JOURNAL OF VIROLOGY, Oct. 1969, p. 323-338
Vol. 4, No. 4

Early Events of Virus-Cell Interaction in an Adenovirus System

KARL LONBERG-HOLM[1] AND LENNART PHILIPSON
Department of Microbiology, The Wallenberg Laboratory, Uppsala University, Uppsala, Sweden

Received for publication 27 June 1969

The interaction of ^{32}P-labeled adenovirus type 2 and HeLa or KB cells has been examined during early infection. The kinetics of virus uncoating to deoxyribonuclease-sensitive products, the partial characterization of three such products by gradient centrifugation, and the distribution of these products in the extranuclear and nuclear portions of infected cells are reported. The results are compatible with the following model. Extracellular virus attaches to a receptor on the plasma membrane. The membrane-bound virus has a half-life of less than 15 min and is transformed to a partly uncoated product which is free inside the cell and about half of which rapidly enters the cell nucleus. This is rapidly transformed, in both cytoplasm and nucleus, to a membrane-bound virion "core." The proteins of the bound "core" are then removed from the intact virus deoxyribonucleic acid (DNA). In the nucleus, viral DNA is the main product and there the overall sequence is completed in about 2 hr.

The interaction of virion and cell can be examined in considerable detail with the human adenoviruses, because adenovirus can be readily labeled with isotopes (10, 12, 21, 24), purified and maintained in relatively stable infective pools (3, 21). Attachment of labeled virus to cultured human cells is also rapid and efficient (12, 21). In addition, there is available a wealth of background information on virion morphology, chemical composition, and also on the specific biological activities of some of the individual virion proteins (for reviews, *see* 17 and 26).

The attachment of adenovirus type 2 or 5 to HeLa or KB cell suspensions occurs at specific receptor sites which are also able to bind, and be inactivated by, adenovirus type 2 fiber antigen. The attachment reaction can occur either in vivo or in vitro and there are over 10^4 receptor sites for adenovirus type 2 on the plasma membrane of the cell (22).

It has been shown that much of the deoxyribonucleic acid (DNA) of cell-attached type 2 or 5 adenovirions becomes accessible to digestion by deoxyribonuclease (E.C. 3.1.4.5) within less than about 30 min at 37 C and that the direct product of this "uncoating" is not naked viral DNA (12, 21). Furthermore, Sussenbach has shown that one uncoating product of adenovirus type 5 has lost only a small fraction of its total original protein (27). Until now there have been no reports of biochemical studies of the entry of the parental adenovirus genome into the cell nucleus.

The present study of the interaction of adenovirus type 2 with HeLa and KB cells describes the first step in uncoating and provides some indirect evidence that it may occur at the plasma membrane. About half the product of the first uncoating step is shown to gain rapid entry into the cell nucleus where its DNA is subsequently stripped of the virion protein.

Our results are interpreted with the aid of the hypothetical sequence: (A) free extracellular virus, (B) virus-receptor complex, (C) partly uncoated intracellular virus, (D) virus core complexed with cell material, and (E) intact intracellular viral DNA.

In addition to these naturally occurring components, a product (B') is found which arises from the disruption of B during cell breakage by detergent and which appears similar to free virus.

The virion-derived components are partially characterized and the kinetic pattern of their formation in the extranuclear and nuclear portions of the cell is presented.

MATERIALS AND METHODS

Virus production. Nonradioactive adenovirus type 2 of the prototype strain was produced in KB cells in spinner culture and purified as already described (22).

[1] Recipient of special Public Health Service research fellowship award SF3-AI-6864 from the National Institute of Allergy and Infectious Diseases. On leave of absence from E. I. Dupont Central Research Department, Wilmington, Del.

Purified virus was dialyzed against and stored in 0.25 M sucrose containing 0.02 M tris(hydroxymethyl)-aminomethane(Tris)-chloride buffer (*p*H 7.4), 0.001 M $MgCl_2$ and 0.5% *n*-butanol (21), at about 10^{13} particles/ml. ^{32}P-virus was produced in KB cell-monolayer cultures in 32-oz. (900 ml) prescription bottles in a way similar to that already described (22), except that the multiplicity of infection was increased to about 20 fluorescent focus units per cell. The cells were covered with 30 ml of Eagle's minimal essential medium (MEM) containing 10% calf serum for 18 to 20 hr and then washed, and the medium was replaced with 20 ml of citrate-MEM (MEM with citrate replacing phosphate) in which the arginine had been increased from 0.6 to 1.0 mM and containing 10% dialyzed calf serum. The infected cells were harvested after the cytopathic effect was complete at about 56 hr after infection. ^{3}H-thymidine- and ^{3}H-arginine-labeled virus was produced in a similar manner, except that the citrate medium was replaced with normal MEM containing 10% undialyzed calf serum. In the case of arginine-labeling, the arginine concentration was reduced to 0.02 mM. The labeled virus was purified as described previously (22) and double-labeled suspensions were prepared by mixing separately labeled ^{32}P- and protein-labeled pools.

Fluorescent focus assay. Adenovirus infectivity was assayed by fluorescent focus formation (20, 28) as in a previous report (22). Dilutions of virus were made in phosphate-buffered saline, and samples were used for infection within 1 hr after dilution. Rabbit antiserum against adenovirus type 2 hexon was made by U. Pettersson of this department, and fluorescein-labeled antiserum against rabbit immunoglobulin G was purchased from Microbiological Associates, Bethesda, Md. The results are expressed in fluorescent focus units (FFU).

Cells and incubations. HeLa or KB cells were grown in spinner culture as previously reported (21). These were usually kept at 10^5 to 3×10^5 per ml and were harvested by centrifugation. After the first centrifugation, the cells were washed by resuspending to 1/10 the original volume in Eagle's spinner medium with 2% calf serum and sedimenting at low speed (10^3 to 3×10^3 $g \times$ min) so that much of the cell debris remained in suspension. For virus attachment, cells were suspended at 10^7 to 3×10^7/ml in spinner medium containing 2% calf serum.

Cells were never incubated with the virus suspension for longer than 30 min. For longer incubations, the unattached virus was removed by diluting the initial cell suspension 10-fold with cold medium and sedimenting the cells (2×10^3 $g \times$ min). The cells were then incubated further at 37 C at 10^7, or less, per ml.

Cell counts. Cell counts were made in a hemocytometer chamber with 10 large squares equal to 1 mm^3 of cell suspension. Because an incorrect factor had been used, the cell counts reported in a previous paper (22) were high by a factor of 2.5 and all data derived from this must be corrected by this factor.

Cell disruption and fractionation. All work was done at 0 to 4 C. In some experiments cells were sonically treated in hypotonic buffer by treatment for 15 to 30 sec with the 100-w ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., London, England). When the only subsequent step was an assay for deoxyribonuclease digestability, the buffer was 0.05 M Tris-chloride (*p*H 7.4) containing 0.02 M $MgCl_2$, and cells were at about 10^6/ml. When, however, the virus-derived components were to be characterized by gradient centrifugation, the cells were at 10^7/ml in 0.05 M Tris-chloride (*p*H 8.1) containing 0.002 M ethylene diamine tetraacetic acid (EDTA).

For subcellular fractionation the general procedure of Penman (18) as modified by Penman, Smith, and Holtzman (19) was followed with some modifications. Cells were swollen by treatment for 10 min in RSB [0.01 M NaCl, 0.01 M Tris-chloride buffer (*p*H 7.4), and 0.0015 M $MgCl_2$] at 4×10^6 to 14×10^6/ml. The cells were broken with a precalibrated tight-fitting all glass Dounce homogenizer (Kontes Glass Co., Vineland, N.J.). Nuclei were sedimented, washed by mechanical rocking for 15 min with 0.5% Tween-40 in RSB and sedimented. They were resuspended in RSB and sedimented directly and then resuspended in RSB. Tween-40 and DOC (sodium deoxycholate) were added to 1 and 0.5%, respectively, as described by Penman (18), before the final pelleting. The nuclei were sedimented at each step for 1,660 $g \times$ min. Pooled supernatant fluids were combined as the "extranuclear fraction." The "nuclear" pellet contained 25% or less of the total cell protein.

The complete sequence outlined above is referred to as procedure I. In some experiments the 15-min wash with 0.5% Tween-40 and the subsequent RSB wash were omitted (procedure II). The use of Tween-40 alone does not disrupt the virus-receptor complex (22) and, in some cases, to get an extranuclear fraction with intact complex, only the first low speed supernatant fluid of disrupted cells was pooled with the supernatant fluid from the 0.5% Tween-40 wash (procedure III).

Procedure II, which omits two washes, gives essentially the same distribution of radioactivity in cells infected with ^{32}P-labeled adenovirus as does the complete procedure I. Omission of the DOC-treatment (procedure III) reduces the ^{32}P in the extranuclear fraction by roughly 5 to 10% of the total cell associated counts. Control experiments have shown that in procedure I there may be around 20% breakage or nonspecific leakage from the nuclei during fractionation, whereas only 1 to 2% of either added intact virus or labeled virion-derived material from an extranuclear fraction can enter the nuclear fraction nonspecifically.

The pooled extranuclear fractions were sonically treated before analysis, except in the case of procedure III. The nuclei were sonically treated, usually in RSB at less than 2×10^7 cell equivalents per ml. However, in those cases where sucrose-gradient analyses were made, the nuclei were sonically treated in 0.05 M Tris-chloride (*p*H 8.1) containing 0.002 M EDTA; the extranuclear fraction was also adjusted to 0.004 M EDTA before sonic oscillation.

Extraction of viral DNA from infected cells. Alkali-denatured viral ^{32}P-DNA was extracted from cells in a way designed to prevent breakage by shear. Infected cells (10^7) were spun into a pellet in a 12-ml conical

centrifuge tube, and the total volume of the pellet and undrained liquid was about 0.2 ml. A small amount of ^{3}H-thymidine-labeled adenovirus type 2 was added as a source of marker DNA. Cold saline (0.5 ml) was added, and the cells were resuspended with a "vortex" mixer. A solution (0.7 ml) of 1% sodium dodecyl sulfate in 0.1 M EDTA (*p*H 8.2) was gently mixed with the cell suspension at room-temperature, the tube was chilled in ice, and 5.3 ml of cold 0.25 M NaOH was added. The tube was then stoppered and rocked gently on a mechanical rocker at 15 cycles per min for 30 min at 4 C. Samples were removed for rate-zonal centrifugation with a broken-tip pipette.

Ultracentrifugation. Spinco L1 and L2 ultracentrifuges and MSE Superspeed ultracentrifuges were employed. Gradients and rotors were precooled, and all runs were at about 4 C. "Small" gradients in 5-ml tubes were run in the Spinco 50-L, 50-1, or MSE 3- by 5-ml swingout rotors, and "large" gradients (20-ml tubes) were run with the swingout rotor (3 by 20 ml). All *g*-values given are calculated for the center of the tube. Small gradients were always punctured and sampled dropwise from the bottom, and large gradients were sampled from the bottom with the aid of a fractionator made to take three gradients simultaneously and consisting of a mechanical elevator, 16-gauge steel inlet tubes, a three-channel peristaltic pump, and a fraction collector (B. Öberg and P. Krantz, *unpublished*).

CsCl-gradient analysis was made in 5-ml tubes with, unless noted otherwise, 3.6-ml linear preformed gradients onto which were placed samples of 1 ml. All CsCl solutions were made with 0.02 M Tris-chloride buffer (*p*H 7.5). Unless noted otherwise the gradients were about 1.3 to 1.4 g/cm^3 and the samples were adjusted to 1.17 to 1.20 g/cm^3 with CsCl beforehand. Densities were measured at room temperature, usually with a micropycnometer. In a few experiments, the refractive index was used. Providing that the samples are from sonically disrupted material, up to 1.3×10^7 equivalents of purified nuclei or about 6×10^6 equivalents of whole cells can be run in a small gradient.

Sonic extracts of whole cells or of subcellular fractions could be reproducibly separated by rate zonal centrifugation in sucrose gradients, provided a high concentration of salt was present. The gradients routinely contained 10 to 25% sucrose, 1 M NaCl, and 0.02 M Tris-chloride buffer (*p*H 8.1), "high ionic strength gradients." Small linear gradients of 4.2 ml were used with sample volumes between 0.1 and 0.25 ml. Large gradients of 18 ml were also used, usually with 0.6-ml samples containing 6×10^6 equivalents of sonically treated cells. In addition to the use of high ionic strength, we have usually also added 0.5% Nonidet P-40 (NP-40) plus 0.5% Tween-40 to the sample before sucrose gradient centrifugation to improve the resolution of component D. We have preferred to use NP-40 rather than DOC. The latter is present in the subcellular fractions at low concentrations in procedure I and II, but at higher concentrations it can give erratic results.

For special purposes, other types of sucrose gradients were employed. For separation of virion "cores" produced by urea treatment, samples containing urea were dialyzed 1.5 hr against 0.25 M sucrose, 0.002 M Tris-chloride (*p*H 7.5), and 0.0002 M EDTA and layered over a 4.4-ml gradient of 10 to 25% sucrose containing this same Tris and EDTA composition. For the analysis of labeled virion-derived DNA, 18-ml gradients of 10 to 25% sucrose containing 0.2 M NaOH and 0.8 M NaCl were made in "polyallomer" tubes.

Deoxyribonuclease digestion. Samples containing labeled adenovirus DNA were treated with deoxyribonuclease to determine the extent of virion "uncoating." This was performed in about 1-ml volume with a final concentration of 0.02 to 0.04 M $MgCl_2$ and about 0.05 M Tris-chloride buffer (*p*H 7.5) and with 0.1 mg of deoxyribonuclease (DNase-I, amorphous, from beef pancreas; Sigma Chemical Co., St. Louis, Mo.). The samples were incubated at 37 C for 30 min and then cooled and precipitated with 2 mg of bovine serum albumin (fraction 5) and 0.1 mg of degraded herring sperm DNA (both from Sigma) as carrier. Trichloroacetic acid was added to 7 to 10%. The trichloroacetic acid-precipitated deoxyribonuclease-treated samples were always left at 4 C for 16 to 20 hr before collecting the precipitate by low speed centrifugation. Under these conditions the ^{32}P-DNA from labeled virus which had been disrupted by 0.2 M NaOH is digested to the extent of 85 to 90%.

When CsCl gradients were to be tested for deoxyribonuclease-sensitivity, alternate fractions were diluted directly with 1 ml of buffer containing $MgCl_2$ and enzyme and then incubated and precipitated without transfer from the original tube. This was necessary because of aggregation and adsorption of the diluted virion products after CsCl-gradient centrifugation.

Assay of radioactivity. Samples were counted for 5 min or longer with a Packard scintillation spectrometer model 3375. Most samples were first precipitated with carrier and trichloroacetic acid, and the precipitate was dissolved in 0.5 ml of 0.3 M NaOH and rinsed into counting vials with 0.5 ml of water. Each was mixed with 10 ml of dioxane-based counting solution (solution 3; 6) which contained an equal volume of dry "Cab-O-Sil." The "spill" of ^{32}P-counts into the lower energy channels was corrected when necessary by a factor determined in each experiment. In a few cases, one-drop fractions from sucrose-gradient separations were counted in total and without acid precipitation after dilution with 1 ml of 0.1 M NaOH. Samples containing only ^{32}P were also sometimes counted on planchets in a Tracerlab Multimatic thin window GM counter for 5 min each, or were counted by Čerenkov radiation as previously described (22).

Representation of data. For summation of the counts in the separated components of sucrose gradients, zones were separated either between two fractions or at a fraction. CsCl gradients were fractionated with variable sample volumes, and smoothed curves were drawn for the counts per drop multiple versus the accumulated drop number. Designated areas were integrated with a planimeter. This type of representa-

tion and integration must give a distortion at places where the fraction volume changes, or where there are large changes in radioactivity, but these errors are of secondary importance relative to the precision of the overall method and to the extent to which we relied upon the quantitative results.

Chemical assays. Protein was determined by the method of Lowry et al. (13) with bovine serum albumin as a standard. The samples were first dialyzed in the cold against 0.1 M NaOH, and the bags were weighed before and after dialysis to permit correction for volume changes. DNA was determined by the Giles and Myers modification of the diphenylamine reaction (2).

Miscellaneous. NP-40 was a gift from the Swedish Shell Corp. of Stockholm. Tween-40 was purchased from the Kebo Corp. of Stockholm. "Cab-O-Sil" M-5 thixotropic gel powder was purchased from the Cabot Corp., Boston, Mass.

RESULTS

Infectivity of purified type 2 adenovirus. Table 1 lists properties of some purified nonradioactive pools of adenovirus type 2; three pools were assayed for protein and DNA, and the percentage of DNA was calculated on the assumption that adenovirus type 2 is composed entirely of DNA and protein (3). The percentage of DNA is in reasonable agreement with the value of 13% reported by Green and Piña (3). The concentration of virus particles was calculated from the protein concentration with the data of Green, Piña, and Kimes for the amount of protein per particle (4), and the relationship of particle number to light adsorption was determined. It was found that one optical density (OD) unit ($A_{257nm} - A_{300nm}$ in buffer containing 0.25 M sucrose, uncorrected for light scattering) was roughly equal to 7×10^{11} particles/ml.

TABLE 1. *Nonradioactive pools of adenovirus 2*

Pool[a]	Per cent DNA[b]	OD units/ml[c]	Particles/OD unit[d]	Per cent particles leading to FFU[e]
I	13.8	23	6.7×10^{11}	1.5
II	13.6	18	7.6×10^{11}	2.6
III	15.3	35	6.9×10^{11}	2.5
Avg. of above	14.2		7.1×10^{11}[f]	
Avg. of 21 separate pools				3.8 (1.5–8.1)

[a] Purified adenovirus type 2 stored in sucrose buffer.
[b] DNA content assuming virion composed entirely of DNA and protein.
[c] $A_{257\ nm}$ to $A_{300\ nm}$, uncorrected for scattering.
[d] Number of particles calculated assuming 1.52×10^8 daltons of protein per virion (4).
[e] FFU per 100 particles as estimated assuming one OD unit = 7×10^{11} particles.
[f] Maizel, White, and Scharff (14) dissolved purified adenovirus in SDS to avoid light scattering and obtained a factor of 11×10^{11} particles per OD unit ($A_{260\ nm}$).

The factor of 7×10^{11} particles per OD unit was used to estimate the particle concentration of all other purified pools employed in these experiments. Also, by using this factor, it was found that the average efficiency of FFU formation was 3.8% in 21 nonradioactive pools (with a range of 1.5 to 8.1%. In control experiments it was shown that, under the conditions of the FFU assay, only about 50% of the infective virus particles are attached during 1 hr. This means that the FFU per particle should be multiplied by at least two to give absolute efficiency of plating.

^{32}P-adenovirus type 2 was produced with about 2×10^{-6} disintegrations per min per particle. With 25 separate ^{32}P-labeled pools it was found that, on the average, 2.4% of physical particles produced FFU (0.6 to 7.0 range).

Early uncoating of adenovirus. The kinetics of attachment and uncoating of adenovirus type 2 and 5 have been reported by Philipson (21) and Lawrence and Ginsberg (12), respectively. We have extended these studies by examining the behavior of type 2 during the first minutes after infection. Figure 1 shows that the attachment of ^{32}P-labeled virus to HeLa cells commences at a rapid rate but that the formation of "uncoated" (deoxyribonuclease-sensitive) virus shows a relative lag of 4 to 5 min. This was seen reproducibly in both HeLa and KB cells.

This behavior is predicted by a simple kinetic model in which cell-attached intact virus undergoes a first-order reaction leading to a partially uncoated product. To test this, the smoothed uptake curve for coated plus uncoated virus (Fig. 1; x) was used to calculate the uncoated virus (y). This assumed that $y_0 = 0.7\%$, and arbitrary values for the first-order rate constant. These calculations were made by Jan Johansson of the Department of Computer Sciences, University of Uppsala, who used the "Runga-Kutta" method and values of x taken from the smoothed curve at 0.2 min or longer intervals. The dashed curve (Fig. 1, y) is for $K = 0.065$ min^{-1}.

The experimental points for uncoated virus, with which the theoretical curve should be compared, are the per cent of the total cell-associated ^{32}P which is also sensitive to deoxyribonuclease digestion. In addition, these values have been multiplied by 1.43 on the assumption that the first step in the uncoating reaction gives a product which is 70% deoxyribonuclease-digestable. During the first 15 min, there is a reasonably good fit between the theoretical first-

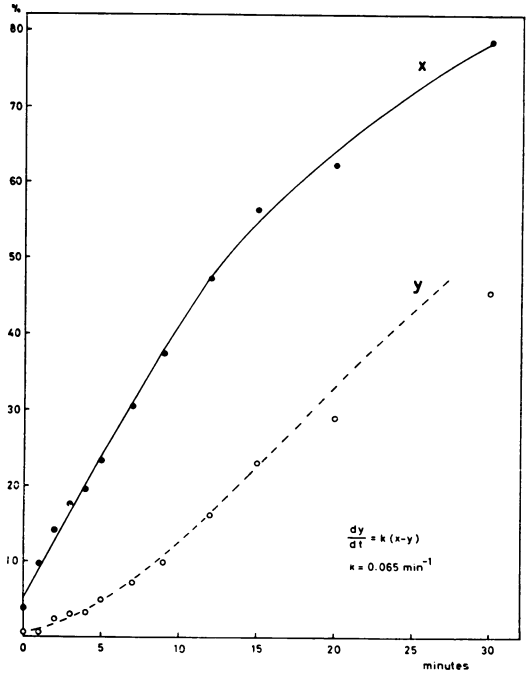

FIG. 1. *Uptake and uncoating of ^{32}P-adenovirus type 2 by HeLa cells. Washed HeLa cells (2×10^7/ml) were incubated with ^{32}P-virus at a particle multiplicity of 1.3×10^3. Portions of 0.4 ml were mixed with 10 ml of cold medium and the cells were separated from unattached virus by low speed centrifugation and sonically treated, and portions were treated with deoxyribonuclease. All values are given as per cent of total trichloroacetic acid-insoluble counts in the suspension. Deoxyribonuclease-sensitive counts were calculated by the difference between digested and undigested samples. X is the arbitrarily smoothed curve for cell-associated ^{32}P (●). Y is the theoretical curve for cell-associated uncoated virus computed from the first-order equation with $K = 0.065$ min^{-1} and with values taken from x; $y_0 = 0.7\%$. The observed values for cell-associated deoxyribonuclease-sensitive ^{32}P (○) have been multiplied by 1.43 to correct for incomplete deoxyribonuclease digestability.*

order curve with $K = 0.065$ min^{-1} and the corrected experimental points. In this case the half-life for the deoxyribonuclease-insensitive cell-associated virion must be around 11 min.

Entry of virus into the extranuclear and nuclear portion of the cell. The entry of coated and uncoated virus into the extranuclear and nuclear fractions of the cell was investigated in detail. The complete cell fractionation procedure used here (procedure I) gives "clean" nuclei at the expense of the purity of the extranuclear fraction.

Labeled virus enters the extranuclear fraction more rapidly than the nuclear fraction. Figure 2a shows an experiment in which KB cells were infected for 30 min with ^{32}P-adenovirus. The extranuclear fraction, which contains disrupted cell membranes and vesicles as well as cytoplasm, rapidly takes up ^{32}P. At early times much of the label must represent receptor-bound virus. The nuclear fraction shows an initial lag, but at 30 min there is almost as much nuclear as extranuclear ^{32}P. In a total of 18 various experiments in which both KB and HeLa cells have been employed and in which the distribution of ^{32}P was determined after 30 min of incubation by fractionation with procedure I or II, an average of 41% of the total cell-associated ^{32}P was in the nucleus (the range was 27 to 53%).

The "uncoated" viral ^{32}P in the extranuclear fraction always undergoes a rather slow and steady increase during a 30-min incubation, whereas that of the nuclear fraction increases in parallel with the total nuclear counts. In Fig. 2a roughly 37% of the extranuclear and 61% of the nuclear counts are sensitive to deoxyribonuclease by 30 min.

In another experiment with KB cells in which the distribution of total and of uncoated viral DNA was followed after the initial 30-min attachment period (Fig. 2), there are no dramatic changes but the radioactivity of the nuclear

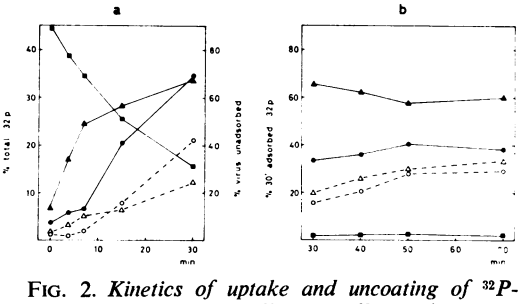

FIG. 2. *Kinetics of uptake and uncoating of ^{32}P-adenovirus type 2 in KB-cells. KB cells (10^7/ml) were infected at a particle multiplicity of about 5×10^3. Samples were fractionated by the complete procedure I. (a) Uptake during a 30-min attachment period. Samples were mixed with 2 volumes of cold media and the cells were pelleted. Radioactivity is given as per cent total trichloroacetic acid-insoluble counts in the suspension [unattached virus (■), extranuclear (▲), and nuclear (●) radioactivity]. The open symbols show the deoxyribonuclease-digestable ^{32}P in the corresponding cell fractions. (b) A separate experiment showing the behavior of cell-associated virion ^{32}P after an initial 30-min attachment period. There was 50% uptake in the initial incubation; the cells were washed and resuspended at 4×10^6/ml and incubated for 40 additional min. Radioactivity is given as per cent of the total trichloroacetic-insoluble ^{32}P in the suspension of washed and reincubated cells. Symbols are as in Fig. 2a, except that the trichloroacetic acid-insoluble ^{32}P released from the cells is also shown (■).*

fraction usually increases slightly at the expense of the extranuclear fraction, and this process is reversed at later times. Uncoated virus increases in both fractions, and after 40 min of additional incubation, the nuclear fraction is more than 75% deoxyribonuclease digestable.

Very little trichloroacetic acid-precipitable ^{32}P is released into the medium during subsequent incubation (Fig. 2b). In most of the experiments, only the trichloroacetic acid-precipitable counts are determined. In a few cases (for example, see the legend to Fig. 10) the trichloroacetic acid-soluble counts have been determined as well. In general, about 10% of the viral DNA which is cell-associated at 30 min is degraded to trichloroacetic acid solubility during a subsequent 90 min of incubation. This does not represent a widespread degradation of all virion DNA, however, as is shown by centrifugation analysis of virion DNA recovered from cells (Fig. 10); it may represent a complete breakdown of a fraction of the infecting particles by a side reaction.

HeLa cells were usually preferred to KB cells for investigations of early viral eclipse because of their greater resistance to mechanical damage. Control experiments have shown that there is little, if any, difference between the behavior of labeled virus in the two cell types; in both cases there is the same rapid pattern of entry of uncoated DNA into the nucleus.

Analysis of in vivo virion products by CsCl-gradient centrifugation. Experiments were made to characterize the cell-associated virion products. Cells infected for 30 min with labeled virus were broken by Dounce homogenization, and the crude nuclei were washed with 0.5% Tween-40 (procedure III). This should produce minimal disruption of the virus-receptor membrane complex, component B. Figure 3a shows the CsCl-gradient centrifugation pattern observed. There is almost no free virus (density about 1.34 g/cm^3) and much of the radioactivity is near the top of the tube (the location expected for B). A new peak, component C, also appears at about 1.35 g/cm^3. In similar experiments C was found to be from 50 to 80% digestable with deoxyribonuclease. Treatment of the extranuclear extract with DOC releases a component (B′) which is resistant to deoxyribonuclease and which appears about the density of free virus, but there is little additional C (Fig. 3b). Although B′ is infective (*unpublished data*) it is possible that B′ is not identical with the starting virus.

At early times after infection, C and B′ constitute the major cell-associated virion products as determined by CsCl-gradient centrifugation. Figure 4 shows the extranuclear and nuclear portion of cells incubated 15 min with virus and

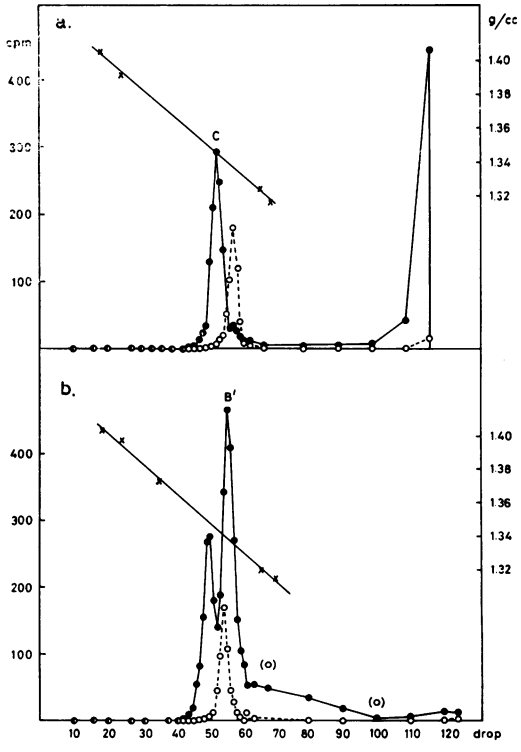

FIG. 3. *CsCl-gradient centrifugation of the extranuclear fraction of cells infected 30 minutes with ^{32}P-adenovirus. HeLa cells (1.2 × 10^7/ml) were incubated at 37 C with ^{32}P-adenovirus type 2 at a particle multiplicity of 8 × 10^8. There was 30% uptake of radioactivity in the washed cells. The extranuclear fraction was produced without DOC treatment (procedure III) and was not sonically treated. ^3H-thymidine-adenovirus type 2 was added as a reference marker and 3 × 10^6 cell equivalents were separated on small gradients for 6 × 10^5 g × hr. Counts per minute per drop are shown versus accumulated number of drops in the gradient. Symbols: ●, ^{32}P; ○, ^3H-marker. The bottom of the gradient is to the left. (a) The extranuclear fraction was adjusted to about 1.2 g/cm^3 with saturated CsCl and layered directly on a preformed CsCl gradient. (b) The extranuclear fraction (containing about 0.2% Tween-40) was adjusted to 0.4% DOC and incubated 3 min at 37 C before adding CsCl and placing onto the gradient.*

fractionated with the aid of DOC (procedure II). Most of the B′ is located in the extranuclear fraction.

Cesium chloride density gradient centrifugation has also been used in attempts to separate other uncoated products of ^{32}P-labeled adenovirus. Figure 5 shows some typical separations of the extranuclear and nuclear fractions of infected cells after 30 min and after 30 min plus 45 min of additional incubation. A significant amount of

radioactivity is not recovered in the gradients. A new component or mixture of components appears, particularly in the nuclear fraction; this is marked E in Fig. 5. The major component in this area will be discussed later. It has been found in other experiments that this component is not at isopycnic density after the centrifugation employed (6×10^5 $g \times$ hr) but is undergoing rate zonal sedimentation. At later incubation times, the resolution of C and B′ from other radioactive material becomes poorer. The large amount of radioactivity at the tops of the gradients may indicate that not all of B is disrupted to B′ under the conditions of this experiment, but, as discussed below there are also other virion components which are bound to membrane material. CsCl-gradient analysis is mainly useful for the determination of B′ and C at early times in infection, but even at 30 plus 45 min it shows that some B′ remains in the system (Fig. 5b).

Analysis of in vivo virion products by sucrose-gradient centrifugation. Rate-zonal centrifugation in sucrose gradients was also used to separate virion products from infected cells. Figure 6 shows an analysis of sonically treated cells which had been infected for a total of 75 min with ^{32}P – ^{3}H-arginine double-labeled virus. There are three peaks of ^{32}P, and these are indicated by B′ plus C, D, and E. These differ in digestability by deoxyribonuclease in the order given.

The radioactivity derived from the ^{3}H-arginine-labeled virus admixed with the infecting ^{32}P-labeled particles does not parallel the nucleic acid in the D or E region. Similar results are obtained with virus labeled with ^{14}C-leucine (not shown). Therefore, there are separate protein components sedimenting in the region of D and E. These are not examined in detail, and we use the letters D and E to designate only the nucleic acid-bearing moieties.

It was found that the resolution of component D from B′ plus C is poor without the addition of either NP-40 or DOC. It was also found that added intact marker virus co-sediments with peak B′ and C and that when this peak is rerun on CsCl gradients it yields largely the B′ and C components already described (not shown). It is thus not possible to resolve A, B′, and C by rate-zonal centrifugation in 1 M NaCl. On the other hand, this method permits us to examine the formation of products beyond C.

Characterization of component D. The nucleic acid containing material designated as D was difficult to characterize because it behaved as though it were bound to cellular membrane material. In separate experiments not shown here, D was obtained from gradients of non-detergent-treated sonic cell extracts, and it was found to have a low buoyant density of about 1.2 g/cm³ in CsCl. If the sonicates had been treated with detergents before sucrose-gradient centrifugation, D showed a broad and nonreproducible distribution when rerun on CsCl (1.2 to 1.35 g/cm³, not shown). This indicates either that D is so "sticky" that it can re-associate in the gradient with lipoproteins or that it is not completely freed by 0.5% NP-40 or DOC.

Figure 7 shows the behavior of D from a sucrose-gradient separation of disrupted cells treated with NP-40 (insert) when it is banded on CsCl after a second NP-40 treatment. This second detergent treatment, after rate-zonal centrifugation, gives a product which has a buoyant density averaging about 1.36 g/cm³ and which is broadly dispersed. Similar results are obtained with DOC-treated D. The sharp ^{3}H-peak at about 1.3 g/cm³ is present whether or not detergents are added after sucrose-gradient centrifugation and is apparently from the

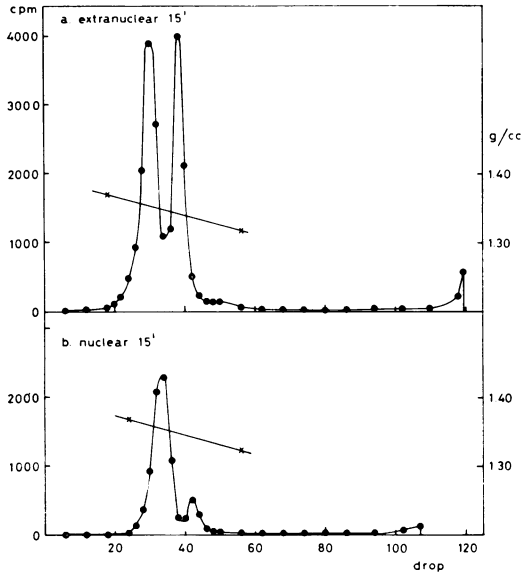

FIG. 4. *CsCl-gradient centrifugation of extranuclear (a) and nuclear (b) fractions of cells infected 15 min with ^{32}P-adenovirus. HeLa cells (3×10^7/ml) were incubated 15 min with ^{32}P-adenovirus type 2 at 3×10^3 particles per cell. These were washed (60% uptake) and the cells were fractionated by procedure II. About 43% of the cell-associated counts were in the nuclear fraction. Samples equivalent to 3.3×10^6 (a) and 2.7×10^6 (b) fractionated cells were centrifuged for 6×10^5 g × hr on small CsCl gradients and fractions were precipitated with trichloroacetic acid. Counts per minute per 2 drops are shown versus total accumulated drops. About 8 and 29% of the total ^{32}P were recovered in the alkaline tube washings of a and b, respectively. The bottom of the gradient is to the left.*

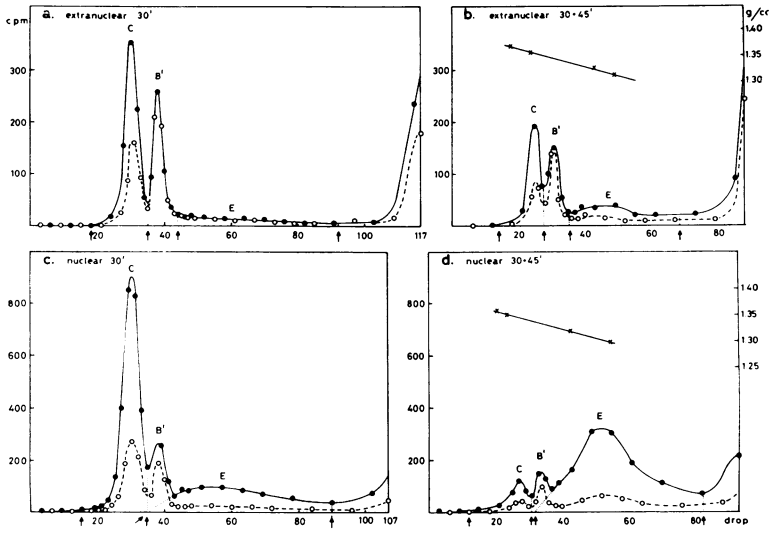

FIG. 5. *CsCl-gradient centrifugation of the extranuclear and the nuclear fractions of cells infected 30 and 75 min with ^{32}P-adenovirus. HeLa cells (2×10^7/ml) were incubated at 37 C for 30 min with ^{32}P-adenovirus type 2(1×10^4 particles/cell) and then washed with cold medium. There was 39% uptake. Part of the cells were then incubated for an additional 45 min at 4 × 10^6/ml. The complete procedure I was used for cell fractionation, and about 33% of the total cell-associated counts were found in the nuclear fractions. The fractions were separated on small preformed CsCl gradients for 6×10^5 g × hr. Trichloroacetic acid-precipitable counts per minute per drop are shown versus accumulated drop number. Symbols: (●) untreated, (○) deoxyribonuclease treated. The bottom of the gradient is to the left. Dotted lines and arrows divide the gradients into arbitrary areas for integration. (a) Extranuclear fraction from 4.3×10^6 cells infected 30 min. The area of the solid curve represents 65% recovery of the counts applied to the gradient. (b) Extranuclear fraction, 4.3×10^6 cells infected 30 min and incubated an additional 45 min; 44% recovery. (c) Nuclear fraction, 1.3×10^7 cells infected 30 min; 86% recovery. (d) Nuclear fraction, 1.3×10^7 cells infected 30 min and incubated for an additional 45 min; 70% recovery.*

protein component which sediments in sucrose slightly faster than, but overlapping D.

It can be calculated from the ratio of ^3H to ^{32}P in the infecting virus (4.1 at the time of counting the CsCl gradient) that the buoyant density banded D has only about 33% of the original virus-arginine. Detergent-treated component D requires a longer centrifugation to reach isopycnic density than does C or B'. The CsCl gradient was run for 2.1×10^6 g × hr (Fig. 7). When this was reduced to 6×10^5 g × hr the average buoyant density was slightly lower (gradients not shown).

The behavior of virion "core," derived by urea treatment of intact virus (15), was examined for comparison. Some of the same double-labeled virus used in the experiment shown in Fig. 8 was dialyzed 21 hr with agitation against 3 M urea containing 0.002 M EDTA and 0.002 M Tris-chloride, adjusted to *p*H 7.5. The "core" was then separated on a low-ionic strength gradient (Fig. 8, insert). Under these conditions the core sediments 5 to 6 times more slowly than does intact virus in 1 M NaCl. The sucrose-gradient banded core contains about half the original ^3H-arginine of the virus. It was found that the urea core is also "sticky" towards cell material. This is illustrated in Fig. 8; some of the core was mixed with sonically treated nonlabeled cells. Tween-40 and NP-40 were subsequently added and the mixture was banded on CsCl for 2×10^6 g × hr. The nucleic acid-containing component bands at about 1.36 g/cm^3 and contains roughly 29% of the original arginine. Some of the labeled arginine has also been displaced or replaced and is found at lower densities. There must be some cell material adhering to the core because in other gradients (for example, Fig. 9c) it was found that, when no cell material is added, the mean density is between 1.44 and 1.47 g/cm^3. When cell sonic extract was added without further detergent treatment, most of the core was lighter than 1.3 g/cm^3 (not shown). In conclusion, since in vitro cores behave similarly to the in vivo component D, we tentatively consider component D to be virus core which is associated with cell membrane material.

Characterization of component E. Component

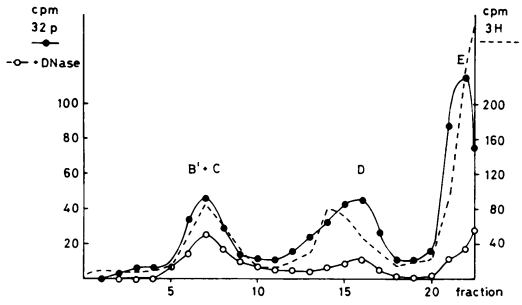

FIG. 6. *Sucrose-gradient centrifugation of sonicated cells infected with doubly labeled adenovirus. HeLa cells (3 × 10⁷/ml) were infected with ³²P and ³H-arginine double-labeled adenovirus type 2 (2 × 10³ particles/cell) for 30 min and then washed with cold medium. The apparent uptake was 62 and 57% for ³²P- and ³H-labeled virus. The cells were then reincubated (10⁷/ml) for 45 min. The sonic extract of the cell pellet was adjusted to 0.5% Tween-40 and 0.5% NP-40 and 6 × 10⁶ cell equivalents were separated on a large-sized high ionic strength sucrose gradient 3.7 × 10⁶ g × min. Of each fraction, 200 μliters were precipitated with trichloroacetic acid directly (●, ³²P; dashed curve, ³H) and 200 μliters were treated first with deoxyribonuclease (○, ³²P). Eleven per cent of the total ³²P could be recovered from the pellet. The bottom is to the left.*

E from sonically disrupted cells was shown to have properties compatible with its being "naked" and sheared viral DNA. Under the usual conditions for CsCl-gradient centrifugation, ³²P-labeled E taken from sucrose gradient sediments to the position occupied by E in FIG. 5 (*unpublished data*). This was investigated further in Fig. 9 in which E from cells infected with ³²P – ³H-arginine double-labeled virus was recentrifuged for different periods on steep (1.3 to 1.6 g/cm³) CsCl gradients. The ³²P-labeled E migrates from a position of density less than 1.35 g/cm³ at 6 × 10⁵ g × hr (Fig. 9a) to one of over 1.5 g/cm³ at 2 × 10⁶ g × hr (Fig. 9b). At the latter time it is probably still sedimenting toward higher density and it is not associated with any significant ³H-arginine label. This indicates that E is not fragmented core, and for comparison Fig. 9c shows the isopycnic separation of urea-core produced in vitro from the same starting virus as used in Fig. 9a, b.

The sedimentation rate of E on sucrose gradients is higher if sonic oscillation is omitted during the isolation and the nuclei are "dissolved" directly in 1 M NaCl, 0.02 M Tris-chloride buffer, *p*H 8.1 (not shown). Further experiments were made to determine by indirect means if E in vivo is, or contains, intact viral DNA.

Alkali-denatured adenovirus type 2 DNA has been shown by Green and co-workers (5) to be noncircular and with a sedimentation coefficient of 34*S*. Figure 10 shows an experiment in which alkali-denatured ³²P-adenovirus DNA has been extracted from cells infected for zero and 2 hr with ³²P-adenovirus and separated by rate-zonal centrifugation in an alkaline sucrose gradient. Thymidine labeled virus was added to provide marker DNA. After 2 hr there is no major change in the sedimentation rate of the bulk of the infecting virus DNA even though more than 33% is expected to be present in component E (see below). There is, at the same time, however, a breakdown of a discrete portion of the infecting virus DNA to trichloroacetic-soluble products (12% of the 30 min cell-associated ³²P in the

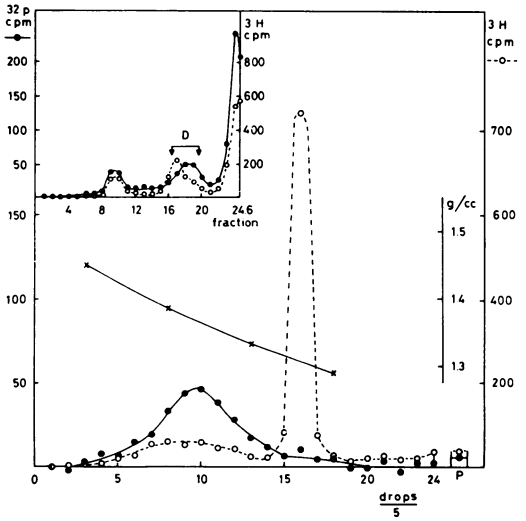

FIG. 7. *Further characterization of component D. HeLa cells (2.4 × 10⁷/ml) were incubated 30 min with ³²P- and ³H-arginine double-labeled adenovirus type 2 at 1.1 × 10⁴ particles/cell and then washed with cold medium. The apparent uptake was 38% for both isotopes. The cells were then incubated 45 min more. The sonic extract of the cell pellet was adjusted to 0.5% each of Tween-40 and NP-40 and 6 × 10⁶ cell equivalents were separated on large-sized high ionic strength sucrose gradients for 3 × 10⁶ g × min (insert). Portions (50 μliters) were counted in total, and the D component was pooled. A sample of D equivalent to that obtained from 2 × 10⁶ cells was adjusted to 0.5% each of Tween-40 and NP-40 and then to about 1.17 g/cm³ with saturated CsCl (final volume 0.97 ml). This was then separated on a 1.45 to 1.31 g/cm³ preformed CsCl gradient for 2.1 × 10⁶ g × hr and 5-drop fractions were precipitated with TCA and counted for 5 min. Counts per minute per 5 drops (●, ³²P; ○, ³H) are shown versus fraction number. P indicates the counts recovered by an alkaline wash of the centrifuge tube, and the bottom is to the left. At the time of counting Fig. 7, the ratio of ³H to ³²P for the starting virus was 4.1 (3.4 for the insert).*

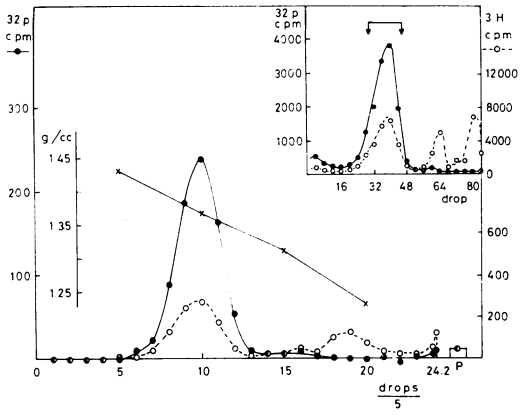

FIG. 8. *Gradient centrifugation of ^{32}P — ^{3}H- arginine double-labeled "urea-cores" from adenovirus type 2. Double-labeled virus (the same used in Fig. 7; 1.9 × 10^{11} particles in 100 μliters) was dialyzed against 3 M urea. This was separated, after a second dialysis against a low density buffer, on a low ionic strength sucrose gradient for 9.7 × 10^{6} g × min as described in Materials and Methods. Every fourth drop was counted in toto, and counts per minute per drop is plotted against total accumulated drops (insert). The pellet contained 2.5 and 2.4% of the total ^{32}P and ^{3}H of the sample. The indicated fractions were pooled, a sample (8 × 10^{9} virion equivalents) was mixed with HeLa cell sonic extract (2 × 10^{6} cell equivalents) in 0.05 M (pH 8.1) Tris-chloride containing 0.002 M EDTA, and the mixture was adjusted to 0.5% Tween-40 and 0.5% NP-40 (final volume 200 μliters). After 30 min at 0 C, this was diluted to 1 ml with added dilute CsCl to a final density of 1.17 g/cm³. The sample was then centrifuged 2.1 × 10^{6} g × hr on a small (1.45 to 1.31 g/cm³) CsCl gradient. Fractions were collected, precipitated with trichloroacetic acid and counted (counts per minute per 5 drops; ●, ^{32}P; ○, ^{3}H). P indicates counts recovered by an alkaline wash of the centrifuge tube, and the bottom is to the left. At the time of counting Fig. 8 the ratio of ^{3}H to ^{32}P in the starting sample was 4.1 (3.4 for the insert).*

experiment shown in Fig. 10). Since the bulk of the viral DNA remains intact, it is concluded that the major ^{32}P-labeled components described in this report do not represent fragmented breakdown products of the virus.

Kinetics of virus-cell interaction. Isopycnic centrifugation on preformed CsCl gradients is not a very satisfactory method for studying the kinetics of the overall process A to E. Except for early periods after infection it will be difficult to measure the amount of B′ or C in infected cells because partly dissociated D may tend to sediment to the same part of the gradient. Also E is not at isopycnic density and is so broadly dispersed that it may overlap the other components. Even so, attempts were made to use data from CsCl-gradient analyses for kinetic purposes. The components were divided from each other in the arbitrary manner indicated by dotted lines and arrows in FIG. 5. Figure 11 gives the amount of virion-^{32}P in the integrated areas as the per cent of the total ^{32}P of the cell. This is subject to considerable error as discussed in Materials and Methods; there was also only 44 to 86 per cent recovery of radioactivity (Fig. 5).

The results of three experiments of the type shown in Fig. 5 and 11 are in rough agreement. The extranuclear fraction contains more B′ than the nuclear fraction, and the total B′ decreases during incubation. There is also roughly as much C in the nuclear as in the extranuclear fraction at 30 min. Component E increases at the expense of B′ plus C, and in 2 out of 3 experiments there is clearly more E in the nuclear than in the extranuclear fraction.

Figure 12 gives the separations in a kinetic experiment employing sucrose-gradient sedimentation of the extranuclear and nuclear fractions of HeLa cells. In control experiments (not shown) labeled virus was added to extranuclear and nuclear fractions from unlabeled cells before sonic oscillation and 7 to 14% of the label trailed from the B′ plus C into the D region. However, at times later than 15 min most of the radioactivity in this region cannot be due to trailing.

The gradients of Fig. 12 were counted without trichloroacetic acid precipitation so that values for E may be somewhat high, since trichloroacetic acid-soluble products should also remain at the top of the gradient. This should not be sufficient, however, to qualitatively alter the distribution pattern of E between the nuclear and extranuclear fraction.

Figure 13 shows the quantitative distribution of the radioactivity between the three major peaks of the sucrose gradients of Fig. 12. At 15 min, 33% of the total ^{32}P is in the nuclear B′ plus C peak and by difference 41% is in the extranuclear B′ plus C peak. This can be interpreted with reference to Fig. 4b, which indicates that at 15 min C alone makes up the major portion of the nuclear B′ plus C. Roughly at least 50% of the total C in the cell must then have gained entry into the nucleus at this early time.

There appears to be a precursor-product relationship between B′ plus C and D, but some B′ plus C also appears to be refractory to the transformation; this is located primarily in the extranuclear fraction.

The kinetics of E differs from that of D. Formation of E is not complete in the cytoplasm and the extranuclear E, given by difference in Fig. 13, does not appear to increase after 50 min of total incubation. In the nucleus, however,

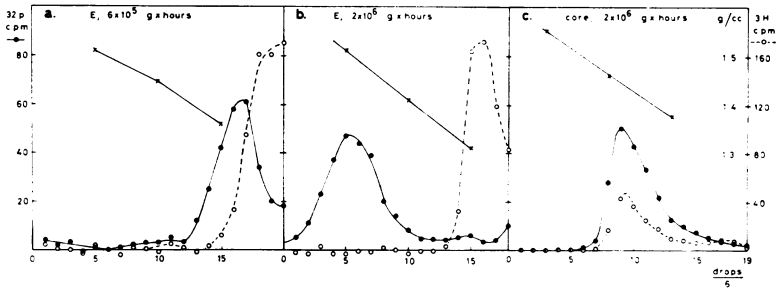

FIG. 9. *CsCl-gradient centrifugation of component E and comparison with "urea-cores." (a, b). Rate zonal separation of E. HeLa cells were infected with ^{32}P and ^{3}H-arginine double-labeled virus for a total of 75 min in the same way as in Fig. 7. The cells were sonically treated and 6 × 10^6 cell equivalents were separated (without added detergents) with 3.7 × 10^6 g × min centrifugation on a large-sized high ionic strength sucrose gradients (separation not shown). The top 11% of the gradient was used as E. This was adjusted to about 1.2 g/cm³ with CsCl and 0.8-ml portions were centrifuged on 4.0-ml CsCl gradients (1.3 to 1.6 g/cm³) for (a) 6 × 10^5 and (b) 2 × 10^6 g × hr. Trichloroacetic acid-precipitable counts per min per 6 drops; the bottom is to the left. In both a and b, less than 5% of the total ^{32}P or ^{3}H could be recovered in the alkaline wash of the centrifuge tube. (c) Isopycnic separation of the core. The same double-labeled virus used in a and b was also used for the production of purified core as in Fig. 8 (sucrose gradient separation not shown). A sample of this was adjusted to about 1.2 g/cm³ with CsCl and 0.8 ml was centrifuged as was b, above. At the time of counting (Fig. 10c), the original virus mixture had a ^{3}H to ^{32}P ratio of 2.46.*

there is a clear precursor product relationship from C to D to E.

DISCUSSION

Possible limitations of the biochemical investigation of virus-cell interactions. Our experiments have employed purified adenovirus type 2 in suspensions of known concentration. These were usually labeled with ^{32}P, in which case the infectivity and label have already been shown to attach to cells at the same rate (21). Only about 2.4 to 3.8% of labeled or unlabeled particles appear to lead to infection in 3 days, as assayed by fluorescent focus formation. A similar proportion (3%) has been determined by plaque formation by Green, Piña, and Kimes (4). This efficiency is a minimum value, because in our assay only 50% of the infectivity attaches to the cells and also because some virions may attach either to injured cells or cells which later become detached during infection or staining. We estimate then that roughly 6 to 10% of the attached virions may be able to produce infection. The biochemical studies, as discussed below, show that in the major sequence of virion transformations about 30% of the DNA of attached virions enters the nucleus intact and is stripped of virion proteins. The adenovirus system thus shows a ratio of 3:5 between the number of virions passing through the main biochemical pathway and the successfully infective particles; this is comparable to the vaccinia virus system and better than in several other systems (9). Consequently the observed sequence of events could represent the "true" infective process, provided that additional blocks occur at later stages of the infective cycle.

In conclusion, the efficiency of infection is high enough to make it likely that early intermediates in the infective process can be seen biochemically. It is not high enough, however, to make it absolutely certain that the entire main pathway of biochemical interaction must be part of the infective cycle. This same limitation would also be encountered in an electron microscopic study.

Overall pattern of early virus-cell interaction. The events during the first 2 hr of infection with adenovirus type 2 have been represented as a hypothetical network in FIG. 14. Only DNA-containing components are represented. In these studies, the observed speed and synchrony of the passage of A to E is surprising. Thousands of virus particles are attached to each cell in less than 30 min. The membrane-attached particles (B) are transformed with a half-life of less than 15 min to a partly uncoated product (C) which is free inside the cell. There is no evidence that intact free intracellular virus is an intermediate. Less than 20% of the attached virus appears to be refractory to uncoating; although this fraction might represent intravesicular virions, it must also contain virus which is attached to nonviable cells or cell fragments.

C is able to enter the nucleus freely, as shown by the kinetics of entry of uncoated viral DNA and also by CsCl gradient analysis of the nuclear fraction. The passage of C into the nucleus is arbitrarily represented as reversible in Fig. 14.

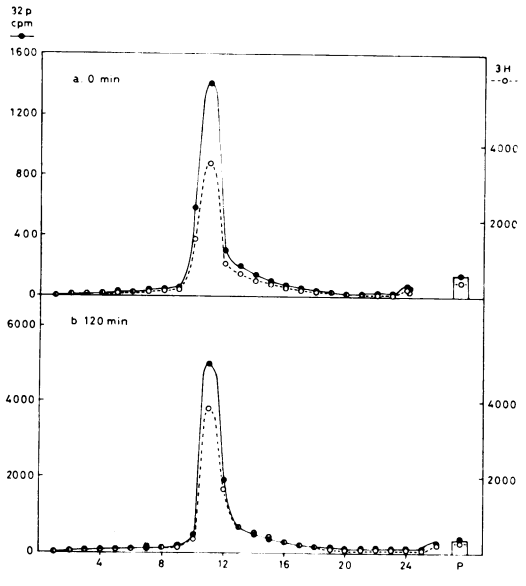

FIG. 10. *Sedimentation of alkali-denatured ^{32}P-adenovirus type 2 DNA, before and 2 hr after infection. ^{3}H-thymidine marker has been added to both samples. Trichloroacetic acid-insoluble counts per min per unit of fraction are shown versus fraction number; the bottom is to the left, and P indicates activity recovered in a rinse of the tube. (a) Zero time control. The DNA of 1 × 10^{7} HeLa cells and of added ^{32}P-adenovirus (5.7 × 10^{9} particles) plus ^{3}H-thymidine-adenovirus (6.6 × 10^{10} particles) was extracted exactly as described in Materials and Methods and about 1 ml was analyzed on a large alkaline sucrose gradient. (b) DNA from infecting virus. HeLa cells (3 × 10^{7} per ml) were infected with ^{32}P-adenovirus at a particle multiplicity of 3.4 × 10^{3}. (The starting virus contained 0.5% of its ^{32}P in a trichloroacetic acid-soluble form.) After 30 min the cells were washed and 62% of the ^{32}P remained with the pellet. The cells were then diluted to 5 × 10^{6} per ml and reincubated for 90 min and analyzed as in (a). Of the total ^{32}P in the suspension of washed and reincubated cells, 2% was released to the incubation medium in a trichloroacetic acid-precipitable form and 6% was released in a trichloroacetic acid-soluble form; 86% was cell-associated and in a trichloroacetic acid-precipitable form and 6% was cell-associated but trichloroacetic acid-soluble (as determined after NaOH-treatment).*

The transformation of C to D is relatively rapid in both the nuclear and extranuclear portions of the cell. The properties of D suggest that it may be composed of virion cores which are bound to cellular material; the binding to cellular material may occur either during the disruption of the cells or in vivo.

The transformation of D to E appears to be most efficient or fastest in the nucleus where it is almost completed after a total of 2 hr of incubation. All or most of the core protein is removed in the formation of component E, and it is represented as naked viral DNA in the schematic diagram. The transformations A through E are discussed in greater detail below.

Rate of the primary step in uncoating. The kinetics of both uptake and uncoating of ^{32}P-labeled adenovirus type 2 were studied simultaneously with the same cell suspension (Fig. 1). The adenovirus system allows this because at high cell density the rate of attachment and of uncoating to deoxyribonuclease sensitivity is of the same magnitude. There appears to be a relative lag in the formation of uncoated virus.

A simple first-order kinetic model predicts the shape of the curve (Fig. 1), and the apparent lag may merely reflect the sequential nature of attachment and uncoating. There is a reasonably good fit during the initial 15 min of incubation with the first-order model if the rate constant is chosen as 0.065 per min, i.e. the half-life of attached but otherwise intact virus is 11 min. If the data had not been corrected for a partial deoxyribonuclease sensitivity of the product, the first-order model would also fit, at early times, with a half-life of 15 min. This also agrees with the rapid uncoating observed by Philipson with adenovirus type 2 (21) and by Lawrence and Ginsberg with adenovirus type 5 (12).

The first-order model cannot be correct in detail because the primary product of uncoating is modified to other products of greater deoxyribonuclease sensitivity and also because the population of attached virions is not homogene-

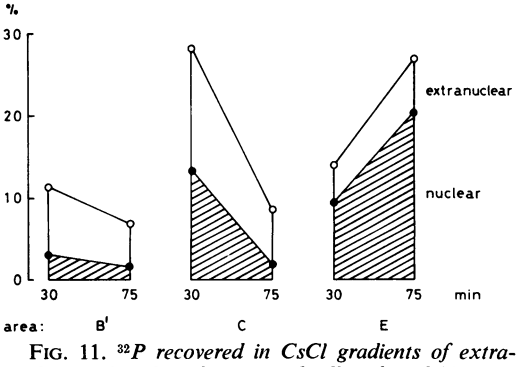

FIG. 11. *^{32}P recovered in CsCl gradients of extranuclear and nuclear fractions of cells infected 30 or 30 plus 45 minutes with labeled virus. The areas designated as B', C, or E in Fig. 5 are shown as per cent of total cell-associated trichloroacetic acid-precipitable counts. The upper lines give totals from extranuclear plus nuclear fractions, whereas the lower lines (with shaded areas) give the nuclear recovery only. Duplicate samples of nuclear extracts were analyzed, and their average is used in Fig. 11.*

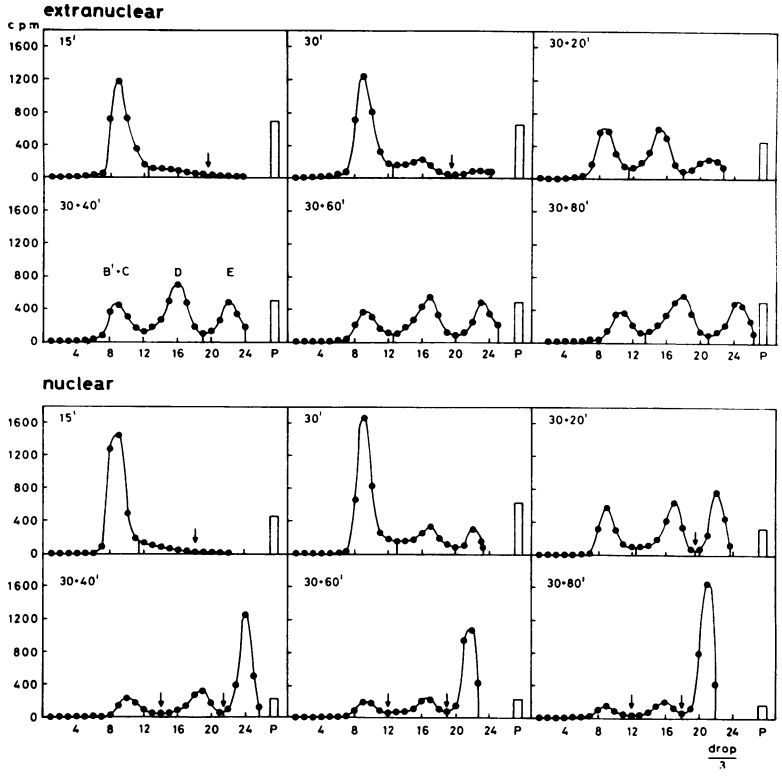

FIG. 12. *Sucrose-gradient analysis of extranuclear and nuclear fractions of cells infected various times with ^{32}P-adenovirus. Samples of HeLa cells (2×10^7 cells in 0.67 ml) were incubated at 37 C with ^{32}P-adenovirus type 2 at a particle multiplicity of 3×10^3. These were washed with cold medium at 15 or 30 min and resuspended in 4 ml of medium and again incubated for 0, 20 min, or longer as indicated. (There was 73% uptake at 30 min.) The pelleted cells were fractionated by procedure II and 0.25 ml of the extranuclear fraction (1.04×10^6 cell equivalents) or 0.15 ml of the nuclear fraction (1.36×10^6 cell equivalents) was sedimented on small high ionic strength sucrose gradients for 1.7×10^6 g × min. The 3-drop-sized fractions were counted directly on planchets. Counts per minute per 3 drops are shown versus fraction number. The 0.1 M NaOH washings of each tube are also shown (P) and the bottoms are to the left.*

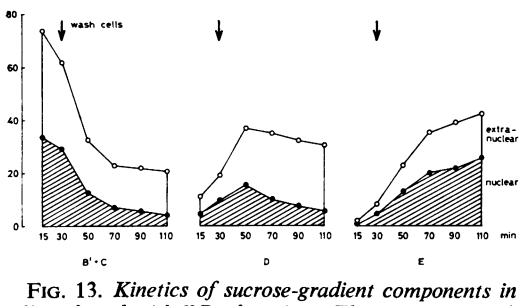

FIG. 13. *Kinetics of sucrose-gradient components in cells infected with ^{32}P-adenovirus. The counts in each gradient of Fig. 12 were divided as indicated. The radioactivity recovered in each component is expressed as percentage of the total cell-associated counts and is shown as a function of incubation time. (All samples incubated longer than 30 min were washed free of unattached virus at 30 min). Symbols: ●, nuclear fraction; ○, sum of nuclear and extranuclear fraction.*

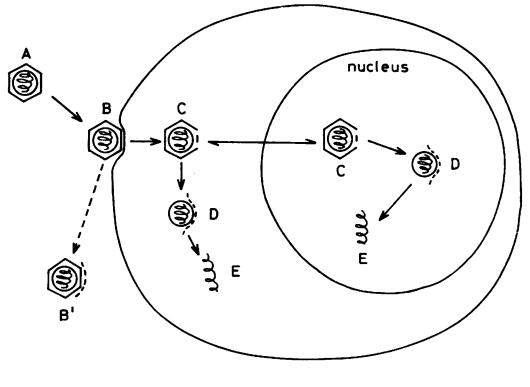

FIG. 14. *Diagram of a hypothetical sequence of events during the first two hours of infection with adenovirus type 2. (Only DNA containing components are shown).*

ous and some attached virus appears to be refractory. The upward deviation of the theoretical curve after 15 min suggests that the latter effect is of greater importance. As an alternative to the simple model indicated in Fig. 14, there may be several steps such as penetration and translocation between attachment and uncoating. Any one of such steps would then be rate limiting.

First products of virus-cell interaction. The virus-receptor complex (B) is found when a virion reacts with a specific receptor site on the plasma membrane and there is evidence that this is mediated by the fiber proteins (22). The complex can be disrupted by detergent or sonic oscillation to give a product, B′, which is deoxyribonuclease-resistant and which has a buoyant density close to the of the starting virus. B′ could still contain a fragment of the plasma membrane as is drawn in Fig. 14; this could be as small as the erythrocyte-receptor for adenovirus type 7 which Neurath et al. have solubilized with DOC (16), or it could be larger, depending upon the extent of membrane disruption.

B′ could easily be mistaken for free virus (Fig. 3b) but with gentle cell disruption it is possible to show that there is little, if any, free intact virus in the cytoplasm (Fig. 3a). It is also possible that there is some virus within intracytoplasmic vesicles which are not broken by gentle homogenization; however, such particles must be considered as encapsulated rather than "free" in the cytoplasm. The absence of free intact virus in the cytoplasm is in accord with the lack of kinetic evidence for more than one intermediate between A and C. The evidence then suggests that virions are uncoated as they are released from the membrane into the cytoplasm. More conclusive evidence for such a mechanism can probably only be obtained with the development of an efficient in vitro system.

Component C appears to be unbound to cellular material. It is analogous to the partly uncoated product Sussenbach has recovered from HeLa cells infected with adenovirus type 5 (27). The extent to which it is digestable by deoxyribonuclease is variable, but often it appears to be less sensitive than that of other virion products; as Sussenbach has already shown, C lacks only a small portion of the total protein of the virion. When intact virus is dialyzed against certain buffers, a deoxyribonuclease-sensitive product is formed in vitro and this is missing much of the total penton antigen of the virion (23; Prage et al. *unpublished*). The in vitro product is somewhat more sensitive to deoxyribonuclease and is slightly higher in buoyant density than is C. By analogy, C is drawn in Fig. 14 as missing some vertex material; Sussenbach also suggested that penton antigen is missing from C (27).

Entry of viral DNA into the cell nucleus. A significant finding in this study is that a deoxyribonuclease-sensitive product from ^{32}P-labeled adenovirus type 2 migrates rapidly into the cell nucleus (Fig. 2). After 30 min of incubation, an average of 41% of the cell-associated ^{32}P has entered the KB or HeLa cell nucleus and much of this is uncoated. This is probably not the result of an artifact, since control experiments indicated that artifacts in distribution are likely to occur as losses from the nuclear fraction. Furthermore, an electron microscopic study (8) of nuclei prepared by the methods used in this work showed that even the outer portion of the nuclear membrane is removed by the detergents.

Lawrence and Ginsberg also observed that about 40% of the ^{32}P from labeled adenovirus type 5 gains entry, after cell attachment, into what was described as a crude nuclear fraction of KB cells (12). Hochberg and Becker have reported the uncoating and entry of labeled DNA from herpesvirus into BSC cell nuclei (7). Only uncoated virus entered the nucleus, and in this case much of the virion protein remained in the extranuclear fraction. The overall process was considerably slower than that described in the present study of adenovirus type 2.

In an electron microscopic study of the interaction of adenovirus type 7 with HeLa cells, Dales (1) observed what appeared to be cellular entry by pinocytosis ("viropexis"). The virions remained encapsulated for several hours, but eventually a few apparently intact virions were released and were thought to enter into and to be uncoated within the nucleus. Such a process is not suggested by the kinetics of the adenovirus type 2 system as studied by biochemical techniques.

There is relatively little B′ associated with the nuclear fraction of cells infected with adenovirus type 2 (Fig. 11). At 15 and 30 min after attachment begins, roughly 50% of all component C is within the nucleus (Fig. 4 and 11). In the diagram of Fig. 14, component C is represented as being the only form in which viral DNA enters the nucleus.

Transformation of C to D. Much of component C present after a 30-min attachment period is used up within 45 min as shown by CsCl-gradient analysis (Fig. 11). Although sucrose-gradient analysis does not separate B′ from C, the disappearance of the B′ plus C peak in the nuclear fractions may be used to indicate the disappearance of C because the nucleus contains little B′. This indicates that more than half of the nuclear C is used up in the period between 30 and 50

min (Fig. 13), during which time some C is probably still entering the nucleus, and thus the in vivo life expectancy of C may be about the same as that of B.

D appears to be formed as a product of C (Fig. 13). It probably also contains some complexed cell material because of its low apparent buoyant density. Even after D is given a second detergent treatment, after sucrose-gradient separation, it has a buoyant density in CsCl of about 1.36 g/cm^3 with a broad dispersion (Fig. 7). About 33% of the original protein-arginine of the infecting virion is left associated with the DNA, as shown by double labeling. This in turn probably means that even more than 66% of the virion protein has been removed, since the capsid proteins are the most likely to have been removed and they are relatively poor in arginine (U. Pettersson and S. Höglund, Virology, *in press*). The buoyant density of an adenovirion lacking more than 66% of its protein is likely to be greater than 1.42 g/cm^3 (25). Cell protein or lipid or both must still be associated with D after detergent treatment.

A number of studies have shown that adenovirions contain a core composed of internal proteins and DNA (11, 15, 23, 24). Soluble "cores" have been obtained by treatment of intact virions with acetone (11) or urea (15), and we have modified and used the urea method to produce material for comparison with the components found in vivo. If the urea core is exposed to disrupted cells it picks up lipid-containing material and then bands in CsCl at a low density. Even after treatment with detergent, the resulting complex still bands at about 1.36 g/cm^3 (Fig. 8), which is considerably lighter than pure core (Fig. 9c). Because they are both "sticky" towards cell material and because they both retain a similar proportion of the virion arginine, D is pictured in Fig. 14 as an in vivo core and it is also shown complexed with an intracellular membrane.

Formation of component E. The formation of E is fastest, or most efficient, in the nucleus where it is almost completed in 2 hr and where the kinetics indicate that D is the precursor (Fig. 13). The evidence suggests that E also occurs outside of the nucleus, but the possibility of some leakage from the nuclear fraction during cell disruption makes it difficult to estimate this quantitatively.

Component E is largely free of protein from the virion core as shown by rate-zonal centrifugation in CsCl (Fig. 9). E is represented in Fig. 14 as naked DNA, although it is likely that it will form ionic bonds with cellular proteins. It is also shown as intact because in infected cells E is formed more rapidly than the virion DNA can be degraded (Fig. 10). Because E is sheared during cell disruption or fractionation, it is not known whether it is complexed with membrane material at one or a few points along its entire length or also whether a small portion remains associated with virion protein.

The removal of protein in the formation of E is probably an active process. We are currently testing the hypothesis that this may entail a chemical modification of the basic core proteins.

ACKNOWLEDGMENTS

This investigation was supported by the Damon Runyon Memorial Fund, the Swedish Medical Research Council, and the Swedish Cancer Society. We thank Helena Hessle and Eva Hjertson for their capable technical assistance and Jan Johansson and K. K. Kannan for their generous help with numerical analysis.

ADDENDUM IN PROOF

After submission of this report, we were informed of the study of C. Morgan, H. S. Rosenkranz, and B. Mednis entitled "Structure and Development of Viruses as Observed in the Electron Microscope. X. Entry and Uncoating of Adenovirus," which is to be published in the *Journal of Virology*. The results appear to be comparable, in spite of the fact that different types of adenovirus were used. Presumably, our B component corresponds to structures shown in Fig. 4, 5, and 6 of Morgan et al. The C component is probably the spherical form with the granular core illustrated in their Fig. 7–21, whereas our D component may be the dense core visible in the particles of Fig. 38–41. Whether the E component has been visualized is uncertain. The electron microscopic evidence suggests that our "nuclear" C component may not be located within the nucleus but rather that it is attached to the outer nuclear membrane as in Fig. 32, 36, and 37 of Morgan et al.

Recent experiments of ours indicate that component E is not entirely free within the nucleus. When nuclei from infected cells, which contain mainly E, are lysed directly in CsCl solution, most of the labeled DNA floats to the top of an ultracentrifuge gradient into which added free viral DNA is banded. This may be interpreted to indicate that the unsheared DNA is membrane bound.

LITERATURE CITED

1. Dales, S. 1962. An electron microscope study of the early association between the mammalian viruses and their hosts. J. Cell Biol. 13:303–322.
2. Giles, K. W., and A. Myers. 1965. An improved diphenylamine method for the estimation of DNA. Nature (London) 206:93.
3. Green, M., and M. Piña. 1963. Biochemical studies on the adenovirus multiplication. IV. Isolation, purification and chemical analysis of adenovirus. Virology 20:199–207.
4. Green, M., M. Piña, and R. C. Kimes. 1967. Biochemical studies on adenovirus multiplication XII. Plaquing efficiencies of purified human adenoviruses. Virology 31:562–565.
5. Green, M., M. Piña, R. Kimes, P. C. Wensink, L. A. MacHattie, and C. A. Thomas, Jr. 1967. Adenovirus DNA. I. Molecular weight and conformation. Proc. Nat. Acad. Sci. U.S.A. 57:1302–1309.

6. Hayes, F. N. 1963. Solutes and solvents for liquid scintillation counting. Packard Technical Bulletin no. 1, revised. Packard Instrument Co., Downers Grove, Ill.
7. Hochberg, E., and Y. Becker. 1968. Adsorption, penetration and uncoating of herpes simplex virus. J. Gen. Virol. 2:231–241.
8. Holtzman, E., I. Smith, and S. Penman. 1966. Electron microscopic studies of detergent treated HeLa cell nuclei. J. Mol. Biol. 17:131–135.
9. Joklik, W. K. 1965. The molecular basis of the viral eclipse phase, p. 44–96. *In* J. L. Melnick (ed.), Progress medical virology, vol. 7, Karger, Basel.
10. Lacy, S., and M. Green. 1965. Biochemical studies on adenovirus multiplication. VII. Homology between DNA's of tumorigenic and nontumorigenic human adenoviruses. Proc. Nat. Acad. Sci. U.S.A. 52:1053–1059.
11. Laver, W. G., H. G. Pereira, W. C. Russell, and R. C. Valentine. 1968. Isolation of an internal component from adenovirus type 5. J. Mol. Biol. 37:379–386.
12. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. J. Virol. 1:851–867.
13. Lowry, O. H., N. J. Rosebrough, A. F. Farr, and R. J. Randall. 1951. Protein measurements with the Folin-phenol reagent. J. Biol. Chem. 193:265–275.
14. Maizel, J. V., Jr., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A and 12. Virology 36:115–125.
15. Maizel, J. V., Jr., D. O. White, and M. D. Scharff, 1968. The polypeptides of adenovirus II. Soluble proteins, cores, top components and the structure of the virion. Virology 36: 126–136.
16. Neurath, A. R., R. W. Hartzell, and B. A. Rubin. 1969. Solubilization and some properties of the erythrocyte receptor for adenovirus type 7 hemagglutinin. Nature (London) 221:1069–1071.
17. Norrby, E. 1968. Biological significance of structural adenovirus components. p. 1–43. *In* W. Arber et al. (ed.), Current topics in microbiology and immunology, vol. 43. Springer-Verlag, New York.
18. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. 17:117–130.
19. Penman, S., J. Smith, and E. Holtzman. 1966. Ribosomal RNA synthesis and processing in a particulate site in the HeLa cell nucleus. Science 154:786–789.
20. Philipson, L. 1961. Adenovirus assay by the fluorescent cell-counting procedure. Virology 15:263–268.
21. Philipson, L. 1967. Attachment and eclipse of adenovirus. J. Virol. 1:868–875.
22. Philipson, L., K. Lonberg-Holm, and U. Pettersson. 1968. Virus-receptor interaction in an adenovirus system. J. Virol. 2:1064–1075.
23. Prage, L., U. Pettersson, and L. Philipson. 1968. Internal basic proteins in adenovirus. Virology 36:508–511.
24. Russell, W. C., W. G. Laver, and P. J. Sanderson. 1968. Internal components of adenovirus. Nature (London) 219:1127–1130.
25. Russell, W. C., R. C. Valentine, and H. G. Pereira. 1967. The effect of heat on the anatomy of the adenovirus. J. Gen. Virol. 1:509–522.
26. Schlesinger, R. W. 1969. Adenoviruses: the nature of the virion and of controlling factors in productive or abortive infection and tumorigenesis. p. 1–61. *In* K. M. Smith and M. A. Lauffer (ed.). Advances in virus research, vol. 14. Academic Press Inc., New York.
27. Sussenbach, J. S. 1967. Early events in the infection process of adenovirus type 5 in HeLa cells. Virology 33:567–574.
28. Thiel, J. F., and K. O. Smith. 1967. Fluorescent focus assay of viruses on cell monolayers in plastic Petri plates. Proc. Soc. Exp. Med. 125:892–895.