Three Size-Classes of Intracellular Adenovirus Deoxyribonucleic Acid¹

B. T. BURLINGHAM² AND W. DOERFLER

The Rockefeller University, New York, New York 10021

Received for publication 26 January 1971

When human adenovirus type 2 or 12 infects cells, either productively or nonproductively, three classes of viral deoxyribonucleic acid (DNA) are found within the cells: (i) viral DNA which cosediments with DNA extracted from infectious adenovirions at 31.3S for adenovirus type 2 and at 29.0S for adenovirus type 12, (ii) viral DNA which sediments at about 18S, and (iii) viral DNA which sediments at >45S and is apparently integrated into the cellular DNA. A precursor-product relationship is suggested as a working hypothesis; the intact viral DNA is hydrolyzed to slowly sedimenting DNA and the slowly sedimenting DNA is integrated into the cellular DNA. Both the parental and the newly synthesized viral DNA are altered by this route. The intact viral DNA within the cells apparently is cleaved into the slowly sedimenting DNA by a preformed enzyme.

Adenovirions are composed of a double-stranded deoxyribonucleic acid (DNA) molecule (19) and, reportedly, nine proteins (23, 24). The DNA molecule has a molecular weight of 20×10^6 to 25×10^6 daltons, is linear and is free from single-strand breaks (13, 19, 32).

Specific types of human adenoviruses may infect cells productively or nonproductively (see review by Green, reference 16). The type of virus-cell interaction apparently is determined by the type of cell and the type of adenovirion. Productive infection by adenoviruses is characterized by: (i) stimulation of DNA synthesis of the host cell followed by inhibition of host cell DNA, ribonucleic acid (RNA), and protein synthesis, (ii) production of infectious adenovirions, and (iii) cytopathic changes of the host cell. Abortive infection by adenoviruses is characterized by: (i) a stimulation of cellular DNA synthesis, (ii) absence of replication of virus or synthesis of viral DNA, (iii) synthesis of early proteins, (iv) integration of viral DNA into the host cell genome, and (v) transformation of a portion of the cells.

The amount of adenovirus DNA synthesis during infection has been characterized by using

density-label techniques with equilibrium sedimentation and DNA-DNA hybridization (16). Hodge and Scharff (20) have identified by zonal sedimentation the newly synthesized DNA of adenovirus type 2 (Ad2) between 5 and 17 hr after productive infection of HeLa cells. One class of adenovirus DNA which cosediments with the DNA extracted from adenovirions has been described in productive infection. Cells nonproductively infected with adenovirus type 12 (Ad12) contain at least two species of viral DNA, intact DNA from the parental virions, and viral DNA integrated into the host genomes (10, 12, 36).

This report describes three classes of viral DNA found both in productively and nonproductively infected cells: (i) a class of DNA which sediments at >45S, (ii) a species of DNA which cosediments with DNA extracted from infectious adenovirions, and (iii) a class of DNA which sediments at 18S. During productive infection, both the parental and the newly synthesized DNA appear in all three classes.

MATERIALS AND METHODS

Virus strains and cell cultures. Preparations of human Ad12 strain Huie were supplied by the American Type Culture Collection and by Wallace Rowe. Ad2 strain adenoid 6 was obtained from the American Type Culture Collection. The identity of every virus preparation was confirmed by the buoyant density of the virions and the buoyant density and sedimentation coefficient of the DNA extracted from the purified virions.

KB cells (CCL17) were obtained from the American

¹ Part of this report was taken from a thesis submitted by B. T. B. in partial fulfillment of the requirement for the Doctor of Philosophy degree from The Rockefeller University, New York, N.Y. A preliminary report was presented at the 1969 Annual Meeting of the Federation of American Societies for Experimental Biology.

² Present address: Department of Cell Biology and Pharmacology, School of Medicine, University of Maryland, Baltimore, Md. 21201.

Type Culture Collection and were grown in suspension culture in Spinner medium supplemented with 5% calf serum. Spinner medium is Eagle's (15) medium without CaCl_2 and enriched with 100 μmoles of each nonessential amino acid per liter.

Baby hamster kidney (BHK-21) cells, clone 13, were given to us by N. Karabatsos of the Rockefeller Foundation Virus Laboratory. The BHK-21 cells were grown as monolayers by using reinforced Eagle's medium (1) supplemented with 10% Tryptose phosphate broth (Difco) and 10% calf serum.

Human embryonic lung (HEL) cells derived from a fetal lung digested with trypsin were supplied by P. Choppin of The Rockefeller University. The HEL cells were grown as monolayers in reinforced Eagle's medium.

Primary human embryonic kidney (HEK) cells were purchased from Microbiological Associates and grown in Eagle's medium supplemented with 5% calf serum.

HeLa (S3) cells were obtained from L. A. Caliguiri of The Rockefeller University. HeLa cells were grown in Spinner medium supplemented with 5% calf serum.

Production and purification of adenovirions. Adenovirus was produced in KB cells by a modification of the procedure described by Green and Piña (17, 18). Suspension cultures of KB cells were infected with Ad2 or Ad12 at a multiplicity of infection of about 100 plaque-forming units (PFU)/cell. When the viral DNA was isotopically labeled, 1 μCi of thymidine-6- ^3H (>25 Ci per mmole, Amersham/Searle) or 0.5 μCi of ^{14}C -formate (50 to 62 μCi per mmole, Amersham/Searle) per ml was added to the culture immediately after adsorption. The labeled virus preparations will be referred to as ^3H -Ad2 and ^3H -Ad12 or ^{14}C -Ad2 and ^{14}C -Ad12.

Forty-eight hours after infection, the cells were disrupted by ultrasonic treatment. The adenovirions were purified by equilibrium sedimentation in CsCl density gradients by using a modification of the technique described by Doerfler (10, 11). The clarified supernatant fluid from the homogenized infected cells was extracted twice with equal volumes of trichlorotrifluoroethane. S40 centrifuge tubes (Beckman) were prepared with 6.0 g of CsCl and 1 ml of glycerol; the tube was filled to 13.5 ml with extracted supernatant fluid and was centrifuged at 4 C for a minimum of 11 hr at $80,000 \times g$. The virus was purified further by equilibrium centrifugation in CsCl gradients three times.

A 1-liter preparation (3.5×10^8 cells) of KB cells yielded 5 to 15 mg of purified Ad2 virions or 1 to 2 mg of purified Ad12 virions. Nearly 90% of the infectivity found in the supernatant fluid from the sonically treated cells was recovered as purified virus. No adeno-associated viruses were detected in CsCl equilibrium gradients or by electron microscopy. The degree of virus purification was determined by isotope dilution experiments. No more than 0.01 ng of KB cell protein and 0.02 ng of KB cell DNA were present per μg of purified Ad2. The $A_{260}/A_{280} = 1.31$ (Ad2) or 1.28 (Ad12). These values have not been corrected for scattering. The adenovirions were usually quantitated by the A_{260} and by infectivity titer. $A_{260} = 1$ is equivalent

to 180 μg of viral protein and 25 μg of viral DNA per ml and about 2×10^{10} PFU/ml. The buoyant density of Ad2 is $1.3345 \pm 0.0016 \text{ g/cm}^3$ ($N = 57$) and of Ad12 is $1.3224 \pm 0.0017 \text{ g/cm}^3$ ($N = 24$).

Preparation of adenovirus DNA. Viral DNA was prepared by the method described by Doerfler (11) with minor modifications. The purified virions (1 to 10 mg/ml) were dialyzed extensively against either TE buffer or phosphate-buffered saline (PBS, reference 14) at 4 C. TE buffer is 0.01 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (pH 7.2) and 0.001 M ethylenediaminetetraacetate (EDTA). The dialyzed virus was stored at 4 C for about 10 days or until the virus precipitated. During storage, the virus became more susceptible to proteolytic digestion. The virus was incubated at 37 C with 0.1 volume of Pronase solution for 30 to 60 min. The Pronase solution contained (per ml): 1 mg of Pronase (Calbiochem), 2 mM β -mercaptoethanol, 100 mM Tris-hydrochloride (pH 7.2), and 50 mM EDTA. Protein impurities were degraded by preincubation of the Pronase solution at 37 C for 30 min before storage. The digested virus suspension was extracted three times with phenol and two times with diethyl ether. The DNA was dialyzed against five changes of 1,000 volumes of TE buffer or PBS.

More than 90% of the viral DNA was recovered by this method. When ^{35}S -L-methionine (240 μCi per mmole, Amersham/Searle)-labeled virus was extracted, less than 0.02% of the label, and thus presumably less than 0.02% of the protein, remained associated with the viral DNA. Isotope dilution experiments indicated that each microgram of DNA extracted from purified Ad2 DNA may contain 0.073 ng of KB cell protein and 0.02 ng of KB cell DNA. The A_{260}/A_{280} of the DNA is 1.98 to 2.00. The buoyant density of Ad2 DNA is $1.7137 \pm 0.0015 \text{ g/cm}^3$ ($N = 9$); the sedimentation coefficient of the native DNA is $31.3 \pm 0.12\text{S}$ ($N = 8$). The buoyant density of Ad12 DNA is $1.7048 \pm 0.0018 \text{ g/cm}^3$ ($N = 12$), and the sedimentation coefficient of the native DNA is $29.0 \pm 0.39\text{S}$ ($N = 17$) and of the denatured DNA is $31.9 \pm 0.43\text{S}$ ($N = 6$). The viral DNA was usually quantitated by absorbancy measurements at 260 nm ($A_{260} = 1$ is equivalent to 47.1 μg of viral DNA/ml).

Zonal sedimentation in sucrose density gradients. The size of the viral or cellular DNA was determined by a modification of the zonal sedimentation method described by Burgi and Hershey (4). Linear 5 to 20% (w/v) sucrose density gradients were used. The neutral sucrose solutions were made in 1.0 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.2), and 0.001 M EDTA. The alkaline sucrose gradients were made in 0.3 M NaOH, 0.7 M NaCl, and 0.001 M EDTA. When Ad2 DNA was incubated with a 20% neutral sucrose solution, no single- or double-strand scissions were detected.

Marker DNA, usually ^{14}C -Ad2 DNA characterized for homogeneity in the analytical ultracentrifuge, was added, and 0.5 ml of the DNA samples was carefully floated onto the top of the sucrose gradient. The gradients were then centrifuged under isokinetic conditions at $300,000 \times g$ for 1 to 2 hr at 4 C in a Beckman SW56 rotor. After centrifugation, seven-drop

fractions, about 0.160 ml each, were collected from the bottom of the tube directly into scintillation vials. Bray's (3) scintillation fluid (10 ml) was added directly to the samples and the radioactivity was determined.

Data from experiments with two radioisotopes were processed by the discriminator ratio method devised by Okita et al. (27). The data were corrected for the effect of the gradient, and a third-order interpolated fit of the data points was constructed and plotted. The sedimentation coefficient for each maximum was calculated by the method of Martin and Ames (25) and Burgi and Hershey (4) from the reference DNA. The molecular weight of the native and alkaline-denatured DNA was calculated by the method described by Studier (31).

Preparation of cellular DNA. Cellular DNA was labeled by growing the cells for 48 to 72 hr in medium which contained ^3H -thymidine or ^{14}C -formate. The cells were pelleted and washed once with PBS and resuspended at a concentration of 10^6 cells/ml in 0.01 M Tris-hydrochloride (pH 8.1) and 0.005 M EDTA. The cells were lysed with sodium dodecyl sulfate, (SDS), digested with 0.1 volume of Pronase solution, and extracted with phenol (10). The mean density of the DNA solution was adjusted to 1.70 g/cm³ with CsCl. After centrifugation at $80,000 \times g$ for about 6 hr, fractions were collected from the bottom. After dialysis, the cellular DNA had an A_{260}/A_{280} of about 2.0.

DNA-DNA hybridization. The DNA-DNA hybridization procedure described by Denhardt (9) was slightly modified and combined with the wash procedure described by Packman and Sly (28). Schleicher and Schuell type B-6 filters were heated to 80 C in $6\times$ SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) for 15 min so the entire filter was wettable. The filters were washed with 5 ml of $6\times$ SSC.

The DNA in $6\times$ SSC was denatured by heating for 10 min in a boiling-water bath and quickly cooled by dilution into 100 volumes of ice-cold $6\times$ SSC. About 3 μg of the denatured DNA was adsorbed to the filter. Adsorption was more than 90% complete. The filter was washed with 10 ml of $6\times$ SSC, dried overnight in a vacuum desiccator, and then placed in an oven at 80 C for 2 hr. The DNA was fixed onto the filter, and less than 1% of the DNA eluted from the filter during the hybridization. The filter was preincubated in glass scintillation vials for 6 hr at 65 C in 1 ml of PM [PM = 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrrolidone (Sigma), and 0.02% bovine serum albumin (Armour) in $3\times$ SSC]. After incubation with PM, filters without DNA could adsorb less than 0.05% of the DNA in the incubation solution.

The labeled DNA in $6\times$ SSC which was hybridized with the DNA on the filter had been sheared by ultrasonic treatment to about 10S fragments, denatured by heating, and then quickly frozen in a dry ice bath. About 0.25 ng (1,000 counts/min) of this denatured labeled DNA was added to the vials which contained the filters with DNA attached. After 12 hr of incubation at 65 C, the filters were washed six times with 20 ml of 0.01 M Tris-hydrochloride buffer (pH 8.1). After washing, the nonspecifically adsorbed label was less than 0.1% of the input radioactivity. The amount of input DNA was determined after drying the DNA on

a filter. The amount of radioactivity was determined in toluene-Liquifluor (New England Nuclear Corp.) scintillation fluid, and the per cent of input radioactivity hybridized with the DNA on the filter was calculated.

Protein and DNA determination. Protein concentrations were determined chemically with the method described by Lowry et al. (22). Crystalline bovine serum albumin was used as a standard. DNA concentrations were determined chemically with the method described by Burton (6). Calf thymus or salmon sperm DNA was used as a standard.

RESULTS

Uptake and elution of Ad2 or Ad12. The fate of the parental adenovirus DNA can be followed within the cells infected with purified adenovirions which contain ^3H -labeled DNA. Monolayers of cells on petri plates (60 mm in diameter) were infected with labeled virus, and the total intracellular DNA was extracted at various times after infection. The size of the labeled DNA was determined in alkaline and neutral sucrose density gradients. The cells on mock-infected plates were suspended with trypsin-EDTA and counted in a hemocytometer. Each plate contained between 10^6 and 5×10^6 cells, and the multiplicity of infection was about 10^4 to 10^5 PFU per cell or about 10^6 to 10^7 adenovirus particles per cell. However, only a small portion of the inoculum was adsorbed by the cells.

The virus inoculum for each plate usually contained about 10^6 counts/min but only about 1,000 to 2,000 counts/min remained associated with the cells after adsorption and exhaustive washing with PBS. Figure 1 shows the amount of label associated with the cells found in the medium when BHK cells are infected with ^3H -Ad12. At 12 hr after infection, 44% of the label is eluted into the medium. More than 98% of the label found in the medium was acid-precipitable and, furthermore, nearly half of the label is present in infectious particles. Thus, a portion of the adenovirions associated with the cells is eluted in infectious form during such experiments.

More than 90% of the label associated with the cells is acid-precipitable. Similar data for uptake and elution are found when BHK, HEK, KB, HEL, and HeLa cells are infected with either Ad2 or Ad12.

Metabolism of the parental Ad2 or Ad12 DNA in cells. The total intracellular DNA was extracted and the size of the labeled DNA from the parental virus was determined by zonal sedimentation. The sedimentation pattern of the label extracted from BHK cells 36 hr after infection with ^3H -Ad12 is shown in Fig. 2. Three size-classes of labeled DNA are found both in alkaline and neutral sucrose gradients: (i) a compo-

FIG. 1. Loss of tritium label from BHK cells infected with Ad12. Monolayers which contained 4.7×10^6 BHK cells were inoculated with purified ^3H -Ad12. The inoculum for each monolayer contained 5×10^6 counts/min and 1.1×10^3 PFU per cell. After a 2.5-hr adsorption period, the excess inoculum was removed from the cells by exhaustive washing, and fresh medium was added. After various intervals of incubation, the medium was removed and the cells on the dish were washed five times. The cells were lysed with 0.5% SDS and were digested with Pronase. The acid-precipitable tritium in the cell extract and the medium was determined.

nent which sediments rapidly, in this experiment at about 60 to 70S; (ii) a component which co-sediments with the marker DNA; and (iii) a component which sediments at about 14 to 18S. Each of the classes of DNA observed in this experiment apparently is homogeneous. The three size-classes of DNA found in the alkaline gradients have the sedimentation coefficients expected from their sedimentation behavior in neutral gradients. This type of pattern is found for each of the cell lines, BHK, KB, HEK, HEL, and HeLa cells, when infected with either Ad2 or Ad12.

Characteristics of the three classes of Ad2 and Ad12 DNA found in infected cells. The three classes of DNA were isolated from alkaline sucrose density gradients, sheared, and identified by hybridization with cellular and viral DNA. For this experiment, HEK cells were productively infected with Ad2 and were used since the uptake of adenovirus is highest in these cells and enough labeled DNA for accurate hybridization assays could be obtained. The results of the DNA-DNA hybridization are listed in Table 1.

FIG. 2. Sedimentation pattern of labeled DNA extracted from BHK cells infected with ^3H -Ad12. Monolayers of 3.2×10^6 BHK cells were infected with Ad12 labeled with thymidine-6- ^3H at a multiplicity of infection of 4×10^3 PFU per cell. The monolayers were extensively washed after adsorption, and fresh medium was added. After 36 hr of incubation, the monolayers were washed and the cells were lysed with 0.5% SDS. The cell lysate was digested with Pronase and was extracted three times with phenol. Marker ^{14}C -Ad12 DNA was added to the sample and the size of the labeled DNA was analyzed by zonal sedimentation in sucrose density gradients at pH 7.2 and 12.5. The direction of sedimentation is to the left.

The BHK cells were infected with ^3H -Ad12 and were disrupted at various times after infection by freezing and thawing three times. One-half of each sample was incubated at 37 C for 60 min with 100 μg of pancreatic deoxyribonuclease. The DNA species from the deoxyribonuclease-treated sample and untreated control were extracted and analyzed by velocity sedimentation in neutral sucrose gradients. These results are summarized in Table 2. The results of the control experiments indicate that essentially only uncoated viral DNA is hydrolyzed by pancreatic deoxyribonuclease.

The three classes of DNA found in infected cells are characterized as follows. (i) *The fast sedimenting class of adenovirus DNA.* The sedimentation coefficient of the fast sedimenting Ad2 or Ad12 DNA varied between 45 and 80S. Apparently this variation was due to mechanical breakage of the cellular DNA. The fast sedimenting

component is also found in alkaline sucrose gradients. The sedimentation coefficient for the denatured form of this DNA agrees with that predicted from the sedimentation coefficient of the

TABLE 1. Identification of the labeled DNA in human embryonic kidney (HEK) cells infected with Ad2^a

Class of ³ H-DNA from alkaline gradient	³ H-DNA from HEK cells annealed to DNA on filters at various times postinfection (%)			
	Ad 2 DNA fixed to filter		HEK cell DNA fixed to filter	
	8 hr	24 hr	8 hr	24 hr
HEK cellular	0.2	0.4		22.0
>45S	6.0	15.0	3.0	<0.1
34S	64.0	37.0	<0.1	<0.1
20.5S	44.0	67.0	<0.1	<0.1

^a Monolayers which contained 2.1×10^6 HEK cells were infected with ³H-Ad2 at a multiplicity of infection of 210 PFU/cell. At 8 and 24 hr after infection, cells were lysed and the DNA was extracted. The three classes of labeled DNA were separated by zonal sedimentation in alkaline sucrose density gradients. Each class of DNA was then hybridized with viral and cellular DNA and the per cent of the labeled DNA bound to the filter was calculated.

native DNA. Thus, the DNA which contains the label has few single-strand breaks. The label associated with the fast sedimenting DNA specifically hybridizes with the viral RNA. The fast sedimenting DNA isolated from HEK, BHK, and KB cells infected with either Ad2 or Ad12 had a similar pattern of hybridization. Between <0.1 and 5% of the label anneals with cellular DNA; however, the hybridization to cellular DNA is variable. In control experiments ¹⁴C-Ad2-DNA was added prior to lysing the cells growing in monolayers in the presence of ³H-thymidine; the total DNA was extracted and sedimented in neutral and alkaline sucrose gradients. No viral DNA was associated with the fast sedimenting cellular DNA. This control makes block sedimentation of free viral DNA unlikely. All these data indicate that the fast sedimenting label constitutes viral genetic material.

When the total cellular DNA of infected cells was treated with deoxyribonuclease (Table 2), almost all of the fast sedimenting label was digested. Thus, the fast sedimenting DNA is probably all uncoated within the cell.

(ii) *The species of adenovirus DNA which cosediments with the intact viral DNA.* The labeled DNA which cosediments with the Ad12 marker DNA sediments as a symmetrical peak in both alkaline and neutral sucrose gradients at 33 and

TABLE 2. Sensitivity of parental adenovirus DNA to pancreatic deoxyribonuclease in infected cells^a

Samples	DNA sedimenting at ^b					
	>45S		29S		25.5 to 3S	
	Control	Deoxyribonuclease treated ^c	Control	Deoxyribonuclease treated ^c	Control	Deoxyribonuclease treated ^c
Ad12 DNA			94.9	0		
Ad12 DNA and BHK cell extract			83.1	0		
Ad12 virus			95	90.4	4	0
Ad12 virus and BHK cell extract			69	62.5	12	0
Extracts of BHK cells infected with Ad12 after infection for						
3 hr	2.1	0.2	56	10	12	0
5 hr	8.8	0	75	11	18	0
7 hr	11	0.4	55	0	34	0
24 hr	15	0.7	34	0	20	0

^a A plate which contained 10.3×10^6 BHK cells was infected with ³H-Ad12 at a multiplicity of infection of 2.1×10^3 PFU/cell (617,000 counts/min). After incubation for various times, cell extracts were prepared by freezing and thawing the cells three times. The extracts were incubated at 37°C for 60 min with 100 µg of pancreatic deoxyribonuclease. The sample was made 0.5% sodium dodecyl sulfate, digested with Pronase, and extracted with phenol. The size of the DNA was analyzed by zonal sedimentation in neutral sucrose gradients. The values listed are the per cent total of DNA found in each size-class.

^b Relative amounts of the species of DNA.

^c In deoxyribonuclease-treated samples, nearly all of the label sedimented at less than 3S.

29S, respectively. According to Studier's (31) calibration, a native double-stranded DNA molecule which sediments at 29S at neutral pH has a sedimentation coefficient of 33S after denaturation at alkaline pH. Thus, it appears that this species of DNA is double-stranded DNA with essentially no single-strand breaks. Similarly, the DNA extracted from cells infected with Ad2 sediments at 34S in alkaline and at 32S in neutral sucrose.

The data in Table 1 indicate that this species of DNA hybridizes only to viral DNA and not to cellular DNA. The decreased hybridization at 25 hr after infection may be due to competition from newly synthesized unlabeled Ad2 DNA in the productively infected cells. The size of the DNA and the results of the DNA-DNA hybridization experiment identify the DNA isolated from infected cells which cosediments with the marker DNA as intact, double-stranded Ad2 or Ad12 DNA. The data in Table 2 indicate that all of the DNA extracted from Ad12 is sensitive to digestion with deoxyribonuclease, whereas only 4.8% of the DNA in the adenovirions is sensitive to deoxyribonuclease. When the virions are incubated at 37°C with the BHK cell extract, about 20% of the viral DNA becomes susceptible to deoxyribonuclease. The partial degradation of the virion during the *in vitro* incubation and exposure of the viral DNA may be explained by the action of proteolytic cellular enzymes.

Five hours after BHK cells are infected with Ad12, essentially all of the viral DNA is susceptible to deoxyribonuclease. The data suggest that all of the Ad12 particles found in the cells are uncoated. The uncoating may not be synchronous since 10% of the DNA remains resistant to deoxyribonuclease until 5 hr after infection. This resistance may be due to adsorbed particles outside the cell or to particles in lysosomes or vacuoles (7, 8, 26).

(iii) *The slowly sedimenting class of adenovirus DNA.* The viral DNA which sediments slower than the marker DNA is symmetrically distributed in both neutral and alkaline sucrose gradients. The respective sedimentation coefficients are 18.5 and 20.5S. The sedimentation coefficient of denatured DNA expected for a double-stranded intact DNA sedimenting at 18.5S would be 20.5S according to Studier's equation (31). Thus, the slowly sedimenting DNA species is a double-stranded DNA molecule with few, if any, single-strand breaks. The intact native Ad2 DNA has a calculated molecular weight of 24.9×10^6 daltons. The molecular weight of the 18.5S native DNA fragment is 5.4×10^6 daltons, and the molecular weight of the 20.5S denatured DNA fragment is 2.8×10^6 daltons.

The slowly sedimenting DNA hybridizes only to Ad2 DNA and not to cellular DNA (Table 1). All of the slowly sedimenting DNA is susceptible to digestion with deoxyribonuclease (Table 2). Thus, the slowly sedimenting DNA is native viral DNA without single-strand breaks which has a molecular weight about one-fourth of that of the intact adenovirus DNA.

Thus, all three size-classes of DNA can be digested by deoxyribonuclease and are uncoated. All are double-stranded DNA molecules with few single-strand breaks. The label in the fast sedimenting DNA hybridizes to both cellular and viral DNA but anneals with viral DNA to a much higher extent. The cosedimenting and slowly sedimenting DNA molecules hybridize only to viral DNA. Thus, it can be concluded that the labeled portion of the three classes of intracellular DNA is derived from the parental DNA of Ad2 or Ad12.

Changes in the amounts of the three classes of adenovirus DNA during infection. Although there is only one species of DNA in the adenovirions which infect the cells, at 36 hr after infection, three different size-classes of viral DNA are found in the cells. This result is observed in four different cell lines, in BHK, KB, HEL, and HEK cells infected with either Ad2 or Ad12.

Monolayers of BHK cells were infected with ^3H -Ad12. Infection of BHK cells with Ad12 is nonproductive, and viral DNA replication cannot be detected (11). The total intracellular DNA was harvested at various times after infection and analyzed on neutral and alkaline sucrose gradients. The relative amounts of the three classes of DNA at various times after infection were determined from the relative areas under the curves (Fig. 3).

During the first 10 hr after infection, nearly all of the intact Ad12 DNA sediments at 29S. A substantial amount of the 18S DNA first appears at 12 hr after infection. As the amount of intact DNA declines, the 18S species rapidly increases to a maximum amount at 24 hr after infection. At that time, the 18S DNA represents 45% of the labeled intracellular DNA. At 48 hr after infection, the amount of 18S parental DNA declines to about 8% of the labeled intracellular DNA. The fast sedimenting species of labeled DNA first appears in small amounts at 12 hr after infection. At 24 hr after infection, the amount of fast sedimenting DNA rapidly increases as the amount of 18S DNA declines. At 72 hr after infection, 90% of the labeled Ad12 DNA in the cell is found as the rapidly sedimenting form, 8% as the 18S form, and 1 to 2% as intact Ad12 DNA.

Since the hybridization data demonstrate that the label in the three DNA species is viral DNA,

FIG. 3. Temporal variation in the amounts of parental adenovirus DNA in three size-classes found in BHK cells infected with ^3H -Ad12. Monolayers of 5.4×10^6 cells were infected with 3.6×10^3 PFU per cell of ^3H -Ad12.

these data suggest that the parental Ad2 or Ad12 DNA is cleaved into 18S fragments which may become linked to the fast sedimenting cellular DNA, presumably as the integrated form described previously (10, 12). Thus, the 18S DNA may be the precursor of the integrated form of the Ad12 DNA.

Size of the newly synthesized DNA in cells infected with adenovirus type 2. The size of the newly synthesized DNA in cells productively infected with Ad2 was also determined. Confluent monolayers of BHK and KB cells were infected with purified unlabeled Ad2 at 116 and 160 PFU/cell, respectively. Control cells were mock-infected with PBS. At intervals, monolayers were pulse-labeled for 2 hr with $1 \mu\text{Ci}$ of ^3H -thymidine per ml. At the end of the pulse, the total intracellular DNA was extracted and analyzed in neutral and alkaline sucrose density gradients. The distribution of newly synthesized DNA resembled that obtained for parental DNA (Fig. 2). The labeled DNA in the mock-infected cultures all sedimented rapidly.

The radioactivity was determined in a portion of each fraction from the alkaline sucrose gradients. The DNA in the alkaline sucrose gradients was divided into three classes: (i) fast sedimenting DNA ($>45\text{S}$), (ii) intact Ad2 DNA (33S), and (iii) slowly sedimenting DNA ($<25\text{S}$). The relative amounts of these three classes of DNA are shown in Fig. 4.

The pulse labeling was started at 10 hr after infection. At that time, the 33S species of DNA constituted 50 to 75% of the newly synthesized DNA. The amount of 33S DNA which was newly

FIG. 4. Size of the newly synthesized DNA in KB cells infected with Ad2. Monolayers of KB cells were infected with 160 PFU of Ad2 per cell. After various periods of incubation, infected monolayers were pulse-labeled with $1 \mu\text{Ci}$ of ^3H -thymidine per ml for 2 hr. Then the total intracellular DNA was extracted and analyzed in alkaline sucrose density gradients. The relative amount of each of the three classes of DNA was calculated. The time plotted is the midpoint of the pulse interval.

synthesized slowly declined. Late in infection, the slowly sedimenting DNA became the predominant species. Throughout the course of the experiment, very little label was found in the fast sedimenting DNA although a small amount of newly synthesized fast sedimenting DNA appeared at 16 to 18 hr after infection.

The fractions in each class from alkaline sucrose gradients were pooled, and the DNA was hybridized with cellular and with viral DNA. The total amount of DNA in each size-class which annealed with cellular and viral DNA is listed in Table 3.

The maximum amount of viral DNA synthesis occurs between 14 and 20 hr after infection. The hybridization data show that very little cellular DNA is synthesized in the KB and BHK cells later than 18 hr after infection. The DNA synthesized in mock-infected cells does not hybridize with viral DNA. All of the newly synthesized cellular DNA sediments rapidly.

The temporal variation in the size of the newly synthesized viral DNA is similar to that of the parental DNA because three classes of DNA of comparable sizes are found. A portion of the newly synthesized intact viral DNA is fragmented to slowly sedimenting DNA. It is striking that most of the DNA in the fast sedimenting class found later than 18 hr after infection hybridizes with viral DNA (Table 3).

The occurrence of fast sedimenting viral DNA which has been newly synthesized can be explained by the following mechanisms. (i) Newly synthesized viral DNA is integrated into cellular DNA during productive infection. (ii) Superhelical circular viral DNA is formed during produc-

TABLE 3. *Incorporation of ^3H -thymidine into KB cells infected with Ad2 and identification of the newly synthesized DNA^a*

Cells	Time after infection or mock-infection (hr)	Counts/min incorporated into new DNA	Per cent of ^3H -labeled DNA from alkaline sucrose gradient, hybridizing with various types of DNA on filter, of size					
			>45S		34S		<25S	
			KB	Ad2	KB	Ad2	KB	Ad2
Mock-infected KB	10-12	14,401	37	<0.1	42	2.1		
	33-35	17,234	28	<0.1	34	<0.1		
Ad2-infected KB	10-12	72,299	11	0.3	9.4	94	0.4	84
	12-14	143,604	14	3.1	<0.1	83	0.3	79
	14-16	184,143	21	2.6	<0.1	89	0.1	74
	16-18	180,057	16	4.0	0.2	84	0.1	48
	18-20	184,752	<0.1	50	0.1	76	0.4	55
	22-24	112,066	2.0	21	0.2	91	0.2	54
	24-26	92,136	0.4	54	0.1	67	<0.1	73
	30-32	11,317	1.1	69	<0.1	84	0.2	81
	33-35	8,160	0.5	71	<0.1	78	<0.1	87
	42-44	4,213	0.1	73	<0.1	69	0.3	69
Control DNA from KB cells			67	0.2				
DNA from Ad2			0.3	84				

^a Monolayers of 2.6×10^6 KB cells were infected with 160 PFU/cell of Ad2. At various times after infection, $1 \mu\text{Ci}$ of thymidine- $6\text{-}^3\text{H}$ per ml was added to the medium for 2 hr. Then, the cells were washed and lysed and the DNA was extracted and separated into classes by zonal sedimentation in alkaline sucrose gradients. The DNA in each size class was then hybridized with viral and cellular DNA and the per cent of the labeled DNA hybridizing was calculated.

tive infection. Superhelical DNA sediments faster than linear DNA in alkaline sucrose density gradients (2, 33). (iii) Linear polymers of viral DNA are synthesized. A combination of possibilities (i) to (iii) might also apply. The available data do not clearly distinguish between these alternatives.

Effect of chemical inhibitors of macromolecular syntheses on the metabolism of parental Ad12 DNA. When macromolecular syntheses in infected cells are inhibited, the importance of newly synthesized viral or cellular products in the formation of the fast or slowly sedimenting species of viral DNA can be determined. The synthesis of DNA, RNA, or protein in BHK cells in monolayers was inhibited by the addition of 5 mM thymidine, 5 μg of actinomycin D, or 2.5 μg of cycloheximide per ml of medium. At these concentrations, the synthesis of DNA, RNA, or protein was inhibited 75 to 90% (Table 4). The per cent of inhibition was determined from the incorporation of ^3H -cytidine, ^3H -uridine, or ^3H -leucine in control cultures. The cells were infected with labeled Ad12; the intracellular DNA was extracted at 12 hr after infection and analyzed by neutral sucrose density gradients. The amount of each class of viral DNA

TABLE 4. *Effect of chemical inhibitors on the metabolism of adenovirus DNA^a*

Inhibitor	Per cent inhibition of synthesis			Per cent of parental DNA in size-class		
	DNA	RNA	Protein	>40S	29S	18S
None.....	0	0	0	12	67	15
Thymidine (5 mM).....	75	15	4	9	70	19
Actinomycin D (5 μgml).....	67	79	62	0	84	15
Cycloheximide (2.5 μgml).....	62	76	90	0	83	16

^a Chemical inhibitor was added to the medium of monolayers of BHK cells 2 hr before infection. The monolayers were infected in the presence of inhibitor with 4.2×10^3 PFU/cell of ^3H -Ad12. After adsorption for 2 hr, the cells were exhaustively washed, and medium which contained the inhibitor was added. The monolayers were exhaustively washed again 12 hr after infection, and the cells were lysed. The DNA was extracted and analyzed by zonal sedimentation. The percentage of DNA in each size-class was calculated.

found in cells from inhibited and control cultures is listed in Table 4.

The cells were treated with the inhibitors for 14 hr. Thymidine was a relatively specific inhibitor of DNA synthesis; however, actinomycin D and cycloheximide caused a major reduction in DNA, RNA, and protein synthesis. Inhibition of the cells with thymidine had little effect on the formation of the fast or slowly sedimenting viral DNA. However, both cycloheximide and actinomycin D inhibited the formation of the fast sedimenting DNA species but had little effect on the formation of the slowly sedimenting species of viral DNA.

These results suggest that DNA, RNA, and protein syntheses are not required for the formation of the 18S species of viral DNA and that an endonuclease may be preformed within the cell or the adenovirus. However, since the inhibition of synthesis is not complete, the lack of inhibition of the endonuclease must be interpreted cautiously. The results regarding the fast sedimenting viral DNA are similar to the results of Doerfler (12) which show that inhibition of DNA synthesis does not affect integration.

Effect of inactivation of virus on the metabolism of the parental viral DNA. Does the virus play an essential role in the formation of the slowly sedimenting viral DNA? Adenovirions labeled with ^3H -thymidine were inactivated by (i) heat treatment, (ii) ultraviolet light, (iii) formaldehyde, or (iv) β -propiolactone.

The labeled Ad2 and Ad12 were heat-inactivated at 56 C for 30 min (29, 34). At 7 hr after infection, the size distribution of the viral DNA was determined in BHK, HEL, and KB cells which were inoculated with heat-inactivated virus. The data in Fig. 5 show that nearly all of the labeled DNA found in the BHK cells inoculated with heat-inactivated ^3H -Ad12 sediments at 16.6S. Similar findings are obtained with KB and HEL cells. Only very small amounts of the label sediment fast, cosediment with the marker DNA, or do not sediment at all. The DNA sediments at 16.6S as a symmetrical peak.

When the DNA from the heat-inactivated Ad12 used as inoculum was analyzed, it sedimented at 18.6S. No DNA sedimented faster than or cosedimented with the 29S marker. These results clearly indicate that the adenovirus DNA is cleaved during the inactivation period and that the size of the viral DNA is only slightly reduced within the BHK cells.

Russell et al. (30) have shown that the DNA of adenovirions heated for 1 min to 56 C becomes accessible to pancreatic deoxyribonuclease. Thus, the change in size of the Ad12 DNA from 18.6 to DNA of 16.6S found in the infected cells could have occurred in the cell without further uncoating of the viral DNA. The data of Morgan et al.

FIG. 5. Metabolism of parental viral DNA in BHK cells inoculated with heat-inactivated adenovirus. Monolayers of 9.35×10^6 BHK cells were inoculated with ^3H -Ad12. This preparation had an infectivity titer of 1.7×10^{10} PFU per ml, but, after heating at 56 C for 30 min, the infectivity titer was <10 PFU per ml. The intracellular DNA was extracted 7 hr after infection and the size of the DNA was determined by zonal sedimentation in neutral sucrose gradients (top). The DNA from a portion of the heat-inactivated virus used as inoculum was also extracted and analyzed (bottom).

(26) and Chardonnet and Dales (7, 8) suggest that virus particles which had been inactivated by physical or chemical means were sequestered in the lysosomes and were not uncoated normally. This further decrease in size (18.6 to 16.6S) may be due to an endonuclease found in BHK cells by Koh et al. (21).

The data in Table 5 summarize the results of an experiment in which the uptake and elution of heat-inactivated Ad12 and the sedimentation coefficients of the intracellular labeled DNA were determined 7 hr after inoculation of BHK, HEL, and KB cells. Depending on the type of cells used, the uptake of the heat-inactivated virus is increased 3- to 30-fold over the uptake of control virus. The amount of label which elutes from the cells inoculated with inactivated virus during the 7-hr incubation period is increased to about one-third to one-half of the adsorbed label. The enhanced uptake and elution of the heat-inactivated virus may be explained by the formation of viral aggregates. Size differences of the viral DNA (14 to 18.6S) found in different cell lines (Table 5) may be explained by cellular nucleases (Burlingham and Doerfler, *in preparation*).

The effects of inactivation of the virus by ultraviolet light, formaldehyde, and β -propi-

TABLE 5. *Uptake and elution of heat-inactivated adenovirus and sedimentation coefficient of intracellular viral DNA^a*

Adenovirus	Uptake of Ad12 (counts/min)			Label eluting into medium (counts/min)			Sedimentation coefficient of labeled DNA in cell		
	BHK cells	HEL cells	KB cells	BHK cells	HEL cells	KB cells	BHK cells	HEL cells	KB cells
Control Ad12 ^b	528	228	1,852	150	0	250	29S (100) ^c	29S (90) 18.5S (10)	45S (1) 29S (94) 14S (4)
Heat-inactivated Ad12 ^d	14,792	8,020	5,880	4,700	3,100	2,000	16.6S (92)	18.6S (100)	17S (94)

^a BHK cells: 9.35×10^6 ; HEL (human embryonic lung cells): 5×10^5 ; KB cells: 8×10^5 .

^b Infectivity titer: 1.7×10^{10} PFU per ml.

^c Values in parentheses express per cent.

^d Heated to 56 C for 30 min; infectivity titer: < 10 PFU per ml. The monolayers were exhaustively washed 7 hr after infection, and the cells were lysed. The amount of acid-precipitable label in the medium and in the cellular extract was determined. The DNA was extracted and analyzed by zonal sedimentation in neutral sucrose gradients.

lactone were also studied. The experimental conditions and the results of these experiments are summarized in Table 6. The DNA extracted from the ultraviolet light- and formaldehyde-inactivated Ad12 has the same sedimentation pattern as the DNA from the control virus preparation. However, 20 to 30% of the DNA from Ad12 treated with β -propiolactone sedimented at about 21S. Ultraviolet light which acts mainly on the viral DNA has no effect on the size distribution of the intracellular Ad12 DNA. Formaldehyde and β -propiolactone interact with both viral DNA and proteins. Formaldehyde inhibits the formation of the slowly sedimenting DNA, but the β -propiolactone enhances the formation of the slowly sedimenting DNA. These findings suggest that the viral proteins may be involved in the formation of the slowly sedimenting DNA.

DISCUSSION

High multiplicities of infection were required to determine the metabolism of the parental DNA. Only 0.1 to 0.2% of the labeled adenovirions were adsorbed to or taken into the cells. Thus, the cell may select particular particles from the population. Control experiments suggest that such a selection is unlikely. An inoculum of ³H-Ad12 was adsorbed to one monolayer for 2 hr and then was transferred to a new monolayer. The same amount of label was adsorbed and the sedimentation pattern of the intracellular labeled DNA was similar in four monolayers to which adenovirions had been adsorbed sequentially from the same inoculum. Furthermore, no heterogeneity could be detected in the purified adenovirions or their DNA by physical analysis.

A large fraction of the adsorbed virions is

TABLE 6. *Fate of adenovirus DNA in cells inoculated with virus inactivated by ultraviolet (UV) light, formaldehyde, or β -propiolactone*

Determination	Per cent uptake	Per cent of intracellular DNA which sediments at:		
		>45S	29S	<25S
Ad12 inoculum				
Control ^a			94	
UV-inactivated ^b			91	
Formaldehyde-inactivated ^c			96	
β -Propiolactone-inactivated ^d			72	26
BHK cells				
Control	0.36	4	80	12
UV-inactivated	0.28	1	81	14
Formaldehyde-inactivated	0.06		92	
β -Propiolactone-inactivated	0.12		72	25
HEL cells				
Control	0.18	2	78	14
UV-inactivated	0.13	1	73	18
Formaldehyde-inactivated	0.11		97	
β -Propiolactone-inactivated	0.28		74	24
KB cells				
Control	0.23	4	83	12
UV-inactivated	0.12	2	76	19
Formaldehyde-inactivated	0.08		90	3
β -Propiolactone-inactivated	0.10		68	28

^a Infectivity titer: 4×10^9 PFU/ml.

^b Infectivity titer: 20 PFU/ml. Inactivated by irradiation from a General Electric G 15T8 germicidal lamp 15 cm above the virus suspended in 0.5 ml of buffer in a 60-mm petri dish.

^c Infectivity titer: <10 PFU/ml. Inactivated by dialysis against 1,000 volumes of 1% formaldehyde at 4 C for 18 hr and then dialyzed against four changes of PBS.

^d Infectivity titer: <10 PFU/ml. Inactivated by dialysis against 1,000 volumes of buffer containing 4 g of β -propiolactone per liter at 4 C for 18 hr and then dialyzed against four changes of PBS. Monolayers of cells were inoculated with the control and inactivated virus. The total intracellular DNA was extracted and analyzed 7 hr after infection. Infection of cells, extraction of DNA, and analysis of DNA as in Table 5.

eluted as intact infectious virions from the cells. This elution makes quantitative interpretation of the data difficult, especially at times soon after infection. The elution leads to asynchronous metabolism. Moreover, a fraction of the virions are probably sequestered into lysosomes and are not metabolized in the cell as the bulk of the virions (7, 8, 26). Thus, the estimates of the amount of intact parental DNA includes the DNA of these nonmetabolized particles. Most of the label that is lost from the cells reappears in the medium as infectious virions which may have adsorbed to but may not have penetrated into the cell. However, most of the loss due to elution occurs in the first 8 hr after infection. This loss of intact cell-associated virions probably increases the slope of the curve for the decline of the 29S DNA that actually is intracellular (Fig. 3). The 18S or the fast sedimenting DNA species could not be recovered from the medium.

However, since all of the intracellular viral DNA is sensitive to pancreatic deoxyribonuclease 7 hr after infection, nearly all of the viral DNA must be uncoated. Presumably, the intracellular DNA which sediments rapidly represents the integrated form of the adenovirus DNA described previously (10, 12). Since an equal amount of labeled marker DNA was added to the sample before sedimentation and little or no marker was found in the fast sedimenting DNA, the fast sedimenting DNA does not result from block sedimentation or collision with the tube wall (Fig. 2). The data from the alkaline sucrose gradients support the claim that the fast sedimenting DNA is covalently linked to large fragments of cellular DNA. Since the productively infected cells as well as the abortively infected cells contain fast sedimenting, labeled, viral DNA, covalent linkage of the viral genomes to the cellular genome may not be an unusual or special event which occurs only in nonproductive infection. However, the finding of fast sedimenting viral DNA in productively infected cells can be explained by mechanisms different from integration. Winters and Khoobyarian (35) suggest a persistence of the adenovirus genome in productively infected rabbit cells which have slowly stopped synthesizing infectious adenovirions but still synthesize viral antigens.

The labeled DNA which cosediments with the marker DNA extracted from purified adenovirions must represent the parental adenovirus DNA which remains unchanged in size. Since this DNA also cosediments with the marker DNA in alkaline gradients, it has few, if any, single-strand scissions. This DNA could be derived from uncoated viral DNA in the cell, from intact virions which are adsorbed onto the

surface of the cell, or from partly uncoated virions. Since this DNA is entirely sensitive to pancreatic deoxyribonuclease at 7 hr after infection, it probably represents uncoated parental viral DNA within the cell. At times from 2 to 24 hr after infection, in all of the cell types investigated the intact DNA is the predominant form of parental DNA found. The slowly sedimenting DNA sediments as a symmetrical peak in sucrose density gradients at both pH 7.6 and 12.5.

The labeled intracellular forms of DNA were identified by DNA-DNA hybridization. The DNA which cosedimented with the viral marker and the slowly sedimenting DNA were unambiguously identified as viral DNA. The hybridization of the fast sedimenting DNA to both viral and cellular DNA could best be explained by postulating that the random fragments made by ultrasonic treatment of cellular DNA which contains integrated viral DNA could contain both labeled viral DNA and unlabeled cellular DNA. The low level of hybridization could be explained by the low concentration of labeled viral DNA in a large amount of unlabeled cellular DNA. A shear fragment which contained mainly or entirely viral DNA would hybridize better to viral DNA on the filter since the competition is reduced or absent and the relative amount of homology is higher. This explanation agrees with the physical data on the integration of Ad12 DNA into BHK cell DNA presented previously (10, 12, 36). However, in productively infected cells, the existence of a newly synthesized oligomeric or circular form of viral DNA cannot be rigorously excluded. The fast sedimenting DNA which hybridizes with the adenovirus DNA could represent a small amount of oligomeric form of viral DNA in the cell. There is a small amount (1 to 3%) of DNA in the alkaline sucrose gradients which sediments at about 45S and thus represents molecules which contain 75×10^6 to 100×10^6 daltons of DNA. This DNA hybridizes only to viral DNA.

The results of productive infection of BHK cells with Ad2 or nonproductive infection with Ad12 were similar. The fast sedimenting form of adenovirus DNA is present in cells productively or nonproductively infected with Ad12. In cells productively infected with Ad2, a fast sedimenting species of newly synthesized DNA is found which may represent integrated viral DNA. The results presented further suggest that the formation of the fast sedimenting DNA and the 18S DNA is a virus-regulated phenomenon since the same classes of viral DNA and the same relative amounts of each class of DNA are found in different cell types infected with Ad2 or Ad12.

It is postulated that the slowly sedimenting intracellular form of viral DNA is formed by a specific endonuclease. The homogeneity of the cleavage product, its specific size, and the absence of single-strand scissions support an enzymatic attack on the viral DNA. The data from experiments with chemically inhibited cells or inactivated virions suggest that the slowly sedimenting viral DNA arises through endonucleolytic cleavage of the intact viral DNA by a preformed enzyme. Furthermore, the heat-inactivation experiments suggest that the endonuclease activity is associated with the virions. Results communicated in a subsequent paper will demonstrate that endonuclease is found in extracts of infected cells and that this endonuclease is a virus-coded product and is associated with purified virions (Burlingham and Doerfler, *in preparation*). Furthermore, it will be shown that this endonuclease is associated with the penton, a subunit of the protein coat of the virion (5).

ACKNOWLEDGMENTS

This investigation was supported by grant E-565 from the American Cancer Society, by Public Health Service grant AI-03445 from the National Institute of Allergy and Infectious Diseases, and by contract AT(30-1)-3983 from the U.S. Atomic Energy Commission. B. T. B. was the recipient of a Public Health Service postdoctoral fellowship from the National Institute of Allergy and Infectious Diseases (AI 31662-02). W. D. is the recipient of a Career Scientist Award from the Health Research Council of the City of New York (I-620).

LITERATURE CITED

- Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. *Virology* 26:100-113.
- Bode, V. C., and A. D. Kaiser. 1965. Changes in the structure and activity of λ DNA in a superinfected immune bacterium. *J. Mol. Biol.* 14:399-417.
- Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1:279-285.
- Burgi, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. *Biophys. J.* 3:309-321.
- Burlingham, B. T., W. Doerfler, U. Pettersson, and L. Philipson. 1971. Adenovirus endonuclease: association with the penton of adenovirus type 2. *J. Mol. Biol.*, *in press*.
- Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62:315-323.
- Chardonnet, Y., and S. Dales. 1970. Early events in the interaction of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. *Virology* 40:462-477.
- Chardonnet, Y., and S. Dales. 1970. Early events in the interaction of adenoviruses with HeLa cells. II. Comparative observations on the penetration of types 1, 5, 7, and 12. *Virology* 40:478-485.
- Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.
- Doerfler, W. 1968. The fate of the DNA of adenovirus type 12 in baby hamster kidney cells. *Proc. Nat. Acad. Sci. U.S.A.* 60:636-643.
- Doerfler, W. 1969. Nonproductive infection of baby hamster kidney cells (BHK21) with adenovirus type 12. *Virology* 38:587-606.
- Doerfler, W. 1970. Integration of the deoxyribonucleic acid of adenovirus type 12 into the deoxyribonucleic acid of baby hamster kidney cells. *J. Virol.* 6:652-666.
- Doerfler, W., and A. K. Kleinschmidt. 1970. Denaturation pattern of the DNA of adenovirus type 2 as determined by electron microscopy. *J. Mol. Biol.* 50:579-593.
- Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines of poliomyelitis viruses. *J. Exp. Med.* 99:167-182.
- Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* 130:432-437.
- Green, M. 1970. Oncogenic viruses. *Annu. Rev. Biochem.* 39:701-756.
- Green, M., and M. Piña. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. *Virology* 20:199-207.
- Green, M., and M. Piña. 1964. Biochemical studies on adenovirus multiplication. VI. Properties of highly purified tumorigenic human adenoviruses and their DNA's. *Proc. Nat. Acad. Sci. U.S.A.* 51:1251-1259.
- 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.
- Hodge, L. D., and M. D. Scharff. 1969. Effect of adenovirus on host cell DNA synthesis in synchronized cells. *Virology* 37:554-564.
- Koh, J. K., A. Waddell, and H. V. Aposhian. 1970. The synthesis and breakdown of nucleic acids in mammalian cells transformed by oncogenic viruses. I. Purification and properties of an endonuclease from baby hamster kidney cells transformed by polyoma virus. *J. Biol. Chem.* 245:4698-4707.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- 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.
- 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.
- Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* 236:1372-1379.
- Morgan, C., H. S. Rosenkranz, and B. Mednis. 1969. Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. *J. Virol.* 4:777-796.
- Okita, G. T., J. J. Kabara, F. Richardson, and G. V. Leroy. 1957. Assaying compounds containing H³ and C¹⁴. *Nucleonics* 15:111-114.
- Packman, S., and W. S. Sly. 1968. Constitutive λ DNA replication by λ c_{ir}, a regulatory mutant related to virulence. *Virology* 34:778-789.
- Rafajko, R. R., and J. C. Young. 1964. Thermal and pH stability of adenovirus types 12, 14, and 18. *Proc. Soc. Exp. Biol. Med.* 116:683-685.
- 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.
- Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* 11:373-390.
- van der Eb, A. J., and L. W. van Kesteren. 1966. Structure

- and molecular weight of the DNA of adenovirus type 5. *Biochim. Biophys. Acta* 129:441-444.
33. Vinograd, J., and J. Lebowitz. 1966. Physical and topological properties of circular DNA. *J. Gen. Physiol. (Suppl.)* 49:103-125.
34. Wilcox, W. C., and H. S. Ginsberg. 1963. Protein synthesis in type 5 adenovirus-infected cells. Effect of *p*-fluorophenyl-alanine on synthesis of protein, nucleic acids, and infectious virus. *Virology* 20:269-280.
35. Winters, W. D., and N. Khoobyarian. 1970. Fate of adenovirus types 2 and 12 in infected serial cultures of non-primate origin. *J. Gen. Virol.* 8:95-104.
36. zur Hausen, H., and F. Sokol. 1969. Fate of adenovirus type 12 genomes in nonpermissive cells. *J. Virol.* 4:256-263.