Temperature-sensitive Events in the Growth of Variola Virus in HeLa Cells

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SUMMARY

The multiplication of variola virus in HeLa cells was examined under one-step growth conditions at 35° and at 40°. At 35°, after an eclipse of 10 hr, exponential growth proceeded for 10 to 12 hr with final yields of 200 to 500 pk.f.u. per cell; at 40° there was no growth of infectious virus. In contrast, vaccinia virus grew equally well at either temperature. The temperature-sensitive phase of the growth cycle was delineated by experiments involving temperature-shift. The first sensitive event was at 4 hr after infection, the time at which virus DNA synthesis normally begins at 35°, and the last was within 1 hr of the onset of virus maturation. Other features of the growth cycle at 40° were the absence of virus DNA production, the delayed appearance of the LS-antigen complex and the haemagglutinin, and the absence of any evidence of particle formation. Analysis of the temperature-shift experiments suggested that production of the LS-antigen complex and particle development were both directly involved in the late event.

INTRODUCTION

Each member of the vaccinia-variola subgroup of pox viruses has its own characteristic ceiling temperature for growth on the chorioallantois of the chick embryo (Bedson & Dumbell, 1961). For example, vaccinia virus grows at temperatures up to 41° whereas variola major ceases to grow above 38.5°. Studies on the nature of the inhibition produced by temperature under these conditions suggested that the block was reversible and occurred late in the virus growth cycle (Dumbell & Bedson, 1964, and unpublished observations) but it was not possible to determine more precisely the nature of the temperature-sensitive events involved. The present study of the growth of variola major virus in tissue culture cells is an extension of this investigation. We report experiments which delineate the extent of the temperature-sensitive period and which suggest that in this system the virus functions concerned may be both early and late in the growth cycle. It is to be expected that this study of a naturally temperature-sensitive pox virus will complement studies which are being made with artificially derived temperature-sensitive pox viruses obtained either with the aid of mutagens (Sambrook, Padgett & Tomkins, 1966) or by passage under selective conditions (Kirn, Braunwald & Scherrer, 1965). At the same time, since the naturally occurring pox viruses clearly differ in their ceiling temperatures, their study should extend our knowledge of the differences between them and may lead to a better understanding of their varying pathogenicity.
METHODS

Viruses. A master stock of the HINDEN strain of variola major virus (Downie & Dumbell, 1947) was grown on the chorioallantoic membrane and stored at \(-20\)° in 50\% (v/v) glycerol. Working stocks were prepared from this by a single passage in HeLa cells. Harvests from disrupted infected cells were cleared of cellular debris, concentrated by centrifugation at 11,000 g for 40 min. followed by re-suspension in 0.004 M-phosphate buffer pH 7.2 containing penicillin and streptomycin (phosphate buffer) and stored at \(-70\)°.

Stocks of the CL strain of vaccinia virus were maintained in the same way. Satisfactory working stocks contained \(10^{9.5}\) to \(10^{9.8}\) pk.f.u. per ml. and had particle to infectivity ratios of the order of \(10\) to \(1\).

Virus assay. Titrations of infectivity were made by means of pock counts in groups of 5 or 6 12-day chick embryos prepared for inoculation as described by McCarthy & Dumbell (1961). Particle counts were made by the loop drop method (Watson, 1962), the virus being first inactivated by exposure to ultraviolet light.

HeLa cells. Cells of the 'Bristol' line (obtained in the first instance from Dr T. H. Flewett of the Regional Virus Laboratory, East Birmingham Hospital, and on a subsequent occasion from Dr J. E. M. Whitehead of the Public Health Laboratory, Coventry) were propagated in Eagle's medium containing 10\% tryptose phosphate broth and 10\% calf serum (ETC) and maintained during experiments in Eagle's medium containing 2 or 10\% calf serum (EC). For virus growth curves, confluent monolayers were prepared in 6 cm. diameter borosilicate Petri dishes. These were seeded with 4 to 5 \(\times 10^6\) cells in 10 ml. ETC and used 2 to 3 days later when they contained 5 to 7 \(\times 10^6\) cells. Cytological observations were made in coverslip cultures maintained within 1.8 cm. diameter stainless-steel rings stuck to the glass with a paraffin+wax mixture. These were seeded with 1 to 2 \(\times 10^5\) cells in 1 ml. ETC 1 to 2 days before use.

Virus growth curves. Infection was made at an input multiplicity of 10 to 20 pk.f.u. per cell. After removal of ETC from the monolayers, virus was added in 0.2 ml. of EC containing 0.02 M-MgCl\(_2\) (Joklik, 1964). Adsorption was for 1 hr at 35°; control experiments showed that it did not matter whether this period was spent at 35° or 40° and the lower temperature was therefore used routinely. After adsorption the residual inoculum was removed and replaced by 10 ml. of EC. The cultures were incubated at either 35° or 40° in a humidified atmosphere flushed with CO\(_2\) in incubators fitted with internal fans (Laboratory and Electrical Engineering Co., Nottingham, England). Temperatures were maintained within \(\pm 0.2\)° of that required; in many experiments continuous readings were made throughout the incubators and in mock cultures with a miniature recorder (Grant Instruments Ltd, Toft, Cambridge). When cultures were 'shifted-up' from 35° to 40° the transition to 39° was achieved in 10 min. but 20 to 25 min. were required to reach 40°. On 'shifting' cultures 'down' from 40° to 35°, 35.5° was reached in 10 min. and the transition was completed in 15 to 20 min.

The proportion of cells infected was determined by two methods in pilot experiments. Coverslips were placed in the bottom of the Petri dishes and cells in the monolayer formed on these were examined for cytoplasmic DNA inclusions 6 hr after infection; monolayers were trypsinized 4 to 6 hr after infection and the cells plated
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after treatment with rabbit-pox antiserum, for the development of infective centres. Both methods indicated that 90% or more of the cells became infected.

Points on the growth curves were timed from the first addition of virus. Each point was established by harvesting intracellular virus from two cultures. Samples taken both early and late in the growth cycle established that extracellular virus was a negligible fraction of that present within the cells. After removal of EC and the addition of 0.5 ml. of phosphate buffer per dish, the cells were detached from the glass with a rubber policeman, transferred to 5 ml. screw-capped bottles and stored at −20°. Before titration, samples were thawed and the cells disrupted by ultrasonic vibration in an ‘Electrosonic’ bath (Surgical Equipment Supplies Ltd, London).

Certain experiments were made employing 5-fluorodeoxyuridine (FUDR) (a gift from Roche Products Ltd, Welwyn Garden City, Herts.) at a final concentration of 10⁻⁵.⁷ M.

Virus antigens. After titration, growth curve specimens were examined by immuno-diffusion and micro-immunodiffusion in gels of 1% agar in 0.85% NaCl. The antisera were from rabbits hyperimmunized with rabbit pox virus or with vaccinia virus. Also used was a serum from a rabbit immunized by intravenous injection of a preparation of vaccinial LS antigen (Shedlovsky & Smadel, 1942) and a reference preparation of the same material.

The presence of haemagglutinin was detected in titrations employing a 1% suspension of vaccinia-agglutinable fowl cells in 1% normal rabbit serum.

Acridine orange staining. Coverslip cultures were drained, washed, fixed in Carnoy’s fluid, stained with acridine orange 0.05% in citrate buffer pH 3.0 to 3.4, and examined in a Zeiss u.v. microscope.

Electron microscopy. Cells were detached from the Petri dishes with a rubber policeman, washed once in buffered saline and fixed in 1% gluteraldehyde in 0.1M-phosphate buffer pH 7.0 for 1 hr. After washing overnight in 0.1M-cacodylate buffer pH 7.0, the cells were post-fixed in 1% osmium tetroxide in Zetterquist’s solution. After dehydration through a graded series of alcohols, the cells were embedded in 9 to 1 mixture of butyl- and methyl-methacrylate partially prepolymerized by heat. Sections were cut on a Porter–Blum LT2 ultramicrotome, stained with lead hydroxide and uranyl acetate, and examined in a Siemens Elmiskop I.

Autoradiography. Coverslip cultures held under appropriate conditions were given a 30 min. pulse with [³H]thymidine (³H c/m-mole; 1 μc/ml). They were then drained of medium, washed 10 times in saline, fixed in acetone, dried in air and overlaid with Kodak AR10 stripping film. The films were developed in D 19b developer after 3 to 5 days at 4°.

RESULTS

The effect of temperature on the growth of variola virus in HeLa cells

To determine standard temperature conditions for use in this investigation, the growth of variola virus in HeLa cells was first examined at a number of temperatures in the range 35° to 40°. At 35° the yields obtained were from 200 to 500 pk.f.u. per cell. Growth also occurred at 38° but at this temperature maximum yields were only about 10% of those at 35°. The results at 39° were less clear-cut but in most experiments there was evidence of slight growth with yields of up to 5 to 10 pk.f.u. per cell. No growth was obtained at 39.5° and 40°. On the basis of these results 35° and 40° were
chosen as the permissive and restrictive temperatures. Representative growth curves
at these temperatures were constructed (Fig. 1a). At 35° there was satisfactory ex-
ponential growth following an eclipse period of about 10 hr, whereas at 40° there was
a steady slow decrease throughout the period of observation, which was on occasion
extended up to 96 hr.

Experiments with vaccinia virus under similar conditions strongly suggested that
the inhibition of variola at 40° was virus-specific and not due to a failure of the cells
to support virus growth at this temperature. Thus the growth curves of vaccinia at
35° and 40° were practically identical (Fig. 1b). These curves do, however, differ in
several minor respects from the growth curve of variola at 35°. With vaccinia the
latent period was clearly shorter, the growth curves being 3 to 4 hr in advance of that
of variola, and the final yields of virus, about 100 pk.f.u. per cell, were consistently
lower.

Fig. 1. One-step growth curves in HeLa cells of (a) variola virus and (b) vaccinia virus at
35° (○—○) and at 40° (●—●).

The timing of temperature-sensitive events in the growth cycle

Experiments to determine the extent of the temperature-sensitive period closely
followed the plan established by Lwoff and his colleagues in their study of the tem-
perature-sensitivity of poliomyelitis viruses (Lwoff, 1962). Observations were made of
the effects on subsequent virus growth of 'temperature-shifts' both 'up' from 35° to
40° and 'down' from 40° to 35°.

In the first series of experiments groups of infected cultures were held at 40° for
different periods of time and then 'shifted-down' to 35°. At intervals thereafter samples
were taken for analysis of growth curves in comparison with cultures maintained
continuously at 35° (Fig. 2). Cultures 'shifted-down' 4 hr after infection produced
virus at the same time and rate as control cultures held at 35° throughout. When the
'shift-down' was made later than 4 hr there was a delay in the onset of virus produc-
tion. The length of this delay was roughly equal to the period in excess of 4 hr spent
at 40°. Thus cultures 'shifted-down' at 6, 8 and 12 hr after infection were delayed by
2, 3 to 4 and 7 to 8 hr respectively. It was thus concluded that events in the first 4 hr
of infection proceeded normally at 40° but that further progress was then blocked by
the intervention of a temperature-sensitive step.
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In the second series of experiments groups of infected cultures were incubated at 35° for different periods of time and then 'shifted-up' to 40°. Samples were taken at intervals thereafter and their virus content determined (Fig. 3). A 'shift-up' rapidly halted the production of infectious virus, no matter at what point the restraint was applied. If the 'shift-up' was made before the end of the eclipse no infectious virus was produced. If it was made later when virus maturation was already proceeding, then further growth was prevented. Because of the slow fall seen in most of the curves after the 'shift-up' to 40°, it was not possible to decide from these experiments just how rapidly the switch to 40° became operative. However, in a further experiment with a 'shift-up' at 12 hr and samples taken first at hourly and then at 2 hr intervals, only the 13 hr sample showed any increase above the amount of virus present at 12 hr. In the 13 hr sample the rise was itself barely significant and equivalent to less than 1 hr of growth at 35°.

Other features of the growth of variola virus at 35° and 40°

Virus particles. Particle counts were made on many of the growth curve specimens. In none of the situations investigated, whether involving straightforward inhibition at 40°, temperature-shifts, or the use of FUdR (see below) was there any evidence of a significant production of non-infectious particles. There were, however, certain minor but consistent changes in the quality of virus recovered at different times after infec-
tion. These are illustrated in Fig. 4 with the particle/infectivity (P/I) ratios for the particular 35° and 40° growth curves of Fig. 1(a). At 35° there was a fall in quality during the eclipse phase, P/I ratios rising from an initial value of 8 to values of 20 at 6 to 10 hr. In other experiments values as high as 40 were seen at this stage. During virus growth quality improved and the P/I ratio returned to less than 10 by 20 hr. At 40° there was the same or in some instances a slightly greater decline in quality during the eclipse and, in the absence of virus growth, it was possible to follow the continuation of this process with P/I ratios rising to 50 by 28 hr and to over 100 by 48 hr.

![Growth curves of variola virus in HeLa cells 'shifted-up' from 35° to 40° at different times after infection.](image)

The changes in quality seen at 35° resemble those previously described for vaccinia (Smith & Sharp, 1960) and for herpes simplex virus (Russell et al. 1964). In the latter instance it was also clearly established that the particles produced early in the growth phase were only of low infectivity. With pox viruses, however, the changes in quality are much less dramatic and it is difficult to be certain of this point, but our data are on the whole consistent with the same conclusion.

**Synthesis of virus DNA.** The synthesis of virus DNA was examined principally by means of the inhibitor FUdR (Salzman, 1960). Growth curves of 'functional' virus DNA were constructed by examining the 24 hr yields of infectivity in cultures to which FUdR had been added at intervals after infection. The growth of 'functional' DNA at 40° was determined in the same way but the cultures were 'shifted-down' from 40° to 35° at the time of addition of FUdR (Fig. 5). The synthesis of virus DNA
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Fig. 4. Virus particle to infectivity ratios during the growth cycle of variola in HeLa cells at 35° (○—○) and at 40° (●—●).

Fig. 5. The growth of variola virus 'DNA' at 35° (●—●) and 40° (△—△) shown in relation to the one-step growth curve at 35° (○—○).
at 35° commenced at about 4 hr and its curve of production ran parallel to and about 7 hr in advance of that of the virus. At 40° the position was strikingly different and either little or no virus DNA was produced. It was not possible to distinguish between these alternatives because of the rather erratic base line of inhibition obtained with FUdR. Others have observed that inhibition with FUdR is incomplete and subject to local variation in conditions (Easterbrook, 1963; Baxby & Rondle, 1965). The question was therefore re-examined by cytological studies of the development of cytoplasmic centres of DNA synthesis in infected cells.

In coverslip cultures incubated at 35° and stained with acridine orange, green-staining cytoplasmic inclusions were first visible in a few of the cells 3 to 4 hr after infection. Over the following hours inclusions were seen in an increasing proportion of cells and by 7 hr they were present in 95%. The inclusions themselves progressed from pin-point size at 3 to 4 hr to larger round bodies at 5 to 6 hr which thereafter became irregular in shape and tended to fuse with one another and to stain less intensely. These inclusions were not observed in cells incubated at 40°. Autoradiographs made 6 hr after infection following a 30 min. pulse with [3H]thymidine provided similar results. A high proportion of the cells incubated at 35° showed typical clusters of grains in their cytoplasm and these were absent from cells incubated at 40°. By contrast nuclear labelling at 40° was not noticeably different from that at 35°.

**Virus antigens.** Selected growth curve specimens were examined after titration for the presence of virus antigens by immunodiffusion using hyperimmune rabbit-pox antisera. The first line of precipitation was given by specimens taken at 5 to 6 hr and there were two or sometimes three lines from specimens at 7 to 8 hr. However, this 'early' pattern was apparently the same whether incubation was at 35° or 40° and a clear difference between the two temperatures was not found until 11 to 12 hr. Specimens from 35° then gave a number of further lines, the most prominent of which were two shown to be associated with LS-antigen complex by reference to an LS preparation and a specific anti-LS serum. These lines did not appear at 40° until very much later, being barely discernible at 26 hr and only clearly present at 36 and 48 hr.

Further information was obtained from the study of specimens from the temperature-shift experiments (Fig. 2, 3). These were examined in microimmunodiffusion tests with the anti-LS serum. In 'shifts-down' from 40° to 35° the first appearance of LS antigen was delayed whenever there was a delay in the onset of virus growth and these two features always retained the close relationship seen in the 35° growth curve. 'Shifts-up' from 35° to 40° made at 8 and 10 hr before the onset of virus growth suppressed the normal appearance of LS antigen. In order to examine the results of a 'shift-up' at 12 hr, when LS antigen was already present, rough quantitative measurements were made of the development of LS antigen both at 35° and following the 'shift-up'. Serial twofold dilutions of the specimens were tested to find the highest dilution which could still give a line of precipitation. Whereas at 35° there was a progressive increase in LS antigen, the 'shift-up' to 40° rapidly prevented any further increase (Table 1).

A limited study was also made of the production of haemagglutinin. At 35° no haemagglutinin was detected at 11 hr or in earlier specimens. The titre then rose rapidly from 1/4 at 14 hr to 1/256 at 20 hr and thereafter to 1/1024 at 26 hr and 1/2048 at 48 hr. At 40° haemagglutinin production was severely curtailed, there being none at 26 hr and a titre of only 1/16 at 48 hr.
Table 1. *The development, as shown by gel diffusion, of LS antigen in cells infected with variola virus and incubated either at 35° or 'shifted-up' to 40° after 12 hr at 35°*

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The numbers are the reciprocals of the maximum dilutions of antigen giving a line. ND, Not examined.

**Ultrastructural appearances in thin sections of infected cells.** The development of variola virus in HeLa cells at 35° followed a course similar to that described for vaccinia in L cells (Dales & Siminovitch, 1961). In infected cells incubated at 40° areas of 'viroplasm' developed (Pl. 1a) but there was a suggestion that these were less dense than those formed at 35°. No further development took place at 40°.

An attempt was made to investigate the late temperature-sensitive event by examining cells at 24 hr after a 'shift-up' from 35° to 40° at 7 hr. As expected from the corresponding growth curves (Fig. 3), these cells did not appear to contain any fully mature virus particles. Several of the early stages of particle formation were observed (Pl. 1b). The predominant form was that of the large immature particle of elliptical section bounded by a complete double membrane, and a number of these particles contained an eccentric nucleoid. Later stages of development involving condensation of the nucleoid were not seen. The process of development of variola virus particles is clearly temperature-sensitive and can be arrested in mid-course. However, it is not possible to decide whether only one or several stages of development are temperature-sensitive. In the first place the degree of synchrony achieved in these experiments was never complete; even in cells incubated at 35° for 24 hr it was possible to find a few particles in relatively early stages of development. Secondly, the predominant form present may be critically related to the time of applying the restraint and our results refer only to 'shifts-up' to 40° made at 7 hr.

**DISCUSSION**

The one-step growth curve of variola virus in HeLa cells appears to differ in certain respects from those of other pox viruses, notably rabbit pox and vaccinia viruses, which have been studied under similar conditions. In making such comparisons one must remember that results in suspension culture are different from those achieved in monolayer (Joklik, 1966) and that due allowance must be made for variation caused by different host cells and different strains of virus. Nevertheless, variola virus seems to have a slower and more prolonged growth cycle than these other viruses. Its eclipse phase of about 10 hr contrasts with one of 6 to 8 hr for vaccinia (Joklik & Rodrick, 1959; Kirn *et al.* 1965) and 4 to 6 hr for rabbit pox virus (Appleyard & Westwood, 1964; Appleyard & Zwartouw, 1965), and these differences are supported by our own findings with vaccinia (Fig. 1b) and rabbit pox virus (unpublished). The same relationship is shown by these viruses in their growth on the chick chorioallantois, but,
(a) Part of a section of a variola-infected HeLa cell which had been incubated at 40° for 24 hr. A localized area of granular material ('viroplasm') is lying close to the nuclear membrane.

(b) Part of a section of a variola-infected HeLa cell which had been incubated for 7 hr at 35°, 'shifted-up' to 40° and harvested 18 hr later. All the particles are immature. A number of stages of development can be seen.
whereas variola virus has consistently given the highest yields in HeLa cells, the position on the chorioallantois is reversed and the yield of variola virus is then lower than that of either rabbit pox or vaccinia virus (Hahon & Friell, 1962). The longer eclipse phase seen with variola virus has both advantages and disadvantages for the present study. On the one hand it may allow a clearer temporal separation of different viral functions and make the short delays in temperature-shifts less critical. On the other hand opportunities for the development of asynchrony are that much greater.

Comment may also be made about the particular restrictive temperature used in this work. On the chorioallantois the growth of variola virus is prevented at 39° (Hahon, Ratner & Kozikowski, 1958; Bedson & Dumbell, 1961) but a slightly higher temperature was found necessary in HeLa cells. This is in keeping with earlier observations that the ceiling temperature of a virus is in part determined by the host involved (Bedson, 1964; Gurvich & Marennikova, 1964). The actual choice of restrictive temperature, which could in this instance have been any temperature from 39.5° upwards, may be very critical if multiple temperature-sensitive events are involved and if these events do not all have the same ceiling temperature. This difficulty should not arise in studies with single-step temperature-sensitive mutants.

Our results clearly demonstrate that more than one temperature-sensitive event is involved in the present situation. In the first place there is the very long period of temperature sensitivity extending from 4 hr to within 1 hr of virus maturation. The duration of this period is very similar to that observed by Kirn et al. (1965), with a 'cold' strain of vaccinia derived by continuous passage at low temperatures and therefore unlikely to be a single-step mutant. In the case of variola virus the onset of the temperature-sensitive period has been timed to the stage at which replication of virus DNA normally commences and this, together with the inhibition of DNA replication observed at 40°, strongly suggests that the earliest temperature-sensitive event is intimately concerned with this process. The temperature sensitivity of the replication of virus DNA has been the subject of further study and will be discussed in greater detail elsewhere (Bedson & Cruickshank, in preparation). The evidence that later events are also involved and that these are quite separate from this early event is necessarily less direct, but the point seems clearly established by the experiments involving 'shift-up' to 40°. These show that a block can be rapidly imposed at a time when much of the virus DNA has been made and virus production is even in progress. In its timing the late block is strongly reminiscent of the late fluorphenylalanine-sensitive event described with vaccinia (Salzman, Shatkin & Sebring, 1963) and which we have also observed with variola virus (unpublished results). The production of 'late' virus antigens, the assembly and/or maturation of virus particles and probably also the production of haemagglutinin all seem to be implicated in the late block. The findings in respect of the LS-antigen complex are of particular interest, for there is evidence to suggest that it may be a major surface component of the virus particle (Craigie & Wishart, 1936; Smadel & Rivers, 1942; Marquardt, Holm & Lycke, 1965). Although this protein may itself be temperature sensitive in our sense, it seems possible that its delayed production at high temperature is part of a more widespread failure to synthesize 'late' viral antigens. If this were so, a regulatory function might be involved and it could be argued that the late block was an indirect consequence of the early event. However, parallel studies with alastrim virus have shown that the late block may be found in the absence of any early temperature-sensitive event (Cooper &
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Bedson, to be published). Kirn and his colleagues (personal communication) in further work with their ‘cold’ strain of vaccinia have likewise found good evidence for the complete separation of ‘early’ and ‘late’ temperature-sensitive events.

It is to be expected that the explanation of temperature-sensitive events will be found in terms of essential thermolabile proteins, for this was shown to be so in earlier work with Neurospora (Horowitz & Leupold, 1951) and in more recent work with bacteriophages following the lead given by Epstein et al. (1963). In view of the current findings concerning the ‘early’ and ‘late’ proteins of pox viruses (Salzman & Sebring, 1967; Wilcox & Cohen, 1967) it is not surprising that temperature sensitivity should be found both early and late. The same pattern is also evident in studies of temperature sensitivity with a number of other animal viruses (Burge & Pfefferkorn, 1966; Cooper, Johnson & Garwes, 1966; Zebowitz & Brown, 1967).

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REFERENCES


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