1952, v199, 127.
2. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 1931, v25, 117.
3. Krebs, H. A., and Eggleston, L. V., *ibid.*, 1940, v34, 442.
4. Handler, P., *J. Biol. Chem.*, 1945, v161, 53.
5. Busch, H., and Potter, V. R., *ibid.*, 1952, v198, 71.
6. Drury, D. R., *Am. J. Physiol.*, 1935, v111, 289.
7. Busch, H., and Potter, J. R., *Cancer Research*, 1953, v13, 168.
8. Lifson, N., and Stolen, J. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v74, 451.
9. Lee, J. S., and Lifson, N., *J. Biol. Chem.*, 1951, v193, 253.
10. Hayaishi, O., *ibid.*, 1955, v215, 125.

Received August 8, 1956. P.S.E.B.M., 1956, v93.

Cultivation of Type 2 Dengue Virus in Rhesus Kidney Tissue Culture.* (22692)

SUSUMU HOTTA† AND CHARLES A. EVANS.
Department of Microbiology, University of Washington, Seattle, Wash.

It was reported(1) that mouse-adapted type 1 dengue virus of the Mochizuki and Hawaiian strains multiplied in trypsinized rhesus kidney tissue cultures, and produced a cytopathogenic effect. It was also demonstrated that degeneration of infected cells was inhibited by type specific anti-dengue immune rabbit serum, thus making possible virus neutralization tests in tissue culture. In the present paper, experiments are reported in which type 2 dengue virus was cultivated in tissue culture.

Materials and methods. Tissue cultures employed consisted of trypsinized rhesus kidney cells(2). Technical details including preparation and incubation of cultures, constituents of medium, etc., were described previously(1). Type 2 dengue virus of the New Guinea C strain was received on Jan. 25, 1955, from Dr. Edwin H. Lennette in the form of 20% mouse brain homogenate in 30% normal rabbit serum frozen with dry ice. It was stated that this material represented the 19th mouse brain passage, and that its LD_{50} titer was $10^{-6.2}$ at time of harvest, Nov. 11, 1953. The virus was kept in dry ice chest until used. On Feb. 1, 1955, the frozen material was thawed at room temperature, and diluted with Hanks' balanced salt solution. Its infectivity was measured by injecting 2 to 3-week-old white mice intracerebrally with 0.02 ml of 10-fold dilutions. The mortality ratios of injected mice were 4/4 for the 10^{-3} dilution and 0/4 each for dilutions of 10^{-4} through 10^{-8}.

Results. Serial passage through tissue culture: Seven cultures were inoculated with 0.2 ml of above-mentioned virus diluted 10^{-3}. After 10 minutes 1.8 ml of culture medium was added to each tube. The cultures were incubated in the stationary state at 35°C. Half or whole volume changes of medium were made usually every 2 to 3 days, to keep the pH of the fluid phase in the range of 7.2 to 7.6. Eleven days after beginning of incubation a portion of culture fluid was taken from each tube and pooled with fluids from other similar tubes. 0.2 ml of the mixture was then inoculated into new cultures with subsequent addition of 1.8 ml of medium to each tube. In this manner serial passages through tissue cultures were performed at intervals of 7 to 15 days. The virus content of the inoculum for each subculture was assayed by injecting 0.02 ml of 10-fold dilutions into mice. The results obtained are summarized in Table I. The specimens used for tissue culture passage and virus titration contained no agent cultivable on ordinary bacteriological culture media.

The virus was cultivated in this tissue culture system for 18 passages during 176 days.

* Aided by contract with Office of Naval Research, grants from Eli Lilly Co. and State of Washington Fund for Research in Biology and Medicine.

† Present address: Microbiological Institute, Kyoto University, Kyoto, Japan.

TABLE I. Serial Transmission of Type 2 Dengue Virus of New Guinea C Strain through Rhesus Kidney Tissue Culture.

Passage in tissue culture	Mortality ratios of mice inoculated with tissue culture fluid diluted 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Cumulative period of cultivation, days	Calculated dilution of original infected mouse brain based on passage and medium-change
1	2/3	1/3*	2/3	1/3	0/3	0/3	11	$10^{-4.9}$
2	3/3	2/3	0/3*	1/3	0/3	0/3	25	$10^{-6.8}$
3	0/3	0/3	0/3	0/3	0/3	0/3	38	$10^{-8.7}$
4	2/3	2/3	1/3	1/3	0/3	0/3	50	$10^{-12.0}$
5	1/3	0/3	2/3	1/3	0/3	0/3	65	$10^{-13.8}$
6	2/3	1/3	1/3	0/3	0/3	0/3	74	$10^{-15.2}$
7	0/3	1/3	1/3	0/3	0/3	0/3	81	$10^{-16.5}$
8	2/3	2/3	1/3	0/3	0/3	0/3	92	$10^{-18.1}$
9	1/3	0/3	2/3	0/3	0/3	0/3	100	$10^{-19.4}$
10	1/3	2/3*	0/3*	1/3	0/3	0/3	110	$10^{-21.3}$
18	3/3	2/3	0/3	2/3	1/3	0/3	176	$10^{-41.5}$†

* One of the surviving mice showed paralytic signs and recovered.
† For 1 ID_{50} in 18th passage.

One ID_{50} in the 18th passage represented a dilution of $10^{-41.5}$ of the original infected mouse brain homogenate. As reported by other investigators(3), mortality ratios of mice inoculated with the New Guinea C strain virus were erratic.

Morphological changes: Inoculated cultures were examined with ordinary light microscope. They showed cellular degeneration which was indistinguishable from that produced by type 1 dengue virus(1). Five days to one week after inoculation of virus, the cells were dark and spherical. This was followed by their detachment from the glass surface. After about 2 weeks only a relatively small part of the cell population remained. After 3 to 4 weeks practically all cells had disappeared. It was confirmed again in our experiments that uninoculated control cultures never showed widespread destruction of cells; changes resulting from prolonged incubation were limited. A large population of cells of normal appearance remained in uninfected cultures incubated 4 weeks or more.

Parallel titrations of virus in mice and in tissue cultures: The titers of cultivated virus were compared in mice and in tissue cultures. The inoculum was 0.02 ml for mice by the intracerebral route, and 0.2 ml for tissue cultures containing 1.8 ml of medium. The results obtained are given in Table II. In these limited tests the highest dilution which caused death of mice was greater than that producing definite degeneration of tissue culture cells.

Neutralization test in tissue culture: Immune serum was obtained by injecting a rabbit repeatedly with the New Guinea C strain of virus that had been propagated in mouse brain and had never been passed in tissue culture. The procedure for immunization was similar to that used in obtaining anti-type 1 immune serum(1). Serum collected from the rabbit prior to immunization served as a control non-immune serum. The serum was inactivated by heating at 56°C for 30 minutes, and stored at —10°C until used. Type 1 Mochizuki and Hawaiian strains of virus and

TABLE II. Parallel Titrations of Type 2 Dengue Virus of the New Guinea C Strain in Mice and in Tissue Cultures.

Passage in tissue culture	Inoc. into*	Dilution of infected tissue culture fluid 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10	Mice		1/3†	2/3	0/3	1/3	0/3	0/3
	Tissue cultures		3/3†	3/3	0/3	0/3	0/3	0/3
18	Mice	1/3	3/3	2/3	0/3	2/3	1/3	0/3
	Tissue cultures	3/3	3/3	3/3	2/3	0/3	0/3	0/3

* Inoculum: 0.02 ml in mice intracer., and 0.2 ml in tissue cultures with 1.8 ml of medium.
† Numerators indicate No. of mice showing characteristic signs of infection or tissue culture tubes showing definite degeneration. Denominators indicate No. of mice or tissue culture tubes inoculated.

TABLE III. Neutralization Tests in Tissue Cultures of Type 1 and Type 2 Dengue Viruses.

Virus					Serum*		
Type	Strain	Passage in tissue culture	ID_{50} for tissue culture/0.2 ml	Dilution of infected tissue culture fluid	Anti-Mochizuki immune	Anti-New Guinea C immune	Control†
1	Mochizuki	43	$10^{-6.0}$	100-fold	0/5‡	5/5	5/5
	Hawaiian	25	$10^{-5.25}$	"	0/5	5/5	5/5
2	New Guinea C	18	$10^{-2.25}$		5/5§	0/5	5/5

* Diluted 1:5 with 0.1% lactalbumin hydrolysate medium. Aliquots of this mixed with equal volumes of virus suspension.

† Taken before immunization from a rabbit used for immunization against New Guinea C strain virus.

‡ Numerators indicate No. of tissue culture tubes showing definite degeneration. Denominators indicate No. of tissue culture tubes inoculated. The culture fluid used for changing medium included 1% of immune or control serum.

§ Degeneration of tissue cultures inoculated with mixture of type 2 virus and anti-type 1 serum was milder than that in control cultures exposed to type 2 virus and non-immune serum.

anti-Mochizuki immune rabbit serum were used to determine heterologous reactions. In performing the neutralization tests a given concentration of cultivated virus was mixed in equal volume with serum diluted 1:5 in 0.1% lactalbumin hydrolysate medium. After incubation at 37°C for 1 hr and at 4°C for 1 more hr, 0.2 ml of the mixture was inoculated into tissue culture tubes with subsequent addition of 1.8 ml of medium. Examples of results obtained are shown in Table III.

It was demonstrated that the viruses were neutralized by the homologous serum, but not by the heterologous serum. It appeared, however, that the degeneration shown in tissue cultures inoculated with the mixture of type 2 virus and anti-type 1 serum was a little milder than that shown in control cultures exposed to virus and non-immune serum.

Summary and conclusions. 1) Mouse-passaged type 2 dengue virus of the New Guinea C strain was cultivated in trypsinized rhesus kidney tissue cultures grown directly on glass surfaces and incubated in the stationary state at 35°C. The virus was maintained in this system of tissue culture through 18 passages during 176 days. Culture fluid from the 18th passage representing a $10^{-41.5}$ dilution of the original infected mouse brain was infective for mice. 2) The infected culture cells exhibited characteristic degeneration indistinguishable from that associated with infection of tissue cultures with type 1 dengue virus. Infectivity of the virus was neutralized by specific antiserum as indicated by the fact that the degeneration was suppressed completely by homologous immune rabbit serum, but not by heterologous serum. It appeared that the cellular degeneration produced by type 2 virus mixed with anti-type 1 serum was a little milder than that produced by the same virus mixed with the control non-immune serum. Although further studies are necessary to clarify the antigenic relationships of type 1 and type 2 dengue viruses, it is possible that the reduced degeneration was due to a partial neutralization by heterologous antibody.

1. Hotta, S., and Evans, C. A., *J. Infect. Dis.*, 1956, v98, 88.

2. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

3. Meiklejohn, G., England, B., and Lennette, E. H., *Am. J. Trop. Med. and Hyg.*, 1952, v1, 51.

Received August 9, 1956. P.S.E.B.M., 1956, v93.