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THE EFFECT OF TEMPERATURE ON THE *IN VITRO* TRANSCRIPTASE REACTION OF BLUETONGUE VIRUS, EPIZOOTIC HAEMORRHAGIC DISEASE VIRUS AND AFRICAN HORSESICKNESS VIRUS

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ABSTRACT

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Virions of bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV) and African horsesickness virus (AHSV) can be converted to core particles by treatment with chymotrypsin and magnesium. The conversion is characterized by the removal of the 2 outer capsid polypeptides of the virion. The loss of these 2 proteins results in an increase in density from 1,36 g/mℓ to 1,40 g/mℓ on CsC1 gradients. The BTV, EHDV and AHSV core particles have an associated double-stranded RNA dependent RNA transcriptase that appears to transcribe mRNA optimally at 28 °C. It was found, at least in the case of BTV, that this low temperature preference is not an intrinsic characteristic of the transcriptase, but is due to a temperature-dependent inhibition of transcription at high core concentrations.

INTRODUCTION

Bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV) and African horsesickness virus (AHSV) are all classified as orbiviruses, a subgroup of the Reoviridae (Verwoerd, Huismans & Erasmus, 1979). These viruses consist of 10 segments of double-stranded RNA (dsRNA) which are surrounded by a double layer protein coat (Verwoerd, Els, De Villiers & Huismans, 1972; Bremer, 1976; Huismans, Bremer & Barber, 1979). Each segment probably contains the information for the synthesis of one virus specific polypeptide.

During infection each of the 10 dsRNA segments is transcribed into a mRNA molecule of the same polarity as the plus strand of the dsRNA (Huismans & Verwoerd, 1973). The RNA dependent RNA polymerase involved in this reaction is not a normal cellular enzyme, but is part of the protein coat of the virus (Verwoerd & Huismans, 1972).

The BTV-associated transcriptase is activated *in vitro* by the removal of the 2 outer capsid polypeptides, P2 and P5, from the virion. The optimum temperature for the *in vitro* transcription reaction of BTV has thus far always been reported as 28 °C (Verwoerd & Huismans, 1972; Van Dijk & Huismans, 1980) as opposed to the 45 °C of other dsRNA containing viruses such as reovirus (Skehel & Joklik, 1969) and rotavirus (Cohen, 1977). It is not yet known whether the low temperature optimum of the BTV *in vitro* transcription reaction is a characteristic of all orbiviruses. Because of the difficulties experienced in both the purification of these viruses and in the removal of the outer capsid polypeptides (Huismans *et al.*, 1979), no *in vitro* transcription has been reported yet for orbiviruses other than BTV.

In this paper we report a method for the *in vitro* activation of the EHDV- an AHSV-associated transcriptases. We also investigated the temperature dependence of these enzymes. In the case of BTV we were able to demonstrate that the core concentration plays an important role with regard to the optimum temperature of the transcription reaction.

MATERIALS AND METHODS

Cells and viruses

Baby hamster kidney cells (BHK) were obtained from the American Type Culture Collection and grown as monolayers in Roux flasks or roller bottles in modified Eagle's medium (Verwoerd, Oellermann, Broekman & Weiss, 1967). The medium was supplemented with 5% bovine serum.

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The following virus strains were used: an avirulent strain of BTV serotype 10; type 3 (Abney strain) reovirus; AHSV type 3 obtained from Dr B. J. Erasmus of the Virology Section of the Veterinary Research Institute, Onderstepoort, and the New Jersey strain of EHDV serotype 1 (Barber & Jochim, 1975) which was obtained from the Arthropod-borne Animal Diseases Research Laboratory, USDA, Science and Education Administration—Agricultural Research, Denver, Colorado, USA.

The viruses were propagated in monolayer BHK cells. BTV and reovirus were purified by the method described by Huismans (1979). In the case of EHDV and AHSV the purification was modified according to the method of Huismans *et al.* (1979).

Preparation of ^{3}H-labelled BTV and ^{14}C-labelled reovirus

BHK cell monolayers in roller bottles (1×10^8 cells/bottle) were infected with either BTV or reovirus at a multiplicity of infection of 40 plaque-forming units (PFU) per cell. Four hours after infection the medium in each bottle was replaced with 5 mℓ of Eagle's medium, containing 4,4 μCi ^{3}H-uridine/mℓ in the case of BTV and 0,1 μCi ^{14}C-uridine/mℓ in the case of reovirus. After 24 hours an additional 15 mℓ of Eagle's medium was added. The cells were harvested after another 20-hour incubation period and the virus purified as described by Huismans (1979).

Isopycnic CsC1 density centrifugation

CsC1 was added to solutions of viral particles in 0,1 M Tris-HC1 pH 8,0 an pH 7,0, respectively, until the refraction index of the solutions corresponded to a density of 1,37 g/mℓ. Centrifugation was performed in a SW 50.1 rotor at 40 000 rpm for 27 hours at 4 °C. The gradients were fractionated in 2 or 3 drop fractions from the bottom of the tube. The refraction index of every 2nd fraction was determined. Radioactivity of the fractions was determined by counting 20 $\mu\ell$ of each fraction in the presence of 5 mℓ Triton X100 scintillation fluid (66% Toluene v/v; 33% Triton X100 v/v; 0,2% PPO w/v; 0,04% POPOP w/v) in a Packard Tricarb Scintillation Counter.

Preparation of core particles

A partially purified virus suspension (i.e. virus suspension which was not purified on sucrose gradients) obtained from approximately 1×10^{10} infected BHK cells was incubated for 60 min at 37 °C in a mixture containing 100 mM Tris-HC1 pH 8,0; 100 μg/mℓ chymotrypsin and 600 mM $MgC1_2$. After incubation, the virus particles were recovered by centrifugation through a 40% sucrose cushion in a SW 27 rotor at 27 000 rpm for 120 min at 4 °C. The virus material in the pellet was further purified by isopycnic CsC1 density centrifugation

at pH 8,0. Core particles banded at a density of 1,40 g/mℓ. The bands were usually collected with a syringe, diluted with 2 mM Tris-HC1 pH 8,0 and concentrated by pelleting through a 1 mℓ 40% sucrose cushion in a SW 50.1 rotor at 45 000 rpm for 45 min at 4 °C. The pellet was suspended in approximately 1,5 mℓ of 2 mM Tris-HC1 pH 8,0 and kept at 4 °C until use. Alternatively, the CsC1 bands were not concentrated but only desalted on a prepacked Sephadex G-25M column (Pharmacia Fine Chemicals, PD-10).

Polyacrylamide gelelectrophoresis

Electrophoresis was performed in a slabgel unit constructed as described by Studier (1973). The electrophoretic procedure was essentially the same as that described by Laemmli (1970). The concentration of acrylamide in the separating gel was 12,5%, and in the stacking gel 5%. The gels were run for 16 hours at 200 volts. They were stained in Coomassie Brilliant Blue G250 solution (Anderson, Cawston & Cheeseman, 1974) and destained in 4% acetic acid at 50 °C. The gels were dried on a Whatman filter paper No. 3 under vacuum on a heated gel-drying apparatus.

Assay for RNA polymerase activity

Transcriptase activity was usually assayed in a standard transcription mixture (150 $\mu\ell$) which contained the following: 1,7 mM each of ATP, GTP and CTP; 100 mM Tris-HC1 pH 8,0; 6 mM $MgC1_2$; 7,5 mM phosphoenolpyruvate; 0,1 mg pyruvate kinase/mℓ; 2 mM dithiothreitol; 2 mM $MnC1_2$; 0,25 mM S-adenosyl-L-methionine (SAM) and ^{3}H-UTP as 40 μCi/mℓ (specific activity 23 mCi/mmole). Core particles were added to a final concentration of 0,5 A_{260}-units/mℓ. This transcription mixture was incubated for 4–5 hours at 28 °C, unless otherwise indicated. After the incubation period duplicate samples (65 $\mu\ell$ each) were spotted on Whatman No. 3 filter paper discs (2 cm in diameter). The discs were then washed successively 3 times for 10 min in cold 10% TCA (10 mℓ/disc), twice in cold 5% TCA, and once in cold water. Water was removed by a rinse in cold 95% ethanol and a rinse in ether as described by Bergmann & Lodish (1979). The discs were dried in an oven for 30 min at 90 °C, and then counted in a toluene scintillation fluid in a Packard Liquid Scintillation Counter.

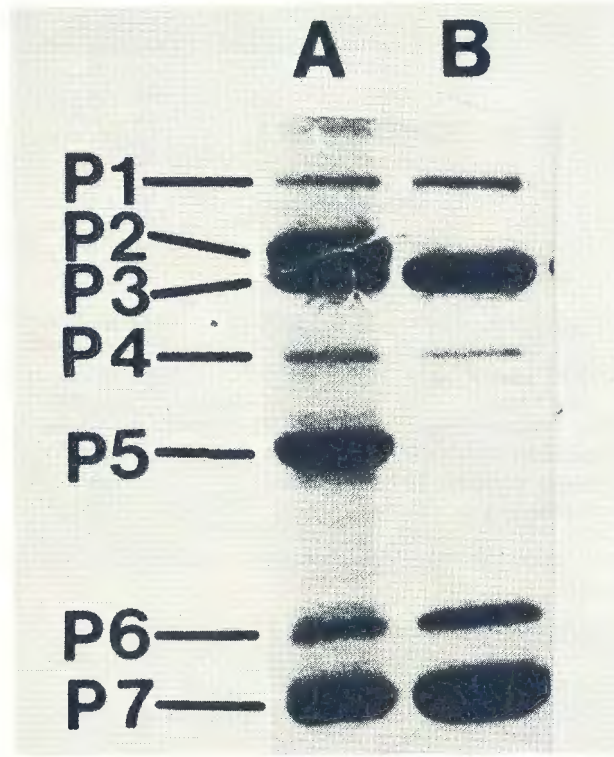

FIG. 2 Gelelectrophoretic analysis of (A) BTV control showing all 7 structural polypeptides of the virion and (B) BTV particles with a CsC1 density of 1,40 g/mℓ

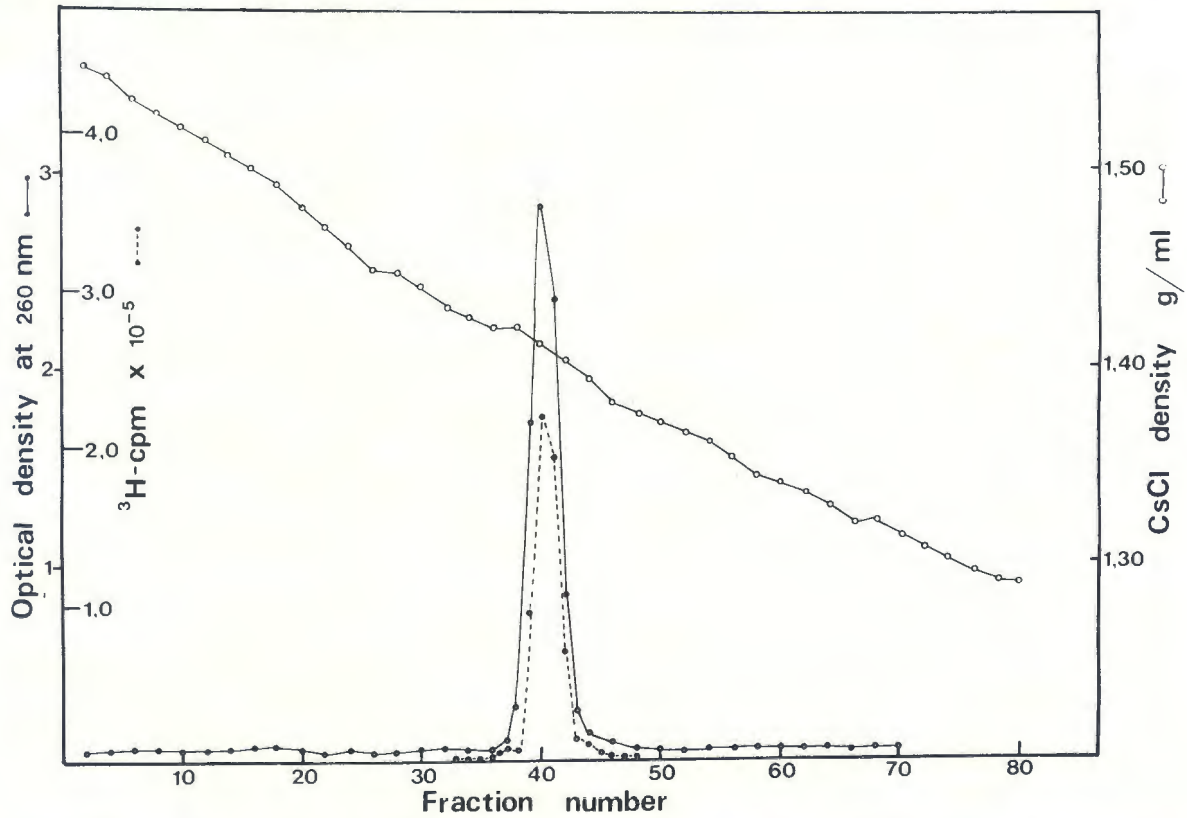

FIG. 1. The density in CsC1 of BTV particles with *in vitro* activated transcriptase activity. The optical density of the particles in the different fractions was measured at 260 nm (●———●) and the transcriptase activity assayed as the ^{3}H-cpm incorporated from ^{3}H-UTP into acid-insoluble material (●--------●)

RESULTS

Density of the BTV subviral particle with in vitro *transcriptase activity*

Treatment of BTV virions with chymotrypsin and Mg^{++} removes the outer capsid polypeptides and unmasks the viral associated transcriptase (Van Dijk & Huismans, 1980). The density of these particles with *in vitro* transcriptase activity was determined by treating 4,5 mg of BTV virions with chymotrypsin and Mg^{++} and then subjecting them to isopycnic CsC1 gradient centrifugation as described under Materials and Methods. Each fraction of the gradient was analysed for optical density at 260 nm and *in vitro* transcriptase activity. The result is shown in Fig. 1.

The single optical density peak at a CsC1 density of 1,40 g/mℓ coincides exactly with the peak of transcriptase activity. Gelelectrophoretic analysis of the BTV particles recovered from the peak fractions (Fig. 2) shows that they have the typical polypeptide composition of core particles, i.e. they lack the 2 outer capsid polypeptides P2 and P5.

Density of BTV virions and cores

The 1,40 g/mℓ density of the BTV particles with transcriptase activity differs significantly from previously published densities of BTV cores (Verwoerd *et al.*, 1972; Martin & Zweerink, 1972). Since this difference was possibly due to the way in which the BTV cores were prepared, we reinvestigated the density of BTV virions and cores using the method of Verwoerd *et al.* (1972) to prepare the core particles. According to this method virions are converted to cores on CsC1 gradients at a pH of 7,0.

Tritium-labelled BTV virions were centrifuged to equilibrium on isopycnic CsC1 gradients at pH 8,0 and pH 7,0. As a density control, ^{14}C-labelled reovirions were included in both gradients. The density of reovirus is 1,36 g/mℓ and it is not affected by changes in the pH of CsC1 gradients (Skehel & Joklik, 1969). Fig. 3 shows the distribution of ^{3}H- and ^{14}C-labelled virus material in the gradients.

On the pH 8,0 gradient the ^{3}H-labelled BTV particles had a density of 1,36 g/mℓ and on the pH 7,0 gradient a density of 1,40 g/mℓ. On both gradients the ^{14}C-labelled reovirus particles banded at 1,36 g/mℓ, a result which is in agreement with the published value. Polyacrylamide gelelectrophoresis confirmed that the virus particles recovered from the 1,40 g/mℓ fractions of the pH 7,0 gradient contained only the 5 BTV core polypeptides. The 1,36 g/mℓ fractions of the pH 7,0 gradient contained only the structural polypeptides of reovirus, whereas the 1,36 g/mℓ peak of the pH 8,0 gradient consisted of a mixture of BTV and reovirus structural polypeptides (result not shown).

The in vitro *uncoating of EHDV sand AHSV*

African horsesickness virus and EHDV were partially purified and treated with either chymotrypsin alone or with a combination of chymotrypsin and Mg^{++}, according to the procedures described under Materials and Methods. The virus particles were recovered by centrifugation through a sucrose cushion and the proteins were analysed by electrophoresis. The result is shown in Fig. 4. Since the viruses were only partially purified and it is not possible to distinguish between the minor viral polypeptides and the remaining cellular polypeptides, only the major viral polypeptides are indicated.

Treatment with chymotrypsin alone removes polypeptide P2 from AHSV and EHDV virions (Fig. 4 lanes B and E), while the combination of chymotrypsin and Mg^{++} removes both P2 and P5 (Fig. 4, lanes C and F).

Isopycnic CsC1 density centrifugation of the virus material recovered after treatment with chymotrypsin and Mg^{++} yields highly purified EHDV and AHSV core particles. These core particles have a density of 1,40 g/mℓ (result not shown) and *in vitro* transcriptase activity.

The influence of temperature on the in vitro *transcription reaction of BTV, AHSV and EHDV*

BTV, EHDV and AHSV core particles were prepared from partially purified virus material as described under Materials and Methods. The *in vitro* transcriptase activity of the different core particles was assayed as described under Materials and Methods, except that the incubation temperature was varied from 15 °C to 50 °C. The result is shown in Fig. 5.

The transcriptase reaction in all 3 cases has a similar temperature dependence. In all 3 cases maximum transcriptase activity was found at 28 °C and almost identical temperature-activity curves were obtained.

The influence of core concentration on the in vitro *transcription reaction of BTV*

Van Dijk & Huismans (1980) have shown that the transcription reaction of BTV is affected by the concentration of the cores in the reaction mixture. As it is not yet known to what extent this effect is influenced by temperature, the temperature of the BTV transcriptase was investigated at core concentrations of 2 A_{260}-units/mℓ; 0,625 A_{260}-units/mℓ and 0,125 A_{260}-units/mℓ. The BTV cores were prepared as described under Materials and Methods and were passed before use through a small column of Sephade G-25M equilibrated with 2 mM Tris, pH 9,0. Transcriptase activity was assayed at temperatures which varied from 20 °C–47 °C by using standard transcription mixtures of 400 $\mu\ell$ from which 25 $\mu\ell$ samples were spotted on filter paper discs every 30 min during a 6-hour reaction period. A 25 $\mu\ell$ sample of the 0,125 A_{260}-units/mℓ transcription mixture contained $3{,}125 \times 10^{-3}$ A_{260}-units of BTV cores. The ^{3}H-cpm incorporated into acid-insoluble material by this amount over a 6-hour period at different temperatures is shown in Fig. 6A. To compare this result with those obtained at the other 2 core concentrations, the results in Fig. 1B and 1C were plotted to show the incorporation by exactly the same amount of cores under the conditions indicated.

The degree to which temperature affects the transcriptase reaction depends greatly on the core concentration. At a core concentration of 0,125 A_{260}-units/mℓ there is only a small difference between the reaction rates at 37 °C and 28 °C, though the reaction appears to proceed best at 31 °C. At a core concentration of 0,625 A_{260}-units/mℓ the reactions at 28 °C and 31 °C are about the same, but there appears to be inhibition of the transcriptase reaction at 37 °C. At the highest core concentration, 2 A_{260}-units/mℓ, the reaction is strongly inhibited at 37 °C and comes to a halt after only 2 hours while at the lower temperatures this inhibition is much less pronounced and the reaction proceeds somewhat better at 28 °C and 24 °C than at 31 °C.

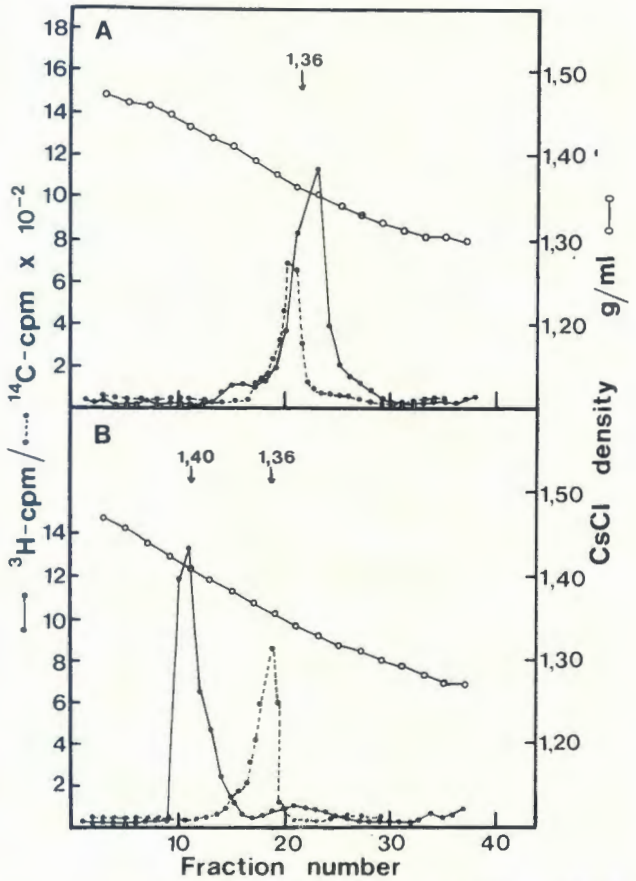

FIG. 3 CsC1 density analysis of ^{3}H-labelled BTV and ^{14}C-labelled reovirus on a (A) pH 8 CsC1 gradient and (B) pH 7 CsC1 gradient

The same tendency is reflected by the results obtained at the other temperatures. At the highest core concentration the reaction at 20 °C proceeds almost as well as the reaction at 28 °C, but there is virtually no transcription at 41 °C beyond 1 hour after the commencement of the reaction. At the low core concentration, 0,125 A_{260}-units/mℓ, the reactions at 20 °C and 41 °C are about the same.

DISCUSSION

In contrast to bluetongue virus, orbiviruses such as EHDV are stable on CsC1 gradients at neutral pH (Huismans *et al.*, 1979). The previously described method for the preparation of cores (Verwoerd *et al.*, 1972) is therefore not generally applicable to all orbiviruses. This led us to investigate the possibility of using an alternative method to prepare cores from purified or partially purified EHDV and AHSV preparations. We found that chymotrypsin, such as has been described for BTV (Van Dijk & Huismans, 1980), specifically removes outer capsid polypeptide P2 from both the EHDV and AHSV virions, while a combination of chymotrypsin and Mg^{++} removes P2 together with the other outer capsid polypeptide, P5. This provides a method for the *in vitro* preparation of orbivirus cores, which is likely to be applicable to many more, if not to all, orbiviruses. The core particles of BTV, EHDV and AHSV that are obtained after treatment with chymotrypsin and Mg^{++} have a density of 1,40 g/mℓ as opposed to virions which have a density of 1,36 g/mℓ. An additional advantage of this method is that, since partially purified virus material can be used, time is saved and the difficulties experienced in completely purifying these viruses eliminated.

The *in vitro* transcription reaction of EHDV and AHSV is temperature-sensitive and, as indicated in Fig. 5, appears to proceed optimally at a temperature of 28 °C. This is in agreement with the result previously found for BTV (Verwoerd & Huismans, 1972). In this respect the orbiviruses seem to resemble cytoplasmic polyhedrosis virus of the silkworm *Bombyx mori* (Lewandowski, Kalmakoff & Tanada, 1969) and wound tumour virus (Black & Knight, 1970). Other dsRNA-containing viruses such as reovirus (Skehel & Joklik, 1969) and rotavirus (Cohen, 1977) have a temperature optimum of approximately 45 °C for *in vitro* transcription. A low temperature preference for *in vitro* transcription, however, is also common among a wide variety of completely different viruses such as vesicular stomatitis virus (Huang, Baltimore & Bratt, 1971), Newcastle disease virus (Huang *et al.*, 1971), Sendai virus (Stone, Portner & Kingsbury, 1971) and influenza virus (Raghow & Kingsbury, 1976).

It is clear from the results in Fig. 6, however, that the apparent low temperature preference of the BTV transcriptase is not an inherent characteristic of the enzyme itself, but that the results can be explained by a temperature-dependent inhibitory effect of the concentration of cores in the reaction mixture.

We have previously reported on the core concentration-dependent inhibition of the transcriptase reaction of BTV (Van Dijk & Huismans, 1980). However, it was not realized at that time that the degree of inhibition is influenced by the temperature. At very low core concentrations the transcriptase reaction proceeds as well as, if not better than, at temperatures as much as 7° above 28 °C. If the core concentration is increased, the transcriptase reaction is inhibited, but much less so at the lower than at the higher temperature, creating the impression that the transcriptase has a low temperature preference.

The results in Fig. 6 also indicate that the core-dependent inhibition of the transcriptase reaction mainly affects the continuation of the transcription reaction, i.e. reinitiation or chain elongation. At the core concentrations investigated there is very little difference in the rate of transcription during the first hour after the commencement of the reaction. After this, at a time dependent on the core concentration, the rate of transcription levels off until it completely ceases. This inhibition is to some extent counteracted by a decrease in temperature.

For the quantitative synthesis of BTV mRNA in an *in vitro* reaction it is of practical advantage to work at the highest possible core concentration. Therefore, the optimum temperature for the quantitative *in vitro* synthesis of BTV mRNA remains 28 °C. This, however, is not to be taken as evidence that the transcriptase itself has a low temperature optimum.

Inhibitory factors such as core concentration are unlikely to play an important role in the transcriptase reaction in the infected cell. This explains why there is no evidence for a low temperature requirement for *in vivo* transcription and why labelling of *in vivo* synthesized BTV mRNA is normally carried out successfully at 37 °C (Huismans, 1970; Verwoerd & Huismans, 1973).

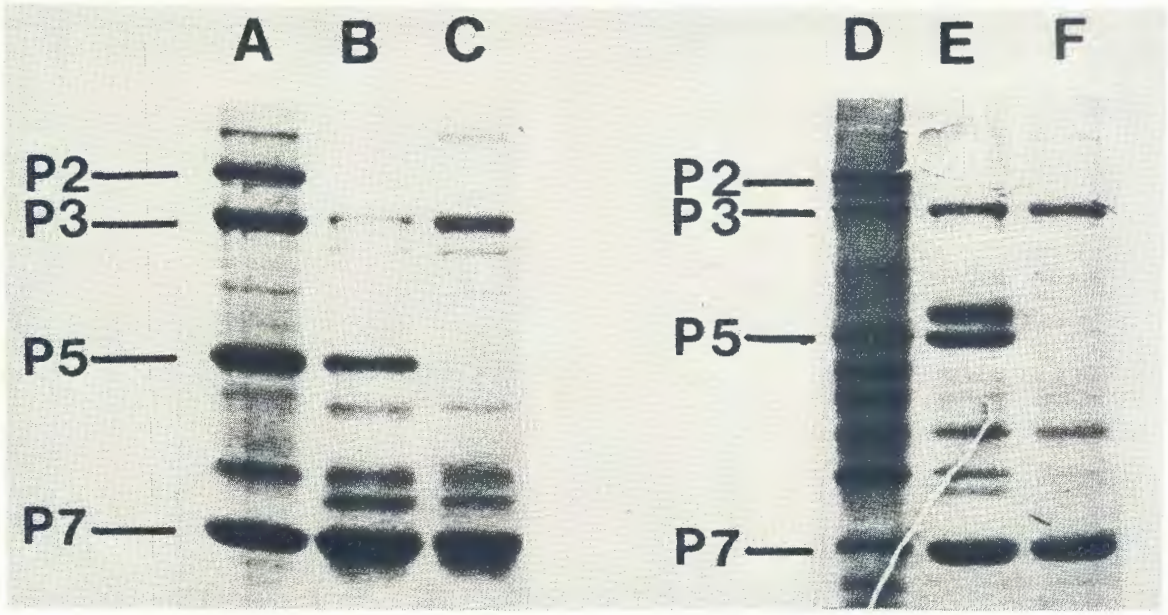

FIG. 4 Gelelectrophoretic analysis of partially purified (A) AHSV, (B) AHSV after treatment with chymotrypsin, (C) AHSV after treatment with chymotrypsin and Mg^{++}, (D) EHDV, (E) EHDV after treatment with chymotrypsin and (F) EHDV after treatment with chymotrypsin and Mg^{++}

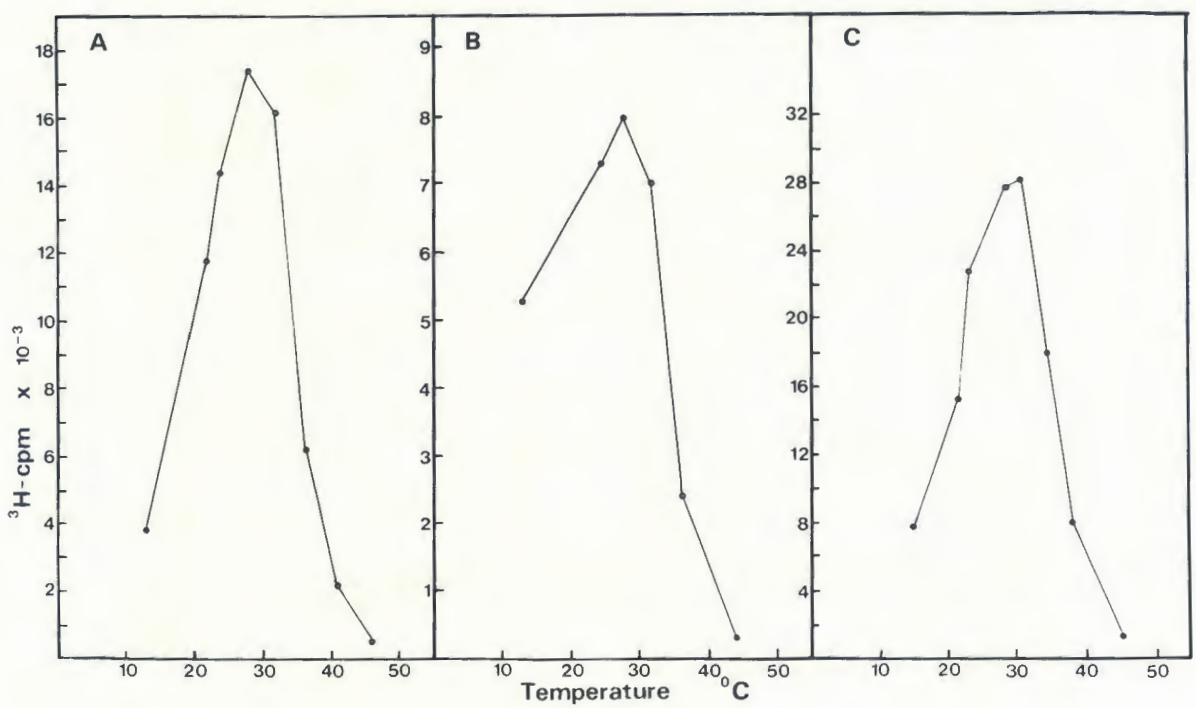

FIG. 5 The *in vitro* transcription reaction of (A) AHSV, (B) EHDV and (C) BTV at different incubation temperatures

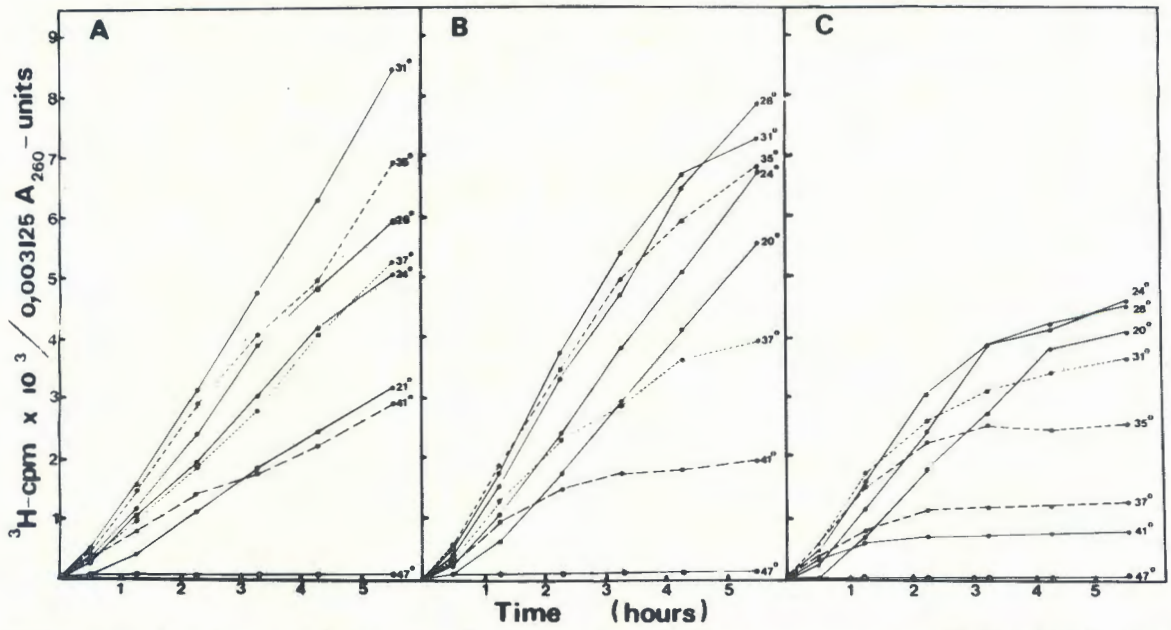

FIG. 6 The temperature dependence of the BTV *in vitro* transcription reaction at 3 different core concentrations. All the indicated points are the average values of 2 determinations (A) 0,125 A_{260}-units BTV cores/mℓ, (B) 0,625 A_{260}-units BTV cores/mℓ and (C) 2.0 A_{260}-units BTV cores/mℓ

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