APPLIED MICROBIOLOGY, Aug. 1968, p. 1245-1250
Vol. 16, No. 8

Production of Highly Labeled Adenoviruses

J. PAUL BURNETT, A. O. SUMMERS,[1] J. A. HARRINGTON, AND A. C. DWYER
Lilly Research Laboratories, Indianapolis, Indiana 46206

Received for publication 8 May 1968

A method is described for increasing the incorporation of radioactive thymidine into adenovirus deoxyribonucleic acid by the use of amethopterin. In addition, a modified procedure is presented for the preparation of highly purified adenoviruses. This procedure, which employs enzymatic digestion of cellular debris, obviates the necessity for fluorocarbon treatment of crude virus suspensions, and routinely provides excellent recovery of virus.

The objective of the work described in this report was the production and purification of adenoviruses with very high specific radioactivity in the viral deoxyribonucleic acid (DNA). Such DNA is useful in many types of investigation, particularly in attempts to detect the viral genome, or parts thereof, in adenovirus-induced tumor cells. Tritium-labeled adenovirus DNA has been prepared previously by the addition of tritiated thymidine (^{3}H-TdR) to infected cell cultures (7, 8). We investigated the use of amethopterin, a folate antagonist (1), and found it effective for increasing the incorporation of exogenous ^{3}H-TdR into viral DNA. Amethopterin inhibits the cellular synthesis of purines and of thymidine; this effect can be reversed by the addition of a combination of adenosine, glycine, and thymidine (6). Because the drug also induces a large increase in thymidine kinase in cell cultures (12), it was thought that this would facilitate the incorporation of ^{3}H-TdR into adenovirus DNA.

Our use of published procedures (5, 13) for the purification of adenoviruses frequently led to low recoveries of virus when concentrated suspensions of infected cells were processed. Because it appeared that the losses occurred primarily during the Genetron extraction of the crude virus suspension, we devised an alternative procedure that does not employ the halocarbon, and routinely provides good yields of virus.

MATERIALS AND METHODS

Materials. A KB cell line was provided by Maurice Green of St. Louis University, Henle's Intestine 407 cell line was obtained from the American Type Culture Collection, and human embryonic kidney (HEK) primary cultures were obtained from Flow Laboratories, Rockville, Md. Adenoviruses type 7 (Gomen) and type 12 (Huie) were obtained from the American Type Culture Collection. Cesium chloride was purchased from American Potash & Chemical Corp., Los Angeles, Calif., and from Harshaw Chemical Co., Cleveland, Ohio. Unlabeled nucleosides were obtained from Sigma Chemical Co., St. Louis, Mo., and ^{3}H-TdR was obtained from New England Nuclear Corp., Boston, Mass. Enzymes were purchased from Sigma Chemical Co. and from Worthington Biochemical Corp., Freehold, N.J. Amethopterin (4-amino-N^{10}-methyl-pteroyl glutamic acid) was a product of the Lederle Laboratories Division of the American Cyanamid Co., Pearl River, N.Y.

Growth of virus. Unlabeled virus was prepared in monolayer cultures in 32-oz bottles and grown in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated fetal calf serum. At the time of infection, the medium was removed from the cell sheets and the virus was added at multiplicities of 1 to 10 $TCID_{50}$/cell in a small volume. Adsorption was allowed to proceed for 30 min to 1 hr. Eagle's MEM with 5% heat-inactivated fetal calf serum, nonessential amino acids, and additional arginine (105 mg/liter) was then added. After 36 to 48 hr, the infected cells were dislodged by gentle shaking and recovered by centrifugation. Cell pellets were then suspended in 1% of the original volume of medium and stored at −90 C until use.

Radioactive virus was produced in monolayer or suspension cultures by use of a modified Eagle's MEM (12) with 5% dialyzed fetal calf serum. For suspension cultures, the same medium, containing Earle's salts for suspension cultures (3), was substituted for the modified Eagle's monolayer medium. Modified Eagle's medium contained, in addition to the usual ingredients, 2×10^{-5} M inositol, 5×10^{-5} M adenosine, and glycine and serine each at 10^{-4} M. All virus pools and cell cultures were examined routinely and found to be free of mycoplasma.

Enzymatic hydrolysis of cellular debris. A concentrated suspension (about 10^8 cells/ml) of KB cells was homogenized and treated with various enzymes to measure their ability to degrade cellular materials. All enzymes tested were added individually at a concentration of 100 μg/ml; cofactors were added as

[1] Present address: Institute for Molecular Virology, Saint Louis University School of Medicine, St. Louis, Mo. 63103.

necessary. After 45 min at 37 C, the suspension was centrifuged for 20 min at 32,000 × *g* and the supernatant fluid was removed. The precipitate was suspended in a known volume of buffer. The total amount of nitrogen remaining in the supernatant fluid and precipitate was determined. Samples of the supernatant fluid were then homogenized with Genetron 113 to determine whether any further nitrogen-containing materials could be removed.

Chemical determinations. DNA was quantitated by the procedure of Scott et al. (11), or by Burton's diphenylamine reaction (2). Nitrogen content was determined by the Kjeldahl method (10).

Infectivity measurements. Bioassays of adenovirus preparations were made by a tube dilution or plaque method in primary or secondary monolayer cultures of HEK cells. Eagle's (MEM) medium containing 5% heat-inactivated fetal calf serum and additional arginine (105 mg/liter) was used; 0.9% purified agar (Difco) was added for plaque assays.

Radioactivity measurements. ^{3}H and ^{14}C determinations were made by dissolving the samples in NCS™ Solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill.) or in Hydroxide of Hyamine 10X (Packard Instrument Co., Inc., Downers Grove, Ill.), and counting, in toluene containing Liquifluor (New England Nuclear Corp.), in a Nuclear-Chicago Mark IV or a Packard Tri-Carb liquid scintillation spectrometer. Quench corrections were made by the addition of an internal standard.

RESULTS

Radioisotopic labeling: effect of amethopterin on virus production. Amethopterin, at a concentration greater than 10^{-7} M, drastically inhibits adenovirus type 12 production in I-407 cells (Table 1). This effect, however, can be effectively reversed by the addition of thymidine. The data in Table 2 further indicate that the cells may be pretreated with amethopterin for at least as long as 24 hr to deplete cellular TdR levels. The addition of thymidine may be delayed as long as 4 hr after infection without significantly affecting virus yield. In other experiments, thymidine has been withheld up to 7 hr after infection without decreasing the virus production. On the basis of these data, a concentration of 10^{-6} M amethopterin was chosen for use in further experiments. For reversal, a concentration of 6.25×10^{-6} M thymidine was ordinarily employed. As can be seen from the data in Table 3, this concentration is safely below the levels of thymidine that inhibit the production of adenoviruses.

Tritium-labeled adenovirus type 12. Infected

TABLE 1. *Effect of amethopterin on adenovirus type 12 production in I-407 cells*[a]

Amethopterin concn	Thymidine concn	Total virus produced
M	*M*	$TCID_{50}/ml$[b]
10^{-5}	0	$10^{3.8}$
10^{-6}	0	$10^{6.1}$
10^{-7}	0	$10^{7.7}$
0	6.25×10^{-7}	$10^{7.7}$
0	6.25×10^{-6}	$10^{7.9}$
10^{-5}	6.25×10^{-7}	$10^{3.8}$
10^{-5}	6.25×10^{-6}	$10^{3.1}$
10^{-6}	6.25×10^{-7}	$10^{5.9}$
10^{-6}	6.25×10^{-6}	$10^{7.5}$
10^{-7}	6.25×10^{-7}	$10^{7.8}$
10^{-7}	6.25×10^{-6}	$10^{7.7}$
0	0	$10^{7.8}$

[a] Cultures were infected at low multiplicity and incubated with or without amethopterin and thymidine. All cultures were harvested at 4+ cytopathic effect.

[b] Titrations performed in HEK cells.

TABLE 2. *Effect of amethopterin pretreatment of I-407 cells on subsequent production of adenovirus type 12*

Amethopterin concn	Thymidine concn	Time of thymidine addition (hr post-amethopterin treatment)	Virus produced[a]
M	*M*		$TCID_{50}/ml$
0	0		$10^{7.0}$
0	6.25×10^{-6}	0	$10^{7.5}$
1×10^{-6}	0		$10^{5.6}$
1×10^{-6}	6.25×10^{-6}	0	$10^{7.6}$
1×10^{-6}	6.25×10^{-6}	20	$10^{7.6}$
1×10^{-6}	6.25×10^{-6}	24	$10^{7.6}$

[a] Cells were infected at 20 hr after amethopterin treatment. Titrations were performed in HEK cells.

TABLE 3. *Effect of thymidine on adenovirus type 7 replication*[a]

Thymidine concn	Virus yield
M	$TCID_{50}/ml$
10^{-2}	$10^{5.2}$
10^{-3}	$10^{5.8}$
10^{-4}	$10^{6.8}$
10^{-5}	$10^{6.8}$
10^{-6}	$10^{6.5}$
10^{-7}	$10^{6.4}$
10^{-8}	$10^{5.8}$
Control (no thymidine)	$10^{5.8}$

[a] Replicate groups of cultures were infected at a multiplicity of 10, and Eagle's MEM with various concentrations of thymidine was added. All cultures were harvested 36 to 48 hr postinfection (4+ cytopathic effect). Titrations were performed on virus released after repeated freeze-thaw cycles.

cultures treated 24 hr before infection with 10^{-6} M amethopterin, and reversed 7 hr after infection with 6.25×10^{-6} M radioactive thymidine produced virus with a very high specific radioactivity in the DNA. This use of amethopterin inhibition of endogenous thymidine synthesis affords a two-fold or threefold increase in specific activity in the viral DNA (Table 4).

Purification of the virus. Preliminary experiments showed that the infectivity of adenoviruses was stable in the presence of several proteolytic enzymes. Of the proteolytic enzymes tested, only pronase and trypsin caused a significant decrease in virus titer. In addition, ribonuclease, deoxyribonuclease, and crude snake venom (*Crotalus adamanteus*) had no detectable effect on infectivity (Table 5). To evaluate the ability of these enzymes to degrade cell debris, cell homogenates were incubated with the enzymes, and particulate material was separated from soluble nitrogen-containing materials by centrifugation as indicated in Table 6. Although subtilisin, α-chymotrypsin, and collagenase apparently were effective in solubilizing the cellular nitrogen-containing debris, a mixture of α-chymotrypsin, subtilisin, and snake venom appeared optimal. The failure of subsequent homogenization with Genetron 113 to reduce significantly the total nitrogen content of the supernatant fluids supports the efficacy of the enzyme digestion as a replacement for the fluorocarbon treatment.

Although data for the purification of adenovirus type 7 are cited for illustration, identical results were obtained with adenovirus type 12. The following purification scheme has been successfully used with all adenovirus types so far tested, including types 2, 3, 5, 7, and 12, as well as a number of simian adenoviruses.

Step one: cell breakage. The infected cell pellet, which had been suspended and frozen in 1% of the original volume of medium used for virus growth (the volume at this point routinely is 100 ml for the harvest of 200 32-oz bottle monolayer cultures), was thawed quickly and sonically treated with a Branson 20-kc probe-type Sonifier (model S125) with cooling to maintain a temperature of 6 to 8 C. At an instrument setting of 4 or 5, from 5 to 10 min was required to reduce

TABLE 4. *Production of ^{3}H-adenovirus type 12*[a]

Culture type	Amethopterin	Specific activity of ^{3}H-TdR	Disintegrations per min per μg of virus DNA
	M	μc/μg	
Monolayer	0	30	1.35×10^6
Monolayer	0	27	1.54×10^6
Monolayer	10^{-6}	27	3.29×10^6
Suspension	0	6.7	0.50×10^6
Suspension	10^{-6}	6.7	1.26×10^6

[a] I-407 cultures were incubated 24 hr prior to infection, with or without amethopterin, and ^{3}H-TdR (6.25×10^{-6} M) was added 7 hr postinfection. The infected cells were harvested 36 to 48 hr after infection, and purified virus was prepared by the method described in the text.

TABLE 5. *Effect of enzymes on adenovirus type 7 infectivity*

Enzyme[a]	Remaining infectivity (log $TCID_{50}$)	
	10 min	45 min
None	5.2	5.4
Deoxyribonuclease + ribonuclease	4.8	5.2
Chymotrypsin	5.5	5.5
Lecithinase	5.4	5.4
Lysozyme	5.8	5.5
Pronase	4.5	4.2
Trypsin	4.5	3.8
Subtilisin	5.5	5.5
Collagenase	5.6	5.8
Snake venom (*Crotalus adamanteus*)	5.4	6.2

[a] All enzymes were used at a final concentration of 100 μg/ml. Cofactors and *p*H were adjusted as necessary.

TABLE 6. *Effect of enzymes on KB cellular debris*

Enzyme[a]	Percentage of total nitrogen		
	Pellet	Supernatant fluid	Supernatant fluid after Genetron extraction
None	53	47	32
α-Chymotrypsin	22	78	66
Subtilisin	23	77	64
Collagenase	38	62	55
Lecithinase	47	53	37
Snake venom (*Crotalus adamanteus*)	53	47	47
Mixture I[b]	26	74	64
Mixture II[c]	45	55	41
Mixture III[d]	21	79	75

[a] Samples incubated with enzymes for 45 min at 37 C.

[b] Mixture I: subtilisin, collagenase, and snake venom.

[c] Mixture II: lecithinase and lysozyme.

[d] Mixture III: chymotrypsin, subtilisin, and snake venom.

the intact-cell content to less than 2 to 5%, as noted microscopically.

Step two: enzyme treatment. Pancreatic ribonuclease and deoxyribonuclease I were each added at 100 μg/ml of cell suspension, and the mixture was incubated and stirred for 30 min at 37 C. Crude snake venom was then added at 100 μg/ml, and incubation was continued for 15 min. Lastly, subtilisin and α-chymotrypsin were added at 100 μg/ml each and the mixture was incubated for 30 min. After this treatment, a marked clearing of the suspension was apparent. Particulate debris remaining after this incubation was removed by centrifugation for 5 min at 2,500 × *g* in the Sorvall centrifuge at 4 C.

Step three: concentration and density gradient centrifugation. The remainder of the purification is similar to an earlier procedure (6), except that CsCl was substituted for RbCl. The virus supernatant fluid of step two was layered onto a 5-ml cushion of 45% CsCl in 0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4, and centrifuged in a Spinco SW25.1 rotor at 54,000 × *g* or in an International SB110 rotor at 69,500 × *g* for 2 hr at 4 C. The supernatant fluid was discarded, and the virus that formed a band in the upper portion of the CsCl cushion was recovered. After adjustment of the density to 1.35 g/ml, the virus fraction was centrifuged in the Spinco SW50 rotor at 165,000 × *g* for 14 to 16 hr at 4 C. The band of whole particles was recovered and recentrifuged to equilibrium in CsCl, 1.35 g/ml. In the second gradient, only a single band was evident. This was collected from the bottom of the tube and dialyzed against 0.15 M NaCl, 0.01 M Tris (*p*H 7.4), and 0.001 M EDTA, and stored at −90 C until use.

The purity of the virus preparations, as judged by electron microscopy, is very good (Fig. 1). In addition, no separation of infectivity and radioactive material (when labeled virus preparations were purified) could be detected after repeated centrifugation in a CsCl gradient (Fig. 2). As a further test of this procedure, uninfected cell homogenates labeled with either ^{3}H-TdR or ^{14}C-valine and ^{14}C-leucine were added to unlabeled crude virus-cell homogenates, and purification of the virus was carried out as described. When the virus was mixed prior to purification with host cells labeled with ^{3}H-TdR or ^{14}C-leucine and ^{14}C-valine, less than 0.0004% of the initial tritium was present in the final virus (Table 7). Less than 0.013% of the initial ^{14}C remained in the final preparation. The total plaque-forming units (PFU) recovered represented 82.5% of the initial crude preparation.

It is very important to delay removal of cellular particulate debris by centrifugation until after the enzyme treatment if maximal virus yields are desired. Losses up to 60% have been observed when low-speed centrifugation was carried out prior to the enzyme treatment. These losses are especially significant when purifying virus from very concentrated (10^{8}/ml, or greater) cell suspensions.

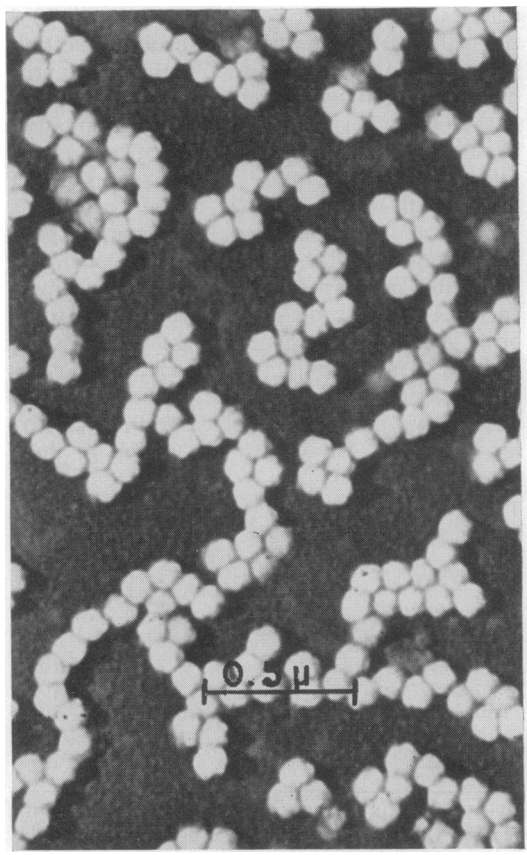

FIG. 1. *Electron micrograph of adenovirus type 12 purified by the procedure described in the text.* × *40,000.*

DISCUSSION

We have prepared highly radioactive, purified adenoviruses grown in the presence of amethopterin and ^{3}H-TdR. There was no decrease in virus yield, and the specific activity of the viral DNA was enhanced two- to threefold over that obtained without the use of amethopterin. The increased specific activity of the preparation cannot be ascribed to contamination of the virus with highly labeled cell debris because repeated density gradient centrifugation demonstrates no further separation of label and infectivity. Furthermore,

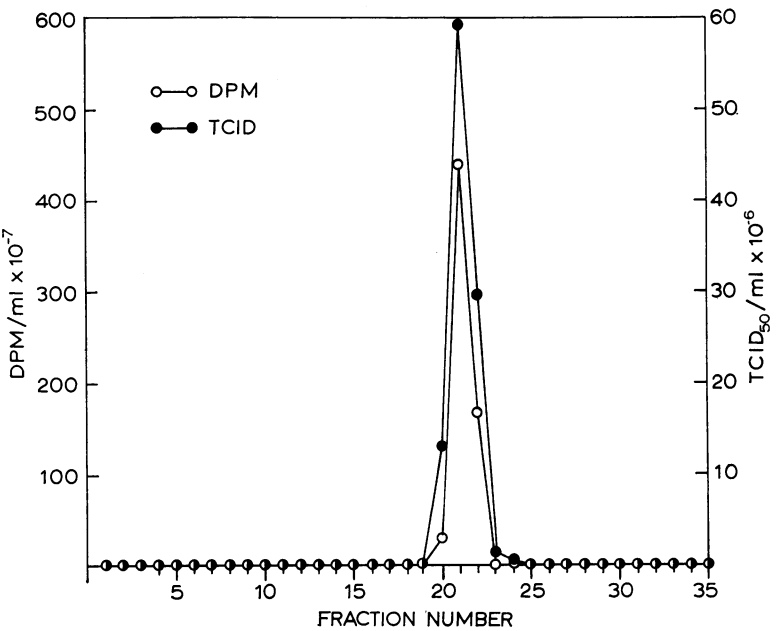

FIG. 2. *CsCl equilibrium density gradient profile of ^{3}H-adenovirus type 12 after two cycles of gradient centrifugation.*

TABLE 7. *Purification of unlabeled adenovirus type 7 from radioactive cell debris*[a]

Fraction	Total PFU	Total disintegrations/ min of ^{14}C	Total disintegrations/ min of ^{3}H
Sonically treated cell suspension	2.38×10^{11}	6.73×10^{7}	3.07×10^{8}
Sonically treated cell suspension after enzyme treatment	2.31×10^{11}	6.80×10^{7}	3.05×10^{8}
Purified virus	1.98×10^{11}	9.0×10^{3}	1.3×10^{8}
Isolated DNA (845 μg)		0	170

[a] Unlabeled, infected cells were mixed with uninfected cells labeled with ^{3}H-TdR or ^{14}C-leucine and ^{14}C-valine. Purification was performed as described in the text.

the purification procedure employed has been shown to be highly efficient in removing cell DNA.

The concentration of amethopterin chosen was determined on the basis that it effectively inhibited thymidine synthesis, as shown by its effect on virus production, and could be reversed with a low concentration of ^{3}H-TdR. Lower concentrations of amethopterin did not routinely provide good inhibition, and higher concentrations required levels of thymidine for reversal that would have been too large for economical use of highly radioactive ^{3}H-TdR. Also, a high concentration of thymidine might possibly have inhibited adenovirus synthesis (4).

We have found the methods used in this study to be generally applicable to both human and simian adenoviruses. It might be expected, also, that amethopterin would be applicable to the production of other highly labeled DNA viruses. Our results with amethopterin compare favorably with those reported by Luborsky et al. (9) on the use of 5-fluorodeoxyuridine (FUdR) for the enhanced incorporation of ^{3}H-TdR into polyoma virus DNA. These workers reported that the ^{3}H-DNA produced was three times more highly labeled when a combination of FUdR and ^{3}H-TdR was used than when ^{3}H-TdR alone was added to infected cultures. However, 50% less polyoma DNA was obtained in the presence of the drug.

We have found our purification method most useful in the preparation of virus for subsequent DNA extraction. Because potent proteolytic enzymes are used in the procedure, the absolute integrity of the virus-coat protein may be ques-

tioned. Although we have not attempted to evaluate this possibility in detail, the retention of infectivity and the morphology of the purified particles, as shown by electron microscopy, suggest that the extent of damage must be small. In addition, viruses purified by this procedure have been used as antigen for the production of antibody having a very high complement fixation and neutralizing titer, with retention of type specificity.

Apparently, the use of these enzymes promotes the release of adenovirus from the cellular debris. If the particulate cellular debris is removed from the virus suspension prior to enzyme treatment, a drastic reduction in yield is noted. This suggests either physical entanglement of virus in the cellular debris or a possible attachment of adenoviruses to some cellular component. We have not noted such large effects on yield when using more dilute suspensions of infected cells for purification. This may be due in part to the fact that the cells are more thoroughly homogenized when sonically treated at dilute concentrations, thus achieving a more complete breakdown of cellular components.

The usefulness of our procedure for the purification of human adenovirus types 2 and 7 has been substantiated recently in another laboratory (W. W. Wiese, *personal communication*).

ACKNOWLEDGMENTS

We thank L. F. Ellis for the electron-microscope examination of the purified virus preparations.

This investigation was supported by Public Health Service contract PH43-65-570 from the National Cancer Institute.

LITERATURE CITED

1. Bertino, J. R. 1963. The mechanism of action of the folate antagonists in man. Cancer Res. **23**:1286–1306.
2. 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.
3. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.
4. Green, M. 1962. Biochemical studies on adenovirus multiplication III. Requirement for DNA synthesis. Virology **18**:601–613.
5. Green, M., and M. Piña. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. Virology **20**:199–207.
6. Hakala, M. T. 1957. Prevention of toxicity of amethopterin for sarcoma-180 cells in tissue culture. Science **126**:255.
7. Lacy, S. S., and M. Green. 1964. Biochemical studies on adenovirus multiplication. VII. Homology between DNA's of tumorigenic and nontumorigenic human adenoviruses. Proc. Natl. Acad. Sci. U.S. **52**:1053–1059.
8. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. J. Virol. **1**:851–867.
9. Luborsky, S. W., D. Lorenz, D. Reynolds, J. E. Verna, E. Usdin, and P. T. Mora. 1967. On the production of highly labeled tumor virus deoxyribonucleic acid. Proc. Natl. Acad. Sci. U.S. **57**:1286–1293.
10. Miller, G. L., and E. E. Miller. 1948. Determination of nitrogen in biological materials. Anal. Chem. **20**:481–488.
11. Scott, J. F., A. P. Fraccastoro, and E. B. Taft. 1956. Studies in histochemistry. I. Determination of nucleic acids in microgram amounts of tissue. J. Histochem. Cytochem. **4**:1–10.
12. Stubblefield, E., and G. C. Mueller. 1965. Thymidine kinase activity in synchronized HeLa cell cultures. Biochem. Biophys. Res. Commun. **20**:535–544.
13. Valentine, R. C., and H. G. Pereira. 1965. Antigens and structure of the adenovirus. J. Mol. Biol. **13**:13–20.