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# High Pressure Effects on the Endothermic Association of Tobacco Mosaic Virus Protein

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**Abstract.** Tobacco mosaic virus protein in phosphate buffer pH 6.5–7.0 ($I = 0.1$ M) shows endothermic polymerization accompanied by water release of the capsomers. At protein concentrations $c \sim 2$ mg/ml the transition temperature is $T^* = 20 \pm 1°$ C. As indicated by the increase of the partial specific folume ($\Delta V_2 = 0.0049 \pm 0.0003$ cm$^3$/g) in going from A-protein to helical rods at pH 6.50, the assembly reaction is expected to be inhibited by high pressure; the corresponding isobars of the endothermic polymerization should be shifted to higher $T^*$ values.

Turbidity measurements at pressures $1 < p < 1{,}500$ bar are in agreement with the given hypothesis: both, double discs and helical rods are found to be dissociated at elevated pressure, the latter showing somewhat higher stability. At 700 bar the transition temperature of helix formation is shifted by 14° C to higher temperatures.

Complete reversibility of the pressure dependent dissociation-association without "hysteresis" proves the process to represent a true equilibrium. At low temperatures and high pressures the association equilibrium is shifted to a molecular weight distribution with $\bar{M}_w < M$ (A-protein). Increased cooperativity in the transition A-protein $\rightarrow$ helical rods, as well as an apparent inversion of the sign of the reaction volume at high temperatures and pressures are caused by pressure induced pH shifts. Adjusting the pH at high pressure to the value at ambient pressure allows to eliminate both effects.

The product of association at high pressure differs in its conformation from the end product obtained from the endothermic polymerization at 1 bar and subsequent pressure application.

**Key words:** Association – High pressure – Hydrophobic interactions – Polymerization – Tobacco mosaic virus protein

## Introduction

The self-assembly of tobacco mosaic virus (TMV), and the related process of reversible "polymerization" of its coat protein (TMV-P) is considered to be an

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entropy driven process [1] caused by a significant contribution of intersubunit hydrophobic interactions [2]. Experimental evidence confirming this mechanism has been obtained from the endothermic nature of the association reaction [3] and from direct measurements of the water release [4], as well as the decrease in apparent specific heat accompanying quaternary structure formation [5]. Consistent with these results is the observed change of the partial specific volume which is found to be positive for the polymerization reaction: $\Delta V_2 = +0.0049 \pm 0.0003$ ml $\cdot$ g$^{-1}$ [4].

Based on the observations given one would expect that elevated hydrostatic pressure has a significant inhibitory effect on the polymerization reaction of TMV-protein. For the complete virus Lauffer and Dow [6] have shown that high pressures cause separation of the coat protein from the viral RNA, which is accompanied by coagulation and/or denaturation. In the present study pressures up to 1.5 kbar have been applied to investigate structural changes of TMV coat protein in the range of the reversible transitions between the different association states in the „phase diagram" of the protein [7, 8]. As predicted on the basis of the previous thermodynamic data, a pronounced pressure dependence of the transition temperature, $T^*$, of the endothermic helix formation is observed: at 0.7 kbar $T^*$ is found to be increased by 14° C. The product of association at high pressure differs in its conformation from the end product obtained from endothermic "polymerization" at ambient pressure and subsequent pressure application.

Obviously the pressure induced structural changes affect both the assembly and the three-dimensional folding of the protein subunits.

## Materials and Methods

The common strain of tobacco mosaic virus [TMV(vulgare)] was isolated by differential centrifugation with two depigmentation steps [9] or according to Leberman [10]; it was kept in 10 mM EDTA pH 7 at ~ 2° C. Tobacco mosaic virus-protein (TMV-P) was prepared by the acetic acid method [11]; the isoelectric precipitate was dissolved in 0.1 M phosphate buffer pH 8.5, and clarified by high speed centrifugation (2 h at 100,000 *g*). Portions of this stock solution were dialyzed in the cold against buffers of desired pH, and clarified by centrifugation, if necessary. Individual samples were prepared for each set of experiments; the protein was never kept for longer than 10 days.

The concentrations of virus and protein were measured spectrophotometrically [4]: DMR 10, and PMQ II (Zeiss), 118 (Cary). To determine the state of association of the protein the turbidity at 320 nm was measured in a Gilford 2 400 S spectrophotometer [12]. Slow reactions were monitored using a Servogor SRE 541 recorder (Metrawatt); for fast reactions a visigraph FR 301 (San Ei, Japan) was applied.

Thermostated cuvettes with temperature control $\pm$ 0.05° C were used throughout.

For high pressure turbidity measurements a modified transmission cell (according to Lüdemann and Mahon [13]) was applied [14], for quench

experiments with subsequent conformational analysis an autoclave according to Schade et al. [14]. Circular dichroism spectra were monitored in a Jouan-Roussel Dichrographe II with high sensitivity accessory. To characterize the state of association sedimentation velocity runs were performed in an analytical ultracentrifuge (Beckman, Model E). Chemicals were of A-grade purity, bidistilled water was used troughout.

## Results and Discussion

### *1. High Pressure Effects Under Isothermal Conditions*

TMV-P forms a variety of aggregates depending on pH, ionic strength and protein concentration [7, 8, 15]. Increased hydrostatic pressure in the range of up to 1 kbar causes dissociation of all of these assemblies. Starting from helical rods of variable length (produced by varying rates of heating [16]), the final dissociation product shows the limiting turbidity value for the "A-protein" at low temperature

$$A_{320} = 0.025 \pm 0.005 .$$

As shown in Fig. 1a this limiting turbidity is independent of the starting conditions (temperature, rate of pressure application) within the limits of error. The observed dissociation is found to be fully reversible: reducing the pressure in steps back to atmospheric pressure yields a protein that is indistinguishable from the starting material. The respective experiments were performed under conditions avoiding drastic temperature changes, by stepwise increasing the pressure with an interval of 20 min between the single steps. This procedure allows optimum equilibrium conditions to be established [16]. No hysteresis due to overshoot effects could be detected; the small deviations at 26° C cannot be considered significant. In Fig. 1B similar experiments with the TMV-P double disk as starting material are shown. Since in this case the limiting particle weight cannot differ by more than a factor of 10–30 (assuming A-protein or the monomer as the limiting dissociation product) a higher protein concentration was used. To provide a homogeneous population of double disks the protein ($c_{TMV\text{-}P}$ = 10.0 mg/ml) in phosphate buffer pH 7.0, 0.1 M ionic-strength, was kept at 25° C for 24 h. Under these conditions one single 21 ± 1S peak was observed in the ultracentrifuge, with no significant percentage of either A-protein or high molecular weight aggregates. As in the case of helical aggregates elevated pressure causes reversible dissociation with limiting turbidity values close to the monomer. The pressure of half-dissociation is slightly below the value observed for helical rods at pH 6.5.

Preliminary kinetic experiments prove the dissociation to be a complex reaction with at least two consecutive steps: applying pressure jumps from 1–500 bar, a precursor reaction with a halftime < 1 s and a consecutive reaction in the $10^2$ s range were observed. Since the adiabatic compression causes severe temperature changes no quantitative evaluation was attempted [23].

## 2. *Endothermic Association Under Isobaric Conditions*

As indicated by the traces in Fig. 1 heating at fixed pressure is expected to generate reversible polymerization. Since there is no appreciable association below 10° C under the given experimental conditions [4, 7, 16], the experiments were performed in the temperature range from 10 to ~ 30° C. After measuring

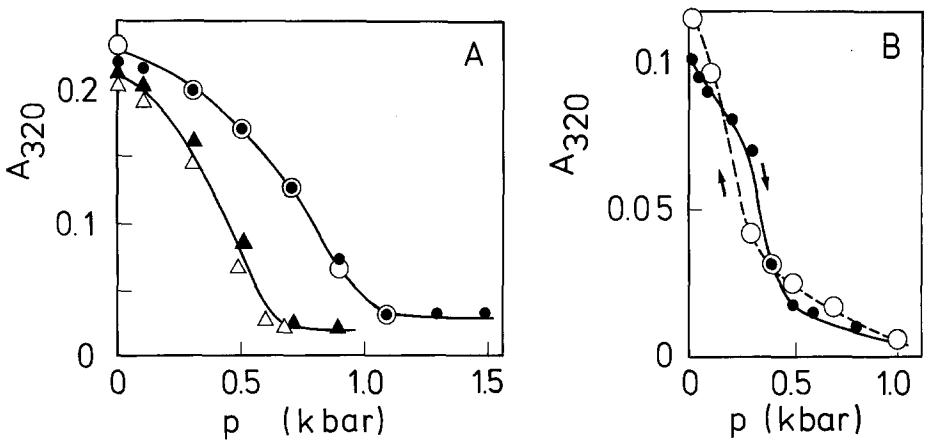


**Fig. 1A and B.** Pressure induced dissociation of tobacco mosaic virus protein. **A.** Initial state at atmospheric pressure: helical rods. Phosphate buffer pH 6.50, $I = 0.1$ M. $c_{TMV\text{-}P} = 2.2$ mg/ml. △, ▲ 26.2° C; ○, ● 29.7° C. **B.** Initial state at atmospheric pressure: double disks. Phosphate buffer pH 7.00, $I = 0.1$ M. $c_{TMV\text{-}P} = 10$ mg/ml. 25.0° C. Closed (open) symbols refer to increasing (decreasing) pressure

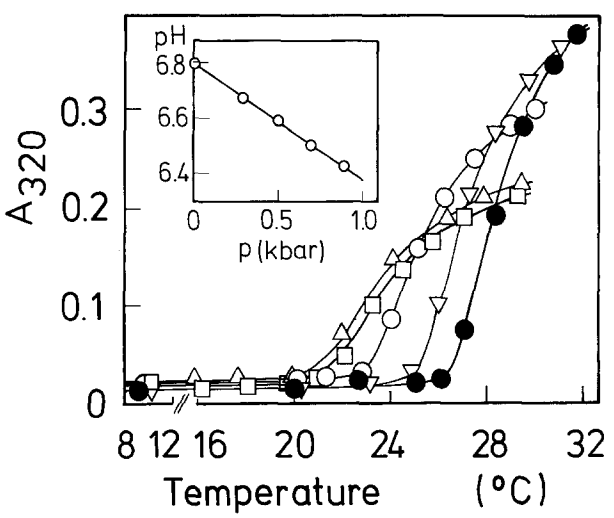


**Fig. 2.** Endothermic association (A-protein → helical rods) of tobacco mosaic virus protein at various pressures. Phosphate buffer pH 6.50, $I = 0.1$ M. $c_{TMV\text{-}P} = 2.2$ mg/ml. △, 1 bar; □, 0.3 kbar; ○, 0.5 kbar; ▽, 0.7 kbar; ●, 0.9 kbar. *Insert:* Effect of pressure on the pH of the given buffer

the protein concentration and the initial turbidity at 320 nm the protein was equilibrated in the high pressure transmission cell. The temperature was increased stepwise at a heating rate of about 0.1° C/min using an equilibration period of 20 min. Monitoring the turbidity change during the whole heating process showed that within the time intervals given the association equilibrium was established.

Reversal of the endothermic association was provided by cooling to 10° C. In order to avoid artefacts due to partial denaturation ("ageing") a fresh sample was used for each isobar. The respective results, including the endothermic polymerization at normal atmospheric pressure, are shown in Fig. 2.

In general increasing pressure is found to shift the association equilibrium at low temperatures to values below the molecular weight distribution characteristic for the A-protein. Corresponding to the positive reaction volume $\Delta V$ for the association reaction the transition curve at 0.3 kbar is shifted to a higher temperature by ~ 1° C compared to the profile at atmospheric pressure. At high temperatures and pressures an apparent inversion of the sign of $\Delta V$ is observed; at the same time the cooperativity of the transition increases significantly and the limiting value of the average particle size (reflected by $A_{320}$) increases with increasing pressure.

The foregoing observations cannot be interpreted in terms of pressure and temperature dependent changes of the reaction volume of the protein. Instead, the pressure dependence of the ionization of the buffer system has to be taken into consideration. As shown by Neuman et al. [17] the ionization volume of phosphoric acid and its anions is strongly negative so that increasing pressure promotes dissociation causing a pH shift of $\Delta$pH = −0.44 pH units per kbar at room temperature. As a consequence, the foregoing experiments implicitly contain the pH as an additional variable (cf. insert, Fig. 2). Considering the specific association conditions the pressure induced pH change shifts the system into the domain of helical rod formation [7].

The increased cooperativity and association yield in this domain has been observed previously [4, 18].

To eliminate the pH effect two alternative approaches can be applied: either buffers with negligible ionization volume can be used (e.g., *Tris* or imidazole [19]), or the pressure induced $\Delta$pH can be compensated applying the original phosphate buffer at an initial higher pH which is then shifted to the desired pH at some elevated pressure.

Since TMV-P is less stable in *Tris* or imidazole buffer, compared to phosphate buffer (which has always been used as standard solvent in the given context), the second approach was applied. TMV-P in phosphate buffer pH 6.5 associated at atmospheric pressure was compared to TMV-P in the same buffer at pH 6.8 which at $p$ = 0.7 kbar is shifted to pH 6.5. From Fig. 3 it is evident that application of a pressure of 0.7 kbar increases at constant pH the association temperature by 14° C, without changing the transition profile significantly. The difference seen when comparing the two traces for $p$ = 0.7 kbar in Figs. 2 and 3 can be attributed to the pH difference only; both the temperature shift and the increased slope correspond quantitatively to the results obtained from systematic pH dependent experiments [18].

As mentioned earlier, high pressure dissociation of helical aggregates is reversible. Within the limits of error, the respective initial and final turbidity values are identical for A-protein, as well as its association products. One remarkable additional result in this connection is the stabilizing effect of high pressure on the native structure of the protein. As shown in all previous experiments, TMV-P is found to exhibit anomalously high thermal stability under elevated pressure. This effect becomes obvious if the pressure is released at high temperature: reducing the pressure at 36° C from 0.7 kbar to 1 bar results

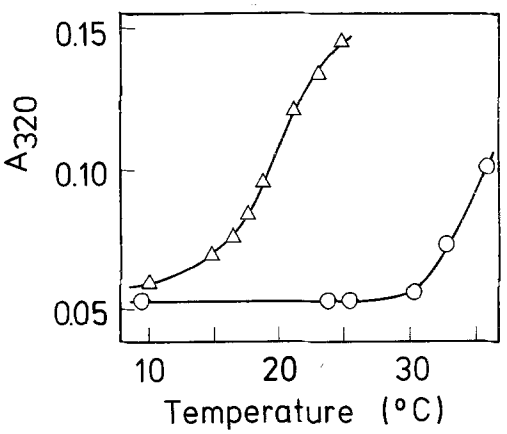


**Fig. 3.** Endothermic association (A-protein → helical rods) of tobacco mosaic virus protein at 1 and 700 bar, and "constant pH". Phosphate buffer $I = 0.1$ M. $c_{TMV\text{-}P} = 2.2$ mg/ml. △, 1 bar, pH 6.50; ○, 700 bar, initial $pH_{1\,bar} = 6.80$. The pressure induced pH shift leads to $pH_{700\,bar} = 6.5$ (cf. insert Fig. 2)

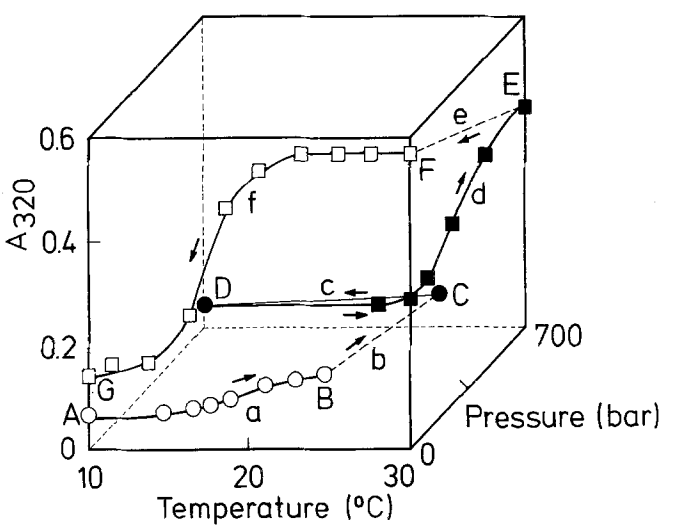


**Fig. 4.** Temperature and pressure effects on the endothermic association of tobacco mosaic virus protein. Starting conditions: phosphate buffer $pH_{1\,bar} = 6.50$, $I = 0.1$ M, $c_{TMV\text{-}P} = 2.6$ mg/ml. A, and C, D, G: A-protein, and related depolymerized states; B, E, F: helical assemblies of varying conformation and/or quaternary structure. Broken lines refer to pressure changes; no experimental points were taken at intermediate pressures. See text

in immediate formation of unspecific aggregates of the protein. This is illustrated by reaction (E) in Fig. 4. The fact that thermal denaturation is characterized by a significant excess volume has been shown previously by independent dilatometric and spring balance experiments [20].

### 3. *Product of Isothermal and Isobaric Dissociation-Association*

A comparison of the isothermal and isobaric experiments summarized before (cf. Figs. 1 and 2) proves that the sequential application of pressure and temperature on TMV-P leads to different products depending on the sequence of pressurization and heating. The difference becomes immediately apparent from the respective association states reflected by the final values of $A_{320}$:

$$\begin{array}{l}\text{Helical rods (1 bar, 30° C)} \xrightarrow{1\text{ bar} \rightarrow 0.9\text{ kbar}} \text{A-protein (or subunits)} \quad (0.9\text{ kbar}, 30°\text{ C}) \quad (1)\\ A_{320} = 0.23 \qquad\qquad\qquad\qquad A_{320} = 0.06\end{array}$$

$$\begin{array}{l}\text{A-protein (or subunits)} \quad (0.9\text{ kbar}, 10°\text{C}) \xrightarrow{10°\text{ C} \rightarrow 30°\text{ C}} \text{helical rods (0.9 kbar, 30° C)} \quad (2)\\ A_{320} = 0.015 \qquad\qquad\qquad\qquad A_{320} = 0.33\end{array}$$

In both experiments the final pH is shifted to some extent (e.g., from pH 6.5 to about 6.2); however, the final conditions are identical with respect to all parameters involved. The two reaction schemes clearly indicate that it depends on the pathway of the reaction whether at 30° C and 0.9 kbar tobacco mosaic virus protein is present in a distribution of helical rods of variable length, or in a distribution similar to "A-protein".

It is wellknown from a number of studies [15, 16, 21] that the mode of association of TMV-P under certain conditions may be significantly influenced by the "history" of the protein.

In order to gain some insight into the relative stability of the different states an experiment with the following sequence of $p$ and $T$ changes was performed (pH 6.50, 0.1 M ionic strength, $c_{\text{TMV-P}} = 2.6$ mg/ml):

(*a*) isobaric endothermic polymerization at 1 bar: 10 → 25° C,
(*b*) isothermal compression at 25° C: 1 → 700 bar,
(*c*) isobaric depolymerization at 700 bar by cooling: 25 → 10° C,
(*d*) isobaric endothermic polymerization at 700 bar: 10 → 30° C,
(*e*) isothermal decompression at 30° C: 700 → 1 bar,
(*f*) isobaric depolymerization at 1 bar: 30 → 10° C.

The result, illustrated in Fig. 4, shows: firstly, that neither of the intermediate states is identical to any other state in the reaction sequence; secondly, that high pressure dissociation at low temperature (state D) is characterized by an average particle size below the initial A-protein (state A); thirdly, that the endothermic

polymerization starting from state D differs qualitatively from the endothermic polymerization at atmospheric pressure.

It is obvious that pressure induced $\Delta$pH effects are involved in this experiment, as in the experiments given previously in Fig. 2. While reaction (*a*) occurs at constant pH (pH 6.5), (*b*), (*d*), and (*e*) are accompanied by pH shifts of $\pm$ 0.3 pH units for $1 \leftrightarrow 700$ bar. Therefore, the significant difference is the fact that the endothermic polymerization at 1 bar and 700 bar occur at slightly different pH. Taking into account the sensitivity of the self-assembly of TMV-P to pH changes, this is clearly of importance. In conclusion, the direct transition from state C (or C′) to the more stable state E is not possible unless intermediary cooling and subsequent re-heating are applied.

Apart from the difference in the association behaviour it was not possible to obtain other structural characteristics for this "inhibition". Attempts to characterize the states B and E by direct spectral analysis failed because, for different reasons, circular dichroism and fluorescence measurements at elevated pressure cannot be obtained under the given experimental conditions. Due to the reversibility of pressure induced structural changes (cf. Fig. 1), spectral characteristics referring to state F (measured immediately after decompression of E) do not necessarily reflect the state at high pressure. However, comparing the near UV CD spectra of states B and F a decrease of dichroic absorption at $250 < \lambda < 280$ nm by a factor $\sim 0.6$ is observed. Since metastable states caused by pressure induced pH changes would generate the opposite effect, this observation may indicate structural differences suggested previously.

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## References

1. Lauffer MA (1975) Entropy driven processes in biology. Springer, Berlin Heidelberg New York
2. Kauzmann W (1959) Some factors in the interpretation of protein denaturation. Adv Protein Chem 14: 1–63
3. Lauffer MA, Ansevin AT, Cartwright TE, Brinton CC Jr (1958) Polymerization-depolymerization of TMP-protein. Nature 181: 1338–1339
4. Jaenicke R, Lauffer MA (1969) Polymerization-depolymerization of TMV-protein. XII. Further studies on the role of water. Biochemistry 8: 3083–3092
5. Sturtevant JM, Velicelebi G, Jaenicke R, Lauffer MA (1981) A scanning calorimetric investigation of the polymerization of the coat protein of TMV. Biochemistry (in press)
6. Lauffer MA, Dow RB (1941) The denaturation of TMV at high pressure. J Biol Chem 140: 509–518

7a. Durham ACH, Finch JT, Klug A (1971) States of aggregation of TMV protein. Nature [New Biol] 229: 37–42

7b. Durham ACH, Klug A (1971) Polymerization of TMV protein and its control. Nature [New Biol] 229: 42–46

8. Butler PJG, Durham ACH (1977) TMV protein aggregation and the virus assembly. Adv Protein Chem 31: 188–247
9. Boedtker H, Simmons NS (1958) The preparation and characterization of essentially uniform TMV particles. J Am Chem Soc 80: 2550–2556

10. Leberman R (1966) The isolation of plant viruses by means of "simple" coacervates. Virology 30: 341– 347
11. Fraenkel-Conrat H (1957) Degradation of TMV with acetic acid. Virology 4: 1–4
12. Smith CE, Lauffer MA (1967) Polymerization-depolymerization of TMV protein. VIII. Light scattering studies. Biochemistry 6: 2457–2465
13. Lüdemann H-D, Mahon WAS (1969) Absorption spectra at high temperatures and pressures IV: A new optical high pressure cell for corrosive liquids. High Temp High Pressures 1: 215–220
14. Schade BC, Lüdemann H-D, Jaenicke R (1980) Reversible high-pressure dissociation of lactic dehydrogenase from pig muscle. Biochemistry 19: 1121–1126
15. Vogel D, de Marcillac GD, Hirth L, Gregori E, Jaenicke R (1979) Size distribution in the higher stages of polymerization of the A-protein of TMV (vulgare). Z Naturforsch 34c: 782–792
16a. Schuster TM, Scheele RB, Khairallah LH (1979) Mechanism of self-assembly of TMV protein: I. Nucleation-controlled kinetics of polymerization. J Mol Biol 127: 461–485
16b. Shire SJ, Steckert JJ, Schuster TM (1979) Mechanism of self-assembly of TMV protein: II. Characterization of the metastable polymerization nucleus and the initial stages of helix formation. J Mol Biol 127: 487–506
17. Neuman RC Jr, Kauzmann W, Zipp A (1973) Pressure dependence of weak acid ionization in aqueous buffers. J Phys Chem 77: 2687–2691
18. Srinivasan S, Lauffer MA (1973) Polymerization studies on protein from the Dahlemense Strain of TMV: Light scattering and related studies. Arch Biochem Biophys 158: 53–66
19. Jaenicke R (1981) Enzymes under extremes of physical conditions. Annu Rev Biophys Bioeng 10 (in press)
20. Jaenicke R (1971) Volume changes in the isoelectric heat aggregation of serum albumin. Eur J Biochem 21: 110–115
21a. Durham ACH, Vogel D, de Marcillac GD (1977) Hydrogen-ion binding by TMV protein polymers. Eur J Biochem 79: 151–159
21b. Vogel D, Durham ACH, de Marcillac GD (1977) Metastable aggregates in the polymerization of TMV protein. Eur J Biochem 79: 161–171

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