protein are increased after 6-TG administration.

In guinea pigs, some investigators have reported failure to suppress antibody production during administration of 6-mercaptopurine(13), although suppression of production of allergic encephalomyelitis and of delayed hypersensitivity was observed by others(14). In the present work, administration of 6-MP (50 mg/kg body weight; 50% mortality) produced neither loss in body weight nor decrease in serum gamma globulins, whereas administration of 6-TG (5 mg/kg body weight; 100% mortality) produced decreases in guinea pig gamma globulin concentration. It will be of interest to determine whether a difference is found in the effect of 6-MP and 6-TG on guinea pig gamma globulin concentration as well as on antibody production when 6-MP and 6-TG are administered at dosages which produce comparable mortality.

Summary. Administration of 6-mercaptopurine and 6-thioguanine decreased serum gamma globulin concentrations in rabbits and produced an increase in concentration of a β_2 protein as measured by agar and paper electrophoresis. Immunoelectrophoretic studies confirmed the increase in the β_2 globulin and allowed detection of a "γ_x" precipitin line which was masked by the gamma globulin precipitin line prior to treatment.

Dr. David Alling did the statistical analyses and his help is gratefully acknowledged. The authors wish to thank Dr. Sheldon Dray for supplying purified rabbit gamma globulin, the sheep antisera and helpful criticisms; and Mr. Stanley Ward and Mr. Eurmal Exum for expert technical assistance.

1. Sterzl, J., Holub, M., *Czech. Biol.,* 1957, v6, 75.
2. Schwartz, R., Stack, J., Dameshek, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 164.
3. Nathan, H. C., Bieber, S., Elion, G. B., Hitchings, G. H., *ibid.,* 1961, v107, 796.
4. Wolff, S. M., Goodman, H. C., *J. Clin. Invest.,* 1962, v41, 1413.
5. Kabat, E. A., Mayer, M. A., *Experimental Immunochemistry,* 2nd Ed., Chas. C Thomas, 1961, p559.
6. Spinco Model R, Paper Electrophoresis Manual, RIM-4, Spinco Division, Beckman Instruments Co.
7. Uriel, J., Grabar, P., *Ann. Inst. Pasteur,* 1956, v90, 427.
8. Grabar, P., Williams, C. A., *Biochim. Biophys. Acta,* 1953, v10, 193.
9. Scheidegger, J. J., *Intern. Arch. Allergy,* 1955, v7, 103.
10. Wilcoxon, F., *Biometrics,* 1945, v1, 80.
11. Wolff, S. M., Goodman, H. C., unpublished observations.
12. Hirschfeld, J., *Acta Path. et Micro. Scand.,* 1959, v46, 229.
13. Genghof, D. S., Battisto, J. R., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 933.
14. Hoyer, L. W., Good, R. A., Condie, R. M., *J. Exp. Med.* 1962, v116, 311.

Received November 29, 1962. P.S.E.B.M., 1963, v112.

Quantitative Studies of Japanese B Encephalitis Virus in Hamster Kidney Cell Cultures.* (28063)

JOHNG S. RHIM† AND WILLIAM MCD. HAMMON
Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pa.

The development of a plaque assay by Dulbecco and Vogt(1) led to quantitative studies with animal viruses equivalent in precision to those made with bacterial viruses. Plaque titration of infectious particles has proven to be virtually essential for quantitative studies in present day virology. Certain of the arthropod-borne viruses (arboviruses), notably

* This work was carried out under the sponsorship of the Commission on Viral Infections, Armed Forces Epidemiological Board, and was supported by U. S. Army Medical Research and Development Command, Dept. of the Army.

† Fellow supported by a training grant from Nat. Inst. Health, U.S.P.H.S. Present address: Louisiana State Univ., International Center for Med. Research and Training, Apartado 688, San Jose, Costa Rica.

among the B group have not been readily amenable to this technic. Hamster kidney cell cultures (HKC), among the most susceptible for certain of these have proved particularly difficult for the plaque technic(2,3).

This report describes the details of a plaque assay for precise quantitation of Japanese B encephalitis (JBE) virus. Control of environmental variables, and precision attained are given. Comparative sensitivity of plaque production in cultures of HKC and chick embryo cell cultures (CEC) has been studied. Titers obtained in plaque forming units (PFU), in TCD_{50} by the cytopathic endpoint, and in intracerebral titration (LD_{50}) in mice, are compared.

Materials and methods. Virus. The Nakayama strain of JBE virus was used in its 46th passage in mouse and 28th passage in HKC cultures. The stock virus was prepared in HKC in lactalbumin hydrolysate medium (4) with 4% calf serum (maintenance medium) and harvested after 3 days of incubation. Cultures were frozen with an equal amount of inactivated (56° 30 min) normal calf serum (INCS) and thawed to disrupt residual cells and release intracellular virus. Following freezing and thawing, cultures were harvested, centrifuged at 2000 rpm for 20 minutes, pooled and stored frozen in small sealed ampoules at about −70°C (mechanical freezer).

Tissue culture. Monolayer cultures of HKC and CEC were grown in 3 oz. bottles with lactalbumin hydrolysate medium. Growth medium consisted of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (BSS) with 4% calf serum, 1% glutamine, 1% vitamin stock solution (100X concentrated for Eagle's basal medium) and antibiotics(5). Maintenance medium consisted of the same as the growth medium except that it contained only 0.5% concentration of glutamine and vitamin stock solution. Phosphate buffered saline (PBS)(1) was used for washing cultures.

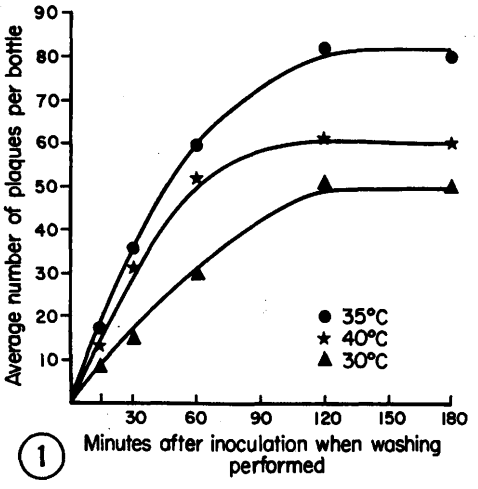
Diluent. Bovine albumin, 0.2% in 0.5% lactalbumin hydrolysate medium was used as diluent for titrating virus.

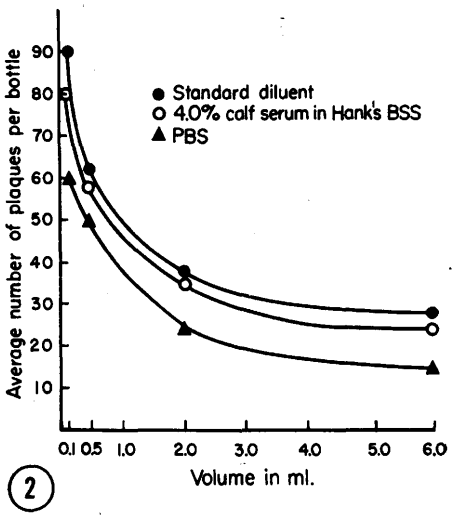
Virus assay. After removing the growth medium and washing twice with PBS, drained monolayers of HKC were inoculated with 0.1 ml of selected dilutions of virus in diluent. After an appropriate incubation period and with 2 further washings as described, 6 ml of the first agar overlay were added. After the agar solidified the cultures were turned over and kept at 37°C.

The first agar overlay was made by mixing equal parts of 3.0% agar in sterile distilled water and the following: 2% lactalbumin hydrolysate in Hanks' BSS (4X concentrated, without either phenol red or $NaHCO_3$) 25 ml; INCS 4 ml; $NaHCO_3$ (4%) 3 ml; antibiotics 1 ml; sterile water 17 ml. After 4 days of incubation a second overlay of 6 ml was added. The composition of the second overlay was similar to the first except that it contained neutral red to give a concentration of 1:30,000. Plaque counts were made on the sixth day after seeding the virus.

Experimental results. Effect of time of adsorption at different temperatures. Experiments were designed to test the time necessary for adsorption to take place. Virus was seeded in 0.1 ml volumes into different sets of drained bottle cultures and incubated at 30°C, 35°C and 40°C. At the end of 15, 30, 60, 120 and 180 minutes aliquot cultures were washed and the first overlay then added. The results after the second overlay (Fig. 1) show that 50% of the maximum adsorption obtained occurred in about 30 minutes at the higher temperatures and approached a maximum at 2 hrs. The highest efficiency of adsorption occurred at 35°C.

Effect of volume and diluent on virus inoculum. The effect of volume of inoculum and of different diluents on plating efficiency was examined. Equal aliquots of the same suspension of virus were diluted to a final volume of 0.1 ml, 0.5 ml, 2.0 and 6.0 ml, using diluent, maintenance medium and PBS. The total of each of these volumes was used to seed a bottle culture. Two bottles were used for each volume and each diluent. After 2 hours adsorption, the fluid was removed and the double overlay applied as described above. The plating efficiency with each diluent was highest when the virus was present in the smallest volume employed (0.1 ml) and it decreased with increase in the volume





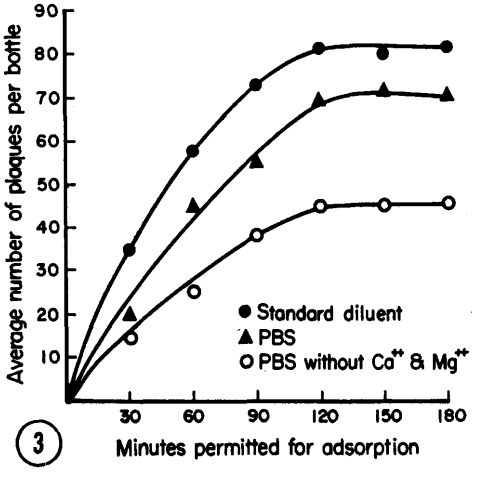


FIG. 1. Effect of time of adsorption and temperature on number of plaques.

FIG. 2. Effect of varying volume of a constant amount of virus with different diluents.

FIG. 3. Effect of diluents including Ca^{++} and Mg^{++} on adsorption.

inoculated (Fig. 2). The 2 diluents containing serum protein were of about equal value, while PBS yielded poorer results.

Effect of amino acids and divalent cations. Experiments were carried out to compare the rate of viral adsorption using standard diluent (containing glucose, an enzymatic hydrolysate of highly nutritional protein, plus the salts required for cell growth and 0.2% bovine albumin as stabilizer) with phosphate buffered saline, with and without Ca^{++} and Mg^{++}.

The results given in Fig. 3 show that adsorption was probably less complete without Mg^{++} and Ca^{++}. The most complete adsorption was obtained with the diluent which has been employed as standard in the JBE virus plaque assay system.

Plating efficiencies for cultures washed before and after exposure to virus. The effect of washing cell layers with PBS at pH 7.0 before and after incubation with virus was studied. Washings were carried out with 5 ml of PBS. Table I shows that either type of washing alone or in combination did not alter the plaque counts.

Possibility of infection of the cell layer by diffusion of virus through the agar overlay. Experiments concerned with the possible diffusion of JBE virus through the overlay were performed. Maintenance medium was removed from several cultures. They were then layered with either 3.0 or 4.0 ml volumes of non-nutrient agar fluid. After gelation, these cultures, along with others without agar overlay were exposed for 120 minutes to 0.1 ml inocula containing approximately 30 PFU. Agar which would normally represent the first agar overlay medium was then added and brought the total volume of overlay on each infected culture to 7.0 ml prior to the additional incubation for plaque development. The "second" agar overlay was added by the same method described

TABLE I. Effect on Plating Efficiency of Washing Cultures Before and After Exposure to and Incubation with Virus.

Washing procedure			
Before exposure	After exposure* and incubation	PFU per bottle	Avg PFU per bottle
None	None	43, 36	39.5
"	Once	32, 40	36
Once	None	45, 35	40
"	Once	37, 27	32
Twice	Twice	33, 33	33

* Incubation for 2 hr at 37°C.

above. An average of 30 plaques developed on control plates, while none was found in cultures which had been protected with agar barriers prior to inoculation with virus.

TABLE II. Reproducibility of Plaque Assay on the Same Virus Suspension.*

Exp No.	Dilution factor	No. of plaques per bottle	Avg No. of plaques per bottle	Virus titer (PFU/ml)
1	1×10^{-6}	99, 73, 80	84	8.4×10^{-7}
2	1×10^{-6}	88, 82	85	8.5×10^{-7}
3	1×10^{-6}	77, 89	83	8.3×10^{-7}
3	2×10^{-6}	58, 40	49	9.8×10^{-7}
3	4×10^{-6}	20, 30	25	10.0×10^{-7}
3	8×10^{-6}	14, 12	13	10.4×10^{-7}
4	10×10^{-6}	8, 10	9	9.0×10^{-7}

* Different ampoules of the same virus stock were assayed by the plaque technic. Each numbered experiment was performed on a different day. Each culture was inoculated with 0.1 ml of virus dilution.

Reproducibility and precision of plaque assay. Reproducibility of the plaque assay was determined by repeating the titration on a separate frozen ampoule of the same virus stock on different days. Plaque assay by this method was quite reproducible, particularly when the inoculum was diluted to contain not more than about 50 PFU per bottle (Table II).

Sensitivity of the plaque method (PFU) compared with the cytopathic endpoint tissue culture infective dose and the intracerebral titration in weanling mice. Comparative titrations were made of 2 JBE viruses at different levels of mouse and HKC adaptation by 3 different methods to determine the relative sensitivity of each method. The results are shown in Table III. The titers by plaque assay were in almost all instances similar to those of the tube culture method and varied with the mouse titer as might be expected on the basis of the passage level in mice or in HKC.

Comparative sensitivity of JBE virus plaque production in monolayers of HKC and CEC. To determine the comparative susceptibility of JBE virus plaque production in HKC and CEC, simultaneous titrations were carried out with the same virus stock in HKC and CEC. The results of several experiments (Table IV) show that HKC were uniformly slightly more sensitive than CEC, the average ratio being 1:0.6.

Discussion. The HKC plaque assay for JBE virus described above is shown to be precise, reproducible and relatively sensitive. The sensitivity of the plaque assay is at least equal to that of CPE in monolayers of HKC and when using HKC adapted virus is superior to that of the intracerebral titration in the weanling mouse, and of plaques in CEC.

Bachtold *et al.*(7) in work with foot and mouth disease virus state, "that more

TABLE III. Sensitivity of Plaque Assay Compared with Tube Culture Titration and Intracerebral Titration in Mice.

Strain and passage level		Log virus titer/ml		Ratio of titer
Mouse-HKC	PFU	TCD_{50}	LD_{50}	(PFU:TCD_{50}:LD_{50})
Nakayama				
48-26	7.8	7.7	8.0	1:0.8:1.6
48-27	7.3	7.5	7.6	1:1.6:2.0
48-28	7.9	8.0	7.7	1:1.2:0.6
OCT-541*				
0-2	6.8	6.5	7.0	1:0.5:1.5
0-30	7.3	7.3	6.5	1:1.0:0.16
0-55	8.2	8.3	6.7	1:1.2:0.03

* Mosquito isolate(3).

TABLE IV. Comparative Titers of Japanese B Encephalitis Virus (Nakayama Strain)* by Plaque Assay in HKC and CEC.

Tissue	No. of plaques per bottle	Avg No. of plaques	PFU/ml	Ratio to HKC
HKC	99, 73, 80, 88, 82	85	8.5×10^7	1.0
CEC	52, 51, 72, 40, 43	52	5.2×10^7	.6

HKC = Hamster kidney cell.
CEC = Chick embryo cells.
* Nakayama strain HKC passage 28.

plaques were formed when adsorption was carried out at 37° and 43° than at lower temperatures suggests that physical adsorption is not the rate-determining step in virus invasion. Primary attachment of virus to cell layer by forces of physical adsorption theoretically should proceed as well or better at the lower temperatures. Therefore, initiation of infection may be more dependent on chemical or enzymatic reactions with positive temperature coefficients." The better results obtained with JBE virus at 35° and 40° than at 30° might support the same hypothesis.

In experiments on the effect of volume and composition of virus inoculum on plating efficiency, the data indicate that higher efficiency of plating resulted when virus was suspended in small volumes. This result was most likely due to the increased opportunity for virus to come in contact with cells before thermal inactivation occurred. Also data show that bovine albumin was required for maximum efficiency. It was found that bovine albumin could be used as stabilizer as well as serum in arbovirus storage(11) and in overlay medium(5,12). It was found also that there was greater recovery of plaque forming units when bovine albumin was added to the diluent in adenovirus plaques in grivet monkey kidney cells(13). The loss of plaque forming units on dilution in salt solution may have been due in part to adsorption of virus particles to the glass of the container. Bovine albumin is an excellent desorbing agent(14).

The experiments on plating efficiency (Fig. 3) showed that plaques were produced in the absence of added Ca^{++} and Mg^{++} but that these cations were required for maximum virus adsorption. Puck and co-workers(6) had shown that Ca^{++} and Mg^{++} promote adsorption of bacteriophage to host cells, but the effect seems to be equivocal for enteroviruses (7,8,9) and reovirus(10). No reports have been observed for the arboviruses.

Table I shows that washing cells, before or after virus inoculation and adsorption, alone or in combination, did not alter the plaque counts. Bachrach *et al.*(15) obtained similar results with foot and mouth disease virus in the primary bovine kidney cell system. It was found that cellular monolayers need not be washed with buffer at pH 7.0 prior to infection even though the pH of the nutrient fluid on such cultures before infection is frequently as low as 6.7, an environmental condition which might inactivate much of the applied virus before its adsorption to cells(15). The absence of such inactivation on unwashed monolayers might be explained on the basis that residual fluid (*i.e.*, approximately 0.5 ml) remaining after aspiration of the major portion probably rapidly loses carbon dioxide to bring the pH into the range (7.0 to 7.5) which is more suitable for virus stability(15).

A recent finding of Kanda Inoue *et al.*(12) that particles of mouse adapted JBE virus, unadsorbed at the time of agar overlay, subsequently diffused through the agar and produced plaques, but unadsorbed HKC adapted virus was unable to produce plaques, led to the diffusion experiments reported here. The virus used in our experiment was mouse adapted and subsequently hamster adapted.

That washing of cell layers after exposure to virus was not required indicates that virus not adsorbed by the time agar was poured was subsequently unable to initiate plaques. This conclusion, confirmed by the agar barrier experiments in which less than 1 particle in 30 survived diffusion through the thickness of a 3 ml agar layer, is opposite to Youngner's findings(16) that poliovirus diffuses through agar to infect monkey kidney monolayers. It is interesting to know why JBE virus, which is about as small or smaller in size, does not appear to behave similarly. The failure to detect diffusion of JBE virus

through agar may be due to its instability at 37°(2), and to inactivation by agar or aqueous agar extracts(17).

Summary. Japanese B encephalitis virus on hamster kidney cell cultures has been studied by the plaque method, and the following results obtained. 1. The effect of some environmental variables on plating efficiency: a. Maximum adsorption was obtained by 120 minutes incubation at 35°C. b. Optimal conditions for initiating plaques were obtained by seeding the virus in a small volume of lactalbumin medium containing bovine albumin. c. Cations were required for maximum virus adsorption but plaques were produced in the absence of added Ca^{++} and Mg^{++}. d. Washing cultures with phosphate-buffered saline (pH 7.0) before and after exposure to virus was not required, and it was shown that plaques developed only from virus adsorbed on cells before agar was applied. 2. The titers obtained by plaque assay were in almost all instances similar to those of the tube culture method and varied with the mouse titer as might be expected on the basis of the passage level in mice or in HKC. 3. Hamster kidney cells proved slightly more sensitive for plaque assay than chick embryo cells, when HKC adapted virus was used.

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

2. Diercks, F. H., Hammon, W. McD., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 627.

3. Rohitayodhin, S., Hammon, W. McD., *J. Immunol.,* 1962, v89, 823.

4. Melnick, J. L., in *Diagnostic Procedures for Virus and Rickettsial Diseases,* 2nd ed., p142, *Am.* Public Health Assn., 1956.

5. Rhim, J. S., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 887.

6. Puck, T. T., Garen, A., Kline, J., *J. Exp. Med.,* 1951, v93, 65.

7. Bachtold, J. G., Bubel, H. C., Gebhardt, L. T., *Virology,* 1957, v4, 582.

8. McLaren, L. C., Holland, J. J., Syverton, J. T., *J. Exp. Med.,* 1960, v112, 581.

9. Wallis, C., Melnick, J. L., *Virology,* 1962, v16, 122.

10. Rhim, J. S., Melnick, J. L., *Tex. Rep. Biol. Med.,* 1961, v19, 851.

11. Olitsky, P. K., Yager, R. H., Murphy, L. C., *U. S. Armed Forces Med. J.,* 1950, v1, 415.

12. Kanda Inoue, Y., Iwasaki, T., Kato, H., *J. Immunol.,* 1961, v87, 337.

13. Tytell, A. A., Torop, H. A., McCarthy, F. J., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 916.

14. Valentine, R. C., Allison, A. C., *Biochem. Biophys. Acta,* 1959, v34, 10.

15. Bachrach, H. L., Callis, J. J., Hess, W. R., Patty, R. E., *Virology,* 1957, v4, 224.

16. Youngner, J. S., *J. Immunol.,* 1956, v76, 288.

17. Schulze, J. T., Schlesinger, R. W., *Fed. Proc.,* 1962, v21, 455.

Received December 3, 1962. P.S.E.B.M., 1963, v112.

Experimental "Lactational" Mammary Gland Growth in the Rat as Measured by DNA.* (28064)

DAVID R. GRIFFITH† AND C. W. TURNER
(With technical assistance of Mary E. Powell)
Department of Dairy Husbandry, University of Missouri, Columbia, Mo.

Previous studies in this laboratory have employed desoxyribonucleic acid (DNA) as a quantitative index of mammary gland development during pregnancy, lactation, and involution(1,2,3). It had been considered that mammary gland growth occurred primarily during the first ⅔ of pregnancy, followed by gradual initiation of lactation during the final trimester(4). By estimating DNA of the mammary glands, it was found that DNA increased significantly during last ⅓ of pregnancy and between day 1 and 3 of lactation indicating cellular proliferation continues during early stages of lactogenesis in

* Contribution from Mo. Agr. Exp. Sta. Journal Series No. 2444. Approved by the Director. This investigation was supported by grants from U.S.P.H.S. and Am. Cancer Soc.

† Public Health Service Research Fellow of Nat. Cancer Inst.