Suspended Cell Tissue Cultures for Study of Virus Growth Kinetics.* (20438)

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The embryonated hen's egg, the deembryonated egg, and, more recently, tissue cultures using the chick embryo chorioallantoic membrane have been widely used in the study of the growth of influenza virus(1-13). The present investigation was undertaken to appraise the suspended cell tissue culture for the quantitative determination of the multiplication of this agent. Attention has been focused on early virus production as measured by titration of infectivity in tissue culture flasks.

Materials and methods. Virus. The PR8 strain of influenza A virus propagated in chick chorioallantoic membrane tissue culture has been used in all experiments. This agent was received from Dr. Frank Horsfall 15 years ago and has been maintained since then by allantoic passage in the chick embryo. The stock virus consisted of the fluid removed from cultures inoculated 36 to 48 hours previously with 0.1 ml of infected allantoic fluid diluted 10^{-3}. It was stored at —70°C in glass-sealed ampules.

"Titration" flasks. For titration of viral infectivity at various intervals tissue culture flasks such as those used by Enders and his coworkers(14,15) were prepared with scissor-minced chorioallantoic membrane tissue from 9-11 day chick embryos. Three ml of a mixture consisting of 3 parts Hanks' balanced salt solution and one part Simms' ox serum ultrafiltrate was added to each 25 ml Erlenmeyer flask. The medium also contained 50 units of penicillin and 50 μg of streptomycin per ml. A 50% suspension of the minced tissue was prepared by centrifuging the fragments at 1000 rpm for 5 minutes and adding the required volume of Hanks-Simms' solution. An equal number of drops (2 or 3) of the well agitated suspension was delivered into each flask from a large bore capillary pipette avoiding the first and the last few drops in the pipette. Inspection of the flasks revealed that tissue could be distributed in this way with less than a 2-fold variation in quantity (counts of fragments) from flask to flask. The flasks were stoppered tightly and incubated at 35°C.

"Reaction" flasks. To avoid significant changes in the volume of the culture medium that would result from the repeated removal of aliquots for titration from cultures containing small quantities of fluid a larger system was employed for the study of virus multiplication. To Erlenmeyer flasks of 250 ml capacity 21 ml of Hanks' balanced salt solution (without serum ultrafiltrate) containing 50 units of penicillin and 50 μg of streptomycin per ml were added. The source of tissue was chorioallantoic membrane from 9-day embryos. Pooled membranes were minced, and the tissue was washed 5 times with Hanks' solution. After each washing the tissue was sedimented by centrifugation at low speed. Seven times as much tissue was employed as in the cultures used for titration. After addition of the virus the flasks were stoppered tightly and incubated at 35°C.

Experimental results. I. *Parallel titrations in tissue cultures and chick embryos.* On the same day parallel titrations of viral infectivity were carried out in tissue culture and in embryonated eggs using a series of dilutions based on a factor of $10^{0.5}$. In each titration the same volume of each dilution was added within a few minutes to groups of flasks and inoculated into the allantoic cavity of embryonated eggs. The endpoint was determined by testing the freshly harvested egg fluid from each egg or tissue culture for the presence of hemagglutinin. The results of 2 parallel titrations are summarized in Tables I and II. Two different preparations of virus were titrated.

II. *Growth of virus in tissue culture following introduction of small inocula.* The increase in infectivity of the supernatant fluid

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TABLE I. Procedures and Results of Simultaneous Titrations of Influenza PR8 Virus in Tissue Cultures and Eggs.

	I	II
Age of chorioallantoic membrane used in flasks	10 days	9 days
Incubation period before inoculation of eggs	12 "	11 "
Inoculum vol used for eggs and for flasks	0.1 ml	0.2 ml
Period inoculated eggs incubated before harvesting	2 days	2 days
Period inoculated flasks incubated before harvesting	6 "	6 "
Titer* in flasks	$10^{-3.4}$	$10^{-4.8}$
Titer* in eggs	$10^{-4.4}$	$10^{-5.3}$

* All infectivity endpoints were calculated by the method of Karber, G., *Arch f. Exp. Path. u. Pharmakol.*, 1931, v162, 480.

TABLE II. Comparison of Tissue Culture and Egg Titration of Influenza PR8 Virus.

Dilution	I		II	
	Eggs	T.C.	Eggs	T.C.
$10^{-2.5}$	6/6	6/6	—	—
$10^{-3.0}$	5/6	5/6	7/7	5/5
$10^{-3.5}$	5/6	3/6	7/7	5/5
$10^{-4.0}$	4/6	0/6	7/8	5/5
$10^{-4.5}$	3/6	0/6	6/8	3/5
$10^{-5.0}$	2/6	0/6	5/8	1/5
$10^{-5.5}$	—	—	2/8	0/5
$10^{-6.0}$	—	—	2/8	1/5
$10^{-6.5}$	—	—	1/7	—
Titer	$10^{-4.4}$	$10^{-3.4}$	$10^{-5.3}$	$10^{-4.8}$

from tissue culture flasks has been studied during the first 72 hours after inoculation. Influenza virus was mixed with the tissue culture medium at 35°C and the mixture was immediately added to the washed and minced tissue in the reaction flask. A sufficient quantity of virus was added to yield a final concentration of 10 ID_{50} (for tissue culture) per ml of the medium. At frequent intervals 0.5 ml of the supernatant fluid from the reaction flask was withdrawn and promptly titrated in tissue cultures. The samples were centrifuged immediately in a Sorvall centrifuge at 13,000 rpm for 10 minutes to remove tissue elements. Two-fold serial dilutions of the supernatant fluids were prepared with isotonic phosphate buffer pH 7.1 and aliquots inoculated into titration flasks. Usually 3 cultures, although occasionally only 2, were used for each dilution; in a few instances 5 or more were employed. After 5 to 7 days the supernatant fluids from each titration flask were tested for hemagglutinin. In every case titration flasks were inoculated within an hour after a sample was withdrawn from the reaction flask. For infectivity titrations of fluids a series of dilutions based on a factor of 2 were employed.

After several preliminary experiments in which the approximate ranges of infectivity were determined at 2-hour intervals following inoculation, large-scale experiments were carried out in which the variation in the titer in one reaction flask was determined during a period of 36 to 72 hours. The results of 2 such experiments are presented in Fig. 1 and 2. The titers are expressed as ID_{50} per 0.1 ml (for tissue culture) of supernatant fluid from the reaction flask. The inoculum for the reaction flask consisted one ID_{50} per 0.1 ml of medium. This was verified by titration of a sampled aliquot in tissue culture flasks immediately after addition of the medium containing virus to the tissue in the reaction flask. The vertical lines drawn through the points represent the variation in titer that might be expected to occur in different determinations of the infectivity on a single specimen of virus. In repeated experiments the titer of a given fluid did not vary by more than a factor of 2.

Discussion. The results of infectivity titrations of influenza PR8 virus in tissue culture flasks and embryonated eggs have been compared. It appears that the egg is a more sensitive indicator of the presence of virus than the tissue culture as employed in these experiments. Infectivity endpoints in cultures are sharp but the endpoints in eggs are higher by a factor of $10^{0.5}$ or $10^{1.0}$. The possibility of increasing the sensitivity of tissue culture infectivity titrations by agitation or rocking or of adopting another type of culture such as the roller tube has not been tested. Fluids were tested for hemagglutinin at any time from the fifth to the ninth day after inoculation of the flasks. Hemagglutinin had not emerged after the fifth day, and no positive flasks became negative for hemagglutinin on standing until the ninth day. Dilution steps based on a factor of 2 have been successfully employed using 3 or more flask replicates for each dilution. For relative estimates of virus

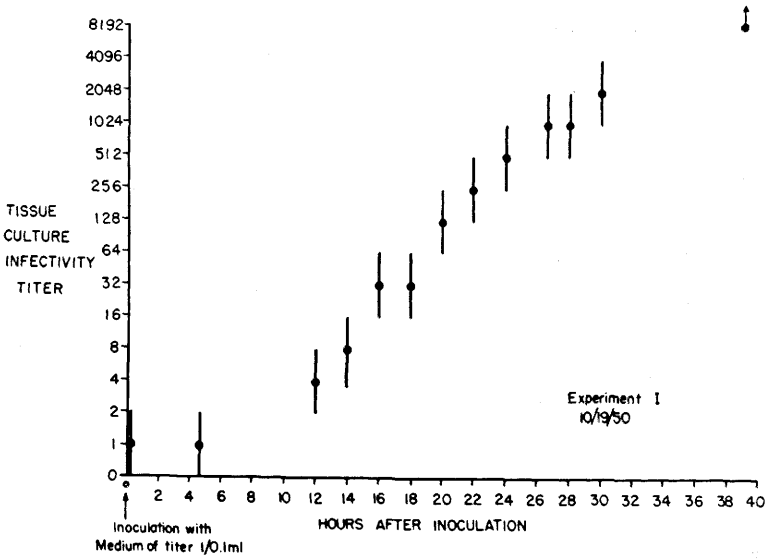

FIG. 1. Growth of influenza PR8 virus in chick embryo chorioallantoic membrane suspended cell tissue culture (large flask).

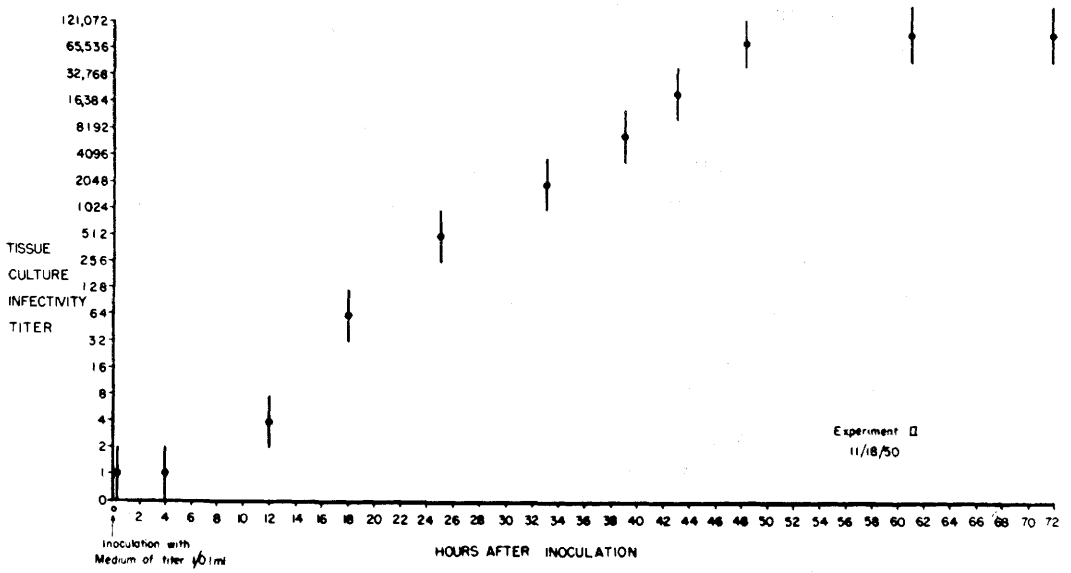

FIG. 2. Growth of influenza PR8 virus in chick embryo chorioallantoic membrane suspended cell tissue culture (large flask).

infectivity tissue culture flasks provide a uniform testing system, simple to prepare in large numbers and easy to manipulate. Six to 8 flasks may be prepared from a chorioallantoic membrane derived from one 10-day embryo.

The growth curves in Fig. 1 and 2 illustrate the reproducibility of results that may be obtained with the technics here described. The 2 experiments were conducted one month apart employing the same infecting dose of virus. Several other similar experiments gave equally consistent results. Since these early growth curves are consistently reproducible it may be concluded that the procedure may be employed as a means of determining the effect

of variation in the physical conditions or composition of the culture medium on viral multiplication. Even relatively small changes in the shape of the curve would appear to be significant.

Several curves reflecting the decay of virus infectivity at 35°C and at various pH ranges in Hanks' balanced salt solution with the addition of antibiotics but in the absence of tissue have been determined for different initial concentrations of virus. After 4 hours there was no measurable loss of virus infectivity, but after 24 hours this had declined by a factor of $10^{0.7}$ from an initial titer of $10^{-4.3}$. Although it may be objected that such decay curves may not represent the loss of infectivity occurring in reaction flasks *in the presence of living tissue,* the correction of the growth curves of Fig. 1 and 2 for decay as determined in the absence of tissue might be justified.

For accurate growth kinetic studies of mammalian viruses similar to those available to workers with bacteriophage the suspended fragment tissue culture is far from the ideal, *i.e.,* cultures of uniformly suspended pure strain cells which may be accurately quantitated. Progress toward this ideal has been made by Evans and her coworkers(16,17) who have developed pure tissue culture lines from single cells and succeeded in producing quantitatively uniform replicate cultures by counting the viable cell nuclei in cell suspensions of their tissue inocula. But for the present, the simple suspended fragment technic here employed provides a technically uncomplicated and fairly accurate means for the quantitative evaluation of the multiplication of certain viruses in tissue culture.

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