in mice during the past 10 years, none of these 3 viruses have been recovered from these sources.

Satisfactory results with the N test have been obtained with Lukuni virus only, and it has not been possible to carry out serosurveys with Bushbush and Ieri viruses. Limited studies with Lukuni virus have demonstrated the presence of neutralizing antibody to this virus in the serum of Trinidadians. Many undiagnosed human fever cases occur in Trinidad and 1 or more of these 3 viruses may be responsible for some of the cases. The medical or other importance of the 3 viruses remains to be determined.

Summary. Seven virus strains, all isolated from forest mosquitoes collected in eastern Trinidad between 1955 and 1959, have been shown to represent 3 unrelated new viruses, designated Bushbush, Ieri and Lukuni. Neutralizing antibody to Lukuni virus has been demonstrated in human serum. No relationship has been found between either Bushbush or Ieri virus and recognized arboviruses, but Lukuni virus has been found to be related to Anopheles A virus in complement-fixation and neutralization tests. Some of the biological properties of the 3 viruses are described.

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A Plaque Method for Titration of Frog Viruses Using Starch Gel Overlay.* (32010)

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In the course of studies on the Lucké renal adenocarcinoma of the leopard frog (*Rana pipiens*) 3 laboratories have reported isolation of viruses from frog tissues. The development of plaquing methods using solid overlay permits the accurate quantitative study of these new agents.

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TABLE I. Poikilothermic Cell Lines.

Species	Organ of origin	Designation
Fathead minnow *(Pimephales promelas)*	tail	FHM(4)
Rainbow trout *(Salmo gairdneri)*	gonad	RTG(5)
Bull frog *(Rana catesbiana)*	tongue	FT(6)
Box turtle *(Terrapene carolina)*	heart	TH-1(*)
Side-necked turtle *(Podocnemius unifilis)*	heart heart	PH-1(*) PH-2(*)
Gecko *(Gekko gecko)*	heart lung	GH-1(*) GL-1(*)

* Clark, unpublished.

Granoff *et al*(1,2) isolated 14 viruses from *Rana pipiens*, FV-1 to FV-3 and FV-8 to FV-18. They were isolated from spontaneously appearing plaques which formed in primary cell cultures of normal frog kidneys, from homogenates of frog renal tumors, and from normal frog kidney and liver homogenates inoculated into fathead minnow cell cultures. Rafferty(3) isolated 4 frog viruses, FV-4 to FV-7, from the pooled urines of tumor-bearing frogs and from frog tumor extracts. Clark (unpublished) has isolated 5 viruses, LT-1 to LT-4, from the red eft stage of newts *(Triturus viridescens)* which had been inoculated with cell suspensions from Lucké frog tumors, and L-4 from a Lucké tumor cell suspension inoculated into a turtle cell line (TH-1). The frog viruses of Granoff and of Clark have been shown to grow readily in a variety of poikilothermic and homothermic cell cultures(1,2, Clark unpublished). Rafferty's viruses have a markedly restricted host range and have been grown with some difficulty(3).

In this paper a plaque assay system will be described for 5 frog viruses. The efficacy of methylcellulose, starch gel, and agar as overlay materials will be compared in a variety of poikilothermic cell cultures.

Materials and methods. Viruses. Five viruses were studied: the LT-1, LT-2, LT-3 and L-4 viruses isolated in this laboratory, and FV-1 virus supplied by Dr. Granoff. All virus stocks were maintained and prepared in the turtle cell line, TH-1, and stored at —70°C in a mechanical freezer.

Cell cultures. Continuously propagated cell lines from fish, amphibians, and reptiles were used. Table I shows the origin of the 8 cell lines studied. The fathead minnow cells were purchased from Baltimore Biological Laboratories. The rainbow trout gonad cells were purchased from Microbiological Associates. The frog tongue cell culture was kindly supplied by Dr. Wolf. The 5 reptilian cell lines were originated in this laboratory. The 2 fish cell lines were grown and maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). All reptile cell lines were grown and maintained in Eagle's basal medium (BME) with Earle's basic salt solution and 10% FCS. Frog cells were grown in a hypotonic medium described by Wolf(6). The reptile cell lines were incubated at 30°C, except for TH-1 which was incubated at room temperature (approximately 23°C). Fish and amphibian cell lines were also incubated at room temperature. Monolayers for plaque and overlay studies were prepared in disposable plastic bottles (Falcon) with a surface area of approximately 25 cm^2.

Preparation of overlay materials. Noble agar was prepared by the method of Dulbecco and Vogt at a final concentration of 1%(7). Methylcellulose was prepared by the method of Rapp *et al*(8), as modified by Schulze and Schlesinger(9). Starch gel in a final concentration of 10% was prepared by a modification of the method of DeMaeyer and Schonne(10), using 10X rather than 5X concentrated media to permit the addition of an increased volume of FCS. Noble agar was also employed after treatment to remove sulfated polysaccharides (Righthand, V.F., personal communication). The agar was washed for 24 hours with 0.9 N sodium chloride and dried on a Buchner funnel. This extraction was repeated for a total of 3 times. The agar was then washed with 95% ethanol and acetone. Overlays were prepared using the nutrient media employed for growth. In the case of the frog medium, which could not be prepared at the 10 X salt concentration needed for starch gel overlay, MEM made hypotonic by diluting 4:5 with distilled water was substituted as suggested by Wolf (personal communication).

Cultures were incubated in the upright position, since methylcellulose was not firm enough to permit incubation in the inverted position. A 5 ml volume was used for both primary and secondary overlays.

Preparations were stained with neutral red added to a second overlay in an amount sufficient to attain a concentration of 1:50,000 in the total overlay.

Results. Evaluation of overlay materials. For each cell line, starch gel, methylcellulose, Noble agar and extracted agar were evaluated for their effect, as overlay materials, on cell survival and appearance. Starch gel was found to be the most satisfactory overlay material. Although cell survival was equally good using methylcellulose, the fact that it could not be pipetted easily and that it was not firm at room temperature made it less desirable than starch gel. Both extracted and non-extracted agar were found to be toxic for all cell lines except fathead minnow. The quality of the cultures was independent of the type of material used for the second overlay.

Susceptibility of poikilothermic cells to frog viruses. Five frog viruses were titrated in tubes of 8 poikilothermic cell lines. Each of the viruses tested was found to be cytopathic for each cell line. When compared to the other cell lines, the titers using TH-1 cells were from 10^1 to 10^3 TCD_{50} higher.

Plaque assays. Duplicate monolayer cultures of poikilothermic cell lines were inoculated with 0.5 ml of each virus dilution. After adsorption for one hour at room temperature, the inoculum was decanted and 5 ml of overlay was applied. A second staining overlay of 5 ml was added at 5 days. When LT-2 virus was studied, the second overlay was applied at 10 days.

All viruses formed plaques when grown in TH-1 cells, using starch gel overlay. The plaques were circular, with irregular margins, and were approximately 1 mm in diameter (Fig. 1). Microscopically, pale granular rounded-up cells mixed with a few dye-containing cells were visible within the plaques. The plaque titer of LT-1 virus in TH-1 cells under Noble agar was reduced by 20 to 46% when compared to starch gel overlay. When extracted agar was used, this titer reduction

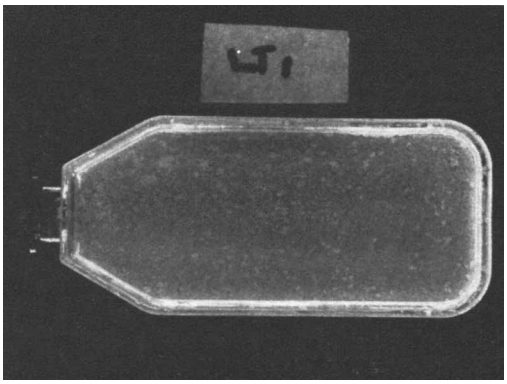

FIG. 1. LT-1 virus plaques in TH-1 cell culture under starch gel overlay.

was not evident. Starch gel was adopted as the overlay material to be used for further studies of plaque formation.

The timing of initial appearance and maximal titers of plaques and growth of plaque size were studied employing LT-1 virus and TH-1 cells. The monolayers were examined 24 hours after application of the staining overlay. Plaques could be detected microscopically (100 $\times$ magnification) in cultures stained on day 1. Plaques visible to the naked eye were first seen in cultures stained on day 3. The maximum plaque number was reached by day 5, and remained constant through day 20. The diameters of 20 plaques in one bottle were measured with a vernier caliper and the mean diameter calculated. The plaques stained on the third day of incubation had a mean diameter of 0.4 mm. Plaque size continued to increase through day 20, when the mean diameter was 1.7 mm. The plaque diameters increased at a somewhat slower rate in the presence of neutral red. Hence, the second overlay was routinely applied between the 5th and 7th days. When the majority of the plaques had attained a diameter of 1.0 mm, it became evident that there was a second population of small plaques, 0.5 mm or less, comprising approximately 10% of the population. This phenomenon was observed with LT-1 and FV-1 viruses in TH-1 and FHM cells.

Six replicate titrations of LT-1 virus using a 10^{-5} dilution of the same stock resulted in a mean plaque number of 43, with a standard deviation about the mean of 2.3.

TABLE II. Plaque Titrations of Frog Viruses in Poikilothermic Cell Lines.

Virus	Cell lines						
	FHM	RTG	FT	TH-1	PH-1	GH-1	GL-1
LT-1	$10^{6.9}$*	$10^{4.5}$	$10^{5.8}$	$10^{6.9}$	0	$10^{4.7}$	$10^{2.5}$
LT-2	nd	$10^{2.0}$	nd	$10^{6.6}$	nd	$10^{4.0}$	nd
LT-3	nd	nd	$10^{6.6}$	$10^{7.2}$	0	nd	nd
L-4	$10^{4.3}$	nd	$10^{6.5}$	$10^{7.1}$	nd	nd	nd
FV-1	$10^{6.0}$	$10^{2.3}$	$10^{6.1}$	$10^{6.3}$	0	$10^{5.4}$	nd

* Plaque forming units per 1.0 ml. nd = not done.

There was a linear relationship between the concentration of virus inoculated and the number of plaques formed. Virus producing typical cytopathic effect was recovered from Pasteur pipette aspirates of plaques in TH-1 cells, but not from cells in a plaque-free area in the same culture flask.

The plaque forming unit (pfu) titer of each virus in several cell lines, using starch gel overlay, is shown in Table II. TH-1 appeared to be the most sensitive cell line, paralleling results in fluid culture. The pfu titers obtained were very close to those expected from the TCD_{50} titers obtained in fluid culture. There were two exceptions. Despite the fact that all viruses showed cytopathic effect in PH-1 cells maintained in fluid culture, there were no plaques observed in this cell line under solid overlay. In addition, the pfu titers of LT-2 and FV-1 viruses in RTG cells were below those expected from the TCD_{50} values.

Except for LT-2, all of the viruses formed plaques in TH-1 cells at the same rate and of the same size as that described above for LT-1. The LT-2 plaques were smaller, attaining a diameter of 0.3 mm on day 10 and 0.4 on day 20.

Discussion. The use of starch gel as a solid overlay material provides a reliable plaque assay system which is well tolerated by fish, amphibian and reptilian cell lines, and does not appear to depress the plating efficiency of the system for frog viruses. TH-1 was the most sensitive cell line studied. The optimum plaque system for studying frog viruses utilized starch gel as the overlay material. The staining overlay was applied between 5 and 7 days to assure maximum plaque number and convenient plaque size. The superiority of starch gel over other solidifying agents was demonstrated by comparing cell quality when overlaid with various materials.

The 5 frog viruses studied showed cytopathic effect in all of the poikilothermic cell lines. The fact that the highest TCD_{50} and pfu titers were found with TH-1 cells may be a reflection of adaptation. In this laboratory, all virus stocks have been prepared only in TH-1 cells (LT-1 for 10 passages, FV-1 for 2 passages, and others for 4 passages). The frog viruses tested formed plaques in all of the cell lines with the exception of PH-1 cells. The frog viruses replicate in PH-1 cells maintained in fluid culture and produce typical cytopathic effect. Two workers have shown analogous situations to exist in homothermic systems. Farnham(11) described cytopathic effect and no plaque formation induced by herpes simplex in HeLa cells. This was ascribed to the fact that herpes simplex infected HeLa cells continue to incorporate neutral red. Rouse, Bonifas and Schlesinger(12) described the ability of adenovirus to cause a cytopathic effect and yet fail to produce plaques in several cell lines. They demonstrated that this effect was caused by a nutritional deficiency induced by PPLO contamination, and they were able to produce plaques by adding arginine to the overlay media or eliminating the PPLO infection. The factors preventing plaque formation by frog viruses in PH-1 cells have not been explained.

The reduction in plaque titer of LT-1 virus grown in TH-1 cells under agar overlay may be related to inhibition of the virus by sulfated polysaccharides present in Noble agar. DeMaeyer and Schonne(10) demonstrated inhibition of herpes simplex and vaccinia in rat embryo cells by agar when compared to starch gel. In a prior report of plaquing frog viruses, Granoff(1) described reduction of plaque titer by 30 to 70% when agar was compared to a fluid overlay system. Granoff described a fluid overlay plaque system, employing crystal violet stain after 4 days' incubation. In the present study, using starch gel overlay, 10% of the plaques were conspicuously small. If this variation in plaque size occurs in a fluid overlay system, it may be difficult to distinguish between secondary plaques and small plaques.

The plaque assay system described has

satisfied the following qualifications for validity: The plaques have been shown to be virus-associated by forming only in the presence of virus and by recovery of virus only from the plaque sites. Repeated plaque titrations with the same virus stock have given variation in plaque numbers of less than 15%. A linear relationship has been demonstrated between virus inoculum and plaque number. Specific immune sera have been shown to neutralize frog virus plaque formation (Lehane, unpublished).

Summary. A reliable plaque assay system has been developed for several recently isolated frog viruses. Cell lines from reptiles, amphibians, and fish were tested for their ability to be maintained under various solid overlay materials. Starch gel was found to be the most useful overlay material. The most sensitive assay system was shown to be *Terrapene* heart (TH-1) cell culture.

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Vitamin D-Induced Calcium Binding Factor in Rat Intestinal Mucosa.* (32011)

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It is now accepted that one of the chief functions of vitamin D is to enhance the intestinal absorption of calcium(1,2). However, the mechanism of action of vitamin D is still open to question, although several theories have been proposed(2). One of these is that the vitamin may stimulate the synthesis or operation of a carrier, possibly a protein, which facilitates the movement of calcium across the intestinal mucosa. There is some evidence indicating that vitamin D may act in a synthetic process since it has been shown that actinomycin D, an inhibitor of protein synthesis, prevents vitamin D-induced hypercalcemia in rats(3) and the vitamin D-stimulated absorption of calcium in rats(4) and chicks(5). Puromycin also inhibits the vitamin D-stimulated absorption of calcium in rats(6). In addition, Norman(7) has recently reported an increase in the synthesis of RNA, presumably mRNA, in chick intestinal mucosa after vitamin D_3 administration. However, Harrison & Harrison(8), on the basis of their studies, suggested that actinomycin D acts on the calcium active transport system *per se* and not specifically at the vitamin D-stimulated site.

Wasserman and Taylor have provided evidence suggesting that there is a substance present in the supernatant fraction of intestinal mucosal homogenates from vitamin D-treated rachitic chicks that depresses the uptake of Ca^{45} by the homogenate debris(9,

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