Characterization Studies of the Pichinde Virus—a Member of the Arenavirus Group[1] (35330)

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Chronic virus infection of the natural host is observed in a limited number of groups of viruses. The characterization of the infection of mice by lymphocytic choriomeningitis virus (LCM) has been extensive; however, detailed analysis of the LCM virus has proven difficult. Recently, a number of viruses which resemble LCM have been isolated, and members of this group of LCM-like viruses have been classified as the arenaviruses (1).[2] Trapido and Sanmartin (2) isolated one of these viruses from a large rodent, *Oryzomys albigularis*, in the Pichinde Valley of Columbia, South America (3, 4). The Pichinde virus produces a chronic inapparent infection in the natural host. This virus, unlike certain other members of the virus group, does not appear to produce illness in man and thus might serve as a model for analysis of the properties of the arenaviruses. The results of the present study indicate that Pichinde virus replicates well in tissue culture and is easily assayable. The size, ether lability, and characteristics of its response to inhibitors of nucleic acid synthesis were found to be similar to those described for LCM virus.

Material and Methods. Tissue cultures. Vero cells, a stable line of African green monkey kidney cells, were grown in medium 199 supplemented with 5% fetal bovine serum, 0.75 g/liter of sodium bicarbonate, and antibiotics (100 units of penicillin and 100 μg of streptomycin/ml). The concentration of sodium bicarbonate was increased to 1.5 g/liter for cells grown in petri dishes. Maintenance medium consisted of Eagle's medium supplemented with 2% fetal bovine serum, 1.5 g/liter of sodium bicarbonate and antibiotics. Primary rabbit kidney cells and BSC-1 cells (5) were grown as previously described (6) in Eagle's medium supplemented with 10% fetal bovine serum.

Viruses and assay. Pichinde virus, strain AN 3739, originally isolated from *O. albigularis* and passaged 12 times in baby hamster brain, was kindly supplied by Dr. Carlos Sanmartin, Cali, Colombia. Virus stocks were prepared in Vero cells and experiments were conducted using virus passed once or twice in Vero cells. The virus was assayed in Vero cells grown in petri dishes by the plaque counting method. The medium was removed from the petri dishes and 0.2 ml of inoculum was added to the cell monolayer. After adsorption, infected monolayers were overlaid with 4 ml of medium containing Eagle's medium supplemented with 10% fetal bovine serum, 2.25 g/liter of sodium bicarbonate, antibiotics (100 units of penicillin, 100 μg of streptomycin, and 50 units of Mycostatin/ml) and 1.5% Bacto-agar (Difco). After 3 days incubation at 37° in a 5% CO_2 in air atmosphere another 4 ml of medium, which contained 3 ml of 1:1000 neutral red/100 ml, was added and plaques were counted 24 hr later. In the latter part of this study, the addition of 0.5 ml of 1:1000 neutral red/plate instead of 4 ml of second overlay medium after 3 days incubation of the infected Vero cells was found to yield more distinct plaques, although no significant differences in the number or size of plaques was found

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[2] Arenavirus was approved as the group name by the International Committee on Nomenclature of Viruses (personal communication). Arenovirus was suppressed because it might be confused with adenovirus, particularly in oral communication.

between these two methods. The same method was used to assay the virus in BSC-1 cells and primary rabbit kidney cells. In addition, Pichinde virus was assayed by the hemadsorption negative plaque technique (6). Sindbis virus, strain AR 339, grown in chick embryo fibroblast cells, and type 1 herpesvirus KOS strain, grown in human embryonic kidney cells, were used in some experiments as control viruses. These viruses were assayed in Vero cells and primary rabbit kidney cells, respectively, by the plaque counting method.

Virus adsorption. Vero cell monolayers in petri dishes were inoculated with 0.2 ml of virus diluted in maintenance medium. The cultures were incubated at 37° in a 5% CO_2 atmosphere and at intervals three plates were washed twice with cold maintenance medium. After washing, the monolayers were covered with overlay medium and the adsorbed virus were enumerated by the formation of plaques.

Thermal inactivation. Stock virus was diluted in maintenance medium or Tris buffer containing 0.2% fetal bovine serum (pH 7.2) and incubated at different temperatures in a water bath. Samples were taken at intervals and assayed for surviving virus.

pH sensitivity. One-tenth ml of virus was diluted with 0.9 ml of Tris–HCl buffer containing 2% fetal bovine serum at pH 3.4 to 9.0. The virus was further diluted 10-fold with Tris–HCl buffer containing 2% fetal bovine serum at the same pH as the initial diluent. Each of these 1:100 suspensions of the virus was incubated for 2 hr at room temperature and then assayed for surviving virus.

Filtration of virus particles. Stock virus diluted 1:10 or 1:100 in Tris buffer containing 2% fetal bovine serum (pH 7.2) was serially passaged through Millipore membranes which had or had not been pretreated with 10% fetal bovine serum before filtration of virus (7); the filtrates were assayed for virus.

Density gradient centrifugation. Linear density gradients of sucrose and of cesium chloride (CsCl) were used. Sucrose (5 to 70%) was prepared in 0.01 *M* Tris buffer (pH 7.2) containing 0.001 *M* ethylenediaminetetraacetic acid (EDTA) (8). The sucrose gradients were prepared by layering 0.4 ml of 70% and 0.3 ml of 5 to 65% in increments of 5%. The gradients were held at 4° to allow for diffusion of the layers and 0.5 ml of virus sample was placed on the preformed gradient. Centrifugation was carried out for 2 hr at 36,000 rpm in a Spinco Model L2 centrifuge using an SW50 rotor. CsCl was dissolved in the Tris buffer–EDTA solution and the gradients were prepared by layering 0.8 ml of CsCl at densities 1.10, 1.20, 1.30, and 1.40 onto 1 ml of a cushion of CsCl at a density of 1.50. A 0.5-ml sample of the virus was added to the top of the gradient and centrifuged for 18 hr at 25,000 rpm. Fractions (20 drops each) were collected by piercing the bottom of the centrifuge tube; and the refractive index and infectivity of each fraction was measured.

Results. Formation of plaques. Pichinde virus formed plaques in Vero cells. If neutral red was included in the initial overlay, very small plaques appeared 2 days after infection, and the number of plaques increased until day 4. The plaque number did not increase significantly after 5 days, although the plaque size did continue to increase. The plaque size was always larger in the cultures which did not receive neutral red in the first overlay medium. Figure 1 shows the linear relationship between the number of plaques and the virus concentrations, indicating that one plaque was produced by one infectious

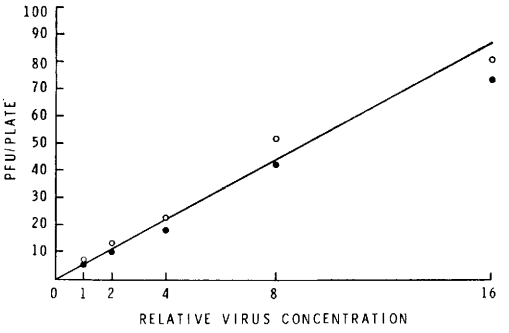

FIG. 1. Linear response of Pichinde virus plaque formation in Vero cells: Serial twofold dilutions of virus suspension were prepared in maintenance medium; 0.2 ml of each dilution were inoculated onto Vero plates and overlaid after adsorption and plaques were counted after 4 days of incubation.

particle of virus.

BSC-1 cells were found to produce virus plaques under agar overlay medium and also by the hemadsorption negative plaque method. However, titers of infectious virus obtained in assays in BSC-1 cells using agar overlay was almost 10-fold less than simultaneous assays performed in Vero cells. By the hemadsorption negative plaque method, 100-fold less infectious virus was found. Primary rabbit kidney cells infected and overlayed with agar were also found to produce plaques after 4-days incubation; however, the sensitivity to the virus was again almost 100-fold less than that of Vero cells.

Replication of Pichinde virus in Vero cells. The replication of Pichinde virus in Vero cells was examined by infecting cell monolayers in culture tubes with virus at an input multiplicity of 20. After 1-hr adsorption, the monolayers were washed 3 times with maintenance medium and incubated at 37° with 1 ml of maintenance medium. Extracellular virus and cell-associated virus from triplicate tubes were harvested at intervals and assayed for infectivity. After an eclipse period of about 8 hr, the virus began to replicate rapidly and maximum titers were reached in cell-associated virus at 24 hr. Maximum titers of extracellular virus were observed at 36 hr after infection (Fig. 2).

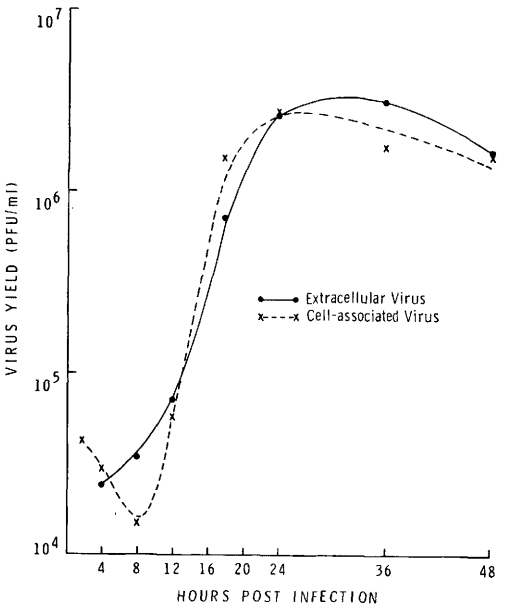

FIG. 2. Replication of Pichinde virus in Vero cells: Vero cell monolayers in culture tubes were infected with Pichinde virus at an input multiplicity of 20. After 1-hr adsorption of virus, the monolayers were washed 3 times with maintenance medium and incubated at 37° with 1 ml of maintenance medium. At intervals, extracellular virus and cell-associated virus from triplicate tubes were harvested and assayed for infectivity.

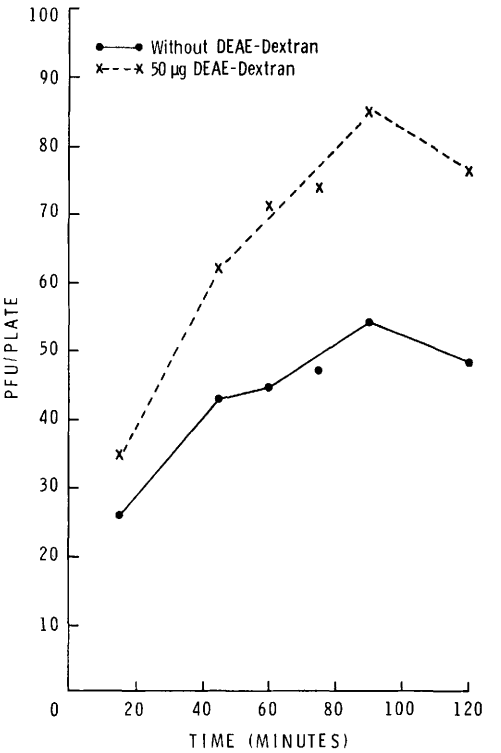

FIG. 3. Adsorption of Pichinde virus: Vero cell monolayers were inoculated with 0.2 ml of virus suspension and incubated at 37°. To measure the effect of DEAE-dextran during adsorption, 50 µg/ml were added to virus just prior to inoculation. At intervals triplicate samples were washed twice with cold maintenance medium and overlayed with agar medium.

Adsorption of Pichinde virus and effect of DEAE-dextran on virus plaquing efficiency. Adsorption of virus was compared in the cultures with and without 50 µg/ml of DEAE-dextran. As shown in Fig. 3, adsorption of the virus proceeded until 90 min after inoculation in both cultures, followed by a slight decrease in the number of plaques obtained with longer periods of incubation. The

amount of virus absorbed throughout the time course was apparently higher in DEAE-dextran treated cultures than in nontreated cultures. In other experiments, the effect of DEAE-dextran on virus plaquing efficiency was compared among the cultures: (a) pretreated with different concentrations of the compound for 1 hr before infection, (b) cultures treated with the compound during virus adsorption only, and (c) cultures overlaid with agar medium containing the compound. Pretreatment of cells with DEAE-dextran before infection resulted in a slight increase in the number of plaques only at a concentration of 10 μg/ml, while higher concentrations caused a gradual decrease in plaquing efficiency (Fig. 4). The treatment of cells with DEAE-dextran during virus adsorption only resulted in marked increases in the number of plaques between the concentrations of 10 to 100 μg/ml. No significant increase in the number of plaques was observed with the addition of DEAE-dextran to the agar overlay medium.

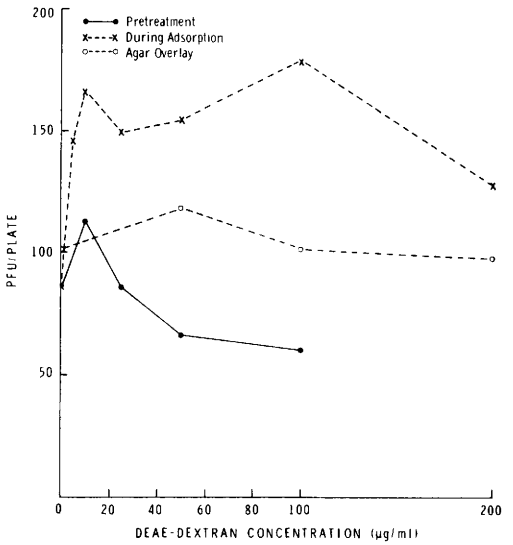

FIG. 4. Effect of DEAE-dextran on Pichinde virus plaquing efficiency: (●) The cells were pretreated with maintenance medium containing DEAE-dextran for 60 min. The cells were then washed and infected. After 90-min adsorption the monolayers were washed twice to remove unadsorbed virus and overlayed. (x) The effect of DEAE-dextran during adsorption was measured by adding virus suspensions to DEAE-dextran to yield the concentration of 5, 10, 25, 50, and 100 μg/ml. Cell monolayers were then infected and methods following adsorption previously described were followed. (○) DEAE-dextran was added to the agar overlay medium to yield final concentrations of 50, 100, 200, and 500 μg/ml.

TABLE I. Thermal Inactivation of Pichinde Virus.

Temp (°)	Time of incubation (hr)	Virus titer (PFU/ml)	Recovered (%)
4[a]	0	2.8×10^4	—
	24	2.6×10^4	95
	48	2.1×10^4	75
	72	1.6×10^4	58
25[a]	0	2.8×10^4	—
	2	2.3×10^4	82
	4	2.0×10^4	73
	8	2.0×10^4	73
	(min)		
37[b]	0	1.9×10^4	—
	30	1.9×10^4	100
	60	1.6×10^4	85
	90	1.3×10^4	68
	120	1.1×10^4	58
	180	3.4×10^3	18
	360	3.0×10^2	2
56[a]	0	1.3×10^4	—
	10	6.0×10^2	3
	20	2.5×10^2	1
	30	3.5×10^1	0.1
	60	0	0

[a] Virus diluted in maintenance medium was incubated at 4, 25, or 56°; and samples were taken at the times indicated.

[b] Virus was diluted 10-fold in Tris buffer with final serum concentration of 0.2% and incubated at 37°.

Stability of Pichinde virus. Ether sensitivity, thermal stability, and pH stability of the virus were examined. Incubation of the virus for 18 hr at 4° with equal volumes of ethyl ether resulted in a complete loss of infectivity, indicating that the virus had a lipid-containing envelope.

Under the conditions of the experiments, Pichinde virus seemed to be relatively stable (Table I). Over 50% of infectivity was recovered after 3 days at 4°, 8 hr at 25°, and 2 hr at 37°. Virus infectivity was rapidly lost at 56°.

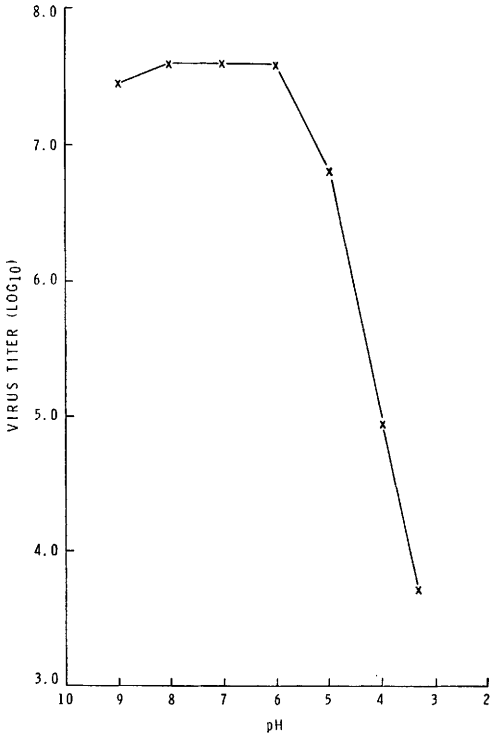

FIG. 5. Effect of pH on the infectivity of Pichinde virus: Virus suspension was diluted in Tris–HCl buffer solution (pH 3.4–9.0), containing 2% fetal bovine serum. The diluted virus was incubated at room temperature for 2 hr, then the pH was adjusted to 7.0 in cold maintenance medium and virus titer was determined.

The effect of pH on virus infectivity is shown in Fig. 5. The virus was very stable in the pH range 6.0–9.0; however, holding the virus at pH 3.4 for 2 hr at room temperature resulted in more than a 1000-fold decrease in infectivity.

Effects of inhibitors on the replication of Pichinde virus in Vero cells. To determine the nucleic acid content of the virus, the effect of inhibitors of nucleic acid synthesis on the replication of virus was tested. All inhibitors were added to the cultures at the end of a 1-hr adsorption period. The effects of the inhibitors in the replication of the virus are shown in Table II. In the presence of the DNA inhibitors 5-iododeoxyuridine (IudR) and cytosine arabinoside (ara-C) the replication of Pichinde virus was not affected, whereas the replication of type 1 herpesvirus, a DNA virus, was inhibited by more than 99% by both IudR and ara-C. The addition of 4 and 1 μg/ml of actinomycin D to the cultures infected with Pichinde virus resulted in more than 99 and 80% inhibition of virus replication, respectively. The concentration of 0.4 μg/ml of actinomycin D did not effect the replication of virus. Toxic effect caused by the addition of actinomycin D was not observed in the cultures during this time. The replication of Sindbis virus, used as a control of a resistant RNA virus, was not inhibited with 4 μg/ml of actinomycin D. The drug 6-azauridine, which is known to inhibit RNA synthesis (9), did inhibit Pichinde virus replication more than 90% between the concentrations of 1 and 5 μg/ml.

Size of Pichinde virus. Estimates of the size of Pichinde virus were obtained by filtering the virus through various size Millipore membranes (7). The virus readily passed 450-mμ sized membranes and some infectivity was recovered after passing the virus through 100-mμ pore size membranes (Table III). No infectivity passed through membranes of 50-mμ pore size.

Buoyant density of Pichinde virus. Pichinde virus was found to be stable in both sucrose and CsCl. Recovery of input from sucrose gradients approached 100% and the recovery from CsCl gradients was about 75%. As shown in Fig. 6, the peak of infectivity in a sucrose density gradient corresponded to a density of 1.18 g/cm^3. More than 50% of the virus was recovered in the peak fraction. Similar results were obtained by CsCl density gradient centrifugation, except that the density of the peak of infectivity was slightly higher (1.19–1.20 g/cm^3).

Discussion. Pichinde virus was found to replicate well in Vero cells and 10^8 PFU/ml of virus infectivity was obtained. The virus can be readily assayed in Vero cells by plaque formation after 4-days incubation. It can also be assayed in BSC-1 cells and primary rabbit kidney cells; however, Vero cells, which are defective in their ability to make interferon (10), were more sensitive to the virus and the most distinct plaques were produced in these cells. Other members of the Tacaribe complex of viruses, such as Ma-

TABLE II. Effects of Nucleic Acid Inhibitors on the Replication of Pichinde Virus in Vero Cells.[a]

Inhibitor	Conc (μg/ml)	Pichinde virus (PFU/ml)	Pichinde virus Inhibition (%)	Herpesvirus (PFU/ml)	Herpesvirus Inhibition (%)	Sindbis virus (PFU/ml)	Sindbis virus Inhibition (%)
IudR	0	7.5×10^6	—	5.6×10^7	—	NT[d]	
	75[b]	7.5×10^6	0	NT		NT	
	25	7.5×10^6	0	NT		NT	
	10	7.5×10^6	0	1.1×10^4	>99	NT	
ara-C	0	8.4×10^6	—	5.6×10^7	—	NT	
	10	8.6×10^6	0	2.5×10^2	>99	NT	
	1	1.2×10^7	0	NT		NT	
Actinomycin D[c]	0	7.5×10^6	—	NT		2.2×10^6	—
	4	5.0×10^4	>99	NT		4.1×10^6	0
	1	1.2×10^6	84	NT		5.5×10^6	0
	0.4	7.5×10^6	0	NT		NT	
6-Azauridine	0	1.9×10^5	—	NT		NT	
	5	1.7×10^3	>99	NT		NT	
	2.5	1.6×10^3	>99	NT		NT	
	1	2.1×10^4	98	NT		NT	

[a] Vero cell monolayers in test tubes were infected with virus at an input multiplicity of 20. Following 1-hr adsorption of virus, the monolayers were washed and 1 ml of maintenance medium containing the inhibitor was added. At 24 hr postinfection, triplicate tubes from each sample were harvested and assayed for total virus after 3 cycles of freezing and thawing.

[b] Sample was harvested at 36 hr after infection.

[c] Virus was carefully assayed under the conditions designed to eliminate photoinactivation by actinomycin D.

[d] NT = not tested.

chupo, Junin, and Amapari, have also been found to replicate in Vero cells and to be assayable by plaque formation after 5 days or more incubation (11–13). A cell line of human lymphoblastoid cells was reported to be more beneficial in obtaining high titers of virus infectivity for Machupo virus (11).

TABLE III. Filtration of Pichinde Virus Through Millipore Membranes.

Filter size ($m\mu$)	Titer of virus, Expt.: 1[a]	2	3
Unfiltered	1.9×10^{4}[b]	1.8×10^4	4.4×10^5
450	—	—	2.6×10^5
450	1.0×10^4	1.2×10^4	1.6×10^5
220	4.5×10^3	3.4×10^3	8.5×10^3
100	3.3×10^2	2.0×10^2	1.6×10^2
50	$<10^1$	$<10^1$	$<10^2$

[a] Expt. 1. Stock virus diluted 10^{-1} in Tris buffer and serially passed through untreated membranes. Expt. 2. Stock virus diluted 10^{-1} in Tris buffer containing 2% fetal bovine serum and filtered serially through membranes pretreated with 10% fetal bovine serum. Expt. 3. Virus stock diluted 10^{-2} in Tris buffer containing 2% fetal bovine serum and filtered as in Expt. 2.

[b] Plaque forming units/ml.

The replication of Pichinde virus in Vero cells was rather slow. A latent period of approximately 8 hr was observed and maximum yields of virus were not obtained until 26 to 36 hr postinfection. These growth characteristics were similar to that of other arenaviruses, LCM (14) and Junin (15), although a difference in growth rates among LCM virus strains has been noticed (14).

The size of Pichinde virus was estimated by filtration through Millipore membranes; and the results suggested a minimum particle size of between 50 and 100 mμ. Electron microscopic studies have revealed that LCM virus particles are pleomorphic, varying from about 50 mμ to more than 200 mμ (16). Machupo virus, another member of the arenavirus group (1), was found to be mor-

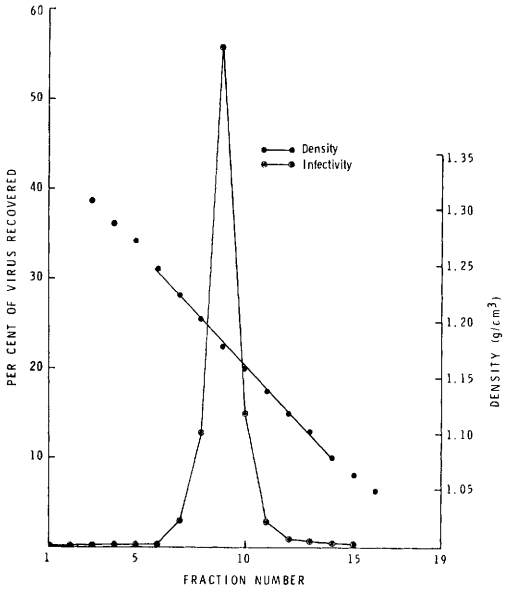

FIG. 6. Buoyant density of Pichinde virus in sucrose density gradient. One half ml of Pichinde virus was centrifuged in linear sucrose density gradient (5 to 70%) prepared in 10^{-2} *M* Tris containing 10^{-3} *M* EDTA. Centrifugation was done in an SW 50 rotor for 2 hours at 36,000 rpm at 4°C. Fractions (20 drops each) were collected by piercing the bottom of the centrifuge tube. The density of each fraction was calculated from the refractive index and the virus infectivity was quantified by the formation of plaque in Vero cells.

phologically similar to LCM and a mean diameter of 110 mμ was reported (11).

Pichinde virus appears to be relatively stable against the effects of temperature, multiple cycles of freezing and thawing (unpublished data), and the sucrose and CsCl gradients. Although a small difference in thermal stability can be seen among LCM virus (14, 17), Machupo virus (18), and Pichinde virus, this might be due to different experimental conditions, especially the different protein contents in the virus diluent and virus concentration. Pichinde virus is similar to Machupo (19) and LCM virus in its lipid-solvent sensitivity.

The effects of nucleic acid inhibitors on the replication of virus are of interest. The fact that the DNA inhibitors, IudR and ara-C, had no effect on virus replication seems to suggest that DNA synthesis is not necessary for the production of virus and that the nucleic acid of the virus is probably RNA. In addition, the virus incorporates uridine but not thymidine (unpublished data). Actinomycin D suppressed the virus replication by more than 80% at a concentration of 1 μg/ml, although 0.4 μg/ml of actinomycin D did not inhibit virus replication. These results are very similar to the effects of inhibitors on LCM virus replication (20, 21). Since Junin virus replication has been reported not to be affected with 1 μg/ml of actinomycin D (15), it cannot be assumed at present that all members of the arenavirus group have a similar response to actinomycin D. The effect of actinomycin D on virus replication in other members of the arenavirus group will have to be studied.

The stability of Pichinde virus in sucrose and CsCl density gradients allowed determination of the density of the virus. Densities of 1.18 g/cm^3 in sucrose gradients and 1.19 to 1.20 g/cm^3 in CsCl were repeatedly obtained. The lability of LCM virus in certain gradient materials and the strain differences in stability of this virus (14, 22) has hampered the biophysical characterizations of the virus. A density of 1.17 g/cm^3 in potassium tartrate gradients has been reported (14). In addition to the common biological, serological, and morphological characteristics, which have recently been demonstrated in this new taxonomic group of viruses (1, 4, 11, 23), we can add that arenaviruses have a density of approximately 1.18 g/cm^3.

Summary. Pichinde virus, a virus capable of producing a chronic infection, was found to replicate in Vero cells and to form plaques in these cells. The virus had a latent period of approximately 8 hr and maximum titers were reached by 24 to 36 hr after infection. Adsorption of the virus was enhanced in the presence of 10 to 100 μg/ml of DEAE-dextran. Pichinde virus was found to be relatively thermostable, stable between pH 5.5 to 9.0 and sensitive to ethyl ether. Virus replication was not effected by the DNA inhibitors, IudR and ara-C; however, virus replication was inhibited by actinomycin D at concentrations of 1 and 4 μg/ml. The virus was stable in sucrose and cesium chloride and densities of 1.18 and 1.19 to 1.20 g/cm^3,

respectively, were obtained in gradients made of these two substances.

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