[CONTRIBUTION FROM GIBBS LABORATORY, CHEMISTRY DEPARTMENT, HARVARD UNIVERSITY]

The Preparation and Characterization of Essentially Uniform Tobacco Mosaic Virus Particles

BY HELGA BOEDTKER[1] AND NORMAN S. SIMMONS[2]

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A reproducible method of preparing tobacco mosaic virus (TMV) having essentially uniform length has been developed. Solutions of such samples have been examined by light scattering, flow birefringence, viscosity and sedimentation methods. As a result of these measurements we have verified the relatively narrow size distribution and conclude that TMV has a molecular weight of 39.0 ± 1.2 million, a length of 3000 Å. and an effective diameter of 149 Å. It appears that the length of the hydrodynamically equivalent ellipsoid, about 3600 Å., is significantly greater than the actually particle length of 3000 Å. as a result of the inability of the ellipsoidal model to represent accurately a cylinder.

Tobacco mosaic virus (TMV) has been the subject of numerous investigations since Stanley's original isolation and crystallization of the virus almost a quarter of a century ago.[3] Recently there has been a renewed interest in the TMV particle largely because of the results obtained by Fraenkel-Conrat and Williams[4] on the one hand, and by Gierer and Schramm[5] on the other. The former authors reported that TMV could be split into its two components, protein and ribose nucleic acid (RNA), then recombined or reconstituted into a particle which not only bore a remarkable resemblance to the original virus but which proved to be infective. Shortly thereafter both Gierer and Schramm and Fraenkel-Conrat showed that the infectivity resided in the RNA component alone.[5,6] Hence for the first time it was possible to prepare highly purified RNA solutions which retained at least part of the biological activity. An accurate determination of the size and shape of the infective molecule, as well as the number of these per virus particle, have of course become important problems. However, the solution of these problems requires a more reliable means of preparing uniform TMV, and a more reliable characterization of the virus particle than has heretofore been available. This has been the aim of the present investigation.

There have been many previous investigations of the size and shape of TMV, both in the solid state and in solution. Indeed, the X-ray diffraction measurements first of Bernal and Fankuchen[7] and later of Franklin[8] and Caspar[9] have established that the virus has a diameter of 152 Å. And the numerous electron microscope investigations, in particular those by Williams,[10] indicated that the length of the fundamental virus particle is probably close to 3000 Å. The results of physical measurements on TMV solutions are less certain, however. Although a sedimentation constant of about 185 S. had been reported by several investigators, published values of the intrinsic viscosity, the flow birefringence lengths and molecular weight varied considerably. The discrepancy was largely due to the difficulty of preparing unaggregated virus solutions. And in those cases in which the molecular weight was measured directly (*e.g.*, by light scattering), no attempt was made to determine the degree of homogeneity of the virus solutions examined. Since most TMV preparations are aggregated to some extent, it must be assumed that a TMV solution is polydispersed unless evidence is given to the contrary. Thus far only two investigators have reported obtaining highly uniform preparations,[11,12] and only one of these[12] studied the preparation by several different physical chemical methods. The results described below are in substantial agreement with those obtained by Watanabe,[12] differing only by the wider interpretation made possible by the additional measurements made in this study.

The difficulty of obtaining nearly monodisperse preparations of TMV is well known to all who have worked with this virus. Indeed, even when an occasional monodisperse preparation was obtained, the reason for its greater degree of uniformity was not known, and the preparation could not be reproduced.[12,13] The first problem, therefore, was to find a preparative method which would yield relatively monodisperse TMV in solution and which could be reproduced at will. After a brief description of the production of the virus, and of the physical chemical methods used, this report will be divided into two parts: the first will deal with the method of preparation, its reproducibility and the stability of the resultant virus solutions; the second will present a detailed characterization of the TMV particle in solution as determined by sedimentation, viscosity, flow birefringence and light scattering measurements.

Experimental Methods

Production of TMV.—Young White Burley tobacco plants were infected with a common strain TMV (U-1) in potassium phosphate buffer, *p*H 7.3. Concentrations of about 4×10^{-6} g./cc. were used. The original strain was kindly supplied by Dr. Don Caspar (Yale University). Leaves were infected by rubbing lightly with cheesecloth soaked in the virus solution. Some three weeks after infection, the leaves were harvested and immediately placed in a deep freeze (−20°).

(1) Public Health Service Postdoctoral Fellow, 1955–1957.

(2) On leave from the Atomic Energy Project, University of California at Los Angeles.

(3) W. M. Stanley, *Science*, **81**, 644 (1935).

(4) H. Fraenkel-Conrat and R. C. Williams, *Proc. Nat. Acad. Sci.*, **41**, 690 (1955).

(5) G. Schramm and A. Gierer, *Nature*, **177**, 702 (1956).

(6) H. Fraenkel-Conrat, THIS JOURNAL, **78**, 882 (1956).

(7) J. D. Bernal and I. Fankuchen, *J. Gen. Physiol.*, **25**, 111, 147 (1941).

(8) R. E. Franklin and A. Klug, *Biochim. Biophys. Acta*, **19**, 403 (1956).

(9) D. Caspar, *Nature*, **177**, 475 (1956).

(10) R. C. Williams and R. L. Steere, THIS JOURNAL, **73**, 2057 (1951).

(11) H. K. Schachman, *ibid.*, **73**, 4808 (1951).

(12) I. Watanabe and Y. Kawade, *Bull. Chem. Soc. Japan*, **26**, 294 (1953).

(13) H. K. Schachman, private communication.

Concentration Determinations.—Concentrations were determined by measuring the optical density of the solutions at 265 mμ using 1 cm. cells in a Beckman Model DU spectrophotometer. The extinction coefficient in g./100 cc. is 30.6. This is based on a dry weight determination of the concentration of a 1% solution of TMV which had been exhaustively dialyzed against distilled water. An aliquot of TMV was frozen and lyophilized dry for 24 hr. in a tared vessel. The vessel was then transferred to an Abderhalden pistol where it was dried for 24 hr. over P_2O_5 at room temperature under high vacuum. The temperature was then raised to 56° with refluxing acetone for 24 hr. Finally the temperature was raised to 110° with refluxing toluene for 24 hr. After the pistol had been allowed to cool to room temperature, dry air was admitted and the vessel quickly stoppered, removed and weighed after the outside glass surface had reached equilibrium with the atmosphere.

Intrinsic Viscosity.—TMV solutions exhibited a definite gradient dependence when the viscosity was measured in a special multigradient viscometer[14] covering a gradient range from 25 to 150 sec.$^{-1}$. For this reason, the intrinsic viscosity was determined by measuring each concentration at a series of gradients (usually four), plotting the log of the specific viscosity against gradient and extrapolating to zero-gradient. The resultant value of the reduced specific viscosity thus obtained was found to be independent of concentration over the concentration range measured (0.6 to 0.12 g./100 cc.).

Flow Birefringence.—The extinction angle, χ, and the birefringence, Δn, were measured with a Rao Birefringence of Flow Instrument[15] over a gradient range from 50 to 5000 sec.$^{-1}$ in a room thermostated at either 20 ± 2 or 25 ± 2°. χ was found to be independent of concentration between 0.5 and 0.05 g./100 cc.

Sedimentation Constant.—Sedimentation was observed in a Spinco Model E Ultracentrifuge both at 5400 and at 17250 r.p.m. Measurements at the higher speed were performed over a concentration range of 0.06 to 0.6 g./100 cc. using the schlieren optics and as low as 0.012 g./100 cc. using ultraviolet absorption optics. The sedimentation constant was computed and corrected to that for water at 20° as described previously.[16]

Light Scattering.—Light scattering measurements were made in a slightly modified Brice–Speiser photometer, as described previously.[14,17] Only erlenmeyer flask-shaped cells were used. Solvent was clarified by filtering through an ultrafine sintered glass filter. TMV solutions were freed from dust and extraneous matter by brief centrifugation (about 30 min.) in a Spinco Model L Ultracentrifuge at low speeds (7000 r.p.m.). In this way, dust free solutions were obtained while the concentration of the solution was reduced less than 10% due to sedimentation. A concentration range from 6 to 30 × 10^{-6} g./cc. was covered.

Refractive Index Increment.—The specific refractive index increment, dn/dc, was determined with a Brice–Speiser differential refractometer. Solutions were first dialyzed against distilled water, their concentration determined by dry-weight, and dilutions made up gravimetrically. A value of 0.194 ± 0.001 was found for TMV in water at a wave length of 4360 Å.

Purification Procedure

Soon after the original isolation of TMV by Stanley,[3] Wyckoff demonstrated the advantages of differential centrifugation over chemical methods in preparing relatively uniform solutions of TMV,[18,19] and this method has been used almost exclusively ever since. The uniform preparations reported by Schachman and Watanabe were both prepared in this way, the only differences being in the choice of solvent. Wyckoff reported that the uniformity of his preparations was markedly improved when distilled water was used throughout the preparations. Schachman, however, observed aggregation in solutions prepared from water alone and reported that the best results were obtained when TMV was twice centrifuged from 0.1 *M* phosphate buffer,[20] then from water, while Watanabe followed the 0.1 *M* cycles with 0.01 *M* phosphate buffer. Because of the success of these earlier preparations, the purification steps were followed in detail. Unfortunately, the product obtained in each case exhibited two peaks sedimenting at 190 and 240 S., respectively, when viewed in the ultracentrifuge. After these initial failures, some of the preparative details were altered and the purification tried again. Each step was followed by studying the sedimentation pattern in the ultracentrifuge. This procedure revealed that the formation of the faster sedimenting component was actually taking place during the purification. Similar conclusions have been reached by Reichmann[21] who succeeded in examining the crude sap by flow birefringence and found nearly monodisperse solutions which became polydispersed after several cycles of high and low speed centrifugation.

Once we were convinced that aggregation was occurring during the preparation itself, a series of remedies (adding a chelating agent, working at higher *p*H) were tried. As a result, a successful preparative method evolved, the details of which are given below.

The frozen[22] leaf pulp (about 300 g. at a time) was pulverized with a mortar and pestle in the cold room. Four grams of $Na_2HPO_4 \cdot 7H_2O$ was added to each 100 g. of pulp to maintain the *p*H of the crude sap near neutrality during and after thawing. When the pulp was thawed thoroughly, one part of Johns-Manville Hyflo Super-Cel was added to 20 parts of pulp. The sap was then separated from the solid debris by centrifuging in a basket rotor in an International Centrifuge (Model BE) using strips of Schleicher and Schuell 404 filter paper. The brown juice was collected in iced erlenmeyer flasks. About 600–700 cc. of juice was obtained from 1000 g. of wet pulp. A series of low and high speed centrifugations followed. The low speed step consisted of a 20 minute centrifugation in a Sorval SS angle centrifuge at 8200 r.p.m. The supernatant from the latter was then centrifuged for 150–180 minutes (depending on the approximate virus concentration) at 18750 r.p.m. in the no. 21 rotor of the Spinco Model L ultracentrifuge. The supernatant was then discarded and the pellet was covered with fresh solvent and allowed to dissolve slowly by standing overnight in the cold room. Usually three cycles were sufficient to produce pure, colorless virus solutions. The critical aspect of the purification procedure seems to be the solvent used. Our best results were obtained by taking up the pellet at the end of the first cycle in a solvent that was 0.1 *M* sodium phosphate buffer, and 0.01 *M* Versene, *p*H 7.1, and at the end of the second and third cycles in distilled water. The effect of adding distilled water was to produce a solution having a *p*H of 7.65 ± 0.05. The attainment of this *p*H at low ionic strength is apparently sufficient to prevent dimer formation and to dissociate those formed as a result of the first purification cycle.

The fact that TMV does aggregate during the first cycle, when it is dissolved in a relatively high ionic strength buffer, was detected when we followed each purification step by measuring the flow birefringence and the sedimentation constant. At the end of the first purification step, the TMV exhibited a normal and a fast sedimenting component and had a flow birefringence length of 5200 Å. (at 1100 sec.$^{-1}$). After this solution was spun down and the pellet dissolved in distilled water, however, the sedimentation diagram showed only a single sharp peak and the flow birefringence length had dropped to 3600 Å. (at 1100 sec.$^{-1}$). As only a small amount of virus was lost during this step, we believe that the dimer was converted to the monomer when the TMV was dissolved in distilled water. If, however, the virus pellet was allowed to dissolve in phosphate buffer (either 0.1 or 0.01 *M*) a second time, an irreversible aggregation occurred, and the faster sedimenting component

(14) A. Holtzer, H. Benoit and P. Doty, *J. Phys. Chem.*, **58**, 624 (1954).

(15) J. T. Edsall, A. Rich and M. Goldstein, *Rev. Sci. Instr.*, **23**, 695 (1952).

(16) H. Boedtker and P. Doty, THIS JOURNAL, **78**, 4267 (1956).

(17) H. Boedtker and P. Doty, *J. Phys. Chem.*, **58**, 968 (1954).

(18) R. W. G. Wyckoff, J. Biscoe and W. M. Stanley, *J. Biol. Chem.*, **117**, 57 (1937).

(19) R. W. G. Wyckoff, *ibid.*, **121**, 219 (1937).

(20) H. K. Schachman and W. J. Kauzmann, *J. Phys. Colloid Chem.*, **53**, 150 (1949).

(21) E. Reichmann, private communication.

(22) The possibility that freezing the leaves caused the virus to aggregate was considered and tested. One batch of leaves was divided into two parts immediately after harvesting. Half the leaves were frozen, the other not. Identical results were obtained, leading us to conclude that freezing does not affect the uniformity of the resultant solution.

remained even after the virus was spun down again and then dissolved in water.

Because successive centrifugations from water caused the *p*H to drop to below 7, resulting in some aggregation, we adopted the procedure of buffering the TMV after it had dissolved in water. It is in this respect mainly that our method differs from that outlined by Schachman.[20] For example the solution was made 0.01 *M* Versene, *p*H 7.55, before the third and 0.01 *M* sodium phosphate, *p*H 7.5, before the fourth ultracentrifugation step. Whenever possible, the fourth cycle was omitted, and the TMV solution stored in the 0.01 *M* phosphate buffer until used.

The criteria for uniformity include sedimentation diagrams (Fig. 1) and flow birefringence measurements. The latter was found to be by far the most sensitive to small amounts of aggregated particles. In Fig. 2 the gradient dependence of χ is shown for five different TMV preparations, prepared at different times from different batches of leaves. The data include all the preparations which have been carried out since the steps outlined above were worked out. The substantial agreement we have obtained on successive preparations, as shown by Fig. 2, seems clear proof that it is now possible to prepare reproducible and relatively uniform TMV solutions.

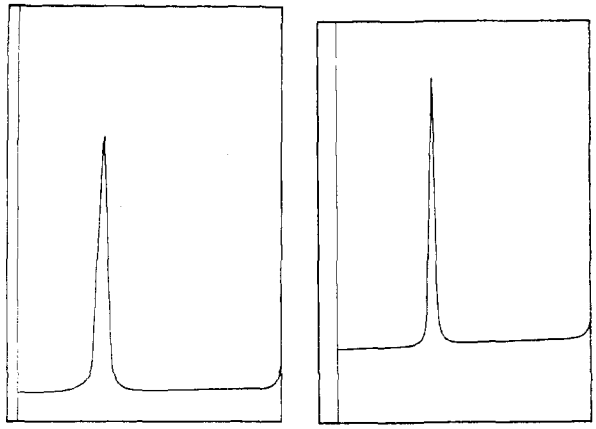

Fig. 1.—Sedimentation diagrams of TMV solutions: (a) sample A-4, 0.24 g./100 cc., 5400 r.p.m., 229 min.; (b) sample I, 0.085 g./100 cc., 17250 r.p.m., 20 min.

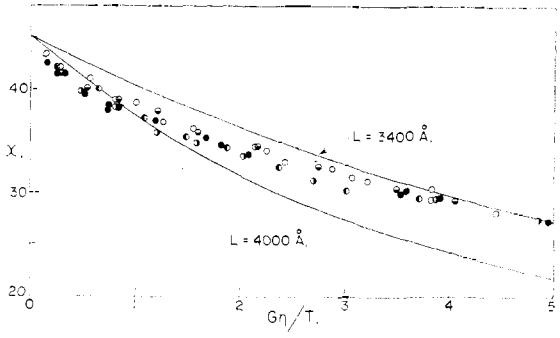

Fig. 2.—Extinction angle as a function of $G\eta/T$: ● A-,4 ◑ D, ◐ E, all in 0.01 *M* phosphate, *p*H 7.1; ◒ FG in 0.01 *M* versene, *p*H 7.5, O I in 0.02 *M* phosphate, *p*H 7.55.

Moreover the preparations described proved remarkably stable. Indeed no change could be detected in either the sedimentation diagram or the flow birefringence data on a solution measured immediately after preparation and after six months storage in 0.01 *M* phosphate buffer, *p*H 7.5, at 5°.

The Size and Shape of Uniform TMV Particles

Although TMV solutions have been subjected to almost every possible physical chemical measuring device, the most complete examination of TMV to date[12] did not include either a light scattering measurement of the molecular weight or flow birefringence data, while the most complete flow birefringence investigation[23] and light scattering measurements[24,25] were performed on samples of questionable uniformity. The following data were obtained therefore, in the hope that the combination of tools used would lead to a more precisely defined picture of TMV.

Light Scattering.—The light scattering examination of two different TMV preparations, A-4 and I, produced almost identical results as shown in Table I.

TABLE I

LIGHT SCATTERING DATA ON TMV

Preparation	Mol. wt., million	Length (Å.)
A-4	38.2	3200
I	40.8	3200

These results were obtained from Zimm-type plots such as the one shown in Fig. 3 and by fitting the limiting slope of the experimentally obtained

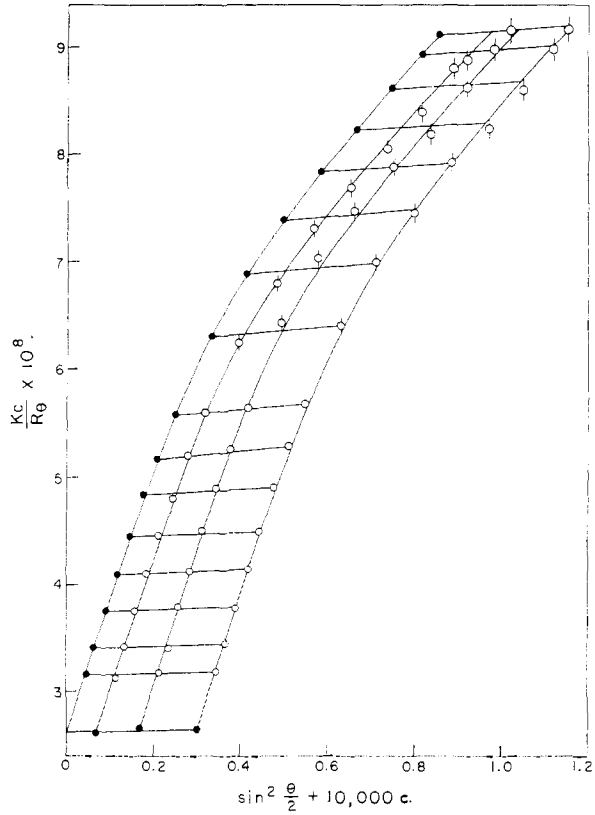

Fig. 3.—Light scattering of TMV solutions: Sample A-4, 0.01 *M* phosphate buffer, *p*H 7.1; M = 38,200,000, L = 3200 Å.; O, experimental data, ●, extrapolated points.

reciprocal particle scattering function with that calculated for a rod of definite length (Fig. 4).[16] The molecular weights obtained from light scattering correspond to a weight average while the

(23) J. W. Rowen and W. Ginoza, *Biochim. Biophys. Acta*, **21**, 416 (1956).

(24) G. Oster, P. Doty and B. H. Zimm, THIS JOURNAL, **69**, 1193 (1947).

(25) G. Oster, *J. Gen. Physiol.*, **33**, 445 (1950).

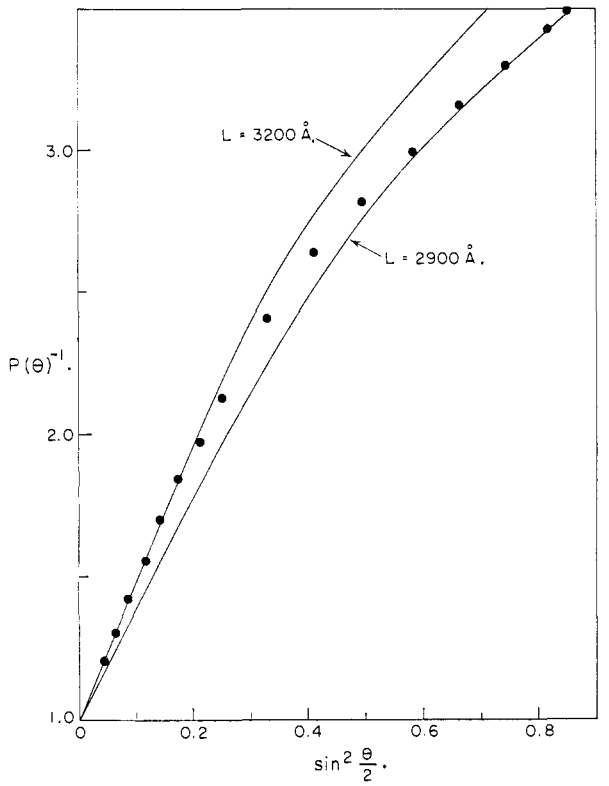

Fig. 4.—Reciprocal particle scattering factor of TMV solution: sample A-4, ● experimental points, ——— theoretical scattering curves.

lengths are a $[z(z + 1)]^{1/2}$ average.[26] The relatively good fit of the experimental data to the theoretical curve for L = 3200 Å. leaves little doubt that TMV is a rigid rod-shaped particle. The slight deviation at high angles where the data fits the L = 2900 Å. can only be interpreted as the result of a small amount of polydispersity. It has been shown that the reciprocal scattering curve for a polydispersed collection of molecules will always lie below that for a monodispersed system of equal length.[27] The scattering is heavily weighted by the longer particles at lower angles but these make a relatively small contribution at high angles. Other deviations from rod-like behavior (*e.g.*, flexibility) would cause the high angle points to lie *above* rather than below the theoretical curve. The fact that the difference between the high and the low angle data is no more than 10% indicates that the size distribution is quite narrow. Additional evidence for this is presented below and in the discussion at the end.

Although the light scattering results reported above are in apparent agreement with those previously reported,[24,25] the facts are otherwise. The value of dn/dc used in the former work was much lower than that measured and used here. Making this correction would lead to a much lower molecular weight.

Flow Birefringence.—Having determined the length and weight of TMV by light scattering, it is interesting to see how the latter values agree with those determined from hydrodynamic measurements. As we mentioned in the first part of this report, the most sensitive measure of polydispersity of TMV solutions is the data obtained from flow birefringence. As was shown in Fig. 2, all five of the preparations which we examined had some particles with lengths greater than 3400 Å., that is, as the gradient approaches zero, the experimental values fall on or below the theoretical curve corresponding to L = 4000 Å., while at gradients of 1000 sec.$^{-1}$ ($G\eta/T$ = 3.46) or greater, the experimental curve coincides with the theoretical curve for L = 3400 Å. To illustrate this point more clearly, lengths have been calculated from the data using the Peterlin equation

$$a = [3kT(-1 + 2 \ln 2a/b)/16\pi\eta_0\theta]^{1/2}$$

where a is the length of the semi-major axis, k is the Boltzmann constant, T the absolute temperature, a/b the axial ratio (obtained from viscosity measurements), η_0 the viscosity of the solvent and θ the rotary diffusion constant. As readily can be seen in Fig. 5, the lengths of two different TMV

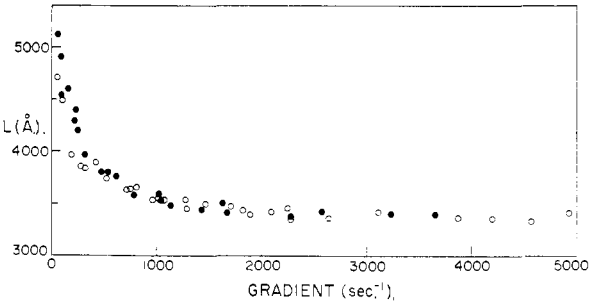

Fig. 5.—Flow birefringence lengths of TMV solutions: ● sample A-4, 0.01 M phosphate buffer, pH 7.1; O sample I, 0.02 M phosphate buffer, pH 7.55.

preparations are essentially identical over the entire gradient range. Indeed a value of L ± 3500 ± 140 Å. will fit all the data from 600 to 5000 sec.$^{-1}$. The appearance of longer lengths at gradients lower than 600 sec.$^{-1}$ means that a very few longer species were present even in our best preparations. As similar results have been obtained on the crude sap,[21] we can only conclude that occasional linear aggregates are present in the leaf itself.

It should be noted that while the length obtained from this investigation (3500 Å.) is in good agreement with the average value (3350 ± 250 Å.) previously obtained from measurements at 5000 sec.$^{-1}$ on eight different strains,[23] all these lengths are substantially longer than those obtained from electron microscopy. Indeed the high gradient flow birefringence lengths, which are comparable to a weight average, are longer than the much more highly weighted average lengths obtained from light scattering. Thus polydispersity cannot explain the high values obtained from hydrodynamic measurements in both this and other investigations.

Intrinsic Viscosity.—The intrinsic viscosity was measured on one preparation (A-4) in 0.01 M phosphate buffer, pH 7.1, and a value of 0.367 ± 0.006 (in units of 100 cc./g.) was obtained. The similarity of the preparations as indicated by light

(26) P. Doty and A. Wada, to be published in THIS JOURNAL.
(27) M. Goldstein, *J. Chem. Phys.*, **21**, 1255 (1953).

scattering and flow birefringence results ensure that this value is a representative one. Although previous results for the intrinsic viscosity have varied from 0.25 to greater than 0.6, those reported on relatively monodispersed solutions were 0.32[20] and 0.365,[12] in good agreement with that reported here. Since both past and present investigations leave little doubt that TMV is a rigid rod, it is possible to use the intrinsic viscosity to determine the axial ratio using the Simha equation[28] if the density and % hydration are both known. Assuming the latter to be zero and using the partial specific volume, 0.73,[29] one calculates an axial ratio of 23.8. However, this value does not correspond to what one would calculate from the X-ray diameter of 152 Å. and electron microscope length of 3000 Å. Such a comparison may not be valid, however, because the viscosity axial ratio is obtained by assuming the TMV particle to be an ellipsoid of revolution even though its geometrical shape more closely resembles that of a right cylinder, and there is no *a priori* reason why the axial ratios of the two should be exactly equal.

Sedimentation Constant.—Whereas flow birefringence measures the length, and viscosity the axial ratio, the diameter of the TMV particle can be obtained from the sedimentation constant. In Fig. 6, the reciprocal of $s^{\circ}_{20,w}$ is plotted as a function of concentration. From the intercept one obtains a value of 188 S. for the sedimentation constant at infinite dilution. This is in excellent agreement with the values obtained by Lauffer[29] and others. The length of the semi-minor axis, b, can be calculated from Perrin's equation

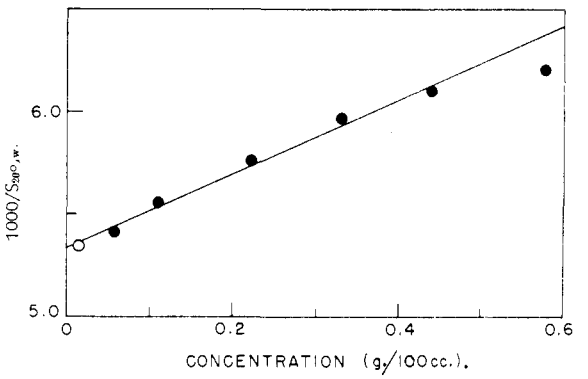

Fig. 6.—Reciprocal sedimentation constants of TMV solutions: ● sample A-4, schlieren optics; ○, sample 1, ultraviolet absorption optics.

$$\frac{s^{\circ}_{20,w}\eta_0 v}{1 - \bar{v}\rho} = \frac{2}{9} b^2 \ln 2\frac{a}{b}$$

Using $v = 0.73$, $a/b = 23.8$, in this equation one obtains a value of $2b$ of 154 Å. When combined with the axial ratio, this would give us a length of 3660 A. for the ellipsoid. As this value agrees with that obtained from flow birefringence, our results are internally consistent. But although the value of $2b$, which is identical with that reported by other investigators, agrees with the X-ray diameter, such a comparison must be questioned because one would expect the cylindrical diameter to be smaller than that of an equivalent ellipsoid.[16,30] Fortunately no such ambiguity is involved in the molecular weight, which can be calculated directly from $s^{0}_{20,w}$ and (η)[31] and is 37.9 million, checking the light scattering molecular weight nicely.

Discussion

Thus far evidence for the relative uniformity of the TMV preparations discussed in this report has been based upon physical chemical data. Before discussing these results in more detail however, mention must be made of the length and length distribution obtained from electron microscope measurements. Careful measurements obtained by Dr. Hall on preparation I indicate that the number average length is 3020 Å. and that over 85% of the 201 particles measured had lengths between 2800 and 3200 Å.[32] This corroboration of a monomeric TMV length of 3000 Å. is particularly important because Williams' value has recently been questioned on the basis of the calibration of the polystyrene spheres.[23] Dr. Hall's results would indicate that Williams' calibration had been correct. In addition to settling the uncertainty that had arisen about the electron microscope length of TMV, Hall's results are important because, unlike Williams' study which was made on the crude sap,[10] this investigation was made on a purified preparation. We therefore have direct evidence that our preparations have an unusually narrow size distribution. Moreover, physical chemical measurements were made on the identical solutions which were examined in the electron microscope, and we are therefore in an excellent position to make a direct comparison between the results obtained on the two.

This investigation has shown that the molecular weight of TMV, determined by light scattering and by sedimentation-viscosity, is 39.0 ± 1.2 million. This agrees nicely with recent chemical and X-ray determinations which have placed the weight of a particle 3000 Å. long at 38.4 to 40.7 million, depending on whether one takes the molecular weight or a subunit as 17,000 or 18,000.[33] It is also in good agreement with the values reported by Watanabe[12] (M = 42 million) and by Schramm and Bergold[34] (M = 40.7 million) based on sedimentation and diffusion measurements. The fact that these investigators using different methods have obtained the same value for the molecular weight seems to leave little doubt about the correct molecular weight of TMV. We can offer no simple explanation for the 25% higher result obtained by Williams[35] especially since it now appears that his calibration was correct.

The question of the molecular weight having been discussed, we now take up a comparison between the dimensions of TMV obtained by different

(28) R. Simha, *J. Phys. Chem.*, 44, 25 (1940).
(29) M. A. Lauffer, THIS JOURNAL, **66**, 1188 (1944).
(30) P. Doty, A. M. Holtzer and J. H. Bradbury, *ibid.*, **78**, 947 (1956).
(31) H. A. Scheraga and L. Mandelkern, *ibid.*, **75**, 179 (1955).
(32) C. E. Hall, *ibid.*, **80**, 2556 (1958).
(33) R. E. Franklin, A. Klug and K. C. Holmes, "The Nature of Viruses," Little, Brown and Company, Boston, Mass., 1957, pp. 39–51.
(34) G. Schramm and G. Bergold, *Z. Naturf.*, **2b**, 108 (1947).
(35) R. C. Williams, R. C. Backus and R. L. Steere, THIS JOURNAL, **73**, 2162 (1951).

methods. First the light scattering length of 3200 ± 160 Å. agrees quite well with the electron microscope length of 3020 Å. It is somewhat misleading, however, to compare these two figures because while the latter is a number average, the former is a $[z(z+1)]^{1/2}$ average and will thus be extremely sensitive to the presence of any particles longer than the mean. By assuming that the electron microscope distribution was truly representative of the distribution of particle sizes in solution, we were able to calculate an electron microscope $[z(z+1)]^{1/2}$ average length of 3340 Å. This value also agrees well with the light scattering length and indicates that, in the present case, the deviations from uniformity were so minor that the error introduced by polydispersity was within the ±5% precision of the measurement. It is because of this precision, however, that we have not gone to the additional effort of taking into account the effect of finite thickness of the rods on the determination of length from the angular distribution. The method for doing this is available,[36] but it is a very small correction when the axial ratio is greater than 5 and need not be considered until more precise measurements are possible.

If we now compare the light scattering and electron microscope length with that obtained from hydrodynamic measurements, we find the agreement is not good. The high gradient flow birefringence length (3500 Å.) and that obtained from a combination of sedimentation and viscosity (3660 Å.), while they agree with one another, are appreciably larger than 3000 Å. In particular, the flow birefringence length which corresponds to a weight average length should certainly be shorter than the light scattering length. In fact, the weight average length computed from the electron microscope distribution is only 3120 Å. Since polydispersity alone cannot explain the discrepancy between the lengths obtained from the hydrodynamic measurements and those obtained from either light scattering or electron microscopy, the explanation must lie elsewhere. We believe the answer lies in the failure of the ellipsoid model to accurately represent a cylinder. In other words, the major axis of the hydrodynamically equivalent ellipsoid is not precisely equal to the length of the TMV cylinder.

Next we wish to discuss the diameter of the TMV particle. Density distributions obtained from X-ray studies tell us that TMV is a cylinder with a helically grooved surface and a central hole of radius 20 Å. Part of the virus surface lies 91 Å. from the central axis[37] while other surface regions are only 65 Å. from the center.[30] In the dry state, however, the center-to-center separation of the particles is 152 Å., and we take this to approximate the average or "effective" diameter determined by X-ray studies. Such an average diameter can be calculated for a smooth cylindrical shell having a molecular weight of 39 million and a length of 3000 Å. if the density is known. Assuming that the correct value of the density is 1.31,[33] we calculate a diameter of 149 Å. If instead we had used the reciprocal of the partial specific volume for the density, namely, 1.37, the calculated diameter would have been 147 Å. Consequently the value obtained is only slightly sensitive to the precise value of the density. And it is now clear that the calculated diameter is significantly smaller than the maximum diameter of TMV of 182 Å. The volume of a cylindrical shell having a diameter of 182 Å. is 1.5 times greater than that of one having a diameter of 149 Å. This means that TMV occupies only two thirds of the space inside its cylindrical envelope. Assuming that the remaining volume is filled with water, we calculate an internal hydration of 0.38 g. of water per gram of TMV. It should be emphasized that this internal hydration does not affect the molecular weight measured by light scattering or by sedimentation and viscosity.[31] Moreover, X-ray diffraction data have shown that the wide angle spacing of TMV diagrams is insensitive to the water content.[7] Therefore both the length and the diameter of the particle must be the same regardless of whether the virus is in solution or totally dry.

The cylindrical diameter also can be calculated from the sedimentation and viscosity data if we equate the volume of a 3000 Å. long cylinder to that of the hydrodynamically equivalent ellipsoid. Using a density of 1.37, we have already calculated the latter to have the dimensions of 3660 by 154 Å. If instead we use a density of 1.31, we obtain the dimensions of 3730 by 157 Å. The cylinder equivalent to the latter ellipsoid would have a diameter of 144 Å., which is certainly in good agreement with the value calculated from the light scattering and electron microscope data. However, this simply means that the volume of the two geometric solids is the same within experimental error and verifies the concurrence of the results obtained by different methods. It should be noted, however, that *neither* the length nor the diameter of the TMV particle could have been obtained directly from hydrodynamic measurements alone. In conclusion we find that the helically grooved and hydrated TMV particle can be approximated by a hydrodynamic ellipsoid of 3700 by 157 Å. but is more accurately represented by a smooth cylinder of 3000 by 149 Å.

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NOTE ADDED IN DEC. 1957.—Since this paper was written, two additional preparations of TMV have been made and were found to be quite monodispersed when examined by flow birefringence and sedimentation. Indeed, the flow birefringence

(36) N. Saitô and Y. Ikeda, *J. Phys. Soc. Japan*, **6**, 305 (1951).
(37) R. E. Franklin, private communication.

data coincided with the $L = 3400$ Å. curve down to gradients of 100 sec.$^{-1}$ We attribute this greater homogeneity to prolonged exposure (3 hr.) to 0.01 M Versene at room temperature prior to the third high speed centrifugation. In all other details, the preparative procedure was identical with that outlined above.

CAMBRIDGE 38, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Lengths of Tobacco Mosaic Virus from Electron Microscopy[1]

BY CECIL E. HALL

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A sample of tobacco mosaic virus showing a high degree of uniformity as to length which had previously been studied by physical-chemical techniques was examined and measured with the electron microscope. From a measured population of 200 particles 85% have lengths between 2800 and 3200 Å. and 75% have lengths between 2900 and 3100 Å. The most frequently occurring length is between 2950 and 3050 Å. Methods and precautions taken in making these measurements are described. These results are in excellent agreement with previous electron microscope results.

A sample of tobacco mosaic virus (TMV) prepared by Dr. H. Boedtker who has reported the physical-chemical measurements of this same material[2] has been examined and measured by electron microscopy. Although TMV has been observed on innumerable occasions by electron microscopy, good correlation of results with hydrodynamical data has been lacking. It therefore seems worth while to report the electron microscope results in some detail insofar as they may be of value in the interpretation of the data from indirect methods. It is also interesting to compare our new results with the electron microscope work of Williams and Steere[3] since the two sets of data are in excellent agreement although the methods differ appreciably.

Experimental

A volume of the TMV solution was diluted with double-distilled water containing polystyrene latex to a final concentration of about 0.5 mg./ml. of virus and sprayed from a high pressure gun similar to that described by Backus and Williams[4] onto the surface of freshly cleaved mica. The polystyrene latex (Dow Chemical Company) has an average diameter of 880 Å. and was present in an amount of 0.06 mg./ml. It was employed as an aid on focussing and not for magnification calibration. The surface was shadowcast with 2.4 cm. of 0.1 mm. Pt wire at a shadow-to-height ratio of 10:1, coated normally with 0.5 mg. of SiO evaporated from a distance of 12 cm. to provide rigidity, coated with 0.5% collodion and stripped on water. The stripped specimens were supported on 200-per-inch nickel screens.

Before the specimens were observed with the electron microscope (RCA Type EMU3B, 100 kv.), the microscope was calibrated by recording 5 images of an SiO replica of a 15,000-line-per-inch diffraction grating on a 2 × 10 inch plate which provided a total of 10 spacings (magnification about 12,500 ×). Two plates of TMV showing 10 different fields were then recorded and the calibration procedure repeated. Since the calibration of the instrument drifts as it warms up, the average of the two calibration plates was used for the TMV. Owing to the highly hydrophilic nature of a cleaved mica surface, sprayed droplets spread over a very large area which makes it impossible in general to include an entire droplet in a single field of view. The recorded fields were therefore random samples from droplets covering large areas. Subsequent plates were also recorded in the same manner with a calibration before and after the TMV specimens. Before each plate was recorded, hysteresis effects in the lenses were removed by turning objective and projector lenses to their maximum current values and back to operating value 4 times.

Electron micrographs containing well-dispersed particles were enlarged to a total magnification of 66,000 to 67,000 × as calculated from the calibration micrographs. TMV particles were measured (to the inside of the cap on shadowed ends) with a scale graduated in 0.5 mm. divisions and grouped in intervals of 0.25 mm. All particles whose two ends could be seen without ambiguity were measured. Since magnifications were close together (66,500 ± 500), measurements from all prints were added together to be plotted on the same millimeter scale. It was not necessary to correct for field distortion as was done by Williams and Steere because this effect introduces an error of less than 1% at the magnification employed.

Results

The length-distribution for 201 particles is shown in Fig. 1 where a scale in Ängströms has been superimposed in place of the millimeter scale on the basis of a magnification of 66,500 ×. The graph shows that the sample consists to a large extent of particles whose lengths are very close to 3000 Å. A better visual demonstration of the relative number of particles contained within various length classes is provided by the cumulative plot in Fig. 2 which shows that although there are a few particles present with random lengths above and below the peak value, about 75% of the particles are within the range 2900 to 3100 Å. In view of the fact that random observational errors which might be as high as ±2% in some instances would contribute to broadening of the distribution, the degree of uniformity is remarkable. That the observed breadth around the maximum does not result entirely from calibration error is evident from the fact that observed lengths vary within a given field by about the spread shown on the graphs. Possibly there is some distortion of the particles on drying, but the rigidity of the support might be expected to keep this to a minimum. It would appear that the most frequently occurring length for particles dried in this fashion is very probably within the range 2950 to 3050 Å. There is no evidence for any special tendency to form dimers although a few particles having about double the most frequent length do occur.

(1) This work was supported by a research grant C217I(C5) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

(2) H. Boedtker, THIS JOURNAL, **80**, 2550 (1958).

(3) R. C. Williams and R. L. Steere, *ibid.*, **73**, 2057 (1951).

(4) R. C. Backus and R. C. Williams, *J. Applied Phys.*, **21**, 11 (1950).