STUDIES ON PNEUMONIA VIRUS OF MICE (PVM) IN CELL CULTURE

I. Replication in Baby Hamster Kidney Cells and Properties of the Virus*,‡

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Plate 19

(Received for publication 29 March 1967)

Pneumonia virus of mice (PVM) was first isolated as a latent agent from apparently healthy mice (1, 2), and pulmonary disease was produced only by intranasal inoculation of lung material and serial blind passage. Evidence of a high incidence of inapparent PVM infection has been found in a number of laboratory mouse colonies from a variety of sources (2–4), and antibodies have also been detected in other mammalian species (3, 5).

The virus is highly thermolabile (2, 6), ether sensitive (7), and combines with a lung tissue component from a variety of mammalian species (8). PVM agglutinates mouse and hamster erythrocytes (9, 10), but unless special precautions are taken when harvesting infected lungs (11), combination of the virus with the tissue component occurs, and hemagglutination can be detected only after the virus is freed by treatment such as heating to 70°C (9).

The multiplication of PVM in the mouse lung is inhibited by polysaccharides obtained from several bacteria (12), and the first example of successful chemotherapy of a virus infection in an animal was reported in 1951 by Ginsberg and Horsfall (13) who used the capsular polysaccharide of Friedländer bacillus type B in mice infected with PVM.

PVM has been propagated in minced chick embryo cultures (1, 2) and grown to low titer in primary cultures of suckling hamster kidney cells (14), but little is known about the replication, structure, or chemistry of the virus.

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* Supported by Research Grants AI-05600 and AI-03445 from the National Institute of Allergy and Infectious Diseases and by Public Health Service Grant GM-577 from the Institute of General Medical Sciences.

‡ Presented in part at the 50th Annual Meeting of the Federation of American Societies of Experimental Biology. 1966. *Fed. Proc.* **25**: 311.

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The present communication describes the multiplication of PVM in a line of baby hamster kidney (BHK21) cells, and the cytological changes induced in these cells as shown by cytochemical and immunofluorescent staining. The paper which follows describes electron microscopic studies of the virus and virus-infected cells (15). Evidence will be presented suggesting that PVM is an RNA virus that replicates in the cytoplasm and resembles the myxoviruses in structure and replication.

Materials and Methods

Cell Cultures.—The BHK21 baby hamster kidney cell line (16) was obtained in its 56th passage from Dr. N. Karabatsos of The Rockefeller Foundation Virus Laboratory, who had obtained it in its 45th passage from Dr. M. Stoker. The cells were grown in a modified Eagle's medium with 10% unheated calf serum and 10% tryptose phosphate broth. The BHK21-F cell line (17) is a heteroploid variant of the BHK21 line, and is highly susceptible to fusion by the simian parainfluenza virus SV5 (17) and by visna virus (18). The HKCC cell line is a continuous line of adult Syrian hamster kidney cells which were obtained from Dr. C. H. Calisher of Microbiological Associates, Inc. BHK21-F and HKCC cells were propagated in reinforced Eagle's medium (19) with 10% calf serum and 10% tryptose phosphate broth.

Virus.—PVM was propagated in 3-wk old Rockefeller University Swiss mice by intranasal inoculation of infected mouse lung suspension containing $10^{4.4}$ MS_{50} (50% maximum score) of virus. 4 days after inoculation, surviving animals were sacrificed, and the lungs were removed under sterile conditions and homogenized. A 20% lung suspension was prepared in Eagle's minimum essential medium (20) with 1% bovine plasma albumin (BSA) (Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.) and stored at −60°C until used.

PVM was also propagated in baby hamster kidney cells by the inoculation of confluent monolayers (approximately $6 \times 10^{6.0}$ cells) in 60-mm plastic Petri dishes with 0.5 ml of undiluted virus suspension from either mouse lung or previous tissue culture passage. After adsorption for 3 hr at 37°C, the cultures were incubated in a modified Eagle's medium (21) with 10% calf serum at 37°C in humidified 5% CO_2 in air. At harvest, BSA was added to a concentration of 0.5%, and the supernatant medium was collected and stored at −60°C.

Immune Sera.—Antisera to PVM grown in the mouse lung and in BHK21 cells were prepared by injecting adult rabbits intravenously with 10 ml of virus suspension containing approximately 1280 hemagglutinating (HA) units, followed in 9 days by the intraperitoneal administration of 640 HA units. The rabbits were bled 9 days later, and sera were heated at 56°C for 30 min before use.

Assay of Infective Virus in Cell Culture.—Tenfold dilutions of virus were prepared in reinforced Eagle's medium with 0.5% BSA, and held in an ice bath until inoculation. Confluent monolayers of BHK21 cells in 60-mm plastic Petri dishes were washed twice with phosphate-buffered saline (PBS), pH 7.2 (22), and inoculated with 0.5 ml of virus, six monolayers per dilution. After 3 hr at 37°C, 5.0 ml of modified Eagle's medium with 1% inactivated calf serum was added and the cultures were incubated at 37°C for 10 or 11 days. Medium from each plate was then harvested and tested for hemagglutinin. 50% infectivity end points ($TCID_{50}$) were calculated by the method of Reed and Muench (23).

Infectivity titrations in the mouse were performed as described by Horsfall and Curnen (6), except that virus dilutions were prepared in reinforced Eagle's medium with 0.5% BSA. The results of animal titrations were expressed as 50% mortality (LD_{50}) or 50% MS_{50} endpoints based on the extent of pulmonary consolidation (24).

Hemagglutination and hemagglutination-inhibition titrations were performed as previously described (25) using a 0.8% suspension of freshly prepared mouse erythrocytes in PBS with 0.25% BSA. End points were read after 2 hr at 25°C.

Neuraminidase from *V. cholerae*, 500 units/ml, was obtained from Calbiochem, Los Angeles, Calif. A unit is defined as that amount of enzyme which splits 1 μg of *N*-acetylneuraminic acid from a glycoprotein substrate obtained from human serum (fraction I, Dische) in 15 min at 37°C.

Staining Procedures.—BHK21 cells were grown to confluent monolayers in plastic Petri dishes containing 18-mm square glass cover slips and inoculated with tissue culture—grown PVM at a multiplicity of a 0.5 $TCID_{50}$ per cell; controls were inoculated with medium. Modified Eagle's medium with 10% inactivated calf serum was added after an adsorption period of 3 hr, and the cultures were incubated at 37°C. At 24, 48, 72, and 96 hr after inoculation, replicate cover slips were harvested; one set of cover slips was fixed in Zenker's fluid for 60 min and stained with hematoxylin and eosin (26); a second set was fixed in Carnoy's fixative and stained with 0.05% acridine orange stain (27); a third set was washed with PBS, fixed in acetone, and stained by the indirect fluorescent antibody technique (28). The PVM rabbit antiserum, normal rabbit serum, and goat anti-rabbit globulin conjugated with

TABLE I

Multiplication of PVM in Hamster Kidney Cell Lines

Time	Virus yield, hemagglutinating units/ml		
	Cells		
	BHK21	BHK21-F	HKCC
days			
3	4	<4	<4
5	512	<4	<4
7	1024	128	<4
10	4096	1024	32
12	8192	8192	32

fluorescein isothiocyanate (Microbiological Associates, Inc., Bethesda, Md.) used in fluorescent antibody studies were absorbed with acetone-extracted guinea pig liver powder and BHK21 cell powder. Fluorescent antibody and acridine orange—stained cells were examined and photographed with a Zeiss Ultraphot II photomicroscope using an Osram 200 watt high pressure mercury lamp, a BG12 exciter filter, and an OG4 barrier filter.

RESULTS

Multiplication of PVM in Hamster Kidney Cells

To determine whether PVM would multiply in BHK21, BHK21-F, or HKCC cells, replicate monolayers in 60-mm Petri dishes, containing 6–10 $\times$ 10^6 cells, were inoculated with 8 $\times$ 10^4 MS_{50} of virus grown in mouse lung, and incubated at 37°C. At intervals, the medium was removed and assayed for PVM hemagglutinin, and fresh medium was added.

The results shown in Table I indicate that virus replication occurred in all three cell types, although the yield from HKCC cells was very low. Hemagglutinin was produced most rapidly and in largest amount by BHK21 cells; therefore, this cell line was chosen for use in subsequent experiments.

Serial Passage of PVM in BHK21 cells.—

12 serial passages of PVM were made in BHK21 cells, inoculating in each case undiluted virus-containing medium from the preceding passage. Virus was harvested at 4 or 5 days after inoculation.

The yield of infective PVM in the first passage was $1.0 \times 10^{6.0}$ $TCID_{50}$ per ml, and in the last passage, $6.3 \times 10^{6.0}$; the hemagglutination titers were 512 and 1024, respectively, and there was little variation in yield in the intervening passages. The $TCID_{50}$ per HA ratios ranged from $10^{3.3}$ to $10^{4.1}$, with a mean of $10^{3.7}$. The moderate differences in the $TCID_{50}$ per HA ratios of these passages probably reflect the thermal lability of this virus, as well as its mechanical fragility, as will be discussed below.

A number of experiments were carried out in BHK21 cells using various media and incubation at temperatures varying from 33°C to 37°C, and it was concluded that the highest virus yields were obtained by incubation at 37°C in modified Eagle's medium with 10% calf serum which had been inactivated at 56°C for 30 min.

These results in BHK21 cells are in contrast with those of Tennant and Ward (14) who found, in primary cultures of suckling hamster kidney, a maximum titer of $10^{3.5}$ $TCID_{50}$ on the first passage and a decrease by the eighth passage to only $10^{1.8}$ $TCID_{50}$.

Adsorption of PVM to BHK21 Cells.—

Since a plaque assay of PVM is not yet available, determination of the rate of adsorption of infective virus BHK21 cells was not attempted, but the adsorption of hemagglutinin was studied using the precise fractional hemagglutination titration method of Horsfall and Tamm (29). Replicate monolayers of BHK21 cells were washed three times, inoculated with 0.5 ml containing 256 hemagglutinating units of virus, and incubated at 37°C; at 30-min intervals the inoculum was removed and assayed for unadsorbed hemagglutinin. The geometric means of two determinations were taken for each time point.

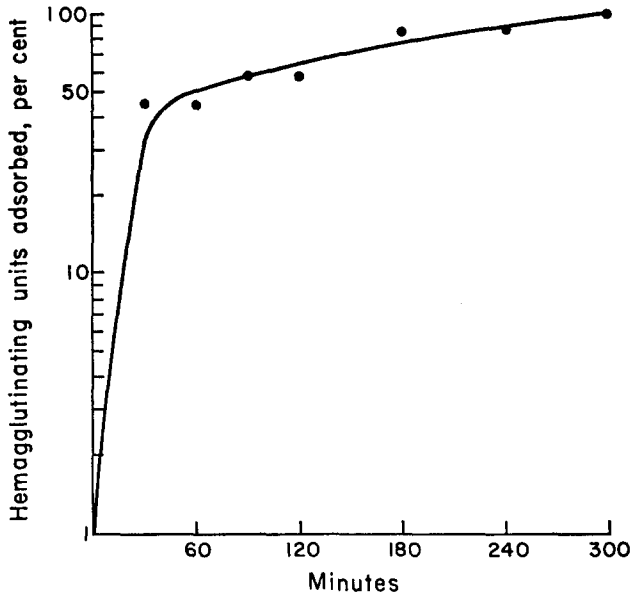
As shown in Text-fig. 1, 3 hr were required for 80% adsorption to occur, and this time was selected as the adsorption period in subsequent experiments.

Kinetics of PVM replication in BHK21 cells.—The amount of infective virus obtained from BHK21 cells is not sufficient to permit inoculation of cells at a high enough virus/cell multiplicity to give a single cycle growth curve, and it has not been possible to concentrate this labile virus without loss of infectivity. However, virus stocks were obtained with an infectivity titer sufficient to inoculate at a multiplicity of 0.5 $TCID_{50}$ per cell, and this was done in all the following experiments on PVM replication.

Replicate monolayers were washed twice with PBS and inoculated with virus. After adsorption for 3 hr at 37°C, the inoculum was removed, the cells were washed twice with PBS; modified Eagle's medium with the 10% calf serum was added, and the dishes were incubated at 37°C. At intervals, cells from two dishes were scraped from the plastic surface,

cells and supernatant medium were harvested, and BSA was added to a concentration of 0.5%. Samples were rapidly frozen and thawed three times, and centrifuged at 1500 *g* for 10 min at 4°C to sediment debris. The supernatant was stored at −60°C until assayed for infectivity and hemagglutinin.

The results of such an experiment are shown in Text-fig. 2. The maximum titer of infective PVM was obtained at 72 hr, and there was a decline after 96 hr. The peak titer in this experiment of 4×10^6 $TCID_{50}$ per ml represents a yield of approximately 4 $TCID_{50}$ per cell; however, this must be taken as a



TEXT-FIG. 1. Adsorption of PVM hemagglutinin to BHK21 cell monolayers at 37°C.

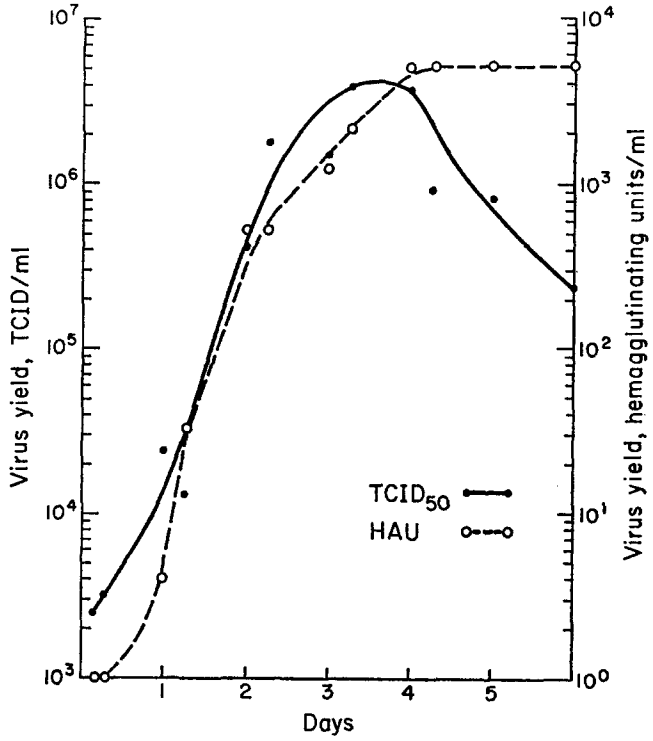
minimum yield because of the extreme thermal lability of the virus. Text-fig. 2 also shows that the yield of PVM hemagglutinin continued to rise after the titer of infective virus had begun to decline, reflecting the previously known fact that hemagglutinating activity is a much more stable property than infectivity.

Effect of Halogenated Deoxyuridines on PVM Replication.—To investigate the type of nucleic acid in the PVM virion, the effect of 5-fluoro-2′-deoxyuridine (FUDR), 5-bromo-2′-deoxyuridine (BUDR), and 5-iodo-2′-deoxyuridine (IUDR) on the replication of the virus was determined. Each of these compounds inhibits the synthesis of DNA viruses (30–33).

Replicate monolayers of BHK21 cells were inoculated with PVM at a multiplicity of 0.5 $TCID_{50}$ per cell, and the yield of virus after incubation for 4 days at 37°C with each of the

halogenated deoxyuridines present in the medium was determined. Vaccinia, a DNA virus, was included as a control in each experiment.

As shown in Table II, there was no significant inhibition of the replication of either infective virus or hemagglutinin by any of the compounds, suggesting that PVM is an RNA virus.



TEXT-FIG. 2. Growth of PVM in BHK21 cells at 37°C. Monolayers were inoculated at a multiplicity of 0.5 $TCID_{50}$ per cell.

Cytopathic Changes in PVM-Infected Cells.—In BHK21 cells inoculated at a multiplicity of 0.5 $TCID_{50}$ per ml, cytopathic effects consisting of scattered rounding of cells followed by detachment from the surface, began to appear around 48 hr after infection. Although rounded cells and areas devoid of cells were apparent after this time, much of the cell monolayer was still intact after 96 hr. At about 48 hr after infection, eosinophilic cytoplasmic inclusions were detected in hematoxylin- and eosin-stained preparations, and these inclusions became large and numerous by 72–96 hr (Fig. 1).

Acridine orange stains double-stranded DNA and RNA orthochromatically green, and single-stranded RNA and DNA metachromatically flame red under

the staining conditions employed in the present experiments (34–37). PVM-infected BHK21 cells stained with acridine orange 48 hr or more after infection showed accumulations of intensely stained, bright red material in the cytoplasm, which in size, shape, and distribution corresponded to eosinophilic cytoplasmic inclusions described above. There was no evidence of virus-induced nucleic acid in the nuclei of infected cells.

Fluorescent antibody–stained infected BHK21 cells showed viral antigen localized in the cytoplasm. Antigen was first detected at 24 hr; clusters of cells containing antigen were surrounded by cells without specific fluorescence, reflecting the multiple cycles of infection under the conditions employed. Maximum staining was seen at 72 hr (Fig. 2). No evidence of viral antigen in the nuclei was detected.

TABLE II

Lack of Effect of Halogenated Deoxyuridines on the Multiplication of PVM in BHK21 Cells

Compound	Virus yield*	
	Infective virus, $TCID_{50}$/ml	Hemagglutinating units/ml
None	5.6×10^6	182
BUDR, 10^{-4} M	4.5×10^6	182
FUDR, 10^{-5} M	2.9×10^6	128
IUDR, 10^{-5} M	3.9×10^6	182

In parallel experiments each compound caused >96% inhibition of vaccinia virus multiplication.

* Harvested at 4 days. Means of two experiments.

The results of these studies with acridine orange, fluorescein-labeled antibody, and halogenated deoxyuridines, suggest that PVM is an RNA virus that replicates in the cytoplasm; and that the RNA is a single-stranded molecule.

Properties of PVM Grown in BHK21 Cells

Hemagglutination by PVM Grown in BHK21 Cells.—To investigate whether PVM grown in cell culture is combined with a tissue component which inhibits hemagglutination, as is the case with PVM in mouse lung suspension, the effect of heat on virus grown in cells and in the mouse lung was determined. Both released virus, found in the supernatant medium of cultured BHK21 cells, and cell-associated virus, obtained after harvesting the cells and freezing and thawing three times, were employed.

As shown in Table III, the hemagglutinating activity of PVM grown in BHK21 cells did not increase after heating, in contrast to that of virus from the mouse lung. That PVM produced by BHK21 cells can combine with a

component of mouse lung was demonstrated by mixing the virus with mouse lung homogenate and assaying for hemagglutinin (Table IV). These results indicate that PVM grown in cell culture is not combined with a cellular inhibitor that prevents hemagglutination, and thus is similar to the free virus Curnen and Horsfall obtained by carefully perfusing the infected mouse lung (11, 38).

Incubation of a 0.8% suspension of murine erythrocytes with 8 units of neuraminidase did not prevent agglutination by 8 hemagglutinating units of PVM grown in BHK21 cells, whereas 1 unit of enzyme prevented agglutination

TABLE III

Effect of Heat on Hemagglutination by PVM Grown in Mouse Lung and in BHK21 Cells

Source of virus	Hemagglutination titer	
	Unheated	70°C, 30 min
Mouse lung	<8	512
BHK21 cells, released virus*	1024	512
BHK21 cells, cell-associated virus‡	512	512

* In supernatant medium.

‡ Obtained after freezing and thawing cells 3 times.

TABLE IV

Inhibition by Mouse Lung Homogenate of Hemagglutination by PVM Grown in BHK21 Cells

Treatment of virus*	Hemagglutination titer	
	Unheated	70°C, 30 min
PVM + medium	512	256
PVM + mouse lung homogenate	<4	256

* Virus was mixed with equal volumes of reinforced Eagle's medium with 0.5% BSA or a 10% (w/v) suspension of mouse lung homogenate, held at 37°C for 30 min, centrifuged at 1500 *g*, and the supernatant titered.

of such erythrocytes by 8 units of the Lee strain of influenza B virus. This suggests that a neuraminic acid–containing receptor is not required for virus adsorption; however, the possibility cannot be excluded that the virus adsorbs to a receptor that contains neuraminic acid residues in linkages that are either insusceptible or inaccessible to the neuraminidase used in these experiments.

Antigenic Similarity of PVM Grown in BHK21 Cells and the Mouse Lung.— In early experiments it was found that a 1:2048 dilution of antiserum against PVM grown in the mouse lung neutralized 3000 $TCID_{50}$ of virus which had undergone 7 passages in BHK21 cells. To investigate whether serial passage in cell culture had resulted in any demonstrable antigenic changes in the virus,

PVM from the 10th passage in BHK21 cells and from the mouse lung were compared in cross hemagglutination-inhibition titrations with their corresponding rabbit antisera. As shown in Table V, no significant antigenic differences were detected.

Virulence in Mice of PVM Grown in BHK21 Cells.—The ability of PVM, which had undergone 10 passages in cell culture, to cause pulmonary consolida-

TABLE V

Antigenic Similarity of PVM Grown in Mouse Lung and BHK21 Cells

Rabbit antiserum against virus grown in	Hemagglutination-inhibition titer*	
	Virus grown in	
	Mouse lung	BHK21 cells
Mouse	3620	3620
BHK21 cells	5120	7240

* Geometric means of two experiments.

TABLE VI

Comparison of the Infectivity in Cell Culture and in Mice of PVM Grown in Mouse Lung and in BHK21 Cells

Source of virus	Infectivity titer*		
	In BHK21 cells	In mice	
	$TCID_{50}$	MS_{50}	LD_{50}
Mouse lung	6.5×10^5	6.4×10^5	1.8×10^5
BHK21 cells‡	6.3×10^6	1.5×10^4	5.0×10^3

* Per gram of mouse lung or per milliliter of virus-containing medium.

‡ 10th passage virus.

tion and death in mice was investigated by comparing virus grown in BHK21 cells with virus from the mouse lung with respect to LD_{50} and MS_{50} titers in mice, and infectivity titers ($TCID_{50}$) in BHK21 cells. As shown in Table VI, the $TCID_{50}$ and MS_{50} titers of virus grown in the mouse lung were similar, indicating that BHK21 cells are as sensitive an indicator of PVM from the mouse lung as is the mouse itself. On the other hand, the virus grown in BHK21 cells had an MS_{50} titer over 100-fold lower than the $TCID_{50}$ titer, and also a lower LD_{50} titer. Thus, after 10 passages in cell culture, the virus appears to be less virulent, though still capable of inducing pulmonary disease and death if given in sufficient amounts.

Attempts to Concentrate and Purify PVM.—

In attempts to obtain concentrated virus suspensions for single cycle multiplication experiments and for study of the properties of the virion, PVM was subjected to centrifugation in various gravitational fields and to ammonium sulfate precipitation. Results of representative experiments are shown in Table VII.

In repeated experiments, centrifugation resulted in very poor recovery of infectivity. Initial experiments were done at 79,000 *g* because of the previously reported size of 40 mμ (11); however, after it was found by electron microscopy that the virus particle is 80–120 mμ in diameter (15), sedimentation at 20,000 *g* was also carried out without successful recovery of infectivity. Ammonium sulfate precipitation also failed to give a satisfactory recovery of infective virus.

TABLE VII

Loss of Infectivity and Increase in Hemagglutinating Activity of PVM after Centrifugation or Ammonium Sulfate Precipitation

Procedure	PVM recovered	
	Infective virus	Hemagglutinating units
	%	%
20,000 *g*, 180 min	13	210
64,000 *g*, 180 min	26	820
79,000 *g*, 180 min	4	228
Ammonium sulfate	7	430

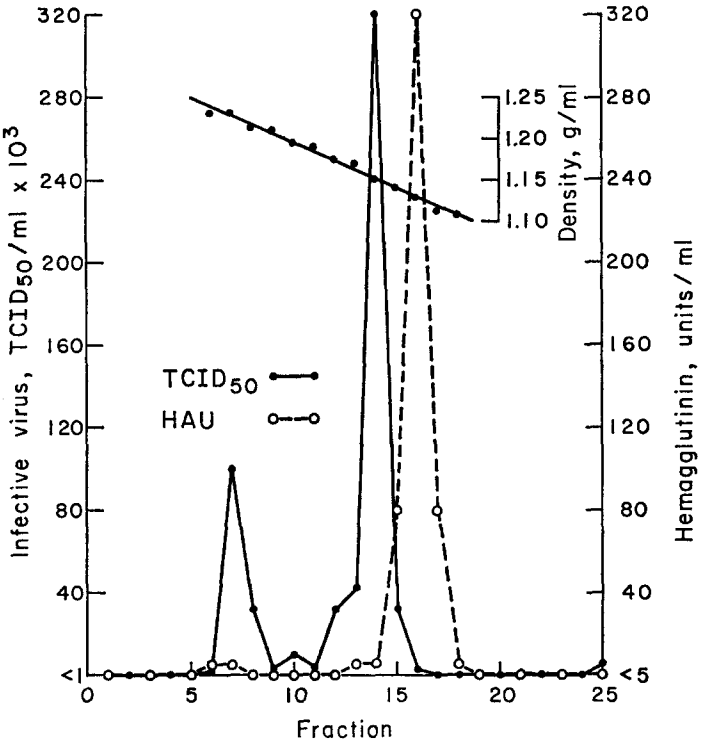
Although recovery of infectivity was very low, greater than 100% recovery of hemagglutinin was consistently obtained, a result which suggests that the labile virus particles may have fragmented during the procedure, resulting in a loss of infectivity but an increase in the number of particles capable of hemagglutination. A loss of infectivity accompanied by an increase in hemagglutination titer is known to occur after disruption of influenza virus particles by ether (39–41).

Density Gradient Centrifugation.—PVM was subjected to equilibrium zonal centrifugation in potassium tartrate in an attempt to purify the virus and determine its buoyant density.

A virus suspension was used which had an infectivity titer of 3.2×10^6 $TCID_{50}$ per ml, a hemagglutination titer of 1024, and a $TCID_{50}$/HA ratio of $10^{3.5}$. Virus, 0.4 ml, was layered onto a preformed continuous 5–50% (w/w) gradient of potassium tartrate in 0.1 M phosphate buffer, pH 7.0, containing 0.001 M EDTA, and centrifuged at 100,000 *g* for 3 hr in a Spinco SW39L rotor. Three drop fractions were collected from below and assayed for infectivity and hemagglutinin.

Text-fig. 3 shows the results of a typical experiment in which approximately 50% of the infectivity added to the gradient was recovered. Infective virus was

found in two peaks, with the major portion in a single fraction with a density of approximately 1.15, and a smaller amount at a density of approximately 1.23. The hemagglutination titers of the fractions which contained the two peaks of infectivity were low, i.e. 5 units per ml. The $TCID_{50}$/HA ratio of the lighter, major peak of infectivity was $10^{4.8}$, and that of the heavier, smaller peak $10^{4.3}$. Although *most* of the infectivity was in the lighter peak, there was



TEXT-FIG. 3. Equilibrium zonal centrifugation of PVM in potassium tartrate. Virus suspension was placed on a preformed gradient and centrifuged at 100,000 *g* for 3 hr at 4°C.

no visible band in the gradient at that point; however, the relatively small amount of infective virus in the heavier peak was associated with a distinct band of flaky opalescent material, suggesting that the lighter fraction with a density of 1.15 contained infective virus sedimenting as such, while the heavier band consisted of virus adsorbed to or combined with, some cellular component which was responsible for the visible material.

In contrast to the distribution of infective virus, most of the hemagglutinating activity was found in a sharp peak at a density of 1.13, and there was very little associated infectivity. The $TCID_{50}$ per HA ratio of the fraction with peak

hemagglutinating activity was only 10^1. Thus, most of the hemagglutinating acitivity was associated with noninfective particles, which probably represented fragments of the envelope of this labile virus, or virus particles which had lost their nucleoprotein internal component. As shown in the accompanying paper (15), PVM is structurally similar to the myxoviruses, and hemagglutination by noninfective fragments of these viruses is well established.

DISCUSSION

Although PVM has been studied extensively in the mouse, a suitable cell culture was not previously available, and thus knowledge of the nature of the virus and its multiplication has been limited. The ability to propagate PVM in BHK21 cells has provided the means of studying the virus at the cellular level, and significant information has been gained regarding the physical and chemical properties of the virus, and its site of replication and mode of assembly. The availability of a cell culture system should also facilitate an investigation of the mechanism of the inhibition of PVM multiplication by bacterial polysaccharides.

The lack of effect of halogenated deoxyuridines on PVM replication indicates that it is an RNA virus, and the metachromatic, red staining with acridine orange suggests that the viral RNA is single-stranded. Both the cytochemical and immunofluorescence studies have produced evidence that the virus replicates in the cytoplasm, a conclusion which is also supported by the electron microscopic studies reported in the following communication (15). A comparison of the hematoxylin-eosin– and acridine orange–stained preparations of PVM-infected cells suggests that the eosinophilic cytoplasmic inclusions present in such cells contain RNA; and electron microscopic observations are compatible with the conclusion that these inclusions represent collections of viral internal component or nucleocapsid (15). Although all the available data indicate that PVM replicates only in the cytoplasm, the possibility cannot be completely excluded that a transient nuclear phase in the synthesis of some viral component or precursor occurs but has not been detected.

Curnen and coworkers (11) in studies of the sedimentation velocity of PVM hemagglutinin in sucrose solutions concluded that the hemagglutinating particle was 40 mμ in diameter and had a density of 1.13 g/ml. However, they were unable to assay the virus by infectivity after centrifugation, and emphasized that the 40 mμ size was a minimum estimate which represented the *smallest* hemagglutinating particles present in appreciable quantity. Our electron microscope studies (15) indicate that PVM particles, many of which are long filaments, are 80–120 mμ in diameter and possess an envelope covered with projections. The envelope is acquired at the cell surface where the virus particle matures by a process of budding from the cell membrane. Thus PVM is structurally similar to the myxoviruses. It is easily disrupted by centrifugation or precipitation, which results in a loss of infectivity and an increase in hemagglu-

tinating activity. It is known that disruption of myxoviruses yields fragments which hemagglutinate (39–41), and fragmented or disrupted PVM particles provide an explanation for the 40 mμ hemagglutinin described by Curnen and coworkers (11). This interpretation is supported by the present finding that infective virus has a density in potassium tartrate of 1.15, whereas noninfective hemagglutinin has a density of 1.13, which is identical to that reported for the 40 mμ hemagglutinin by the above authors.

The separation in a gradient of lighter, noninfective hemagglutinin from infective virus appears to reflect extreme fragility of PVM. The thermal lability of this virus was demonstrated very early (2, 6) and its fragile nature has been manifest in the present studies in the inability to concentrate it by usual means, and in the disruption of unfixed virus during negative staining for electron microscopy (15). The finding that stock virus preparations had an average $TCID_{50}$/HA ratio of $10^{3.7}$, whereas infective virus, separated from noninfective hemagglutinin by gradient centrifugation, had a ratio of $10^{4.8}$, indicates that there is much noninfective hemagglutinin in virus stocks.

SUMMARY

Pneumonia virus of mice (PVM) has been serially propagated in a line of baby hamster kidney (BHK21) cells. A maximum titer of 6.3×10^6 $TCID_{50}$ per ml was obtained, and there was little variation in yield on serial passage.

PVM grown in BHK21 cells was antigenically similar to virus obtained from the mouse lung, but was somewhat less virulent for the mouse after 10 serial passages in these cells. Virus produced by BHK21 cells agglutinated mouse erythrocytes without prior heating or other treatment.

Sedimentation of PVM in the ultracentrifuge or precipitation by ammonium sulfate resulted in a loss in infectivity but an increase in hemagglutinating activity, presumably due to disruption of the virus particle. In a potassium tartrate density gradient, the major portion of infective virus sedimented at a density of approximately 1.15, and noninfective hemagglutinin, at a density of approximately 1.13. Stock virus preparations appear to contain a large amount of noninfective hemagglutinin.

The replication of PVM was not inhibited by 5-fluoro-2′-deoxyuridine, 5-bromo-2′-deoxyuridine, or 5-iodo-2′-deoxyuridine. Infected cells contained eosinophilic cytoplasmic inclusions which showed the acridine orange staining characteristic of single-stranded RNA. Foci of viral antigen were observed in the cytoplasm of infected cells by fluorescent antibody staining.

The results suggest that PVM is an RNA virus that replicates in the cytoplasm.

The authors wish to thank Mrs. Karen Funk and Mr. Salvatore Spatola for excellent technical assistance.

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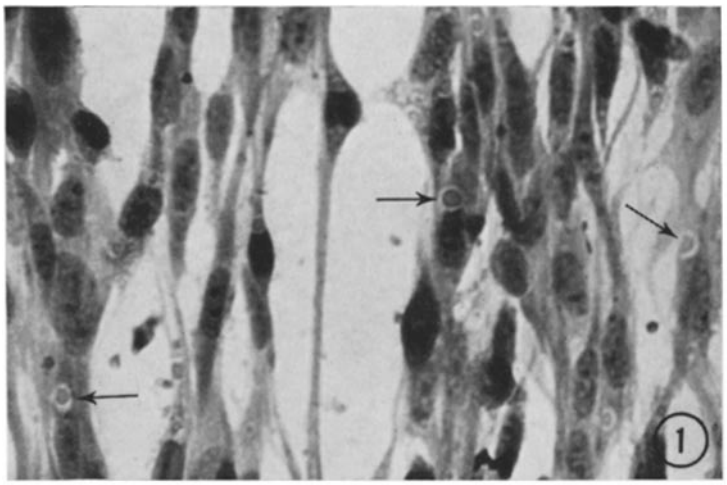
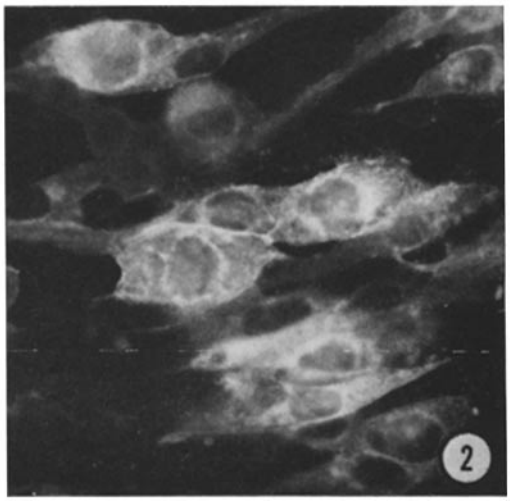
EXPLANATION OF PLATE 19

FIG. 1. Eosinophilic intracytoplasmic inclusions (arrows) in BHK21 cells 72 hr after infection with PVM at a multiplicity of 0.5 $TCID_{50}$ per cell. Hematoxylin and eosin stain. × 460.

FIG. 2. PVM antigen in the cytoplasm of BHK21 cells 72 hr after infection with PVM at a multiplicity of 0.5 $TCID_{50}$ per cell. Stained by the indirect immunofluorescence technique. × 580.





(Harter and Choppin: Replication of PVM in cell culture)