

ADSORPTION AND MATURATION OF POLIOVIRUS IN SINGLY AND MULTIPLY INFECTED HELa CELLS

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The disappearance of an inoculum in an experimental virus-host complex (eclipse phenomenon) is well documented for many systems (1-6). In some cases, the appearance of new intracellular virus has been shown to precede release into the surrounding medium or tissue. Examples of this are influenza virus in chorio-allantoic membrane (1), T2 bacteriophage in *Escherichia coli* B (4), and more recently in mammalian cell culture systems, Western equine encephalomyelitis virus (WEE) in chicken fibroblasts (6), and Type I poliovirus in monkey kidney epithelium (7). In these instances analysis of the events in a single cycle of virus infection suggests that the infecting particle disappears, imparts its message to the synthesizing mechanisms of the cell, and that new virus appears after a characteristic time lapse. The present experiments were designed to study the phases of a single cycle of poliovirus multiplication in monolayer cultures of HeLa cells. The findings confirm and extend the earlier reports of Howes and Melnick in monkey kidney cells (7) and by Maassab *et al.* in HeLa cells (8).

Methods and Materials

Virus.—A single large pool of a Type I Mahoney strain of poliovirus was prepared by infecting cell cultures in 1 liter bottles in the absence of nutrients except Earle's saline (9) and 2 mM glutamine.

Cell.—A clonal strain (S-3) of HeLa cells originally isolated by Puck (10), and obtained from Microbiological Associates, Bethesda, Maryland, was grown in monolayer culture in Eagle's medium (11) supplemented with 10 per cent whole human serum until the time of experiments.

Infection Procedure.—Replicate cell cultures growing as monolayers in T-30 culture flasks (12) were used for experiments 72 to 96 hours after planting at which time cell counts by the method of Sanford *et al.* (13) had reached 2.5 to 3.0×10^6 per flask. After infection the flasks were incubated for 30, 60, or 120 minutes at 37°C. on a rocking platform which rotated 30° every 40 seconds promoting the uniform distribution of inoculum. For high multiplicities, *e.g.* virus/cell ratio of 10:1, 1 ml. of stock inoculum was used, while for low multiplicities, 1 ml. of a 1:100 or 1:150 dilution was used. Samples of the supernatant fluid at the conclusion of the adsorption period, as well as of the original inoculum incubated in the absence of cells, were titrated to determine the number of plaque-forming units (P.F.U.) adsorbed per flask.

Excess virus was removed by washing four times with Earle's saline. Flasks were then refed with 10 cc. of warm Earle's saline containing 5 mM glucose and 2 mM glutamine and returned to the incubator at 37°C. It has been shown that these two nutrients together with the salts in Earle's saline, permit maximal viral yields from monolayer cultures of HeLa cells (14). Duplicate flasks were taken for cell-rupturing procedures on conclusion of washing and re-feeding and at intervals thereafter. Samples of the supernatant fluid from each flask were pooled immediately prior to the rupturing procedures ("free virus") and samples of the suspension taken on completion of treatment ("total virus").

Cell-Rupturing Procedures:

(a) *Sonic Vibration.*—The viral titer of a stock suspension was unaffected by a 5 minute exposure to sonic vibration from a 9 KC Raytheon sonic oscillator. The combined contents

TABLE I
Variability in Poliovirus Yield and Plaque Assay in HeLa Cells

Replicate flasks	Viral titer in 18 hr. supernatants P.F.U./10 ⁻⁶ cc.	Average	Standard deviation
Flask 1	52, 48, 40, 36, 31, 49	42.7	±8.3
Flask 2	39, 39, 40, 41, 42, 46, 39	40.9	±2.4
Flask 3	47, 37, 29, 36, 37	37.2	±5.8
Flask 4	36, 33, 16, 29, 32, 33	29.8	±7.2

The supernatant fluids from four replicate flasks containing 5.0×10^6 cells infected at a high multiplicity (10:1) were harvested for titration 18 hours after infection. Samples were diluted in duplicate and at least two plates inoculated with each dilution tested. The data indicate the reliability of a single titration to be ±20 per cent.

of two infected flasks were given the 5 minute sonic vibration treatment at varying time periods after inoculation. Cells were harvested for treatment by scraping them into the overlying medium with a rubber policeman. Between uses the sonic vibration chamber was cleaned by rinsing with a disinfectant, then with distilled water, and dried with acetone.

(b) *Freeze-Thaw Technique.*—Three cycles of rapid freezing and thawing were carried out by immersing flasks alternately in an acetone-dry ice bath and a 37°C. water bath. Approximately 2 to 3 minutes per cycle were required for complete freezing and thawing.

Viral Assay:

Viral suspensions were assayed by a modification of the Dulbecco and Vogt technique (15) using HeLa cells rather than monkey kidney epithelium.¹

2.0×10^6 HeLa cells per 60 mm. Petri dish or 5.0×10^6 cells per 100 mm. dish were planted in Eagle's medium supplemented with 10 per cent whole human serum and 0.2 per cent lactalbumin hydrolysate (acid). Cultures were refed after 24 hours and used 48 hours after planting. Cells were washed free of human serum, inoculated with a dilution of virus suspension in Earle's saline, and overlaid with a medium consisting of 2 parts Eagle's medium 1.5 times concentrated, 1/20,000 neutral red and 4 per cent horse serum, and 1 part 3 per cent bacto-agar (Difco). Because of early death of the underlying cell sheet Noble's agar could not be used. Plaques were counted at 48 and 72 hours. Table I illustrates the reliability of repli-

¹ We are grateful to Dr. B. Mandel, Division of Infectious Diseases, The Public Health Research Institute of the City of New York, Inc., for suggestions which aided in the use of this technique.

TABLE II
Comparison of Sonic Vibration and Freeze-Thaw Procedures for Release of Poliovirus from HeLa Cells Early and Late in the Infectious Cycle

Treatment	Total virus in culture*					
	1.5 hrs. postinfection			8 hrs. postinfection		
	Dilution	P.F.U./ml.	Titer/ml.†	Dilution	P.F.U./ml.	Titer/ml.†
Sonic vibration	10 ⁻³	108, 120	1.1 × 10 ⁵	5 × 10 ⁻⁶	110, 107	2.2 × 10 ⁷
	10 ⁻⁴	9, 13		5 × 10 ⁻⁷	10, 9, 9	
Freeze-thaw	10 ⁻³	120, 121	1.1 × 10 ⁵	5 × 10 ⁻⁷	24, 14,	3.5 × 10 ⁷
	10 ⁻⁴	10, 11			15	

Replicate HeLa cell cultures infected at a high multiplicity (10:1) were given the indicated treatment 1.5 and 8 hours postinfection. Titrations of the resulting suspensions gave total virus in a culture.

* Not shown in table are values for free virus in supernatant fluid at time of cellular disruption. Free virus amounted to less than 1 per cent of total virus at 1.5 and 8 hours in this experiment.

† Each flask contained 10 cc.

TABLE III
Adsorption of Poliovirus to HeLa Cells and Recovery by Cellular Disruption

Experiment	Adsorption of inoculum					Recovery of adsorbed virus from washed cultures		
	Cells/Flask	Adsorption time	Residual virus titer/ml.*	P.F.U. adsorbed		Total P.F.U. per flask		Recovery of adsorbed virus†
				Per flask	Per cell	Supernatant before freeze-thaw	Suspension after freeze-thaw	
1	2.7 × 10 ⁶	0§	8.0 × 10 ⁷	3.3 × 10 ⁷	13	4.5 × 10 ³	1.2 × 10 ⁶	3.9
		1 hr.	4.6 × 10 ⁷					
2	2.0 × 10 ⁶	0§	1.2 × 10 ⁸	4.0 × 10 ⁷	20	1.4 × 10 ⁴	2.0 × 10 ⁶	5.0
		30 min.	8.0 × 10 ⁷					
		1 hr.	7.1 × 10 ⁷					
		2 "	7.4 × 10 ⁷	4.9 × 10 ⁷	24	1.8 × 10 ⁴	1.5 × 10 ⁶	3.1
			4.6 × 10 ⁷	23	2.0 × 10 ⁴	2.8 × 10 ⁶	6.1	
3	2.0 × 10 ⁶	0§	8.3 × 10 ⁵	4.1 × 10 ⁵	0.20	8.0 × 10 ¹	1.2 × 10 ⁴	2.9
		30 min.	4.2 × 10 ⁵					
		1 hr.	3.6 × 10 ⁵					
		2 "	3.2 × 10 ⁵					
			5.1 × 10 ⁵	0.26	1.7 × 10 ²	1.0 × 10 ⁴	2.0	

* 1 ml. inoculum used per flask.

† Per cent recovery, total P.F.U. per flask after freeze-thaw/P.F.U. adsorbed per flask.

§ Inoculum incubated for 1 hour at 37° in absence of cells.

cate titrations of given samples, as well as the range of variation in yields between replicate infected flasks.

RESULTS

Sonic Vibration versus Freeze-Thaw.—As can be seen in Table II sonic vibration and the freeze-thaw technique were found to be equally effective in recover-

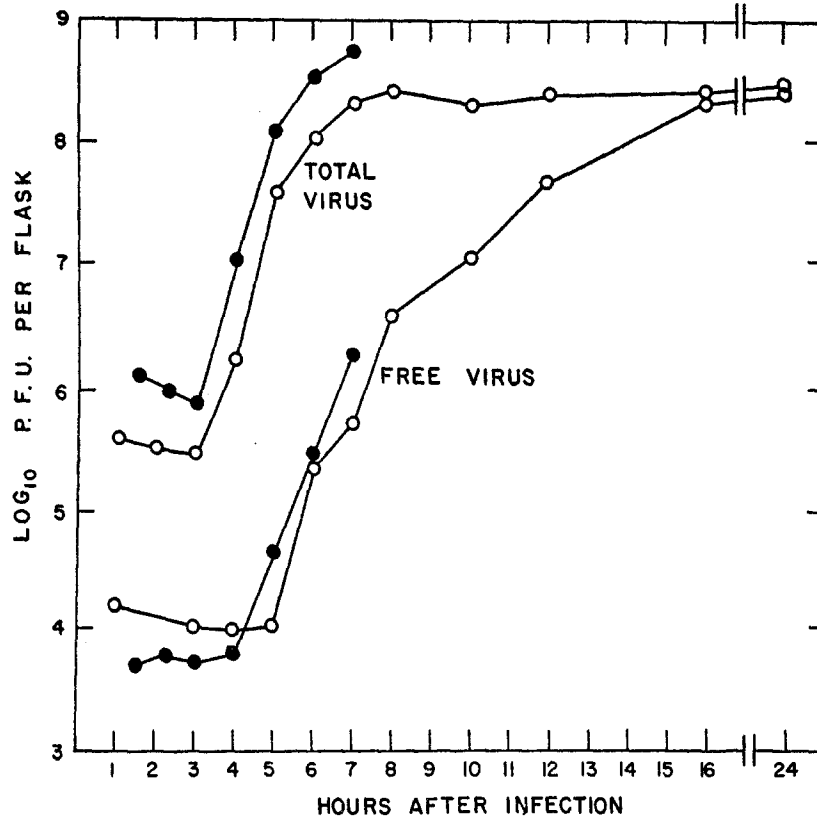


FIG. 1. Increase in total virus (intracellular virus) prior to release as free virus (extracellular virus) in Type I poliovirus-HeLa cell system. ●, adsorbed multiplicity of 13:1, ○ adsorbed multiplicity of 24:1.

ing virus from infected cells either early or late in course of infection. In that Table the "1.5 hours postinfection" column represents virus which remained in or on the cells after adsorption and four washes with Earle's saline. The 8 hour value represents new virus, since this was 10 times as much virus as had been originally adsorbed in this experiment. After the equal efficacy of sonication and freeze-thaw procedures had been established only the freeze-thaw procedure was used in most of the experiments described.

Adsorption of Virus.—At virus/cell multiplicities of 1:30 to 20:1 approximately 30 to 40 per cent of a viral inoculum was adsorbed in 1 hour by 2.5 to 3.0×10^6 cells on a 33 cm.^2 surface (Table III). This agrees with the data of Youngner for adsorption of poliovirus on monkey kidney epithelium at low multiplicities (16). As seen in Table III, varying exposure time from 30 minutes

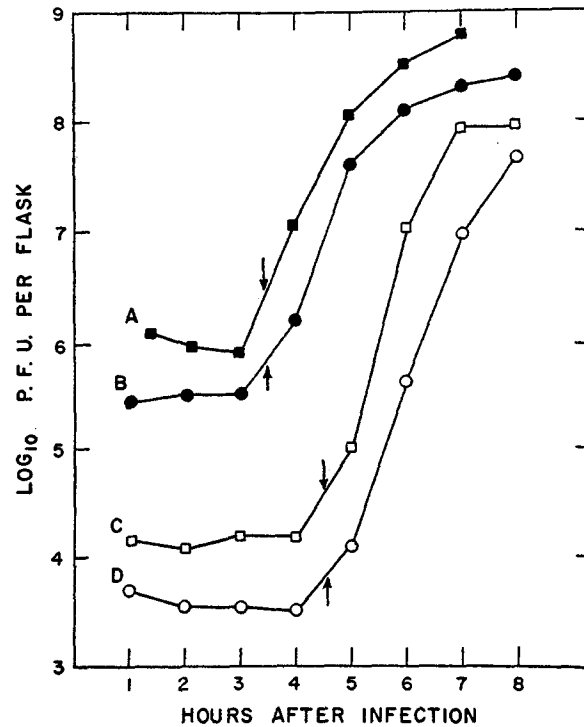


FIG. 2. Appearance of intracellular virus (virus released by cellular disruption) from multiply and singly infected cells. A, virus cell multiplicity 10:1, B, virus cell multiplicity 20:1, C, virus cell multiplicity 1:4, D, virus cell multiplicity 1:30. Arrows indicate onset of intracellular virus accumulation.

to 2 hours had only slight effect on adsorption. About 2 to 6 per cent of the virus adsorbed was recoverable from the cells by disruption immediately after adsorption and removal of excess virus. It is clear that this was adsorbed virus, since the last wash fluid prior to the sonic vibration or freeze-thawing contained only 1 per cent or less of that recoverable after cellular disruption. The recoverable fraction of adsorbed virus did not seem to vary with the length of the adsorption period or the effective multiplicity. The latter fact is particularly surprising for in some experiments (*e.g.* Experiment 3, Table III) multiplicities of 0.2 adsorbed viruses per cell indicate that any infected cell received only one P.F.U.

New Virus Formation.—After infection at a high multiplicity, the amount of total virus in a culture remained constant for 3 hours, and of this total only 1 per cent or less was free virus (Fig. 1). Between the 3rd and 4th hour after infection total virus began to increase, and continued to increase at a logarithmic rate until 6 or 7 hours, by which time the maximum virus yield was reached. The liberation of virus into the supernatant medium did not begin until between the 5th and 6th hour, 1 to 2 hours after it began to appear in the cells. Release proceeded at a slower rate than production, so that maximal levels of free virus were obtained only after 16 hours of infection. At 6 hours, for instance, the cells contained 1000 times as much virus as there was in the overlying fluid.

After infection with low multiplicities, total virus did not begin to increase until between the 5th and 6th hour postinfection or 1 hour later than with high multiplicities (Fig. 2). Once initiated, the rates of appearance seemed to be independent of multiplicity. Although not shown in Fig. 2, liberation into the medium again began approximately 1 hour after new virus appeared in the cells. Free virus was followed through only 8 hours in these cultures where large numbers of cells were uninfected by the original inoculum, and a second cycle of infection was certain to ensue.

DISCUSSION

As with many other viral systems, virtually all of the infecting virus disappeared in these experiments, although 2 to 6 per cent of adsorbed infecting virus remained intact and could be recovered on cellular disruption. In explaining the residual virus, the following possibilities may be considered: (a) the recoverable virus may initially be loosely attached to the cells. If this were the case one might expect either subsequent elution or attachment and disappearance as plaque-forming units. Elution of any significance, however, does not occur (Fig. 1), since there is no rise in free virus in the early hours of infection. If firm attachment and disappearance as plaque-forming units do take place in this portion of the viral population, then new virus must arise to take its place, because the amount of cellular virus is constant for 3 hours postinfection. (b) This fraction may be attached to cells which for some reason destroy virus slowly or fail to inactivate it at all, and in consequence fail to become infected. The fraction of cells involved would be too small to detect either by plating infected cells or attempting to prove a 5 per cent decrease in the total expected yield. (c) In experiments with higher multiplicities it is possible that only the first few adsorbed particles disappear as infectious units. If this were the case, however, one would not expect residual virus in cells infected with such low multiplicities that any infected cell would have only 1 adsorbed virus. (d) Finally, since a plaque-purified strain of virus was not used in these studies, it may be that because of a genetic difference 5 per cent of the population is adsorbed but resists destruction as plaque formers. This has not been tested.

In agreement with previous investigations (7, 8), these experiments demon-

strate a lag of 1 to 2 hours between the synthesis of poliovirus and the onset of release. In consequence, the "total virus" is 100 to 1000 times the amount of the "free virus" throughout the most active period of virus production. This is in contrast to the case of WEE virus² and chicken fibroblasts, in which the average intercellular life of a completed P.F.U.³ is about 1 minute (6). Two factors could make free virus appear falsely low in these experiments, (a) read-sorption and (b) intercellular trapping of released virus within the monolayer. It seems apparent, however, from these studies and others on adsorption by monolayers (16) that this is a relatively inefficient process, especially when there is a large excess of medium diluting the released virus.

With regard to trapping, if the free surface of monolayers releases virus in proportion to its area, as suggested by Howes and Melnick (7), then one might expect that from 20 to 40 per cent of virus would make its egress directly from cells into fluid. Intercellular trapping therefore could account for at most $2\frac{1}{2}$ to 5 times as much virus as had been released during the first 3 to 8 hours of infection, while the observed disparity between total and free virus is 1000 to 1. It thus appears that the relatively long intracellular life of the poliovirus is real.

Another comparison with the WEE chicken fibroblast system is of interest. Dulbecco has shown that the latent period (time from adsorption to extracellular appearance of new virus) in suspensions of chicken fibroblasts is shorter in multiply infected cells than in singly infected ones (17). This is confirmed for the HeLa cell-polio system here, and in addition it is further shown that the length of the eclipse period (time from adsorption to intracellular appearance of new virus) is also decreased by high multiplicity. Furthermore, it would appear that the shortening of the eclipse with high multiplicities is the controlling factor in the shortening of the latent period.

There are a number of possible explanations of this phenomenon. If there were more than one site of viral replication in these cells, then in a singly infected cell the first virus produced might be undetectable because it would immediately attach to and activate other sites, while in the case of a multiply infected cell intracellular virus might be detected earlier because all sites would begin producing simultaneously. Another possible explanation would lie in a mass action effect of many viruses on a single viral synthesizing apparatus. In either case however, once intracellular virus appeared it could continue to increase at the same rate, in accordance with the observed data.

SUMMARY

Studies with polio-infected HeLa cells using cell disruption techniques during the early stages of infection have shown that:

² Western equine encephalomyelitis virus.

³ Plaque-forming unit.

The inoculum of poliovirus adsorbed on HeLa cells is largely inactivated.

Infective virus accumulates in a monolayer sheet of cells in advance of release into the medium.

The eclipse period is shorter in multiply infected cells than in those infected with a single particle.

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