1. Meschia, G., Am. J. Phys. 181, 1 (1955)
2. McCarthy, E. F., J. Physiol. (London) 104, 443 (1946).
3. Meschia, G., Yale J. Biol. Med. 27, 206 (1954).
4. Hepp, O., Z. Ges. Exptl. Med. 99, 709 (1936).
5. Rehm, W. S., Science 100, 364 (1944).

Received Nov. 13, 1968. P.S.E.B.M., 1969, Vol. 131.

Abortive Infection of Human Cell Cultures by a Canine Adenovirus (33810)

WARREN D. GEHLE[1] AND KENDALL O. SMITH
Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

Adenovirus replication is usually limited to cell cultures originating from the naturally susceptible host (1). Infection of cells from other species often results in an abortive growth cycle. For example, incomplete replication of human adenovirus 4 in canine cells was described by Carmichael (2). He found that inoculation of canine kidney cells with adenovirus 4 resulted in the synthesis of viral DNA and viral antigens which differed from those produced by adenovirus 4 in human cells. The multiplication cycle was incomplete, since no infectious particles were produced.

Certain exceptions to the incomplete replication of adenoviruses in heterologous cells have been reported. Human adenoviruses have been propagated in cells derived from various animals (3–6). More recently, differences in the abortive cycle of various human adenoviruses in simian cells have been reported (7). These abortive infections resulted in either progressive disappearance of the virus, or in a low-level *de novo* production of infectious particles which never exceeded the input. Replication of several human adenoviruses is enhanced by simian virus 40 or by simian adenovirus 15 in simian cells (8, 9). In these cases a "helper" virus is necessary for adenovirus replication in the heterologous system. Infection of hamster cells by adenovirus 12 results in morphological alteration of the cells (transformation), enhancement of the ability of the cells to divide, and the synthesis of adenovirus 12 specific complement fixing antigens *without infectious virus* (10, 11).

Nonhuman adenoviruses have been propagated in human cells with similar results (12–14). The canine adenovirus, infectious canine hepatitis virus (ICHV), was shown by Govaerts (12) to grow to low titers in several human cell lines when incubated over a period of 4–8 days. More recently, ICHV was shown to be an effective "helper" for the synthesis of adeno-associated virus in human cells (15). The present report describes a nonparallel, *de novo* synthesis of antigens and infectious particles on serial passage of ICHV in human cell cultures.

Materials and Methods. Virus strain. The ICHV was obtained from the American Type Culture Collection and passed once in primary canine kidney cells. It was verified as ICHV by neutralization using rabbit anti-ICHV serum prepared against the Cornell strain.[2]

Cell cultures. Human amnion (HA, strain FL) cells and the Madin Strain of canine kidney cells (MDCK) were used for viral growth. Eagle's minimal essential medium with 10% fetal bovine serum and 0.03% glutamine was used as nutrient medium for the growth of cell monolayers. One hundred units of penicillin and 40 μg of streptomycin/ml were used, and aureomycin (6μg/ml) was

[1] This work was submitted to the Microbiology Department of the University of Maryland in partial fulfillment of the requirements for the degree of Master of Science.

[2] Kindly provided by Dr. Nicola Tauraso of the Laboratory of Virology and Rickettsiology, Division of Biologics Standards, National Institutes of Health.

often added to this to prevent growth of mycoplasma (these cells were later found to be free of mycoplasma). Occasionally 60 μg of neomycin/ml were used alone.

Inoculation and growth of virus. Aliquots of ICHV were thawed at 36°, then treated in a Raytheon sonic oscillator to disrupt the cells and disperse the virus. When necessary, dilutions of the virus were made in nutrient medium before inoculation. After removal of the nutrient medium from the cells, a known volume of the virus suspension was inoculated onto the cell cultures. These cultures were incubated at 36° for 1 hr with rotation every 10–15 min. The inoculum was removed and the cells were rinsed three times with 10 ml of medium. Nutrient medium was added and the cultures were incubated at 36° for 44–48 hr before harvesting. The infected cells were harvested by scraping them off the glass and into the nutrient medium with a rubber policeman. This material was stored at −20° or in an ice bath for short periods of time. Replicate cell cultures were used for experiments in which the inoculum contained a varying number of infectious particles per cell.

Complement fixation titrations. The method of performing complement fixing (CF) antigen titrations has been previously described (16). In order to compare CF antigen concentrations in different experiments, results were expressed as the number of CF antigen units contained in 10^6 cells. A CF antigen unit is defined as the reciprocal of the highest antigen dilution giving a 3–4+ reaction with the optimal concentrations of anti-ICHV serum.

Infectivity titrations. A fluorescent-focus (FF) assay was employed to titrate ICHV infectivity (17). Briefly, the method consisted of inoculating tenfold dilutions of sonic treated material into small plastic petri dishes containing a monolayer of MDCK or HA cells. After adsorption for 1 hr at room temperature, nutrient medium containing 0.25% special agar-Noble was overlayed on the cells to inhibit virus spread, thereby localizing the fluorescing foci. The cultures were then incubated at 36° in a CO_2 incubator for 2 days. Cell cultures were fixed and stained, and the fluorescing foci were counted. The infectivity of the sample was calculated as described by Thiel and Smith (17). The terminal tube dilution (CPE) method was also employed for infectivity titrations (18).

The infectivity titer obtained in MDCK cells was used as a measure of the infectious virus in a sample. Multiplicity of infection (M) is defined in this work as the FF titer of virus in MDCK cells/total number of HA or MDCK cells.

Immunization of rabbits. Immune serum was prepared in rabbits by injecting purified ICHV subcutaneously as previously reported by Smith (18). This serum was pooled and found to have a CF titer of 1:320 and a neutralization titer of greater than 1:1000 against $10^{4.5}$ infectious units of ICHV.

Precipitation analysis by double diffusion in agar gel (Öuchterlony). The method has been reported previously (19). Briefly, an aqueous solution of 1% special agar-Noble containing 1% sodium azide was poured into plastic petri dishes. A Feinberg gel cutter (Colab No. 1815) was used to construct a center well and four smaller circumferential wells. The serum was placed in the center well, and the antigens were placed in the circumferential wells 1 day later. The test was read in 2–4 days.

Titration of ICHV tumor antigen. The MDCK and HA cell cultures which had been inoculated 24 hr previously with ICHV at a multiplicity of 10 were frozen at −70° until tested by Dr. Padman Sarma[3] for ICHV tumor antigens.

Results. Antigen and virus production in canine and human cells. Canine cultures were infected at a multiplicity of 40, incubated for 48 hr, and then frozen. Samples were later titrated for CF antigen and infectious virus. The upper part of Fig. 1 shows the virus yield per canine (MDCK) cell after each of three serial virus passages. The results indicate that each passage produced approximately the same relative quantity of CF

[3] National Institute of Allergy and Infectious Diseases, National Institutes of Health.

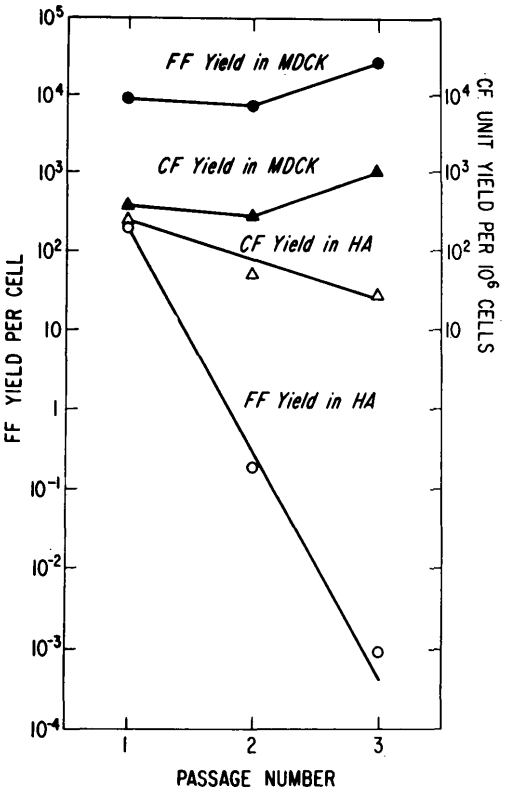

FIG. 1. ICHV antigen and infectivity yields obtained for each of three serial passages of ICHV in MDCK and HA cultures.

antigen (CF yield) and infectious virus (FF yield).

The ICHV infection of human cells was studied similarly, although the multiplicity of infection became quite low upon serial passage because of the steady decline in virus yields. The lower part of Fig. 1 shows that the yield of infectious virus decreased rapidly with each passage, while the CF yields did not decrease in such a manner. This nonparallel production of CF antigen and infectivity in human cells differs sharply from the parallel yields obtained by serial passage in canine cells.

The input infectious virus was compared with the yield of CF antigen per cell in these experiments. The number of infected canine or human cells (calculated on the basis of M)[4] required to synthesize a given amount of CF antigen is shown in Table I. It appears that the number of infected cells necessary to produce a constant amount of CF antigen decreased with each abortive passage of ICHV in human cells.

The effect of multiplicity of infection (M) on CF antigen and virus production. A sample of ICHV of known titer was serially diluted and inoculated onto canine and human cells so as to give various input multiplicities. These cultures were harvested after 48-hr incubation and samples were assayed for CF antigen and infectious virus. The results are presented in Fig. 2. For $M \geqq 1$ in the canine cells, a plateau was reached in the synthesis of both CF antigen and infectious virus. However, in the human cells, a M of 10 resulted in a substantially greater production of infectious virus than a M of 1. The nonparallel production of CF antigen and infectious virus was again noted (lower portion, Fig. 2), and the difference was most apparent in the region of low $M(<1)$.

The effect of multiplicity of infection on the initiation of fluorescing foci. Serial dilutions of ICHV were inoculated simultaneously onto human and canine cells, and the cells were incubated for 96 and 48 hr, respective-

TABLE I. A Comparison of the Number of Infected Cells Required to Produce 10 CF Units of Viral Antigen in MDCK and HA Cells.

Virus source	Cell inoculated	Input (FF/cell)	Yield (CF units/10^6 cells)	Infected cells required to produce 10 CF units
MDCK	MDCK	0.4	80	5×10^4
MDCK	HA	0.4	10	4×10^5
HA P-2	HA	0.014	30	5×10^3

[4] See "Materials and Methods" for definition of multiplicity.

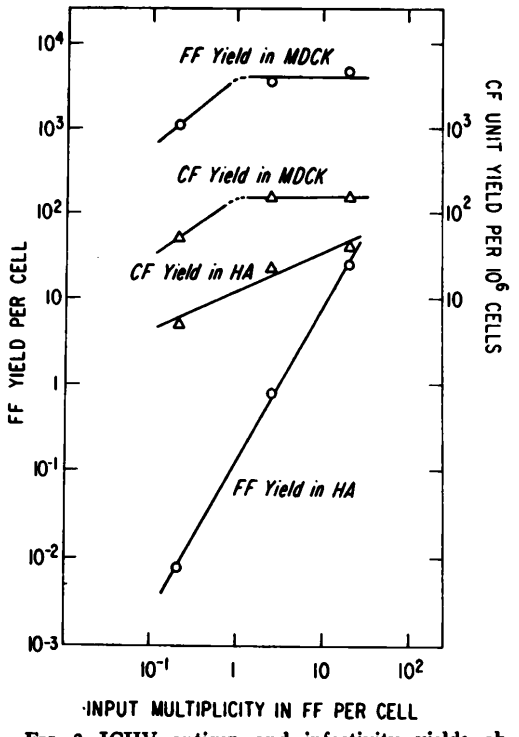

FIG. 2. ICHV antigen and infectivity yields obtained in MDCK and HA cultures for various input multiplicities.

ly. Even though the incubation period was extended to 96 hr for human cells (one complete growth cycle of ICHV in human cells as determined in other experiments), there was a substantial difference in the FF titers obtained in the two cell lines. The ICHV titrations in canine cells were approximately 230-fold higher than those obtained in human

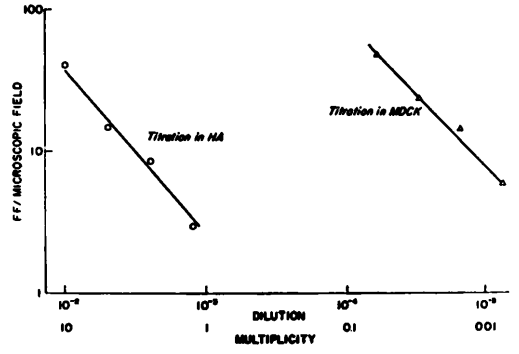

FIG. 3. Simultaneous FF titrations of ICHV in MDCK cells (cells fixed 48 hr postinoculation) and HA cells (cells fixed 96 hr postinoculation). The virus source was MDCK cells.

cells. The dose–response relationship in the human cells indicated the kinetics of a single event (Fig. 3). However, the multiplicity of infection was high in the region required for fluorescing cell counting. Only one HA cell in 40 showed any fluorescence at a M of 10 (based on MDCK titrations), indicating the very low efficiency of ICHV in its ability to induce fluorescence in human cells.

De novo *synthesis of antigens and virus in human cells.* Several experiments were performed to determine whether the CF antigen and infectious virus recovered from human cells were (1) the result of *de novo* synthesis or (2) carry-over from the original inoculum. The HA cell monolayers were inoculated with ICHV, the inoculum was removed, and the cells were harvested immediately or after 48-hr incubation. These materials were then assayed for CF antigen. Table II shows that all the CF antigen activity of the inoculum was recovered after a 1 hr absorption period. When the cells were harvested immediately, they did not react in the CF test (suspended to give about 8 $\times$ 10^5 cells/ml), indicating that the human cells did not absorb viral CF antigen in significant amounts. When the cells were harvested in 48 hr, however, a large yield of CF antigen (a 25-fold increase over the inoculum) was obtained, indicating *de novo* synthesis.

The ICHV was serially passed in human cells as before, but an anti-ICHV serum rinse was used with each passage to neutralize residual virus. The CF antigen and FF yields from these cultures showed the same nonparallel production of viral antigen and infectious virus. A similar experiment using canine cultures showed that the antiserum rinse

TABLE II. Absorption and Yield of ICHV CF Antigen by HA Cells.

ICHV grown in HA cells	CF units
P-1 (original inoculum)	32
P-1 (inoculum 1 hr after absorption)	32
P-2 (48-hr harvest)	800*

* This represents a 25-fold increase over the amount inoculated; replicate cultures harvested at 1 hr after absorption showed no CF reaction undiluted.

also had no effect on the production of either ICHV CF antigen or infectious virus.

The abortive growth cycle of ICHV in HA cultures was studied in sufficient detail to determine if the input virus went through eclipse prior to the recovery of infectious virus. This study showed that ICHV had an eclipse phase lasting about 24 hr and grew to low titers in 4 days. We conclude, on the basis of these experiments, that CF antigen and infectious virus production in human cells was the result of virus infection and not the result of inoculum carry-over from one culture to another.

Comparative titrations by FF assay and terminal tube dilution (CPE). By comparing FF titrations with terminal tube dilution (CPE) titrations of identical virus dilutions in canine cells, it was thought that evidence might be obtained concerning the possible presence of defective virus particles capable of inducing only the synthesis of viral antigens and not infectious virus ($CF^+ I^-$ particles). However, these titrations gave almost identical end points (Table III). This suggests that if the hypothetical $CF^+ I^-$ particles were present, then (i) their titers were lower than the titers of infectious particles ($CF^+ I^-$); or (ii) they induced the synthesis of much less viral antigens than $CF^+ I^+$ particles and the quantities of viral CF antigen produced per cell were too small to be detected by the fluorescent antibody technique; or (iii) the particles were $CF^+ I^-$ *only for HA cells.*

TABLE III. A Comparison of Infectivity Titers by the FF Assay and Terminal Tube Dilution (CPE) Assay.

	Infectivity yield (assayed in MDCK cells)	
Cell line inoculated[a]	FF assay	Terminal tube dilution assay
MDCK	2.7×10^9	2×10^9
HA	5.9×10^6	1×10^7

[a] The input multiplicity was 20, and the virus source was ICHV grown in MDCK.

Comparison of viral antigens synthesized in canine and human cells. Viral antigens from canine and human cells were compared by the Ouchterlony agar gel diffusion test. Two antigenic components were resolved in both the ICHV infected canine and human cells, and they appeared to be identical.

The ICHV infected human cells were tested for the presence of tumor antigen. Antibody to ICHV tumor antigen (present in tumor-bearing hamster sera) reacted with the infected human cells in the CF test. Uninfected human cells gave no reaction. Similar results were also obtained using ICHV infected and uninfected canine cells. None of the above experiments revealed any *qualitative* differences between antigens synthesized in the two cell systems.

Comparison of infectious virus synthesized in human and canine cells. Aliquots of ICHV synthesized in HA or MDCK cells were simultaneously passed (at identical M) in canine cultures. After 48 hr incubation, these cultures were harvested and the infectivity titers were determined by the FF assay. No significant difference was noted in the virus yields, indicating the ability of human cell-synthesized ICHV to replicate in canine cells as well as canine cell-synthesized ICHV.

Discussion. Serial passage or various multiplicities of ICHV in human cells resulted in a nonparallel production of CF antigen and infectious virus (Figs. 1 and 2). By the third passage of ICHV in HA cells or at lower infection multiplicities, a much smaller input of infectious virus was required to produce a given amount of CF antigen than was required in the homologous canine cell system (Table I and Fig. 2). This phenomenon could not be accounted for by carry-over of infectious virus or antigens from the inoculum because the viral materials in infected human cells were shown to be the result of *de novo* synthesis. [This confirms an earlier report by Govaerts (14) concerning the *de novo* replication of ICHV in human cells over a long time period.] Any explanation which might account for the difference in the synthesis of CF antigen and infectious particles in the heterologous system must take into account the observation that this differ-

ence increases with each serial passage; that is, it is a nonparallel difference.

One possible explanation for this phenomenon might be the selection of a virus population which is better suited to replicate in human cells than in canine cells. A loss in ability of human cell-synthesized virus to replicate in canine cells could account for the apparent rapid decrease in the recovery of infectious virus, since canine cells were used to assay for the presence of infectious virus. This explanation seems unlikely, however, for two reasons. There was little if any CPE or spread of virus from cell to cell in the second and third passages of ICHV in human cells. Also, human cell-synthesized ICHV (which could be detected by the FF assay) showed no difference in its ability to replicate in canine cells.

Another possible explanation for the phenomenon of nonparallel viral antigen and infectious virus production in human cells is the presence of defective virus particles which are capable of inducing only the synthesis of CF antigen and not the synthesis of infectious virus (CF^+ I^- particles). Multiplicity of infection experiments in human cells indicated that large inocula are required in order to initiate significant infectious virus synthesis. The CF yield per human cell was less dependent upon infection by large amounts of virus. This supports the explanation that CF^+ I^- particles were present in the inoculum. We attempted to confirm this explanation by comparing the CPE dilution end point with the FF assay (a measure of antigen producing foci) using canine cells, but we found that the end points were the same. Such a comparison in human cells might reveal the presence of CF^+ I^- particles which are defective *only for human cells*; but a CPE dilution end point titration in human cells is not feasible since there is no observable secondary spread of the virus in these cells. (This is understandable because of the low yield of ICHV per cell. Even if a few human cells were producing very high yields of ICHV, and the virus were released efficiently, the number of such susceptible cells in a culture would be so small that no marked CPE would be seen.) In view of these observations and the recent reports of defective SV-40 (20, 21), we feel that the presence of defective ICHV particles is a possible explanation for the phenomenon we have observed, *i.e.*, the nonparallel production of viral antigens and infectious particles in human cells.

These findings make it clear that ICHV interacts with human cells to produce substantial amounts of tumor antigen, viral antigens and low levels of infectious virus. No qualitative differences were seen between antigens produced in human and canine cells. Failure of human cells to produce infectious virus efficiently could be due to either defective synthesis of viral DNA or failure in the assembly of viral components.

Summary. The abortive infection of human amnion cells by infectious canine hepatitis virus (ICHV) was studied. Within 48 hr after inoculation, large quantities of complement fixing (CF) antigen were produced, although infectious virus yields were small. Serial passage of ICHV in human cells, with harvests every 48 hr, resulted in a rapid decrease in infectious virus, but a relatively slow decrease in CF antigen production. If the amount of input infectious virus is taken as a reference, calculations indicate that the ratio of CF antigen per infectious unit increased with each serial passage. This phenomenon was most noticeable after the first serial passage in human cells, or when low multiplicities of infection with ICHV were used. Viral antigens and infectious virus recovered from human cell cultures were found to be the result of *de novo* synthesis. The mechanism of the phenomenon of the nonparallel production of viral antigens and infectious virus in human cells is unknown, but possible explanations are suggested.

The authors are indebted to Dr. Frank M. Hetrick for his helpful suggestions during the progress of the work and his valuable criticisms during the preparation of the manuscript.

1. Pereira, H. G., Huebner, R. J., Ginsburg, H. S., and Van der Veen, J., Virology **20**, 613 (1963).

2. Carmichael, L. E., Am. J. Vet. Res. **26**, 15 (1965).

3. Rowe, W. P., Huebner, R. J., Hartley, J. W.,

Ward, T. G., and Parrott, R. H., Am. J. Hyg. 61, 197 (1955).

4. Guerin, L. F. and Guerin, M. M., Proc. Soc. Exp. Biol. Med. 96, 322 (1957).

5. Kelly, B. and Pereira, H. G., Brit. J. Exptl. Pathol. 38, 396 (1957).

6. Warren, J. and Cutchins, E. C., Virology 4, 297 (1957).

7. Rapp, F., Jerkofsky, M., and Vanderslice, D., Proc. Soc. Exptl. Biol. Med. 126, 782 (1967).

8. Rapp, F. and Melnick, J. L., Progr. Med. Virol. 8, 349 (1966).

9. Naegele, R. F. and Rapp, F., J. Virol. 1, 838 (1967).

10. McBride, W. D. and Wiener, A., Proc. Soc. Exptl. Biol. Med. 115, 870 (1964).

11. Yamane, I. and Toshihisa, K., Nature 213, 187 (1967).

12. Govaerts, A., Arch. Ges. Virusforsch. 10, 395 (1960).

13. Hammon, W. McD., Yohn, D. S., Casto, B. C., and Atchison, R. W., J. Natl. Cancer Inst. 31, 329 (1963).

14. Sharon, J. and Pollard, M., Nature 202, 1139 (1964).

15. Smith, K. O. and Gehle, W. D., J. Virol. 1, 648 (1967).

16. Sever, J. L., J. Immunol. 88, 320 (1962).

17. Thiel, J. F. and Smith, K. O., Proc. Soc. Exptl. Biol. Med. 125, 892 (1967).

18. Smith, K. O., J. Immunol. 94, 976 (1965).

19. Feinberg, J. G., Immunol. 2, 346 (1959).

20. Altstein, A. D., Sarycheva, O. F., and Dodonova, N. N., Virology 33, 744 (1967).

21. Uchida, S., Yoshiike, K., Watanabe, S., and Furumo, A., Virology 34, 1 (1968).

Received Dec. 19, 1968. P.S.E.B.M., 1969, Vol. 131.

Fate of Labeled Polyoma Virus DNA in Cell Cultures* (33811)

BRUCE A. MAURER,[1] KENNETH WERTMAN,[2] AND IRVING YALL
(Introduced by W. S. Jeter)

Department of Microbiology, University of Arizona, Tucson, Arizona 85721

The use of bacteriophage, labeled by radioactive tracers, has proven to be an efficient tool in determining the fate of viral components in a bacterial host (1, 2). This technique has been applied more recently to the determination of the intracellular fate of isotopically labeled polyoma virus capsid and nucleic acid employing autoradiography (3). The early events in adenovirus type 5 infection of HeLa cells have been studied employing ^{14}C and ^{3}H labeled capsid protein and deoxyribonucleic acid (DNA) (4). We are reporting the results of experiments designed to determine the fate of purified polyoma virus containing isotopically labeled DNA in infected cells by detecting radioactivity in specific fractions of the cells. This study employed primary mouse embryo tissue culture (METC) cells, the majority of which undergo a productive infection (5,6) and BHK/21 cell cultures, in which very few of the cells produce viral antigen (7).

Materials and Methods. Virus. The SE polyoma virus was donated by Dr. Bernice Eddy and was serially propagated in monolayer cultures of primary METC cells. The cells were established from 10 to 15-day-old mouse embryos and were propagated in a growth medium containing medium 199 supplemented with 10% fetal calf serum (FCS). The maintenance medium contained medium 199 supplemented with 2% FCS. The virus was harvested from infected cultures employing receptor-destroying enzyme (RDE) according to the method of Crawford (8). Virus assay was performed by the tube hemagglutination test using washed 0.5% guinea pig erythrocytes. Titrations were incubated over-

* Supported in part by NIH Training Grant No. 5T01A100122-07 from the National Institute of Allergy and Infectious Diseases and American Cancer Society Institutional Grant No. IN-80 to the University of Arizona.

[1] Present address: Roswell Park Memorial Institute, Cell and Virus Building, Buffalo, New York.

[2] Deceased, March 30, 1967.