Purification and Characterization of Tobacco Mosaic Virus

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Since the isolation and crystallization of tobacco mosaic virus (TMV), by Stanley[1] studies on physicochemical properties of viruses have greatly advanced, especially by taking advantage of the ultracentrifuge for purification. Ultracentrifugal purification of TMV now seems to be a well-established method; a preparation with high degree of homogeneity is, nevertheless, not easy to obtain. Only a few comprehensive studies on the properties of TMV particles are found in the literature which are based on simultaneous measurements of various physicochemical characteristics of a purified solution. In this paper the results obtained by the authors during the last few years[2] are shown, concerning the ultracentrifugal purification of TMV and characterization of purified preparations, by various means among which a sample of high degree of homogeneity is included.

Experimental

A. Purification of TMV.

1. Ultracentrifuge.—The ultracentrifuge used is an air-driven, vacuum-type one[3] first constructed in Japan at the end of 1948, some details of which were reported previously.[4][5]

2. Materials.—Tobacco leaves infected with tobacco mosaic disease were kindly supplied by Dr. J. Hitaka of the Tobacco Experimental Station at Hatano, Kanagawa Prefecture. Xanthi was used for the starting plant in our earlier experiments, but it was found that brownish pigments were not completely removed even after several cycles of alternate high and low speed centrifugations. In later experiments, therefore, we used mainly the infected leaves of White Burley, for it was found much easier to remove colored materials by centrifugation only.

The virus studied here is believed to be the ordinary strain of tobacco mosaic virus.

3. Purification Procedures.—Of the several preparations obtained, the purification methods of TMV-8 and -9 are described, which follow essentially those of Stanley.[6]

Young White Burley tobacco plants were harvested about two or three weeks after inoculation with TMV. The leaves were frozen in a refrigerator and ground through a meat chopper after three weeks (TMV-8); or they were frozen with solid carbon dioxide and ground the following day (TMV-9). On grinding, solid K_2HPO_4 was added amounting to 2.5% of the fresh weight of the leaves to maintain the *p*H of the expressed juice near neutrality. The juice was clarified by centrifugation and then subjected to three (TMV-8) or five (TMV-9) cycles of alternate high and low speed centrifugations. The high speed runs to obtain the virus pellets were made for one hour at 30,000 R. P. M. (65,000×g) or for half an hour at 36,000 R. P. M. (95,000×g), and those of low speed to remove heavier impurities, at 9,000 to 12,000 R. P. M. (6,000 to 10,000×g) for about 15 minutes. The solvents used to dissolve the pellets of TMV were 0.1M phosphate buffer at *p*H 7.0 for the first two cycles and 0.01M for the others. The final pellets so obtained were practically pigment-free. They were dissolved in 0.01M phosphate buffer at *p*H 7.0 and stored in a cold room near 0°C.

4. Yields.—Biological assays by the half leaf method on *Nicotiana glutinosa* were performed only qualitatively, and it was found that no serious loss or inactivation of TMV had occurred during the isolation processes by comparing the infectivity of purified preparation with that of original juice.

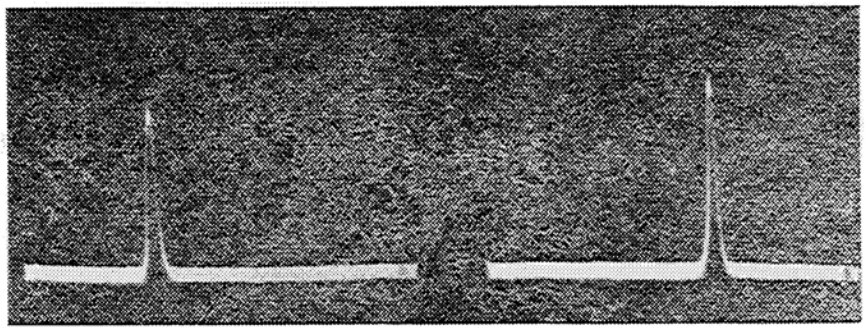
The yields of purified virus were found to be about the same order as that reported by other workers[7][8][9] and are shown in Table 1.

Table 1
Yields of Purified TMV

Sample	Total amount of TMV obtained, g.	Mg. TMV per g. of original plant	Mg. TMV per cc. of plant juice
TMV-8	0.030	1.0	1.2
TMV-9	1.07	1.4	1.8
	(1.5)	(2.0)	(2.5)

( )···Including less pure TMV

(1) W. M. Stanley, *Science* **81**, 644 (1935).
(2) A brief preliminary report, I. Watanabe, Y. Otsuka, and S. Mizushima, *Repts. Radiation Chem. Research Inst., Tokyo Univ.*, No. 5, 10 (1950).
(3) E. G. Pickels, *Rev. Sci. Instruments*, **9**, 358 (1938).
(4) S. Sasaki and O. Taniguchi, *J. Japan Soc. Mech. Eng.*, **54**, 255 (1951).
(5) I. Watanabe and Y. Kawade, *Chemistry and Chem. Industry*, **5**, 585 (1952).
(6) W. M. Stanley, *J. Am. Chem. Soc.*, **64**, 1804 (1942).
(7) W. M. Stanley, in R. Doerr und C. Hallauer, "*Handbuch der Virusforschung*", I, Vienna (1938), pp. 447—546.
(8) H. K. Schachman and W. J. Kauzmann, *J. Phys. Colloid Chem.*, **53**, 150 (1949).
(9) F. C. Bawden, "*Plant Viruses and Virus Diseases*", Waltham (1950).



← Ascending → Descending

Fig. 1.—Electrophoretic pattern of 0.44% solution of TMV-9 in 0.2 ionic strength phosphate buffer at *p*H 7.7[16]

B. Homogeneities and Properties of Purified TMV.

Some chemical and physical properties of purified TMV have been examined to test its homogeneity and to determine size and shape of the virus particles.

1. Chemical Analyses.—An aliquot of the purified stock solution (TMV-9) was dialyzed against distilled water, and precipitated by acidified ethanol. The precipitate, washed and dried, was dissolved in weak alkali and submitted to analyses for nitrogen by the micro Kjeldahl method, phosphorus by King's method,[10] and pentose nucleic acid by the orcinol method.[11] Dische's diphenylamine test[12] for DNA was found entirely negative. The results are shown in Table 2 and are in good agreement with the values reported by previous investigators.[9][13]

Table 2

Chemical Analyses of Purified TMV
(Sample: TMV-9)

	N %	P %	N/P	PNA %	DNA %
	16.6	0.51		6.2_7	0.0
	16.7	0.50		5.8	0.0
Average	16.7	0.51	33	6.0	0.0

2. Physical Measurements. — Electrophoretic measurements of purified preparations were carried out in a Tiselius-type[14] apparatus, equipped with a Svensson optical system,[15] in phosphate buffers of various ionic strengths at *p*H 7.7 and the details will be published elsewhere.[16] In all preparations examined, except in the case only a single boundary was found in the electrophoretic pattern of TMV-8, and the boundary remained very sharp during the course of electrophoresis (forexample, see Fig. 1). A small amount of an unidentified slower component was seen in the pattern of TMV-8. The electrophoretic mobilities were calculated from the descending patterns and in Table 3 are shown the representative data of TMV-9 at 0°C.

Diffusion constants were measured refractometrically by the Svensson schlieren method[15] using a Neurath-type[17] diffusion cell under

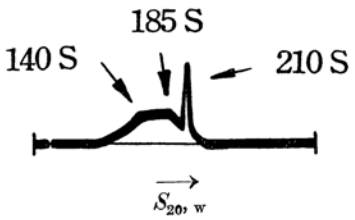


Fig. 2.—Tracing of sedimentation pattern of 0.51% solution of TMV-8 in 0.01M phosphate buffer at *p*H 7.0[18]

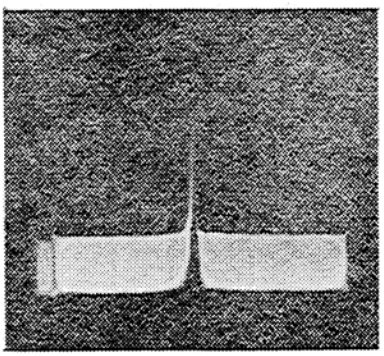


Fig. 3.—Sedimentation pattern of 0.61% solution of TMV-9 in 0.01M phosphate buffer at *p*H 7.0[18]

(10) E. J. King, *Biochem. J.*, **26**, 292 (1932).
(11) S. E. Kerr and K. Seraidarian, *J. Biol. Chem.*, **159**, 211 (1945).
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(13) W. M. Stanley and M. A. Lauffer, in T. M. Rivers, "*Viral and Rickettsial Infections of Man*", Philadelphia (1948), pp. 18—66.
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(15) H. Svensson, *Kolloid-Z.*, **87**, 181 (1939).
(16) I. Watanabe and N. Ui, in preparation.
(17) H. Neurath, *Science*, **93**, 431 (1941).
(18) I. Watanabe, Y. Kawade, and S. Mizushima, *J. Chem. Soc. Japan, Pure Chem. Sect.*, **73**, 828 (1952).

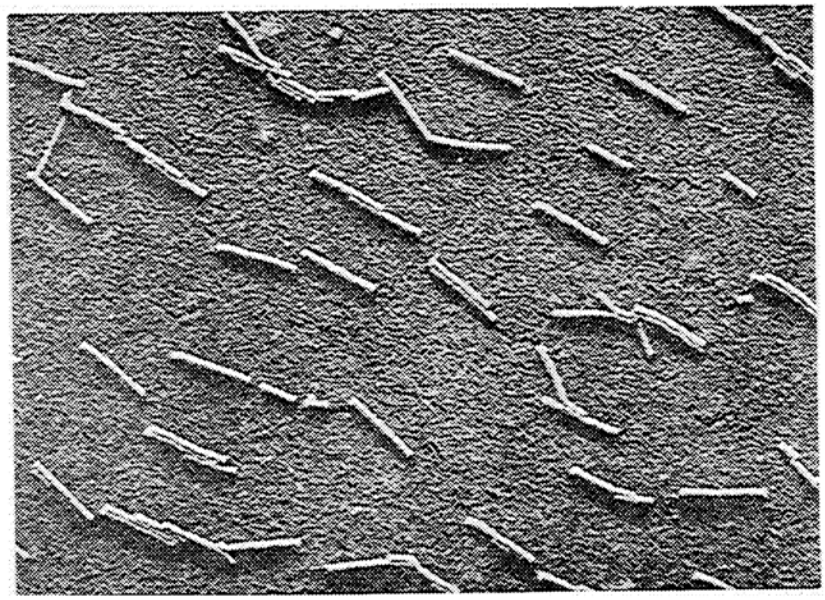

Fig. 4.—Typical electron micrograph of centrifugally purified TMV (TMV-9) photographed by Japan Electron Optics Co., Tokyo. Magnification 35,000 and chromium shadow cast.

various conditions in phosphate buffers. The most reliable value of diffusion constant of the preparation of TMV-9 is shown in Table 3.

Sedimentation studies were carried out in 0.01M phosphate buffer at *p*H 7.0 by using an air-driven analytical ultracentrifuge.[18] TMV-8 proved very inhomogeneous and showed three components in sedimentation pattern (see Fig. 2). The sedimentation constants ($s_{20,w}$) of these three peaks were 210, 185, and 140 S (S: Svedberg unit), when corrected for viscosity of the solution, and would correspond roughly to dimer, monomer, and half-length particle of TMV, respectively. On the other hand, TMV-9 gave a single sharp boundary shown in Fig. 3, suggesting a high degree of homogeneity. It may be stated that such an exceedingly sharp boundary of TMV has not been reported except by Schachman.[19] The sedimentation constant was calculated to be 157S, corrected for viscosity of the solvent, and 185S, corrected for viscosity of the solution according to Lauffer[20] (see Table 3). The latter value is in close agreement with those reported by other workers[5][20][21] for monomeric TMV.

Viscosity was measured in 0.01M phosphate buffer at *p*H 7.0 by an Ostwald-type viscosimeter with low velocity gradient (about 100 sec.$^{-1}$). TMV-8 gave a volume fraction intrinsic viscosity of about 40. This slightly low value of intrinsic viscosity as compared with that of TMV-9 is probably due to the presence of a large amount of shorter (half-length) particles in TMV-8 (see Fig. 2). The viscosity of TMV-9 showed a considerable decrease with time; the intrinsic viscosity at equilibrium state was estimated to be 50 (Table 3).

Electron microscopic observations were made on various samples and in Fig. 4 is shown one of the representative micrographs of purified TMV-9. No extraneous materials have been observed in any. It is apparent that the particles of length about 300 mμ predominate in number in each case. Shorter and longer

Table 3
Physical Data of Purified TMV
(Sample: TMV-9)

Electrophoretic Mobility (at 0°C. in phosphate buffer of ionic strength 0.2 and *p*H 7.7)	$u_D = -7.0_5 \times 10^{-5}$ cm.^{2}/sec. volt
Diffusion Constant	$D_{20,w} = 0.4 \times 10^{-7}$ cm.^{2}/sec.
Sedimentation Constant	$s_{20,w} = 185$ S* (corrected by solution viscosity) = 157 S (0.5%) (corrected by solvent viscosity)
Intrinsic Viscosity (Volume Fraction)	$[\eta]_V = 50$
(Partial Specific Volume)	($V = 0.73$)**

* S is a Svedberg unit and a sedimentation rate equal to 10^{-13} cm./sec. in a unit centrifugal field.

** The value obtained by Lauffer.[22]

(19) H. K. Schachman, *J. Am. Chem. Soc.*, **73**, 4808 (1951).
(20) M. A. Lauffer, *J. Am. Chem. Soc.*, **66**, 1195 (1944).
(21) G. Schramm und G. Bergold, *Z. Naturforsch.*, **2b**, 10 (81947).
(22) M. A. Lauffer, *J. Am. Chem. Soc.*, **66**, 1188 (1944).

Table 4
Size and Shape of TMV Particles
(Sample: TMV-9)

Methods	Mol. Wt. (10^6)	Frictional Ratio, f/f_0	Axial Ratfo, a/b	Length, a (mμ)	Diameter, b (mμ)
Sedimentation and Viscosity	37	2.2	24	310	13
Sedimentation and Diffuion	42	2.3	28	360	13
Diffusion and Viscositys	53	2.2	24	360	15
Electron Microscope and X-Ray Diffraction	45		20	300	15.2*

* The value obtained by Bernal and Fankuchen from the X-ray diffraction experiments[25]

particles are, however, always present; and even in our most homogeneous preparation, TMV-9, the distribution is broader than reported by American investigators.[22][23][24] Electron micrographs of the original extract for TMV-7 were also taken and it was found that a considerable amount of shorter rods had been already present in plant juice. It might, therefore, be difficult, if not impossible, to ascribe the presence of the short rods entirely to the break-up of the monomer virus rods due to mechanical stresses during centrifugation.

3. Size and Shape of TMV Particles.—The physical data mentioned above are summarized in Table 3. Size and shape of TMV can be determined by various combinations of viscosity, diffusion, and sedimentation data in conjunction with the partial specific volume.[22] The results of the various methods of determining the dimensions of the particles of TMV-9 are shown in Table 4. In these calculations Lauffer's value[22] of partial specific volume was temporarily adopted. It can be seen that the agreement between the various methods is excellent considering the experimental errors and uncertainties in the calculation methods of determining the size and shape of such highly asymmetric particles. It must be, however, borne in mind that these values are not conclusive, since the concentration dependence of sedimentation constant has not yet been measured exactly in our case and the determinations of various physical constants have not been made at the same time. The latter consideration seems important because of the observed change in the viscosity of solutions of TMV-9 with the lapse of time.

Discussion

In the ultracentrifugal purification of viruses, it seems primarily important to take care of the choice of suitable starting materials. In fact, it was found in our experiments that the leaves of Xanthi contained pigment which was difficult to remove from the virus fraction and that in this respect White Burley was superior to Xanthi. It is also essential to remove heavier materials as completely as possible in the first clarification of the expressed juice. Otherwise, green impurities remaining in the turbid supernatant, once sedimented by high speed centrifugation and redissolved in buffer solution, become somewhat difficult to be removed by subsequent centrifugation and complicate the further purification procedure.

It is clear from the results of chemical and physical measurements on purified TMV that the purity of centrifugally isolated TMV, especially of TMV-9, was very high, so to say, in a chemical sense. The homogeneities of preparations, however, were not always so good. TMV-9 showed a single sharp boundary both in sedimentation and electrophoretic patterns. On the contrary, three peaks appeared in the sedimentation pattern of TMV-8. But this marked heterogeneity was not detected in the electrophoretic measurements, which showed only one main peak except a presence of a small amount of slower component. This apparent discrepancy in sedimentation and electrophoretic patterns of TMV-8 may be ascribed to some difference of sensitivities of sedimentation and electrophoretic analyses in detecting the heterogeneity of length of rod-like particles such as TMV. Another possible explanation, however, cannot be excluded; that is, a certain change in molecular state might have occurred during the preservation, since the electrophoretic measurements were made immediately after the purification of TMV-8 while the ultracentrifugal analysis was performed nearly three months after it. This marked heterogeneity found in sedimentation pattern could not be detected also in diffusion pattern of TMV-8, the measurements of which were made at the same time as the electrophoretic experiments.

TMV-9, on the other hand, showed no evidence of heterogeneity and a single sharp boundary was obtained both in sedimentation and electrophoretic patterns, as mentioned above*; nevertheless, a rather broad distribution was observed in the electron micrographs. In view of the high sensitivity of ultracentrifuge in detecting hetero-

(23) G. Oster, C. A. Knight, and W. M. Stanley, *Arch. Biochem.*, **15** 279 (1947).
(24) R. C. Williams and R. L. Steere, *J. Am. Chem. Soc.*, **73**, 2057 (1951).
(25) J. D. Bernal and I. Fankuchen, *J. Gen. Physiol.*, **25**, 111 (1941).

* Even after storage in a cold room near 0°C. for more than six months, TMV-9 still retained a fairly good homogeneity in sedimentation pattern.[18]

geneity (presence of short rods) of TMV[19] and a possible change of distribution in length during the preparation of specimen for electron microscopic examination, it might be not unreasonable to conclude that TMV-9 has a high degree of homogeneity and that shorter rods observed in electron micrographs are, at least in part, artefacts. It must be remembered, however, that there still remain other possibilities concerning the origin of these short rods which are seen also in other samples; first, they may be present in the original expressed juice and secondly, they may arise from the break-up of the longer particles as a result of mechanical stresses during the preparation.[26]

It is rather curious that TMV-8, although purified under very similar conditions as TMV-9, showed the marked heterogeneity. In this respect, it would be necessary to point out that in the case of TMV-8, the leaves were frozen in a refrigerator and treated three weeks after the harvest, while in TMV-9, the harvested leaves were frozen with solid carbon dioxide and treated in the following day. These differences in the time of storage of leaves might have caused the unexpected difference in homogeneities of TMV-8 and -9. It seems very desirable, anyway, to start the purification of virus as soon as possible after the harvest of leaves.

Schachman and Kauzmann[8] studied the aggregation effect of phosphate buffer during the purification and storage of TMV and found that isolation of TMV by centrifugation in 0.1M phosphate followed by water leads to the best preparation. Considering their experimental results, however, we used 0.01M phosphate buffer instead of water and the final solution was also stored in the same buffer. Very good results were obtained in this case too. But the solution stored in 0.01M phosphate buffer showed considerable decrease in viscosity with the lapse of time, as mentioned above. This change in viscosity seems very interesting, but other measurements must be performed during storage before a clear explanation can be made about it.

Electrophoretic mobility, diffusion constant, sedimentation constant, and intrinsic viscosity were determined on the most homogeneous preparation, TMV-9. The size and shape of TMV particles were also calculated by various combinations of diffusion constant, sedimentation constant, and intrinsic viscosity and an excellent agreement was found between the results of these indirect physico-chemical procedures and direct observation with the electron microscope.

Since the degree of homogeneity of a purified TMV preparation with respect to the length of the rod-like particle is known to vary various factors, including the purification procedures employed, the nature of the solvent used for preservation, etc.,[27] it is necessary to assess the degree of homogeneity of the specimen when its physicochemical properties are to be studied. Among the numerous investigations on the determination of size and shape of TMV, those of Lauffer[22] and of Schramm and Bergold[21] have been probably the most comprehensive in this respect, but even their samples were apparently inhomogeneous judging from the sedimentation patterns obtained by them. Studies on homogeneous solution of such highly asymmetric particles will be greatly valuable in establishing the hydrodynamic theory underlying the measurements of sedimentation, diffusion, viscosity, etc.

Summary

Tobacco mosaic virus (TMV) was purified by differential centrifugation in a vacuum-type, air-driven ultracentrifuge, first constructed in Japan.

The yield of purified TMV was high and about 2 mg. per cc. of the original plant juice.

Chemical analyses and physico-chemical examinations (including electrophoresis, sedimentation, diffusion, viscosity and electron microscope measurements) were carried out on the purified preparations to test their purities and homogeneities. It was found that the purity of ultracentrifugally isolated TMV was very high in a chemical sense. Although the homogeneity with respect to the length of particles was not always so good, one preparation, TMV-9, was found to have a high degree of homogeneity by the sedimentation and electrophoresis patterns. The size and shape of TMV particles were determined on this preparation by several methods.

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(26) T. Sigurgeirsson and W. M. Stanley, *Phytopathology*, **37**, 26 (1947).

(27) See, e. g., refs. (26), (24), and (8).