Replication of Virus Plaques. (26756)

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The replica plating technic devised by Lederberg and Lederberg(1) has been found to be a most useful tool in research dealing with various facets of bacterial genetics. With the progress made in the field of the genetics of animal viruses, stimulated by the advances in tissue culture and plaque technics(2), it seemed that a similar replica plating technic would be useful in animal viral research. This report concerns the development of such a technic.

Materials and methods. Virus and Tissue culture. Both Eastern (EEE) and Venezuelan (VEE) equine encephalomyelitis viruses were used. The former was obtained from the Communicable Disease Center, U. S. Public Health Service. It was a human strain that has been passed in mice and embryonated eggs. The latter was obtained from a donkey that died of encephalitis, and was passed in embryonated eggs. Chick embryo working seeds were prepared by the usual methods(3). Chick embryo fibroblasts were prepared from 10-day-old chick embryos by the trypsinization method of McClain and Hackett(4). They were used in a suspended cell plaque assay method described below.

Plaque assay. The plaque assay method was a modification of the second method described by Cooper(5) which involved infecting cells previously suspended in an agar overlay from an inoculum placed on the hardened agar surface. Five ml of nutrient agar (4), which contained 0.5% gelatin instead of albumin plus 5% calf serum, was poured as a base into 60-mm Petri dishes. An appropriate volume of chick fibroblasts prepared previously in concentrations ranging from 10-25 million per ml were centrifuged (at 1500 rpm for 5 minutes), resuspended, and mixed in a volume of nutrient agar at 43°C to give a concentration of 50 million cells per ml. One and a half ml of this suspension in agar was poured as an overlay into the Petri dishes and allowed to harden. The infection by virus before incubation at 37°C could be carried out with all materials held at room temperature, but a significant and reproducible increase (3×-6×) in sensitivity of the plaque assay by this procedure was achieved as follows: Plates containing cells were refrigerated for 20 minutes before use. Dilutions of virus seed were made in cold beef heart infusion broth. Fifteen-hundredths ml of appropriate dilutions were inoculated onto the cold plates and spread immediately by rotating the plates. The plates were then placed in the refrigerator for 30 minutes, withdrawn, and incubated right side up for 48 hours at 37°C in a humidified atmosphere containing 5% CO_2. Plaques could be seen by oblique light, but better visualization was obtained by adding a 1/10,000 aqueous solution of neutral red to the plates. It is important in this method of plaque assay that all plates be kept on level surfaces during all manipulations to assure an even distribution of plaques.

Replication of plaques. Replication was carried out in a manner analogous to that described by Lederberg and Lederberg(1) for bacterial colonies. A piece of sterile velveteen was tightened securely over an inverted No. 10 rubber stopper by a celluloid ring. The stopper was connected to a brass base by a metal rod (Fig. 1). The master plate, whose plaques were not developed by addition of neutral red, was inverted and lowered on to the velveteen to make the imprint, removed, and the replica plates in turn inverted and lowered to touch the velveteen. Incubation was carried out at 37°C. After replication, the plaques on the master plate were developed and treated with mercuric chloride as described below.

"Fixing" of plaques. Because plaques fade after a few days, it became desirable to find a simple method to "fix" the location of the plaques and at the same time increase the contrast so that they could be counted and/or photographed when convenient(6). It was found that 2-5% solutions of mercuric

FIG. 1. Apparatus used for replication.

chloride poured over the agar and held for one-half hour before being poured off deepened the red background color which gave better contrast. Plaques so treated remained in excellent condition for several months. The increased intensity of the red color is probably due to precipitation of the dye *in situ,* since in the test tube solutions of the dye precipitate in the presence of mercuric chloride.

Results. The results in Table I show that the number of plaques formed with VEE and EEE virus was approximately inversely proportional to the dilution plated and that approximately the same satisfactory precision was indicated as in the results reported for the monolayer technic of Dulbecco and Vogt (7). In comparative studies of the suspended cell assay technic, and the monolayer assay procedure, 5 times more VEE virus was detected by the former method; the sensitivities of the 2 technics were equal for EEE virus.

Since virus was inoculated on the surface of the plates in this modification of the suspended cell plaque technic, it was reasoned that the resulting plaques should provide the proper conditions for quantitative replication. The results (Fig. 2) showed that except for plaques on the rim of the plate, which the velveteen did not touch, quantitative replication was achieved. The replicas of the already large VEE plaques were considerably larger than the original plaques (Fig. 2), partly because the plaques of this virus characteristically continue to enlarge on prolonged incubation. In contrast, the plaques of a mutant of VEE virus(8) are minute, and, like the wild type (r+)T-even coliphages, do not increase in size after 48 hours' incubation; they, therefore, replicated with minimum enlargement. After 3 replications, the plaques of the parent VEE virus became very large, probably because of spread of liquid and virus by the velveteen, which had become wet. If more than 3 replications are desired with satisfactory results (*e.g.,* for testing for host range mutants or recombinants), one can use as many as 3 velveteens on the same master plate with 3 replicas made from each.

Certain preliminary experiments showed that, as in mouse neutralization tests, EEE and VEE virus did not cross react in suspended cell plaque neutralization tests in which single virus particles failed to form plaques on antiserum-containing plates. These results permitted us to test whether replication of plaques was feasible for the screening of different antigenic types, such as might be used in recombination experiments.

Both VEE and EEE viruses were placed in discrete spots on the surface of agar plates containing the suspended chick fibroblasts. After 48 hours' incubation, the master plates containing plaques of both viruses were replicated in turn on separate plates containing approximately a 1/400 final dilution per plate of EEE or of VEE monkey antiserum, respectively. The plaque neutralization

TABLE I. Proportionality between Number of Plaques and Concentration of Virus.*

Virus strain	Dilution	No. of plaques on each plate	Avg No. plaques per plate	Titer per ml
VEE	4×10^{-7}	30; 35; 30; 21	29	5×10^{8}
	2×10^{-7}	14; 15; 15; 18	16	6×10^{8}
	1×10^{-7}	3; 6; 1; 5	4	4×10^{8}
EEE	4×10^{-7}	75; 38; 56; 60	57	9×10^{8}
	1×10^{-7}	15; 8; 12; 15	13	9×10^{8}

* 0.15 ml of appropriate virus dilution was inoculated on each 60-mm Petri plate.

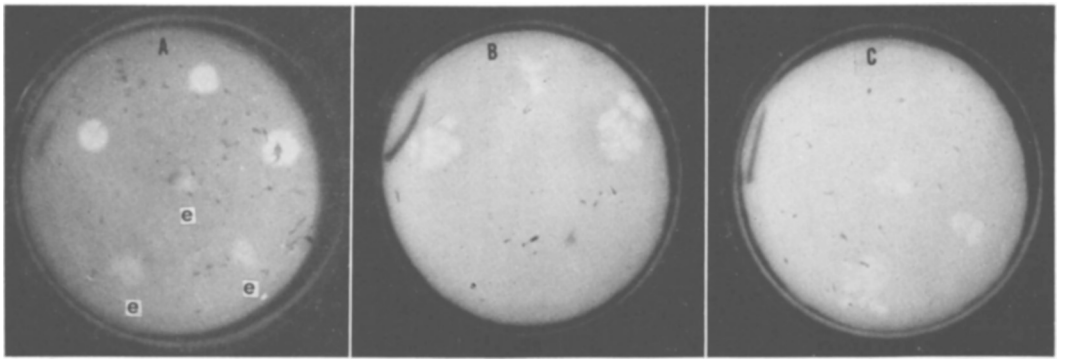

FIG. 2. Replication of virus plaques. A. Master plate showing clear plaques of VEE virus and smaller, turbid plaques of EEE virus. Latter are difficult to photograph and have been marked with an "e" beneath each plaque. B. Replica of A on plates containing EEE antiserum. C. Replica of A on plates containing VEE antiserum.

titers of the undiluted antisera were previously found to be over 1/1000 for each. The results of this experiment are shown in Fig. 2. The master plate shows the plaques of both viruses. EEE virus plaques are smaller and more turbid in appearance, and more difficult to see than are plaques of VEE virus by the suspended cell technic. By the monolayer technic, only size distinguishes the 2 virus strains. On plates containing VEE virus antiserum, the EEE plaques were quantitatively replicated in their expected geographical position, with the complete exclusion of the VEE virus plaques. Corresponding results were obtained for VEE plaques replicated on plates containing EEE virus antiserum.

The results of the above experiments suggest that replication of virus plaques for various purposes is feasible.

Discussion. The choice of the method finally selected for plaquing the viruses for replication was made after the standard monolayer method and Cooper's first suspended cell method(5,9) were found to be unsatisfactory. In the former no plaques could be replicated. The latter procedure involves mixing the virus and cells with agar before pouring it as an overlay. A poor efficiency of replication of viral plaques was obtained (20%-50%) using this assay method. Apparently, the viruses in the plaques beneath the surface either in the monolayer or in Cooper's first suspended cell procedure do not diffuse in sufficient amounts to the surface to permit quantitative replication. Cooper's second suspended cell method, which involves the infection of cells from the inoculum placed on the agar surface, was therefore modified to make it sensitive and precise for quantitative work and to make quantitative replication of plaques feasible. The present replication technic was developed with small Petri plates to illustrate the feasibility of the technic. Some of the disadvantages of the method stemming from formation of larger, overlapping plaques on the replicas could be minimized if larger plates were used. Similarly, means could be devised to place a removable artificial inside rim in the Petri dish so that plaques would not form on the true rim of the plate; the velveteen replication could then be made quantitative.

Summary. One of the 2 suspended cell plaque technics described by Cooper(5) has been modified so that its sensitivity and precision for plaque assay of VEE and EEE virus are satisfactory for general purposes. In addition, the modified suspended cell technic permitted nearly quantitative replication of virus plaques. Feasibility of the method in simulated systems for screening of host range or antigenic type recombinants (or mutants) was demonstrated.

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1. Lederberg, J., Lederberg, E. M., *J. Bact.*, 1952, v63, 399.

2. Dulbecco, R., *Proc. Nat. Acad. Sci.*, 1954, v99, 747.
3. Hardy, F. M., Brown, A., *J. Bact.*, 1961, v81, 20.
4. McLain, M. E., Hackett, A. J., *Virology*, 1959, v9, 577.
5. Cooper, P. D., *ibid.*, 1955, v1, 397.
6. ———, *ibid.*, 1959, v7, 467.
7. Dulbecco, R., Vogt, M., *J. Bact.*, 1952, v63, 399.
8. Hardy, F. M., Hearn, H. J., Jr., *Am. J. Hyg.*, 1961, in press.
9. Cooper, P. D., *Virology*, 1961, v13, 153.

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Response of Growing Rats to Diets Varying in Magnesium, Potassium and Protein Content. (26757)

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Hypomagnesemia accompanied by convulsions is a disturbance which occurs sporadically in ruminants grazing on young, rapidly growing forage(1). A relationship has been observed in the field between nitrogen content of the grass on which cattle graze and incidence of tetany(2). High potassium diets(3) and diets high in protein and potassium(4) reduce serum magnesium and increase fecal excretion of magnesium in sheep. There is retention of calcium in the viscera and kidneys of rats during magnesium deficiency(5,6) and in the yellow elastic fiber of the endocardium, aorta and jugular vein in calves fed low-magnesium diets(7). Colby and Frye(8,9) reported an increase in severity of magnesium deficiency in rats fed high calcium, high potassium or high protein (50%) diets. It was desired to study the effects in rats of high potassium and/or high protein diets on growth, blood electrolytes and pathological anatomy of low or normal dietary magnesium.

Methods. Weanling albino rats of the Cornell strain were used in a factorial arrangement of treatments. Rats were kept in individual wire-mesh cages. Diets (Table I) were provided *ad libitum* and distilled water was available at all times. Feed consumption and weight gains were recorded weekly. One-half of the rats from each group were sacrificed after 28 days and the remainder after 56 days on test by ether anesthesia. Blood was collected by heart puncture into heparinized tubes for serum calcium and magnesium and whole blood potassium determination. Whole blood potassium was determined in duplicate after some modifications of the method of Mosher(10). Five tenths of one ml of whole blood were diluted 1:100 with distilled water for analysis. Serum calcium and magnesium were determined by using a modification of the method of Walser(11). The following organs and structures from each rat were prepared for histological examination: proximal epiphysis of tibia, parietal bone, brain (transverse section through thalamus and cerebellum), thyroid, parathyroid, adrenals, esophagus, trachea, stomach (esophageal and fundic sections), duodenum, jejunum, colon, liver, pancreas, spleen, bone marrow, lymph glands, kidneys, ureter, urinary bladder, skin, myocardium, skeletal muscle, lungs; cross section through hilus, including lungs, bronchi, aorta, mediastinum and thymus. Bones were fixed in 10% formaldehyde and soft tissues in Bouin's fluid. Decalcification was with Perenyi (5 parts 100% HNO_3 and 95 parts 80% ethyl alcohol). Samples were paraffin-embedded, sectioned at 6 micra and stained with H and E stain.

Results and discussion. The data for growth, feed consumption and efficiency of

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