Temperature-sensitive Events in the Growth of Alastrim Virus in Chick Embryo Cells

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SUMMARY

The growth of alastrim virus in chick embryo cells was studied under one-step conditions at various temperatures. Limited growth was found at 37 °C but there was none at 38 °C or above. Temperature-shift experiments suggested that at 38 °C only late events were temperature-sensitive. Virus DNA synthesis, induction of early enzymes and the production of early antigens were all substantially normal at 38 °C. In a study of late events at 38 °C, particle formation was found to be almost completely inhibited. Although a few immature particles were seen by electron microscopy of thin sections, cytoplasmic DNA labelled with [3H]-thymidine did not become resistant to DNase and particles containing DNA were not seen after centrifuging on sucrose density gradients. There was no late rise in DNA-dependent RNA polymerase activity. Late antigen production at 38 °C appeared normal in agar gel diffusion studies both in the time of appearance and in the number of lines present, but the LS-antigen complex was slightly reduced in amount. Production of haemagglutinin was completely suppressed at 38 °C. It is concluded that the major effect of temperature on the growth of alastrim virus is to inhibit a very early stage in particle formation.

INTRODUCTION

Members of the vaccinia–variola group of poxviruses can be differentiated on the basis of their ceiling temperature for growth on the chick chorioallantoic membrane (CAM) (Bedson & Dumbell, 1961). Later work with alastrim virus on the CAM (Dumbell & Bedson, 1964) suggested that temperature sensitivity was determined by a late event in the virus growth cycle. However, Cruickshank & Bedson (1968) found the growth of variola major virus in HeLa cells at 40 °C to be inhibited by at least two temperature-sensitive (ts) events, one occurring early and the other late. The early event prevented the synthesis of virus DNA and involved the virus-induced DNA polymerase (Bedson & Cruickshank, 1969), but attempts to study the late ts event were hampered by the possibility of interference from the early event.

The experiments reported here were made in an attempt to shed further light on the nature of the late ts event. The suggestion that early ts events might not complicate the picture with alastrim virus in the chick embryo host at 38 °C has been re-examined and has led to a detailed study of the temperature sensitivity of this virus in primary cultures of chick embryo cells.

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METHODS

Virus. A master stock of the Butler strain of alastrim virus (Fenner & Burnet, 1957) was obtained from its seventh passage on the CAM since isolation. Working stocks were prepared by a single passage in HeLa cells, partially purified, concentrated and stored at -70 °C as described by Cruickshank & Bedson (1968).

Cell culture. Primary cultures of chick embryo cells were obtained by trypsinization of 12-day embryos and propagated in Eagle's medium containing 10% tryptose phosphate broth and 10% calf serum (ETC). The tryptose phosphate broth was omitted from maintenance medium for virus growth curves (EC10) and the calf serum reduced to 2% in experiments involving the incorporation of [3H]-thymidine (EC2).

Virus growth curves and virus assay. The general design of growth-curve experiments, assays of infectivity, particle counts and the temperature characteristics of the incubators were those described by Cruickshank & Bedson (1968). In brief, one-step growth conditions were obtained by infecting monolayer cultures at an input multiplicity of 20 p.f.u./cell and incubating them at the required temperature in a humidified atmosphere containing about 5% CO2. Harvests of intracellular virus were made at intervals after infection and titrated by pock counts in 12-day chick embryos. Particles were counted by the loop-drop method (Watson, 1962).

The proportion of cells infected in these experiments was determined either by acridine orange staining of monolayers on coverslips placed in the Petri dishes or by infectious centre assay. Both methods showed that only 70 to 75% of the cells were infected, but this proportion appeared to be a maximum since it was also achieved with an input multiplicity of 5 p.f.u./cell and was not increased by doubling the multiplicity to 40 p.f.u./cell.

Electron microscopy. Cells were detached from the Petri dishes with 0.5 mM-EDTA, washed in buffered saline and then fixed, embedded, sectioned and examined as described by Cruickshank & Bedson (1968).

Virus antigens. Growth-curve specimens were examined by immunodiffusion in gels of 1% agar and 0.9% NaCl. More concentrated preparations, made by disrupting cells at a concentration of 10⁸/ml, were used in micro-immunodiffusion tests (Crowle, 1958). Haemagglutinin was titrated with a 1% suspension of vaccinia-agglutinable fowl cells in saline containing 1% normal rabbit serum (McCarthy & Downie, 1953).

Antisera. Rabbits were immunised with the CL strain of vaccinia using extracts from infected RK13 cells in Freund's incomplete adjuvant (after the method of Watson et al. 1966). A serum absorbed with excess 38 °C-antigen was prepared by reacting a 0.5 ml vol. with 4 ml of the more concentrated antigen preparation at 4 °C overnight. After centrifuging at 100000 g for 1 h, the supernatant fluid was concentrated to the original vol. of serum by vacuum dialysis. Serum from a rabbit immunised by intravenous injection of vaccinial LS antigen (Shedlovsky & Smadel, 1942) was also used.

Cell fractionation. The cells, from 2 to 4 x 10⁷/specimen, were removed from the Petri dishes with 0.5 mM-EDTA in phosphate-buffered saline, resuspended in 3 ml of hypotonic buffer (0.01 M-tris/HCl, pH 7.2; 0.004 M-2-mercaptoethanol) and broken in a Dounce homogenizer after swelling for 5 min at 4 °C. One ml of M-sucrose was added and a 1 ml sample removed for counts of nuclei and unbroken cells and for other determinations (whole-cell fraction). The remainder was centrifuged at 400 g for 5 min to deposit nuclei and the supernatant fluid (cytoplasmic fraction) removed. In control experiments with uninfected cells labelled with [3H]-thymidine, between 1 and 3% of whole-cell radioactivity was found in the cytoplasmic fraction. A slightly higher value, 4 to 5%, was obtained with cells prelabelled and then infected.
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Studies of [3H]-thymidine incorporation. The rate of virus DNA synthesis was examined in monolayer cultures pulsed for 1 h in EC 2 containing [3H]-thymidine (2 μCi/dish; 3 Ci/m-mol). Whole-cell and cytoplasmic samples were obtained and their acid-insoluble radioactivity determined by the method of Regan & Chu (1966).

The fate of virus DNA late in infection was examined in cells exposed to [3H]-thymidine (2 μCi/dish) during the period 1 to 10 h and then restored to ETC. At various times thereafter cells were harvested and the cytoplasmic fraction obtained. The resistance to DNase of labelled material in the different samples was determined by incubating a portion with 50 μg pancreatic DNase in 10 mM-MgCl₂ for 1 h at 37 °C before assaying for acid-soluble radioactivity. Controls with labelled DNA from uninfected cells showed that this treatment reduced the acid-insoluble counts to 10 %.

Further analysis was made by centrifuging 0.2 ml samples on 25 to 40 % (w/v) sucrose gradients at 17000 g for 45 min in the SW40 rotor of a Christ Omega II ultracentrifuge. Fractions of 10 drops each were collected from the bottom of the tube and assayed for acid-insoluble radioactivity both before and after treatment with DNase.

Enzyme assays. Cytoplasmic fractions from cells infected at an input multiplicity of 40 pk.f.u./cell were stored at −20 °C. The assay for thymidine kinase activity was based on that of Klemperer et al. (1967). DNA polymerase activity was assayed as previously described (Bedson & Cruickshank, 1969) and DNA-dependent RNA polymerase activity by a method based on that of Kates, Dahl & Mielke (1968). Protein concentrations were determined by the method of Lowry et al. (1951).

Materials. Cytosine arabinoside was obtained from Sigma (London) Chemical Co. Ltd [3H]-thymidine (3 Ci/m-mol) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Pancreatic DNase and 5-bromodeoxyuridine were obtained from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire and puromycin dihydrochloride from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Actinomycin D was a gift from Merck Sharp and Dohme Limited and rifampicin from Lepetit Pharmaceuticals Limited.

RESULTS

The ceiling temperature of alastrim virus in chick embryo cells

The growth of alastrim virus in primary cultures of chick embryo cells was studied at various temperatures. At 35 °C the virus titre fell from an input value of 20 pk.f.u./cell to an eclipse value of about 2 pk.f.u./cell. Then, from 8 h after infection, there was an exponential rise to reach a maximum of about 60 pk.f.u./cell at 24 h with a slightly lower titre at 48 h. In a limited study at 37 °C there appeared to be a slight amount of growth, the 24 h specimen showing a titre of 4 to 5 pk.f.u./cell, but again there was a lower titre at 48 h. At 38 °C, 39 °C and 40 °C no virus growth was detected and specimens taken from 10 h onwards showed only a progressive decline in titre. In subsequent work, 38 °C was used as the non-permissive temperature.

The effects of temperature-shifts

The timing of ts events in the growth cycle was studied in 'temperature-shift' experiments. In the first series, cultures were infected and incubated at 38 °C for periods of 6 to 10 h. They were then transferred to 35 °C and sampled at intervals thereafter. The resultant growth curves were compared with those in cultures maintained at 35 °C and 38 °C throughout (Fig. 1). Incubation at 38 °C for 6, 8 and 10 h had little effect on the subsequent growth
curves and in no instance was there a delay of more than 2 h. Moreover, transfer to 35 °C after 10 h at 38 °C was followed almost immediately by exponential growth. This pattern of results suggests that, at 38 °C, the earliest ts event occurred only just before maturation and certainly not more than 2 h before it.

The existence of a late ts event was also shown in experiments in which cultures infected at 35 °C were transferred to 38 °C at 8 h or more after infection (Fig. 2). Virus growth was rapidly inhibited no matter at what stage the transfer was made. If the restraint was applied during the eclipse phase, no infectious virus was produced. If it was applied when virus maturation was already proceeding, then further growth was prevented.

**Early events in the growth cycle at 38 °C**

Although the ‘temperature-shift’ experiments suggest that early events proceed normally at 38 °C, a number of them were examined directly.

Virus DNA synthesis at 38 °C was indistinguishable from that at 35 °C. At both temperatures the rate of [3H]-thymidine incorporation increased rapidly starting 2 to 4 h after infection, reached a maximum at 8 h and then decreased rapidly to low levels at 14 h. Both DNA synthesis and its regulation therefore took place normally at the restrictive temperature.

A surprising feature of these experiments was the complete lack of inhibition of host-cell DNA synthesis at either temperature (Table 1). This is in contrast to results obtained with other poxviruses and host cells (Joklik & Becker, 1964; Jungwirth & Launer, 1968) and particularly with variola major virus in HeLa cells (Bedson & Cruickshank, 1969). Reciprocal experiments with alastrim virus in HeLa cells and variola major virus in chick embryo cells have shown that the difference is associated with the host cells rather than the virus.
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2.0
1.6
1.2
0.8
0.4
0.0

Fig. 2. Growth curves of alastrim virus in chick embryo cells ○—○, at 35 °C and •—•, at 38 °C after transfer from 35 °C at the times indicated.

Table 1. Rates of nuclear incorporation of [3H]-thymidine in uninfected and in chick embryo cells infected with alastrim virus at 35 °C and 38 °C

<table>
<thead>
<tr>
<th>Specimen</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 °C uninfected</td>
<td>1435*</td>
<td>2438</td>
<td>2787</td>
<td>3099</td>
<td>2874</td>
</tr>
<tr>
<td>35 °C infected</td>
<td>1467</td>
<td>1810</td>
<td>2713</td>
<td>2624</td>
<td>3570</td>
</tr>
<tr>
<td>38 °C uninfected</td>
<td>1540</td>
<td>3004</td>
<td>3119</td>
<td>2364</td>
<td>2621</td>
</tr>
<tr>
<td>38 °C infected</td>
<td>1590</td>
<td>1888</td>
<td>3645</td>
<td>4541</td>
<td>3369</td>
</tr>
</tbody>
</table>

* Whole cell counts less the cytoplasmic counts expressed as ct/min.

Some of the early enzymes concerned with virus DNA synthesis were also examined. The rise in activity of thymidine kinase at 35 °C and 38 °C was almost identical and a 40-fold increase was reached 12 h after infection. DNA polymerase activity in infected cells at 38 °C differed from that at 35 °C. At 35 °C there was an eightfold increase in activity reaching maximum levels at 16 h. At 38 °C, there was only a threelfold increase in activity, most of which was in the first 6 h in parallel to that at 35 °C and there was little change thereafter.

Early virus antigens were analysed in micro-immunodiffusion tests with extracts of cells at 24 h using 10⁻⁴⁶ M-cytosine arabinoside to inhibit late antigen production (Oda & Joklik, 1967). Three lines of precipitation were obtained and no differences were observed between specimens from 35 °C and 38 °C.

Late events at 38 °C

The results presented in the previous sections indicate that early events of the poxvirus growth cycle occur with a reasonable level of efficiency at the non-permissive temperature of 38 °C and that, under these conditions, virus growth is apparently blocked only by the
operation of a late ts event. A detailed comparison was therefore made of the performance of individual late functions at 35 °C and 38 °C.

**Virus particle formation**

Particle counts, made on a number of specimens from the growth curves, revealed that there was no significant production of non-infectious particles at 38 °C. At 35 °C, the number of particles per cell increased from a minimum of about 50 at 8 h to 300 at 24 h. The rise in the number of particles was less steep than the rise in infectivity, the particle to infectivity ratio of 30 at 8 h falling to one of about 6 at 24 h. Such changes in quality are expected in poxvirus infections (Smith & Sharp, 1960; Cruickshank & Bedson, 1968). At 38 °C, none of the specimens gave counts above the 8 h value at 35 °C.

Evidence of the formation of virus particles was also sought in thin sections from infected cells fixed at 30 h. At 35 °C, the asynchrony of infection in monolayers was clearly evident. In any one cell, many stages of particle development were present and even at this late stage of the growth cycle relatively few mature particles were seen (Fig. 3 (a)). At 38 °C, the principal feature was the presence in the cytoplasm of areas of granular material or ‘viroplasm’, the characteristic factory sites of poxvirus infection (Dales, 1963). For the most part these were completely devoid of membrane formation or other signs of particle development (Fig. 3 (b)). However, in a few cells there was an occasional group of large immature particles, most of which were bounded by a complete double membrane (Fig. 3 (c)). None of these had advanced to the stage of possessing an eccentric nucleoid and later forms involving condensation of the nucleoid were not observed. No attempt was made to quantify the data but the results suggest that particle formation at 38 °C was halted at a very early stage and that there was little production of even the earliest immature forms.

**The fate of DNA synthesized at 38 °C**

Newly synthesized poxvirus DNA is at first sensitive to DNase and in a form which remains at the top of sucrose density gradients. Later in infection a fraction develops which is resistant to DNase and some of the DNA is found in particles which centrifuge to lower positions in sucrose gradients (Joklik & Becker, 1964). Both these changes, which are associated with the coating of DNA by protein and its packaging into particles during maturation, were sought in pulse-chase experiments at 35 °C and 38 °C.

At 35 °C, there was a progressive increase in the proportion of DNA resistant to DNase from 12 h onwards, reaching 35 to 40 % at 24 h (Fig. 4). An almost identical curve with samples from cells labelled at 38 °C and then transferred to 35 °C at 10 h indicated that DNA synthesized at 38 °C was handled normally on reversal of the temperature block. However, DNA synthesized at 38 °C remained fully susceptible to DNase when incubation was continued at this temperature.

In another experiment the cytoplasmic fractions were centrifuged on sucrose density gradients. Samples taken immediately after the 10 h labelling period, whether at 35 °C or 38 °C, showed no evidence of particle formation and virtually all the DNA remained at the top of the gradient (Fig. 5a). The 35 °C sample at 17 h gave a peak of radioactivity near the bottom of the gradient (Fig. 5b). This peak presumably contained mature particles as most of it was resistant to DNase (not shown). A similar but more pronounced virus peak was evident in the 24 h sample from 35 °C (Fig. 5c). At 38 °C, neither at 17 h (Fig. 5b) nor at 24 h (Fig. 5c) was there any indication of a virus or sub-virus peak, all the DNA remaining at the top of the gradient.

These results indicate that DNA synthesized at 38 °C is not coated and remains sensitive
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Fig. 3. Parts of sections of chick embryo cells 30 h after infection with alastrim virus. (a) Particles in various stages of development at 35 °C. (b) A typical area of 'viroplasm' at 38 °C. (c) One of the few groups of immature particles seen at 38 °C.
Late enzymes

Rises in the activity of two enzymes, an 'acid' DNase (McAuslan & Kates, 1967) and a DNA-dependent RNA polymerase (Kates et al. 1968) have been reported to be late events in poxvirus-infected cells. Repeated attempts were made to detect 'acid' DNase activity in chick embryo cells infected with alastrim virus, both at 35 °C and at 38 °C, but without success (data not presented).

Studies of the DNA-dependent RNA polymerase showed virtually no activity in the cytoplasm of uninfected cells. In infected cells, both at 35 °C and 38 °C, low levels of activity were found 6 and 12 h after infection, presumably due to the input virus. At 38 °C, this level persisted unchanged up to 24 h, but at 35 °C there was a rapid increase in activity between 12 and 24 h after infection.

Virus antigens

A complex pattern of 11 to 13 lines of precipitation was obtained in micro-immunodiffusion tests with concentrated specimens prepared 24 h after infection (Fig. 6). The inclusion of a specimen grown in the presence of cytosine arabinoside demonstrated that most of the lines represented late antigens. Although there was evidence of differences in concentration, some lines appearing nearer the 38 °C-antigen well than the 35 °C-antigen well, none of the antigens present at 35 °C could be said to be completely absent at 38 °C. This result was supported by the failure of antiserum absorbed with excess 38 °C-antigen to give any lines of precipitation with the 35 °C-antigen.
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Fig. 5. Sucrose density gradient patterns of DNA from the cytoplasmic fractions of infected cells labelled with [H]-thymidine from 1 to 10 h and sampled at 10 h (a), 17 h (b) and 24 h (c). ○—○, incubated at 35 °C; •—•, incubated at 38 °C.

Studies made with the ordinary growth-curve specimens were less sensitive, giving a maximum of seven lines, but the lack of any major differences between comparable specimens from 35 °C and 38 °C showed that there was no significant delay in the appearance of these antigens at 38 °C.

Two known late antigens were examined specifically. The LS complex was detected in gel-diffusion tests by means of a specific anti-LS serum. Although this antigen appeared at the same time at 38 °C as at 35 °C, rough quantitative estimates on 24 h specimens consistently showed higher titres at 35 °C than at 38 °C. Using doubling dilutions the titre at 35 °C was three-fold, while that at 38 °C was three-quarters or occasionally four.
8 or 10 h after infection. Similar results were obtained if cultures were incubated at 38 °C for 8 or 10 h before addition of the drug and return to 35 °C.

Experiments with inhibitors

Since virus DNA, late virus proteins and presumably late messenger RNA were synthesized at 38 °C, it was of interest to examine the effect of metabolic inhibitors added to cultures when switched from 38 °C to 35 °C at 10 h and at 16 h after infection. As was to be expected from the apparently normal synthesis of virus DNA at 38 °C, addition of $10^{-4}$ M bromodeoxyuridine did not prevent subsequent virus growth. By contrast, addition of puromycin ($15 \mu g/ml$) completely suppressed virus growth, indicating the need for further protein synthesis at 35 °C. The significance of this requirement is however difficult to assess, since normal virus growth at 35 °C requires concomitant protein synthesis and addition of puromycin at 10 or 16 h immediately halts the rise in infectivity. Similar difficulties affect the interpretation of the inhibition achieved by adding actinomycin D ($5 \mu g/ml$) or rifampicin ($150 \mu g/ml$) at the time of switch from 38 °C.

DISCUSSION

The ‘cut-off’ temperature of alastrim virus in chick embryo cells was found to be 38 °C in agreement with the results of Bedson (1964) and Gurvich & Marennikova (1964). At this temperature alastrim virus appears to be sensitive only in respect of late events and different therefore from variola major virus which was found to be temperature sensitive in respect of both early and late events when grown in HeLa cells at 40 °C (Cruickshank & Bedson, 1968). However, the two investigations involve differences in non-permissive temperature.
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and in host cell, as well as in virus, and attempts to resolve the interrelationship of these factors in determining 'cut-off' temperatures are described in a separate paper (R. J. Cooper & H. S. Bedson, unpublished observations).

The results of the temperature-shift experiments are similar to those obtained in earlier work with alastrim virus on the CAM (Dumbell & Bedson, 1964) and are most simply explained in terms of one or more late ts events. This interpretation is supported by the apparent normality of those early functions which were examined directly. DNA synthesis, the induction of early enzymes and antigens, the regulation of these events and the switch to late antigen production all proceeded at 38 °C much as at 35 °C. A minor exception was the lower level of DNA polymerase activity at 38 °C which was only 40 % of that at 35 °C. This enzyme may be temperature sensitive and approaching its 'cut-off' temperature at 38 °C but clearly the reduced level of enzyme was sufficient to support normal DNA synthesis at 38 °C.

Examination of late events at 38 °C has shown that the predominant effect is upon particle formation. This is clear from the lack of non-infectious particles, from the fact that virus DNA remains non-particulate and sensitive to DNase and from the appearances in thin sections. The defect in particle formation may also explain the failure to produce DNA-dependent RNA polymerase at 38 °C since this activity has only been detected in maturing virus particles (Kates et al. 1968; Pitkanen et al. 1968). The absence of 'acid' DNase at 35 °C, as well as 38 °C, may be a technical matter and associated with the host cell rather than the virus, for McAuslan et al. (1965) could not demonstrate 'acid' DNase activity in cowpox, rabbitpox or vaccinia-infected chick embryo cells and yet this enzyme has since been shown to be present in highly purified poxvirus particles (Pogo & Dales, 1969).

Haemagglutinin was not produced at 38 °C. This was also a feature of the late ts event of variola major in HeLa cells (Cruickshank & Bedson, 1968). The results obtained with cytosine arabinoside show that DNA synthesis, whether at 35 °C or at 38 °C, is an essential pre-requisite for haemagglutinin production but its relationship to defective particle formation is unclear. The haemagglutinin is not a structural component of the virus (Joklik, 1966) and its synthesis is believed to be regulated independently of particle morphogenesis (Ichihashi, Matsumoto & Dales, 1971), yet in the present study its production has been coupled with this process and dissociated from the production of other late antigens. Comparison with the rifampicin-blocked situation (vide infra) suggests that its production may be closely involved with an early stage of membrane formation.

The substantially normal production of late antigens at 38 °C revealed in gel-diffusion studies does not necessarily mean that the proteins thus detected are normal in function. The full complement of lines can, for instance, still be obtained when virus growth is inhibited with p-fluorophenylalanine (Appleyard & Zwartouw, 1965). Moreover, it is clear that, even if one considers only structural components (Sarov & Joklik, 1972), the number of proteins involved is far greater than can be detected or resolved by the gel-diffusion technique. It remains quite possible that one or more structural proteins are not made at 38 °C and the results obtained with puromycin could be interpreted as supporting this view.

Although the maturation of poxviruses is sensitive to inhibition by a wide variety of agents none appears to give quite the same picture found with alastrim virus at 38 °C. Perhaps the closest parallel is with inhibition by rifampicin where early and late proteins are made but not mature particles or DNA-dependent RNA polymerase (McAuslan, 1969; Moss et al. 1969). However, haemagglutinin is produced in the presence of rifampicin (Ichihashi et al. 1971) and electron microscopy reveals membranes which are the precursors of virus envelopes (Grimley et al. 1970). Very little evidence of membrane formation was seen with alastrim
virus at 38 °C suggesting inhibition at an even earlier stage of particle formation than with rifampicin. This last observation is the one feature of our results which is not entirely consistent with the interpretation of the ts event of alastrim as a late function. The accumulation of virus envelopes that has been observed when late events have been suppressed by a variety of means (see Joklik, 1968) suggests that membrane formation should, at least in part, be regarded as an early event. Thus, the temperature sensitivity of alastrim virus could be explained in terms of one or more of the early structural proteins required for membrane formation and yet still appear as a late event in temperature-shift experiments. Experience with rifampicin has shown that particle formation can proceed very rapidly indeed once the block is removed (Grimley et al., 1970; Pennington, Follett & Szilagyi, 1970). In the case of rifampicin, analysis of radioactively labelled polypeptides has shown that cleavage of a precursor molecule into a core polypeptide is inhibited (Katz & Moss, 1970) and it is possible that analogous experiments with alastrim virus would help to determine more precisely the nature of the ts event.

REFERENCES


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