1. Calcagno, P. L., Rubin, M. L., Mukherji, P. K., Terplan, K. L., *A. M. A. J. Dis. Child.*, 1959, v98, 509.

2. ———, *Proc. of 11th Annual Conference on Nephrotic Syndrome,* 1959, p145.

3. Seegal, B. C., Hsu, K. C., Rothenberg, M. S., Chapeau, M. L., *Am. J. Path.*, 1962, v41, 183.

4. Hiramoto, R., Calcagno, P. L., Pressman, D., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 482.

5. Hammer, D. K., Vazquez, J. J., Dixon, F. J., *Lab. Invest.*, 1963, v12, 8.

6. Smadel, J. E., *J. Exp. Med.*, 1936, v64, 921.

7. Heymann, W., Lund, H. Z., *Pediatrics,* 1951, v7, 691.

8. Conway, E. J., *Micro-diffusion analysis and volumetric error,* D. Van Nostrand Co., Inc., New York, 1940.

9. Bloor, W. R., Pelkan, K. F., Allen, D. M., *J. Biol. Chem.*, 1922, v52, 191.

10. Hiller, A., McIntosh, J. F., Van Slyke, D. D., *J. Clin. Invest.*, 1927, v4, 235.

11. Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.*, 1949, v177, 751.

Received January 23, 1963. P.S.E.B.M., 1963, v114.

Characteristics of a Plaque Method for the Murine Salivary Gland Virus.* (28605)

DONALD HENSON† AND HENRY PINKERTON

Department of Pathology, Saint Louis University School of Medicine, St. Louis, Mo.

The murine salivary gland virus (MSGV) belongs to a group of unclassified mammalian viruses (cytomegaloviruses(1) characterized by unique cytopathic alterations(2), which may be harmlessly confined to the salivary glands, or widely and lethally distributed in other viscera. The murine(3), human(4) and cavian(5) strains have been serially propagated in tissue culture, but quantitative studies have not been reported. The objectives of this report are to describe a rapid, precise, quantitative method for studying MSGV, and to present certain data obtained by its use.

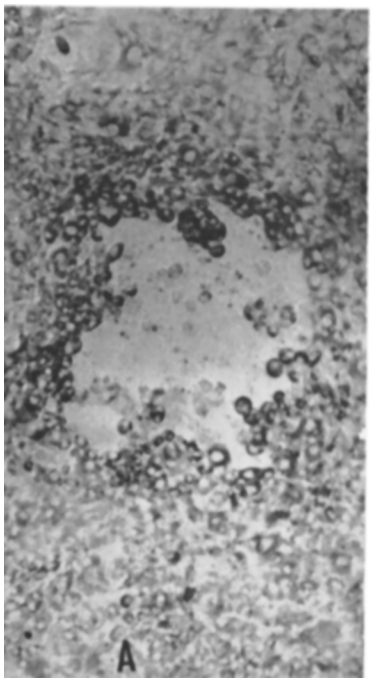
Materials and methods. Cell cultures. Monolayers of mouse embryonic cells, prepared by the method of Youngner(6) from NLW Swiss mice, were grown in 1-liter Blake culture bottles, using 50 ml of a growth medium (GM) composed of 90% Earle's balanced salt solution (BSS), Eagle's amino acid and vitamin mixture(7) in double strength (BME×2), 10% calf serum, phenol red, 100 u/ml penicillin, 100 μg/ml streptomycin and 25 u/ml Mycostatin. Cells were trypsinized and subcultured every 3 or 4 days for 4-8 weeks; then arbitrarily discarded and replaced by new lines. After 2 subcultures, the cells became uniformly fibroblastic in appearance. All cultures were incubated at 37°C without CO_2.

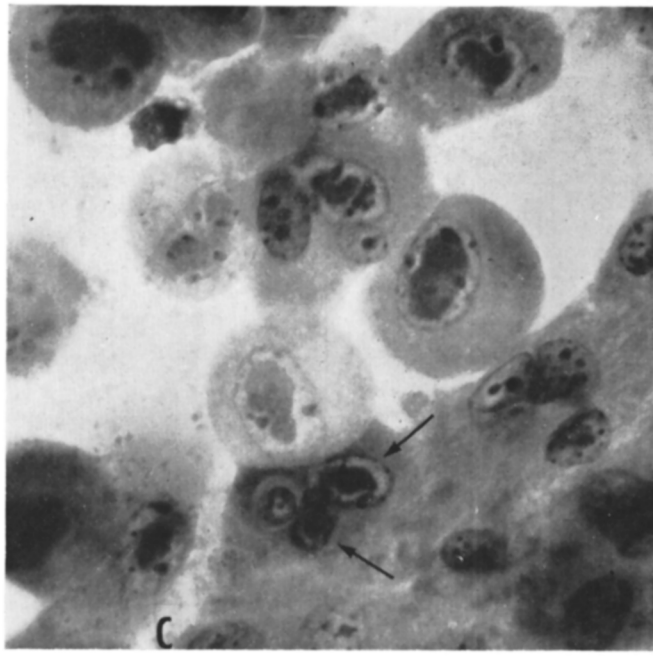
Virus cultivation. Latently infected mice were obtained through the courtesy of Dr. Margaret G. Smith. Using a procedure similar to that used by Smith(3), the above described monolayer cultures were readily infected, nearly all of the cells showing cytopathic effect (CPE) in 2-4 days, in primary as well as subsequent cultures. Serial passage of the virus was made every 3-5 days by transferring 10 ml of fluid from an infected to an uninfected bottle culture. After 38 such passages in 1-liter bottle cultures, always with rapid and generalized CPE because of the high input level, plaque studies were undertaken.

Procedure for plaque formation. Uninfected cells were obtained by trypsinizing densely populated bottle cultures, centrifuging at 500 RMP for 5 minutes, decanting the trypsin, and resuspending the cells in the GM described, with addition of 0.005 M Tris. Five ml of this medium, containing approximately 1.8×10^6 cells, was pipetted into each of a series of flat-bottom 40 mm Petri dishes. Confluent monolayers formed within 24 hours, at which time the medium was removed, and the cells washed with 2 ml of

* Supported by USPHS grant from Nat. Inst. of Allergy and Infect. Dis.

† Present address: Wistar Inst., Philadelphia, Pa.





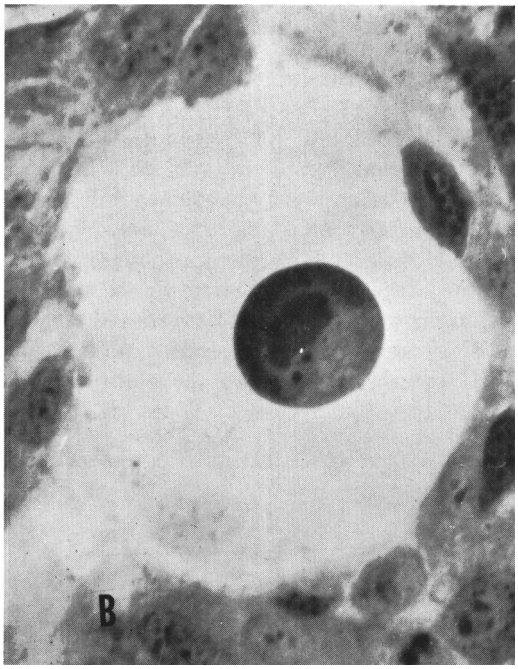


FIG. 1. A. Plaque, accented by neutral red, 6 days after seeding, ×35. B. Single cell plaque, 36 hr after seeding, H and E, ×850. C. Edge of mature 3 day plaque. Arrows indicate centrifugal spread, H and E, ×850.

phosphate-buffered saline (PBS) in preparation for virus inoculation.

Routinely, 1 ml of fluid from a heavily infected bottle culture was suitably diluted (Fig. 2) in PBS supplemented by 2% calf serum, and 0.2 ml from each dilution was pipetted onto the center of the monolayers, using 4 or 5 plates for each dilution. The plates were covered and allowed to stand at 27-30°C for 90 minutes. (Longer preliminary contact periods, up to 180 minutes, did not significantly increase the plaque counts). Five ml of "agar overlay medium" (0.9% agar, approximately 97% BSS containing BME×4, 2% calf serum, antibiotics, phenol red and 0.005 M Tris) was then added to each plate, without removing the unadsorbed virus.

Fixing and staining. For histological study of the plaques, neutral red was omitted, and 10% formalin was poured on top of the agar. After a fixation period of 16-24 hours, the agar was separated from the sides of the dish and peeled from the culture with the aid of a scalpel. The intact monolayer was stained with dilute hematoxylin and eosin or by other methods. (A removable coverslip originally placed on the bottom of the Petri dish facilitated histologic study.)

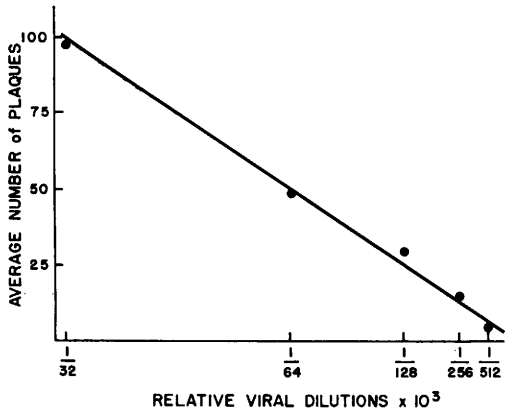


FIG. 2. Proportionality between number of plaques and virus concentration.

Results. Description of plaques. In living cultures containing neutral red, plaques (Fig. 1A) were grossly visible on the 4th day and attained a diameter of 0.2 to 0.8 mm by the 6th day. At this time counts were routinely made at a magnification of 35 ×. Thereafter, up to 12 days, the plaques increased in diameter (up to 2.5 mm) but not in number. Microscopically, plaques first became visible after 36-48 hours incubation (Fig. 1B).

Histologic studies showed the characteristic CPE in nearly all cells within the plaques. The presence of small nuclear inclusions in peripheral cells of otherwise nearly normal appearance (Fig. 1C) indicated centrifugal spread of the infection from cell to cell. Three types of plaques were noted, the first with a central area of complete lysis, the second with a reticulated central zone (Fig. 1C) and the third composed of contiguous inclusion-bearing cells. The significance of these plaque types in unknown. Sequential studies at 4-hour intervals showed steady and rather uniform development from the "single cell plaque" (Fig. 1B) to the grossly visible stage (Fig. 1C). Apparently a single infected cell developed advanced CPE before surrounding cells were altered (Fig. 1B). When undiluted infected GM was used, widespread CPE developed earlier (by the 16th hour) but plaques did not form.

Dilution factor. Using the procedure described for plaque formation, a consistent linear relationship was found between relative viral dilution and number of plaques formed (Fig. 2) indicating that only one infectious unit was necessary to initiate a plaque(8). Approximately 1×10^7 plaque forming units (PFU) per ml were commonly present in GM from infected bottle cultures. When multiple monolayer cultures were inoculated with equal dilutions and read at 6 days, the plaque counts approximated the Poisson distribution.

Effect of calf serum. Viral inoculum serially diluted in PBS and in PBS with 2% calf serum was stored at 29 ± 1°C and periodically assayed for plaque forming units. Fig. 3 shows the salutary effect of calf serum on virus stability, and also indicates that no significant loss of viral titer occurred during the routinely adopted 90-minute preliminary contact period. The size, morphology, time of appearance and number of the plaques were not affected by increasing the calf serum in the agar overlay medium from 2 to 20%.

Production of plaques by virus obtained from salivary glands. Salivary glands aseptically removed from infected mice were homogenized in 10 ml PBS. After centrifugation at 500 RPM for 5 minutes, 1 ml of supernate was serially diluted in PBS with 2% calf serum, and assayed as described above. In size and time of appearance, the plaques were comparable to those produced by the tissue culture adapted virus.

Pathogenicity after prolonged cultivation. During more than 120 serial passages, the PFU per ml of GM and the ability of the

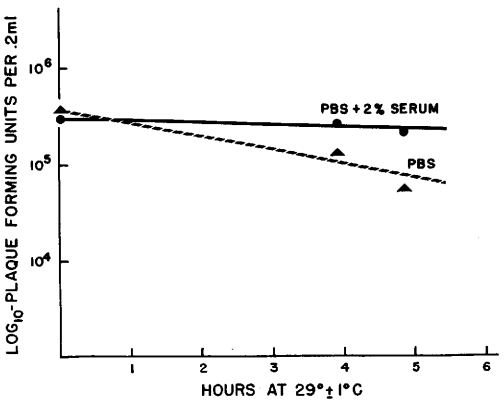


FIG. 3. Stability of virus. (Each point represents arithmetic mean of 4 plates.)

virus to produce CPE with inclusions in mouse embryonic fibroblasts were not significantly altered. Pathogenicity for mice, which was of a high order after the first 3 or 4 passages, was completely lost after about the 36th passage.

Attempts to produce plaques on other cell types. Using the procedure described above for obtaining virus directly from the salivary glands of infected mice, attempts were made to produce plaques on chick embryo cells, HeLa cells, and L cells. These attempts were all unsuccessful; neither plaques nor inclusions were observed after 6 days' incubation in any of these cell types.

Summary. Using a double agar overlay, MSGV produced discrete spherical plaques in mouse embryonic fibroblasts, but not in chick embryo, HeLa or L cells. Plaques appeared about 36 hours after seeding as single large rounded inclusion-laden cells, surrounded by wide clear zones. Centrifugal expansion by progressive involvement of adjacent cells resulted in plaque diameters of 0.2 to 0.8 mm by the 6th day. Fluid medium from infected bottle cultures commonly contained 1×10^7 PFU/ml. A linear relationship was found between plaque counts and virus dilution. The virus was markedly stable when stored for 5 hours in PBS with 2% calf serum, but rapid loss of titer occurred if serum was omitted. Virus titer and CPE production *in vitro* were not significantly altered during 120 passages, but pathogenicity for mice was lost after about 36 passages.

The authors would like to thank Dr. R. W. Schlesinger, Dept. of Microbiology, for his generous assistance.

1. Weller, T. H., Hanshaw, J. B., Scott, D. E., *Virology,* 1960, v12, 130.
2. Smith, M. G., *Progress in Medical Virology,* Karger, Bassal, New York, 1959, v2, 171.
3. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 435.
4. ———, *ibid.,* 1956, v92, 424.
5. Hartly, J. W., Rowe, W. P., Huebner, R. J., *ibid.,* 1957, v96, 281.
6. Youngner, J. S., *ibid.,* 1954, v85, 202.
7. Eagle, H., *J. Exp. Med.,* 1955, v102, 37.
8. Dulbecco, R., Vogt, M., *ibid.,* 1954, v99, 167.

Received February 26, 1963. P.S.E.B.M., 1963, v114.

Effect of B-3-Thienylalanine on Antibody Synthesis. II. Relation of Time of Drug Feeding to Inhibition of Synthesis.* (28606)

MARIANO F. LA VIA
Department of Pathology, University of Colorado Medical Center, Denver

It has previously been reported that B-3-DL-thienylalanine, (B-3-TA), when fed to rats by stomach tube, would inhibit antibody formation if feeding was started 2 days before antigen injection and continued until sacrifice of the animals(1). No inhibition was seen if feeding began on the third day after immunization, even though similar amounts of drug were fed. Addition of the drug during synthesis of antibody *in vitro* showed no depressing effect on the synthetic process as measured by incorporation of radioactive glycine into antibody.

* This work was supported in part by grants from Nat. Inst. of Allergy and Infect. Dis., P.H.S., and from Am. Cancer Soc.

Wissler *et al.*(2) showed that there is a reduction in the splenic cellular proliferation which accompanies antibody formation following B-3-TA feeding. The present work was undertaken in an effort to: a) establish the optimal time of B-3-TA feeding in relation to antigen injection necessary to achieve maximum inhibition of antibody synthesis and b) to investigate further the effect of B-3-TA on the cellular changes which occur in the spleen of the immunized rat(3).

Materials and methods. Sprague-Dawley, young adult, male, albino rats were used in these experiments. Diets were prepared as described by Hruban *et al.*(4). The rats received their total ration in 3 separate feed-