'Shortened Latency' as a Result of Multiple Infection by Vesicular Stomatitis Virus in Chick Cell Culture

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SUMMARY: Above a value of one, repeatedly doubling the multiplicity of infection of chick embryo cells by vesicular stomatitis virus progressively shortened the latent period by about 0.6 hr.; this phenomenon is referred to as 'shortened latency'. Varying the multiplicity above unity with dilute-passage stocks did not interfere with rate of infective virus release, number of cells infected, or final yield, i.e. there was no 'von Magnus' effect or other obvious interference phenomena.

The doubling time for virus release was also about 0.6 hr. This suggested that virus may have been growing as a simple intracellular pool equally accessible to all adsorbing virus, and that 1 particle was released when the pool reached a certain size (perhaps 20-200 units) irrespective of inocula. However, other explanations are possible, and of those allowing experimental test, earlier initial adsorption of virus, multiplicity reactivation amongst a partly inactivated population, more rapid elution of attached virus or more rapid release of accumulated internal virus could not account for shortened latency.

Earlier results (Cooper, 1957*a*) suggested that the release of vesicular stomatitis (VS) virus from completely infected chick embryo cell monolayers occurred earlier when inocula of higher titre were used. More specifically, the latent period (defined here as the time between addition of virus and release of one plaque-forming unit or pfu per infected cell) was shorter at higher multiplicities of infection. Final yields and rates of release were not affected.

These findings are confirmed and extended below. For the present purpose, such a phenomenon resulting solely from varying the multiplicity of infection of dilute-passage stocks (other conditions such as temperature and pH being equal) will be referred to as 'shortened latency'. The effect of undiluted-passage stocks will be considered elsewhere (Cooper & Bellett, to be published). Some quantitative aspects of shortened latency are presented, which have suggested a simple hypothesis for the intracellular increase of viral units; this is elaborated in the discussion of this paper. However, there are several alternative explanations, and an attempt is made to evaluate some of them experimentally.

The possible existence of auto-interference and exclusion, so that not all adsorbed virus may participate in viral reproduction, is very relevant to any explanation of shortened latency. This question is considered separately for VS (Cooper, 1958), and the results suggest that homotypic exclusion does not occur with dilute-passage stocks of this virus. Extensive discussion of exclusion is therefore omitted from the present paper.

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METHODS

Viral assays and stocks, one-step growth curve methods and media were as described by Cooper (1957*a*). When infected monolayer cells were removed with trypsin for virus release in suspension or for assay of infective centres, the inoculum was removed, plates washed twice with phosphate buffered saline (PBS, Dulbecco & Vogt, 1954), 2 ml. 0.7 mg. Armour crystalline trypsin/ml. or 2.5 mg. Difco trypsin/ml. added at 37° for 5 min., cells quantitatively resuspended and chilled, washed twice in PBS, and resuspended in 1 ml. of PBS. Large clumps were allowed to settle for 5 min., and the monodisperse supernatant fluid, containing not less than 50 % of the total cells, was removed for assay.

'*Multiplicity of infection*' relates to the number of pfu adsorbed per cell, and not necessarily to the pfu which actually achieve infection. The multiplicities reached in Figs. 1 and 2 are not dependent upon the plating efficiency of the virus assays (defined as ability to detect infective virus) as the adsorptions for both were carried out in the same manner; an agar cell-suspension method (Cooper, 1955) appeared to detect only 20–40 % more infective virus than did the monolayer method. Therefore 'pfu adsorbed' is regarded as the same as pfu added in the inoculum.

RESULTS

Effect of multiplicity of infection on latent period

Figures 1 and 2 confirm in more detail the earlier suggestion (Cooper, 1957*a*) that higher multiplicities of infection gave shorter latent periods with VS virus growing in chick cells. Thus at higher average multiplicities of infection, on the average each infected cell released its first progeny particle sooner. Rate of release and final yield were not affected; it is therefore noteworthy that no interference phenomena (decrease in release rate or final infective yield) were found. This is characteristic of the dilute-passage stocks used; undiluted-passage stocks show marked interference (Cooper & Bellett, to be published). The experiments shown used the New Jersey serotype, but the same phenomenon was also found with the Indiana serotype.

The experiments of Figs. 1 and 2 were performed with chick cell monolayers of the same batch infected simultaneously with different dilutions of the same high-titre virus stock, thus excluding possible day-to-day variations in cells or virus preparations as the explanation of apparent shortened latency. Shortened latency is not due to re-adsorption effects (possible in the monolayer experiment of Fig. 1) as identical results were obtained in dilute cell-suspensions (Fig. 2) where re-adsorption of released virus was negligible. This similarity also suggests that, in general, re-adsorption is not a significant factor in monolayer release curves, which is to be expected from calculations involving adsorption rates (50 % of virus is adsorbed in 3 hr. from 5 ml., whereas release is doubled every 30 min.). Figure 3 shows that there was a roughly logarithmic relation between multiplicity and latent period; a possible

significance of this is discussed later. The experiment of Fig. 2 (at 37°) was repeated with the exponential release period at 30° (the latent period having been at 37° as before), when the same shortened latency occurred even though the release doubling time was lengthened to approximately 1 hr.

Fig. 1

Fig. 2

Fig. 1. Effect of multiplicity of infection on one-step virus release curves from intact monolayers of the same batch (average pfu/infected cell). Virus was adsorbed for 45 min. from 0.5 ml. by monolayers of 2×10^7 cells, which were then washed 3 times with 5 ml. of medium. Free virus then assayed less than 1 pfu/10 infected cells. Release was allowed to proceed into 10 ml. medium from intact monolayers. Multiplicities of adsorption were 2.0 (○), 2.25 (△), 3.2 (●), 7.0 (▽), 9.25 (▲) and 22.5 (●) pfu/cell. The multiplicities of adsorption 3.2–22 were sufficient to infect all cells in the first cycle; correction in the two lower curves for cells not infected ($pr=0.176$ and 0.135) gave average multiplicities of infection of 2.0 and 2.25, and the curves (but not the points) have been corrected for these infective centre errors.

Fig. 2. Effect of multiplicity of infection on one-step virus release curves from cells in suspension. After infection of the monolayers (all of the same batch) to known multiplicity, cells were rapidly removed with trypsin, washed twice with medium and resuspended in medium at 37° to 10^4 cells/ml., while still early in the latent period. At intervals, samples of medium+cells were frozen unseparated for virus assay. Multiplicities of adsorption were 1.0 (○), 7.0 (▲), 15 (●) and 25 (△) pfu/cell. The culture with the lowest multiplicity of adsorption (1.0) received only 0.15 pfu/cell in the monolayer. Free virus was initially about 10% of the concentration of infected cells. Plating efficiencies (infective centres/total cells) were about 60%, except for multiplicity=1, which was 10%.

Possible explanations for 'shortened latency' shown to be unlikely

Virus elution. Virus may elute from the higher multiplicities of adsorption, giving apparently earlier release; in this case the earlier liberation of virus would be the summation of elution and virus release from an effectively low- or single-multiplicity infection. It is necessary to postulate for this either that

the probability of elution of each adsorbed particle is much greater in multiple than in single infections, or the unlikely case that higher multiplicities depress the final yield by a factor exactly equal to the proportion of virus eluting from lower multiplicities, since elution from low multiplicities would reduce the number of infective centres.

Fig. 3. The relationship between multiplicity of infection and latent period derived from Fig. 1 (○) and Fig. 2 (▲), which each used plates of one batch. Three points (●) are from independent experiments with different batches of plates, and the bar at multiplicity of six represents the extreme range of latent periods observed in Fig. 1 of Cooper (1957*a*).

That elution is not occurring is shown by: (*a*) the exponential nature of the release curves yielding virus in significant excess over that added, and (*b*) when virus growth (in a suspension containing in 1 ml. 10^4 cells previously infected in monolayers to an average multiplicity of 20) was prevented 40 min. after infection by freeze-thaw disrupting the cells or by the addition of 10 mM-NaCN, the 10 hr. virus yield/cell and therefore presumably the elutable virus was less than 0.1 % of the yield/untreated cell. In a number of experiments, the pfu liberated after 1 freeze-thaw of cells infected with high multiplicities was equivalent to 1–10 % of the number of infected cells, or less than 1 % of the virus added (Cooper, 1958); this is of the same order as the intact cells probably present (Cooper, 1957*b*).

Earlier adsorption of a single particle. As the rate of VS virus adsorption is probably independent of virus concentration, on average the time at which each cell receives its final quota of virus should be constant and independent

of multiplicity. However, a higher virus titre in the inoculum would mean that each cell would be likely to receive its first particle in a shorter time. It can be shown that little more virus is adsorbed by monolayers after 30 min. under the conditions used and 50 % is adsorbed in 10–15 min., independently of the concentration; therefore for the longest latent period, i.e. for single infection, half the cells have received their virus by 15 min. and nearly all by 30 min. The inoculum was usually removed and the plates washed at this stage. Thus the higher titres of virus added for the higher multiplicities could not achieve single infection more than 30 min. before the low titres. The latent period could not therefore be shortened in this way by more than 30 min., and there should be little difference in latent periods above multiplicity 2–3; reference to Figs. 1 and 2, where the maximum shortening was 3 hr., and where continuing increase in the multiplicity above 3 still further shortened the latent period, shows that shortened latency is not due to earlier adsorption at higher multiplicities.

Multiplicity reactivation among a partly inactivated virus population. It is very likely from the thermal instability of VS virus (Cooper & Bellett, to be published) that freshly harvested virus preparations contained an excess of virus which was non-infective for this system (i.e. not leading to infective progeny and therefore a plaque); many preparations certainly contained an appreciable proportion, often an excess, of particles 'storage'-inactivated during sojourn at -20° . It can be imagined that these may yield infective progeny under conditions of multiple infection and thereby affect the latent period in certain circumstances. However, direct examination for multiplicity reactivation in the sense of larger recovery of infective centres at high multiplicities of infection compared with low, revealed none in a population containing a tenfold excess of 'storage'-inactivated and an unknown excess of thermally inactivated particles (Table 1). Alternatively, the penetration of the cell by several particles, active or inactive, may provide material, perhaps structural protein or enzymes, which can overcome some bottleneck in virus production and lead to earlier maturation. In this case pretreatment of the cells with inactive particles might affect the latent period. Reference to Fig. 1 of Cooper (1958), where the cells were pretreated with a large excess of 'storage' and uv-inactivated particles, shows no effect on latent period by this means. Similar experiments (Cooper & Bellett, to be published) with an excess of the interfering component present in undiluted-passage stocks also showed no effect on the latent period in those cells able to release virus, and numerous one-step growth curves at single multiplicity (using various stocks and an input multiplicity of 10^{-3} to 0.3) always gave a latent period of 4.0–4.5 hr., showing that the varying multiplicities of thermally inactivated virus thereby achieved can have no marked effect on latent period.

Earlier release of an accumulated pool of mature virus

Experimental manipulation of cells infected with virus have in some hands stimulated virus release; there is evidence that mature poliovirus may accumulate in or on cells (Howes & Melnick, 1957) and be released in a burst (Lwoff,

Table 1. *The lack of effect of dilution of a dilute-passaged seed containing excess of 'storage' and thermally-inactivated virus on the recovery of cells yielding infective progeny (lack of multiplicity reactivation)*

Dilutions of a VS virus stock originally assaying 5×10^8 pfu/ml., and reduced to 6×10^7 pfu/ml. on storage at -20° , were added in 0.5 ml. amounts to each of 7 monolayers containing 2×10^7 cells; after 45 min. the inoculum was removed, plates washed twice with 5 ml. Earle's saline, cells removed with trypsin, chilled, washed once with PBS, re-suspended in 1 ml. and supernatant removed after 5 min. for assay of infective centres on monolayers.

Dilution	Seed		Assay			Infective centres per monolayer		Plating efficiency (%)	
	Multiplicity of infective VS (pfu)	Minimum multiplicity of inactivated VS	Dilution	pfu in		Observed	Expected from pfu added in seed	Observed infective centres	Expected infective centres
				0.1 ml.	0.2 ml.				
Neat	1.5	11	5×10^{-5}	17, 10	—	2.7×10^8	1.08×10^7	25.0	
1/8	0.5	3.6	5×10^{-5}	10, 9	—	1.9×10^8	5.6×10^6	34.0	
1/10	0.15	1.1	10^{-4}	5, 6	—	5.5×10^8	2.5×10^8	22.0	
1/30	0.05	0.36	3×10^{-4}	11, 10	13, 18	2.9×10^8	7.5×10^8	38.7	
1/100	0.015	0.11	10^{-3}	8, 16	20, 22	1.1×10^8	2.5×10^8	44.0	
1/300	0.005	0.036	3×10^{-3}	8, 13	13, 14	2.7×10^8	7.5×10^8	36.0	
1/1000	0.0015	0.011	10^{-3}	6, 5	15, 16	7.0×10^8	2.5×10^8	28.0	

Dulbecco, Vogt & Lwoff, 1955). High multiplicities of infection with VS virus may possibly have so damaged the cell surface that an accumulated pool of infective virus was released sooner than with low multiplicities. However, this would mean that the non-released infective virus associated with singly infected cells must be at least as numerous as the virus released by the higher multiplicity. Thus non-released virus during single infection would have to exceed the released virus by a factor of 10 or more. In fact, precisely the opposite was found by Franklin (1958) for VS virus growing in chick and monkey-kidney cells, i.e. the released virus exceeded the non-released by a factor of 10, as was found for western equine encephalomyelitis virus by Rubin, Hotchin & Baluda (1955). This implies that any virus particle must be released very rapidly once it is mature (becomes infective).

Further evidence against this idea is that in the experiment of Fig. 2 at least 90 % of cells were ruptured before assay by freezing and thawing, so that any non-released pool would be artificially released and included in the total count; it can be seen that the total virus produced by the cells (released plus non-released) still shows shortened latency.

DISCUSSION

Some data of Doermann (1952) suggested that intracellular coli-phage T4 appeared slightly earlier with higher multiplicities, although the absence of marked shortened latency in bacteriophage may be explained by the presence of mutual exclusion. Dulbecco & Vogt (1954) found that the latent period of western equine encephalomyelitis virus was shorter at a higher multiplicity. Liu & Henle (1951) found a similar phenomenon with influenza virus (strain LEE), although this might be accounted for by an enzymic release of attached virus in the same way that multiplicity reactivation was simulated (Henle & Liu, 1951), but later shown probably not to occur in this sense (Cairns, 1955). Kaplan (1957) mentioned an earlier release of herpes simplex virus at higher multiplicities, but as, due to technical difficulties, he appeared in some doubt as to the precise number of infected cells present in these experiments one cannot say whether the latent period, as defined in the present paper, was shortened. Darnell (1958) presented data which suggest a longer latent period in single than in high multiplicity for intracellular poliovirus. However, the reverse applied among the multiple infections (i.e. the latent periods were longer for higher multiplicities) and separate infective centre assays (particularly needed for the single multiplicities) were not given, so that shortened latency was not demonstrated. Dr R. M. Franklin (personal communication) found that L cells, infected with fowl-plague virus and subsequently 'stained' with fluorescent antibody, fluoresced earlier the higher the multiplicity.

The results presented above confirm the existence of the phenomenon of shortened latency in VS virus-infected chick cells. Certain possible explanations of the phenomenon seem to be excluded, namely, elution of virus at the higher multiplicity, earlier infection of the culture with seeds of higher titres, multiplicity reactivation of inactivated virus or pooling of certain

components to produce a complete particle sooner, and an earlier release of an accumulated pool of mature virus at the higher multiplicities. These will not be considered further.

It is demonstrated elsewhere (Cooper, 1958) that homotypic exclusion probably does not occur with dilute-passage VS stocks, although undiluted-passage VS stocks can contain a possibly 'incomplete' form which can exclude homotypic infective VS (Cooper & Bellett, to be published). However, all stocks used had only a limited number of passages (less than three, all with dilute inocula in tissue culture) from a picked single plaque, had a high titre ($1-2 \times 10^9$ pfu/ml.) and showed no depression of yield at higher multiplicities of infection, and so it is believed that they contain negligible amounts of the excluding component. There seems therefore no direct evidence for believing that all adsorbing particles are not participating, at least to some extent, in the growth cycle; until information on homotypic exclusion can be obtained, for example, from genetically labelled strains little further can be said on this subject.

It is proposed to consider briefly some explanations of shortened latency which assume non-exclusion and which did not allow direct experimental test. The earlier intracellular appearance of the first infective virus particle in multiple infection suggests that entry of several particles: (*a*) is equivalent to one particle reproducing for longer, (*b*) enables one particle to start reproducing sooner, or (*c*) permits one particle to reproduce faster. Possibilities (*b*) and (*c*) need not imply mutual exclusion between particles entering a cell, and all three possibilities are not mutually exclusive; (*a*) and (*b*) may well occur together.

If (*a*) is true, for example by multiple contributions to a vegetative pool (suggested by recombination among certain other animal viruses), then the following hypothesis is possible.

Let M = number of contributions to the pool (multiplicity of infection) and assume that increase of viral multiplying units is exponential.

Then r = the number of multiplying units present at the end of the latent period = $M \cdot 2^k$, where $k = (L - d)/g$, and L = length of latent period, d = 'dead-time', during which the virus particle adsorbs, penetrates and organizes into a replicating state, g = doubling time of pool.

Therefore $\log M = -L/g \log 2 + d/g \log 2 + \log r$.

A plot of $\log_{10} M$ against latent period (Fig. 3) gave a straight line of negative slope, indicating that if d is constant during either of the two series of growth curves of Fig. 3 then r is constant for the multiplicities studied. The value of r cannot be determined as d is unknown but the extrapolation to zero latent period indicates that r is less than 200. The ending of some latent periods by 1.5 hr. means that multiplication has at least started in these cells by this time, and if d is constant for all multiplications then d is less than 1.5 hr. and r is greater than 20. Calculations of g from both slopes in Fig. 3 gave values of about 0.6 hr., similar values to those calculated for the release doubling time from the virus release curves (0.54 and 0.60 hr. respectively; most other experiments gave 0.6 hr.).

In other words, doubling the multiplicity should put the pool one generation

ahead. Therefore in the simple case where final yield and rate of multiplication are unaffected, where the content of multiplying units is constant (20–200) when an average of one particle is released per cell, where vegetative growth and release have the same exponential rate, and where the pool is equally accessible to all adsorbing particles, then doubling the input should decrease the latent period by one release-doubling time. These assumptions are all implicit in this hypothesis, which then suggest a rather low probability of maturation of vegetative units compared with the high probability of release of mature virus found by Franklin (1958).

Possibility (b) may be caused by co-operation between particles in penetration or in organizing a replicating site, or in merely giving a greater probability of success in penetrating or in intracellular collision with a suitable site. Cairns (1957) suggested that in influenza virus infections of the allantoic membrane there is a probability of delay after adsorption in starting an infection at single multiplicity, so that each of a series of such infections will start multiplying at very different times. It is likely that a similar probability of delay occurs among particles multiply infecting a cell, so that the higher the multiplicity the sooner virus growth might be started by one of the adsorbing particles. Possibility (c) is allowed because exponential release may be the result of a series of 'bursts' randomly arranged among a large population of cells, and the actual duplication rate need bear no relation to release. It was mentioned above that an accumulation of mature virus was not found, making the 'burst' hypothesis for *release alone* unlikely, but several ways can be envisaged in which wholesale maturation and release could occur very rapidly in individual cells.

These questions are fundamental to our concept of viral growth. Means to investigate some of the alternatives discussed above could be devised, but some more direct method is very desirable.

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