

# Size Distribution in the Higher Stages of Polymerization of the A-Protein of Tobacco Mosaic Virus (*vulgaris*)

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The composition of the A-protein of tobacco mosaic virus (TMV) has been investigated by sedimentation velocity, light-scattering, spectroscopic methods, and thermodynamic calculations, at concentrations from 5 to 20 mg/ml, at temperatures from 7 to 26 °C, at various pH and buffer conditions. Above distinct critical concentrations and temperatures aggregates are formed which sediment near 8S, while the concentration of the smaller aggregates that sediment in the trailing boundary, near 4S, remains approximately constant. We identify the 4S protein with two-layer aggregates (Durham and Klug, *J. Mol. Biol.*, 1972), with weight average molecular weight ( $\bar{M}_w$ ) near 5 subunits, at the lower limit of polymerization. The 8S aggregates are best described by a series of three-layer aggregates, starting with a heptamer (Caspar, *Adv. Protein Chem.*, 1963), but attaining  $\bar{M}_w$  corresponding to at least 12 subunits, at the upper limit. The 4S/8S equilibrium is not significantly changed by a change in pH, nor by the coexistence of higher aggregates (20 – 30S) with residual "A-protein". Three-layer aggregates are more stable than two-layer aggregates, but significantly less stable than would be expected with strictly equivalent bonding; the third layer in the 8S protein disturbs the pairing between the two layers in the 4S protein, and the intersubunit interaction near tryptophan 52 seems to be involved. From the structure, 8S protein should tend to polymerize easier to helices than to disks, in accordance with earlier suggestions (Vogel *et al.*, *Eur. J. Biochem.*, 1977), and corroborated by studies on TMV-mutants.

## Introduction

TMV and its components, though the subject of numerous investigations for many years, are still contributing to our understanding of biological systems [1, 2]. The aggregation behavior of isolated TMV coat protein in many respects may be considered typical for other aggregating systems [3]. The polymorphism of the protein exemplifies the concept of quasi-equivalence of identical monomers built into large structures [4, 5]. Recent investigations have revealed the complexity and many details of the polymerization of the protein, the protein-protein and RNA-protein interactions, and the assembly of the virion [1 – 3, 6 – 21].

Mostly TMV-protein polymerization has been investigated near neutral pH in conditions chosen to be as nearly physiological as possible. In these conditions, the protein polymerizes to disks or related po-

lymers (20 – 30S) [2, 3, 5, 6, 9 – 11, 22, 23], which were shown to be important, together with the RNA, for the *in vitro* reassembly of the virus [1, 2, 16 – 21]. At lower pH values long helical aggregates form ( $\leq 200S$ ) [2, 3, 5, 6, 10]; at slightly higher pH the disks and helices depolymerize to "A-protein" (4 – 8S), a broad distribution of oligomers [2, 3, 5, 10, 12, 22 – 24]. The A-protein is the first state in the aggregation from isolated monomers, and is the starting material for the higher molecular weight aggregates. Though the A-protein has been thoroughly studied by several authors, many different views have been offered about the stability of its components [2, 3, 5, 12, 22 – 25].

In a first attempt to explain the sedimentation behaviour of the A-protein [24] Caspar [5] suggested special stability for "cyclic" trimers (subunits arranged in two layers) and "cyclic" heptamers (subunits arranged in three layers, "six-around-one"), with  $s$ -values of 4 – 4.6S, and 7 – 8.5S, respectively. In later studies the trimer was shown to be a stable configuration at low temperatures under a variety of solution conditions [25]. More recently, Durham and

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Klug [22, 23] fitted sedimentation equilibrium data (at pH 7 and ionic strength 0.1 M below 10 °C and protein concentrations below 9 mg/ml) by a model including monomers and a series of two-layer aggregates, starting from the trimer; in this approach the mean degree of polymerization was found to increase steadily under conditions favouring aggregation, ultimately leading to the disk aggregate. This model which is now widely accepted [1, 26], though conflicting results were reported [12], did not account for other than two-layer aggregates; the general appearance of an "8S" \* aggregate was noticed, under conditions favouring aggregation to a still higher extent [2, 22, 24, 27]. However, frequently in the past it has not been realized that the "8S" species is the normal major component under these conditions of aggregation or assembly, though it was found to be an anomalous state of aggregation in special conditions [27–31].

During an investigation of metastable "overshoot aggregates" [6, 7] near neutrality Vogel *et al.* [11] found critical conditions for the formation of 20–30S aggregates, and the disappearance of the 4–8S material. This suggested that the A-protein is inhomogeneous in a thermodynamic and kinetic sense. From these observations a quantitative reinvestigation of the state of aggregation of the A-protein seemed desirable, at temperatures and concentrations higher than used earlier [22, 23] which at pH 7 would lead to disks and related polymers, though they do not so at pH > 7.4 [2, 22].

In such conditions a rigorous physico-chemical description of the system is difficult because of the effects of non-ideality and aggregation. To overcome this ambiguity various techniques were used to estimate the molecular weights and relative amounts of components. In the present paper we describe the results of experiments using the A-protein of the vulgare strain of TMV. The proposed model following from the given data extends earlier models for the polymerization of TMV A-protein [5, 23]. These studies are the basis of further polymerization studies to be published.

## Materials and Methods

TMV, TMV-protein, and solutions for the measurements were prepared as described earlier [11]. Por-

tions of the stock solutions were dialyzed in the cold against buffer solutions of desired pH, clarified by a short cleaning spin and/or ultrafiltration, kept on ice as long as possible, and thermostatted at the final temperature for about 5 min, before starting a measurement. This time is sufficient for the equilibration of the A-protein (D. Vogel, unpublished). For light-scattering, the cleaning spin was extended to 2 h at 30,000 revs/min, and 0.3 µm pore-size filters were used. All solutions were thermostatted to ± 0.5 °C, or better; for automatic change of temperature a temperature programmer (J. Neimark *et al.*, IBMC, Strasbourg) was used.

For absorbance measurements, recording spectrophotometers Zeiss-DMR 10 and Cary 118, for circular dichroism (CD), Jobin-Yvon-Dichrographes II (with tenfold scale-expansion) and III were used. Molar absorbance difference,  $\Delta a = a_{T_2} - a_{T_1}$ , and molar dichroic absorption,  $\Delta \varepsilon = \varepsilon_L - \varepsilon_R$ , were based on the subunit molecular weight of  $M = 17\,500$ . The ratio of the temperature-induced absorbance difference,  $\Delta a_{\lambda_1}/\Delta a_{\lambda_2}$ , at two different wavelengths  $\lambda$ , was corrected for the effect of temperature on density ( $\rho$ ) and turbidity ( $\tau$ ) (D. Vogel, unpublished), using  $\Delta \rho$  of water (instead of the solvent), and Raleigh's law,  $\Delta \tau_{\lambda} = \Delta \tau_{320\text{ nm}} (320/\lambda)^4$ , respectively. Light-scattering was measured in a Photogoniomètre Fica 50, with benzene as standard, using a refractive index  $n_B$  (20 °C) = 1.499 [33]. The scattered intensity, measured at 546 nm and different angles between 45° and 150°, was converted to  $\bar{M}_w$  by standard formulae [32], with a specific refractive increment for TMV-protein of  $(dn/dc)_{546\text{ nm}} = 0.1859 \text{ ml g}^{-1}$  [34].

Sedimentation velocities were measured in an analytical ultracentrifuge (Beckman, Model E) using 12 and 30 mm double sector cells.  $s$ -values were corrected for water viscosity and 20 °C, and areas under the Schlieren curves were corrected for radial dilution. For quantitative evaluation of the areas, the Schlieren photographs were magnified 20-fold, redrawn by hand, and digitalized ("Summagraphics" Kontron, München, combined with "Facit 4070", Facit, Düsseldorf). A computer programm was used to compare the set of data for a given Schlieren curve  $C_i$  to a reference curve fitting the trailing edge of  $C_i$  to a first approximation. A concentration of 2 mg/ml was chosen for the reference curve. We optimized the fit by multiplying the amplitudes of the reference curve with an adjustable factor, until the difference

\* The terms "4S" and "8S" are used for simplicity, though the sedimentation constants may deviate significantly from these numerical values.

between the trailing edges of the sample and reference Schlieren curves vanished. Areas under the reference and difference curves were determined using a planimeter (Type 30113, A. Ott, Kempten), and each percentage area was converted to concentration by multiplication with the total protein concentration. For a first estimate of monodispersity, the difference curves were fitted according to single Gaussian curves, using the Fletcher and Powell minimization method [35].

The theoretical molecular weight distributions, by extension and modification of the "nucleated two-layer linear condensation model" of Durham and Klug [23] (see discussion) were calculated with the aid of a Texas Instruments TI 59 programmable calculator plus printer PC 100 A, using Newton's method for the search of zeros of functions (TI Solid State Software Program ML-08).

## Results

At pH 8, ionic strength 0.1 M, the A-protein sedimented between 4 and 8 S, depending on the temperature, and without polymerization to disks and higher aggregates (Fig. 1). At protein concentrations above 2 mg/ml and temperatures above 17 °C, the sedimentation patterns were highly unsymmetrical, with a leading quasi-symmetric peak sedimenting at about 7.5 S, and a long trailing edge that remained constant in shape as the protein concentration varied. If the concentrations ( $\leq 2$  mg/ml, at 26 °C) or temperatures ( $< 17$  °C, at 5 mg/ml) were lowered the boundaries grew more and more symmetrical,

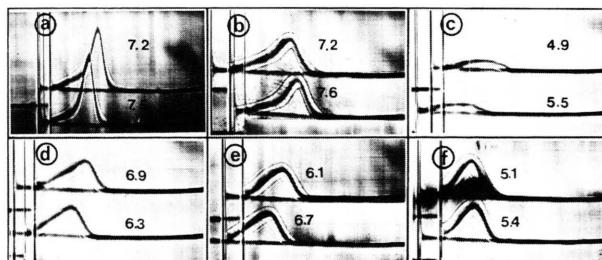


Fig. 1. Schlieren patterns of TMV-A-protein at pH 8.0, ionic strength 0.1 M, in potassium phosphate (lower traces) and sodium pyrophosphate buffer (upper traces). Numbers refer to  $s_{20, w}$  values for the main peaks. 50 740 revs/min, 12 mm cell pathlength, bar angle 76 °, photographs taken 34 min after reaching maximum speed. Measurements at constant temperature (26 °C): a) 10 mg/ml; b) 6 mg/ml; c) 2 mg/ml. Measurements at constant protein concentration (5 mg/ml): d) 20 °C; e) 16.5 °C; f) 15 °C.

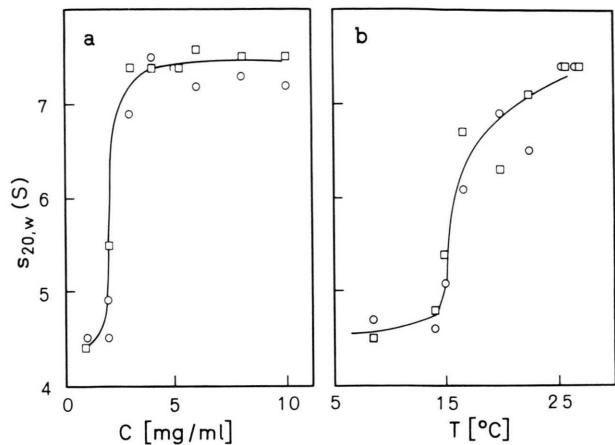


Fig. 2. Sedimentation coefficients (main peaks) of TMV-protein at pH 8.0 as functions of protein concentration at 26 °C (a), and temperature at 5 mg/ml (b), in potassium phosphate (□) and sodium pyrophosphate buffer (○).

while the  $s$ -value decreased abruptly and approached 4.5 S (Figs 1+2). These changes in the pattern and the sedimentation coefficient were observed to be fully reversible on changing temperature or ionic strength, in agreement with earlier findings [22, 27, 31]. An attempt to separate the 4S and 8S components by sedimentation in a fixed partition cell (not shown) failed, suggesting reversibility of the polymerization and re-equilibration of the components: re-sedimenting the material taken from the two compartments of the fixed partition cell resulted in Schlieren patterns indistinguishable from the original ones after correcting for changes in the protein concentration, by the separation. No significant differences were detectable regarding the aggregation behaviour in phosphate and pyrophosphate buffer (Fig. 1, lower and upper traces, and Fig. 2), in contrast to earlier conclusions [30].

Below pH 7.4, disks or related species (20–30 S) were formed, depending on ionic strength and temperature [11, 22, 23]. Fig. 3 summarizes the sedimentation patterns observed during the various aggregation reactions, demonstrating that the changes in pH and the presence of higher aggregates has little influence on the 4S/8S pattern. This pattern is simply determined by the amount of A-protein coexisting with the higher aggregates (Fig. 3 a–c); as at pH 8 where disks are not formed, the 8S peak disappeared when the concentration of the A-protein was decreased below 2 mg/ml. Increasing ionic strength

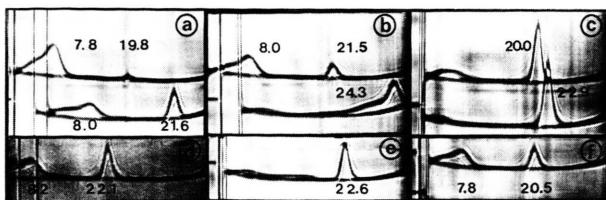


Fig. 3. Schlieren patterns of TMV-protein (5 mg/ml) in potassium phosphate buffer pH 7.32 (a-d) and pH 7.12 (e-f). 59 780 revs/min, photographs taken 40 min (a, b, d-f) and 22 h (c) after attainment of final temperature. a) pH 7.32; 4 °C → 20 °C; ionic strength 0.1 M (upper trace) and 0.2 M (lower trace). b) 4 °C → 24 °C; other conditions as in a). c) 22 h after b). d) 4 °C → 24 °C (30 min) → 20 °C; other conditions as in a). (lower trace). e) pH 7.12; 4 °C → 24 °C (5 min) → 20 °C; ionic strength 0.2 M. f) 4 °C → 24 °C (5 min) → 16 °C; other conditions as in e).

tended to decrease this "critical concentration" for the appearance of the 8S species. The 4S/8S pattern, in the presence of the 20–30S aggregates, was independent of the time and the pathway applied to reach the final temperature (Fig. 3 d–f): the association-dissociation reactions establishing the 4S/8S ratio are "fast", at least as far as sedimentation velocity criteria are concerned.

In Fig. 4, weight average molecular weights,  $\bar{M}_w$ , obtained by light-scattering at different angles of observation, are shown as a function of temperature and protein concentration. Values measured at angles between 75° and 150° were consistent within  $\pm 1.4\%$ , and the ratios of intensities measured at angles of 45° and 135° were generally lower than 1.08, proving large, anisotropic particles not to occur in significant amounts. In the figure,  $\bar{M}_w$  has already been corrected for non-ideality by a common second virial coefficient of  $B = 4.7 \times 10^{-5}$  mol ml g<sup>-2</sup>, as measured for the A-protein of the mutant TMV-A 14 at 20 °C (D. Vogel, in preparation). The A-protein of this mutant sedimented as a nearly symmetrical 8S peak, in conditions where vulgare-A-protein formed the 4S/8S boundary. Thus, the competition between non-ideality and aggregation should be less, and A 14 should be more suitable for an estimate of  $B$ , compared to vulgare, by extrapolation of  $M_{w, obs}^{-1}$  to zero concentration [32]. There should be no problem in using a common  $B$  for both strains, in view of the "fundamentally similar pattern of assembly in all TMV variants" [2], provided their states of aggregation are comparable. This means that the upper limits for  $\bar{M}_w$

shown in Fig. 4 are somewhat more reliable than the lower limits: at the upper limits vulgare protein like A 14 protein sediments mainly as 8S protein (Fig. 1 a) while at the lower limits the 4S entity predominates (Fig. 1 c, f). We do not know the temperature dependence of  $B$  [32] for the 8S aggregates, nor do we know  $B$  for the 4S aggregates. However, since  $B$  is small, its variation is expected to be small, too. The observed  $B$  value is not much higher than the one expected for an uncharged globular protein of the size of a "cyclic heptamer" ( $B = 3.4 \times 10^{-5}$  mol ml g<sup>-2</sup>, cf. [5, 32]). The slight enhancement may be due to a charge effect, but it demonstrates that at moderate ionic strength and moderate net charge the excluded volume does not rise drastically with charge [32, 36]. Likewise, a size effect cannot be excluded (see discussion). At low temperatures and protein concentrations  $\bar{M}_w$  was near to  $5 \pm 1$  subunits, in approximate agreement with ear-

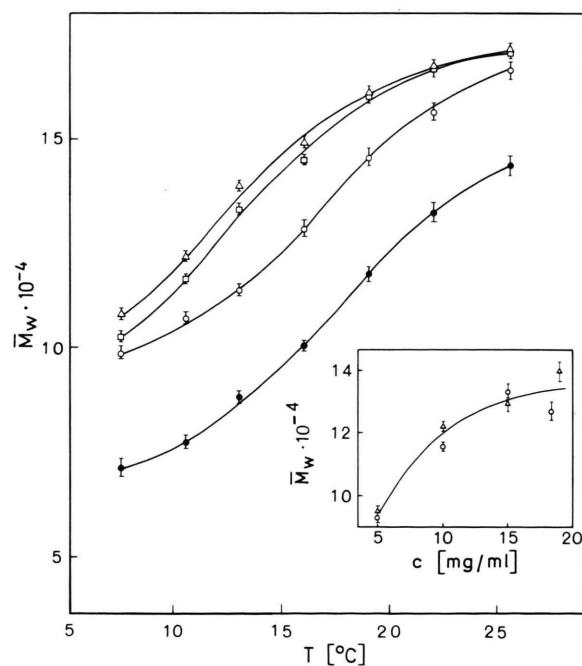


Fig. 4. Weight average molecular weights ( $\bar{M}_w$ ) of TMV-protein as measured by light-scattering, in potassium phosphate buffer solution, ionic strength 0.1 M. Each observation point is the mean of 7 values at different angles of observation, between 75° and 150°, with standard deviations indicated by the error bars.  $\bar{M}_w, obs.$  was corrected for non-ideality by a common second virial coefficient,  $B = 4.7 \times 10^{-5}$  mol ml g<sup>-2</sup> (see text). a) pH 8.0; protein concentrations 5 (●), 10 (○), 15 (□), 20 (△) mg/ml. b, insert)  $T = 10$  °C; pH 8.0 (○) and pH 7.1 (△).

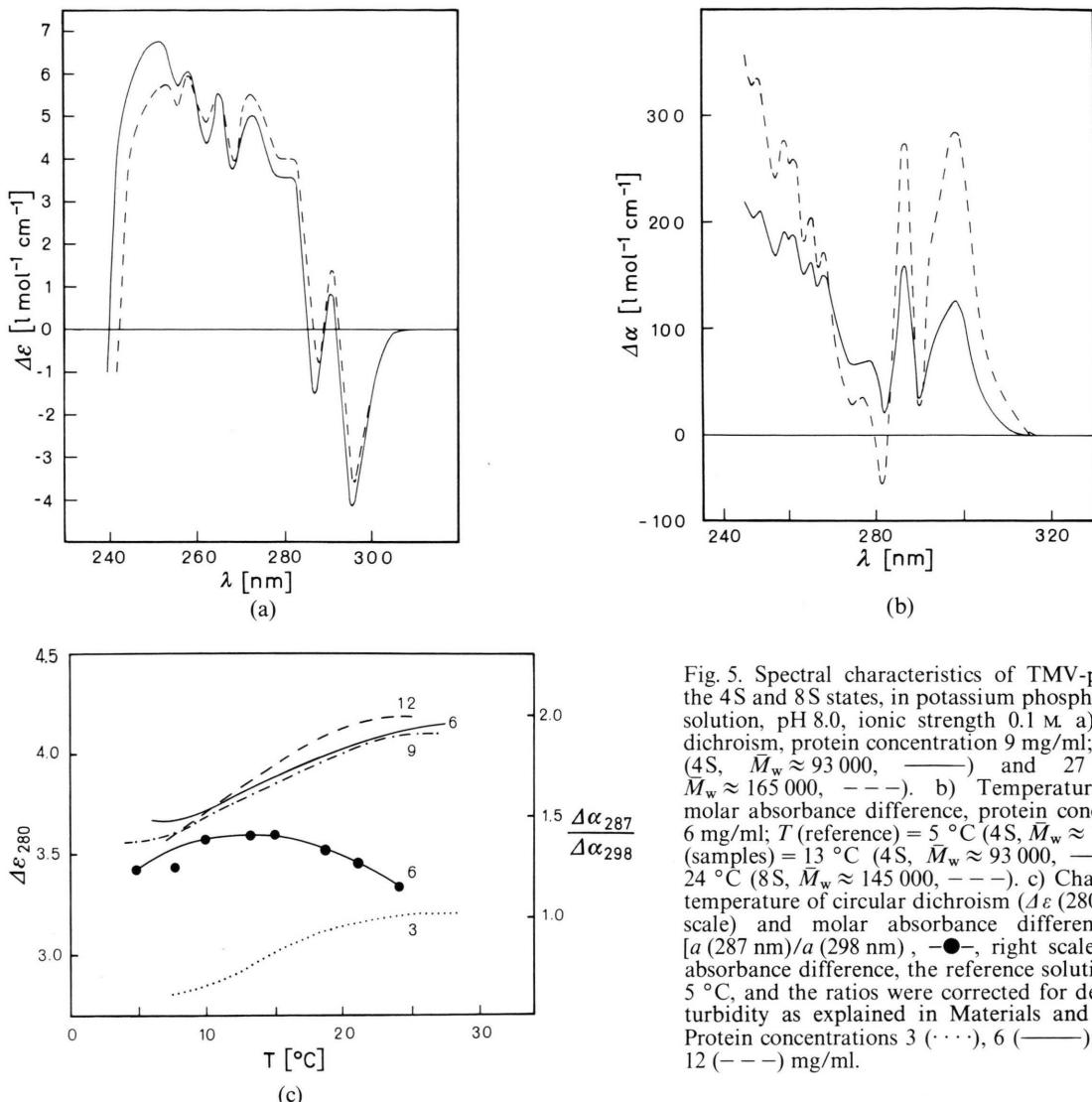


Fig. 5. Spectral characteristics of TMV-protein in the 4S and 8S states, in potassium phosphate buffer solution, pH 8.0, ionic strength 0.1 M. a) Circular dichroism, protein concentration 9 mg/ml;  $T = 4^\circ\text{C}$  (4S,  $\bar{M}_w \approx 93\,000$ , —) and  $27^\circ\text{C}$  (8S,  $\bar{M}_w \approx 165\,000$ , - - -). b) Temperature-induced molar absorbance difference, protein concentration 6 mg/ml;  $T$  (reference) =  $5^\circ\text{C}$  (4S,  $\bar{M}_w \approx 70\,000$ , —) and  $24^\circ\text{C}$  (8S,  $\bar{M}_w \approx 145\,000$ , - - -). c) Changes with temperature of circular dichroism ( $\Delta\epsilon$  (280 nm), left scale) and molar absorbance difference ratios [ $a$  (287 nm)/ $a$  (298 nm), - ● -, right scale]; for the absorbance difference, the reference solution was at  $5^\circ\text{C}$ , and the ratios were corrected for density and turbidity as explained in Materials and Methods. Protein concentrations 3 (· · ·), 6 (—), 9 (---), 12 (— -) mg/ml.

lier studies [23, 27]; at the upper limit,  $\bar{M}_w$  levelled out near an average of 10 subunits (Fig. 4 a). In an experiment (Fig. 4 b) performed with protein from another virus stock than used for Fig. 4 a, the absolute  $\bar{M}_w$  values (at  $10^\circ\text{C}$ ) were slightly higher; again, they levelled out for higher concentrations. Below the temperature where the 20S disk comes into play the differences of the  $\bar{M}_w$ 's at pH 8 and pH 7.1 were insignificant.

In Fig. 5 the spectral properties of TMV protein, as a function of temperature and protein concentration, are summarized. They cannot be evaluated in quantitative terms regarding the partition of the con-

centration of components; however, they show that structural changes parallel the change in aggregation. The change in CD spectra with temperature (Fig. 5 a) is exemplified in Fig. 5 c (left scale) at 280 nm and various protein concentrations. The overall pattern as taken from the CD measurements (Fig. 5 c) is comparable with the light-scattering data presented in Fig. 4 a. The red-shift of the two negative CD-bands at 296 nm and 287.5 nm (Fig. 5 a) which may be attributed to tryptophan-absorption [37] is illustrated more clearly by the temperature-induced absorbance difference (Fig. 5 b): the ratio of the intensities (corrected for  $\varrho$  and  $\tau_\lambda$ ) of

the two positive difference bands at 287 nm and 298 nm (Fig. 5 b) is increased with increasing temperature up to 14.5 °C (at 6 mg/ml); at higher temperature a decrease is observed (Fig. 5 c, right scale). The maximum at 14.5 °C coincides with the temperature where the *s*-value increases at 5 mg/ml (Fig. 2 b); this demonstrates that the change in the state of aggregation is accompanied by a small but significant change in structure.

## Discussion

Though the general occurrence of an "8S" aggregate, first described by Schramm and Zillig [24], had been considered earlier [2, 22, 27], no systematic study of its participation in the A-protein equilibrium thus far was undertaken. The results presented above in fact show that, above distinct critical conditions, an aggregate near 8S constitutes a very normal member of the oligomer distribution; on the other hand, the conditions favouring aggregation used by Durham and Klug [22, 23] were well below these critical conditions, as long as no disks entered the equilibrium. We did not observe significant differences between the Schlieren peaks in phosphate and in pyrophosphate buffer (Figs 1 + 2), nor to the patterns observed earlier in pyrophosphate buffer [30]: the differences supposed were due to the non-comparable solution conditions [22, 23, 27, 30]. This does not exclude the possibility that, at lower pH, the mode and the mechanism of aggregation depend on the kind of buffer used [27].

In phosphate buffer, at pH 7, disks enter the equilibrium between 10 °C and 15 °C [22]; in the study presented here, undertaken at pH 8, no disks at all are present, and the A-protein equilibrium can be followed to higher protein concentrations and temperatures, which in some respect is advantageous: since the pH is no critical parameter in the 4S/8S equilibrium (compare Fig. 1 to Fig. 3, and Fig. 4 b), the results obtained at pH 8 can easily be transferred to pH 7. Into the discussion on the aggregation mechanism near pH 7 thus 4 questions should be introduced which so far were not taken into account: a) are 8S aggregates sufficiently different from 4S aggregates in structure and binding between subunits so that their modes and rates of polymerization can be different? b) if so, what are the relative amounts of 4S and 8S components, in any conditions used for the polymerization studies? c) what are the rate con-

stants for the  $4S \rightleftharpoons 8S$  equilibration? d) can these rates compete with the rates for the polymerization to disks and related compounds, at pH 7, and with the rates with which the temperature, the ionic strength, or the pH values were changed to start this polymerization [6–8, 11]?

As concerns question a) observations were presented earlier [11] which could be explained by a different aggregation behaviour of 4S and 8S aggregates;

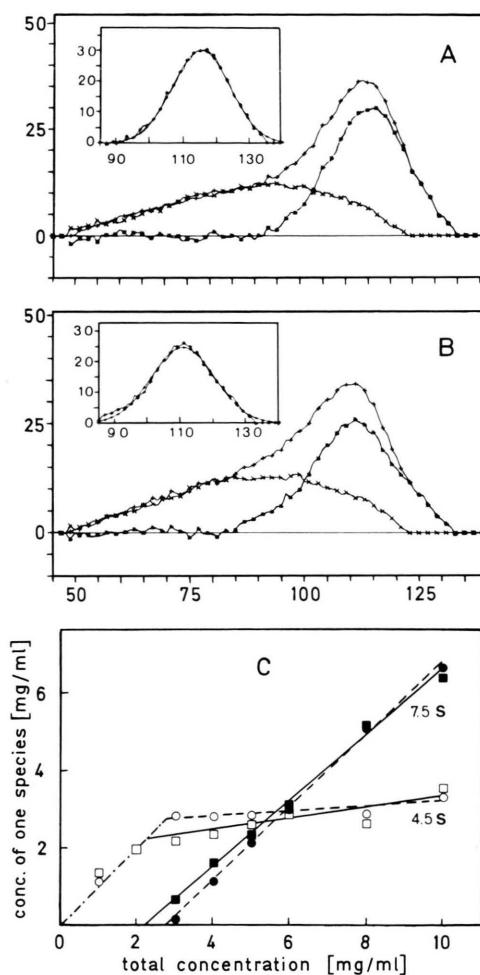


Fig. 6. Computed separation of 4S and 8S components at 26 °C, pH 8.0, ionic strength 0.1 M, in potassium phosphate (a) and sodium pyrophosphate buffer (b). Schlieren curves at 6 mg/ml (+—+—+), 2 mg/ml (x—x—x), and difference curves (\*—\*—\*) are shown (see text). Ten units on the scales correspond to 1 mm on the Schlieren photographs. Insets: Calculated Gaussian curves (+—+—+) compared to the difference curves (◊—◊—◊). c) Amount of 4S (open symbols) and 8S aggregates (closed symbols) as a function of total protein concentration present in potassium phosphate (□, ■) and sodium pyrophosphate buffer (○, ●).

in more detail this will be discussed below, and in a subsequent paper. For question b) the computational procedure described in the experimental section, and exemplified in Fig. 6, gave us a first approximate estimate of the 4S and 8S concentrations in each sample. Schlieren patterns obtained at 2 mg/ml were taken as a reference standard for the trailing boundaries and multiplied by adjustable factors (1.22 for Fig. 6 a and 1.27 for Fig. 6 b) to obtain optimum fit. From this, one may conclude that at 26 °C and a total concentration of 6 mg/ml, the amount in the 4S state was 2.44 mg/ml in phosphate buffer or 2.54 mg/ml in pyrophosphate buffer. As shown by the inserts in Fig. 6 the residual 8S curves can be approximated by single Gaussian peaks. Fig. 6 c, which illustrates the amounts of 4S and 8S material as a function of total protein concentration, suggests that above a critical concentration,  $c_c$ , of about 2.5 mg/ml (very near to the steep increase in S, Fig. 2 a), about 85% of the excess protein is present as 8S aggregates, and that the differences between the two buffers are insignificant.

Certainly this analysis oversimplifies the facts. Possible sources of misinterpretation include interaction of the components during sedimentation [38–40], non-ideality and the Johnston-Ogston effect [41], shifts of the distribution of the 4S two-layer aggregates towards larger particles as a consequence of their slightly increasing concentration [22, 23, cf. Fig. 6 c], and equilibrium displacements, by dilution at the trailing edge of the peak. The apparent homogeneity of the 8S peak, and the probable counteraction of some of the effects suggest that the falsification of the analysis is not very large, but not necessarily negligible. Interaction of the components should be regarded the severest source of error: neglecting diffusion, dilution of a self-associating system should decrease the size and the velocity of the leading peak, with no change at all of the trailing peak until the leading peaks has totally disappeared [38]. However, it was shown [39] that consideration of diffusion and a decrease in monomer molecular weight tends to blur this sharp “bimodality” computed [38]. In our case the monomer molecular weight is still lower than the lowest one used for computation [39], and the gradual distribution of oligomers in our system ( $n = 1, 3, 4, 5, 6, 7 \dots$  [23]), compared to a purely binaric system ( $n = 1, 2; n = 1, 3; n = 1, 4$  [39]), would lead to further blurring. Thus we feel that above the critical conditions the “8S” is the ma-

jer component of the system, but we cannot exclude minor contributions of “8S” below, meaning that the entry of the “8S” may be not so sharp as suggested by Fig. 6 c. For that is some support, by the finding of an “octamer”, at low protein concentrations [12].

If there exist critical concentrations for the formation of 8S from 4S aggregates this resembles phenomena observed in crystallisation, gas-liquid condensation, or micelle formation. As in these cases, near  $c_c$  a very simple relation should hold for the formation constant,  $b$ , of the 8S aggregates in equilibrium with monomers ( $M = 17\,500$ ), of concentration  $c_1$

$$b \cdot c_1 = 1 \quad [42].$$

$c_1$  can be calculated by the equations and the data given by Durham and Klug [23], for any set of critical conditions ( $c_c, T_c$ ) as taken from Figs 2 b, 6 c (phosphate buffer) and 5 c (right scale). Application of this relation, together with the equations of van't Hoff and Gibbs-Helmholtz, to these monomer concentrations  $c_1$  yielded (based on unit activity as standard state [2, 3, 23])

$$\Delta G_{8S} = -8700 \text{ cal (at } 15 \text{ °C)}$$

$$\Delta H_{8S} = +14\,340 \text{ cal}$$

$$\Delta S_{8S} = +80 \text{ e. U.}$$

for the formation of the 8S aggregates. We compare these values to the values obtained by Durham and Klug [23]

$$\Delta G_a = -8\,550 \text{ cal (at } 15 \text{ °C)}$$

$$\Delta H_a = +14\,620 \text{ cal}$$

$$\Delta S_a = +80.4 \text{ e. U.}$$

for the addition of one extra subunit to the end of any two-layer aggregate. The significance of the close correspondence between these sets of data will be discussed below, in connection with structural considerations.

With  $\Delta G_{8S}$  and  $\Delta H_{8S}$  known we can estimate  $c_c$  at any temperature (Table I); with these  $c_c$ 's and the set of thermodynamic data given by Durham and Klug [23], for the two-layer condensation model, we tried to fit the light-scattering data from Fig. 4. There was the problem that, in any condition, even below  $c_c$ ,  $\bar{M}_w$  measured here was lower than given in [23]: protein samples made from virus grown in different laboratories seem to differ slightly in aggregation behaviour (D. Vogel, unpublished); also, dimers were reported to exist [12] which had been omitted

Table I. Critical concentrations ( $c_c$ ) for the formation of three-layer aggregates, and average numbers of subunits per three-layer aggregate, at various protein concentrations ( $c$ ) and temperatures, in potassium phosphate buffer solution, ionic strength 0.1 M, pH 8.

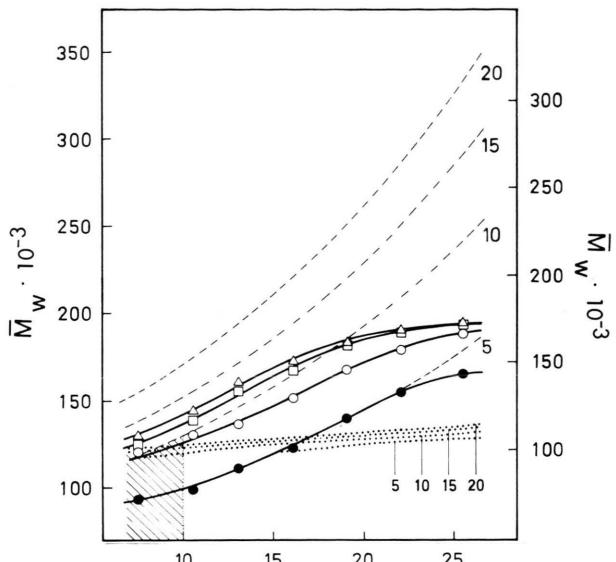
$T$ [°C]	7.5	10.0	15.0	20.0	25.0	26.2
$c_c$ [mg/ml]	9.5	7.6	4.9	3.2	2.2	2.0
$c$ [mg/ml]	Average number of subunits					
5	—	—	7.0	11.0	11.4	11.2
10	7.0	9.0	9.8	11.4	12.0	12.0
15	7.8	8.9	10.8	11.8	11.6	11.6
20	8.0	8.8	10.7	11.5	11.5	11.4

from Durham and Klug's analysis [23]. On the other hand, the  $s$ -values, the overall shape of the sedimentation patterns, and their variations by changing the conditions of the solutions, as measured in different laboratories (Figs 1 + 3, cf. [11, 22, 24, 30, 31]) are reasonable similar, though not totally coincident. Thus, at this stage of knowledge it seemed reasonable to confine the analysis more to the change in the state of aggregation, less emphasizing absolute values. A simple linear scale-shift of the ordinate was sufficient to bring the measured values in coincidence with the calculated ones, if we remained below  $c_c$  (compare Table I to Fig. 7 a, curves at 5 and 10 mg/ml, or to Fig. 7 b, below 10 mg/ml).

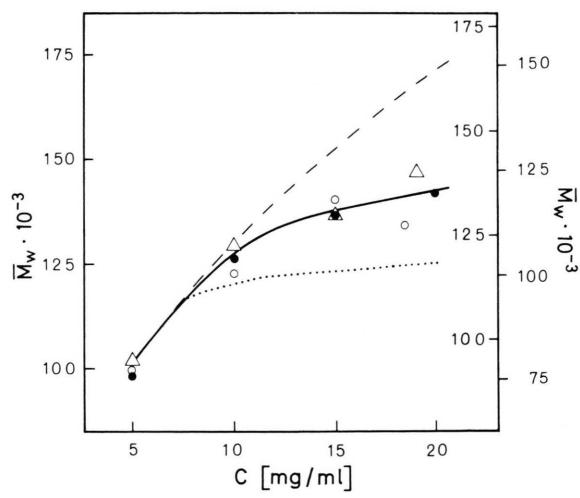
Considering only two-layer aggregates, using Durham and Klug's thermodynamic data [23],  $\bar{M}_w$ , calc. steadily increased with temperature and protein concentration (Fig. 7 a, b, broken lines), and became significantly higher than  $\bar{M}_w$ , obs. (symbols in Fig. 7 a, b), above  $c_c$ .

Next, we adopted Caspar's view [5] that a "cyclic heptamer" would be a stable entity and should sediment near 8S. We introduced this heptamer above  $c_c$  (Table I), using  $c_7 = 0.84 (c - c_c)$  when the concentration of the heptamer is  $c_7 \geq 0$ , if the total protein concentration is  $c \geq c_c$ , and when 0.84 is the slope of Fig. 6 c (phosphate buffer), for the increase of the 8S area above  $c_c$ . The resulting  $\bar{M}_w$ , calc. (Fig. 7 a, b, dotted lines) was much lower than  $\bar{M}_w$ , obs.. A decrease of  $c_7$  by about 75% (changing  $c_c$  or the slope) would be necessary to fit the data, which seemed far beyond the limits of the analysis exemplified in Fig. 6.

In another analysis we abandoned the assumption of an isolated heptamer, instead of this we postulated a series of three-layer aggregates, with the "cy-



(a)



(b)

Fig. 7. Comparison of measured (right scales; symbols as in Fig. 4) and calculated (left scale) weight average molecular weights of TMV-protein. For the ordinate scale shifts see Discussion. a) Effect of temperature, at  $c = 5$ ; 10; 15; 20 mg/ml. (—)  $\bar{M}_w$  calculated with thermodynamic data given in [23]; hatched area: upper range in which measurements [22] for calculations of  $\Delta G$ ,  $\Delta H$ , and  $\Delta S$  [23] were done. (····)  $\bar{M}_w$  calculated, including "cyclic heptamers" above the  $c_c$ 's given in Table I (see Discussion). (—)  $\bar{M}_w$  calculated, including a series of "three-layer aggregates", above  $c_c$  (Table I), with number of subunits per aggregate as shown in Table I. b) Effect of concentration, at  $T = 10$  °C, at pH 8.0 ( $\circ$ ,  $\bullet$ ) and pH 7.1 ( $\triangle$ ).

clic" heptamer as merely the first member: this is in parallel to the extension of Caspar's model [5] made by Durham and Klug [23], who abandoned the assumption of an isolated "cyclic" trimer in favour of a whole two-layer series. The full lines in Fig. 7 a, b fitted best the experimental values, using the thermodynamic data given in [23], the  $c_c$ 's given in Table I, a temperature-independent slope of 0.84, and the average numbers of subunits per three-layer (8S) aggregate were chosen as shown in Table I.

Because of the ambiguities mentioned the numerical values of Table I should be regarded as an approximation. Likewise, we cannot claim that our model comprising two- and three-layer aggregates is unique, as was already stated earlier for the two-layer model [23], but both models seem equally plausible, above and below the critical conditions, and it provides a reasonable possibility for the explanation of several facts. The very existence of a critical concentration is against a single 8S species, but corroborates the existence of a series of larger than two-layer aggregates (*cf.* the theoretical treatment in [42]); already in 1955, Schramm and Zillig [24] estimated a molecular weight of about 260 000 daltons for their vulgare 8S protein, in equilibrium with smaller aggregates; the protein of the mutant A14 tends to sediment with a symmetric 8S peak, its  $\bar{M}_w$  extrapolates to about 254 000 daltons; and we will show that if the A-protein is in the 8S state, its aggregation behaviour will be qualitatively and quantitatively different from that of 4S protein, and that these differences can be explained on a structural basis, assuming three-layer aggregates for the 8S material (D. Vogel, in preparation).

One of the important features of Caspar's model [5] attributed special stability to the "closed" structures like "cyclic" trimers and "cyclic" heptamers, because of their higher bond ( $b$ ) to number-of-subunits ( $n$ ) ratio, together with distortion energy terms, compared to the "linear" aggregates of comparable size. Actually, each three-layer aggregate has a higher ( $b/n$ )-ratio than the corresponding two-layer aggregate, if  $n \geq 7$  (Fig. 8). In the model presented here polymers ( $P$ ) belonging to the two-layer series are represented by  $P_{n \geq 3}^{b=2n-3}$ , and those belonging to the three-layer series represented by  $P_{n=3m+1}^{b=7m-2}$  ( $m \geq 2$ ) would be especially stable ( $n = 7, 10, \dots$ , open circles in Fig. 8), with a closed outer shell, meaning that the subunits at both ends of each particle are arranged convex. The saturation for  $\bar{M}_w$

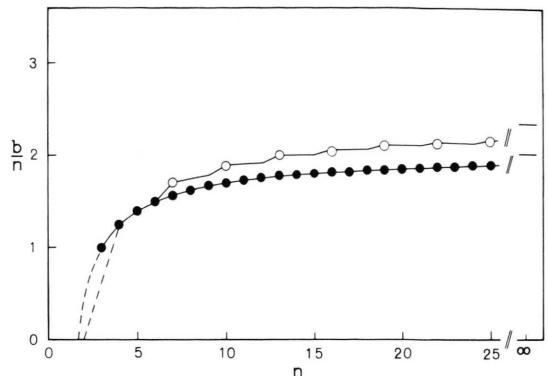


Fig. 8. Number of bonds ( $b$ ) per number-of-subunits ( $n$ ), by extension of the method of Caspar [5], for two-layer ( $\bullet$ —,  $P_{n \geq 3}^{b=2n-3}$ ) and three-layer ( $\circ$ —,  $P_{n=3m+1}^{b=7m-2}$  ( $m \geq 2$ )) aggregates.

near 175 000 daltons (Fig. 4 a), on the average corresponding to 10 subunits on the whole, or to 12 subunits per three-layer aggregate (Table I), may be due to a decrease of the association equilibrium constants, or to an increase of excluded-volume effects, both with particle size. This ambiguity is very common in aggregating systems [43], but the choice for  $B$  as described in Results, and the unfavourable distortion energy term for the middle subunits [5, 23] point at a non-uniformity of the association constants. This, together with sedimentation-specific effects, may act in concert to sharpen the 8S peak, and to simulate its "homogeneity" observed (Fig. 6 b, insets).

Applying Oosawa and Kasai's theoretical treatment of linear and helical aggregations in macromolecules [42] to our model we have to replace their "helical" by our "three-layer" aggregates, and their " $\lambda_{3h}$ ", a nucleus of special steric structure, by another nucleus which, for instance, could be a hexamer, with subunits arranged in three-layers. Addition of a monomer to such a nucleus makes three new bonds [5], with a total gain in free enthalpy,  $\Delta G_{8S}$ , of about -8700 cal. On the other hand,  $\Delta G_a$ , of -8550 cal, corresponds to the formation of only two bonds [23]. That is to say, though three-layer aggregates, compared to two-layer aggregates, are stabilized by an extra bond energy, this stabilization is significantly less than in strictly equivalent bonding. One is tempted to assume that the difference is due to the distortion energy, but the numerical values are not nearly precise enough to justify such a quantita-

tive correlation; the lower the extra stabilization of the three-layer aggregates, the more gradual their entry into the equilibrium would be. On the other hand, the free enthalpy change for the addition of a monomer to the growing helix can be calculated to  $\Delta G_h = -9260$  cal (15 °C) ([44], corrected for printing errors). This close correspondence of  $\Delta G$ 's, for the formation of two-layer ( $\Delta G_a$ ), three-layer ( $\Delta G_{8S}$ ), and helical ( $\Delta G_h$ ) aggregates, both show the delicately poising in equilibrium [44], and gives the strongest support to our analysis.

A pairing, observed between the subunits situated in each upper and lower layer of the disk and related compounds [13, 45, 46], was supposed to stabilize the members of the two-layer A-protein series [23], and to prevent their growth in axial direction. Addition of a third layer should disturb this pairing. A recent report on the 2.8 Å resolution of the disk [15] showed various intra- and, lateral-plus-axial, inter-subunit interactions, including aromatic residues. The axial intersubunit interaction includes the environment of tryptophan 52, which belongs to the buried tryptophan residues [37]; such a residue is affected by the 8S formation, as comes out from the long-wavelength absorption difference bands (Fig. 5 b), and their changes with temperature (Fig. 5 c, right scale) [47]. The environment of tryptophan 52 is completely changed during the transition from disks to helices [15, 37], and the disordering of the pairing may classify the structure of the three-layer aggregates as somehow between disks and helices; this is corroborated by the free enthalpy changes and by the intensity of the CD-spectrum of the 8S aggregates, which is between the intensities of the disks and 4S protein, on one hand, and helices, on the other hand (Fig. 5 a + c, left scale; compare [37]).

From these considerations it is obvious to suggest that, at lowering the pH, 4S aggregates are apt to form directly two-layer disks, and 8S aggregates to

form three-layer disks or helices. Any other mode of reaction must include more complex rearrangement steps. Indications were already presented earlier [11], and evidence will be given in a subsequent paper: in case where the A-protein is nearly exclusively 8S, it tends to make helices, and disks can be made only in special conditions. This is in accord with two very recent reports [48, 49] in which evidence was presented that the initial stages in the formation of overshoot aggregates and helices can be the formation of three-turn helices, sedimenting near 25 S.

Another, more biological significance should perhaps be born in mind: in a sense, three-layer aggregates block the simple, concentration-dependent formation of two-layer disks, by keeping the two-layer aggregates at low concentration. The three-layer aggregates per se need more subunits to build a closed ring structure, with 16 to 17 monomers per turn, than two-layer aggregates. Disks are especially fitted to encapsidate the viral RNA [1, 16–21] or their fragments [50], but, during biosynthesis, a too early protection of the RNA by the coat protein would be undesirable [51].

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