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Intracellular Uncoating of Type 5 Adenovirus Deoxyribonucleic Acid

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Highly purified, ^{32}P-labeled type 5 adenovirus was employed to study "uncoating" of viral deoxyribonucleic acid (DNA)—defined as the development of sensitivity to deoxyribonuclease. Viral infectivity and radioactivity adsorbed to KB cells at the same rate, and significant amounts of ^{32}P did not elute from cells throughout the eclipse period. Kinetic studies of viral penetration, eclipse of infectivity, and uncoating of viral DNA indicated that the three events were closely related temporally, that the rates of each were similar, and that they were completed within 60 to 90 min after infection. Viral penetration, eclipse, and uncoating proceeded normally under conditions which blocked protein synthesis, but they did not occur at 0 to 4 C. Neither viral DNA nor viral protein was degraded to acid-soluble material during the eclipse period. The nature of adenovirus DNA was studied after it was converted intracellularly from deoxyribonuclease-resistant to deoxyribonuclease-susceptible. Intact virions centrifuged in sucrose gradients had a sedimentation coefficient of approximately 800, and viral DNA sedimented as a particle of about 30*S*. Infection of KB cells with purified ^{32}P-labeled virus yielded deoxyribonuclease-susceptible viral nucleic acid which was in particles with sedimentation coefficients of 350 to 450*S*, i.e., greater than 10 times faster than DNA obtained from purified virions which had been disrupted by exposure to *p*H 10.5. When the DNA from disrupted virions was mixed with cell lysates, its sedimentation characteristics were essentially unchanged by the presence of cellular material.

Successful viral replication requires that the infecting virus undergo alterations that will allow the viral nucleic acid to direct synthesis of new virus-specific macromolecules. For the T-even bacteriophages, specialized structures effect the transfer of viral nucleic acid, free from its protein coat, to the interior of the cell (15). However, viruses that infect mammalian cells do not possess such structures, and it appears that these viruses effect the release of the genome from their protective capsid by intracellular processes.

Purified, radioactive-labeled virus has been employed to analyze the intracellular fate of poliovirus (23), mengovirus (17), and poxvirus (20, 21). In the study of poliovirus infection of HeLa cells, extensive elution of ^{32}P-labeled virus occurred, after which most of the remaining cell-associated virus was extensively degraded to acid-soluble material during a 2-hr period, and only about 5% of the intracellular ribonucleic acid (RNA) remained acid-precipitable. It was concluded that adsorbed virus was physically altered, resulting in reversible attachment of the majority of input virus, and that only a small fraction of input virus actually succeeded in initiating infection, whereas the remainder was degraded (23).

In contrast, study of the fate of ^{32}P-labeled purified mengovirus, an agent similar to poliovirus, revealed that parental RNA was conserved physically throughout the eclipse phase. In lysates of infected cells, it was found that newly synthesized progeny RNA was sensitive to ribonuclease, whereas only about 20% of the parental viral RNA was observed to be sensitive to nuclease. In this study, the particle-plaque-forming unit (PFU) ratio was 5, and it was postulated that four of five virions remained unchanged during infection, and that one of five was "uncoated" to yield ribonuclease-sensitive material (17).

Joklik (20, 21), employing radioactive-labeled, purified rabbitpox (RP) virus to infect HeLa

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cells, analyzed the intracellular fate of the infecting viral deoxyribonucleic acid (DNA), protein, and phospholipid. The evidence obtained indicated that the infecting viral DNA was conserved throughout the eclipse period and that the release of poxvirus DNA was a two-stage process. The first stage began immediately after viral penetration, and resulted in breakdown of viral phospholipid and dissociation of about 50% of the viral protein from the parental virion. This step occurred under conditions where host-cell protein and RNA syntheses were inhibited. After 1 hr, the second stage of uncoating was initiated by a newly synthesized "uncoating protein"; after 3 hr, 60 to 70% of the parental viral DNA was converted to a deoxyribonuclease-sensitive form and was considered to be "uncoated."

After infection of KB cells with type 5 adenovirus, infectious progeny virions are not detected until about 14 hr after infection (6). Assembly of infectious virus is preceded by the biosynthesis of viral DNA and protein, beginning approximately 10 and 12 hr, respectively, postinfection (6, 10, 32). The earliest virus-specific biosynthetic event which has been detected is the essential synthesis of RNA at 9 hr after infection (7). This has been demonstrated to be viral messenger RNA (30); (Bello and Ginsberg, *in preparation*).

It was the object of this study to investigate the eclipse of the infecting virion, the release of viral DNA from its protein coat, and the relationship of these events to the relatively long latent period essential for adenovirus replication. To obtain information concerning the physical changes accompanying viral "uncoating," the sedimentation characteristics of viral particles during "uncoating" were analyzed in linear sucrose density gradients.

TABLE 1. *Composition of plaque medium for adenovirus assay*[a]

Ingredient	Amt
10 × concn of Earle's solution without $NaHCO_3$	200 ml
Antibiotic solution (10,000 units of potassium penicillin G and 10 mg of streptomycin sulfate per ml)	20 ml
6% $NaHCO_3$ solution	74 ml
2.5% glutamine solution	20 ml
Lactalbumin hydrolysate	5 g
Peptone (Difco)	10 g
MEM (5) amino acids (100 × concn)	20 ml
MEM vitamins (100 × concn)	4 ml
Distilled water, to final volume of	1,000 ml

[a] Ingredients to prepare 1 liter of 2 × concentrated medium.

The results of these experiments indicated that the eclipse of infectivity and the development of sensitivity of the parental viral nucleic acid to deoxyribonuclease (termed "uncoating") occurred soon after viral penetration. Uncoating of adenovirus DNA was not dependent on new protein synthesis or induction of new enzymes. The results also indicated that for at least 4 hr after infection parental viral DNA which had been converted to a deoxyribonuclease-sensitive state sedimented at about one-half the rate of intact virus, and 10 times faster than free viral DNA.

MATERIALS AND METHODS

Virus. The prototype strain of type 5 adenovirus was used (18). Pools of crude virus were prepared by infecting monolayer cultures of KB cells at a multiplicity of 50 to 100 PFU per cell. At 36 to 40 hr after infection, the cells were harvested and seed virus was prepared as previously described (6, 10). Samples (4 ml) of crude virus were stored at −20 C for use in experiments.

Tissue culture. KB cells in suspension cultures were maintained in the exponential growth phase by use of Eagle's medium, modified for suspension cultures (25) by supplementation with 10% human or calf serum. The cell concentrations of suspension cultures were maintained at 1.5×10^5 to 4×10^5 cells per ml. For infection of cells, chicken serum replaced human or calf serum at the initiation of the experiment.

Plaque assay of viral infectivity. Amounts of 0.2 ml of appropriate viral dilutions were allowed to adsorb to KB cell monolayers in 60-mm culture dishes for 3 hr at 37 C with frequent rocking of the plates to redistribute unadsorbed virus and prevent drying of cells. The plates were then overlaid with 10 ml of plaque (Pl) medium at *p*H 7.3, and incubated at 37 C in a humidified 8% CO_2-air atmosphere. The composition of double strength Pl medium is shown in Table 1. The final overlay medium was prepared by mixing 42.5 parts of twice concentrated Pl medium, 42.5 parts of melted 2% Difco Purified Agar in water, and 15 parts of chicken serum at 44 C. After incubation for 8 days, the plates were overlaid a second time with 4 ml of Pl overlay medium containing neutral red sufficient to give a concentration of 1:50,000 in the total overlay volume. The final plaque counts were made on the 14th day postinoculation. Four monolayer cultures were employed for each viral dilution.

This modified plaque technique for assay of adenovirus was highly sensitive and reproducible; there was a linear relationship between plaque counts and viral concentration, and there was a Poisson distribution of plaque counts on 50 plates inoculated with aliquot samples of a single virus dilution.

Purification of virus. Spinner cultures of KB cells at a concentration of 3×10^5 to 4×10^5/ml were infected at a multiplicity of 100 to 200 PFU per cell and incubated for 36 to 40 hr. The infected cells were harvested by centrifugation and resuspended in a volume of 0.01 M PO_4 buffer (*p*H 7.4) equal to 1/100

of the volume of the original culture. To obtain cell lysis, sodium deoxycholate (DOC) was added to a final concentration of 0.5%. After allowing 30 min for lysis of cells, $MgCl_2$ was added to a final concentration of 0.02 M, and pancreatic deoxyribonuclease to a concentration of 10 μg/ml. After incubation at 37 C for 1 or 2 hr, the mixture was centrifuged at 1,400 $\times$ g for 30 min. The resulting supernatant fluid was then extracted three times with an equal volume of Freon 113 (Trichlorotrifluoroethane; E. I. Dupont de Nemours Co., Wilmington, Del.) at room temperature.

The Freon-treated viral suspension was further purified by a series of three equilibrium density gradient centrifugations. The first centrifugation employed a nonlinear CsCl density gradient (31, 32) consisting of three successive layers: 0.7 ml of CsCl solution of density 1.6 g/cc, 1.2 ml of CsCl of density 1.3 g/cc, and 3.0 ml of viral suspension. The final two centrifugations employed preformed linear CsCl gradients ranging from 1.2 to 1.4 g/cc. The viral band collected from the nonlinear gradient was diluted to a density of less than 1.2 g/cc with water and layered on top of a linear CsCl gradient. The centrifugations were carried out at 5 C in a Spinco model L preparative ultracentrifuge with the use of the SW-39 rotor. The first and second gradients were centrifuged at 34,000 rev/min for 3 hr; the third gradient was centrifuged at 34,000 rev/min for 12 to 16 hr. After each centrifugation, the viral band was collected through a small puncture hole at the bottom of the tube. CsCl was removed from purified virus preparations by filtration through a column (2 $\times$ 15 cm) of Sephadex G-25 developed with 0.15 M NaCl, 0.005 M PO_4, pH 7.2 (PBS). Fractions containing visible virus, recovered in the void volume of the column, were pooled, diluted with PBS to a concentration of approximately 10^{10} PFU/ml, and stored at 4 C.

Preparation of isotopically labeled virus. To label viral DNA with ^{32}P, KB spinner cultures were prepared in Eagle's spinner medium lacking phosphate and containing 10% dialyzed chicken serum. At 6 to 8 hr after infection, carrier-free sodium radiophosphate (^{32}P) was added to a final concentration of 2 to 10 μc/ml. In some cases, 20 ml of Eagle's medium containing PO_4 was added per liter of culture at the time that ^{32}P was added. This addition resulted in higher viral yields, although ^{32}P incorporation into viral DNA was reduced. Cells were harvested 36 hr after infection, and labeled virus was purified by the procedures described above.

To label viral DNA with tritium, 1 μc of thymidine-methyl ^{3}H per ml of culture medium was added at 6 hr postinfection. ^{14}C labeling of virus was accomplished by adding 1 μc of algal protein hydrolysate per ml of culture at 8 hr postinfection. Propagation and purification of ^{3}H- and ^{14}C-labeled virus was carried out as described above.

General experimental conditions for measuring viral uncoating. In experiments designed to investigate the release of viral DNA from its capsid, the following conditions were employed. KB cells in logarithmic growth phase were centrifuged for 10 min at 800 $\times$ g and resuspended in spinner medium at a concentration of 5 $\times$ 10^6 to 10 $\times$ 10^6 per ml. Labeled, purified type 5 adenovirus was added at multiplicities of 200 to 1,000 PFU/cell. Adsorption proceeded at 0 C for 10 to 15 min, at which time approximately 10% of the input virus had become firmly cell-associated. The infected cells were centrifuged for 10 min at 800 $\times$ g and then washed three times with 200 ml of cold medium. This washing procedure removed over 99% of the unadsorbed virus. After washing, the virus-cell complexes were resuspended at a concentration of approximately 10^6 cells/ml of spinner medium which had been prewarmed to 37 C. The time at which cells were resuspended was considered time-zero. At various times, 10-ml samples were withdrawn and transferred to chilled tubes in an ice bath. Cells were sedimented at 800 $\times$ g and washed with 10 ml of cold PBS. The cells were then resuspended in 1 ml of 0.01 M PO_4 (pH 7.0) containing 0.5% DOC and stored at 4 C until assayed for total and acid-soluble radioactivity, and radioactivity rendered acid-soluble after incubation with deoxyribonuclease.

Assay of deoxyribonuclease sensitivity of viral DNA. Uncoating of the adenovirus particle was determined by measuring the conversion of viral DNA from resistance to deoxyribonuclease (intact virus) to sensitivity to the enzyme. The method employed for determination of nuclease sensitivity of viral DNA was a modification of a procedure described by McDonald (27) for assay of pancreatic deoxyribonuclease. The reaction mixture had a final volume of 1.0 ml and contained 45 μM phosphate (pH 7.0), 20 μM $MgCl_2$, 200 μg of deoxyribonuclease I (crystalline, Worthington Biochemical Corp., Freehold, N. J.), and 0.5 ml of infected-cell extract. After incubation of the reaction mixture for 30 min at 37 C, the tubes were placed in an ice bath and 0.1 ml was withdrawn for determination of total radioactivity. To the remaining 0.9 ml, an equal volume of cold 10% trichloroacetic acid was added, and after 30 min in an ice bath the mixture was centrifuged at 2,000 $\times$ g for 30 min. A 0.2-ml amount of the cold trichloroacetic acid supernatant fluid was carefully withdrawn, placed on a planchet, and dried to determine the radioactivity of the acid-soluble material. The quantity of labeled material present in extracts not treated with deoxyribonuclease was determined by omitting the enzyme from the incubation mixture. The results of control studies, presented in Table 2, showed that DOC did not inactivate the infectivity of type 5 adenovirus, and did not render the DNA of intact virus susceptible to the action of deoxyribonuclease. In addition, the level of deoxyribonuclease-sensitive viral ^{32}P in extracts of infected cells prepared either by ultrasonic disruption or by using DOC was the same, and indicated that DOC did not inhibit the ability of deoxyribonuclease to hydrolyze viral DNA.

Measurement of viral penetration. A 0.2-ml amount of a dilution of type 5 adenovirus containing about 1,000 PFU was added to KB cell monolayers in 60-mm culture dishes. After 10 min at 4 C, free virus was removed by washing three times with 6 ml of cold medium. The plates were covered with 2 ml of fresh medium and incubated at 37 C. At intervals, antiviral serum was added to groups of four plates and held at

TABLE 2. *Effect of sodium deoxycholate on type 5 adenovirus*

Treatment of virus	PFU/ml	Counts per min per ml	
		Total	Trichloro acetic acid soluble
None	9×10^9	—	—
DOC (0.5%), 14 hr at 4 C	8.7×10^9	—	—
None	—	6,420	120
DOC (0.5%)	—	7,000	30
Deoxyribonuclease	—	7,060	240
DOC (0.5%) + deoxyribonuclease	—	7,000	260

room temperature for 1 hr. (As antiviral serum, a 1:25 dilution of anti-type 5 adenovirus rabbit serum was used: a 1:50 dilution neutralized greater than 90% of free virus within 30 min at room temperature.) Antiserum was removed by washing three times with 6 ml of medium, and the monolayers were overlaid and incubated as for the plaque assay.

Ultraviolet inactivation of type 5 adenovirus. Purified, ^{32}P-labeled virus was diluted with PBS to a concentration which had an optical density reading of 0.215 at 260 mμ in a Zeiss spectrophotometer; this corresponded to approximately 10^{10} PFU/ml. A 0.5-ml amount of the viral dilution was placed in the center of a 60-mm culture dish and exposed to ultraviolet (UV) radiation from a 15-watt General Electric germicidal lamp at a distance of 10 cm. During UV treatment, the viral suspension was constantly agitated by a tiny magnetic stirring bar. It was found that UV treatment for 2 min under these conditions resulted in a survival of $10^{-4.5}$, and that the DNA of inactivated virus remained insensitive to the action of deoxyribonuclease.

Sucrose gradient centrifugation. The procedures of Martin and Ames (28) were employed for sucrose gradient centrifugation studies. Times of centrifugation refer to the time of the run at the indicated speed, and do not include time for acceleration and deceleration. The bottoms of gradient tubes were punctured, and 3-drop fractions were collected in steel planchets or on filter-paper discs preparatory to assay of radioactivity.

Measurement of radioactivity. Two methods were employed. (i) Samples dried on steel planchets were counted in a windowless gas-flow Geiger counter (Nuclear-Chicago Corp., Des Plaines, Ill). (ii) Samples were collected on filter-paper discs, dried, washed twice with cold 5% trichloroacetic acid, dried, and placed in vials containing 5 ml of a solution of 0.5 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene and 6.0 g of 2,5-diphenyloxazole per liter of toluene. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

Analytical methods. Cell counts were done in triplicate in a hemocytometer. DNA was determined by the diphenylamine method of Burton (1), with calf thymus DNA as the standard. RNA was assayed by the orcinol method, with D-ribose as the standard (3). Protein was measured by the method of Lowry (26), with crystalline bovine serum albumin as the standard. Refractive index measurements were made with an Abbe refractometer, and buoyant density was calculated according to the method of Ifft (19). UV absorbance was measured with a Zeiss spectrophotometer using cells with a 1-cm light path.

RESULTS

Characteristics of purified type 5 adenovirus. An investigation of the fate of radioactive-labeled parental virus after its attachment to susceptible cells required the use of highly purified virus. Consequently, preparations of purified virus were analyzed to insure that contaminating material was not present.

In Fig. 1a, it may be seen that all of the radioactivity appeared in a single band corresponding to a buoyant density of 1.335 g/cc. The band of infectivity was wider and less symmetrical than those of UV absorbance and radioactivity. The skewedness of the infectivity band appeared to reflect the greater sensitivity of the assay for infectivity as compared to the physical determinations, and also to be the result of trailing of about 1% of the infectivity as a consequence of collection from the bottom of the tube. In Fig. 1b, the results of chemical analysis of CsCl gradient fractions are presented. There was no detectable RNA in any fraction; protein and DNA were found only in the fractions containing infectious virus.

Table 3 summarizes the characteristics of purified, ^{32}P-labeled type 5 adenovirus. Of particular importance are the values for trichloroacetic acid-soluble and deoxyribonuclease-sensitive ^{32}P. The level of acid-soluble ^{32}P in purified viral preparations varied from undetectable levels to 3%; treatment with ribonuclease or deoxyribonuclease did not appreciably increase the levels of this material, indicating that the preparations contained very little, if any, contaminating RNA and DNA. Also, these results showed that ^{32}P-labeled DNA in purified virus was protected from the activity of nucleases.

In agreement with the observations of Green (13), it was found that concentrated, purified virus was not stable under a variety of conditions, and low levels of nuclease-sensitive material in purified virus preparations were attributed, in part, to breakdown of viral particles after purification. Frequently, the concentrated purified virus precipitated with loss of infectivity and resistance to deoxyribonuclease during

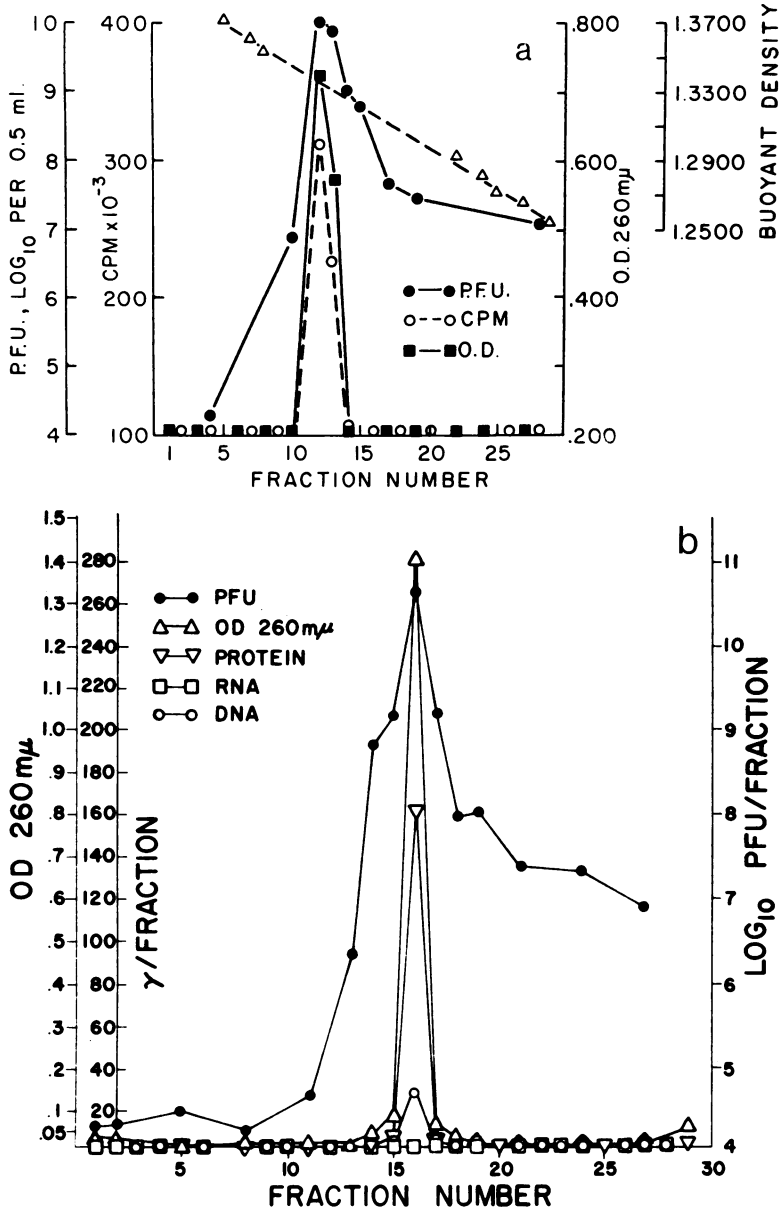

FIG. 1. *CsCl equilibrium density gradient analysis of type 5 adenovirus. Samples (1.5 ml) of purified, ^{32}P-labeled virus were mixed with 3 ml of 1.38 molal CsCl solution and centrifuged for 48 hr at 35,000 rev/min at 5 C by use of an SW 39 rotor. Three-drop fractions were collected from the bottom of the tubes, and the appropriate determinations were made by procedures described in Materials and Methods.*

dialysis to remove CsCl. The use of G-25 Sephadex to remove CsCl proved to be a rapid procedure which avoided the breakdown of virus during desalting. Precipitation of viral suspensions was avoided by dilution to a concentration of less than 10^{10} PFU/ml. Because of the instability of purified virus, the uncoating experiments to be described employed freshly purified virus which was tested for deoxyribonuclease sensitivity prior to each experiment.

It was previously shown that precursors necessary for biosynthesis of adenovirus DNA are derived from intracellular pools from ingredients in the medium, but not from host cell DNA (10). In view of this knowledge, a critical test for the absence of host cell DNA contamination of

TABLE 3. *Characteristics of purified ^{32}P-labeled type 5 adenovirus*[a]

Characteristic	Value
Per cent DNA[b]	15.7 ± 0.4 (3)
PFU/mg of protein	$3.9 \times 10^{11} \pm 1.5 \times 10^{11}$ (3)
PFU/unit of optical density at 260 mμ	3.3×10^{10}/ml ± 1.2×10^{10} (9)
Ratio of optical density at 260 mμ to that at 280 mμ	1.284 ± .012 (6)
Per cent trichloroacetic acid-soluble ^{32}P	1.9 ± 1.6 (5)
Per cent ^{32}P trichloroacetic acid soluble after deoxyribonuclease	2.5 ± 1.4 (5)
Per cent ^{32}P trichloroacetic acid soluble after ribonuclease	1.1 ± 0.3 (2)
Particles/PFU[c]	11.0 ± 5 (4)

[a] Mean values and standard deviations; number in parenthesis is number of preparations examined.
[b] Micrograms of DNA/micrograms of DNA + micrograms of protein.
[c] Calculated as follows: grams of DNA per milliliter, divided by molecular weight of adenovirus DNA, divided by PFU per milliliter, times N, taking 23×10^6 as molecular weight (14); N = Avogadro's number.

TABLE 4. *Purification of type 5 adenovirus synthesized in ^{14}C-thymidine-labeled host cells*

Prepn	Total ^{14}C recovered (counts/min)	Total PFU	Counts per min per PFU	Specific activity of DNA (counts per min per μg)
Infected cell suspension	5.5×10^6	4.0×10^{10}	1.3×10^{-4}	776
Purified virus	94	1.6×10^{10}	5.9×10^{-9}	7
Per cent recovery	0.0017	40	—	—

purified virus was carried out in the following way. KB cells were incubated for 24 hr in the presence of ^{14}C-thymidine. During this time, the cell concentration doubled. The cells were washed and resuspended in medium which was free from radioactive label, and the cells were propagated in "cold" medium for an additional 24 hr, after which they were infected. At 33 hr postinfection, the cells were harvested; a portion of the cell suspension was set aside for assay of ^{14}C and DNA, and the remainder served as starting material for purification of virus. The results of this experiment are presented in Table 4. The overall recovery of infectious virus was 40%, whereas only 0.0017% of the ^{14}C-labeled DNA in the crude suspension was recovered in purified virus. Thus, the purification factor with regard to host cell DNA was at least 2.3×10^4. In addition to providing evidence for the absence of contaminating host-cell DNA in the purified virions, a comparison of the specific activities of host and viral DNA indicated that a maximum of 0.9% of the DNA recovered in purified virus could have been derived from host-cell DNA.

Adsorption of type 5 adenovirus to KB cells. An analysis of the early intracellular events in viral infection required knowledge concerning the initial attachment of viral particles to susceptible cells. Consequently, a study of the characteristics of adsorption of purified ^{32}P-labeled virus to KB cells in suspension was conducted. In addition, since it is known that noninfectious particles are present in purified adenovirus (11), it was important to determine whether all particles, infectious and noninfectious, adsorbed at the same rate, and whether or not elution of a portion of the viral population occurred.

The results of an adsorption experiment, shown in Fig. 2, indicate that viral infectivity

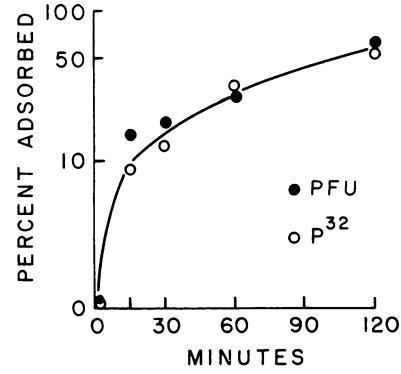

FIG. 2. *Adsorption of ^{32}P-labeled purified type 5 adenovirus. KB cells from a culture in log-phase growth were suspended in spinner MEM at 10^6 cells/ml at 37 C. Purified ^{32}P-labeled virus was added at an input multiplicity of 32. Samples were removed at indicated times and centrifuged; the supernatant fluids were assayed for unadsorbed PFU and for ^{32}P.*

and radioactivity adsorbed at nearly identical rates and to the same extent. Approximately 30% of the virus was adsorbed in the 1st hr; by 2 hr, 40 to 50% of the initial virus was attached to cells. Little additional adsorption occurred after 2 hr. These data are similar to those reported by Green (12) for type 2 adenovirus. The initial rate of adsorption was exponential, and from the data an adsorption constant of 6.1×10^{-0} ml min^{-1} was obtained. (The adsorption constant was calculated from the expression $Vt/Vo = e^{-kct}$, where k is the adsorption constant in milliliters per minute, c is the cell concentration per milliliter, t is time in minutes, Vt is free virus at time t, and Vo is free virus at time 0.)

One-step growth curve of type 5 adenovirus. To relate the early events of the infectious process to the overall growth cycle, a one-step growth curve was studied under the conditions employed in the uncoating experiments to be described. The results are shown in Fig. 3. Immediately after adsorption at 0 C, most cell-associated infectivity was recovered. At this time only 0.1% of virus was free, making it possible to follow the eclipse of infectivity against this low background of infectious virus. At 37 C, there was a rapid eclipse of infectivity so that by 1 hr postinfection only about 3% of the original cell-associated virus was detectable. Further loss of intracellular viral infectivity continued until about 5 hr after infection. Throughout the remainder of the latent period, the levels of infectivity of intracellular and free virus were equivalent, and they were only 0.1% of the original cell-associated virus. The duration of the latent period was 14 hr. Production of intracellular virus then proceeded exponentially so that by 20 hr postinfection approximately 50 PFU per cell had been formed. At that time, virtually all of the newly synthesized virus was intracellular. Viral maturation proceeded until 36 hr postinfection when the maximal intracellular titer was obtained. At this time, the viral yield approximated 4,000 PFU per cell, 98% of which was intracellular.

Intracellular uncoating of viral DNA. A series of experiments were carried out to investigate the nature of the intracellular release of viral DNA from its capsid, and to determine the relationship of viral "uncoating" to other events which occur during viral reproduction. The approach used in these experiments was based on the knowledge that ^{32}P in purified adenovirus was present only in DNA, and that the capsid of intact particles protected the viral DNA from hydrolysis by deoxyribonuclease. The release

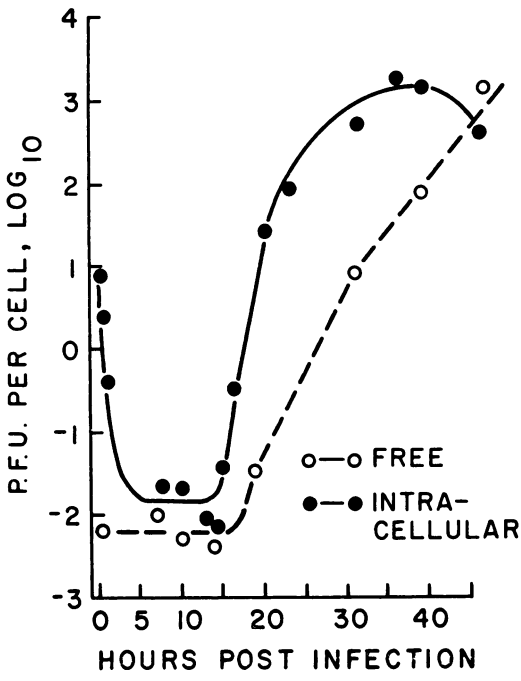

FIG. 3. *One-step growth curve of type 5 adenovirus in a spinner culture of KB cells. Type 5 adenovirus was allowed to adsorb to a suspension of KB cells for 15 min at 0 C. The cells were washed twice with cold medium and resuspended at 115 × 10^5 cells per ml in spinner MEM which had been prewarmed to 37 C. The adsorbed multiplicity was 8 PFU/cell. Samples were withdrawn and assayed for free and cell-associated virus.*

of ^{32}P-labeled parental DNA was determined as described above. In these experiments, "uncoating" refers to intracellular alterations of the viral capsid which render parental DNA accessible to the action of deoxyribonuclease.

Figure 4 illustrates the uncoating of the cell-associated viral DNA and the eclipse of infectivity. Eclipse was rapid and extensive, in accordance with data previously shown in the one-step growth curve. A fraction of virus (19%) was uncoated at 4 C during the 1 hr required for adsorption and washing. At 37 C, intracellular release of viral DNA began almost immediately; uncoating followed eclipse closely, and occurred at a similar rate. Within 1 hr, over 80% of the cell-associated radioactivity had become sensitive to deoxyribonuclease, i.e., was uncoated. The results of this experiment are closely similar to those obtained in four separate experiments carried out in the same way. At time-zero deoxyribonuclease-sensitive viral DNA ranged from 3 to 21%; the maximal level of uncoating observed varied between 80 and 100%.

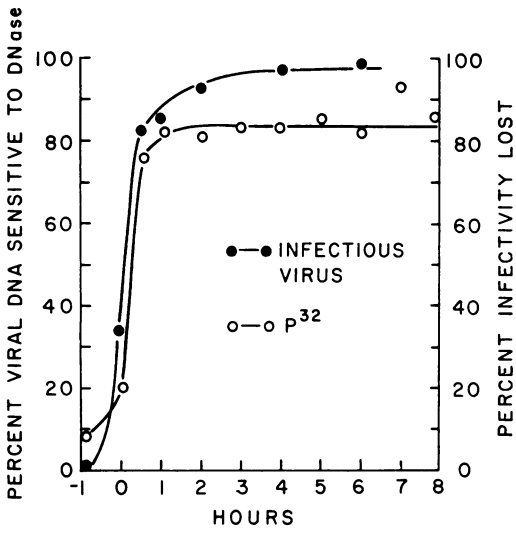

FIG. 4. *Eclipse of type 5 adenovirus and "uncoating" of viral DNA. KB cells were mixed at 0 C with purified ^{32}P-labeled virus (1,000 PFU/cell). After adsorption for 15 min, the cells were washed three times with cold medium, and resuspended in prewarmed spinner MEM. At intervals, 10-ml samples were withdrawn; the cells were washed with cold PBS and resuspended in 1 ml of 0.01 M PO_4, pH 7.0, containing 0.5% DOC. Extracts were then assayed for infectivity, total radioactivity, and deoxyribonuclease-sensitive ^{32}P as described in Materials and Methods.*

Measurements of the distribution of radioactivity in cells and medium were made for each time point (Table 5). Initially, 97% of the virus was cell-associated; gradually, over an 8-hr period, there was an increase in the level of ^{32}P in the medium. When tested for sensitivity to deoxyribonuclease, it was found that up until 2 hr all of this material was trichloroacetic acid-insoluble and resistant to deoxyribonuclease; apparently this was free intact virus not previously removed by washing. The remainder of the ^{32}P which appeared in the medium between 2 and 8 hr consisted of material of which approximately one-half was acid-soluble; the remainder was insoluble but sensitive to deoxyribonuclease. At least part of the small amount of labeled material which gradually appeared in the medium was considered to represent deoxyribonuclease-sensitive material present in the original inoculum, which initially attached to cells and then was slowly released and degraded in the medium. Also, a portion of the extracellular ^{32}P may have resulted from the release of some uncoated or partially uncoated virus from the cells; it is possible that some of this material was derived from cells which had broken down.

TABLE 5. *Distribution of ^{32}P-labeled viral DNA in infected KB cell spinner culture*

Time (hr)[a]	Total counts per min per 0.1 ml[b]	Free counts per min per 0.1 ml[c]	Cell-associated ^{32}P (%)
0	3,100	87	97.2
0.5	3,080	117	96.2
1	3,180	123	96.1
2	3,140	147	95.3
3	3,260	240	92.6
4	3,020	230	92.4
5	2,920	326	88.8
6	2,869	380	96.2
7	2,620	436	84.8
8	2,940	475	83.9

[a] Time 0 is the time at which washed infected cells were resuspended at 37 C.

[b] A 0.1-ml amount of infected cell culture was plated on steel planchets, dried, and counted.

[c] After cells were sedimented by centrifugation at 900 rev/min for 10 min, 0.1 ml of supernatant fluid was plated on steel planchets, dried, and counted.

TABLE 6. *Distribution of cell-associated viral ^{32}P in infected KB cells*

Time (hr)[a]	Per cent cell-associated ^{32}P trichloroacetic acid-soluble	Per cent cell-associated P^{32} trichloroacetic acid-soluble after deoxyribonuclease[b]
0	0.8	19.2
0.5	1.0	76.2
1	1.3	82.0
2	1.4	81.0
3	1.4	83.0
4	1.6	83.0
5	NT[c]	85.0
6	NT	81.5
7	NT	93.0
8	2.2	86.0

[a] Time 0 is the time at which infected washed cells were resuspended at 37 C.

[b] The preparation of cell lysates and assay of deoxyribonuclease sensitivity were as described in Material and Methods.

[c] Not tested.

Approximately 85% of virus remained cell-associated; the distribution of this material is shown in Table 6. Within 60 min, intracellular virus was converted to material that was trichloroacetic acid-insoluble and deoxyribonuclease-sensitive. The maximal amount of DNA observed to become deoxyribonuclease-sensitive was 93%. There are several possible reasons for the failure to observe 100% uncoating. Variation in the extent of uncoating was undoubtedly related to the order of precision and sensitivity

of the assay methods, as well as to differences in the condition of cells, adsorption, washing procedures, and preparation of cell extracts. Small amounts of virus may have attached to sites where penetration did not follow; aggregates or complexes with cellular material may have prevented complete access of deoxyribonuclease to the viral nucleic acid. Joklik (20) observed a maximum of only 67% uncoating in studies of rabbitpox virus.

Only 1% of the cell-associated radioactivity was acid-soluble at time-zero. One hour later when 82% of viral DNA had been uncoated the level of acid-soluble material had not increased; by 8 hr, only 2% of the radioactivity was acid-soluble. These data indicate that the uncoated viral DNA was stable within the cell; i.e., it was not degraded to acid-soluble material.

Relationship between viral penetration and uncoating. Holland (16) observed that eclipse of poliovirus occurred at or near the cell surface, and noted the similarity between the rates of eclipse and penetration, which suggested that these events were closely related. To examine the relationship between penetration and uncoating of adenovirus, the following experiments were carried out.

Monolayer cultures of KB cells in 60-mm culture dishes were employed. An appropriate dilution of purified ^{32}P-labeled virus was added to the monolayers, and after adsorption for 10 min at 4 C free virus was removed by washing. The monolayers were covered with fresh medium and incubated at 37 C. At intervals, plates were removed from the incubator and the extent of viral penetration and uncoating was determined as described in Materials and Methods. To obtain a sufficient level of adsorption of ^{32}P-labeled virus, plates used for uncoating received multiplicities much greater than those utilized to measure penetration, where the number of infectious centers had to be kept sufficiently low to allow counting of plaques. Because of the nature of these experiments, i.e., difficulties in obtaining synchronous adsorption, treatment of large numbers of monolayers, and the noted difference in the inocula, a high order of reproducibility was not obtained. However, the data presented are representative of the results from four separate experiments.

In Fig. 5 it is shown that viral penetration at 37 C occurred rapidly, and was essentially complete within 30 to 60 min after adsorption. Uncoating followed penetration closely; both processes occurred at similar rates, and both were complete by 60 min.

Effect of temperature on viral uncoating. In the case of poliovirus, penetration and irreversible eclipse of infectivity occur at 37 C, but not at 0 C; the temperature dependence of these events suggests the participation of cellular metabolic processes (16). In view of this, the temperature dependence of the uncoating of adenovirus was examined. Purified, ^{32}P-labeled virus was added to KB cells in suspension at 0 C. After 10 min, free virus was removed by washing, and infected cells were resuspended in spinner minimal essential medium (MEM) at 0 C. The spinner culture was kept at 0 C for 3 hr, and then transferred to 37 C. Samples were taken at intervals, and were assayed for infectious virus and viral uncoating.

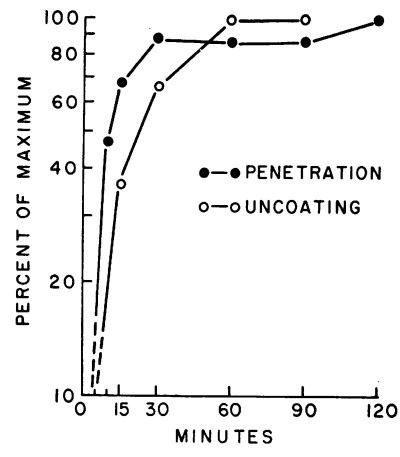

FIG. 5. *Penetration and uncoating of type 5 adenovirus in KB cells. Monolayer cultures of KB cells in 60-mm culture dishes were inoculated with purified ^{32}P-labeled virus. After adsorption for 10 min at 4 C, free virus was removed by washing. The monolayers were covered with 2 ml of fresh medium and incubated at 37 C. At intervals, plates were removed; antibody-resistant infectious centers and uncoating were determined as described in Materials and Methods.*

The results in Table 7 show that, over a 3-hr period at 0 C, detectable uncoating of viral DNA did not occur, nor was there an extensive eclipse of infectivity. Temperature dependence was demonstrated by the rapid loss of viral infectivity and development of deoxyribonuclease sensitivity when the culture was placed at 37 C. The differences in infectivity were considered to be random and due to uncontrolled variations in extraction of virus from cells, and in the plaque assay. The level of free ^{32}P in the medium did not increase during the course of the experiment, and indicated that inhibition of eclipse and uncoating was not the result of elution of virus from the cells.

Although these data are consistent with the notion that uncoating of viral DNA requires metabolic or enzymatic activity, the observed

TABLE 7. *Effect of temperature on eclipse and uncoating of type 5 adenovirus*

Time at 0 C	Time at 37 C after 180 min at 0 C	Per cent uncoated[a]	Per cent loss of infectivity[b]
min	*min*		
0	—	21	0
30	—	16	35
60	—	13	7
120	—	17	20
180	0	17	3
—	15	94	74
—	30	83	75
—	60	90	79

[a] Percentage of total cell-associated ^{32}P sensitive to deoxyribonuclease.

[b] Loss of infectivity present at T_0.

temperature dependence did not prove that this was the case, since it is known that viral penetration did not occur at 0 C (Lawrence and Ginsberg, *unpublished data*), and thus failure to observe eclipse and uncoating at 0 C may indicate only that these events follow penetration and take place intracellularly.

Effect of puromycin on viral uncoating. Joklik (21) demonstrated that the uncoating of poxvirus was blocked by puromycin and actinomycin D. These findings indicated that protein and messenger RNA synthesis were required, and suggested that uncoating was mediated by a host cell enzyme which was synthesized subsequent to infection by poxvirus. In view of these findings for poxvirus, experiments were conducted to determine whether protein synthesis was necessary for uncoating of adenoviruses.

Two spinner cultures were employed: to one culture puromycin hydrochloride was added to a final concentration of 10 μg/ml; the other culture remained as an untreated control. After exposure to puromycin for 2 hr, the cells were mixed with purified ^{32}P-labeled virus. After adsorption, the cells were washed and finally resuspended at 37 C in spinner cultures. In the treated culture, puromycin was present at 10 μg/ml during adsorption, washing, and reincubation at 37 C. Previous experiments showed that, within 1 hr after addition of 10 μg/ml of puromycin, the rate of incorporation of ^{14}C leucine into cellular protein was 10% of untreated control cultures (L. J. Bello, *unpublished data*).

As shown in Fig. 6, preincubation of cells with puromycin at 10 μg/ml for 2 hr before infection and during infection did not alter the rate or the final extent of uncoating. Even when puromycin was employed at concentrations as high as 50 μg/ml, results were obtained similar

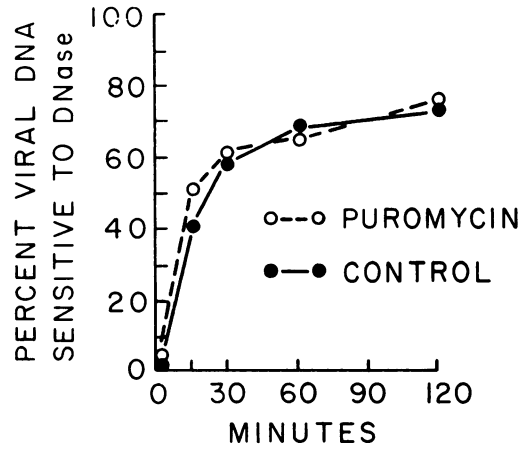

FIG. 6. *Effect of puromycin on uncoating of type 5 adenovirus. Two spinner cultures containing 6 × 10⁷ KB cells in 250 ml were employed. Puromycin hydrochloride (final concentration of 10 μg/ml) was added to one culture; the other culture remained as an untreated control. After 2 hr at 37 C, the cells were centrifuged, resuspended in 10 ml of medium at 0 C, and mixed with purified ^{32}P-labeled virus at an input of 1,000 PFU/cell. After adsorption for 10 min, the cells were washed three times and resuspended at 37 C in spinner MEM. In the treated culture, puromycin was present at 10 μg/ml during adsorption, washing, and incubation. Samples were analyzed by methods described.*

to those shown in Fig. 6. These data indicated that protein synthesis was not necessary for development of deoxyribonuclease sensitivity; thus, if uncoating of adenovirus required cell enzyme activity, this enzyme was not synthesized after the initiation of infection. These experiments, however, did not rule out the possibility that a pre-existing stable cellular enzyme may have mediated the uncoating process.

Uncoating of UV-inactivated virus. Joklik (22) reported that rabbitpox virus inactivated by ultraviolet radiation was not uncoated in HeLa cells; it was postulated that UV treatment affected a viral protein which normally acted as an inducer of the uncoating protein. Although there was no evidence which suggested that adenovirus possessed a similar inducer protein, the uncoating of UV-inactivated adenovirus was investigated to determine whether it was, in fact, adsorbed and uncoated, or whether some UV-sensitive structure was required for uncoating to proceed.

Samples of purified ^{32}P-labeled adenovirus were irradiated for 2 min as described in Materials and Methods, and UV-treated and unirradiated virus were used to carry out uncoating experiments.

TABLE 8. *Uncoating of UV-inactivated[a] type 5 adenovirus*

Time (min)[b]	Per cent viral DNA sensitive to deoxyribonuclease	
	Control[c]	UV-treated[d]
0	16	20
15	89	89
30	80	87
60	95	85
120	78	97

[a] Inactivated to $10^{-4.5}$ survival by the method described in the text.

[b] The time that infected washed cells were resuspended at 37 C.

[c] Total ^{32}P adsorbed was 80×10^3 counts/min, corresponding to 6 PFU per cell.

[d] Total ^{32}P adsorbed was 37×10^3 counts/min, which at a survival of $10^{-4.5}$ corresponded to 0.003 PFU per cell.

The data from a representative experiment, summarized in Table 8, indicate that the rate and extent of uncoating of adenovirus DNA were closely similar for UV-treated and untreated virus. The results show that heavily irradiated virus is effectively uncoated; these data provide evidence that a UV-sensitive viral component is not essential for the uncoating of adenovirus. The fact that the rates and the extent of uncoating of the control virus and of the irradiated virus were closely similar, while the particle to PFU ratios varied between about 10 and 30,000, served to strengthen the postulate that both infectious and noninfectious particles undergo the same changes in the uncoating process.

Fate of ^{14}C-labeled viral protein. The uncoating of virus labeled with ^{14}C-amino acids was examined to provide information as to the nature of alterations which occurred in the viral capsid during the uncoating process. ^{14}C-algal hydrolysate was employed to label virus as described in Materials and Methods. Certain of the amino acids present in ^{14}C-algal hydrolysate are precursors for the de novo synthesis of purines and pyrimidines and result in ^{14}C labeling of viral DNA as well as protein. However, studies with ^{32}P-labeled virus had shown that viral DNA did not become acid-soluble after uncoating unless deoxyribonuclease was added to the cell lysates, so that the appearance of acid-soluble products could be considered to result from degradation of viral protein.

^{14}C-labeled purified virus was adsorbed to KB cells at 0 C for 10 min. The infected cells were washed and resuspended in spinner MEM at 37 C. Less than 1% of the ^{14}C in the preparation of purified virus was acid-soluble. Samples were taken at intervals and assayed for total and trichloroacetic acid-soluble ^{14}C.

TABLE 9. *Fate of viral protein labeled with ^{14}C[a]*

Time (hr)	Total cell-associated ^{14}C counts per min per 0.1 ml	^{14}C acid-soluble counts per min per 0.1 ml[b]
0	285	0[c]
0.25	270	0
0.5	285	5
1	285	0
2	270	5
20	200	0

[a] ^{14}C-labeled purified virus was adsorbed to KB cells at 0 C for 10 min. The infected cells were washed and resuspended in spinner MEM at 37 C. Samples were taken at indicated times and assayed for total and trichloroacetic acid-soluble ^{14}C.

[b] Material soluble in cold 5% trichloroacetic acid.

[c] Background subtracted.

The results presented in Table 9 indicate that the ^{14}C-labeled components of the virus were not converted to acid-soluble material during the time required for uncoating. As late as 20 hr postinfection, ^{14}C-labeled material was not present in the acid-soluble fraction. Hence, uncoating did not involve extensive degradation of the capsid protein and uncoating did not result from activity of proteolytic enzymes. A drop in cell-associated ^{14}C between 2 and 20 hr was accompanied by an increase in acid-insoluble ^{14}C in the medium. This probably represented labeled viral protein released from broken down cells; a portion may have been due to elution of parental virus which had not penetrated into cells.

Sedimentation characteristics of virus and viral DNA. To provide additional information as to the nature of the changes which occurred in parental virions during uncoating, cells infected with purified, ^{32}P-labeled virus were analyzed in linear sucrose gradients. Preliminary experiments were conducted to determine the sedimentation characteristics of purified virus and of viral DNA in sucrose gradients. Purified virus was prepared as described in Materials and Methods. To obtain viral DNA for gradient sedimentation, the purified virus was held for 24 hr in 0.5 M carbonate buffer (*p*H 10.5); this procedure is known to dissociate viral particles into DNA and protein components (32). After treatment at *p*H 10.5, the solution was adjusted to *p*H 7.0 by the addition of 1 M phosphate buffer (*p*H 7.0). Figure 7 shows the results of sedimentation of intact virus and *p*H 10.5 disrupted virus for 5

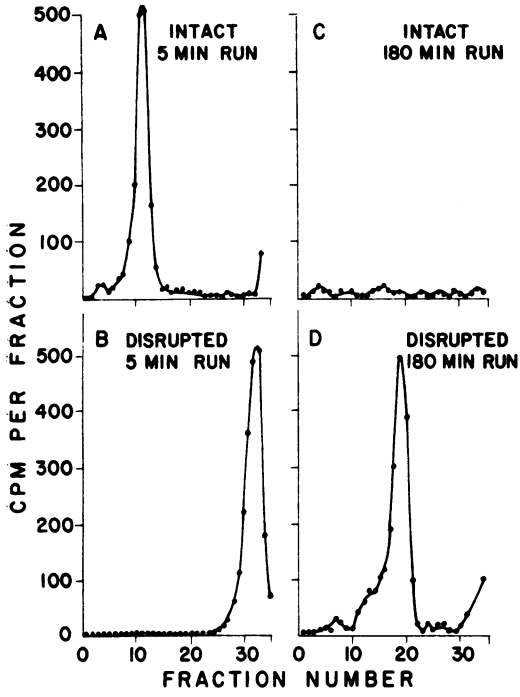

FIG. 7. *Sucrose gradient sedimentation of intact and pH 10.5 disrupted ^{32}P-labeled purified type 5 adenovirus. A 0.1-ml amount of intact or disrupted virus was layered on a 4.5-ml 5 to 20% linear sucrose (in TKE: 0.01 M Tris; pH 7.4; 0.01 M KCl, 0.001 M EDTA) gradient and centrifuged in an SW 39 rotor at 35,000 rev/min, 20 C, for the following times: A and B, 5 min; C and D, 180 min. Three-drop fractions were collected in planchets, dried, and assayed for radioactivity.*

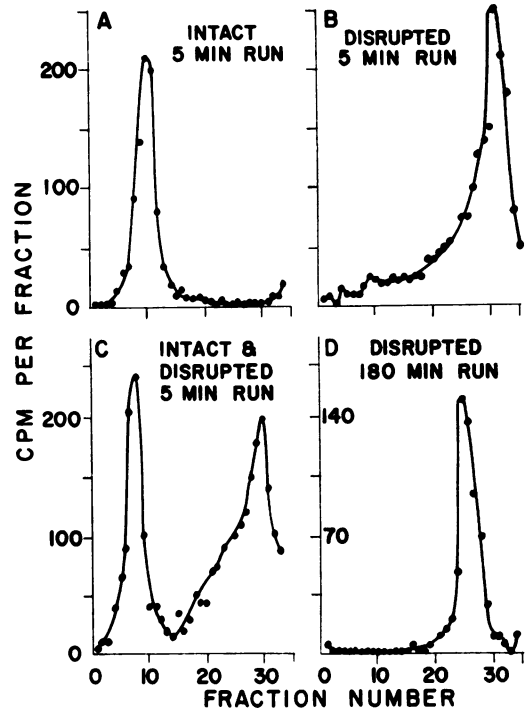

FIG. 8. *Sucrose gradient sedimentation of intact and pH 10.5 disrupted ^{32}P-labeled purified type 5 adenovirus in the presence of KB cell lysate. Intact virus, disrupted virus, or both were mixed with a DOC-prepared lysate of KB cells and allowed to stand for 4 hr. A 0.1-ml amount of the mixture was layered on a 4.5-ml 5 to 20% sucrose (TKE) gradient and centrifuged in an SW 39 rotor at 35,000 rev/min, 20 C, for the following times: A and B, 5 min; C and D, 180 min. Three-drop fractions were collected in planchets, dried, and assayed for radioactivity.*

min in a linear sucrose gradient. Under the conditions employed, intact virus sedimented to the lower third of the tube, whereas the DNA of disrupted virus remained near the top of the gradient (Fig. 7A and B). After centrifugation for 180 min, intact virus sedimented to the bottom of the tube and was not recovered in the gradient; however, the DNA of disrupted virus sedimented about one-third the length of the gradient (Fig. 7C and D). The $S_{20,w}$ for type 2 adenovirus is 795; the sedimentation coefficient of type 2 adenovirus DNA is 30 (11, 14). Based on the time and speeds of centrifugation employed in the experiments, the distances which intact and disrupted type 5 adenovirus sedimented in sucrose gradients corresponded to those expected for particles of approximately 795*S* and 30*S*.

Sedimentation of virus and viral DNA in the presence of cell lysates. To determine whether the presence of cellular material in lysates affected the sedimentation characteristics of intact virus or disrupted virus, the following experiment was carried out. Uninfected KB cells were lysed by adding DOC to a final concentration of 0.5%. Samples of intact virus, *p*H 10.5 disrupted virus, or a mixture of both were mixed with 5 volumes of the cell lysate and allowed to stand for several hours prior to layering on sucrose gradients. The sedimentation profiles of intact and disrupted virions in the presence of cell lysate are shown in Fig. 8. The diagrams indicate that the sedimentation characteristics of intact and *p*H 10.5 disrupted virus were not markedly altered by the presence of cell lysate. In the gradients which were centrifuged for 5 min, there was some broadening of the viral DNA peak which may have been due to association of a small fraction of the DNA with cellular material. The data summarized in Fig. 8 show that the DNA from disrupted virus sedimented

more slowly than shown in Fig. 7. However, this was not observed in other experiments, and was not considered significant.

Sedimentation characteristics of parental virus during uncoating. Extracts of cells which had been infected with ^{32}P-labeled purified virus were prepared at various times while uncoating was proceeding, and the sedimentation characteristics of the labeled material in the extracts was analyzed. To obtain sufficient ^{32}P-labeled viral DNA for analysis, relatively large amounts of cellular material were placed on the gradients. The viscosity of DOC-prepared extracts was high, owing to the presence of cellular DNA, so that adequate resolution of radioactive materials could not be obtained with whole-cell extracts. However, when cells were disrupted by Dounce homogenization in hypotonic buffer and the nuclei were removed by centrifugation, analysis of DOC-treated cytoplasmic extracts of infected cells was possible.

The relevant experiments were conducted as follows. Purified type 5 adenovirus labeled with ^{32}P or ^{3}H-thymidine was prepared as described

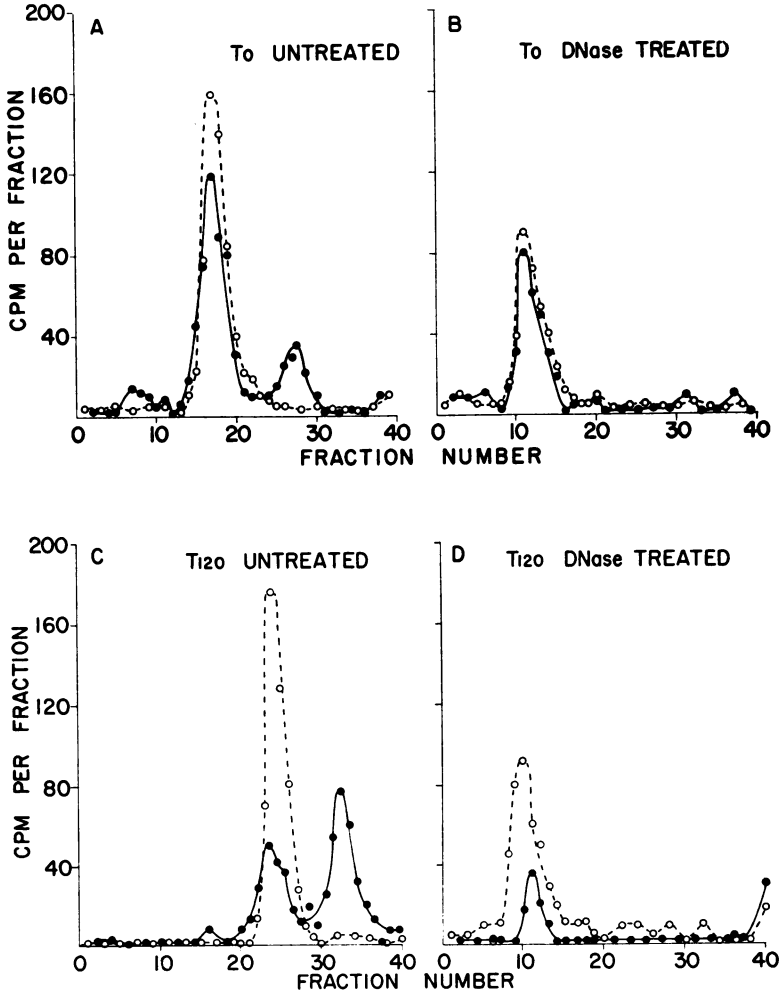

FIG. 9. *Sedimentation on sucrose gradients of intact virions and DNA from uncoated particles at 0 and 120 min after infection. Parental viral ^{32}P, ●; ^{3}H-labeled intact viral marker, ○. A: 0.2 ml of cell extract of time-zero sample was layered on a 10 to 30% sucrose gradient and centrifuged for 5 min at 35,000 rev/min in an SW 39 rotor. B: 0.08 ml of cell extract from time-zero sample was treated with deoxyribonuclease, then layered on a 10 to 30% sucrose gradient, and centrifuged for 5 min at 35,000 rev/min in an SW 39 rotor. C: 0.2 ml of cell extract from 120 min postinfection was layered on a 10 to 30% sucrose gradient and centrifuged for 5 min at 35,000 rev/min in an SW 39 rotor. D: 0.08 ml of deoxyribonuclease-treated 120 min postinfection cell extract was layered on a 10 to 30% sucrose gradient and centrifuged for 5 min at 35,000 rev/min in an SW 30 rotor.*

in Materials and Methods. Less than 1% of the radioactivity in the purified viral preparations was sensitive to deoxyribonuclease. KB cells (300×10^6) were infected at ice-bath temperature with ^{32}P-labeled virus, and adsorption was allowed to proceed for 20 min; the adsorbed multiplicity was 260. The cells were then washed repeatedly with cold medium to remove free virus. Infected cells were resuspended in warm (37 C) medium, and samples were removed at various times. The cells were washed with 25 ml of PBS and then resuspended in 5 ml of hypotonic buffer solution [TKE: 0.01 M KCl; 0.01 M tris(hydroxymethyl)aminomethane, *p*H 7.4; 0.001 M ethylenediaminetetraacetate.] After 5 min at 0 C, the cells were disrupted in a Dounce homogenizer by five strokes with a tight-fitting pestle. Nuclei were removed by centrifugation at 900 rev/min for 5 min. DOC was added to the supernatant cytoplasmic fraction to make a final concentration of 0.5%; 3 ml of chloroform was then added per 5 ml of extract, and the extract was shaken for 5 min and placed at 4 C for 16 hr. After centrifugation at 2,500 rev/min for 15 min, the supernatant fluids were retained for sucrose gradient analysis.

Approximately 60% of the labeled input virus was recovered in the cytoplasmic extract; the remainder was in the crude nuclear fraction which was heavily contaminated with cytoplasm. At time-zero, 15% of the viral DNA in the cytoplasmic extract was sensitive to deoxyribonuclease, and at 2 hr after infection 65% of the virus in the cytoplasmic fraction had been uncoated.

Purified ^3H-thymidine labeled virus was added to cell extracts to serve as a marker for intact virus in each gradient. When extracts were treated with deoxyribonuclease, pancreatic deoxyribonuclease was added to give a final concentration of 50 μg/ml, and $MgCl_2$ was added to a concentration of 0.002 M. The mixture was incubated at 37 C for 3 hr prior to layering on the sucrose gradients.

The sucrose gradient diagrams are presented in Fig. 9. At time-zero (Fig. 9a), the majority of the viral DNA present in infected cell extracts was found to sediment as intact virus about 800*S*; a minor component was present which had a sedimentation coefficient of approximately 400*S*. ^{32}P with the sedimentation characteristics of free viral DNA was not present. Figure 9b shows that the 400*S* material was sensitive to deoxyribonuclease.

At 2 hr postinfection (Fig. 9c and 9d), when approximately 65% of the input virus was uncoated as measured by sensitivity to deoxyribonuclease, 60% of the ^{32}P was found to sediment as deoxyribonuclease-sensitive 400S material. The remaining ^{32}P sedimented with intact ^3H-labeled virus. Again, free viral DNA could not be identified in the gradients. Even when extracts from infected cells were centrifuged for 180 min, free viral DNA (30*S*) was not detected. Analysis of extracts prepared from cells which had been infected for 4 hr produced results which were closely similar to those obtained for extracts prepared at 2 hr after infection.

It was noted that the position in the gradients of intact virus and viral DNA varied in different runs. This variation was probably the result of differences in the times of centrifugation associated with the use of different centrifuges, and variations in the time for acceleration and deceleration. In short runs of 5 min, even slight differences in the time of centrifugation would be expected to affect the position of materials in the gradient significantly. However, the use of instant ^3H-labeled virus as a marker allowed comparison of one gradient with another.

DISCUSSION

Viral "uncoating," as it has been termed, was measured in this and in earlier investigations (20, 21) by noting the time and the rate at which the viral nucleic acid became sensitive to nuclease. In the studies described in this communication, cells were infected with a high multiplicity of purified type 5 adenovirus containing ^{32}P-labeled DNA. Hence, the data presented describe the events in a population of virions, and interpretation of these data rests on the validity of the assumption that the fate of the labeled particles is representative of the infectious virions. The following observations indicate that the assumption is warranted.

(i) The adsorption kinetics were similar when measured by radioactivity or infectivity of the virions. These data imply that all the viral particles, infectious and noninfectious, attached to cells similarly and that in this respect the viral population was homogeneous.

(ii) The kinetics of viral eclipse and the appearance of viral DNA sensitive to deoxyribonuclease were alike. If labeled infectious and noninfectious particles were not similarly affected, the eclipse of infectivity would occur appreciably later than the uncoating as measured. Conversely, if infectious particles were altered before the DNA of the noninfectious particles became deoxyribonuclease-sensitive, eclipse would be detected earlier than the uncoating of the majority of the virions.

Penetration of virions into KB cells appears to be closely associated with eclipse of viral in-

fectivity and development of susceptibility of the viral DNA to deoxyribonuclease. Though these processes occur only to a very limited extent at 0 C, the three begin without delay after infected cells are transferred from 0 to 37 C. They have similar kinetics, and they are complete within about 90 min. It was not possible to be certain whether penetration, eclipse, and uncoating merely follow each other closely in time or whether they have similar mechanisms. Holland (16), studying penetration and eclipse of poliovirus, obtained similar results. The data suggest that after attachment of the virions to the cell membrane the particles are engulfed by a cellular process requiring energy. Within the cytoplasm, probably in the phagocytic vacuoles (2), alteration of the viral capsid occurs, leading to loss of viral infectivity, i.e., viral eclipse. The changes that cause loss of viral infectivity are associated with disruption of the viral capsid, and this process permits access of deoxyribonuclease to the viral DNA, i.e., uncoating. It is possible that penetration, eclipse, and uncoating of both poliovirus and adenovirus are associated with a single cellular process.

Joklik (20, 21), studying the uncoating of another DNA-containing virus, rabbitpox virus, showed major differences from the uncoating of adenovirus when comparable methods were employed for the investigation. Although the process begins immediately after either virus enters the cell, the initial stage of uncoating of rabbitpox virus accomplishes only removal of the outer lipoprotein coat, but this step does not produce deoxyribonuclease-sensitive nucleic acid as it does for adenoviruses. In sharp contrast to adenoviruses, the conversion of poxvirus DNA to a deoxyribonuclease-sensitive form is delayed about 1 hr and protein synthesis is required for this second stage.

The uncoating of adenovirus DNA is temperature-dependent (i.e., it is not initiated at 0 to 4 C), which might suggest that it is accomplished by an enzymatic reaction, perhaps a reaction carried out by an enzyme present in the cell rather than by one that must be synthesized de novo. However, the following evidence indicates that this conclusion is not warranted and that an enzymatic reaction should not be necessary for capsid disruption. (i) Viral penetration requires energy for function of the cell membrane to engulf particles, and this suggests that engulfment was the step blocked at low temperatures. (ii) Although the viral capsid was altered to expose the viral DNA to deoxyribonuclease activity, the capsid proteins were not degraded by the process. (iii) The capsid is not constructed by assembly of capsomers through covalent bonds (30), and the capsid can be readily disrupted into its individual capsomers by exposure of virions to *p*H 10.5 (32), detergents (13), or 8 M urea or 6 M LiCl (Lawrence and Ginsberg, *unpublished data*). Hence, after entrance of viral particles into the cytoplasm, they may be exposed to a combination of conditions which break the intercapsomeric noncovalent bonds so that the capsid is disrupted and the viral DNA is exposed. The conditions required for this process may be generated in the cytoplasmic vacuoles in which the virions are found after engulfment (2).

Electron microscopic study of type 7 adenovirus infection (2) suggested that intracellular virus was found almost entirely within membrane-bound vacuoles. Dales (2) reported that morphological changes of the virions were not detectable. It is possible that alterations of the viral capsid did occur within the cytoplasmic vacuoles but that they were not sufficiently extensive to be detected by electron microscopy. Alternatively, the particles observed within the vacuoles may have entered the cell just before examination, since a very high multiplicity of infection was used and free virus was not removed after an adsorption period. Thus, the proportion of virions which were uncoated (i.e., became susceptible to deoxyribonuclease) might have been relatively small at each observation period, and therefore they could not be distinguished by the methods employed.

At the inception of this investigation, it was considered possible that the relatively long delay of 8 to 10 hr between infection and the initiation of the first detectable biosynthetic events (6, 7, 9, 10) resulted from a prolonged uncoating process. The data presented suggest that this hypothesis was unfounded. However, "uncoating," defined as the alteration of the viral capsid to expose the viral DNA to deoxyribonuclease, may not complete the reactions necessary to permit viral DNA to be transcribed and replicated. The data obtained from sucrose gradient sedimentation analysis of infected cell lysates indicate that at 2 and 4 hr after infection, when maximal uncoating had occurred, the viral DNA which was sensitive to deoxyribonuclease was present in a structure with a sedimentation coefficient of about 400*S* rather than 30*S* of free viral DNA having a molecular weight of 23×10^6 (14).

It appears that adenoviruses, even after viral DNA becomes susceptible to deoxyribonuclease, must undergo further alterations before free viral DNA is liberated. The alternative to this would imply that DNA need not be free from other components to serve as template for DNA

and messenger RNA syntheses. In the case of the poxviruses, it appears that viral cores containing viral DNA which is not susceptible to degradation by deoxyribonuclease can direct the synthesis of some virus-specific RNA (24, 29). For adenoviruses, it seems likely that parental viral DNA, even after it has become susceptible to deoxyribonuclease, is still associated with viral protein. At present, there is no information concerning the ability of the hypothetical adenovirus DNA-protein core to function in viral biosynthesis. This DNA-protein core may require a final stage to complete the process of freeing the viral DNA so that it can function. It is possible that complete separation of viral DNA from coat protein occurs after transport to the nuclear site of viral biosynthesis, and therefore it was not detected in the cytoplasmic extracts which were employed in this study.

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