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The thermal stability of turnip yellow mosaic virus under hydrostatic pressure

A small angle neutron scattering study

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Abstract. The thermal stability of an isometric plant virus, Turnip Yellow Mosaic Virus (TYMV), has been investigated at low and high hydrostatic pressure, using small angle neutron scattering. Contrast variation allowed us to separately observe the structural changes of the protein capsid and the RNA core. The experiments were performed in 0.05 *M Tris* buffer at pD = 8.0 and in 0.05 *M bis-Tris* buffer at pD = 6.0 containing different H_2O/D_2O mixtures (40% and 70% D_2O). It was found that hydrostatic pressure enhances the stability of TYMV. The thermally induced uncoating of RNA as well as structural transitions of the protein capsid are shifted to higher temperature upon increasing the pressure from 5×10^6 Pa to 2×10^8 Pa.

Key words: Turnip Yellow Mosaic Virus – Degradation – RNA-release – Neutron scattering – Hydrostatic pressure

Introduction

Simple isometric viruses consist of a nucleic acid protected by a closed protein shell possessing icosahedral symmetry. Turnip Yellow Mosaic Virus (TYMV) is the prototype of icosahedral plant viruses stabilized by strong protein – protein interactions. The architecture and the properties of TYMV have already been characterized (Kaper 1975; Jacrot et al. 1977; reviewed in: Hirth and Givord 1988). Alkaline degradation (Keeling et al. 1979) and heat degradation experiments (Lyttleton and Matthews 1958; Virudachalam et al. 1985) are well established for studying interacting forces inside the virion. It was pointed out that with increasing pH and increasing temperature the stability of TYMV decreases. The mechanism of decapsidation and degradation can be described as follows:

1. The uncoating of RNA is a process in which a cluster of five to eight protein subunits is released, resulting in the formation of a hole in the capsid surface (Keeling and Matthews 1982; Katouzian-Safadi et al. 1983; Matthews and Witz 1985). The intact RNA then escapes, leaving behind an empty shell lacking a few protein subunits. In the case of alkaline or heat treatment the RNA is degraded into small fragments in a stepwise fashion.

2. The disruption of the empty capsid is characterized by a disintegration into subunits followed by protein denaturation (Virudachalam et al. 1985). This disruption is accompanied by the formation of aggregates.

Hydrostatic pressure is a unique tool for obtaining thermodynamic information about chemical equilibria. High pressure experiments on TYMV up to 4×10^7 Pa (Fahey et al. 1969) and up to 1.5×10^8 Pa (Katouzian-Safadi and Haenni 1986) suggested that pressure alone does not induce any alteration of the virion.

In this work thermally induced release of RNA and disruption of the protein capsid are investigated under high pressure and compared with the results at low pressure. It is shown that hydrostatic pressure has a stabilizing effect on the virion.

To monitor structural changes in the virion, we made use of small angle neutron scattering (SANS), a technique particulary well adapted to observe the low-resolution structure of spherical viruses. The application of contrast-variation by using mixtures of D_2O/H_2O allows separate studies of RNA and protein: In a buffer containing 43 vol% D_2O the neutron-optical contrast with respect to the protein moiety vanishes; in a buffer containing 68 vol% D_2O the contrast with respect to the RNA moiety vanishes (Jacrot 1976).

Material and methods

Virus preparation

TYMV was multiplied in turnip (B. rapa Linn, c.v. Just Right). Plant proteins were precipitated from the sap at pH = 4.8 and viral suspensions were further purified by two cycles of high- and low-speed centrifugations. Pellets

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were suspended in 0.02 *M* sodium phosphate buffer pH = 7.2 containing 0.1 m*M* sodium azide. Virions were separated from natural empty shells also present in the purified suspensions by repeated brief ultracentrifugation or by successive isopycnic centrifugation in CsCl density gradients.

TYMV solutions

When pressure experiments are performed care has to be taken in the choice of buffer. It is necessary that the pH does not change appreciably with increasing pressure. This is the case for *Tris* buffer whose pH shift at increasing pressure up to 2×10^8 Pa is less than -0.05 units (Neuman et al. 1973) or -0.15 units (Kitamura and Itoh 1987). For *bis-Tris* buffer, the corresponding figure is -0.11 units (Kitamura and Itoh 1987). On the other hand the disadvantage of *Tris* buffer is its large temperature coefficient: $d(\mathrm{pH})/dT = -0.025\ \mathrm{K}^{-1}$ (Sigma catalogue 1990, p. 1017) whereas for *bis-Tris* buffer: $d(\mathrm{pH})/dT = -0.008\ \mathrm{K}^{-1}$ (Sigma catalogue 1990, p. 1415).

TYMV was dialysed against 0.05 *M Tris*-HCl buffer containing different amounts of D_2O (40%, 70%) at pD = 8.0; a further sample was dialysed against 0.05 *M bis-Tris* buffer with 70% D_2O at pD = 6.0. The pD of deuterated buffer corresponds to pD = pH + 0.3314 n + 0.0766 n^2; n is the D_2O mole fraction in the solvent (Cuillel et al. 1987). The pD values mentioned in this article correspond to those measured at room temperature. The virus concentration was $c = 10\ \mathrm{mg\ cm^{-3}}$ in 70% D_2O and $c = 25\ \mathrm{mg\ cm^{-3}}$ in 40% D_2O, respectively.

Neutron scattering

SANS measurements were performed on the D11 instrument at the Institut Laue-Langevin (Ibel 1976). A special high pressure cell was used (Lechner et al. 1985) at pressures of 5×10^6 Pa and 2×10^8 Pa. The sample thickness was 1.5 mm and 4.5 mm for solutions containing 40% and 70% D_2O, respectively. A minimum pressure of 5×10^6 Pa was applied to the sample to prevent the formation of air bubbles upon heating, as it proved very difficult to efficiently degas concentrated virus solutions.

With a sample-detector distance of 2.5 m and 10.0 m and a wavelength $\lambda = 1.0$ nm the range of the scattering vector ($Q = (4\pi/\lambda) \sin\theta$; 2θ = scattering angle) was from 0.04 $\mathrm{nm^{-1}}$ to 0.88 $\mathrm{nm^{-1}}$. This Q range includes part of the central maximum as well as the first two subsidiary maxima of the scattering curve of the virion (see e.g. Fig. 1).

The duration of an experiment, including the measurement of the transmission and of the scattering at the two detector settings at a given temperature and pressure, was slightly less than 30 min.

Data analysis

The SANS curves were corrected for solvent and background scattering and normalized to 1 cm cell thickness and to unit transmission.

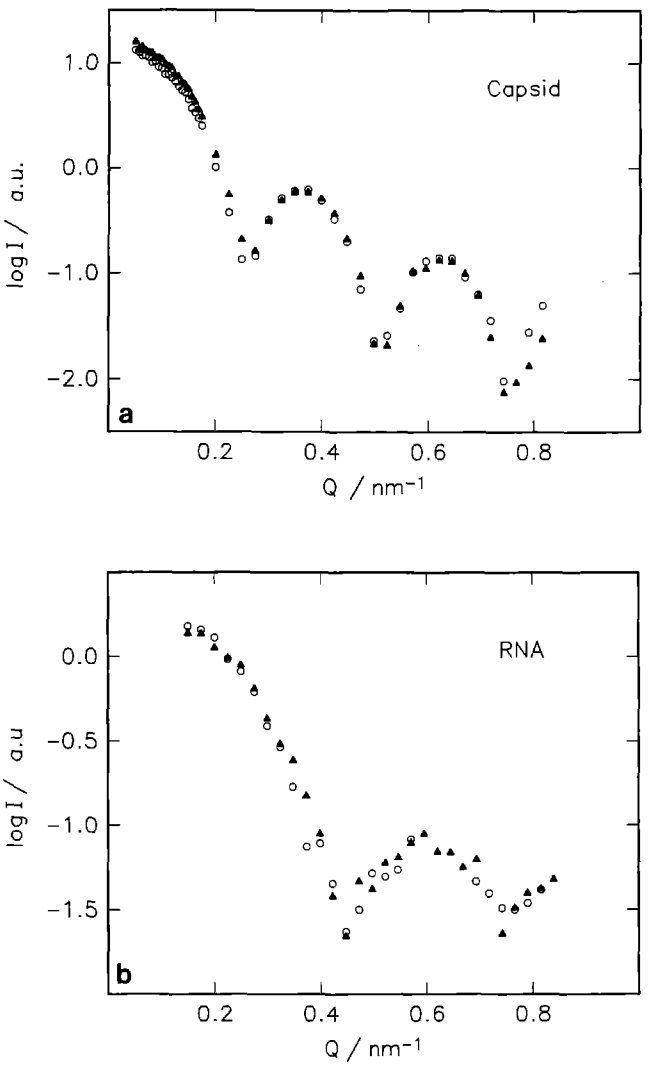


Fig. 1 a, b. Logarithm of the scattered neutron intensity, log $I(Q)$, as a function of the scattering vector in isobar pressure experiments at 40 °C. Buffer: 0.05 *M Tris* at pD = 8.0. o 5×10^6 Pa, ▲ 2×10^8 Pa. **a** 70% D_2O (Capsid), **b** 40% D_2O (RNA)

The scattering function $I(Q)$ of a sphere has a maximum at zero angle and a series of subsidiary maxima of decreasing intensity. For a sphere of uniform density with a radius R the first maximum is at $Q \cdot R = 5.78$ (Guinier 1963). Thus, an increase of size of the sphere is reflected by a shift of the maxima toward smaller angles. For a hollow sphere of the same outer diameter the proportionality factor is smaller than 5.78; it reaches 4.49 for a very thin shell. The intensity at $Q = 0$, $I(0)$, and the radius of gyration R_G were determined by an extrapolation of $\ln I(Q)$ vs Q^2 (Guinier analysis).

Results

General

TYMV was investigated at increasing temperature at two pressures: 5×10^6 Pa and 2×10^8 Pa. We checked that a variation of pressure at 40 °C did not cause any alteration

in TYMV up to 2×10^8 Pa (Fig. 1). At $T < 40$ °C, at low and high pressure, we observed scattering curves similar to those described by Jacrot et al. (1977), providing evidence that native virions in *Tris* buffer at pD = 8.0 also possess a RNA molecule that is located in the central hole of the capsid, with little or no interpenetration with the protein shell. The very small shift of the low angle part of the scattering curves to higher intensities at high pressure can be explained by an increase of the density of particles due to the higher compressibility of H_2O/D_2O (about 3% to 4% per 10^8 Pa) compared to that of the solute, a result noticed previously for proteins (Gavish et al. 1983).

With increasing temperature TYMV loses its stability, which also depends on the pH and ionic strength of the solution. Data concerning the thermal stability in different H_2O/D_2O mixtures cannot be compared directly, even at similar pD values, because the presence of D_2O stabilizes the virions. We have also observed this effect in light scattering experiments (A. Goldbeck and M.D. Lechner, unpublished), and a similar increase of the stability of porcine lactic dehydrogenase under pressure has also been reported (Müller et al. 1982).

Behaviour of RNA

In 40% D_2O the small angle neutron scattering is predominantly due to the viral nucleic acid. Examples of scattering curves at 58 °C are shown in Fig. 2. RNA release by the virion could be monitored directly, by following the changes of scattered intensity in the vicinity of the secondary intensity maximum shown in Fig. 1 b, for instance. Accurate intensity measurements in this Q range, in 40% D_2O buffers where the contrast is very low, do, however, require very long counting rates, with the concomitant risk of slow denaturation of the virion at high temperature, and were therefore not performed.

However, the variation of $I(0)$ with temperature (Fig. 3) clearly shows a transition corresponding to a considerable reduction of the molecular mass of the RNA moiety. As confirmed by analytical ultracentrifugation (not shown) the RNA released upon decapsidation of the virions is degraded by contaminating nucleases. One can, of course, not exclude the possibility that this degradation occurs, or continues to occur, during and after return of the sample to normal conditions, before control experiments could be performed.

Figure 3 shows that the mid-point of this transition is shifted by +4 °C upon increasing the pressure from 5×10^6 Pa to 2×10^8 Pa. It is well known that pressure increases the temperature of helix-coil transitions in nucleic acids. Nevertheless it cannot be excluded that at least part of this shift is due to an effect of pressure on RNase which is known to get denatured under high pressure (Brandts et al. 1970). On the other hand one would expect no activity of RNase at temperatures above 55 °C.

The decrease of $I(0)$ in Fig. 3 monitors the denaturation of viral RNA which is shifted to higher temperatures upon increasing pressure. It is likely that the actual release of the viral RNA by the virion is also shifted to higher temperatures at high pressure as shown by capsid investigations.

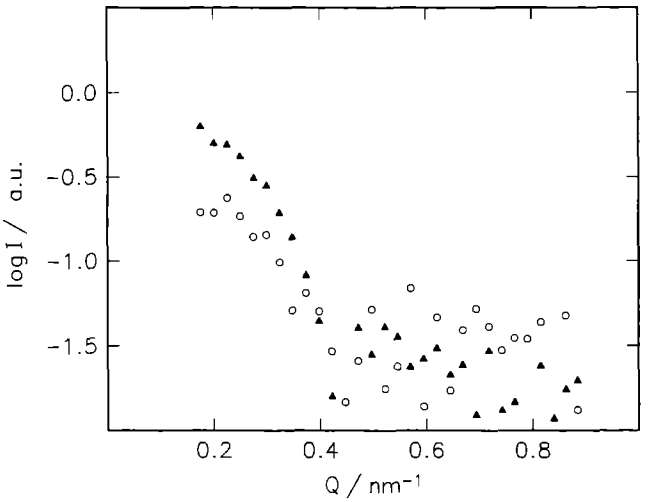


Fig. 2. Logarithm of the scattered intensity as a function of the scattering vector in 40% D_2O at pD = 8.0 at 58 °C. o – 5×10^6 Pa, ▲ – 2×10^8 Pa

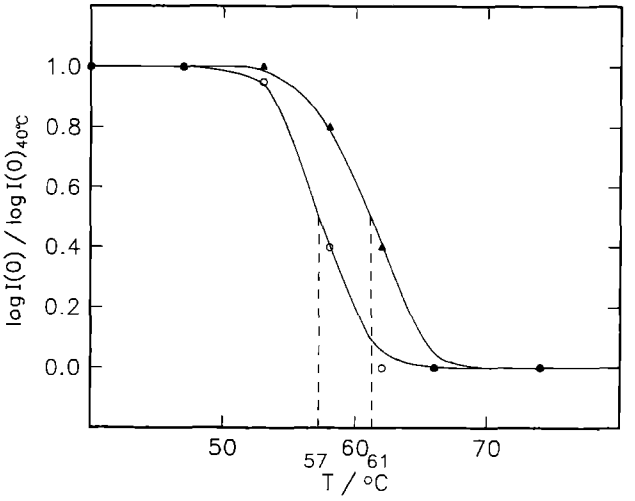


Fig. 3. Logarithm of the scattered intensity at zero angle, log $I(0)$, related to log $I(0)$ at 40 °C as a function of temperature in 40% D_2O at pD = 8.0. o – 5×10^6 Pa, ▲ – 2×10^8 Pa

Investigation of capsid alterations

Changes of the scattering curves in 70% D_2O buffer with increasing temperature reflect changes in the conformation and integrity of the capsid. For a detailed examination of changes in the capsid structure it is useful to analyze four characteristics:

– shifts of the position of the first subsidiary maximum which are due to a change of the radius;
– the decrease of intensity at the first subsidiary maximum which indicates the disruption of the spherical shell structure (Fig. 5);
– changes of intensity at zero angle $I(0)$, which refer to the mass of the particles (Fig. 6);

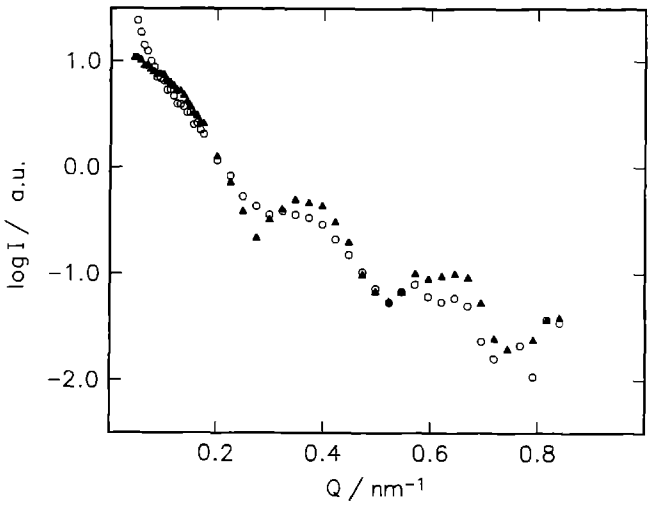


Fig. 4. Logarithm of the scattered intensity as a function of the scattering vector at 62 °C in 70% D_2O at pD = 8.0. o – 5×10^6 Pa, ▲ – 2×10^8 Pa

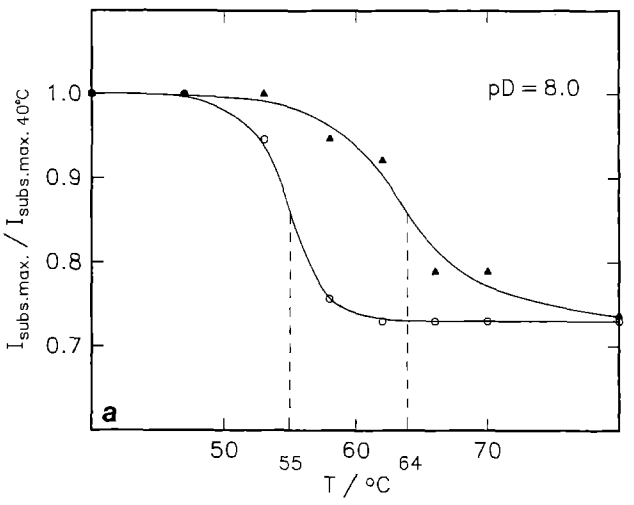
– alterations in the slope of Guinier plots which refer to the radius of gyration.

The first shift was not observed. Keeling and Matthews (1982) reported that the release of RNA is accompanied by a reversible swelling at pH = 11. No swelling effect was obtained under our conditions. A very careful examination of the scattering curves indicates, in fact, that the diameter of the capsid might actually decrease by about 2% upon increasing the pressure to 2×10^8 Pa (data not shown).

Typical examples of scattering curves recorded at low and high pressure at pD = 8.0 at 62 °C are shown in Fig. 4. Figure 5 shows the variation with temperature and pressure of the intensity of the first subsidiary maximum of the scattering curve, compared with that measured at 40 °C: it provides a very sensitive test for the overall integrity of the isometric shell, whereas the intensity scattered at zero angle (Fig. 6) is not only strongly affected by any partial dissociation, but also by the reaggregation of the protein subunits released upon disruption of the shell.

Figures 5 and 6 clearly show that pressure shifted all structural transitions to higher temperature, both at pD = 8.0 and 6.0. Comparison of data corresponding to pD = 8.0 and 6.0 also confirmed that virions are more stable at lower pH.

Upon increasing temperature we first observed a small decrease of $I(0)$ (Fig. 6), without change of the radius of gyration (not shown). $I(0)$ then remained constant over a temperature interval that was small (ca. 3 °C) at low pressure, but this interval increased substantially at high pressure. Since at constant concentration $I(0)$ is proportional to the mass of the scattering particles, this small change may correspond to the loss of a few protein subunits per capsid, since an overall disruption of the capsid would lead to a continuous fall of both $I(0)$ and the radius of gyration. The temperature of this transition at pD = 8.0 was 52 °C at low pressure and 55 °C at high pressure. At higher temperature we observed a considerable increase of the intensity scattered at very low angles, as may be



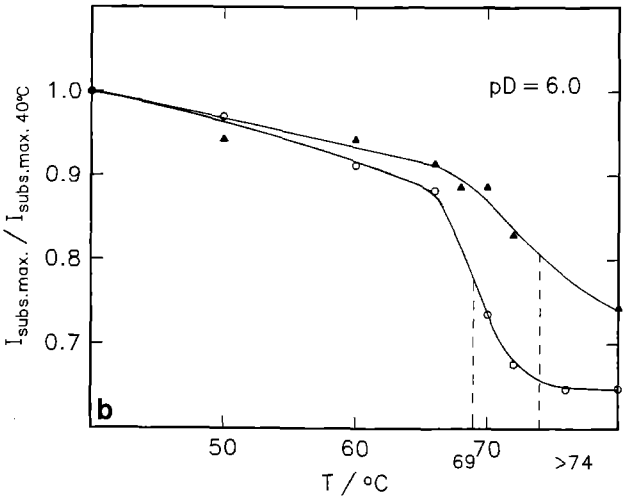


Fig. 5a, b. Scattered intensity of the first subsidiary maximum related to the value at 40 °C as a function of temperature in 70% D_2O **a** pD = 8.0, **b** pD = 6.0. o – 5×10^6 Pa, ▲ – 2×10^8 Pa

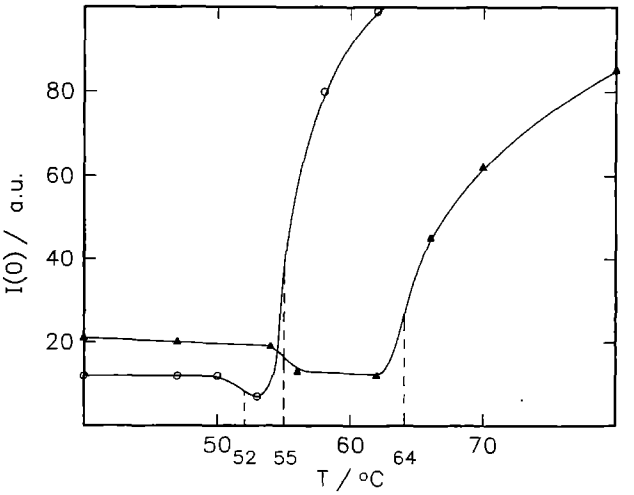


Fig. 6. Scattered intensity at zero angle, $I(0)$, as a function of temperature in 70% D_2O at pD = 8.0. o – 5×10^6 Pa, ▲ – 2×10^8 Pa

seen for instance in Fig. 4. Guinier plots no longer yielded straight lines (not shown), but exhibited a considerable upward curvature that prohibited any linear extrapolation: a typical example of such behaviour may clearly be seen in the very low Q region of Fig. 4. We observed that in such conditions the virus solution became turbid, a further evidence that subunits released upon dissociation of the protein shell formed large polydisperse aggregates. The addition of a reducing agent such as dithioerythritol did not prevent the formation of these aggregates (not shown).

Comparison of Figs. 5 and 6 shows that the formation of protein aggregates is indeed concomitant with the disruption of the protein shell, since at either low or high pressure, the inflection points of the variation with temperature of the intensity of the first subsidiary maximum of the scattering curve corresponded to the temperatures at which $I(0)$ began to rise significantly.

This model of decapsidation of the virion and disruption of the protein shell accounts well for the changes of the scattering curves shown in Fig. 4: at pD = 8.0, $T = 62\,^{\circ}C$, at high pressure, capsids are still intact, or may have released a few protein subunits, a structural change that affects $I(0)$, but not the shape of the scattering curve except very near the minimum separating the central intensity maximum from the first subsidiary maximum (Katouzian-Safadi et al. 1983). At low pressure, disruption of the shells resulted in a considerable filling-in of this minimum, due to the scattering of small protein aggregates, whereas the intensity at low angles had considerably risen, due to the contribution of the scattering of very large polydisperse aggregates.

At pD = 6.0 the transitions were shifted to higher temperatures (Fig. 5b). We did not observe the small decrease of $I(0)$, probably because at low pH the temperature interval separating the release of a few subunits and the disruption of the shell, concomitant with the reaggregation of the released protein subunits, is too small. A similar observation also holds for microcalorimetric measurements with TYMV (K. Mutombo, B. Michels and J. Witz, to be published).

Discussion

The changes with increasing temperature of the small angle scattering curves of TYMV confirmed the stepwise dissociation of the virion upon release of the RNA and the increased stability of the particles at low pH. Comparison of Figs. 3 and 6 shows, however, that in our experiments the mid-temperature of the degradation of RNA, observed in 40% D_2O, is not consistent with that of the release of a few proteins by the capsid, observed in 70% D_2O. The discrepancy could be caused by differences in the stabilizing effect of D_2O or by an effect of D_2O on the activity of contaminating RNases.

Pressure is known to promote the denaturation of proteins and the dissociation of many biologically important protein – protein aggregates (see discussion in: Heremans 1982). TYMV and more generally all viruses belonging to the tymovirus family are stabilized mainly by protein – protein interactions. It is remarkable that at temperatures below 40 °C pressures up to 2×10^8 Pa had no influence on the stability of the virions and that all transitions observed in this study were shifted to higher temperatures upon increasing hydrostatic pressure. This effect concerns RNA – protein interactions as well as protein – protein interactions since the temperature at which the empty shells dissociate, with a concomitant denaturation of the protein subunits, is considerably increased at high pressure (Fig. 6).

An increase of the denaturation temperature has indeed been observed for several proteins such as chymotrypsinogen (Hawley 1971; see also reviews in: Heremans 1982; Weber and Drickamer 1983), but most of the oligomeric proteins investigated have been found to dissociate under pressures (less than 0.5×10^8 Pa) that may be as low as those obtained in an ultracentrifuge (review in: Harrington and Kegeles 1973; see also Table 3 in: Heremans 1982). The only example of an association that is promoted by increasing pressure is that of tRNA-synthetase and tRNA (reviewed in: Harrington and Kegeles 1973), a complex stabilized by strong RNA-protein interactions.

In TYMV, however, RNA-protein interactions are weak, at least under normal pressure (reviewed in: Hirth and Givord 1988). Our results could then imply that these interactions become much more important under high pressure. Pressure could induce the formation of extra hydrogen bonds, either between RNA and protein, or between protein subunits, since the formation of charges or hydrogen bonds is always accompanied by a negative volume change (Heremans 1982). Carboxylic groups of the coat protein may for instance become protonated under pressure and interact with the nucleic acid as they do at low pH under atmospheric pressure. Furthermore, volume changes associated with association of proteins are positive (Zipp and Kauzmann 1973; review in: Heremans 1982), but it is not clear if this statement also holds for the formation of protein-protein interfaces such as those found between coat protein subunits in viral capsids. Such interfaces are mainly stabilized by hydrogen bonds and ionic interactions, and not by hydrophobic contacts between amino acids (discussion in: Harrison 1983).

The behaviour of TYMV is however very different from that of another small isometric virus, Bromegrass Mosaic Virus (BMV), a prototype of virus stabilized mainly by RNA-protein interactions. Silva and Weber (1988) have shown that at 20 °C BMV dissociates reversibly at pressures above 0.6×10^8 Pa, and irreversibly above 1.4×10^8 Pa. Our preliminary small angle neutron scattering studies (to be published) indicate that the diameter of BMV increases substantially upon increasing the pressure to 2×10^8 Pa at 20 °C, and that this swelling is at least partially reversible. In order to unravel the respective contributions of RNA-protein and protein-protein interactions we have now begun to investigate in detail the behaviour of empty shells also present in purified suspensions of TYMV, of BMV at various pH's, and of shells obtained by in vitro reassociation of BMV protein.

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