failed to improve the consumption of these hormones in the feed. The 4 groups of rats injected with Norlutin Enanthate declined similarly in body weight to those fed the other 4 compounds. Gains in body weight were observed in only 3 of the 22 groups of experimental animals.

Summary. The oral effectiveness of 4 progesterone-like compounds to stimulate mammary gland growth (DNA) of ovariectomized rats is reported. Norlutin and Norlutate plus E.B. were ineffective in stimulating increasing DNA in comparison with E.B. alone. Lutoral and Provera plus E.B. at the 1 mg level or above stimulated comparable mammary gland growth to that observed at end of pregnancy or after 1 μg E.B. + 3 mg P. The injection of 1 or 2 μg E.B. plus 3 or 6 mg Norlutin Enanthate was also effective in stimulating DNA values comparable to normal pregnancy. The losses in body weight of most groups during the 19-day feeding period were apparently due to the unpalatable nature of feed containing these compounds.

1. Griffith, D. R., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 448.

2. Moon, R. C., Griffith, D. R., Turner, C. W., *ibid.*, 1959, v101, 788.

Received March 14, 1963. P.S.E.B.M., 1963, v113.

Tissue Culture Cytopathic and Plaque Assays for Mouse Hepatitis Viruses. (28378)

JANET W. HARTLEY AND WALLACE P. ROWE (Introduced by R. J. Huebner)
National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Infectious Diseases, Bethesda, Md.

Although viruses of the mouse hepatitis (MHV) group have been propagated in a variety of tissue cultures(1-8), none of the systems described has been shown to be of broad applicability. In most instances the virus under study did not induce cytopathic changes or did so only after adaptation; the systems in which cytopathic changes occurred were not useful for more than one or two of the laboratory passaged strains, and were insensitive compared to animal inoculation. Manaker *et al.*(5) reported a cytopathic assay in a continuous cell line of C3H mouse liver, clone NCTC 1469(9), for 2 strains, the A-59(5) and MHV-2(10) viruses; however, they did not find this cell line suitable for assay of the MHV-1(11) and H747(12) strains. With slight modifications of Manaker's procedures, principally by substitution of chicken serum-containing maintenance media, we have found the NCTC 1469 line to be useful for assays of all strains of MHV studied, including the various prototype laboratory strains, freshly isolated field strains, and to a limited extent, virus in field specimens, and to be suitable for plaque assays of the great majority of the strains.

Materials and methods. Seed stocks of NCTC 1469 were received from Dr. Robert A. Manaker and Dr. Virginia J. Evans, Natl. Cancer Inst.; the 2 sublines reacted identically, and the latter was used for routine work.* Cell stocks were grown in 2 oz or 32 oz bottles, in a medium consisting of 20% horse serum in NCTC 109(13). Passages were made at 7 day intervals by vigorously shaking the cells into the fluid; passage by scraping cells off the wall yielded cultures which were resistant to infection.

Tube cultures were planted with 4×10^5 cells in 1 ml of the growth medium; 35 mm diameter plastic Petri dishes (Tissue Culture Dish, Falcon Plastics, Los Angeles) with 6×10^5 cells in 1.5 ml; and 60 mm dishes with 1.4×10^6 cells in 3.5 ml. Tube and bottle cultures were tightly stoppered, and Petri dish cultures were kept in a humidified

* The majority of cultures used were produced on contract by Microbiological Associates Inc., Bethesda, Md.

atmosphere with 5% CO_2. Cultures were used 4 or 5 days after planting. Maintenance medium for tube cultures was 5% chicken serum in NCTC 109. Other maintenance media, particularly those containing horse serum, gave marked variability in sensitivity of batches of cells, although the same lot of serum was used repeatedly; in contrast to chicken serum, the horse sera contained inhibitors for mouse hepatitis viruses.

For plaque assays, the cell sheet was washed once with NCTC 109 and once with phosphate buffered saline, pH 7.2. Virus was adsorbed for 45 min. at room temperature, in a volume of 0.2 ml for the 35 mm dishes, and 0.4 ml for the 60 mm dishes. Overlay medium, consisting of equal parts of 1.8% Noble agar and 10% chicken serum in 2 × concentrated NCTC 109, was then added without decantation of the virus; 2 ml and 5 ml of the overlay medium were used for the small and large dishes, respectively. After 2 days in the CO_2 incubator, the agar was covered with 2 ml of 1:20,000 neutral red solution, which was poured off after 2 hours. Plaques were counted on the 2nd and 3rd days after infection, using a dissecting microscope.

All sera used in tissue culture media were inactivated at 56°C for 30 min., and all media contained penicillin (250 U/ml) and streptomycin (250 μg/ml).

Results. Mouse tissue suspensions of all reported laboratory strains tested produced indistinguishable cytopathic effects (CPE) in the NCTC 1469 cell line; these included MHV-1, MHV-3(14), JHM(15), H747, MHV-S(16), as well as A-59. The characteristic early CPE resembled closely that described and illustrated by Manaker *et al.* (5), with predominantly giant cell formation. Two types of giant cells were produced, one about twice the diameter of the normal round cells, and the other 5-10 times the normal diameter. Some early clumping was often observed, but this was not specific. As CPE progressed, the large giant cells tended to disappear, and the remaining cells enlarged, clumped, and detached from the glass.

With low virus dilutions, distinctive CPE was evident within 18 hours, and limiting titration dilutions produced CPE beginning within 2 to 5 days. CPE progressed to complete destruction of the cell sheet in an additional 1 to 3 days. Infected cultures showed no definite difference in pH from control tubes.

Freshly isolated strains from feces of normal mice, tested after 3 to 6 suckling mouse passages, were somewhat erratic, producing CPE in some cell batches but not in others; cultures maintained in chicken serum medium were less variable in this respect than those in horse serum. In the latter medium there was also some difficulty in reproducing the characteristic CPE in continuous tissue culture passage, particularly with recently isolated field strains but also with MHV-1 and H747; this problem could usually be obviated by adding normal mouse brain suspension to the medium, and was not a major difficulty with cultures maintained in the chicken serum medium.

Virus isolations could be made on occasion directly from mouse feces using both tube and plaque procedures; 0.2 ml of a 1.5% suspension of pooled feces from colony "M" Swiss mice(16) produced an average of 180.5 plaques, and mixture with 0.1 ml of a 1:100 dilution of polyvalent MHV hyperimmune mouse serum completely prevented plaque formation.

There appears to be variation in tissue culture tropism between naturally occurring strains, since virus was repeatedly recovered in tissue culture from feces of colony M mice (16), but not from virus-containing fecal pools from other infected colonies, although blind passages were done and suckling mouse passaged viruses from these colonies could be recovered in tissue culture.

Tissue culture fluids were a highly satisfactory source of complement fixing (CF) antigen for all strains, with antigen titers of 1:8 to 1:32. Starr and Pollard(17) have reported production of CF antigen with MHV-1 in explant cultures of mouse kidney, but with lower titers than generally obtained in the present study.

All of the laboratory adapted prototype strains, either as mouse tissue suspensions

TABLE I. Relationship Between Dose of Virus and Number of Plaques.

Virus strain	Virus dilution (reciprocal)	Plaque count: No. plaques per dish	Plaque count: Avg	Log_{10} virus titer (PFU per 0.1 ml)
MHV-S	1×10^3	270, 262	266	5.42
(8th tissue	2×10^3	151, 134	142.5	5.45
culture	4×10^3	72, 71	71.5	5.45
passage)	8×10^3	36, 31	33.5	5.43
JHM	$.32 \times 10^2$	244, 189	216.5	3.84
(mouse brain)	1.0×10^2	59, 84	71.5	3.85
	3.2×10^2	27, 20	23.5	3.87

or tissue culture fluids, produced plaques within 2 to 3 days. Recent isolates generally required passage in tissue culture before giving distinct plaques. With well adapted strains the plaques were usually 1 mm in diameter after 48 hours, enlarging to 2 or 3 mm by the 3rd day although there was some variation with cell batch in the size of plaques produced; plaques produced by the A-59 strain were 3 to 5 mm across at 3 days. Mouse tissue suspensions and early tissue culture passages of recent isolates produced smaller plaques, averaging 0.5 to 1 mm. In recent experiments using Mixture 199 in place of NCTC 109 as the agar-overlay base, virus titers have been consistently 0.2 to 0.4 logs higher, and generally larger and more distinct plaques have been obtained. The dose-response relation was linear (Table I); the second titration in Table I illustrates the degree of variability occasionally encountered. Virus could be recovered from plaques but not from normal areas, and plaque neutralization tests gave inhibition by high dilutions of homotypic mouse antiserum. In Table II is shown the sensitivity of tissue culture and suckling mouse assays for titration of the MHV-S, MHV-1, JHM, and A-59 strains. There were some differences in sensitivity of the two assays with the different strains; passage in tissue culture tended to produce some attenuation of suckling mouse pathogenicity.

The agar-cell suspension plaque assay method described by Cooper(18) also gave satisfactory results but required a larger number of cells than the monolayer technique.

TABLE II. Comparative Titrations of Mouse Hepatitis Viruses in Tissue Culture (NCTC 1469) and Suckling Mice.

Strain	Virus preparation	Infectivity titer (log_{10}): Tissue culture PFU/0.1 ml	Tissue culture $TCID_{50}$/0.1 ml	Suckling mice* LD50/0.1 ml
MHV-S	Suckling mouse brain-liver 6th infant mouse passage	5.30		4.5
	Mouse liver tissue culture, 8th TC passage, after 8 infant mouse passages	5.36	5.2	3.4
MHV-1	Suckling mouse brain-liver, mouse passage line	3.22	3.8	4.5
	Mouse liver tissue culture, 5th TC passage of above	3.51	3.5	3.6
JHM	Weanling mouse brain, mouse passage line	3.86	$\geq$4.2	4.7
	Mouse liver tissue culture, 3rd TC passage of above	3.41	3.8	3.5
A59	Mouse liver tissue culture, 6th TC passage, after 29 TC and 1 infant mouse brain passage	5.63	5.5	3.9

* Titrations in 0-2 day old Swiss mice inoculated by combined intracerebral (0.02 ml) and intraperitoneal (0.03 ml) routes.

Discussion. Although animal assay procedures can be used for virus isolation, titration, and serologic studies of mouse hepatitis viruses, there are many inherent limitations: erratic titrations, high contagiousness, endemic infection of most stocks, and mortality among uninoculated infant mice from uninfected colonies when exposed to carriers (16). The tissue culture system described here has obviated most of these difficulties, in that neutralization tests and virus titrations can be performed with precision, and virus stocks can be plaque-purified and passaged without contamination. The chief limitations of the tissue culture system at present are the relative insensitivity for isolation of many field strains, fluctuation in sensitivity of different batches of cells, and occasional variation in susceptibility of replicate cultures. A potential danger is contamination of the cell line by pleuropneumonia-like organisms (PPLO), since Manaker observed a marked decrease in sensitivity for the A-59 strain of a subline of NCTC 1469 which was contaminated with PPLO(5). To date the sublines used here have remained free of these organisms.

It is of interest that the NCTC 1469 cell line is derived from a C3H mouse, an inbred strain which yields macrophage cultures which are genetically resistant to at least one strain of MHV(4).

Summary. Clone NCTC 1469 tissue culture line of C3H mouse liver, maintained in chicken serum-containing medium, was found to provide sensitive assay systems, based on production of plaques and cytopathic changes, for laboratory adapted and recently isolated strains of mouse hepatitis viruses, as well as for some naturally occurring strains in mouse feces.

Wendell E. Pugh, Worth I. Capps, and Mrs. Joan B. Austin provided valuable technical assistance. We are indebted to Drs. Alan W. Gledhill, Robert A. Manaker, and J. Anthony Morris, and Miss Joan B. Daniels for virus strains.

1. Gompels, A. E. H., Niven, J. S. F., *J. Path. and Bact.,* 1953, v66, 567.

2. Miyazaki, Y., Katsuta, H., Aoyama, Y., Kawai, K., Takaoka, T., *Japan J. Exp. Med.,* 1957, v27, 381.

3. Starr, T. J., Pollard, M., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 97.

4. Bang, F. B., Warwick, A., *Proc. Nat. Acad. Sci. U. S.,* 1960, v46, 1065.

5. Manaker, R. A., Piczak, C. V., Miller, A. A., Stanton, M. F., *J. Nat. Cancer Inst.,* 1961, v27, 29.

6. Mosley, J. W., PROC. SOC. EXP. BIOL. AND MED., 1961, v108, 524.

7. Vainio, T., *ibid.,* 1961, v107, 326.

8. Haff, R. F., *Virology,* 1962, v18, 507.

9. Hobbs, G. L., Sanford, K. K., Evans, V. J., Earle, W. R., *J. Nat. Cancer Inst.,* 1957, v18, 701.

10. Nelson, J. B., *J. Exp. Med.,* 1952, v96, 293.

11. Gledhill, A. W., Dick, G. W. A., Andrewes, C. H., *Lancet,* 1952, v2, 509.

12. Morris, J. A., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 875.

13. McQuilkin, W. T., Evans, V. J., Earle, W. R., *J. Nat. Cancer Inst.,* 1957, v19, 885.

14. Dick, G. W. A., Niven, J. S. F., Gledhill, A. W., *Brit. J. Exp. Path.,* 1956, v37, 90.

15. Cheever, F. S., Daniels, J. B., Pappenheimer, A. M., Bailey, O. T., *J. Exp. Med.,* 1949, v90, 181.

16. Rowe, W. P., Hartley, J. W., Capps, W. I., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 161.

17. Starr, T. J., Pollard, M., *Nature,* 1959, v183, 1690.

18. Cooper, P. D., *Virology,* 1955, v1, 397.

Received March 18, 1963. P.S.E.B.M., 1963, v113.