drome seems warranted. It is undoubtedly enzootic in certain mouse colonies but neither the portal of entry nor mode of transmission are known. A minute particle capable of reproducing the entire organism appears to be the infective component.

The natural disease has been detected with certainty only in mice of the originally inbred Swiss Webster (W) strain. The so-called Bagg mice used by Morris *et al.*(1) were obtained from the Flora O'Grady colony and, as verified by her, are Swiss W. We also recovered the causal agent from the spleens of Swiss W mice secured from the dealer who supplied the originally infected animals. It was later detected in ascitic fluid which appeared in Swiss W mice of another subline, used elsewhere, in studies on murine leukemia. There is no evidence, however, that the agent is enzootic in our random-bred Swiss colony. Whatever the natural distribution of the organism may ultimately prove to be, its unsuspected presence in mice under experimentation may lead to false conclusions.

*Summary.* Transmissible ascites of natural occurrence in Swiss W mice is described. It closely resembles an enzootic murine disease reported by Morris, McCown, and Blount in 1956. Evidence that the causal agent is a microsporidian is presented. Ascitic fluids examined by phase microscopy show small intracellular organisms which on pressure release a filament with an attached globoid structure. An infective component of the organism may remain viable for at least a year in recovered mice.

The helpful counsel of Dr. William Trager is gratefully acknowledged.

1. Morris, J., McCown, J. M., Blount, R. E., *J. Inf. Dis.*, 1956, v98, 306.

2. Wenyon, C. M., *Protozoology*, New York, N. Y., 1926, v1, 734.

3. West, A. F., Jr., *J. Parasit.*, 1960, v46, 747.

4. Levaditi, C., Nicolau, S., Schoen, R., *Ann. L'Inst. Pasteur*, 1924, v38, 651.

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## A Plaque Assay for Myxoma Virus Infectivity.* (27318)

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A cytopathic effect has been observed in plasma clot(1) and monolayer(2) cultures of rabbit kidney epithelial cells infected with myxoma virus. This phenomenon should lend itself readily to the development of a plaque assay and thus offer the possibility of a more quantitative approach to any studies of the myxoma virus-host cell relationship than is presently employed. The conditions suitable for such an assay on monolayer cultures of cells derived from rabbit kidney are described in this report.

*Materials and methods.* The South American Sanarelli strain of myxoma virus, obtained from Dr. Ralph B. Houlihan, Cutter Laboratories, was used. Virus pools were stored in glass sealed ampules at –60°C as a 10% rabbit dermal tumor suspension in Eagle's medium(3) containing 10% calf and 10% lamb serum. They were clarified before storage by low speed centrifugation and by filtration through .01 Selas bacteriologic filters.

The tissue culture systems used were an established line of suckling rabbit kidney cells (SRK) obtained from Dr. D. G. McKercher and primary rabbit kidney cells (RK) grown from trypsinized fragments of kidney tissue freshly excised from young adult animals. Both types of cultures were grown in 10 ml of medium as monolayers in 4-oz prescription bottles and consisted of mixtures of epithelial and fibroblastic cells. Growth medium for SRK cultures was composed of Eagle's medium supplemented with 2 μg/ml of hydrocortisone, 42 μg/ml arginine, 10% calf serum

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and 10% lamb serum. RK cultures prepared essentially by the trypsinization method described by Bodian(4) for monkey kidney tissue, were grown in medium 199(5) containing 20% calf serum. There was no apparent difference in the microscopic appearance or sensitivity to infection by myxoma virus between the two types of cultures.

The plaque assay for myxoma virus infectivity was carried out as follows: Confluent monolayer cultures of either SRK or RK cells in prescription bottles were aspirated to remove growth medium and washed twice with 10 ml of phosphate buffered saline (PBS), pH 7.4(6). Cultures were then inoculated with 0.4-ml volumes of appropriate dilutions of virus suspension in Hanks' solution, rocked to disperse the small inoculum evenly over the cell sheet and incubated at 37°C for 2 hours to permit virus adsorption. Inoculated cultures were then overlaid with 10 ml of growth medium containing 0.6% Noble agar and incubated at 37°C for 7 days. After the development of plaques, the nutrient agar overlay was gently slipped off the cell sheets which were washed once with 10 ml of PBS. They were then fixed and stained for 20 minutes with a dilute solution of carbol fuchsin in methanol(7), washed briefly with tap water and allowed to drain and dry. Plaques, or foci of infection, were readily counted at 7 to 15× magnification under a stereoscopic microscope.

*Results.* Foci of infection first appeared in monolayer cultures of SRK and RK cells at 4 days. They ranged in diameter between 0.5 and 2 mm and consisted of slightly enlarged, densely staining cells contrasting sharply with uninfected background cells. At 7 days affected cells began to degenerate and pull away from the normal cell sheet (Fig. 1). At 12 days degenerate cells usually sloughed off when agar overlay was removed leaving a clear, cell-free plaque. For routine assays infected monolayer cultures were fixed and stained on the 7th day after inoculation.

The rate of adsorption of myxoma virus to RK monolayer cultures was determined at 37°C. Cultures washed twice with PBS were inoculated with a dilution of myxoma virus pool estimated to contain 15 to 20 plaque forming units (PFU) and incubated at 37°C.

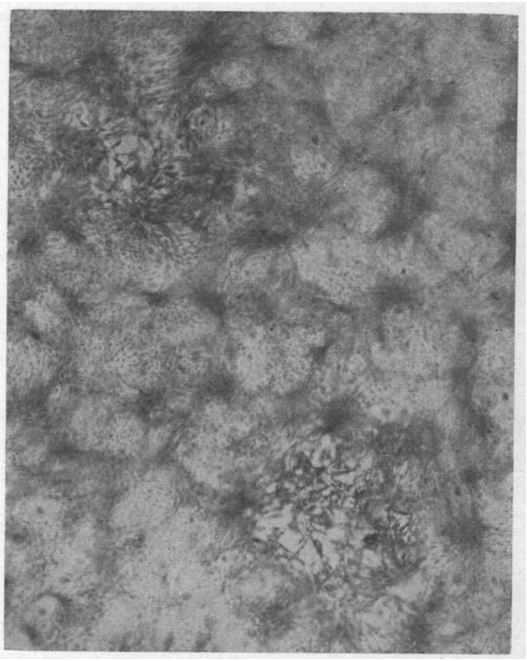
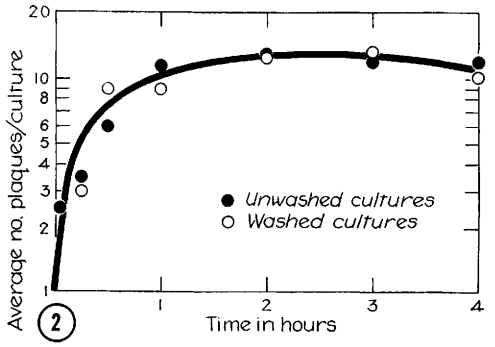
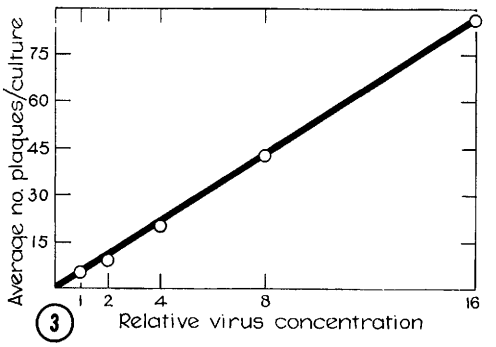


FIG. 1. Two plaques produced by myxoma virus in primary rabbit kidney cell monolayers. Magnification: ×14.

At time intervals up to 4 hours after inoculation, groups of 4 cultures were removed from the incubator. Two were immediately overlaid with nutrient agar while the remaining 2 were aspirated to remove residual inoculum and washed twice with PBS before overlaying with agar. Maximum adsorption occurred at 2 hours as shown in Fig. 2. Since washed cultures exhibited the same apparent rate of adsorption of myxoma virus as the unwashed cultures, it would appear that any unadsorbed virus suspended in the 0.6% agar overlay failed to reach the cell sheet and produce recognizable lesions at 7 days after inoculation.

The proportionality between plaque count and virus concentration was tested over a range of 5 serial 2-fold dilutions. Six replicate platings were made at each dilution and the average number of plaques per dilution was plotted as illustrated in Fig. 3. The curve is linear with a slope close to 1 suggesting that each plaque is initiated by a single virus particle. Further evidence for a one plaque-one virus particle relationship was obtained from replicate platings of myxoma virus samples on both SRK and RK cultures



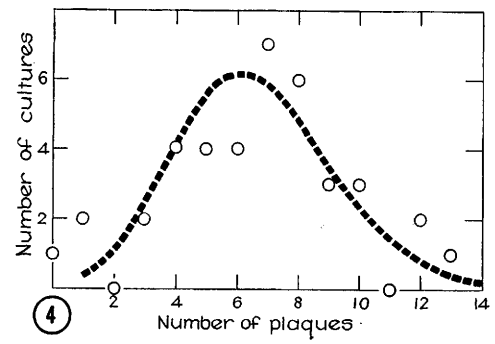




FIG. 2. Adsorption of myxoma virus to monolayer cultures of RK cells at 37°C.

FIG. 3. Relationship between plaque counts and relative myxoma virus concentration.

FIG. 4. Frequency distribution of plaques in SRK cell monolayer cultures. The broken line represents the calculated Poisson curve for independently distributed infectious particles in suspension. Open circles are experimental points.

to determine the frequency distribution of PFU in a series of inocula of equal volumes. In a representative experiment 39 SRK bottle cultures were inoculated with equal amounts of an appropriately high dilution of myxoma virus suspension. The distribution of PFU was determined experimentally for comparison with that predicted by the Poisson law of small numbers as calculated from the mean value obtained. The theoretical plot and the experimentally determined frequency data are illustrated in Fig. 4. The mean PFU per culture was 6.64. A $\chi^2$ test of the discrepancy between the observed and theoretical distribution gave a $\chi^2$ value of 6.46 which has a probability of about 0.6 indicating a satisfactory goodness of fit. The usual interpretation of such data is that the number of plaques represents the number of independently distributed infectious particles in suspension(8).

To test the specificity of plaque formation by the myxoma virus pools used, a neutralization test was carried out using convalescent serum from a brush rabbit (*Sylvilagus bachmanii*) experimentally infected with the California Searsville strain of myxoma virus (courtesy Dr. David Regnery, Stanford University). Serial 10-fold dilutions of convalescent and normal rabbit sera were mixed with equal volumes of virus suspension estimated to contain 30 PFU per inoculum and incubated for 1 hour at room temperature. The mixtures were then assayed for PFU as described above. The results (Table I) indicate that the convalescent serum possessed approximately 100 times the neutralizing potency of the normal serum although the latter also exhibited some non-specific inhibition of plaque formation at high concentration.

In the course of developing the plaque assay, parallel titrations of several pools of myxoma tumor virus were carried out simultaneously in rabbits and SRK or RK tissue culture for comparison of the relative sensitivities of the 2 assay systems. The assay in rabbits entailed intradermal inoculations of

TABLE I. Neutralization of Myxoma Virus* (Sanarelli Strain) by Myxoma Virus (Searsville Strain) Immune Serum.

| Serum | dilution in inoculum | Avg PFU/culture† |
|---|---|---|
| Immune | $10^{-1}$ | .3 |
| | $10^{-2}$ | 2 |
| | $10^{-3}$ | 23 |
| Normal | $5 \times 10^{-1}$ | 2 |
| | $10^{-1}$ | 26 |

* Virus inoculum was estimated to contain approximately 30 PFU.

† Avg No. of plaque forming units of 2 or 3 cultures/serum dilution.

TABLE II. Comparison of Myxoma Virus Infectivity Titers by Plaque and Rabbit Intradermal Inoculation Assays.

| Virus pool | Tissue culture assay system | Infectivity, units/ml PFU* | RID† | PFU/RID |
|---|---|---|---|---|
| 1 | RK | $10 \times 10^6$ | $6.3 \times 10^6$ | 1.6 |
| | | $7.5 \times 10^6$ | $20 \times 10^6$ | .4 |
| | | $9.6 \times 10^6$ | $6.3 \times 10^6$ | 1.5 |
| 2 | SRK | $1.1 \times 10^6$ | $.6 \times 10^6$ | 1.8 |
| | | $1.1 \times 10^6$ | $2.0 \times 10^6$ | .6 |
| 3 | RK | $12 \times 10^6$ | $6.3 \times 10^6$ | 1.9 |
| | SRK | $10 \times 10^6$ | $20 \times 10^6$ | .5 |
| 4 | SRK | $1.1 \times 10^6$ | $2.0 \times 10^6$ | .6 |

* PFU = plaque forming units. See text.
† RID = rabbit infective doses. See text.

0.5-ml volumes of serial half-log dilutions. A range of 6 to 8 dilutions estimated to encompass the endpoint was inoculated per animal. One animal was used for each assay. The highest dilution yielding a tumor was considered the endpoint and the titer was expressed as the number of rabbit infective doses (RID) per ml. RID were calculated as the reciprocal of the product of the endpoint dilution and inoculum volume. The results of 8 comparisons show that the 2 methods are of approximately equal sensitivity. The variability in PFU/RID ratios reflects the greater variability of the animal assay (Table II).

*Discussion.* The observation that myxoma virus produces a cytopathic effect upon rabbit kidney epithelial cells(1.2) has led to the development of a plaque assay using SRK or RK monolayer cultures. That this assay does indeed represent the infectivity of single physical particles is attested to by the finding of a linear dose-response relationship and a random distribution of such particles in suspension in accordance with the theoretical Poisson distribution. This does not exclude the possibility that these infectious units represent aggregates of infectious virus particles. The results show, however, that, if such aggregates exist, they do not dissociate upon dilution.

Maximum adsorption of myxoma virus to RK cells at 37°C occurred within 2 hours. It was found unnecessary to wash away residual inoculum since both washed and unwashed infected cultures yielded the same number of plaques after overlaying with nutrient agar. The 0.6% agar overlay apparently served as an efficient barrier to the diffusion of any embedded viable virus particles to susceptible cells. Thermal inactivation is an unlikely explanation for this observation since an inactivation rate determination at 37°C has revealed a fairly high stability with an infectivity half-life of approximately 2 days (unpublished).

Easy identification of plaques was not possible until the 4th day after inoculation despite the finding, to be reported later, that virus production begins at 10 to 12 hours and reaches a maximum intracellular concentration at approximately 24 hours. Possible explanations for the delay in plaque development may be a slow release and spread of virus from cell to cell under agar or a lag period of several days between intracellular virus production and cell degeneration.

The specificity of plaque formation by the Sanarelli strain of myxoma virus was established by neutralization test using an immune serum obtained from a convalescent brush rabbit (*Sylvilagus bachmanii*) experimentally infected with the California Searsville strain. The sensitivity of the method was found to be equal to that of the rabbit assay and its precision greater. The plaque assay should be applicable, therefore, to more meaningful quantitative studies of myxoma virus-host cell relationship or of virus purification and characterization.

*Summary.* A plaque method of assaying myxoma virus infectivity on SRK and RK monolayer cultures has been described. The validity of this method was established by the demonstration of a linear dose-response relationship and by the observation of an infective particle distribution in suspension which agrees with the theoretical Poisson distribution. The specificity of the assay was confirmed by neutralization test and the sensitivity found to be equal to that of the *in vivo* rabbit assay.

1. Chaproniere, D. M., *Virology,* 1956, v2, 599.
2. Kilham, L., *J. Nat. Canc. Inst.,* 1958, v20, 729.
3. Eagle, H., *Science,* 1959, v130, 432.
4. Bodian, D., *Virology,* 1956, v4, 575.
5. Morgan, J. F., Norton, H. J., Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

6. Dulbecco, R., Vogt, M. J., *J. Exp. Med.*, 1954, v99, 167.

7. Postlethwaite, R., *Virology*, 1960, v10, 466.

8. Luria, S. E., in *General Virology*, John Wiley & Sons, N. Y., 1953, 49.

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# Hypoglycemic Activity of Valeramide, 4-Dimethylamino-N-methyl-2, 2-Diphenyl-, Hydrochloride.* (27319)

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Since the discovery of insulin, numerous chemicals and plant and bacterial extracts have been tested for effectiveness in depressing blood sugar following oral administration. The scope of this search is readily apparent from two recent reviews(1,2). Little success was realized until the orally active hypoglycemic effect of sulfonylureas was rediscovered (3-5). More recently, phenethylbiguanide (DBI) was found to be orally effective in diabetics(6).

Although the sulfonylureas and DBI are effective in some diabetics, their use is primarily in the adult type and the search for compounds with more extensive activity or a different mechanism of action has continued. This report describes studies on the hypoglycemic action of valeramide, 4-dimethylamino-N-methyl-2,2-diphenyl-, hydrochloride (U-6420) (Fig. 1).

*Methods. Intact and adrenalectomized rats.* These methods, which have been described(8), consist of measuring blood sugars 2 hours after oral administration of the compound in CMC vehicle(9). In an experiment determining influence of U-6420 on hyperglycemic effect of epinephrine or hydrocortisone, male rats were adrenalectomized one week before use. They were maintained on standard laboratory chow and 1% sodium chloride drink, and were fasted overnight before use. Hydrocortisone or epinephrine was given subcutaneously 30 minutes after oral administration of the valeramide. Blood sugars were determined on the Autoanalyzer on blood obtained from the vena cava 2 hours after U-6420 administration.

*Eviscerate rats.* Male rats (Charles River) weighing 200 ± 5 g were eviscerated by the method of Russell(10) while under anesthesia induced by intraperitoneal injection of cyclopal sodium (32 mg/kg) and subcutaneously injected phenobarbital sodium (132 mg/kg). Following the operation, the animals were connected to a constant infusion pump *via* the jugular vein and received 40 mg glucose/100 g body weight/hour/1.25 ml saline. U-6420 was injected subcutaneously in 0.5 cc CMC at the time glucose infusion was started. Two hours after treatment blood was obtained from the vena cava and analyzed for glucose by the Autoanalyzer. During the experiment animals were kept in a constant temperature box maintained at 26.5 ± 0.5°C.

*Blood sugar of alloxan diabetic rats.* Alloxan diabetes was produced in fasted intact male rats (Sprague-Dawley) weighing 150-170 g by intravenous injection of 42 mg/kg alloxan monohydrate (Eastman) 2-3 months before use. They were housed in wire metabolism cages and cup fed 10 cc of liquid diet(11) twice a day.

Experimental design was a crossover in which half of the animals served as controls

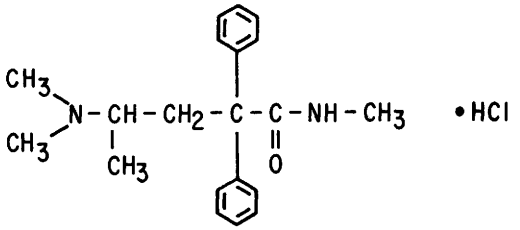


FIG. 1. Structure of valeramide, 4-dimethylamino-N-methyl-2,2-diphenyl-, hydrochloride (U-6420).

* This compound was synthesized and reported to possess oxytoxic and diuretic activity by Moffet *et al.*(7).