Synchrony and the Elimination of Chance Delays in the Growth of Poliovirus

By P. D. Cooper*

Virus Culture Laboratory, Medical Research Council Laboratories, Carshalton, Surrey

(Received 11 June 1964)

SUMMARY

The effect of multiplicity of infection on the growth kinetics of poliovirus suggests that the eclipse period usual for singly infected cells (3.6–3.8 hr) includes variable delays averaging about 1 hr. These delays are overcome at multiplicities above 3, and are sometimes spontaneously absent. They are ascribed largely to chance effects, and lead to markedly asynchronous maturation of virus; in their absence, maturation is almost synchronous.

The kinetics of acid-irreversible eclipse and of the development of antiserum resistance show that about half of the delays must occur during viral penetration; in support of this, virus growth initiated with infective RNA is 0.5 hr less delayed than that of intact virus, although otherwise similar. However, infective RNA synthesis was not detected earlier than 2 hr after infection, even in the absence of chance delays.

INTRODUCTION

The events to be observed during a single cycle of virus growth usually form a reproducible sequence. However, even if the cells are infected simultaneously, all cells may not reach a given part of the sequence at the same time (Cairns, 1957). Such asynchrony occurs in many viral systems, and may at times hinder the study of virus growth as much as non-simultaneous infection of the cells. One cause of asynchrony is the presence of chance hesitations between growth steps (‘random or variable delays’) and it is important in studying virus growth to know the average duration of the viral processes as compared with such delays. It is theoretically possible, for example, that the eclipse period results solely from very large random delays.

This work investigates the extent of delays and asynchrony in the growth of poliovirus in ERK cells, and ways of avoiding them. Of the eclipse period of 3.6 hr which is usual at single multiplicity of infection, nearly 1 hr may be regarded as occupied by random delays. These delays result in substantial asynchrony of virus maturation, and a considerable portion of them appears to occur during stages of penetration. However, random delays and asynchrony are sometimes absent, probably because of factors affecting the state of the cells, which are not understood. Random delays are also eliminated by large multiplicities of infection, which result in considerable synchrony of viral maturation and a reproducibly short eclipse period of 2–6–2.7 hr.

* Present address: Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia.
METHODS

Virus. Poliovirus type 1 (strain Brunenders) was used; virus and infective cells were assayed by the agar cell-suspension plaque method (Cooper, 1961). Infective RNA assays used the procedure described by Cooper (1962). All host cells were from the ERK line grown in medium CSV6 (Cooper, Wilson & Burt, 1959).

One-step growth method. ERK cells were resuspended from bottle cultures with trypsin (2.5 mg./ml.) plus EDTA (2 mg./ml.) to give a monodisperse suspension, and maintained overnight as a suspension culture at 36°. Cells were infected to various multiplicities in suspension at 0°, washed and added to medium at 37°. The pH was controlled at pH 7.3 by replacement of glucose with galactose and by gassing with 5% CO₂ in air. The cells were kept in suspension by a ‘New Brunswick’ type of rotary shaker. The final cell suspension was assayed for total cells (5 - 10 x 10^8/ml.), infective cells (50-80% of total cells when fully infected) and virus free (less than 5% of infective cells); < 10% of the cells were in clumps of 2 or more, and < 5% were non-viable. The multiplicity of infection was determined from assays of the virus added and of the virus recovered after washing the cells. Samples were taken at intervals during virus growth into 2 mg./ml. deoxycholate for assay of cell-associated mature virus. Growth is expressed in terms of plaque-forming units (p.f.u.) produced per infective cell, and the eclipse period equals the time from addition to warm medium to the time of maturation of 1 p.f.u. per infective cell.

Sampling procedure for determination of yield per yielding cell. Portions of infected cell suspensions were taken during 1-step growth, chilled and diluted to 5, 10 and 20 infective cells/ml.; 0.05 ml. samples of these dilutions were added to each cup of an 80-cup haemagglutinin tray, and then frozen in solid CO₂ and thawed. Fresh cells were added to each cup (0.05 ml. containing 10^6 cells), followed by 0.1 ml. of molten agar medium. The cup contents were very rapidly mixed, removed and dropped on to chilled agar base layers contained in 4 in. Petri dishes; the drops were spread somewhat before they set. Eight samples were accommodated per dish, and 600-700 samples were plated in 4-5 hr on 80 Petri dishes. The plates were then incubated as for a normal plaque assay, when the plaques developing in each sample could be counted; only those dilutions in which about 50% of the samples contained plaques were selected for counting.

RESULTS

The effect of multiplicity of infection on the growth of poliovirus. If there are variable delays in a viral growth process caused purely by probability effects, then increasing the multiplicity of infection should decrease the overall chance of delay for any given infected cell (Cairns, 1957). The eclipse period at single multiplicity was delayed to 3-6 hr but increasing the number of adsorbed particles decreased the eclipse period to 2-6-2.7 hr (Fig. 1). These values were quite reproducible, and eclipse periods were never less than 2-6 hr however high the multiplicity (maximum tested equals 80). Figure 2 suggests that the eclipse periods are made up of a component of up to 1 hr which is variable with multiplicity plus an invariable component of 2-7 hr. The shortening of the eclipse period was negligible above a multiplicity of 8. An arithmetical plot (Fig. 3) of the data of Fig. 1 shows that the rate of maturation tends to be constant and that, at very low multiplicities, virus matures.
more slowly. Similar curves were found for influenza virus under conditions of delay and asynchrony (Cairns, 1957). Darnell (1958) and Howes (1959, a, b) reported that higher multiplicities increased the growth rate of poliovirus.

Fig. 1. One-step 'delayed state' maturation curves of poliovirus in suspended ERK cells at 37°. Six cultures from one batch of cells were simultaneously infected with various multiplicities of virus, which are indicated on the figure. The short vertical lines join each curve at the point where the internally matured virus equals the infective cell count (end of eclipse period).

However, in rather less than half of our experiments the growth rate at single multiplicity of infection did not show any delay (Fig. 4): the eclipse period was 2·7 hr, maturation was rapid and exponential in rate, and increasing the multiplicity had no effect. The reasons for this lack of delay are not known, but are suspected to lie in the metabolic state of the cell. In the growth curves of Fig. 4, the increase of infective RNA was also measured for the two cultures, and was the same for both. The RNA curve from the lower multiplicity culture is shown, and indicates
that, even in the absence of delay, an increase in infective RNA was not detected earlier than 2 hr after infection. This aspect is dealt with more fully in the accompanying paper (Cooper, 1964).

![Graph](image)

**Fig. 2.** The relation for poliovirus between the reciprocal of the multiplicity of infection (1/M) and the eclipse period. One experiment (●) is that of Fig. 1; the other (○) is a replicate. Multiplicity of infection in this case equals the average number of particles adsorbed per infected cell (minimum equals 1), and is calculated assuming a Poisson distribution of virus among cells.

**Fig. 3.** One-step ‘delayed state’ maturation curves of poliovirus in suspended ERK cells at 37°. The data are the same as those of Fig. 1, and the multiplicities of infection are indicated on the figure.

*Synchrony and asynchrony in maturation of poliovirus.* The synchrony of maturation was examined by the following direct method, using delayed cultures and cultures in which delay was spontaneously absent or was eliminated by a high multiplicity of infection. One-step growth experiments were performed in which samples of intact cells were taken at times covering the estimated end of eclipse (2.5–4 hr). A series of dilutions of each of these samples was made, and 0.05 ml. of each dilution was added to every cup of an 80-cup haemagglutinin tray. The dilutions were such that, for each sample, at least 1 tray contained an average of about 1 infective cell per two cups. After freezing the samples in the trays and thawing to liberate the virus, the contents of each cup were plated by the procedure already described. The assays from those trays in which about half the cups yielded no or negligible virus enabled the infective virus content of individual cells to be calculated. Three representative experiments are shown in Table 1. In Exp. 1 the eclipse period at single multiplicity of infection was 2.6 hr and virus maturation was rapid and almost exponential, and so random delays were presumed to be absent. The degree of asynchrony was in fact very small; the distribution of virus
among cups was approximately Poissonian, and the number of cups yielding virus at the end of the eclipse period was almost that to be expected from the infective cell assay, so that almost all potentially yielding cells contained some virus at this time.

Table 1. *The distribution of poliovirus among cells of three cultures experiencing different random delay effects*

The one-step growth and sampling procedures are described in the Methods.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Multiplicity of infection</th>
<th>Eclipse period (hr)</th>
<th>Presumed state</th>
<th>Time of sampling (hr)</th>
<th>Yield per yielding cell</th>
<th>Plaques per sample (each sample is presumed to contain only 1 infective cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p.f.u.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

* A replicate experiment performed simultaneously but at single multiplicity had an eclipse period of 3-6 hr.
† Released virus.

In Exp. 2, the maturation rate was slow and the eclipse period was 3.7 hr; random delays were presumed to be present. This experiment showed much more asynchrony than the first in that the yields per cell varied widely and the proportion of cells yielding no virus was higher than that to be expected from the average yield per cup. In Exp. 3, the culture examined had a multiplicity of 5, and an eclipse period of 2-6 hr, while a replicate culture at single multiplicity grown simultaneously had an eclipse period of 3-6 hr. Hence it is presumed that these cultures were in a state in which each adsorbing particle would be subject to random delays but that such delays were overcome for the infective cell by a high multiplicity. Table 1 shows that overcoming the delays in this way also removed the asynchrony. Thus delayed cultures were highly asynchronous in maturing virus, while cultures with little intrinsic delay or in which delay was overcome by high multiplicity were much less asynchronous.

*Delays in entering acid-irreversible eclipse.* Of several stages detected during the penetration of poliovirus, the transition to non-recoverability of infectivity with pH 2-5 buffer ('acid-irreversible eclipse') appears to be the last (Fenwick & Cooper, 1962). The rate of acid-irreversible eclipse in the system used (Fig. 5) shows that the time taken to inactivate the virus in this way can occupy a significant portion of the eclipse period, as 50% of the particles in Exp. A were delayed by at least 0.5 hr. However, the rate of eclipse was not reproducible between experiments (in Fig. 5, curve B, the 50% time was only 0.15 hr) indicating that delays involved in eclipse were variable and were less in some experiments than in others.
Delays in development of antiserum resistance. To see whether delays were introduced at a stage earlier than acid-irreversible eclipse, the rates of development of antiserum resistance of the infective cell were compared at different multiplicities of infection. Multiplicity had a marked effect and the development of antiserum resistance was faster at a high multiplicity (Fig. 6). With 10 p.f.u./cell, the transition of almost all cells occurred within a few minutes. As the maximum hastening of antiserum resistance by high multiplicity was only 20–80 min., this source of delay possibly accounts for rather less than half of the delays usually present. However, in several single-multiplicity growth experiments all infective cells became resistant to antiserum in 1–5 min., indicating that the delays involved in this growth step are also variable between, as well as within, experiments.

Comparison of growth curves with virus and with infective RNA as inocula. If the uncoating of the virion leads to variable delays in the growth cycle, then infection of the cell with uncoated genetic material (infective RNA) should reduce this delay. The eclipse period and the time taken to release 1 p.f.u./cell were both 0.5–0.8 hr shorter when using infective RNA than when using intact virus at single multiplicity (Fig. 7). Hence a considerable portion of the delay occurs during the uncoating of the virus.

A control experiment was necessary for the interpretation of Fig. 7. This was because independent experiments with infective RNA labelled with $^{32}$P showed that as much as 5% of the RNA was taken up by the cells when the RNA was added at
high concentration. Thus it was possible that the virus deriving from infective RNA in Fig. 7 did not contain newly synthesized RNA, but inoculum RNA which had been 'recoated'. This possibility was disproved by preparing two replicate batches of virus labelled with $^{32}$P, using non-radioactive infective RNA (extracted from virus at a concentration of $10^{10}$ (p.f.u./ml.) and non-radioactive intact virus respectively as inocula. The procedure for virus growth and purification described by Fenwick & Cooper (1962) was used; the viral radioactivity was isolated from both preparations by means of a potassium tartrate density gradient, and their specific activities were found to be almost identical.

A

I 1 I
0.01 I
I I
30
60
2
3
4
5
6
7
Minutes at 37° Hours at 37°
Fig. 6

Fig. 6. Effect of multiplicity of infection of poliovirus on the development of antiserum resistance of suspended ERK cells at 37°. Multiplicities were 10 (○), 2.5 (△), and 0.01 (●). Infected cells were diluted at intervals into sufficient antiserum to neutralize 99% of free virus in 10 min. at 4°. After 30 min., the cells were diluted free of antiserum and plated as for virus. The cells surviving as infective centres are expressed as a percentage of cell samples containing no antiserum.

Fig. 7. One-step growth curves of poliovirus initiated by infective RNA (○ = cell-associated virus, or CAV; △ = released virus) and by intact virus (● = CAV; ▲ = released virus). Both sets of cultures had been treated identically before infection, including washing with 0.8 M-Na$_2$SO$_4$ at pH 8.0; they were infected as monolayers at 15°, washed rapidly several times with PBS at 37° in a room at 37°, resuspended with trypsin and versene mixture, and washed before adding to warm growth medium. Time zero equals the time of transition to 37°; care was taken that this transition was sharp and permanent.

DISCUSSION

These data indicate that the normal single multiplicity eclipse period of poliovirus, 3-6-3-8 hr in the system studied, includes variable delays of about 1 hr. At least half but not all of these delays occur during penetration of the virus, probably after the reversible 'neutralization' of virus by cells which occurs with little delay (Fenwick & Cooper, 1962). The term 'penetration' is used here to include all stages up to the final uncoating of the genetic material. The delays lead to considerable asynchrony of maturation. Howes (1959a, b) estimated that there was a total spread of
2–3 hr for the termination times of individual eclipse periods of poliovirus in his system. However, delays and concomitant asynchrony are sometimes spontaneously absent, or can be overcome by a high multiplicity of infection; criteria of such effects are a short eclipse period and rapid maturation. Variations in the extent of delay may explain the differences in kinetics of poliovirus growth apparent between other reports (Dulbecco & Vogt, 1955; Darnell, 1958; Howes, 1959a, b; Holland, Hoyer, McLaren & Syverton, 1960; Darnell, Levintow, Thorén & Hooper, 1961). It is clearly desirable, particularly in biochemical studies of virus growth, to eliminate asynchrony during the infective process, and this now appears to be feasible by use of the method described above. An implication of these findings is that practically all of the 2·7 hr minimum eclipse period of poliovirus must be occupied by viral growth processes, rather than by chance hesitations between processes.

It should be noted that the eclipse periods would also be shortened by high multiplicities if each adsorbing particle were able to replicate autonomously but without delays (Cooper, 1958). In this case the variety of individual multiplicities of infection in a culture would yield considerable asynchrony, again resembling the effects of random delay. However, the acid-irreversible eclipse curves and growth curves resulting from infective RNA indicate that much delay must be present during penetration, and hence that the contribution of autonomous growth to the effects found with poliovirus must be correspondingly limited. The fact that increasing the multiplicity above 3 does not further shorten the eclipse period also indicates that fully autonomous growth is not permitted.

The excellent assistance of Mr H. Cumming and Miss J. Constable is gratefully acknowledged.

REFERENCES


