FACILITATION OF INFECTION OF MOUSE L-CELLS WITH RIBONUCLEIC ACID FROM ENCEPHALOMYOCARDITIS VIRUS*

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Infectious ribonucleic acid (RNA) was first extracted from tobacco mosaic virus by Gierer and Schramm (1956) by treatment of the virus with cold phenol. Subsequently, a number of viruses have been found to yield infectious RNA; however, the efficiency with which extracted RNA is capable of entering the cell is very low as compared with mature virus. Hypertonic sodium chloride treatment of enterovirus RNA to augment its infectivity for hypertonically treated human cells was reported by Sprunt et al (1959). Dubes and Klingler (1961) reported that the infection of primary monkey kidney cells with poliovirus RNA was greatly increased by adding any one of several comparatively water-insoluble substances (which they called facilitators) to the RNA inoculum and depleting the cells of calcium.

Encephalomyocarditis (EMC) virus is one of a number of animal viruses known as the Col-SK group. In experiments reported by Huppert and Sanders (1958) phenol extraction at 4° C of ascites tumor cells infected with mouse-grown EMC yielded 'ribonucleic acid extracts' from extracellular fluid following ultracentrifugation, although the latter was not extracted from the sedimented virus by similar treatment. In studies reported herein the facilitation method of Dubes and Klingler (1961) was employed to infect L-cells with RNA from a large plaque mutant of EMC virus. Moreover, this system was used to test infectivity of extracted supernatant and pellet following ultracentrifugation of tissue culture fluid to determine source of infectious RNA.

Preparation of infectious RNA. Two methods were used for preparation of RNA from EMC virus. The first was the monophasic method, essentially the method of Klingler et al (1959), described as follows: to 7 volumes of water-saturated phenol at 0° C, 93 volumes of EMC virus suspension were added. Following thorough mixing the mixture was left for 5 minutes at 0° C and then extracted 3 times, each time with an equal volume of ether. Excess ether was evaporated

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under air. The second method employed was that of Gierer and Schramm (1956) in which equal volumes of EMC virus and water-saturated phenol were mixed and kept at 0° C for 45 minutes. Aqueous phase was removed, mixed with an equal volume of phenol, and kept at 0° C for 15 minutes. Again the aqueous phase was removed, mixed with equal volume of phenol and kept at 0° C for 5 minutes. The mixture was ether extracted 3 times as in the monophasic method.

Preparation of cells. L-cells were grown in 60 mm Petris in Eagle's (Eagle, 1955) medium supplemented with 10% horse serum in the humidified atmosphere of a continuous-flow incubator in a mixture of 3% CO_2 and 97% air. Sheets were formed in 4 - 7 days. Growth medium was drawn off and plates washed 4 times, each time with 4 ml of phosphate buffered saline (PBS) (Dulbecco and Vogt, 1954) without calcium chloride. A 30-minute incubation period was allowed between second and third washings for calcium depletion of the cells. Cells for control plates were washed similarly except with standard PBS. Cell sheets were observed under low power dissecting microscope; some rounding up of the cells occurred as a result of calcium depletion.

Plaque formation by EMC virus infectious RNA. Calcium-depleted L-cells were infected with EMC virus RNA prepared by either of the two methods described above and suspended in PBS containing 0.25% dibasic calcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$). Control plates received RNA suspended in PBS. Following a one-hour infection period, plates were overlaid with 4 ml per plate of altered Eagle's maintenance medium (Dubes and Wenner, 1957) containing 0.2 mM L-cystine and 0.9% washed agar. Plates were kept 3 days in continuous-flow incubator then stained with neutral red. Results are shown in Table I. The combination of calcium depletion of cells and use of facilitator in inocula greatly increased number of plaques produced from both kinds of RNA preparations.

Table I

Effect of facilitator and calcium depletion on plaque production by preparations of EMC virus RNA on L-cells

Expt. no.	RNA ≠ preparation	Mean no. of plaques/plate* ± S.E.**			
		RNA + $CaHPO_4 \cdot 2H_2O$		RNA + PBS	
		Ca^{++} depleted cells	Control cells	Ca^{++} depleted cells	Control cells
1	Monophasic	58 ±5	6 ±2	2 ±0.6	0
	Extractive	49 ±3	10 ±2	4 ±1	1 ±0.1
2	Monophasic	34 ±2	9 ±1	2 ±0.5	0
	Extractive	29 ±2	4 ±1	0	0

*Average of 10 plates
**S.E. = standard error
≠ = Concentration 0.05

Influence of various facilitators on plaque production by EMC virus RNA. The results of experiments in which chromic oxide and talc were compared with $CaHPO_4 \cdot 2H_2O$ as facilitators of infection of calcium-depleted L-cells with EMC virus RNA are shown in Table II. Each of these compounds (0.5% concentration) resulted in increased plaque formation by RNA.

Table II

Comparison of effect of various facilitators on plaque production by EMC virus RNA on calcium-depleted L-cells

Expt. no.	Mean no. of plaques/plate* ± standard error			
	RNA + $CaHPO_4 \cdot 2H_2O$	RNA + talc	RNA + chromic oxide	RNA + PBS
1	46 ±5	29 ±3	25 ±3	0
2	63 ±8	37 ±2	14 ±2	0
3	29 ±3	18 ±3	19 ±3	0

*Average of 7 plates

Extraction of infectious RNA from intact EMC virus pellet. Tissue culture fluid from EMC virus infected L-cells was centrifuged for 10 minutes at 1,500 rpm; supernatant containing 2 x 10^8 plaque forming units (PFU)/ml was then centrifuged in an analytical ultracentrifuge for 1 hour at 100,000 g. Both the supernatant and pellet were extracted with phenol by method of Gierer and Schramm. The facilitation system described above was used to assay the infectious RNA content in both fractions. $CaHPO_4 \cdot 2H_2O$ at a concentration of 0.5% was used as facilitator. A one-hour infection period at 37° C was allowed. Results are shown in Table III. No infectious RNA was detected in the supernatant; the pellet, however, yielded infectious RNA.

Table III

Plaque production on L-cells by RNA extracted from EMC virus following ultracentrifugation

Material	Titer before phenol (PFU/ml)	Mean no. of plaques/plate* (± S.E.**) after phenol
Infected L-cell tissue culture fluid after 1,500 rpm	2 x 10^8	29 ±5
Supernatant after 1 hr. at 100,000 g	3 x 10^5	0
Pellet after 1 hr. at 100,000 g	5 x 10^8	44 ±8

*Average of 6 plates; inoculum was 0.3 ml of 0.25 concentration of phenol-treated material.
**S.E. = standard error.

These results indicate that infectious RNA of the mutant EMC virus studied was derived, at least mainly, from the intact virus particles. However, Huppert and Sanders (1958) reported results indicating that EMC virus infected ascites tumor cells, or the extracellular fluid into which virus had been released upon cell destruction, contained, in addition to virus, a component capable of virus synthesis when phenol treated. The difference between their results and those shown in Table III may possibly be related to use of a different cell strain and of a mutant EMC virus in this study.

The role of the facilitator in the process of cellular infection by EMC virus RNA appears to be the provision of an artificial cell-entry mechanism, as with poliovirus (Dubes and Klingler, 1961). This is accomplished by adsorption of the RNA on the facilitator which falls onto cells and adheres tightly, thus enabling RNA to enter cells and convey genetic information necessary for virus synthesis.

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