1%) than that of the corresponding virus formed in vivo. In other experiments where the assembled virus was further purified by caesium chloride centrifugations, there was a 1.5 to 2.0 log. increase in the infective particles when compared to the control materials. The quality of the assembled product was examined by considering its sensitivity to deoxyribonuclease. Previous experiments (Russell et al. 1967b) had shown that virus which had lost its pentons and surrounding
hexons by heating at 56° was almost totally sensitive to the enzyme, and it might be assumed that malformed virus particles would be at least partially sensitive to the enzyme. The assembled product appeared to be substantially insensitive to the enzyme, although there was some suggestion of the presence of a small amount of deoxyribonuclease-sensitive material (Fig. 7).

Preliminary studies using the electron microscope have shown that considerable amounts of complete virus particles as well as malformed particles can be seen in the relevant fractions. The significance of these results is now being assessed using particle counting techniques.

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**DISCUSSION**

The aim of the present investigation is to obtain some understanding of the mechanisms involved in the assembly and maturation of adenoviruses. We have been able to demonstrate that assembly of infectious adenovirus from cellular extracts can take place in vitro. The controls were of crucial importance in all these experiments, and in most investigations two control mixtures were used. The first mixture contained extracts of labelled cells infected in E−A medium together with extracts of unlabelled, uninfected cells grown in E+A medium. The second control mixture consisted of extracts of labelled, uninfected cells grown in E−A medium plus either buffer or extracts of unlabelled, infected cells grown in E+A medium. These two controls thus accounted for the possibility that some virus could have...
be made in the arginine-free conditions and for non-specific binding of virus or cellular DNA during the \textit{in vitro} incubation. The major difficulty with the system is that it requires a relatively lengthy assay procedure involving two density gradient centrifugations, and since the controls are always run in parallel, the number of parameters that could be investigated in any one experiment was rather limited. So far, attempts to simplify the assay, while retaining sensitivity, have been unsuccessful. Infectivity assays are satisfactory but more time-consuming. 

The \textit{in vitro} system is obviously still relatively crude, involving cell extracts which may contain partially intact biological systems, particularly of a membranous nature. Thus, it is interesting that these preliminary results show that the assembly process is sensitive to a mild non-ionic detergent and to a fluorocarbon suggesting that a lipid containing membrane might be of some significance in the maturation of the virus. Membranous structures have been shown to be of importance in the replication of poliovirus (Caliguiri & Tamm, 1969), and although the latter virus matures in the cytoplasm and adenovirus in the nucleus, it is likely that the replication and assembly processes are of a similar nature for each virus. One surprising finding was the relative speed at which the assembly process takes place \textit{in vitro} compared to the \textit{in vivo} process (Horwitz, Scharff & Maizel, 1969). Within 15 min. at 32\degree, the various virus precursors had been assembled into infective virus, implying that once the specific virus proteins were available the process was possibly one of independent self-assembly rather than a system depending on protein synthesis or a source of energy. Experiments designed to examine these aspects are under way. In many ways, the properties of the \textit{in vitro} system resemble that described by Phillips (1969) for the assembly of poliovirus empty capsids \textit{in vitro}. In that case, the assembly was complete after 10 to 15 min. at 37\degree, and pre-treatment of the cytoplasmic extracts with metabolic poisons or inhibitors of protein synthesis had no effect on assembly activity.

Previous serological studies (Russell & Becker, 1968) had suggested that a critical step in the maturation of the virus involved a ‘late’ component of the P antigen. Since this particular antigen was apparently not produced in the absence of exogenous arginine, it was suggested that it could be related to the arginine-rich internal components of the virus (Russell \textit{et al}. 1968). The results of the present investigation tend to support these suggestions, e.g. the assembly factor was found to be thermolabile and to be present in the nuclei of infected cells. Moreover, the time of appearance of assembly-rich materials coincided with the time of synthesis of the ‘late’ component of the P antigen (Hayashi & Russell, 1968). Thus, the times after infection when the \textit{in vitro} assembly activity of extracts was greatest corresponded generally to the times when the synthesis of assembly or maturation materials would be expected to be produced in a permissive system \textit{in vivo}, namely after the start of virus DNA synthesis and after the beginning of, or concomitantly with, the synthesis of virus capsid and internal components.

Extracts of infected E + A cells collected up to 12 hr after infection – times before the synthesis of virus capsid antigens – had little or no \textit{in vitro} assembly activity. The diminished \textit{in vitro} assembly activity of the extracts taken at times after 18 hr suggests that this may be a reflexion of what is happening in the infected cells at these times, namely the maturation or assembly materials produced are being used exclusively in the processes of assembling virus and there is little available in the extracts for use in the \textit{in vitro} system. Another possibility is that these assembly factors are simply not being produced in a functional form at these later times. Recent cytochemical studies have shown that the morphological features of this ‘late’ P antigen can be related to the characteristic changes in the pattern of basic proteins in the infected cell (Russell, Brodaty & Armstrong, 1970). We hope to be able to
characterize the assembly or maturation factor further (using the in vitro assay system) by purification and separation of the components in the cell extracts. Since we are now also able to separate the internal virus nucleoprotein containing only one very arginine-rich protein in association with the virus DNA (Russell, McIntosh & Skehel, 1970), it should be possible to test directly the ability of this arginine-rich protein to assemble the infectious particle. The in vitro assembly system also provides a means of examining some of the factors associated with incomplete adenovirus infections. Therefore, it may be possible to show that the ultimate fate of an infected cell, i.e. whether it is killed or latently infected or transformed, might depend, among other things, on its ability to produce factors which can assemble the infectious virus.

Finally, while self-assembly has been described for both bacterial and plant viruses (Kushner, 1969), to our knowledge the in vitro assembly of infectious virus in an animal virus system has not been previously described.

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REFERENCES


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