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THE IDENTIFICATION OF FACTORS CAPABLE OF REVERSING THE CORE-MEDIATED INHIBITION OF THE BLUETONGUE VIRUS TRANSCRIPTASE

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ABSTRACT

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The *in vitro* transcription reaction of bluetongue virus (BTV) is characterized by a core-mediated, temperature-dependent inhibition at high core concentrations and temperatures (Van Dijk & Huismans, 1980; Huismans, Van Dijk & Els, 1987a). It has been found that this inhibition is reversible and that an inactivated transcriptase reaction mixture can be reactivated by lowering the temperature of the reaction from 37 °C to 28 °C. In the same way it is possible to inactivate a reaction by increasing the incubation temperature from 28 °C to 37 °C. It was also found that the inhibition is counteracted by the addition of sucrose or glycerol. At relatively low core concentrations and in the presence of sucrose it is possible to obtain conditions under which transcription at 37 °C is more efficient than at 28 °C. The latter conditions probably reflect much better the *in vivo* temperature optimum for the BTV transcriptase than the *in vitro* conditions at very high core concentrations.

INTRODUCTION

During transcription the 10 double-stranded (ds) RNA genome segments of bluetongue virus (BTV) are individually transcribed into 10 mRNA species. Activation of transcriptase activity requires the conversion of BTV virions to core particles. This involves the removal of the 2 major outer capsid proteins, P2 and P5 (Verwoerd, Els, De Villiers & Huismans, 1972). The resulting core particles are composed of 5 proteins of which P7 and P3 are the 2 major structural polypeptides. The different dsRNA segments of BTV are not all transcribed at a constant rate of chain elongation. It has been found that segment 5 is transcribed at at least double the expected frequency, whereas others, such as segment 10, are transcribed at less than half the predicted rate (Huismans & Verwoerd, 1973). The same result was observed in the case of other orbiviruses, such as the epizootic haemorrhagic disease virus (Huismans, Bremer & Barber, 1979), and this transcription pattern is probably a common characteristic of the Orbivirinae. Differential rates of transcription have also been reported in the case of bovine rotavirus (Bernstein & Hruska, 1981). However, the reovirus dsRNA segments are transcribed in molar amounts inversely proportional to their molecular mass (Skehel & Joklik, 1969).

Another characteristic that distinguishes the BTV transcriptase from that of other Reoviridae is a low temperature optimum at 28 °C, which is in contrast to the 47–52 °C optimum reported for reovirus (Kapuler, 1970) and rotavirus (Cohen, 1977). It has been suggested that a low temperature optimum for *in vitro* transcription might be characteristic for viruses which replicate alternatively or exclusively in poikilothermic organisms (Nuss, 1984). The inhibition of the transcriptase activity at high core concentrations is another characteristic feature of the *in vitro* transcription reaction of BTV (Van Dijk & Huismans, 1980). This inhibition is temperature dependent (Huismans *et al.*, 1987a).

We have investigated different factors that affect inhibition of the BTV transcriptase. Evidence will be presented that the core-mediated inhibition of the transcriptase reaction is reversible and that it can be counteracted by including compounds, such as sucrose in the reaction mixture, or by a reduction in temperature.

MATERIALS AND METHODS

Cells and virus

BHK- and LF-cells were maintained, as described by Huismans *et al.* (1987a). An attenuated strain of BTV serotype 10 was used in the investigation. The virus was propagated in BHK-cells and purified as described by Huismans, Van der Walt, Cloete & Erasmus (1987b).

In vitro *and* in vivo *preparation of BTV core particles*

Core particles were prepared *in vitro* either by the chymotrypsin magnesium method of Van Dijk & Huismans (1980) or by a method based on the solubilization of the BTV outer capsid protein layer in the presence of $MgCl_2$ (Huismans *et al.*, 1987b). Briefly, the latter method involves the following: Purified BTV virions were resuspended in 2 mM Tris-HC1, pH 8,0 at a concentration of 2 mg/mℓ, followed by the addition of an equal volume of 2 M $MgCl_2$. After 30 min at 4 °C, the core particles were collected by centrifugation at 100 000 × g for 120 min at 4 °C through a 40 % sucrose layer. The pellet was resuspended in 2 mM Tris-HC1, pH 8,0 and purified by CsC1 density centrifugation, as described by Van Dijk & Huismans (1980).

Core particles were prepared *in vivo* by infecting an LF-cell suspension culture with purified BTV, as described by Huismans *et al.* (1987a).

In vitro *transcription reaction*

Transcriptase activity was assayed in the standard transcription reaction mixture described by Van Dijk & Huismans (1980). The final concentration of reagents in the transcription mixture was: 1,7 mM each of ATP, GTP and CTP; 100 mM Tris-HC1, pH 8,0; 6 mM $MgCl_2$; 7,5 mM phospho-enol pyruvate; 0,1 mg/mℓ pyruvate kinase; 2 mM $MnCl_2$; 0,25 mM S-adenosyl-L-methionine; 0,5 mg/mℓ bentonite; 40 μCi ^{3}H-UTP (specific activity 23 mCi/mmole); and BTV core particles at 0,5 A_{260}-units/mℓ unless indicated otherwise. Incubation temperatures are indicated in the text. Sucrose was also added to the reaction mixtures as indicated in the text. Reactions were assayed for time-dependent incorporation of ^{3}H-UMP into RNA by spotting a small sample of the reaction mixture onto Whatman 3MM filter paper discs (2 cm in diameter) and determining the amount of ^{3}H-cpm incorporated into acid-insoluble material according to the method of Bergmann & Lodish (1979).

RESULTS

Transcriptase activity of in vivo*-prepared core particles*

The first evidence that sucrose could affect the transcriptase activity of BTV was obtained from an investigation of the transcriptase activity of *in vivo*-prepared core particles. A 500 mℓ suspension of L-cells was infected with BTV, as described by Huismans *et al.* (1987a). After 2 h the cells were harvested and the *in vivo* prepared core particles purified in a sucrose gradient, as

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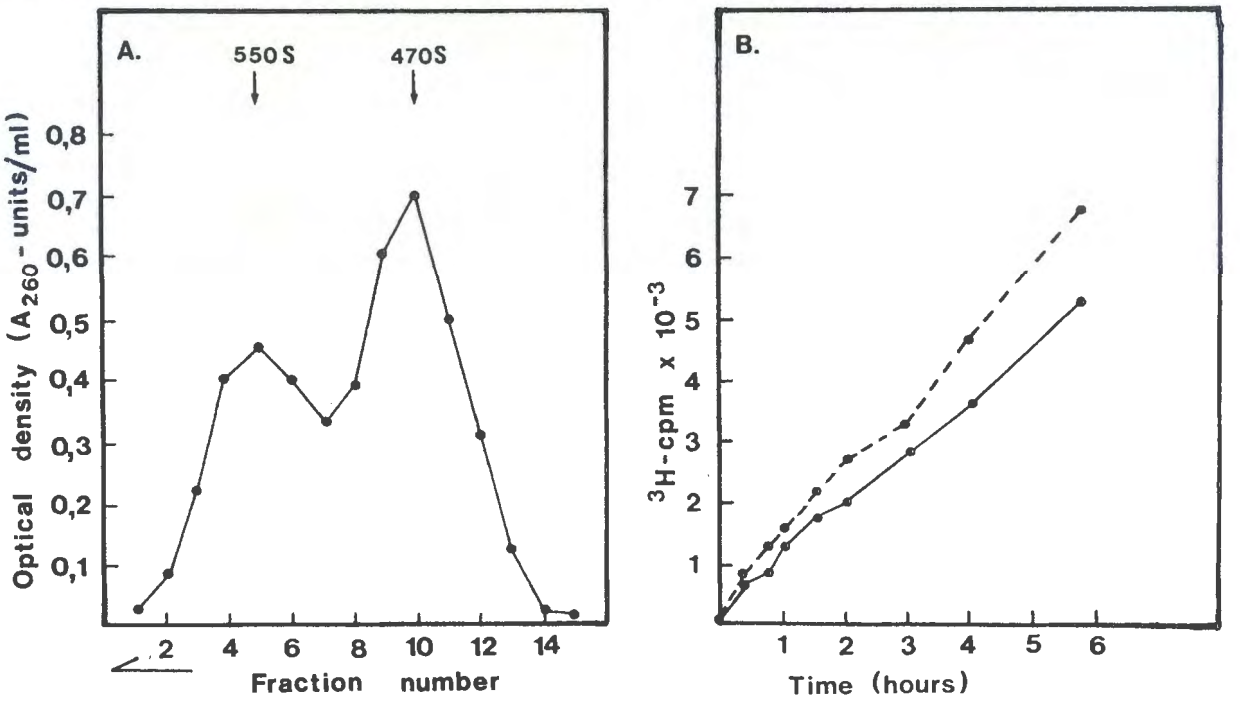

FIG. 1 Sucrose gradient sedimentation analysis (A), and *in vitro* transcription assay (B) of *in vivo* prepared BTV core particles. A: A 500 mℓ suspension culture of mouse LF-cells (5 × 10^6 cells/mℓ) was infected with 12 mg of purified BTV virions. After 2 h at 37 °C the cells were harvested. Cytoplasmic extracts were prepared and viral particles were recovered from these extracts by centrifugation at 100 000 × g for 120 min at 4 °C through a 40 % sucrose layer. The pellets were resuspended in 2 mM Tris-HC1 pH 8,8 and layered on a 4–40 % sucrose gradient in 2 mM Tris. Centrifugation was at 4 °C for 60 min at 100 000 × g. The gradient was fractionated from the bottom and the optical density of each fraction was determined at 260 nm. B: *In vitro* transcriptase activity of *in vivo* prepared BTV core particles in fraction 11 of the sucrose gradient in (A), assayed at 28 °C (●—●), and at 37 °C (●--●).

described in the legend to Fig. 1. The optical density profile of the fractionated gradient (Fig. 1A) indicates the presence of 2 particles with S values of 550S and 470S respectively. The fractions were analysed by SDS-PAGE and the results (not shown) confirmed that the 550S peak consisted of virus particles, whereas the 470S peak contained typical core particles.

Transcriptase activity in the sucrose gradient fractions in the region of the 470S peak was determined at both 28 °C and 37 °C. The reaction mixtures were composed of a 200 μℓ sample of each sucrose gradient fraction and an equal volume of a double concentration transcription reaction mixture. The results obtained with fraction 11 are shown in Fig. 1B. It is evident that the transcriptase activity at 37 °C was slightly higher than at 28 °C. This result seemingly contradicted previous observations on the temperature preference of the BTV-transcriptase (Van Dijk & Huismans, 1982). One possible difference between the current and previous experiments was that the reaction mixtures used in the experiment described in Fig. 1B contained a small amount of sucrose. This observation initiated a more detailed study of the effect of sucrose on the transcription reaction.

Transcriptase activity of BTV-cores in the presence of sucrose

BTV core particles were prepared *in vitro*. The effect of sucrose on the transcriptase activity was investigated by including different concentrations of sucrose in 400 μℓ transcription reaction mixtures. The experiments were carried out at 3 different incubation temperatures (28 °C, 37 °C and 41 °C) and at 2 core concentrations (1,5 A_{260}-units/mℓ and 0,25 A_{260}-units/mℓ). The reactions were assayed by spotting 25 μℓ samples of the reaction mixtures on filter paper discs every 30 min over a 6 h period and determining the ^{3}H-UMP incorporation. The results are shown in Fig. 2.

At the lower core concentration sucrose had no effect on the reaction at 28 °C (Fig. 2A) but it stimulated the reaction at both 37 °C and 41 °C (Fig. 2B & 2C). At the high core concentrations the presence of sucrose stimulated the transcription reaction at all 3 incubation temperatures (Fig. 2D, E & F). An increase in the sucrose concentration resulted in higher levels of stimulation. However, sucrose only had an effect on reactions where inhibition was evident and not when the reaction rate was constant over a 6 h period (Fig. 2A). Since the strongest inhibition was observed at high temperatures in combination with high core concentrations (Fig. 2C, E & F), the largest effect of sucrose was observed under these reaction conditions. It was not determined if sucrose concentrations above 15 % would further enhance ^{3}H-UMP incorporation. It is clear from the result in Fig. 2A and B, however, that at a low core concentration of 0,25 A_{260}-units BTV cores/mℓ the presence of 15 % sucrose enhances the reaction to such an extent that it proceeds more efficiently at 37 °C than at 28 °C. This observation is in agreement with the results illustrated in Fig. 1. However, at high core concentrations (1,5 A_{260}-units/mℓ), the presence of 15 % sucrose is not sufficient to counteract the strong inhibition at 37 °C (Fig. 2E) and the reaction proceeds better at the lower temperature of 28 °C (Fig. 2D) under these conditions.

The stimulatory effect on the *in vitro* transcription reaction does not appear to be unique to sucrose and similar results were obtained when the experiment in Fig. 2 was repeated with glycerol (results not shown).

The effect of a temperature-shift on the in vitro *transcription reaction*

There are at least 2 possible explanations for the inhibition of the *in vitro* transcription reaction at high core concentrations and temperatures. One is that the core particles are unstable under these conditions and that they disintegrate. Alternatively, a reversible inactivation

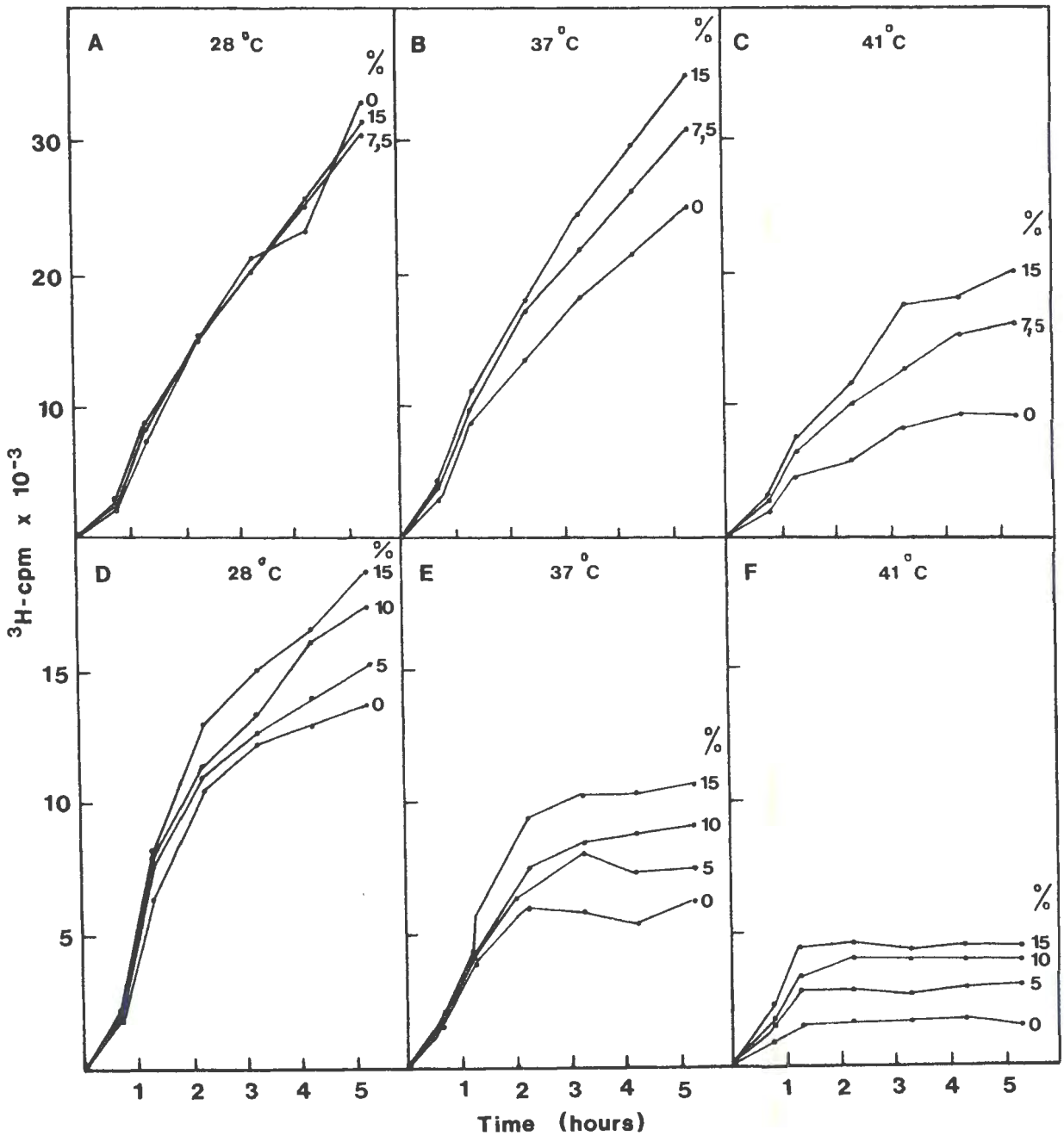

FIG. 2 The effect of varying amounts of sucrose (%) and incubation temperature on the *in vitro* transcription reaction of *in vitro* prepared BTV core particles.
(A), (B), (C): 0,25 A_{260}-units BTV cores/mℓ
(D), (E), (F): 1,50 A_{260}-units BTV cores/mℓ

of the transcriptase reaction could be involved. To investigate this latter possibility, the following temperature-shift experiment was carried out: One half of a 2,4 mℓ transcription mixture, (core concentration of 0,56 A_{260}-units/mℓ) was incubated at 28 °C, while the other half was incubated at 37 °C. After 165 min a 300 $\mu\ell$ sample of the 28 °C reaction mixture was removed and incubated at 37 °C. A similar sample from the 37 °C reaction mixture was incubated at the lower (28 °C) temperature. The remainder of the 2 mixtures were left at the original temperatures. After another 60 min the procedure was repeated. A portion of each of the original mixtures was removed and incubated at the new temperature. Throughout the experiment, 30 $\mu\ell$ samples of the different reaction mixtures were taken, spotted on Whatman 3 MM filter paper and assayed for the incorporation of ^{3}H-UMP. The results are shown in Fig. 3.

During the first 30 min of the reaction the rate of transcription at 37 °C was the same as that at 28 °C. At 90 min, however, the reaction at 37 °C was already severely inhibited and after 165 min no further incorporation of ^{3}H-UMP was observed. The reaction at 28 °C on the other hand continued at its original rate for at least another 4 h. The first shift to a higher temperature after a 165 min incubation period caused an immediate decline in the rate of transcription, and after 1 h very little further ^{3}H-UMP incorporation was observed. On the other hand, the shift to 28 °C in the 37 °C reaction mixture resulted in a very marked reactivation of the transcriptase reaction. The rate of transcription after the shift was almost identical to that of the mixture that was kept at the low temperature throughout the incubation period. The same results were obtained with the temperature shifts at 225 min after the start of the reaction.

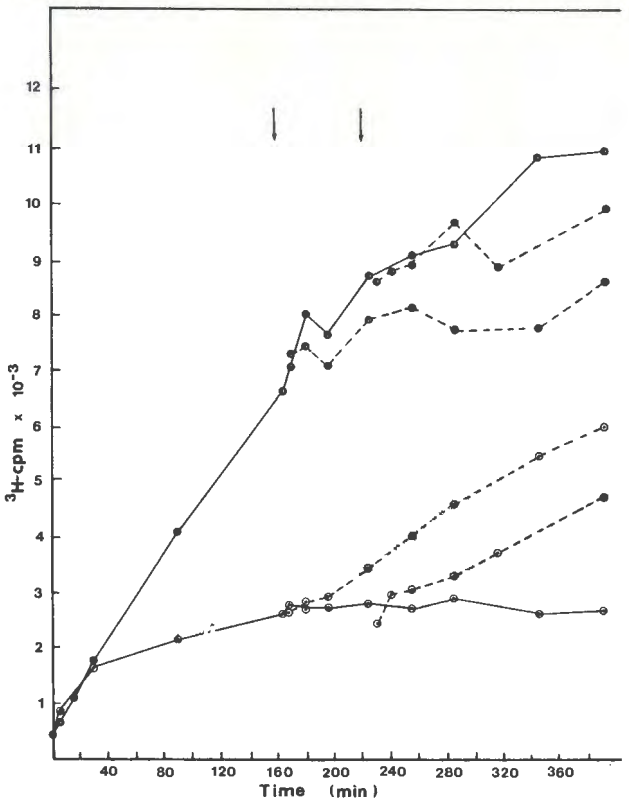

FIG. 3 The effect of a temperature-shift on the *in vitro* transcription reaction of BTV.
(●—●) reaction at 28 °C
(○—○) reaction at 37 °C
(●--●) reaction upshifted to 37 °C
(○--○) reaction downshifted to 28 °C
The arrows indicate when the temperature shifts occurred

DISCUSSION

The results presented in this paper confirmed the results of Huismans *et al.* (1987a) that the transcriptase activity of *in vitro* and *in vivo* prepared core particles is indistinguishable with respect to temperature requirement. At low core concentrations and in the presence of polyhydroxylic compounds, such as sucrose or glycerol, the transcription reaction at 37 °C proceeded at a slightly higher rate than the reaction at 28 °C. Under these conditions linear incorporation of ^{3}H-UMP was observed over a period of at least 5 h. These reaction conditions are probably much closer to the *in vivo* conditions in infected cells than conditions of very high core concentrations.

The low temperature optimum of the BTV transcription reaction therefore applies only to the artificial *in vitro* conditions. The results presented in this paper indicate that the inhibition observed for the *in vitro* conditions can be counteracted by compounds such as sucrose and glycerol.

The stimulatory effect of sucrose on the *in vitro* transcription reaction was observed irrespective of the incubation temperature but applied only to reaction conditions where inhibition of transcription was evident. This seems to suggest that these compounds merely counteracted the inhibition and did not stimulate transcription as such. Polyanions which could overcome a similar inhibition of vesicular stomatitis virus and Sendai virus transcriptases (Carrol & Wagner, 1978; Stone & Kingsbury, 1973) had no effect on the BTV transcriptase (result not shown). Polyhydroxylic compounds, such as sucrose, are known to stabilize proteins and enzyme preparations (Fransler & Loeb, 1974; Valeri, 1975). The report by Van der Walt (1980) that sucrose preserves the infectivity of BTV during freeze-drying, could also provide some support for the hypothesis that sucrose has a stabilizing effect on the antigens and enzymes involved in the transcriptase reaction.

The reversibility of the core-mediated, temperature-dependent inhibition of the transcriptase reaction was also investigated. It was found that the inhibition is completely reversible. This would exclude physical breakdown of the core particles as a cause of the inhibition.

The possibility that aggregation of core particles plays a role in the inhibition also needs to be considered. It has been observed (A. A. van Dijk & H. Huismans, unpublished observations, that at high core concentrations the BTV core particles tend to aggregate strongly. The formation of precipitated complexes could perhaps preclude the physical access of reagents to enter the core particle or the extrusion of newly synthesized mRNA. Such a situation would be similar to the loss of reovirus spikes (White & Zweerink, 1976), or to that in which the core particles are still covered by the outer capsid layer. Aggregation of core particles may be enhanced by elevating the incubation temperature, and it may be reduced by including viscose substances, such as sucrose and glycerol. However, as little is known about the reversibility of core aggregation and even less about the effect of temperature on such aggregates, all explanations can only be speculative.

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