*Acta virol.* **25** : 257—265, 1981

# RESISTANCE OF SEMLIKI FOREST VIRUS PROTEIN SYNTHESIS TO HIGH SALT TREATMENT

S. ERDEI

Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

*Received July 9, 1980; revised April 14, 1981*

*Summary.* — Selective translation of Semliki Forest virus-specific mRNA occurred in virus-infected cells exposed to hypertonic growth medium. The selective resistance of the virus-specific protein synthesis could be detected at a wide NaCl concentration range and was more significant at lowered incubation temperature (28 °C). It is suggested that the translation of the structural proteins encoding subgenomic 26 S RNA is more resistant to the hypertonic initiation block than the translation of the genomic 42 S RNA, which codes for the non-structural viral proteins.

*Key words: Alphavirus; Semliki forest virus; protein synthesis; hypertonic initiation block*

## *Introduction*

A brief exposure of cultured animal cells to hypertonic media results in a reversible block of polypeptide chain inition, while chain elongation, termination and posttranslational cleavage remain unaffected. Upon restoration of isotonicity, synchronous reinitiation of protein synthesis occurs (Saborio *et al.*. 1974). The synthesis of distinct classes of proteins has different sensitivity to hypertonic initiation block (Nuss and Koch, 1976*a*).

Selective inhibition of host mRNA translation was observed in virus-infected cells exposed to hypertonic initiation block (Saborio *et al.*, 1974; Nuss *et al.*, 1975; England *et al.*, 1975; Madore and England, 1975; Oppermann and Koch, 1976; Nuss and Koch, 1976*b*; Garry *et al.*, 1979).

In vesicular stomatitis virus-infected cells the synthesis of individual viral polypeptides is diversely affected by hypertonic treatment (Nuss and Koch, 1976*c*).

These observations led to the suggestion that the enhanced resistance of viral messenger translation to hypertonic treatment reflects an important feature of the mechanism ensuring the preferential translation of viral messenger RNAs in virus-infected cells (Nuss *et al.*, 1975; Carrasco, 1977).

Two major virus-specific mRNA species occur in cells infected with Semliki Forest virus (SFV). The genomic 42 S RNA has been shown to

encode the non-structural proteins which at least in part, are presumably responsible for viral RNA replication. The second major RNA is the subgenomic 26 S RNA, which codes for the structural proteins (for review see Kääriäinen and Söderlund, 1978).

Four non-structural proteins (ns70, ns86, ns72, ns60) have been characterized in SFV-infected cells (for review see Schlesinger and Kääriäinen, 1980). They are mainly synthesized early in the infectious cycle, when the host protein synthesis is not inhibited at all, and their synthesis contributes not more than 3 % of the total protein synthesis (Lachmi and Kääriäinen, 1977). Their identification became possible only after the isolation of certain ts mutants of SFV overproducing the nonstructural proteins (Keränen and Kääriäinen, 1975; Lachmi *et al.*, 1975; Lachmi and Kääriäinen, 1976). By the aid of these mutants it became possible to detect the nonstructural proteins between the in vitro translational products of 42 S genomic RNA (Glanville and Lachmi, 1977; Lehtovaara *et al.*, 1980) and in wild type-infected cells after high salt initiation block (Lachmi and Kääriäinen, 1976).

Late in the infectious cycle, the four main SFV structural proteins become the predominant protein species synthesized in the infected cells: the capsid protein (C) and the glycosylated envelope proteins (E1, E2, E3) (for review see Simons *et al.*, 1980; Schlesinger and Kääriäinen, 1980).

The coexistence of two different virus-specific messenger RNAs with altered biological functions makes SFV-infected cells an attractive system to study the resistance of viral messenger translation to hypertonic initiation block, which is apparently one of the important factors ensuring the preferential translation of virus-specific messages. In cells infected with a closely related alphavirus (Sindbis virus), the increased NaCl concentration in the medium had little effect on the synthesis of alphavirus structural proteins, while the host-specified protein synthesis is strongly inhibited (Waite and Pfefferkorn, 1970; Garry *et al.*, 1979).

We analyzed the protein synthesis in SFV-infected cells under hypertonic conditions. We revealed the optimal conditions for studying SFV structural proteins in the absence of host protein synthesis. Further, our results raised the possibility that the genomic 42 S RNA and the subgenomic 26 S RNA could belong to different classes of messengers in respect of the resistance to hypertonic initiation block. The translation of 42 S RNA seems to be more sensitive to hypertonic conditions than the translation of the structural proteins encoding 26 S RNA.

## *Materials and Methods*

Chicken embryo cells (CEC) were cultured and infected with SFV as described by Keränen and Kääriäinen (1975). In all experiments, the multiplicity of infection was 50 and actinomycin D was omitted from the incubation medium. At a given time during the infectious cycle the minimal essential medium was changed to a medium with increased NaCl concentration. During the exposure to hypertonic medium, a 0.37 MBq/ml $^{35}$S-methionine pulse was given, followed by chase in the presence of excess cold methionine. At the end of the chase the cells were lysed in hot 2 % sodium dodecylsulphate (SDS) and the hot trichloroacetic acid-precipitable counts

were determined as described by Söderlund (1976). Polyacrylamide gel electrophoresis (PAGE) was carried out using a modified Laemmli system (Schlesinger and Schlesinger, 1973). The gels were either processed for fluorography (Bonner and Laskey, 1974) or directly dried and exposed to Kodak X Omat films.

## *Results*

### *The effect of hypertonic medium on the protein synthesis of the host cells and SFV*

We compared the protein synthesis of the host and SFV-infected cells at different temperatures and salt concentrations. SFV-infected and mock-inoculated CEC were exposed to medium containing 200 mmol/l excess NaCl. At different times after the addition of hypertonic medium, a 5-min $^{35}$S-methionine pulse was given to the cultures and the hot trichloroacetic acid-precipitable counts were determined. Concurrently, replicate cultures were incubated and labelled in the same way in isotonic medium and the incorporations in these cultures were taken as 100 % (Fig. 1). The experiments were done at two temperatures routinely used for cultivating SFV and its ts mutants. At 37 °C, the inhibition of protein synthesis in the SFV- and mock-inoculated cultures was rather similar and complete within 40 min, which is in a good agreement with the previous observations (Clegg and Kennedy, 1975; Lachmi and Kääriäinen, 1976). Surprisingly at 28 °C there was a strong difference between the salt sensitivity of the protein synthesis in viral infected and mock-inoculated cultures. Even after long incubation in hypertonic medium (2 hr, data not shown) there was about 40 % residual incorporation in the virus-infected cells.

Subsequently we examined at what slat concentration is manifested the difference between the sensitivity of translation to high salt treatment in SFV-infected and host cells. SFV-infected and mock-inoculated cells were

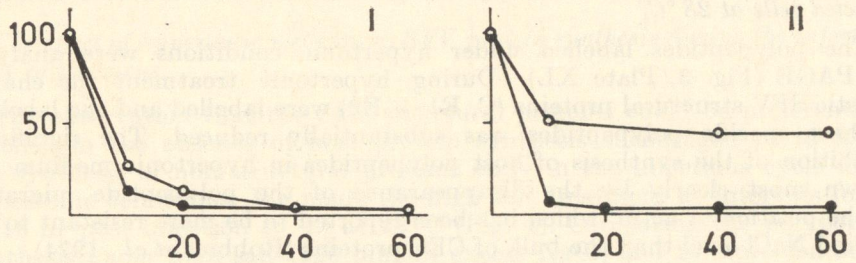


**Fig. 1.**

The time curve of the effect of hypertonic medium on host and viral protein synthesis at 37 °C (I) and 28 °C (II)

A 5-min $^{35}$S-methionine pulse was given to the cultures at intervals indicated on the abscissa (min) after addition of medium containing excess (220 mM) NaCl at 4 hr p. i. at 37 °C or 8 hr p. i. at 28 °C. The $^{35}$S-methionine incorporation was expressed as per cent of incorporation in control cultures kept in growth medium without addition of NaCl (ordinate) ○ — SFV-infected cells; ● — mock-inoculated cells.

incubated in media of different NaCl concentrations for 40 min and then the cultures were labelled for 5 min with $^{35}$S-methionine. Again, duplicate cultures were treated in the same way except that they were incubated in isotonic medium. We could detect the preferential resistance of the protein synthesis in virus-infected cells to hypertonic conditions in a fairly wide concentration

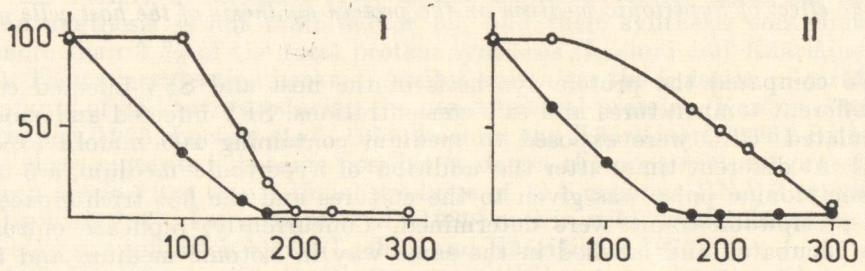


**Fig. 2.**

The concentration dependence of the effect of hypertonic medium on host and viral protein synthesis at 37 °C (I) and 28 °C (II)
A 5-min $^{35}$S-methionine pulse was given to the cultures at 40 min after addition of media containing different amounts of excess NaCl.
Abscissa: additional NaCl (mmol/l).
$^{35}$S-Methionine incorporation and other designations as in Fig. 1.

range (Fig. 2). This resistance was more pronounced at 28 °C and was expressed in a wider concentration range than at 37 °C. Therefore we have focused our attention on the protein synthesis in virus-infected cells under hypertonic conditions at 28 °C.

*Characterization of proteins synthesized under hypertonic conditions in SFV-infected cells at 28 °C*

The polypeptides labelled under hypertonic conditions were analyzed by PAGE (Fig. 3, Plate XL). During hypertonic treatment the characteristic SFV structural proteins (C, E1 + E2) were labelled and the labelling of host specific polypeptides was substantially reduced. The significant inhibition of the synthesis of host polypeptides in hypertonic medium was shown most clearly by the disappearance of the polypeptide migrating at the position of actin, which has been reported to be more resistant to the altered NaCl level than the bulk of CEC proteins (Robbins *et al.*, 1974).

One possible explanation for the greater resistance of viral protein synthesis in hypertonic medium at 28 °C could be that it is due to the slower ribosome run off following the inhibition of initiation by hypertonic treatment at low temperature. It seemed very unprobable that the translation of host and viral messages would be affected in a different way in this respect, and in mock-inoculated cultures both the time and concentration curve of protein synthesis inhibition were very similar at both temperatures. To exclude this possibility, additional control experiments were carried out.

First, we had to know the elongation rate of SFV protein synthesis at 28 °C. In principle, this can easily be estimated after synchronous initiation of translation as described by Saborio *et al.* (1974), but we had to overcome the difficulfy of obtaining complete inhibition of protein synthesis in virus-infected cells at 28 °C. For this reason we have done the high salt treatment at 37 °C. The infected cultures were kept for 40 min in a medium containing additional 220 mmol/l NaCl,which conditions were found optimal for studying the synchronous initiation of translation of SFV proteins after isotonic replacement of medium (Clegg and Kennedy, 1975; Lachmi and Kääriäinen, 1976). After complete inhibition of protein synthesis at 37 °C, we induced the synchronous initiation of translation at 28 °C by replacing the high salt medium with isotonic medium. Giving a different length of $^{35}$S-methionine pulse (2, 5, 10, 20, 30, 45 min) and 30 min chase, from the time of appearance of the individual SFV proteins, we could determine the elongation rate, which was found to be 75 amino acid residues per min at 28 °C. As the longest primary translational product of 26 S RNA is a 130 000 d protein, which accounts for its full coding capacity, the entire translation of 26 S RNA requires 18 min. We could detect the viral protein synthesis even after 60 min incubation in hypertonic medium which is far beyond the 18 min time necessary for the complete run off of ribosomes following the initiation block. Next we analyzed by PAGE the proteins which were labelled at 10, 20, 30, and 40 min after the addition of salt by 5 min $^{35}$S-methionine pulse and 40 min chase. Under these circumstances the consequence of the inhibition of translation initiation would be the preferential C terminal labelling of viral polypeptides, namely that of E1, which is located at the N-terminal end of the polyprotein precursor (Clegg, 1975). The distribution of S$^{35}$-methionine label among the viral proteins was essentially the same in every case, which excludes the possibility that the protein synthesis detected in the infected cells in high salt containing medium at 28 °C was simply due to incomplete ribosome run off following the hypertonic initiation block.

*The effect of hypertonic medium on SFV protein synthesis during the infectious cycle of the virus*

The observation that the SFV structural proteins could be preferentially labelled in the absence of host protein synthesis encouraged us to follow the synthesis of non-structural proteins early in the infectious cycle under hypertonic conditions. We made the high salt treatment at intervals for up to 16 hr post infection (p. i.) at 28 °C (Fig. 4). Comparing the polypeptide synthesis under high salt (220 mmol/l excess NaCl) and isotonic conditions we found that the salt-resisant protein synthesis appeared after 4 hr p. i.

As the synthesis of the non-structural proteins precedes the burst of the structural proteins (Lachmi and Kääriäinen, 1977) we carefully analyzed the labelled polypeptides during the first 4-6 hr p. i. We failed to reveal by PAGE any polypeptides that would be present only in the infected cultures, even when we added such a hot pulse to the salt-treated cultures that the residual 3—5 % virus-specific, non-structural proteins should have been detected.

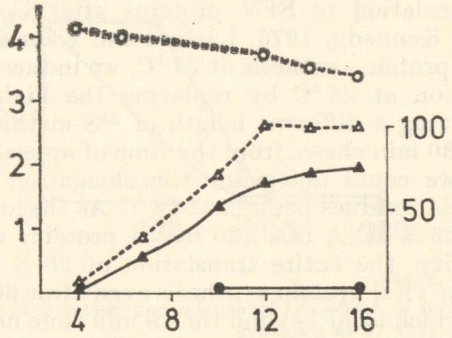


**Fig. 4.**
The effect of hypertonic medium on host and SFV protein synthesis during the infectious cycle of the virus
After infection, 5 min pulses were given at indicated times (abscissa; hr p. i.) to the cultures kept in hypertonic media (170 mmol/l excess NaCl), or in isotonic medium for 40 min.
○ — total $^{35}$S-methionine incorporation in SFV-infected cells in isotonic medium
△ — total $^{35}$S-methionine incorporation in SFV-infected cells in hypertonic medium

Left ordinate: [$^{35}$S] count/min × $10^{-4}$ $^{35}$S-Methionine incorporation in SFV-infected (▲) and mock-inoculated (●) cultures incubated in hypertonic media. The incorporation expressed as per cent of incorporation in control cultures without excess NaCl (right ordinate).

Under isotonic conditions it was also impossible to identify the non-structural proteins in this early stage of infection, because of the high background of the host protein synthesis. They could be identified under isotonic conditions later (after 3 hr p. i. at 37 °C or after 6 hr at 28 °C) (Fig. 5, see Plate XLI). In ts1-mutant-infected cells, a relatively large amount of non-structural proteins is accumulated (Kärenen and Kääriäinen, 1975; Lachmi *et al.*, 1975). The ns86 non-structural protein can be easily seen comparing the labelled proteins of ts1, wild type and mock-infected cells. The identification of the other non-structural proteins (ns60, ns72, ns76) is not unequivocal, because they migrate in the same region of the gel as the p62 precursor of the E2 and E3 structural proteins.

## *Discussion*

In many virus-infected cells exposed to hypertonic growth medium, a preferential inhibition of host messenger translation occurs (Saborio *et al.*, 1974; Nuss *et al.*, 1975; England *et al.*, 1975; Madore and England, 1975; Oppermann and Koch, 1976; Nuss and Koch, 1976*b*; Garry *et al.*, 1979).

Furthermore it has been demonstrated that the translation of the virus-specific messenger RNAs is not the only one displaying relative resistance to hypertonic conditions, but that some other messengers are also significantly more resistant to excess NaCl in the culture fluid. These are either mRNAs for inducible cell proteins such as IgG (Nuss and Koch, 1976*a*) or for proteins needed in large amounts, i. e. chick actin (Robbins *et al.*, 1974). Moreover, there can be differences in the relative sensitivities of individual polypeptide syntheses of the same virus to hypertonic initiation block (Nuss and Koch, 1976*c*).

The effect of media of altered NaCl concentration has also been studied in cells infected with another alphavirus (Sindbis). It has been demonstrated that while changing the NaCl concentration of the growth media had little effect on the synthesis of the virus-specified proteins, it inhibited the synthesis of host proteins. This inhibition was accompanied by the breakdown of polysomes (Garry *et al.*, 1979). The lowered NaCl concentration resulted in reversible blocking of the budding of virus particles (Waite and Pfefferkorn, 1970) and the elevated concentration of NaCl did not change significantly the virus yield (Garry *et al.*, 1979).

We analyzed the protein synthesis in SFV-infected cells. We compared the virus-specific and host protein synthesis in hypertonic media in a wide salt concentration range at two different temperatures. The protein synthesis in virus-infected cells was less inhibited than that in mock-inoculated cells. PAGE confirmed that authentic viral structural proteins were synthesized in the infected cells incubated in hypertonic medium. Besides, at low temperature (28 °C) the selective synthesis of the viral structural proteins occurred in a wider NaCl concentration range than at 37 °C. Kinetic labelling experiments ruled out the possibility that at low temperature the residual protein synthesis in infected cultures under hypertonic conditions was simply due to a slower ribosome run off following the inhibition of initiation of translation.

As the high salt treatment proved to be a very efficient tool for reducing the host-specific protein synthesis in SFV-infected cells, we tried to use it for studying the non-structural proteins early in the infectious cycle. The high background of the host protein synthesis makes impossible the detection of non-structural proteins in the very early stage of infection under isotonic conditions. We treated the infected cultures with medium containing 170 mM excess NaCl at different times p. i. at 28 °C. Analyzing the synthesized proteins in the high salt-treated cultures we could not detect any polypeptides present only in the infected cells during the first 6 hr p. i. At this stage of infection cycle the only virus-specific polypeptides would have been the non-structural proteins. We could detect them under isotonic conditions at a later stage of infection, when the host protein synthesis is already greatly reduced due to the viral infection. The salt-resistant protein synthesis began to appear after 4 hr p. i. at 28 °C, which coincides with the beginning of the viral structural protein synthesis. The proteins synthesized in the infected cultures under hypertonic conditions (170 mmol/l excess NaCl) at a later stage of infection represented the viral structural proteins and their precursors. This observation, together with our failuire to detect the non-structural proteins under hypertonic conditions in the early phase of the infection, could be explained by assuming that the two major virus-specific messenger RNAs in SFV-infected cells represent different classes of messenger RNAs in respect of the resistance to hypertonic initiation block. The translation of 26 S RNA should be more resistant to hypertonic conditions than that of 42 S RNA. The S RNA is translated early after infection and its translation results in the synthesis of non-structural proteins, which are presumably needed for viral RNA replication. The non-structural proteins are present

in small amounts in the infected cells. On the contrary, the 26 S RNA is translated continuously during the late stage of infection and its translation produces the viral structural proteins needed in large quantities for virus production. These differences could imply that these two messenger RNAs should use different strategies for ensuring their proper translation in the infected cells. Their different resistance to hypertonic initiation block could reflect one of the important features of these mechanisms. The 26 S RNA shows higher resistance to hypertonic conditions, which seems to be an inherent feature of certain viral messenger RNAs encoding viral structural proteins.

*Acknowledgements.* I wish to thank Drs. L. Kääriäinen and N. Glanville for their continuous interest and support, and Drs. G. L. Farkas and E. Duda for helpful criticism of the manuscript. Part of the work was performed at the University of Helsinki, and was supported by a grant of the Hungarian Academy of Sciences.

## References

Bonner, W. M., and Laskey, R. A. (1974): A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83—88.

Carrasco, L. (1977): The inhibition of cell functions after viral infection. A proposed general mechanism. *FEBS Letters* **76**, 11—15.

Clegg, J. C. S. (1975): Sequential translation of capsid and membrane protein genes of alphaviruses. *Nature (Lond.)* **254**, 454—455.

Clegg, J. C. S., and Kennedy, S. I. T. (1975): Initiation of synthesis of the structural proteins of Semliki Forest virus. *J. mol. Biol.* **97**, 401—411.

England, J. M., Howett, M. K., and Tan, K. B. (1975): Effects of hypertonic conditions on protein synthesis in cells productively infected with Simian virus 40. *J. Virol.* **16**, 1101—1107.

Garry, R. F., Bishop, J. M., Parker, S., Westbrook, K., Lewis, G., and Waite, M. R. F. (1979): $Na^+$ and $K^+$ concentrations and the regulation of protein synthesis in Sindbis virus infected cells. *Virology* **96**, 108—120.

Glanville, N., and Lachmi, B. (1977): Translation of proteins accounting for the full coding capacity of the Semliki Forest virus 42S RNA genome. *FEBS Letters* **81**, 399—402.

Keränen, S., and Kääriäinen, L. (1975): Proteins synthesized by the Semliki Forest virus and its 16 temperature-sensitive mutants. *J. Virol.* **16**, 388—396.

Kääriäinen, L., and Söderlund, H. (1978): Structure and replication of α-viruses. *Current Topics Microbiol. Immunol.* **82**, 15—69.

Lachmi, B., and Kääriäinen, L. (1976): Sequential translation of nonstructural proteins in cells infected with a Semliki Forest virus mutant. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1936—1940.

Lachmi, B., and Kääriäinen, L. (1977): Control of protein synthesis in Semliki Forest virus infected cells. *J. Virol.* **22**, 142—149.

Lachmi, B., Glanville, N., Keränen, S., and Kääriäinen, L. (1975): Tryptic peptide analysis of non-structural and structural proteins from Semliki Forest virus mutant infected cells. *J. Virol.* **16**, 1915—1629.

Lehtovaara, P., Ulmanen, I., Kääriäinen, L., Keränen, S., and Philipson, L. (1980): Synthesis and processing of Semliki Forest virus-specific nonstructural proteins in vivo and in vitro. *Eur. J. Biochem.* **112**, 461—468.

Madore, H. P., and England, J. M. (1975): Selective suppression of cellular protein synthesis in BHK 21 cells infected with rabies virus. *J. Virol.* **10**, 1351—1354.

Nuss, D. L., and Koch, G. (1976*a*): Variation in the relative synthesis of immunoglobulin G and non-immunoglobulin G proteins in cultured MPC-11 cells with changes in the overall rate of polypeptide chain initiation and elongation. *J. mol. Biol.* **102**, 601—612.

Nuss, D. L., and Koch, G. (1976*b*): Translation of individual host mRNAs in MPC-11 cells is differently suppressed after infection by vesicular stomatitis virus. *J. Virol.* **19**, 572—578.

Nuss, D. L., and Koch, F. (1976c): Differential inhibition of vesicular stomatitis virus polypeptide synthesis by hypertonic initiation block. *J. Virol.* **17**, 283–286.

Nuss, D. L., Oppermann, H., and Koch, G. (1975): Selective blockage of initiation of host protein synthesis in RNA virus-infected cells. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1258–1262.

Oppermann, H., and Koch, G. (1976): On the regulation of protein synthesis in vaccinia virus infected cells. *J. gen. Virol.* **32**, 261–273.

Robbins, P. W., Wickus, G. G., Branton, P. E., Gaffney, B. J., Hirschberg, C. B., Fuchs, D., and Blumberg, P. M. (1974): The chick fibroblast surface after transformation by Rous sarcoma virus. *Cold Spring Harb. Symp. quant. Biol.* **34**, 1173–1180.

Saborio, J. L., Pong, S. S., and Koch, G. (1974): Selective inhibition of initiation of protein synthesis in mammalian cells. *J. mol. Biol.* **85**, 195–211.

Schlesinger, M. J., and Kääriäinen, L. (1980): Translation and processing of alphavirus proteins, pp. 371–392. In R. W. Schlesinger (Ed.): *The Togaviruses*, Academic Press, New York.

Schlesinger, M. J., and Schlesinger, S. (1973): Large molecular weight precursors of Sindbis virus proteins. *J. Virol.* **11**, 1013–1016.

Simons, K., Garoff, H., and Helenius, A. (1980): Alphavirus proteins, pp. 317–333. In R. W. Schlesinger (Ed.): *The Togaviruses*, Academic Press, New York.

Söderlund, H. (1976): The post-translational processing of Semliki Forest virus structural polypeptides in puromycin-treated cells. *FEBS Letters* **63**, 56–58.

Waite, M. R. F., and Pfefferkorn, E. R. (1970): Inhibition of Sindbis virus production by media of low ionic strength: intracellular events and requirements for reversal. *J. Virol.* **5**, 60–71.