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# Intermediates in Adenovirus Assembly

BERIT EDVARDSSON, EINAR EVERITT, HANS JÖRNVALL[1] LENNART PRAGE, AND LENNART PHILIPSON[2]*

*Department of Microbiology, The Wallenberg Laboratory, Uppsala University, Uppsala, Sweden*

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Three intermediates in adenovirus assembly have been defined; nuclear intermediates, young virions, and mature virions. The nuclear intermediates are fragile and heterogenous in size (550S-670S) and withstand separation on ficoll gradients but fall apart upon CsCl gradient centrifugation unless prefixed with glutaraldehyde. They contain both capsid and core structures, and the core structures are preferentially released during purification in CsCl. The precursor polypeptides pVI and pVII are present in the intermediates without any corresponding mature polypeptide. The young virions (Ishibashi and Maizel, 1974) are stable and preferentially confined to the nuclei after cell fractionation. They contain both uncleaved precursor polypeptides and their cleavage products. The mature virions accumulate in the cytoplasm during cell fractionation and contain the final mature polypeptides. Pulse-chase labeling kinetics, focusing on the precursor polypeptides, suggest that these three classes participate in assembly of adenovirus. Tryptic peptide maps establish that polypeptide pVI is the precursor of polypeptide VI, but only a small fraction of polypeptide 26K can in vivo account for polypeptide VIII.

In productive infection with adenoviruses, several classes of particles are produced that differ from mature virions in polypeptide composition and buoyant density in CsCl gradients. One class consists of empty capsids without DNA and is referred to as empty particles or top component (12, 15, 19, 21, 23). They lack polypeptides V and VII, corresponding to the two core proteins of the virion, and contain some polypeptides not present in the virion (12, 19, 24). A second class, which bands close to the empty particles in CsCl gradients, contains fragments of DNA (12, 19) and has been referred to as incomplete particles (19). Several different classes of incomplete particles, varying in DNA content, have been observed (6, 21, 25). All these classes of particles have been proposed to represent intermediates in virus assembly (12, 24), mainly based on pulse-chase labeling kinetics of the different particles in vivo. Additional support for this hypothesis was provided when three polypeptides of the virion were shown to be processed from larger precursor polypeptides. Anderson et al. (1) demonstrated by tryptic peptide maps that the major core polypeptide VII (molecular weight, 18,500) is derived from a precursor pVII that has a molecular weight of 21,000 and suggested that the hexon-associated polypeptide VI (molecular weight, 24,000) likewise is derived from a precursor pVI that is 27,000 in molecular weight. By comparing the immunoprecipitation of polypeptides synthesized in vivo and in an in vitro translation system, Öberg et al. (17) confirmed these relationships and in addition suggested that polypeptide VIII (molecular weight, 13,000) is derived from a large precursor pVIII with a molecular weight of 26,000. All these precursor polypeptides are present in empty and incomplete particles (12, 21, 24), again suggesting a precursor-product relationship between the particle classes and mature virions. It is, however, difficult to establish unequivocally the assembly pathway of adenoviruses in an in vivo system since the major capsid proteins are present in a large excess pool, and only 5 to 20% are incorporated in mature virions (11, 27). Several of the true intermediates may furthermore be fragile and not resist purification procedures involving CsCl gradient centrifugation.

The present investigation demonstrates that some of the particle classes isolated by CsCl gradient centrifugation are artefacts when mild methods to isolate and characterize the intermediates are utilized. The intermediate particles are heterogeneous, with sedimentation rates from 550S to 670S and do not resist banding in CsCl gradients without fixation by glutaraldehyde. By focusing on the precursor poly-

[1] Present address: Department of Chemistry, Karolinska Institutet, Stockholm, Sweden.

[2] Present address: Department of Microbiology, Biomedical Center, Box 581, 75123 Uppsala, Sweden.

peptides that are not present in vast excess, it has been possible to demonstrate a precursor-product relationship between the intermediates and virions by pulse-chase labeling kinetics. In addition there appears to be two classes of virions, one corresponding to young virions (12), which are assembled in the nuclei, and a second class, which during cell fractionation is accumulating in the cytoplasm and represents the mature virion. The assembly process appears to take at least 3 to 5 h for completion.

## MATERIALS AND METHODS

**Virus.** The prototype strains of adenovirus types 2 and 3 (Ad2 and Ad3) were used, and the strains were originally obtained from R. Huebner, National Institutes of Health, Bethesda, Md., and A. Svedmyr, The Municipal Bacteriological Laboratory of Stockholm, Sweden, respectively.

**Cells.** HeLa cells were grown in spinner culture with Eagle spinner medium (8) containing 7% calf serum. KB cells were grown as monolayers in bottles or 35-mm petri dishes in Eagle minimal essential medium containing 15% calf serum.

**Infection and labeling conditions.** Cells were always infected at a multiplicity of 2,000 particles/cell as described by Everitt et al. (10). HeLa cells, mainly used for pulse-chase experiments, were centrifuged at 14 h after infection at 37 C for 5 min at 3,500 × *g* and suspended at a cell density of $2 \times 10^7$ cells/ml in Eagle spinner medium containing only 2.5% of the normal methionine concentration (0.75 μg/ml) but with 7% calf serum. The cells were labeled with 200 μCi of [$^{35}$S]methionine per ml for 15 min, and the label was chased by the addition of cold methionine to 5× the normal concentration. The cells were centrifuged again and resuspended in Eagle spinner medium with the high concentration of methionine.

Labeled virus was prepared in spinner cultures of HeLa cells. The medium was then changed at 8 h after infection, using 2.5% of the normal methionine concentration and 1 μCi of [$^{35}$S]methionine per ml. Cells were harvested at 40 h postinfection. KB cells in monolayers used for isolation of intermediates were labeled at 15 to 20 h after infection using Eagle minimal essential medium containing 2.5% of the normal methionine concentration (0.375 μg/ml), 10% calf serum, 10 μCi of [$^{3}$H]thymidine per ml, and 8 μCi of [$^{35}$S]methionine per ml.

**Virus purification.** Cells were centrifuged at 1,500 × *g* for 30 min, suspended in 0.01 M Tris-hydrochloride (pH 8.1) and frozen at −20 C. After thawing, the cell suspension was sonically treated at 0 C with a 0.5 volume of freon for 30 s, and the mixture was centrifuged at 16,000 × *g* for 10 min. The supernatant was layered onto preformed CsCl gradients ranging from 1.2 to 1.4 g/ml and centrifuged at 52,000 × *g* for 1 h. The virus band was collected and repurified on a preformed CsCl gradient (1.28 to 1.35 g/ml) at 115,000 × *g* for 16 h. The virus suspension was dialyzed against 0.01 M Tris-hydrochloride (pH 8.1) and sedimented for 60 min at 150,000 × *g* through 30% sucrose layered onto a CsCl (1.4 g/ml) cushion. Finally, the virus was dialyzed against 0.01 M Tris-hydrochloride (pH 8.1) and stored in aliquots at −20 C. The CsCl and sucrose solutions were all buffered with 0.01 M Tris-hydrochloride (pH 8.1). Incomplete and empty particles from Ad3 were purified by centrifugation in a self-generating CsCl gradient with an initial density of 1.33 g/ml using a Ti50 angle rotor at 80,000 × *g* for 16 h. The centrifugation was repeated twice and followed by a sedimentation through 30% sucrose. The CsCl gradients used for glutaraldehyde-fixed material contained 0.5% Brij-58.

**Cell fractionation.** The cells were washed with cold phosphate-buffered saline and swelled in reticulocyte buffer (0.01 M NaCl–1.5 mM $MgCl_2$–0.01 M Tris-hydrochloride pH 7.4) for 10 min on ice at a cell density of $6 \times 10^6$ per ml. Nonidet P-40 was added to a final concentration of 0.5%, and the cells were broken by seven strokes in a Dounce B homogenizer. The nuclei were sedimented at 1,000 × *g* for 3 min and suspended in 0.05 M Tris-hydrochloride (pH 8.1) with 2 mM EDTA. The supernatant, referred to as the cytoplasmic fraction, was made 20 mM with regard to EDTA to dissociate polyribosomes, and both fractions were sonically treated at 0 C for 15 s. After sonic treatment, phenylmethylsulfonylfluoride, a proteolysis inhibitor, was added to a final concentration of 2 mM, and both extracts were centrifuged at 16,000 × *g* for 10 min. The supernatants were analyzed on ficoll gradients.

**Ficoll gradient centrifugation.** Ficoll (Pharmacia, Uppsala, Sweden) gradients were used for the isolation of virus and intermediate particles from cell extracts. Gradients containing 5 to 12.5% ficoll in 0.02 M Tris-hydrochloride (pH 8.5) with 1 mM EDTA were centrifuged at 85,000 × *g* for 105 min in an SW27 rotor. The gradients were fractionated from the bottom and analyzed for radioactivity and infectivity.

**Infectivity control.** Infectivity of the virus was assayed by the fluorescent focus assay in KB cells grown in petri dishes (18), and the titer was expressed in fluorescent focus units.

**SDS-polyacrylamide gel electrophoresis.** Samples were precipitated overnight at 4 C with 10% trichloroacetic acid. The precipitate was washed twice with 5% trichloroacetic acid and twice with cold acetone (−20 C). The dry precipitate was dissolved in a sample buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 5% 2-mercaptoethanol (14) and heated to 100 C for 5 min. The acrylamide solutions were prepared as described by Maizel (14) with the following modifications. Exponential slab gels ranging from 10 to 18% polyacrylamide were cast as described by McGuire et al. (13). They were 1.5 mm thick and 70 mm long in a total volume of 10 ml. The 18% acrylamide solution was made in 50% (vol/vol) glycerol, and the mixing volume was 5 ml. A mixture of 5% acrylamide and 0.6% agarose was used as spacer gel. Electrophoresis was carried out for 4.5 h at 100 V in a GE-4 apparatus from Pharmacia. The gels were stained with 0.5% Coomassie brilliant blue R250 in 10% acetic acid and 30% methanol at room temperature and destained in the same solvent.

The polypeptides, which were analyzed by tryptic

peptide mapping, were isolated on 10 or 13% polyacrylamide slab gels. Unlabeled protein (1 mg) was applied and subjected to electrophoresis at 100 V for 2 h. After staining, the polypeptides were reisolated on new gels, and the material in the gel slices was recovered by electrophoresis into a dialysis tubing, using the same electrophoresis buffer and applying 50 V overnight. The recovered polypeptides were subsequently labeled by acetylation.

**Acetylation of polypeptides.** The method for polypeptide acetylation has been described by Cuatrecasas (7). The volume of the sample was around 200 μl. The polypeptides were dialyzed against 0.1 M sodium bicarbonate buffer (pH 8.8) at 4 C overnight. A 5-μCi sample of [$^{14}$C]acetic anhydride (30 mCi/mmol) in 5 μl of benzene was added, and the mixture was vigorously stirred and kept on ice for 90 min. An equal volume of 1.2% agarose in double-strength SDS-sample buffer was added, and the mixture was applied on a slab gel. The labeled polypeptides were eluted from the gel as described above and precipitated with 20% trichloroacetic acid at −20 C overnight. The pellets were washed twice with acetone (−20 C) and dried.

**Glutaraldehyde fixation.** Samples were fixed for 10 min at 0 C with 5% glutaraldehyde (3).

**Preparation of peptide maps.** The precipitated polypeptides were digested directly in the tubes by the addition of TPCK-trypsin (25 μg in 5 μl of 1 mM HCl) and 50 μl of 0.1 M ammonium bicarbonate followed by incubation at 37 C for 4 h. The digests were lyophilized and dissolved in 150 μl of distilled water, and the radioactivity of the samples was counted. The recovery was around 50% of the initial radioactivity, and all peptides were soluble after digestion. Samples from each digest were then resolved by two-dimensional thin-layer electrophoresis and chromatography on plates (20 by 20 cm) coated with 0.5-mm Whatman CC41 cellulose or on precoated cellulose plates (10 by 10 cm) from Merck. The first dimension was electrophoresis (300 V) in pyridine-acetate buffer at pH 6.5 (acetic acid-pyridine-water, 3:100:900, vol/vol/vol) for the small plates (25 min), and at pH 5.5 (acetic acid-pyridine-water, 7:20:970, vol/vol/vol) for the large plates (75 min). The second dimension was ascending chromatography in *n*-butanol-acetic acid-water-pyridine (15:3:12:10, vol/vol) for all plates. Radioactive spots were detected by autoradiography (Fuji medical X-ray films, exposed for 2 to 3 weeks).

**Autoradiography.** The polyacrylamide gels were analyzed by autoradiography by one of the following procedures.

(i) The gel was dried under vacuum at 64 C on a Whatmann 3 MM filter paper and exposed to a Kodak X-ray film (XG/14) for 3 to 5 days at room temperature.

(ii) The gels were treated with 2,5-diphenyloxazol as described by Bonner and Laskey (4). The gels were soaked in dimethylsulfoxide 2× 30 min, then immersed in 4 volumes of 22.2% (wt/vol) 2,5-diphenyloxazol in dimethylsulfoxide for 3 h, and finally immersed in a large volume of water for 1 h to exchange the dimethylsulfoxide. The vacuum-dried gel was then exposed to a Kodak RP Royal "X-omat" film for 1 to 3 days at −70 C. Films were developed with a Kodak X-ray film developer (Kodak D-19) for 5 min at room temperature.

**Radioisotopes and counting procedures.** [$^{35}$S]methionine (200 Ci/mmol) and [$^{3}$H]thymidine (43 Ci/mmol) were obtained from Radiochemical Centre (Amersham, Bucks, England). [$^{14}$C]acetic anhydride (30 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.). The radioactive material was sampled on Whatmann 3 MM filter papers, precipitated with cold 10% trichloroacetic acid, washed twice with 5% trichloroacetic acid and twice with 70% ethanol, and air-dried. The filters were counted in a toluene-based scintillation liquid. Liquid samples were analyzed in Instagel (Packard).

## RESULTS

**Characterization of particles from Ad3.** Indirect evidence has been presented (12, 24) suggesting that the empty and incomplete particles of Ad2 and Ad3 constitute intermediates in virus assembly. In vitro translation of adenovirus RNA has furthermore revealed that at least three of the virion polypeptides (VI, VII, and VIII) are generated from larger precursors (pVI, pVII and pVIII, respectively) (2, 17). It was therefore first necessary to establish whether empty and incomplete particles of adenoviruses contain these precursor polypeptides. To obtain good yields of the different particles Ad3 was used, and the material was purified on CsCl as described above. Virus and incomplete and empty particles from Ad3, labeled with [$^{3}$H]thymidine and [$^{35}$S]methionine for 8 to 40 h after infection, were examined on self-generating CsCl gradients with an initial density of 1.33 g/ml. One gradient contained a mixture of all three particles. Figure 1 shows that the incomplete and empty particles band at densities close to each other, at 1.30 and 1.29 g/ml, respectively, but only the former contained [$^{3}$H]thymidine. Virus has a buoyant density of 1.34 g/ml under these conditions. The polypeptides of virions, incomplete particles, and empty particles were analyzed on exponential SDS-polyacrylamide slab gels. Polypeptides V and VII, which constitute the core proteins, are not present in either empty or incomplete particles (Fig. 2). Both particles contain, however, polypeptides with molecular weights of 27,000 (27K) and 26,000 (26K), corresponding to the size of the precursors to polypeptides VI and VIII, respectively. In empty particles there is a relative abundance of polypeptide 27K, whereas the proportion of 27K to 26K is changed in favor of the 26K in the incomplete particles. The ultimate products of processing, polypeptide VI and VIII, are virtually absent in empty particles. There are several additional polypeptides in empty and incomplete particles (50K, 40K, 33K, and 32K) not present in the

virion. When large amounts of protein are analyzed the 100K polypeptide may also be observed.

**Tryptic peptide maps.** Polypeptides VI and VIII were purified from unlabeled Ad3 virus by SDS-polyacrylamide gel electrophoresis on a 13% preparative slab gel. The 27K and 26K polypeptides were isolated from purified incomplete and empty particles in the same way on 10% SDS-polyacrylamide gels. All four polypeptides were reisolated on SDS gels and labeled with [$^{14}$C]acetic anhydride as described above. Figure 3 shows that all polypeptides consist of single bands after acetylation.

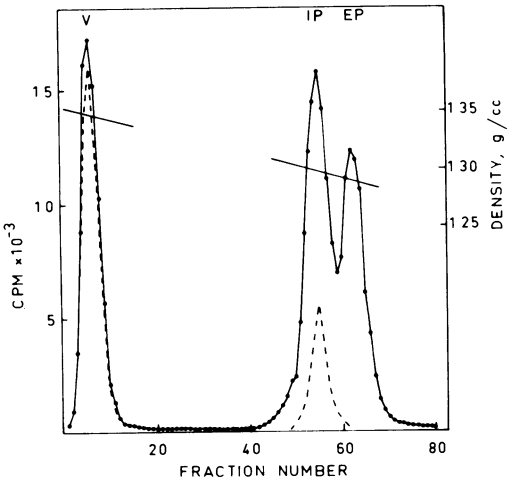



FIG. 1. *Isodensity gradient centrifugation of the three classes of Ad3 particles. An artificial mixture of empty (EP) and incomplete (IP) particles as well as virions (V) were sedimented in the same gradient and generated in an angle rotor as described in Materials and Methods. Symbols: - - -, [$^{3}$H]thymidine; ——, [$^{35}$S]methionine.*

Two-dimensional peptide analyses by cellulose thin-layer electrophoresis and chromatography were performed on several samples from the tryptic digest of each of the polypeptides acetylated with [$^{14}$C]acetic anhydride. The autoradiographs obtained from 50% of the material from each polypeptide digest, applied to 20 by 20 cm plates, are shown in Fig. 4A and B for polypeptides VI and 27K and in Fig. 4C and D for polypeptides VIII and 26K.

It is clear that the autoradiographs obtained from polypeptides VI and 27K are almost identical (Fig. 4A and B), including both the positions and relative intensities of the spots. The patterns indicate that most or all of these peptides must be derived from the same common primary structure. The peptide maps for the 26K and VIII polypeptides show, however, no obvious relationships. Instead, the picture of 26K is reminiscent of that obtained from 27K and

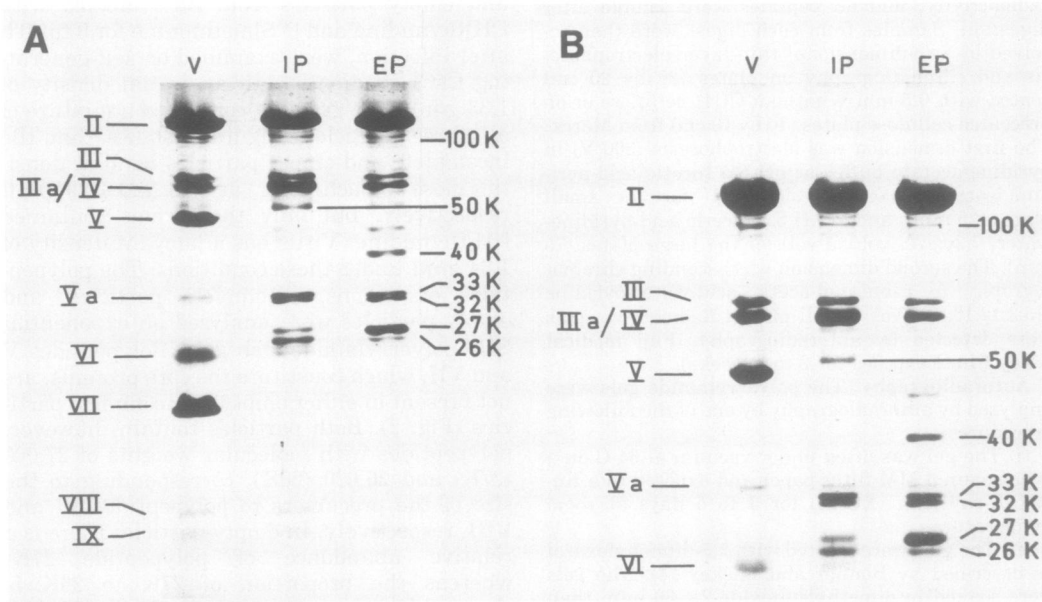



FIG. 2. *SDS-polyacrylamide gel electrophoresis of the polypeptides of the three particle classes of Ad3. Virions (V), incomplete (IP), and empty (EP) particles were analyzed on 13% (A) and 10% (B) slab gels. The stained virion polypeptides are designated according to Everitt et al. (9) and the non-virion polypeptides are identified by their molecular weight × $10^3$ (K).*

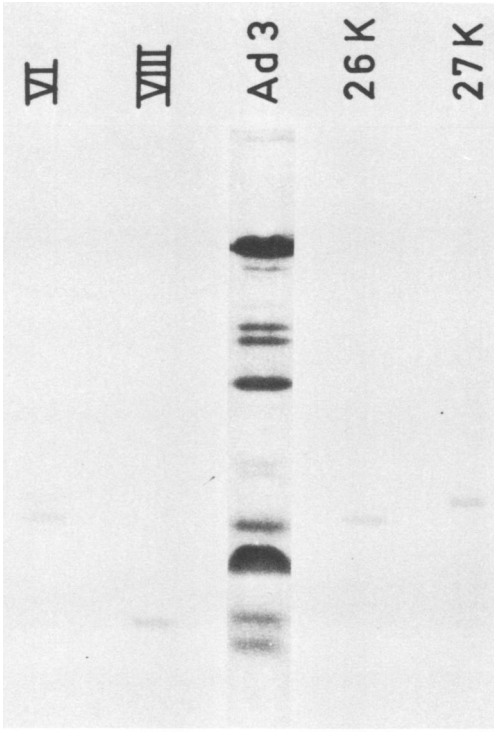



FIG. 3. *Identification of purified acetylated polypeptides on SDS-polyacrylamide gels. The four polypeptides 27K, 26K, VI, and VIII were isolated on 10 or 13% slab gels, acetylated with [$^{14}$C]acetic anhydride, and analyzed on 10 to 18% slab gels together with a virion marker. The designation of the polypeptides is explained in the legend to Fig. 2, and an autoradiogram of the gel is shown.*

VI, although some of the relative spot intensities are different. This finding was consistent when the 26K was isolated from either a mixture of incomplete and empty particles or from incomplete particles. In both cases a simple contamination of 26K during purification by the closely migrating 27K band seems excluded, as shown by SDS-gel electrophoresis (Fig. 3) of the purified products before peptide mapping. The results suggest that only a fraction of the 26K polypeptide can constitute the precursor to polypeptide VIII. The data, however, establish chemically that polypeptide 27K is the precursor to virion polypeptide VI, and the precursor polypeptide may therefore be referred to as pVI as previously demonstrated by immunological techniques (17). At most only a fraction of the 26K polypeptide labeled in vivo seems to be the pVIII entity revealed immunologically, and the majority of the material in this position is probably an additional intermediate in the maturation of polypeptide VI (cf. Fig. 4A and D).

**Isolation of assembly intermediates by ficoll gradients.** Once it was established that some polypeptides in the protein moiety of immature particles were precursors to virion polypeptides, we attempted to isolate particles formed during assembly with mild methods designed to prevent degradation during isolation. For this purpose we chose Ad2, which does not accumulate intermediates in assembly. Pulse-chase analysis of the assembly pathway was therefore facilitated. Zonal centrifugation in ficoll gradients gave the best recovery of unassembled particles.

KB cells in bottles were infected with Ad2 and labeled with [$^{3}$H]thymidine and [$^{35}$S]methionine at 15 to 20 h after infection. The cells were fractionated into cytoplasm and nuclei. Extracts from each fraction of $2 \times 10^7$ cells were layered on ficoll gradients. Mock-infected cells were analyzed in the same way with [$^{35}$S]methionine as the label. Mock-infected cells contain no [$^{35}$S]methionine-labeled particles sedimenting into the gradients under these conditions (Fig. 5B and D). Control experiments with [$^{3}$H]thymidine-labeled mock-infected cells showed that no DNA label sediments with S values larger than 400S under these conditions (data not shown). On the other hand, when extracts from infected nuclei were analyzed two peaks were seen, one sedimenting fast as intact virions at 750S and labeled both in the DNA and in the protein moieties. The slower sedimenting peak appeared to contain less DNA, and it was heterogeneous in sedimentation rate, from 550S to 670S. This second peak is referred to as the intermediate (IM) peak. The gradient with the cytoplasmic extract (Fig. 5C) shows only low radioactivity in the IM peak region and shows a peak corresponding to the virus peak of the nuclear extract, although the sedimentation rate was constantly slightly faster for the cytoplasmic virus. All gradients contain large amounts of labeled material at the top, which is not shown in the figures. Reconstruction experiments with adenovirus cores generated with pyridine (20) revealed that free cores sedimented at the top of the gradient (data not shown). The material in the peak fractions was further analyzed on CsCl gradients and by polyacrylamide gel electrophoresis.

**Density of the particles from ficoll gradients.** The IM peak from nuclear extracts (Fig. 5A) contained fragile particles that fell apart when analyzed in CsCl gradients without fixation, as shown below. Even when left in ficoll at

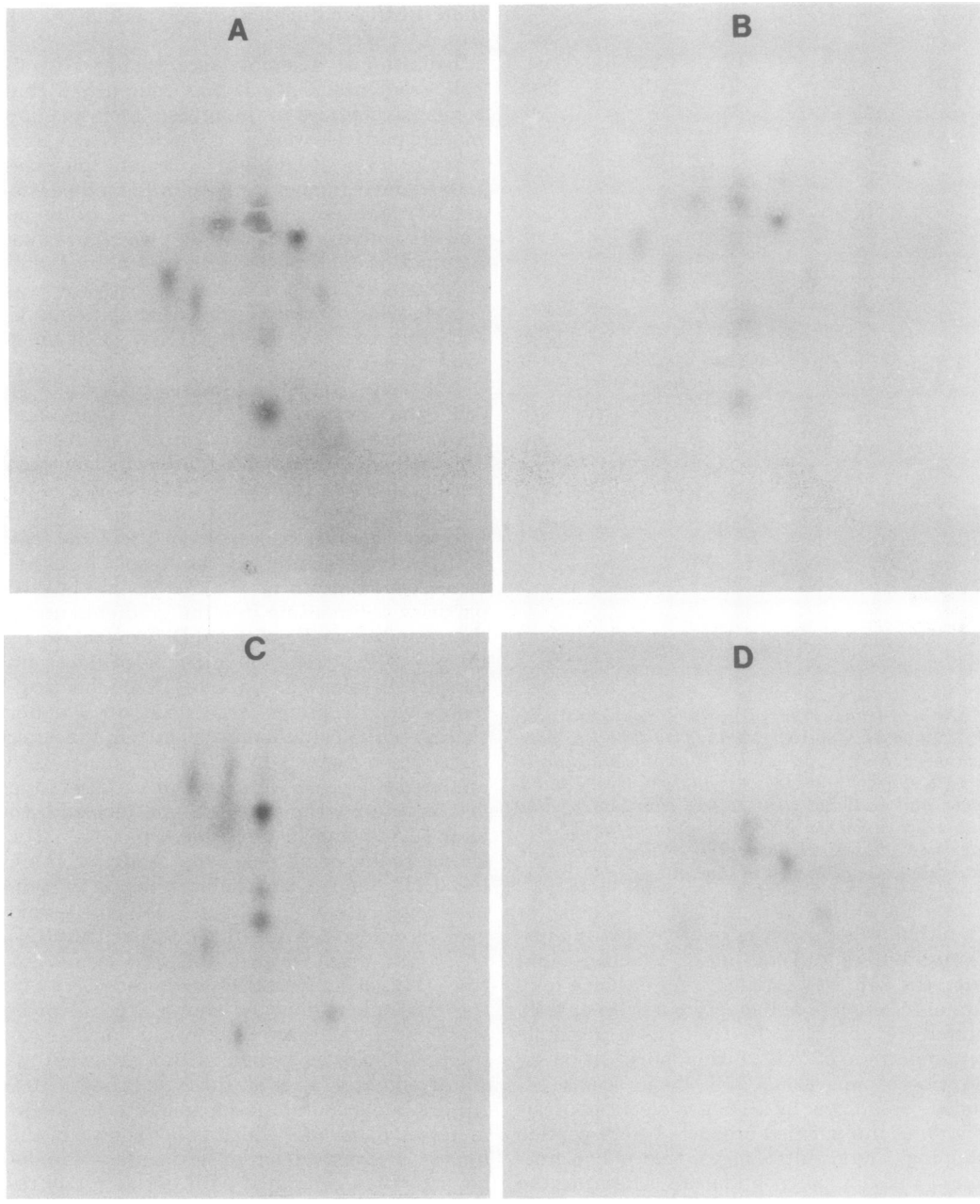



FIG. 4. *Tryptic peptide maps of acetylated polypeptides. The polypeptides were digested by trypsin and separated in two dimensions as described in Material and Methods. (A) Polypeptide VI; (B) polypeptide 27K; (C) polypeptide VIII; (D) polypeptide 26K.*

4 C most of the material in the IM fraction degraded within 48 h. The material from the ficoll gradients was therefore fixed with glutaraldehyde and analyzed on CsCl gradients containing 0.5% Brij-58. Figures 6A and B show the patterns obtained when the virus materials from cytoplasm and nuclei were analyzed. Both contain only one band at the density of virions. The pattern of material from the IM peak (Fig. 6C) shows three distinct peaks in CsCl, one

corresponding to the density of virions but with a more heterogeneous density distribution. The peak with low density contains almost no DNA label, and since this material shows a density of 1.30 g/ml it probably represents empty capsids. The heavy material with a density of 1.39 g/ml has a high ratio between the DNA and protein label. This fraction may contain fragments of a core structure, since pyridine cores fixed with glutaraldehyde band at this density (data not shown). When individual fractions of the IM peak in ficoll gradients were analyzed after glutaraldehyde fixation, the high density fraction increased at lower sedimentation rates, suggesting that the core-like structure was more abundant at low S values. Since artificial

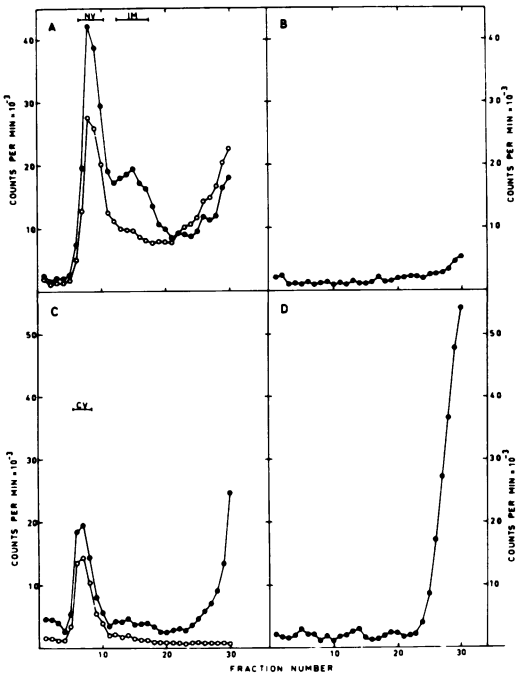



FIG. 5. *Ficoll gradient centrifugation of nuclear and cytoplasmic extracts from adenovirus- and mock-infected KB cells. The cells were labeled from 15 to 20 h after infection with [³H]thymidine and [³⁵S]methionine, and nuclear and cytoplasmic extracts were layered on top of 5 to 12.5% ficoll gradients as described in Material and Methods. The gradients were centrifuged at 85,000 × g for 105 min. The regions corresponding to nuclear virus (NV), intermediates (IM), and cytoplasmic virus (CV) were separately pooled as indicated. (A) Nuclear extract from infected cells; (B) nuclear extract from mock-infected cells; (C) cytoplasmic extract from infected cells; (D) cytoplasmic extract from mock-infected cells. Symbols: ○, [³H]thymidine; ●, [³⁵S]methionine.*

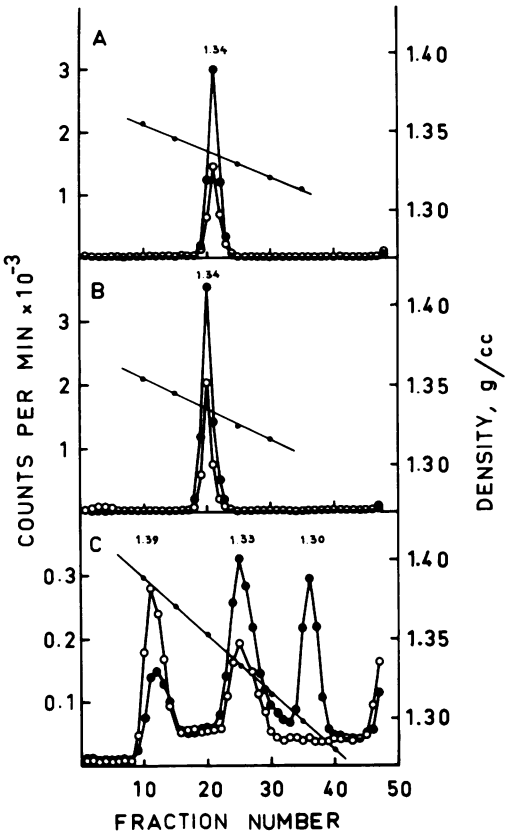



FIG. 6. *Isodensity gradient centrifugation of glutaraldehyde-fixed particles from Ad2 isolated from ficoll gradients. Material from the ficoll gradients were pooled as indicated in the legend to Fig. 5, fixed with glutaraldehyde, and sedimented to isodensity in CsCl gradients. (A) Nuclear virus; (B) cytoplasmic virus; (C) material from the IM peak. Symbols: ○, [³H]thymidine; ●, [³⁵S]methionine.*

cores sediment at the top of the gradient (see above), these findings suggest that the core structure is loosely associated with capsids in the intermediates.

**Polypeptide pattern of the particles.** Material from the three peaks observed in ficoll gradients was further analyzed on SDS-polyacrylamide gels. The fractions indicated in Fig. 5 were pooled, trichloroacetic acid precipitated, and analyzed on exponential slab gels. An autoradiograph of these gels is shown in Fig. 7. When the cells were labeled with [³⁵S]methionine from 15 to 20 h after infection, the virion fraction from nuclear and cytoplasmic extracts showed similar polypeptide patterns. All virion polypeptides are labeled and, in addition, especially in the nuclear fraction, the precursor

polypeptides pVI and pVII are strongly labeled. In the IM material virion polypeptides VI, VII, and VIII contained only low levels of radioactivity. The precursors pVI, pVII, and 26K are strongly labeled, and two more polypeptides can be observed banding in the position corresponding to molecular weights of 100,000 (100K) and 50,000 (50K), respectively. This pattern is

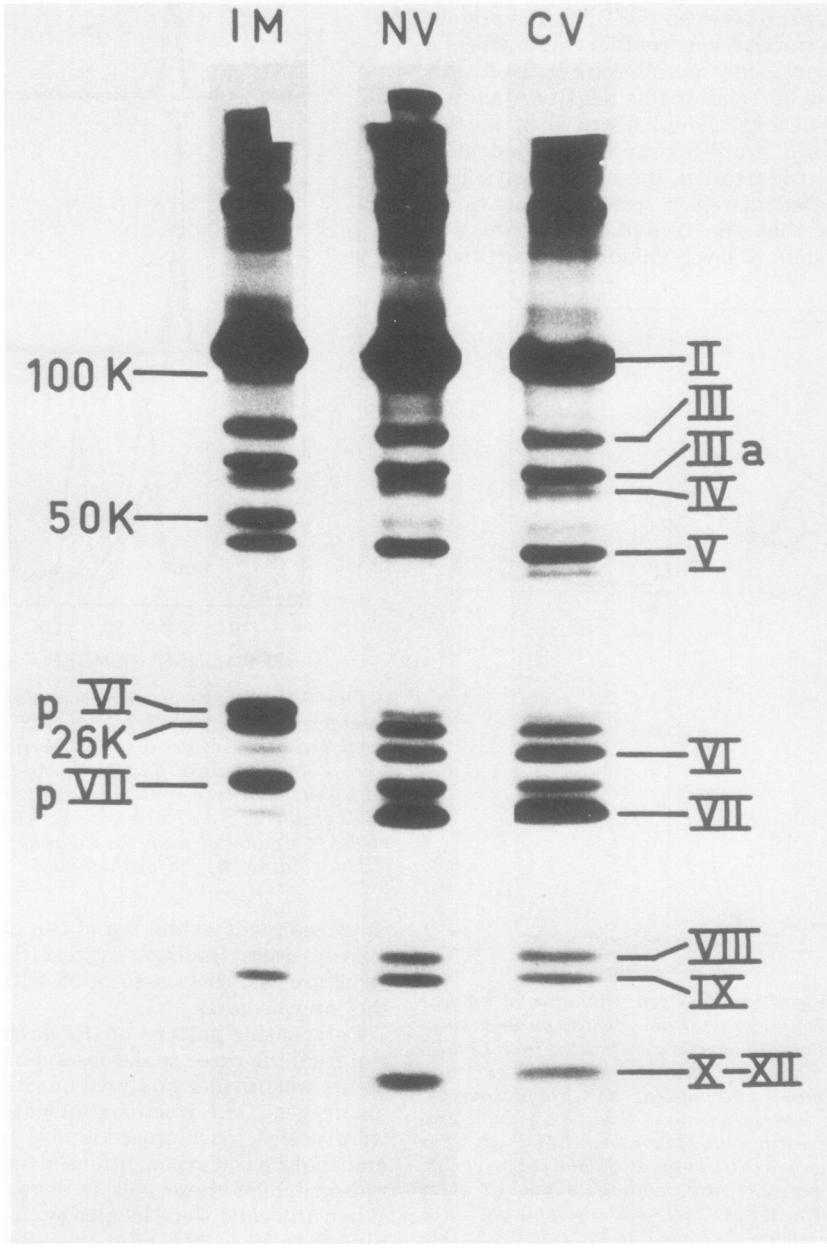



FIG. 7. *SDS-polyacrylamide gel electrophoresis of the Ad2 particle classes from the ficoll gradients. The pooled fractions indicated in Fig. 5 corresponding to the intermediates (IM), nuclear virus (NV), and cytoplasmic virus (CV) were acid precipitated and analyzed on 10 to 18% slab gels. An autoradiogram of the gel is shown. The designation of polypeptides is explained in the legend to Fig. 2. The gel strips contain considerable radioactivity at the top since the samples contained [$^3$H]thymidine in the DNA moiety.*

different from that observed of stained polypeptides in empty particles of Ad3 (Fig. 2), where polypeptides pVII and V are not observed. It is possible that polypeptides V and pVII are constituents of the heavy peak in the CsCl gradients (Fig. 6C). The topography of the 100K and the 50K polypeptides is still unknown.

To make a direct comparison between the intermediates isolated by CsCl gradient centrifugation (Fig. 1 and 2) and those revealed by ficoll gradient centrifugation, Ad3 was also analyzed in ficoll gradients followed by SDS-polyacrylamide gel electrophoresis and CsCl gradient centrifugation.

The same pattern as shown in Fig. 5 was observed for nuclear and cytoplasmic extracts of Ad3 in ficoll gradients. When material from the IM peak was analyzed by CsCl gradient centrifugation with and without fixation with glutaraldehyde, it was revealed that the unfixed core structure is disintegrated during CsCl centrifugation (Fig. 8A and B). Figure 8C finally shows that the labeled polypeptide pattern of the Ad3 intermediate particles differ from those observed from CsCl-purified particles. Most pronounced is the lack of polypeptide V and pVII in the CsCl-purified particles. A comparison of the autoradiograms with the stained polypeptides presented in Fig. 2 reveals furthermore that polypeptide 40K in the particles is unlabeled and therefore probably of host origin.

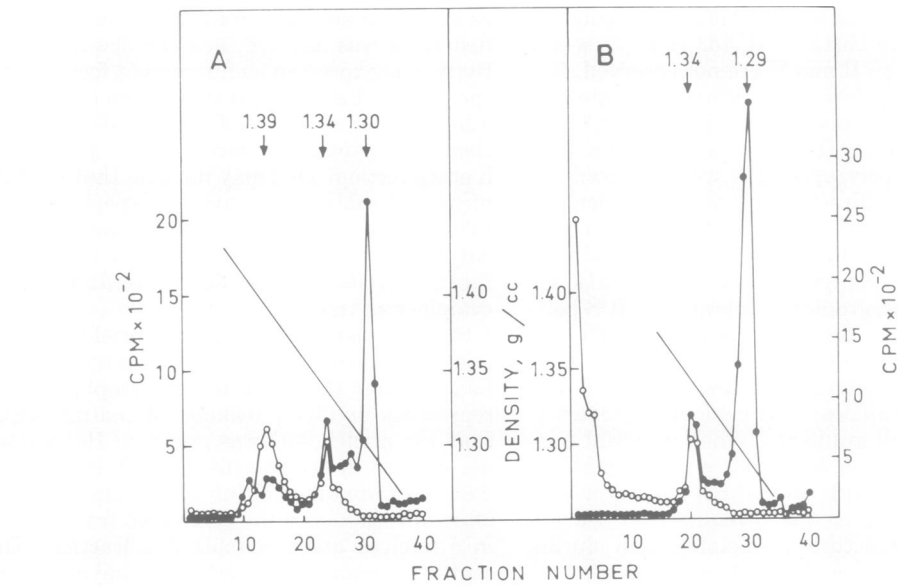
**Infectivity of the precursor particles.** Since the intermediates and the nuclear virus are rich in precursor polypeptides, it was of interest to determine the infectivity of nuclear and cytoplasmic virus and to determine whether the IM material is infective.

KB cells, grown in bottles and infected with Ad2, were labeled with [$^{35}$S]methionine from 15 to 20 h after infection. The cells were fractionated, extracts from the nuclei were subjected to ficoll gradient centrifugation, and the fractions were assayed for infectivity. Figure 9 shows that there is a 30-fold decrease in infectivity from the virus peak to the IM peak. The observed infectivity in the IM region could depend upon the trailing of virus into this region.

The specific infectivity of cytoplasmic and nuclear virus was also investigated. Ad2-infected KB cells were labeled with [$^{35}$S]methionine from 8 to 16 or 8 to 20 h after infection. Under these conditions the virus is assumed to be uniformly labeled, and the amount of virus should be proportional to the amount of radioactivity incorporated. The cytoplasmic and nuclear extracts were prepared, and the virus was purified by CsCl gradient centrifugation as described above. The infectivity of the purified virus was assayed, and the specific infectivity was expressed as fluorescent focus units/cpm. Table 1 shows that there is no significant difference in specific infectivity between nuclear and cytoplasmic virus, either at 16 or 20 h postinfection. This may indicate that nuclear virus that contains precursor polypeptides is as infective as cytoplasmic virus. However, since only around 1% of the particles are infectious, the infectivity may be due to contaminating cytoplasmic virus.

Since virus material was recovered from both nuclei and cytoplasm, it was necessary to establish whether the virus in the cytoplasm only represents artificial leakage of mature virus from the nuclei. Ad2 was grown in HeLa spinner culture under one-step growth conditions. Samples were taken at intervals from 16 to 24 h after infection, and the cells were fractionated in a nuclear and a cytoplasmic fraction. The sonically treated material was assayed for infectivity, and the number of fluorescent focus units per $10^6$ cell equivalents was determined. Figure 10 shows that there is a continuous increase of infectivity in the cytoplasmic fraction but that the nuclear fraction contains more infectivity at the start with a slight decrease at 20 h postinfection, when infectivity of the cytoplasm was still increasing. The ratio of fluorescent focus units between the nucleus and the cytoplasm shows a decrease from 1.2 to 0.4 during this period, suggesting that mature virus is selectively released to the cytoplasm under these conditions. The cytoplasmic virus may therefore represent a more mature fraction of assembled virus than the nuclear fraction.

**Pulse-chase experiments.** Pulse-chase experiments were performed to establish whether the intermediate particles are precursors in virus assembly. HeLa spinner cells were infected with Ad2 and pulse-labeled with [$^{35}$S]methionine for 15 min at 14 h postinfection, followed by a chase for 7 h. Cells ($2 \times 10^7$) were withdrawn at intervals, and the cytoplasmic and nuclear extracts were prepared and analyzed on ficoll gradients. The radioactivity was constant throughout the chase. Figure 11 shows the distribution of label on the ficoll gradients at different times during the chase. After a chase of 1.5 h the radioactivity is mostly confined to the intermediate peak in the nuclear extract. No radioactivity is observed in the cytoplasmic virus peak at this time. The label in the nuclear intermediates decreases to a constant level after 5 to 7 h of chase. Radioactivity in the nuclear virus material increases up to 5 h and is then constant. The cytoplasmic virus is



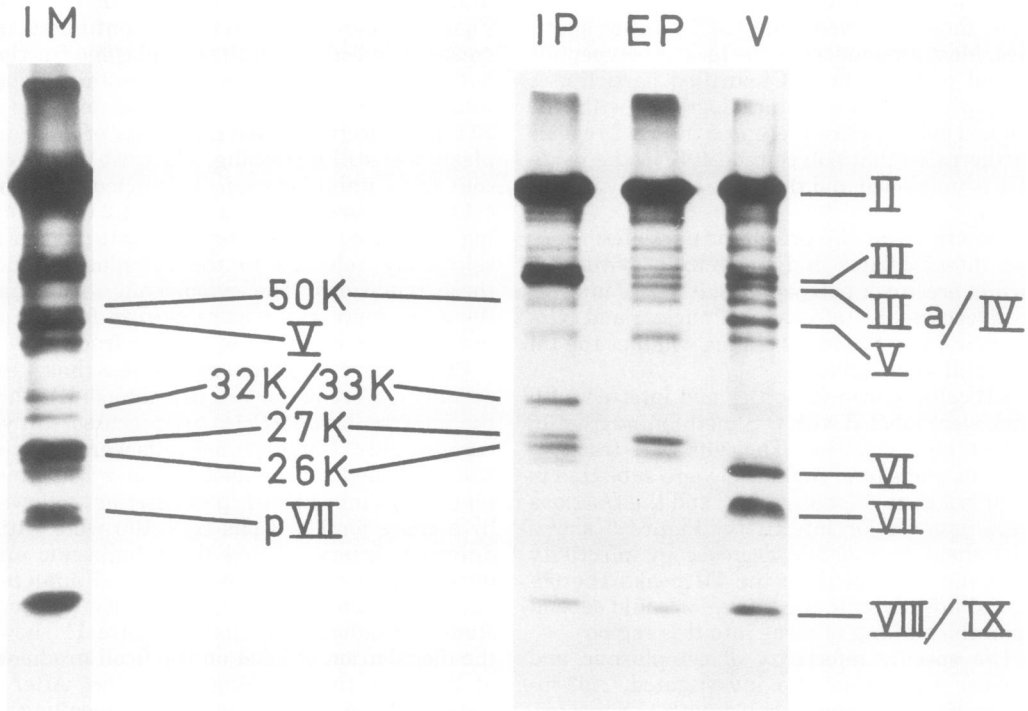




FIG. 8. *Isodensity gradient centrifugation and polypeptide composition of particles from Ad3 isolated on ficoll gradients. Nuclear extracts of ad3 virus-infected cells labeled with [$^3$H]thymidine and [$^{35}$S]methionine from 15 to 20 h postinfection were separated on ficoll gradients, and the intermediate class of particles was fixed with glutaraldehyde and analyzed on CsCl gradients (A) as described in the legend to Fig. 6. A CsCl gradient of unfixed material is included for comparison (B). Symbols: ○, [$^3$H]thymidine; ●, [$^{35}$S]methionine. (C) An autoradiogram of the [$^{35}$S]methionine labeled polypeptide pattern of Ad3 particles recovered from the intermediate fraction (IM) of ficoll gradients. A labeled polypeptide pattern of incomplete (IP) and empty (EP) particles as well as virions isolated on CsCl gradients (see Fig. 2) are included for comparison.*

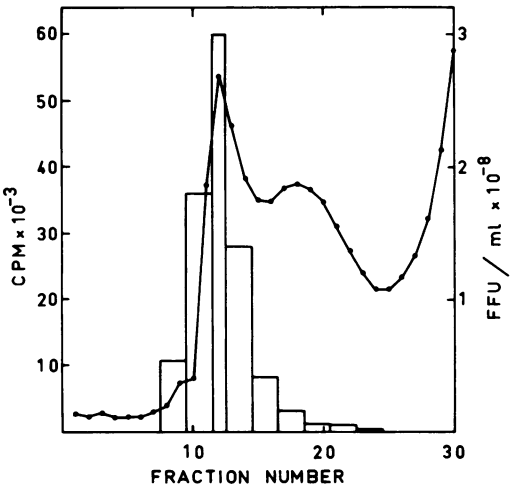



FIG. 9. *Virus infectivity in different size classes across a ficoll gradient. [$^{35}$S]methionine-labeled nuclear extract was sedimented in a 5 to 12.5% ficoll gradient. The fractions were pooled as indicated and assayed for infectivity.*

TABLE 1. *Specific infectivity of nuclear and cytoplasmic adenovirus*[a]

| Time p.i. | Preparation | Nuclear virus (FFU/cpm) | Cytoplasmic virus (FFU/cpm) |
|---|---|---|---|
| 16 | 1 | 342 | 511 |
| | 2 | 491 | 419 |
| 20 | 1 | 304 | 427 |
| | 2 | 370 | 407 |
| | 3 | 442 | 370 |

[a] Cells were labeled from 8 to 16 or 8 to 20 h postinfection (p.i.) with [$^{35}$S]methionine as described in Material and Methods, and nuclear and cytoplasmic virus was purified by CsCl gradients. Infectivity and radioactivity was determined on the preparations, and the specific infectivity is expressed as fluorescent focus units (FFU)/cpm.

first labeled at 3 h after the pulse, and the label rapidly increases throughout the chase. Additional experiments established that radioactivity does not appear in the particles before 30 min of chase and that the radioactivity in the nuclear IM peak reached a maximum at 1 to 1.5 h after the pulse. The nuclear virus peak was labeled first at 1.5 h and showed a maximum at 4 to 5 h. Labeled cytoplasmic virus is only present after 3 h of chase.

These experiments suggest that the nuclear intermediates are precursors in virus assembly and that the newly assembled virus is rapidly released from the nucleus to the cytoplasm during cell fractionation.

**Processing of polypeptides during chase.** The pooled fractions from the pulse-chase experiments shown in Fig. 11 were prepared for SDS-polyacrylamide gel electrophoresis, and an amount of label corresponding to $5 \times 10^5$ cell equivalents was applied from each pool. Figure 12 reveals that the radioactivity of the polypeptides pVI, 26K, and pVII are chased almost completely from the intermediates within a 5-h chase period. The products, corresponding to virion polypeptides VI, VII, and VIII, increase in radioactivity in the virus bands as the chase proceeds. In the nuclear virus peak radioactivity is first observed at 1.5 h after chase, but radioactivity is partially confined to the precursor polypeptides at early times. Late in the chase the cytoplasmic virus is heavily labeled in the bands corresponding to polypeptides VI, VII and VIII but contains only traces of label in the precursors. It was ascertained that the radioactivity in precursor polypeptides are mainly confined to the IM fraction immediately after the chase, with significantly less in the top fraction of the gradients (data not shown).

An attempt to quantitate the radioactivity in the autoradiogram was made to establish the relationship between the precursors and the mature virion products VI, VII, and VIII. Although the method is uncertain, due to nonlinearity in film darkening, the autoradiograms were scanned with a Joyce-Loebl densitometer, and the sum of the peak areas was determined for the polypeptides involved in processing. The

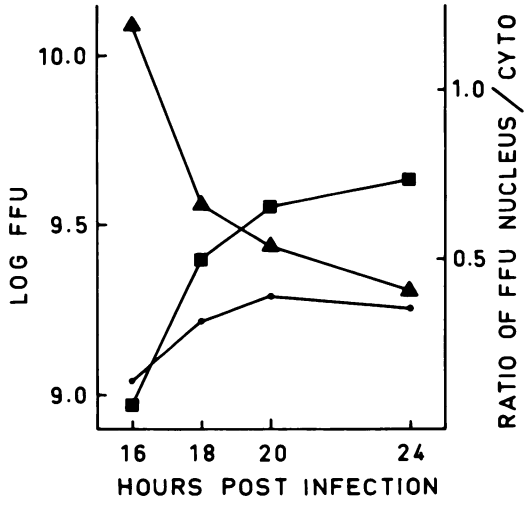



FIG. 10. *Infectivity in nuclear and cytoplasmic extracts at different times after infection with Ad2. Symbols: ●, nuclear infectivity; ■, cytoplasmic infectivity; ▲, ratio of infectivity between nuclei and cytoplasm.*

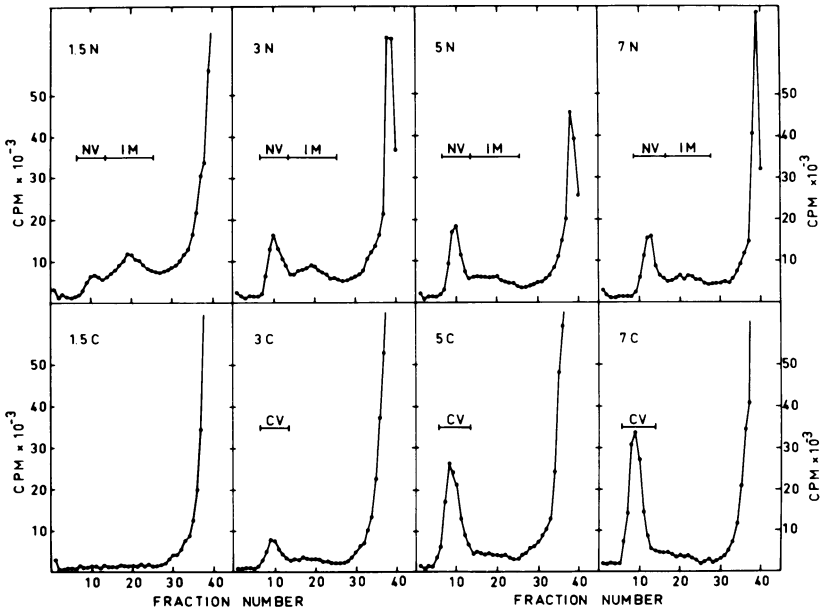



FIG. 11. *Pulse-chase analysis of adenovirus particles by centrifugation in ficoll gradients. Ad2-infected cells were labeled for 5 min with [$^{35}$S]methionine at 14 h after infection and chased with a 200-fold excess of cold methionine. Samples were taken at indicated times for 7 h during the chase and nuclear (N) and cytoplasmic (C) extracts were analyzed on 5 to 12.5% ficoll gradients. Materials from the nuclear virus (NV), cytoplasmic virus (CV), and intermediate (IM) peaks were separately pooled.*

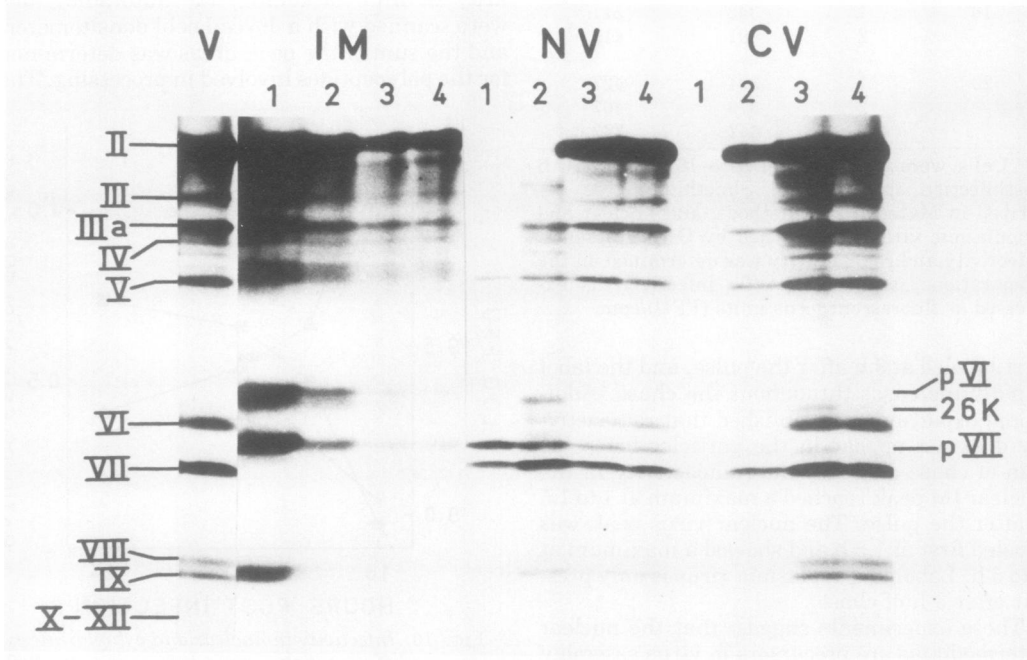



FIG. 12. *SDS-polyacrylamide gel electrophoretic analyses of a pulse-chase experiment. Material from the intermediates (IM), nuclear virus (NV), and cytoplasmic virus (CV) was pooled as indicated in the legend to Fig. 11, precipitated with acid, and analyzed on 10 to 18% SDS polyacrylamide slab gels. Autoradiograms of the gels are shown. The designation 1, 2, 3, and 4 correspond to 1.5, 3, 5 and 7 h of chase, respectively. V denotes a virus marker analyzed in the same slab gel and identical to the CV4 lane.*

kinetics plotted in Fig. 13A shows that most of the radioactivity that disappeared from pVI can be accounted for in polypeptide VI after 7 h of chase. Figure 13B shows that only 10 to 20% of the label that was chased from 26K appeared in polypeptide VIII, which again emphasizes that only a fraction of the 26K can possibly be the precursor to polypeptide VIII. The chase of radioactivity from pVII was almost complete, but the appearance of radioactivity in VII was not quantitative, and only about 50% of the initial pVII radioactivity was observed in polypeptide VII (Fig. 13C). Core fragments generated by sonic treatment may, however, sediment at the top of the gradients and can therefore not be recovered in the IM region. The radioactivity of the precursors is, however, all chased out of the intermediates, in contrast to the ineffective chase of the radioactivity in the major capsid proteins (Fig. 12). The results suggest that the proposed intermediates are precursors in virus assembly.

## DISCUSSION

The present study identifies some of the intermediates in assembly of adenovirus with the aid of pulse-chase labeling kinetics, taking advantage of the fact that the precursor polypeptides pVI and pVII are cleaved to the virion polypeptides VI and VII during the final step in maturation (12). The first intermediate is fragile and heterogeneous in size (550S-670S). They are probably composed of capsids associated with the core of the virus. Most of the core material is, however, released when the same material is purified on CsCl gradients without fixation (Fig. 8). The particles previously isolated by CsCl gradient centrifugation (12, 19, 21, 24, 25) are therefore probably generated during purification and stripped of their core components. The nonvirion polypeptide 50K, which may correspond to $IVa_2$ (9), is associated with the ficoll intermediates and incomplete particles but scarce in empty particles purified by CsCl (Fig. 2 and 8; 24). This polypeptide may therefore be associated with the cores but released at subsequent steps in assembly. One host polypeptide (40K) and two virus-induced polypeptides 33K and 32K may also be associated with the intermediates, irrespective of the purification method. Some of these proteins may serve as scaffolding proteins during assembly. It has not been possible to establish whether the core is introduced into preformed capsids or the capsid is laid down on a preformed core since the intermediates are, as shown here, associated with cores (Fig. 6). Our isolation procedure of the fragile intermediates may not be optimal since sonic treatment was used to release the virus material from the nuclei, which may lead to fragmentation of the core. A method which will release the particles without fragmentation or stripping of the proteins from DNA would be highly desirable.

The second class of particles corresponds to the young virions described by Ishibashi and Maizel (12) that are formed in the nuclei and not readily released to the cytoplasm during cell fractionation. They contain several of the precursor polypeptides, sediment as intact virions at 750S, and are resistant to CsCl gradient centrifugation. The final step in maturation involves cleavage of the precursor polypeptides. The proteolytic activity involved in this step may therefore reside in the young virions (12). The cleavage of the precursor polypeptides is accompanied with a release of the particles to the cytoplasm when cell lysis is carried out at low ionic strength in the presence of detergents. The release of mature virions to the cytoplasm may not occur in vivo, since all particles are confined to the nuclei when infected cells are examined in the electron microscope (5, 16). Therefore, the mature particles may be progres-

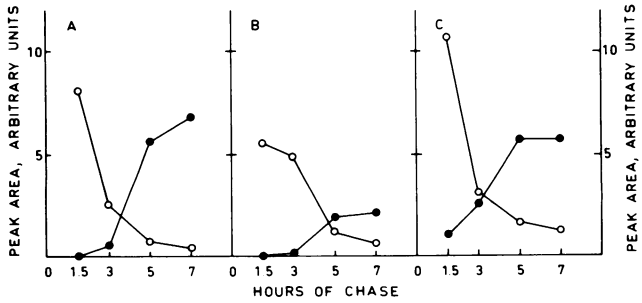



FIG. 13. *Kinetic analysis of the flow of radioactivity from the precursor polypeptides to their products. The autoradiograms shown in Fig. 12 were scanned, and the peak areas in arbitrary units were calculated for the precursors and their products in fractions containing virus and assembly intermediates. They were plotted against the time of the chase. Symbols: (A) ○, pVI; ●, VI; (B) ○, 26K; ●, VIII; (C) ○, pVII; ●, VII.*

sively displaced toward the nuclear membrane, which results in a release through a porous membrane to the cytoplasm during cell fractionation. The release is, however, not unspecific since the released particles contain cleaved precursors and the kinetics of release varies during maturation (Fig. 11). Both nuclear and cytoplasmic virus have, on the other hand, the same specific infectivity (Table 1), which is surprising in the light of the finding that a temperature-sensitive mutant of Ad2 (Ad2*ts*1) is defective in precursor cleavage and therefore noninfectious at the nonpermissive temperature (26). The nuclear and cytoplasmic fractions may, however, be contaminated with each other in the wild type, which could obscure a difference in specific infectivity.

This report has thus established at least three steps in virus assembly: intermediates, young virions, and mature virions. It would be interesting to analyze the temperature-sensitive mutants of Ad5 in this system (28). Preliminary results (B. Edvardsson, S. Ustacelebi, J. F. Williams, and L. Philipson, manuscript in preparation) indicate that only the fiber mutants (Ad5*ts*5, Ad5*ts*9, Ad5*ts*22), and in addition Ad5*ts*19, process their precursor proteins at nonpermissive temperatures, suggesting that virus from these mutants go through assembly to the final step but form noninfectious particles. Analysis on ficoll gradients reveals intermediates but no virus peaks in these mutants, which infers that the virus is unstable and falls apart under restrictive conditions. This interpretation is corroborated by the fact that these mutants shows a lag of 3 to 5 h for virus assembly and synthesize virions de novo in shift-down experiments (Edvardsson et al., manuscript in preparation). All other late *ts* mutants of Ad5 (22, 28) do not process their precursors (Edvardsson et al., manuscript in preparation). In light of the findings presented here it may be more rewarding to look for assembly intermediates among the mutants that are defective in precursor cleavage, since this event occurs very late in virus maturation. The fragility of the assembly intermediates imposes restrictions on the purification of these components and it is probably necessary to design an in vitro system to unravel the details of the assembly pathway. Winters and Russell (29) reported that viral DNA synthesized in an arginine-deficient system could assemble into virions when mixed with extracts from cells maintained in normal medium. A similar system may be required to understand the details of adenovirus assembly.

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