

Replication of Equine Herpesvirus Type 3: Kinetics of Infectious Particle Formation and Virus Nucleic Acid Synthesis

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SUMMARY

The kinetics of equine herpesvirus type 3 (EHV-3) multiplication and of the synthesis of EHV-3 specific DNA and RNA were investigated. A one-step growth curve of EHV-3 in equine epithelial cells from a transitional cell carcinoma was characterized by: (1) a short eclipse period (4 h); (2) an exponential increase in infectious virus between 5 and 10 h post-inoculation; and (3) a slow, inefficient release of newly formed virus into the extracellular fluid. Two hours after infection of cells with EHV-3, the rates of incorporation of specific precursors into total cell RNA or DNA were reduced to 30% and 10%, respectively, of that seen in uninfected cells. With the aid of DNA-RNA hybridization and caesium chloride isopycnic centrifugation techniques, the rates of synthesis of EHV-3 specific nucleic acids at different stages of the virus replication cycle were determined. Virus RNA and DNA synthesis was detectable 2 h after infection and reached maximum levels at an interval (4 to 7 h post-inoculation) corresponding to that period of the virus replication cycle just preceding the time of maximal synthesis of infectious virus.

INTRODUCTION

Since its initial description in 1936 as the etiological agent of an abortigenic disease of horses (Dimock & Edwards, 1936), equine herpesvirus type 1 (EHV-1; equine abortion virus; equine rhinopneumonitis virus) has been the subject of intensive scientific investigations. Replication of EHV-1 in several cell systems and many physicochemical, biological, and immunological properties of both the virus and its subviral components have been described (reviewed by Studdert, 1974 and O'Callaghan, Allen & Randall, 1976). In 1963, Plummer & Waterson (1963) isolated a 'slow' herpesvirus from a horse with a respiratory disease and proposed that the virus, being clearly different from the classic herpesvirus of equine abortion, be called equine herpesvirus type 2 (EHV-2). Since then, a large number of similar, slowly cytopathic herpesviruses have been identified from horses in many parts of the world (Bryans, 1969; Studdert, 1974). Recently, a third species of equine herpesvirus has been identified as the cause of a venereally transmitted progenital disease of horses (Bryans, 1969; Bryans & Allen, 1973). This new equine herpesvirus, designated equine herpesvirus type 3 (EHV-3; Roizman, 1973; Studdert, 1974), has many properties which distinguish it from both EHV-1 and EHV-2. EHV-3 requires cells of equine origin for replication, is not neutralized by antiserum against EHV-1 or EHV-2, and is non-abortigenic for horses. The

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density and base composition of EHV-3 DNA are also distinctly different from those of EHV-1 or EHV-2 DNA (Plummer, Bowling & Goodheart, 1969; Ludwig *et al.* 1971).

In this paper, we present additional characteristics of the replication of EHV-3 by describing the kinetics of infectious particle formation and of the synthesis of virus-specific DNA and RNA. The effects of EHV-3 infection on host nucleic acid synthesis are also described.

METHODS

Virus and cell culture. A continuous line of equine epithelial cells, derived from a transitional cell carcinoma and designated ETCC cells, was used. The development of the cell line, its cultural and karyotypic characteristics, and methods of cultivation have been previously described (Allen & Bryans, 1974). The virus used in this study was the macroplaque variant of the prototype strain (1118) of equine herpesvirus type 3 (EHV-3) which had been cloned by plaque purification. Procedures for propagation and assay of the virus have also been described (Allen & Bryans, 1974).

Virus growth studies. Replicate monolayers of ETCC cells in 25 cm² plastic tissue culture flasks (Falcon Plastics Co., Oxnard, California) were infected with an input m.o.i. of 100 p.f.u. EHV-3/cell. After incubation for 1 h at 37°C to allow for virus attachment, the virus inoculum was removed, and the monolayers were rinsed twice with 10 ml warm (37°C) medium. After the last rinse, 5 ml medium containing 10% rabbit anti(EHV-3) neutralizing serum was added to each culture, and incubation was continued at 37°C for 30 min to neutralize non-penetrated virus. When tested against 10⁶ p.f.u./ml virus, this dilution of antiserum neutralized more than 99.9% of EHV-3 within 30 min. The cells were washed twice more with warm medium to remove antibody and then were incubated at 37°C in 10 ml warm medium. At various times post-inoculation, the cells were scraped into the culture medium and centrifuged for 5 min at 1500 rev/min at 2°C. The cell pellet was resuspended in 10 ml fresh medium; both the resuspended cell pellet and the supernatant fluid were stored at -60°C until assayed. Before assay for infectivity, all samples were treated for 30 s at 2°C with an ultrasonic disintegrator (Branson Instruments, Inc., Stamford, Connecticut) to release cell-associated virus and to break up aggregates of virus particles. The supernatant fluid containing released virus and the sonically disrupted cell pellet containing cell-associated virus were then assayed for infectious virus as previously described (Allen & Bryans, 1974).

Equilibrium centrifugation of DNA. Equilibrium centrifugation of DNA in self-generating caesium chloride gradients was used to separate virus and cellular species of DNA. ³H-labelled DNA, extracted from infected ETCC cells as described by Doerfler (1969), was mixed with 1 to 2 µg ¹⁴C-labelled density marker DNA (uninfected ETCC cell DNA) and was made up to 3.4 ml with 0.01 M-tris-HCl (pH 7.5). Caesium chloride (4.4 g) was added and mixed until dissolved, and the solution (4.5 ml; density = 1.70) was placed in a 5 ml polyallomer centrifuge tube. The tubes were centrifuged at 35000 rev/min (Beckman SW 50.1 rotor) for 48 h at 25°C. Four-drop fractions were collected on to Whatman 3MM filter paper discs which were washed according to the procedure of Bollum (1966). Acid-insoluble radioactivity in the dried discs was determined by liquid scintillation counting. Every 10th fraction of the gradient was collected in tubes, and the buoyant density was calculated from the refractive index by the method of Ifft, Voet & Vinograd (1961).

DNA-RNA hybridization. Radioactive RNA was extracted from infected ETCC cells as described by Scherrer (1969) and was hybridized to denatured EHV-3 DNA immobilized on nitrocellulose membrane filters according to the method of Gillespie & Spiegelman (1965).

Briefly, DNA extracted from purified EHV-3 nucleocapsids (Allen & Bryans, 1976) was diluted with 0.1 × standard saline citrate solution (SSC; 1 × SSC = 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0) to a concentration of 4 µg/ml and placed in a boiling water bath for 10 min to denature the DNA. The solution was cooled quickly to 0°C, adjusted to 4 × SSC, and filtered through 25 mm nitrocellulose membrane filters (B6; Schleicher and Schuell, Inc., Keene, New Jersey). The filters were washed with four 10 ml vols. 4 × SSC and then were heated at 80°C for 4 h *in vacuo*.

Five 5 mm microfilters were cut from each 25 mm filter and were placed in 10 × 75 mm tubes containing 100 µl of radioactive RNA in 4 × SSC-0.1% SDS. Two microfilters were added to each RNA solution, one containing 2 µg EHV-3 DNA and the other containing no DNA. Hybridization was carried out at 65°C for 48 h. At the end of the incubation, the filters were removed and batch-washed four times at 65°C with 200 ml fresh 4 × SSC each time. The filters were then incubated at room temperature with 20 µg bovine pancreatic RNase A/ml (Sigma Chemical Co., St Louis, Missouri), washed three times with 4 × SSC, dried and counted. Radioactivity bound to the blank filters represented less than 0.1% of the input radioactivity and was subtracted from the label bound to the virus DNA-containing filters.

RESULTS

Virus growth curve

A one-step growth curve of EHV-3 in ETCC cells is shown in Fig. 1. An eclipse of infectivity occurred during the first 4 h after infection, when less than 2 p.f.u. of virus/cell could be detected. At 5 h post-inoculation (p.i.), the formation of new infectious virus within the cell was first detected; the first release of infectious virus occurred 1 h later. Between 5 and 10 h p.i., there was a 200-fold increase of cell-associated virus infectivity resulting in a maximum virus yield 10 to 12 h p.i. of 200 p.f.u./cell. Release of newly formed virus from the infected cell occurred slowly and with low efficiency, as less than 10% of the total virus infectivity was recovered in the extracellular fluid.

Kinetics of virus DNA synthesis

To obtain information on the intracellular biosynthesis of virus-specific DNA, the kinetics of synthesis of EHV-3 DNA were investigated. Monolayer cultures of ETCC cells in 75 cm² plastic tissue culture flasks were infected with 100 p.f.u. of virus/cell and then were pulse-labelled with 10 µCi/ml methyl-³H-thymidine (20 Ci/mmol; New England Nuclear Corp., Boston, Massachusetts) for 30 min intervals at various times after infection. Immediately after the pulse, total DNA was extracted from the infected cell cultures with SDS-pronase-phenol (Doerfler, 1969), and the virus and cellular species of DNA were separated by isopycnic centrifugation in caesium chloride gradients as described in Methods.

Preliminary centrifugation of artificial mixtures of ¹⁴C-labelled host DNA and ³H-labelled DNA extracted from purified EHV-3 nucleocapsids indicated that virus DNA, which had a buoyant density of 1.725, was well separated from host DNA with a density of 1.700.

The density profiles of the DNA extracted from ETCC cells pulse-labelled with ³H-thymidine before infection or from 1.5 to 2 h p.i., 2 to 2.5 h p.i., or 5 to 5.5 h p.i. are shown in Fig. 2. Infection with EHV-3 resulted in an almost immediate cessation of host DNA synthesis. Virus DNA replication was first detected between 2 and 2.5 h after addition of virus, reached a maximum activity between 4 and 6 h after infection, and continued to be synthesized at a reduced level until late in the infection cycle.

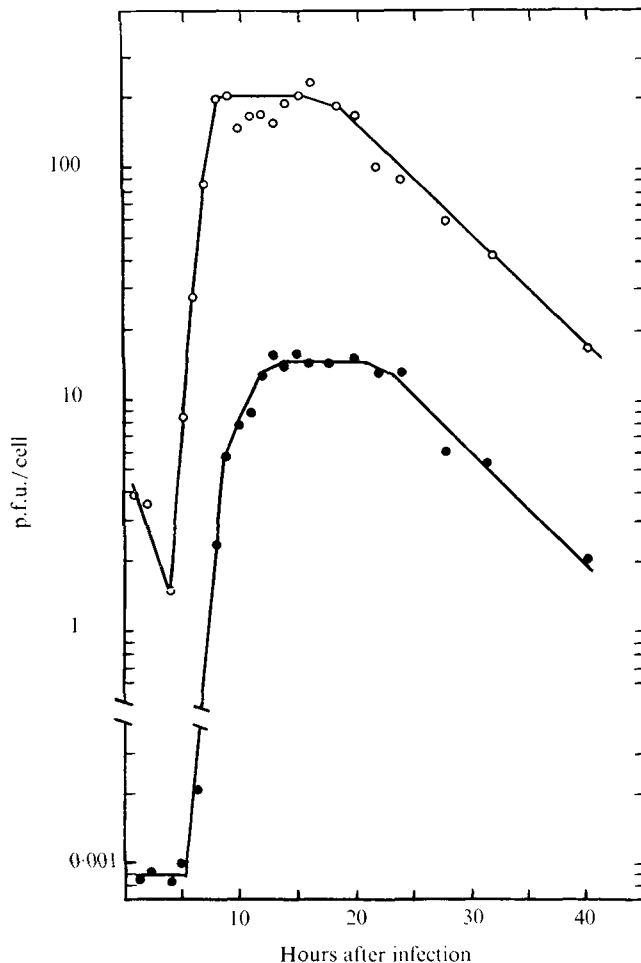


Fig. 1. One-step growth curve of EHV-3 in ETCC cells. Cell-associated virus (○—○); released virus (●—●).

The technique for separation of virus and host DNA made it possible to measure the relative amounts of these two species of DNA made at different times after infection (Fig. 3). As early as 2 h after infection, cellular DNA synthesis was reduced to only 10% of the rate seen in uninfected cultures; only low levels of thymidine were incorporated into host DNA after 2 h p.i. Consequently, the synthesis of EHV-3 DNA accounted for the majority of the total DNA being made at any time after 2 h p.i.

Total RNA synthesis in EHV-3 infected cells

Experiments were done to determine the rate of incorporation of radiolabelled uridine into the total RNA fraction of EHV-3 infected ETCC cells at various times after addition of virus. Infected cells were pulse-labelled with 5-³H-uridine (28 Ci/mmol; New England Nuclear Corp., Boston, Massachusetts) as described by Ball, Van der Berg & Poynter (1973). Replicate monolayers of cells are grown, infected, pulse-labelled, and processed for the assay of acid-insoluble radioactivity while the cells remain attached to the bottom surfaces of glass scintillation vials.

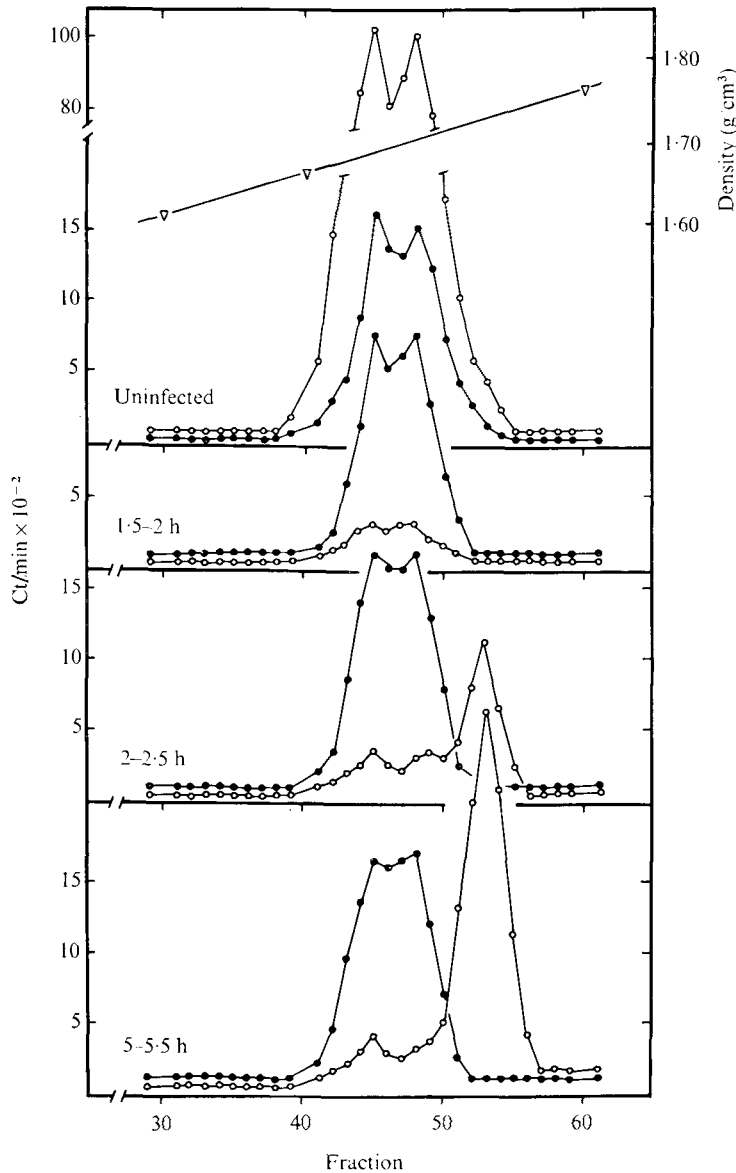


Fig. 2. Buoyant density of the DNA made in uninfected or EHV-3 infected ETCC cells at different times after infection. Total cell DNA was extracted from uninfected or virus-infected cells at the times indicated after a 30 min pulse with $10 \mu\text{Ci}$ of methyl- ^3H -thymidine/ml. The ^3H -labelled DNA ($\circ-\circ$) was mixed with ^{14}C -labelled DNA ($\bullet-\bullet$) from uninfected ETCC cells and was centrifuged to equilibrium in CsCl gradients as described in Methods. The gradients were fractionated and assayed for radioactivity and buoyant density ($\nabla-\nabla$).

Fig. 4 shows the pattern of incorporation of $1 \mu\text{Ci}$ $5\text{-}^3\text{H}$ -uridine/ml during a 30 min pulse of EHV-3 infected ETCC cells at various times after infection with 100 p.f.u. of virus/cell. Incorporation of the labelled precursor diminished sharply after addition of the virus, levelled off between 2 and 7 h p.i., and then entered a phase of steady decline. The temporary plateau in the rate of uridine incorporation between 2 and 7 h p.i. corresponded to that period in the virus replication cycle when the rate of EHV-3 specific RNA synthesis was at a

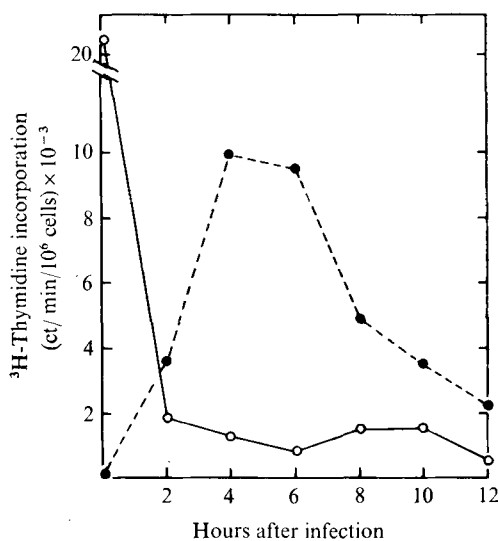


Fig. 3

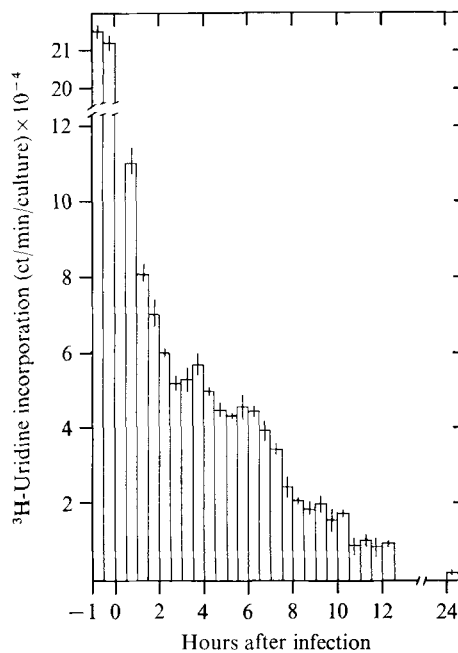


Fig. 4

Fig. 3. Incorporation of ³H-thymidine into EHV-3 and ETCC cell DNA. EHV-3 infected cells were pulse-labelled with ³H-thymidine (10 μCi/ml) for 30 min at intervals after infection. The total DNA was extracted with SDS-pronase-phenol and centrifuged to equilibrium in CsCl gradients as described in Methods. The gradients were fractionated, and the radioactivity represented by EHV-3 specific DNA (●—●) or host DNA (○—○) was plotted against time after infection.

Fig. 4. Incorporation of 5-³H-uridine (1 μCi/ml) into the RNA of ETCC cells at intervals after infection with EHV-3. The vertical bars represent the standard errors of the mean values.

maximum as measured by DNA-RNA hybridization (see below). By 10 to 12 h after infection, when the maximum titres of infectious virus had been synthesized, the rate of total RNA synthesis in infected cells was reduced to less than 10% of that observed for uninfected cultures.

Kinetics of virus-specific RNA synthesis

DNA-RNA hybridization was used to determine the kinetics of synthesis of EHV-3 specific RNA. The details of the hybridization technique have been described in Methods. Preliminary experiments were done to find the optimum conditions of virus DNA-RNA hybridization on filters, to ensure its specificity for the detection of virus RNA, and to define the conditions necessary for obtaining a quantitative determination of virus-specific RNA by DNA-RNA hybrid formation.

The specificity of the hybridization reaction is illustrated in Table 1. Of the added radioactivity of total cell RNA extracted from EHV-3 infected ETCC cells and labelled 4 to 6 h p.i., approx. 10% became bound to membrane filters containing immobilized virus DNA. However, less than 0.1% of the added radioactivity of RNA from uninfected cells did so. Likewise, when EHV-3 DNA-containing filters were incubated with an artificial mixture of ³H-labelled RNA from infected ETCC cells and ¹⁴C-labelled RNA from uninfected cells, only the tritium label bound appreciably to the filters. Non-specific binding of label to blank filters was only a few ct/min above the scintillation counter background.

Table 1. Specificity of DNA-RNA hybridization

RNA	Ct/min bound to:	
	Filter containing 2 μ g EHV-3 DNA	Blank filter
^3H -RNA from EHV-3 infected ETCC cells	9500*	55
^3H -RNA from uninfected ETCC cells	86	50
Mixture of ^3H -RNA from EHV-3 infected ETCC cells + ^{14}C -RNA from uninfected ETCC cells	8900 (^3H) 81 (^{14}C)	62 (^3H) 72 (^{14}C)

* Each value represents the mean of three determinations; the scintillation counter background was 30 to 35 ct/min. The added radioactivity in each case was 100000 ct/min.

The kinetics of the filter hybridization reaction of 5- ^3H -uridine-labelled RNA from EHV-3 infected ETCC cells and filter-bound virus DNA was determined. A plot of ct/min of virus RNA hybridized as a function of incubation time at 65°C indicated that hybridization progressed with time and was virtually complete after 48 h (data not shown).

When filters containing 2 μ g EHV-3 DNA were incubated with increasing concentrations of ^3H -labelled RNA extracted from infected cells, the amount of RNA forming a RNase-resistant hybrid with the virus DNA increased linearly until approx. 20 μ g RNA had been added. These results indicated that 2 μ g filter-bound EHV-3 DNA did not become saturated with infected cell RNA until 20 μ g had been added. Therefore, in all subsequent filter hybridization experiments, no more than 20 μ g RNA was added to each reaction tube.

Having demonstrated the specificity of the DNA-RNA hybridization technique for the quantitative detection of virus RNA sequences, the kinetics of synthesis of EHV-3 specific RNA in infected ETCC cells was studied. Replicate monolayers of ETCC cells in 25 cm² plastic tissue culture flasks were inoculated with 100 p.f.u. of EHV-3/cell. At intervals after infection, duplicate cultures were pulse labelled for 1 h with 20 μCi of 5- ^3H -uridine/ml (28 Ci/mmol). At the end of the pulse, total cell RNA was extracted by the hot phenol-SDS method of Scherrer (1969). Total acid-insoluble radioactivity was determined on 20 μl samples of each final RNA preparation; the remaining RNA was hybridized to EHV-3 DNA on nitrocellulose filters under conditions of DNA excess as described in Methods.

The results, presented in Fig. 5, are expressed either as the total radioactivity hybridizable per culture (Fig. 5*a*) or as the percent of the total incorporated radioactivity per culture which was hybridized (Fig. 5*b*), both as a function of time after infection. EHV-3 specific RNA synthesis was detectable within 1 h after inoculation of cells with virus and represented only 1% of the total RNA synthesized by the cell. The amount of synthesized virus RNA reached a maximum between 4 and 7 h p.i. and then began a steady decline. At the peak of virus-specific RNA synthesis (4 to 7 h p.i.), only 8 to 10% of the total RNA made in the infected cell hybridized to EHV-3 DNA. Although smaller quantities of virus RNA were made very late in infection (10 to 12 h p.i.), this RNA represented a larger fraction of the total RNA made at that time in the infected cell (12 to 15%).

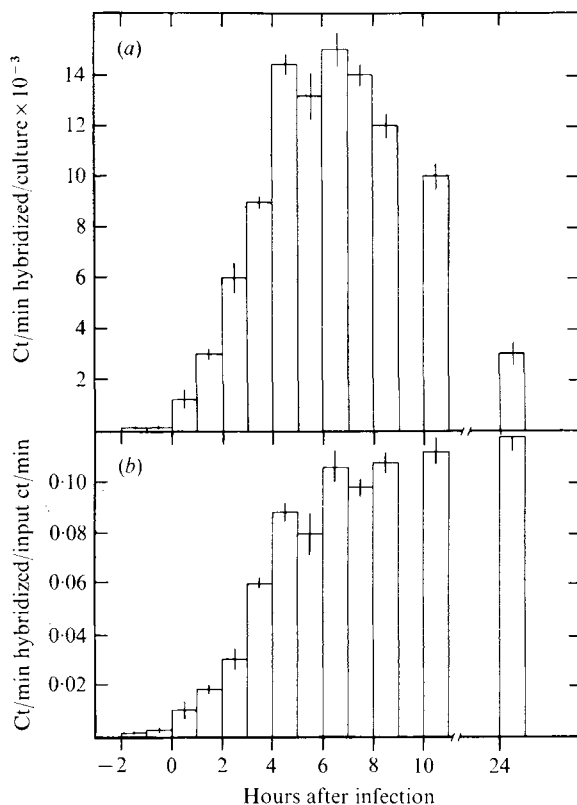


Fig. 5. Kinetics of virus RNA synthesis in ETCC cells infected with EHV-3. ³H-labelled RNA was extracted from infected cells at the times indicated after a 1 h pulse with 5-³H-uridine and was hybridized with 2 µg EHV-3 DNA. The RNase-resistant radioactivity bound to the filters was determined as described in Methods. (a) Results expressed as the total radioactivity hybridized per culture. (b) Results expressed as the percent of the total incorporated radioactivity per culture which was hybridized.

DISCUSSION

These studies have dealt with the time course of formation of infectious virus (EHV-3) and virus-specific nucleic acids and also the effect of EHV-3 infection on host nucleic acid synthesis.

Comparison of the one-step growth cycle of EHV-3 with that reported for EHV-1 (O'Callaghan *et al.* 1968*a, b*; Lawrence, 1971) or EHV-2 (Plummer *et al.* 1969; Studdert, 1974) reveals that EHV-3 is the most rapidly replicating of all the equine herpesviruses. New infectious virus could be detected as early as 5 h after infection of ETCC cells with EHV-3, and maximum titres of infectious virus were obtained 10 to 12 h p.i. The majority (90%) of the infectious progeny virus remained cell-associated and could be recovered only by sonic disruption of the infected cells. This feature of EHV-3 growth is shared by EHV-2 (Bryans, 1969; Plummer *et al.* 1969; Studdert, 1974) but is in direct contrast to EHV-1 replication in mouse L-M cells, in which most of the viral progeny is released into the extracellular medium (O'Callaghan *et al.* 1968*b*). Although the cellular yield of EHV-3 recoverable from the supernatant medium was quite low (20 p.f.u./cell), the amount of total virus made in ETCC cells (200 p.f.u./cell) is comparable to the yield of EHV-1 from infected L-M cells (O'Callaghan *et al.* 1968*b*).

The results show that infection with EHV-3 causes a marked suppression of host nucleic acid synthesis. The inhibition of host DNA and RNA synthesis began very shortly after the entry of virus into the cell and progressed as infection continued. A similar inhibition of host nucleic acid synthesis has been described for other virus-cell systems and appears to be a characteristic feature of herpesvirus infection (Kaplan & Ben-Porat, 1963; O'Callaghan *et al.* 1968*a, b*; Roizman, 1971). The most striking aspect of the inhibition of host macromolecular synthesis by EHV-3, as compared to the inhibition by EHV-1, is the unusual rapidity with which it occurs.

By means of isopycnic centrifugation in caesium chloride and DNA-RNA hybridization, it was possible to measure the rate of incorporation of labelled precursor into EHV-3 specific DNA or RNA at different times after infection. The compressed replicative cycle of EHV-3, discussed above, is reflected in the very early appearance of virus-specific DNA and RNA synthesis in infected cells. Both EHV-3 DNA and RNA could be detected as early as 2 h after infection. Likewise, the time of maximum synthesis of EHV-3 nucleic acids (4 to 6 h p.i.) as well as the decline of virus nucleic acid synthesis occurred earlier than with EHV-1 (O'Callaghan *et al.* 1968*a*; Huang *et al.* 1971).

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