5. Five derivatives of trihydroxy-*iso*butyric acid, hitherto unreported, were prepared and their more important properties determined.

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[CONTRIBUTION FROM THE DEPARTMENT OF ANIMAL AND PLANT PATHOLOGY OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Size and Shape of Tobacco Mosaic Virus Particles[1]

BY MAX A. LAUFFER

I. Introduction

Several physico-chemical techniques have been developed for the determination of the size and physical characteristics of protein particles. These include ultracentrifugation, diffusion and viscosity studies. From various combinations of data obtained by at least two of these methods used in conjunction with the partial specific volume, it is possible to determine not only the molecular weight, but also the size and shape of protein particles. In general, the conclusions drawn from various combinations of the above methods are self consistent. Nevertheless, confirmation of the results by some direct method would enhence the value of such results greatly. It has been pointed out that tobacco mosaic virus protein affords an excellent medium through which to obtain such a confirmation, because the particles of this protein are extremely anisometric and because they are large enough to be seen and measured with fair precision with the electron microscope.[1a] It was shown from the data available in the literature that in a general sort of way the conclusions regarding the size and shape of tobacco mosaic virus protein particles drawn from viscosity, diffusion, and sedimentation results were in agreement with the dimensions measured directly from electron micrographs.[1a] Nevertheless, these data suffered from the limitation of having been obtained in several laboratories on different virus preparations probably in different states of aggregation. It was therefore thought worth while to carry out viscosity, sedimentation and diffusion studies on a limited number of preparations and to compare these results directly with electron micrographs on the same preparations. This report is a description of such studies carried out for the primary purpose of affording a critical test of the general validity of the indirect methods of determining the size and shape of protein particles.

II. Materials

Five preparations of tobacco mosaic virus were used in this study. Preparations A and B were isolated from frozen and ground fourteen-week-old Turkish tobacco plants which had been inoculated with tobacco mosaic virus at the age of nine weeks. Preparation A was isolated by two alternate high and low speed centrifugation cycles. The high speed runs were made for one hour at 24,000 r. p. m. in a Bauer and Pickels air-driven quantity centrifuge.[2] No chemical agent was used at any stage in the treatment. In the isolation of Preparation B, 3 g. of potassium biphosphate was added per 100 g. of pulp before the extraction of the juice, and the two centrifugation cycles were carried out with 0.1 *M* phosphate buffer at *p*H 7 as the solvent instead of water as for Preparation A. Preparation C was an old preparation of unknown history. Preparation D was isolated from a different crop of plants in a manner identical with that for Preparation A. It was used without further purification in viscosity and sedimentation studies. For use in specific volume determinations it was further purified by precipitation with ammonium sulfate and by isoelectric precipitation. It was finally brought to the isoelectric point and dialyzed against distilled water. The ash content was determined to be 1.83%. This further purified material is referred to as Preparation D′. Preparation E, which was used only in specific volume studies, was obtained by subjecting the juice from diseased plants to three high-speed centrifugations, two isoelectric precipitations, two ammonium sulfate precipitations, and, finally, electrodialysis. It was titrated to *p*H 5 with sodium hydroxide to bring it back into solution. The ash content was found to be 1.22%.

III. Partial Specific Volume

In order to be able to determine molecular weight and particle dimensions by indirect physico-chemical procedures and in order to determine particle weights from direct measurements with the electron microscope, it is necessary to know the partial specific volume. Three different values for the partial specific volume of tobacco mosaic virus have been reported. Eriksson-Quensel and Svedberg[3] obtained a value of 0.646. Stanley found a value of 0.77.[4] Bawden and Pirie obtained 0.73 for tobacco mosaic virus and

(1) The data described in this paper were discussed in two papers presented before the Divisions of Biological Chemistry and Colloid Chemistry at the 107th meeting of the American Chemical Society, Cleveland, Ohio, April, 1944.

(1a) M. A. Lauffer and W. M. Stanley, *Chem. Rev.*, **24**, 303 (1939); *Kolloid-Z.*, **91**, 62 (1940).

(2) J. H. Bauer and E. G. Pickels, *J. Exptl. Med.*, **64**, 503 (1936).

(3) I. Eriksson-Quensel and T. Svedberg, THIS JOURNAL, **58**, 1863 (1936).

(4) W. M. Stanley, *J. Phys. Chem.*, **42**, 55 (1938).

two of its strains.[5] Because of this discrepancy, it was thought worth while to reinvestigate this question.

Apparent specific volumes were calculated from densities determined in a 50-cc. pycnometer equipped with a finely graduated thermometer, and from virus protein concentrations determined by drying a weighed amount of solution to constant weight over phosphorus pentoxide in vacuum at 115°. Preparations D′ and E were used. The results are presented in Table I. Since there is no observable trend in the variation of apparent specific volume with concentration, it seems reasonable to regard the average value as a good estimate of the partial specific volume. The figure obtained, 0.73, is in agreement with the results of Bawden and Pirie.

The partial specific volume is a measure of the increase in volume when a gram of dry protein is dissolved in an infinite amount of solvent. It may or may not represent the actual volume of the hydrodynamically effective units in solution. In some of the indirect methods of determining particle dimensions it is not necessary to know the true hydrodynamically effective volume, but in others that quantity is required. The relationship between the hydrodynamically effective volume and the partial specific volume is ambiguous for most protein solutions. The degree of ambiguity in the case of tobacco mosaic virus is considerably less than usual. The X-ray studies of Bernal and Fankuchen[6] show that the internal spacings of completely dried tobacco mosaic virus particles are the same as for particles in solution. Therefore, the virus particles do not bind water by imbibition. Any bound water must be on the surface. It is rather generally believed that the degree of hydration of proteins with molecular weights in the neighborhood of 40,000 is of the order of magnitude of 50% by volume. This would correspond roughly to the amount of water held by a shell the thickness of one water molecule entirely surrounding the particle. It can easily be shown, on the basis of particle dimensions that will be described in subsequent sections, that a shell of water one molecule thick surrounding the tobacco mosaic virus particle would amount to a hydration of only 9% by volume. Hence, it is probable that the potential error involved in the assumption that the partial specific volume is the hydrodynamically effective volume is considerably less for tobacco mosaic virus protein than for most proteins.

TABLE I

THE PARTIAL SPECIFIC VOLUME OF TOBACCO MOSAIC VIRUS

Prepn.	Concn., weight fractions	Apparent specific volume, cc./g.	Temperature, °C.
D′	0.0127	0.724	27.1
	.0058	.712	28.7
	.0028	.725	26.8
E	.0221	.738	27.8
	.0111	.737	27.0

(5) F. C. Bawden and N. W. Pirie, *Proc. Roy. Soc.* (London), **B125**, 274 (1937).

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

IV. Electron Microscopy

Preparations A and B were examined in the electron miroscope through the courtesy of Dr. James Hillier of the R. C. A. Research Laboratories of Princeton. The length of every rod-like particle appearing on the enlarged print of the micrograph for each preparation was measured to the nearest tenth of a millimeter. These lengths were divided by 7400, the magnification factor. The results are shown in the frequency charts of Fig. 1. An estimated error of about 10% may be ascribed to the magnification factor. It may be observed that the lengths of the particles of Preparation A have a unimodal distribution curve with a most frequently occurring value at 270 mμ, while those of Preparation B have a bimodal distribution curve with maxima at 270 mμ and between 405 and 540 mμ. The thickness obtained from the electron microscope is of the order of magnitude of 15 mμ. However, a much more accurate estimate of the thickness of the particles is the value 15.2 mμ, representing the lateral distance between particle centers in dried crystals, obtained from X-ray diffraction studies by Bernal and Fankuchen.[6] Using these values for the dimensions and the reciprocal of the partial specific volume for the density, one can estimate that the molecular weight of the most frequently occurring particles in Preparation A is 40×10^6, with an uncertainty of about 10%.

V. Viscosity

Viscosity measurements were carried out at 25.0° on Preparations A, B, C and D dissolved in 0.1 *M* phosphate buffer at *p*H 7. An Ostwald type of viscometer designed to give an average velocity gradient of about 350 sec.$^{-1}$ was used. This relatively low velocity gradient was achieved by using a capillary tube 150 cm. long with a diameter of 0.09 cm. coiled to allow the average hydrostatic head to be only 4.5 cm. The results are presented graphically in Fig. 2, where the specific viscosity, $\eta/\eta_0 - 1$, is plotted as a function of the concentration of virus in grams per 100 cc. It can be observed that, in the dilute range, specific viscosity is a linear function of virus concentration for all four preparations. The viscosity of Preparation D does not differ significantly from that of Preparation A at low concentrations. At higher concentrations the specific viscosity of Preparation D deviates markedly from a linear function of concentration, but the data follow the equation, $\ln \eta/\eta_0 = 30.5\ C$, with reasonable fidelity up to concentrations of about 1.2 g. per 100 cc. The intrinsic viscosity, which is defined as the limiting value at infinite

dilution of the specific viscosity divided by the volume fraction of virus, may be calculated to be 39.0, 80.7 and 278, respectively, for Preparations A and D, B and C. The value for Preparations A and D is of the same order of magnitude as that which could be calculated from the data of Stanley,[4] and the value for B is approximately the same as that obtained by the author in a previous study.[7] Since it is known that Preparation A is essentially monodisperse and Preparation B is partially aggregated, it may be concluded that the preparation studied by Stanley was probably essentially monodisperse and that studied previously by the author was probably partially aggregated.

It has been pointed out in previous discussions that the ratio of the major to the minor axes of an elongated ellipsoid of revolution considered to be a model of the solute macromolecules can be evaluated from the intrinsic viscosity of that solute by using one of several similar but by no means identical equations derived for that purpose.[1a,7] Since those discussions were published, a more satisfactory theoretical equation has been presented by Simha.[8] When b is the major and a the minor axis of an elongated ellipsoid of revolution and $[\eta]$ is the intrinsic viscosity, the Simha equation can be expressed as

$$\text{Eq. 1} \qquad [\eta] = \frac{(b/a)^2}{15\left(\ln \frac{2b}{a} - \frac{3}{2}\right)} + \frac{3(b/a)^2}{15\left(\ln \frac{2b}{a} - \frac{1}{2}\right)} + \frac{14}{15}$$

It has been shown graphically[9] and it is obvious algebraically that for large values of b/a the Simha equation approaches a much simpler one presented earlier by Onsager.[10] The Onsager equation for rod-like ellipsoids has been expressed as

$$[\eta] = 4/15 \; b^2/a^2 \frac{1}{\ln b/a} \text{ when } b/a \gg 1$$

The axial ratios for tobacco mosaic virus samples A, B and C as calculated by the Simha equation are presented in Table II.[10a] In making these calculations, it was assumed that the virus protein is not hydrated in solution. This assumption was shown in section III to be substantially correct. The average axial ratios for Preparations A and B determined from the electron microscope data of Fig. 1 and X-ray diffraction data are also presented for comparison. It is obvious that the Simha equation gives specific viscosity as a function of the amount of solute and of approximately the square of the axial ratio for very elongated particles. The viscosity of a solution composed

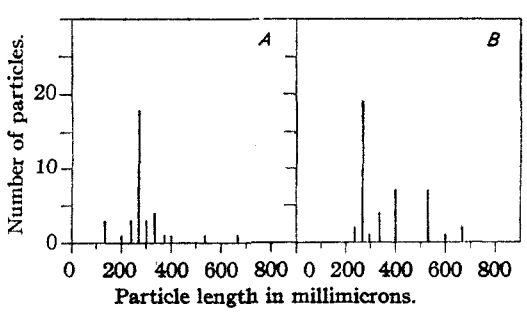

Fig. 1.—Histogram showing the distribution of particle lengths as determined by the electron microscope for an essentially unaggregated (A) and a partially aggregated (B) preparation of tobacco mosaic virus.

TABLE II

THE AXIAL RATIO OF TOBACCO MOSAIC VIRUS PARTICLES

Virus sample	Intrinsic viscosity	b/a (Simha)	b/a electron microscope and X-ray diffraction	b/a diffusion and sedimentation
A	39.0	20.3	23.0	18.6
B	80.7	31.5	28.7	
C	278	64.3		

(7) M. A. Lauffer, *Science*, **87**, 469 (1938); *J. Biol. Chem.*, **126**, 443 (1938).

(8) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

(9) M. A. Lauffer, *Chem. Rev.*, **31**, 561 (1942).

(10) L. Onsager, *Phys. Rev.*, **40**, 1028 (1932).

(10a) The use of viscosity data obtained in a capillary viscometer to determine the axial ratio of tobacco mosaic virus is subject to a possible limitation due to the fact that the particles are oriented to a certain extent in a flowing stream.[7] The measured viscosity ought to be somewhat smaller than that corresponding to random orientation. The Simha equation was derived to interpret the viscosity of a solution of particles randomly oriented. According to the theory of Boeder[11] the degree of orientation of rod-shaped particles in a flowing stream is a function of α, the ratio of the velocity gradient, β, to the rotational diffusion constant, θ. By substituting into the equation

$$\theta = \frac{3kt}{16\pi\eta b^3}\left(-1 + 2\ln\frac{2b}{a}\right)$$

derived by Perrin,[12] one can calculate that θ for tobacco mosaic virus Preparation A is about 640 sec.$^{-1}$ at 25°. Mehl[13] obtained a value of θ of about 50 sec.$^{-1}$ for a preparation of tobacco mosaic virus from measurements of the orientation of the virus in known velocity gradients by using Boeder's theory. It is quite probable that the virus preparation studied by Mehl was aggregated to a certain extent, and should therefore have a considerably lower rotational diffusion constant than Preparation A. Robinson[14] studied a highly aggregated preparation of tobacco mosaic virus in a rotating cylinder viscometer equipped with optical devices for measuring stream double refraction and the orientation of particles. From his orientation results, it is possible to estimate by the Boeder treatment as modified by Peterlin and Stuart[15] that the value of θ at 25° for his virus preparation is a little less than 1 sec.$^{-1}$. Therefore, if the Boeder theory is essentially correct, one would expect our Preparation A to be oriented in a velocity gradient of 350 sec.$^{-1}$ to about the same extent as Robinson's virus was in a gradient of 0.5 sec.$^{-1}$. Robinson's viscosity data show that the intrinsic viscosity of his virus sample at zero velocity gradient is about 5% greater than when partially oriented in a gradient of about 0.5 sec.$^{-1}$. Therefore, one would expect the intrinsic viscosity at zero gradient of our Preparation A to be roughly 5% greater than the measured value. The axial ratio evaluated by means of the Simha equation for Preparation A ought, therefore, to be correct to within 3%, or to allow a wide margin of safety, within 5%. The error for the case of Preparation B ought to be somewhat larger and that for Preparation C is probably very great. The essential point is, however, that the use of a viscometer of the type here described to determine the axial ratios of tobacco mosaic virus preparations in a low state of aggregation does not involve a serious error due to particle orientation.

(11) P. Boeder, *Z. Physik*, **75**, 258 (1932); *J. Rheol.*, **3**, 494 (1932).

(12) F. Perrin, *J. Phys. Rad.*, **5**, 497 (1934); **7**, 1 (1936).

(13) J. W. Mehl, *Cold Spring Harbor, Symp. Quant. Biol.*, **6**, 218 (1938).

(14) J. R. Robinson, *Proc. Roy. Soc.* (London), **A170**, 519 (1939).

(15) A. Peterlin and H. A. Stuart, *Z. Physik*, **112**, 1, 129 (1939).

of particles of several lengths will thus be a function of the weight average of squared axial ratios. Accordingly, the b/a values computed from the electron micrographs and the X-ray data are square roots of weight averages of the squared axial ratios of individual particles. Because of the directness of the methods involved in their computation, the b/a values from electron micrograph and X-ray data are certainly of the right order of magnitude. However, they are not very precise due to the uncertainty in the magnification factor of the micrographs and due to the small sizes of the samples used to establish the averages. It is very significant that the viscosity data as interpreted by the Simha equation gives results in such good agreement with the values calculated by direct means. This is probably the best evidence of the validity of these equations thus far adduced, for all previous tests have compared the viscosity method with other indirect methods.[16] As was pointed out by Bull and Cooper,[17] these previous tests suffer from a dual ambiguity. It is not known how much of the viscosity effect to ascribe to hydration and how much to shape, and it is not known whether to consider the particles as elongated or as flattened ellipsoids. As was shown in a preceding section, there is every reason to believe that hydration plays no more than a minor role in the case of tobacco mosaic virus. Furthermore, the shape of this particle is known to be that of a long cylinder. The only assumption made is that a cylinder behaves hydrodynamically about the same as a prolate ellipsoid of revolution having a minor axis equal to the radius and a major axis equal to half the length. It seems unlikely that this approximation is seriously in error.

VI. Sedimentation Studies

Sedimentation studies were carried out on Preparations A, B, C and D in the manner described in another report.[18] The results for Preparation A are presented in Table I of that paper. As is discussed therein, the sedimentation constant as usually defined varies with the concentration. The limiting value at infinite dilution can be obtained either by extrapolation or by correcting for the viscosity of the solution instead of that of the solvent as is usually done. The limiting value for the sedimentation constant of Preparation A corrected to water at 20° was found to be 185×10^{-13} cm. per sec. per unit field, or 185s, when s designates the Svedberg unit. The limiting value for Preparation D was found to be 187s.[18]

It was shown previously that the molecular weight of tobacco mosaic virus can be determined from viscosity and sedimentation data.[7] From viscosity data the axial ratio can be evaluated by equation 1, and the axial ratio can be substituted into equation 2 derived by Perrin[12] and by Herzog, Illig and Kudar[19]

$$\text{Eq. 2} \quad \frac{f_0}{f} = \frac{(a/b)^{2/3}}{\sqrt{1-(a/b)^2}} \ln \frac{1+\sqrt{1-(a/b)^2}}{a/b}$$

to evaluate Svedberg's frictional ratio, f/f_0. With this factor, the sedimentation constant and the partial specific volume, the molecular weight can be evaluated by using equation 3.

$$\text{Eq. 3} \quad M^{2/3} = 6\frac{f}{f_0} s^0_{20}\pi\eta N(3V/4\pi N)^{1/3}/(1 - V_{20}\rho^0_{20})$$

The symbols are defined by Svedberg and Pedersen.[20] From the viscosity data of Table I for Preparation A, interpreted by means of the Simha equation and used in conjunction with the sedimentation constant of Preparation A and a value for the partial specific volume of 0.73, the molecular weight of the particles in Preparation A was calculated to be 33.2×10^6. This corresponds to a particle 13.6 mμ in diameter and 276 mμ long. Similar calculations made with the data for Preparation D would yield essentially the same result. This value represents a more acceptable figure than that reported originally, because the viscosity and sedimentation data were obtained on the same preparations in this case, but on different preparations in the earlier case. In all liklihood the previous viscosity measurements were carried out on partially aggregated material.

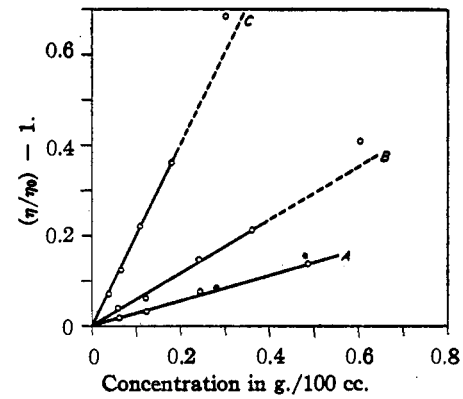

Fig. 2.—Specific viscosity plotted as a function of concentration for tobacco mosaic virus preparations in various states of aggregation: Curve A, ⊙, Preparation A; ●, Preparation D; Curve B, Preparation B; Curve C, Preparation C.

Figure 3 is a refraction gradient diagram representing the boundary of Preparation A after it had sedimented for 12,000 seconds at a speed of 7400 r. p. m. The smooth curve fitting the open circles represents the actual boundary as evaluated by the Lamm scale method.[21] The narrower

(16) J. W. Mehl, J. L. Oncley and R. Simha, *Science*, **92**, 132 (1940).

(17) H. B. Bull and John A. Cooper, *Am. Assoc. Advancement Sci.*, **21**, 150 (1943).

(18) M. A. Lauffer, THIS JOURNAL, **66**, 1195 (1944).

(19) R. O. Herzog, R. Illig and H. Kudar, *Z. physik. Chem.*, **A167**, 329 (1933).

(20) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford, 1940.

(21) O. Lamm, *Z. physik. Chem.*, **A138**, 313 (1928); **A143**, 177 (1929).

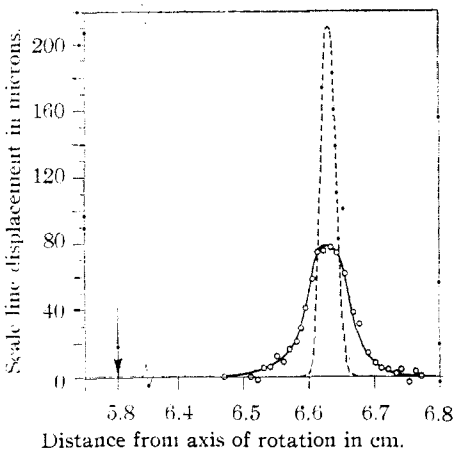

Fig. 3.—Boundary spreading in a sedimentation experiment with Preparation A of tobacco mosaic virus protein. Circles and solid curve represent data obtained by the Lamm scale method, and broken curve represents theoretical spreading due to diffusion alone.

curve represents the boundary that would be obtained if the spreading were due solely to diffusion of the virus. It was obtained in a manner described elsewhere.[22] It can be seen that the boundary spread somewhat more than it should have due to diffusion alone. This result can be interpreted to mean that the particles of virus sample A are not all of exactly the same size, but represent a distribution about a mean size. By a method outlined elsewhere,[22] it can be calculated that the results shown in Fig. 3 are consistent with the assumption that the virus preparation is composed of particles having a distribution of sedimentation rates with a standard deviation of about 4% of the mean rate. From equations 2 and 3 it can be shown that the sedimentation constant of rod-like particles more than 10 times as long as thick should be approximately a linear function of the logarithm of the particle length, if thickness remains constant. On that basis, a standard deviation of sedimentation rate of 4% would correspond to one of 14% in particle length or molecular weight.

Preparation B showed two components in the ultracentrifuge with sedimentation constants calculated in the usual manner of 168 and 193 *s* at a concentration of 0.2 g./cc. When corrected for the viscosity of the solution these became 187 and 216*s*. From the logarithmic dependence of *m* on *s* described in the preceding paragraph, or by an equivalent method described earlier,[1a,7] it can be shown that if rod-like particles with a sedimentation constant of 185*s* and dimensions of 13.6 × 276 mμ, should aggregate end to end to form dimers with dimensions 13.6 × 434 mμ, the sedimentation constant of the dimerized material should be 224*s*. If a reasonable amount of latitude is granted the theory, the observed sedimentation constant of the faster component is sufficiently close to the theoretical value to permit the interpretation that the faster component is composed of end to end dimers of the particles of the first component. On the other hand, the observed result is also consistent with the assumption that the faster component is composed of particles of variable length with an average somewhat less than twice the length of the predominating particles of the more nearly homogeneous preparation. If it is assumed that there are no complications involved in the determination of particle lengths with the electron microscope, the data of Fig. 1 would favor the latter alternative.

Figure 4 is a refraction gradient diagram obtained by the Lamm scale method representing the boundary of Preparation B during the sedimentation experiment. The area under the curve can be apportioned arbitrarily between two essentially symmetrical curves. The relative areas under the two curves are a measure of the relative concentrations of the faster and the slower components. It was found that 39% of the total area can be ascribed to the curve representing the faster component and 61% to that representing the slower component. In other words, the faster and the slower components constitute 39 and 61%, respectively, of the total material. If the faster component is assumed to be a dimer of the slower and if the viscosity is interpreted in terms of the Simha equation, one can calculate from these figures that the intrinsic viscosity of Preparation B should be 72.5. The observed value was 80.7. Considering the fairly high probable error associated with estimating the composition of the solution, this agreement is very good.

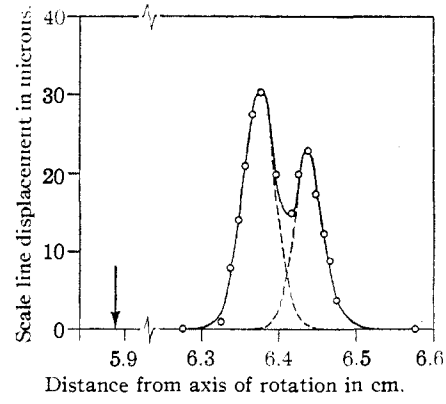

Fig. 4.—Sedimentation diagram obtained by the Lamm scale method of Preparation B of tobacco mosaic virus, showing double boundary.

The sedimentation constant of Preparation C was found to be 204*s* when corrected in the usual manner and 326*s* when corrected for the viscosity of the solution. From the intrinsic viscosity data of Table II, the Simha value for the axial ratio of the particles of Preparation C was calculated to be 64.3. This would correspond roughly

(22) M. A. Lauffer, *J. Biol. Chem.*, **142**, 69 (1942).

to a trimer of the initial particle. Such a trimer should have a sedimentation constant of 240*s*. The discrepancy between this and the observed value may be due to a complication arising from orientation of very large particles both in the viscometer and in the centrifuge. This would result in the viscosity being too low and the sedimentation rate being too high.

VII. Diffusion

Diffusion measurements were made on Preparation A at a concentration of 0.2 g. per 100 cc. in 0.10 *M* phosphate buffer at *p*H 7. A Neurath diffusion cell[23] held in a water-bath at 0.2° was used. The progressive spreading of the boundary was followed by the Longsworth schlieren scanning method.[24] The results are shown in Table III. D_h is the diffusion constant calculated by

TABLE III

DIFFUSION OF TOBACCO MOSAIC VIRUS PROTEIN

Time, sec.	$D \times 10^7$	$D_\mu \times 10^7$
105,500	0.262	0.285
170,000	.273	.262
260,500	.256	.228
345,000	.256	.212
434,000	.289	.269
600,000	.243	.226
692,000	.255	.208
778,000	.265	.241
Average	.262	.241

the maximum ordinate–area method and D_μ is the diffusion constant calculated from the width of the boundary at the inflection points of the refraction gradient curves.[25] From the average of the D_h and D_μ values for 0.2°, a value of D equal to 5.3×10^{-8} sq. cm. per sec. was calculated for tobacco mosaic virus in water at 20°. The viscosity term used in the temperature correction was that of the virus solution. Neurath and Saum[26] obtained a value of about 2.6×10^{-8} and Frampton and Saum[27] reported values of about 4.0×10^{-9} and 4.0×10^{-7} cm.^{2} per sec. for the diffusion constant of tobacco mosaic virus at 20°. Judging by the fact that the intrinsic viscosity of the preparation studied by Neurath and Saum was about the same as that of Preparation B used in this study,[28] their virus preparation was partially aggregated, and the diffusion constant is therefore somewhat too low. The slow-diffusing preparation studied by Frampton and Saum was stated by them to be aggregated. In their case with the higher diffusion rate, the measurements were carried out in 6 *M* urea at about *p*H 7. It has since been shown that tobacco mosaic virus is disintegrated into small fragments void of infectivity under these conditions.[29,30] At least at the time the diffusion experiment was begun virus Preparation A of the present study was an essentially monodisperse solution of the smallest particles definitely known to be associated with tobacco mosaic virus activity. Therefore, the value of 5.3×10^{-8} obtained with it is probably the most nearly correct of those thus far reported.

The molecular weight of a monodisperse material can be calculated from its sedimentation constant, diffusion constant, D, and specific volume by using equation 4.[20]

Eq. 4 $$M = \frac{RTs^0_{20}}{D^0_{20}(1 - V_{20}\rho^0_{20})}$$

From the diffusion, sedimentation and specific volume data of the present study the molecular weight of the particles of tobacco mosaic virus Preparation A can be calculated to be 3.16×10^7. This value is in good agreement with that calculated from viscosity and sedimentation data and with that determined from X-ray and electron optical data. By using the Einstein–Sutherland equation for the diffusion constant of a sphere of known size in conjunction with the measured diffusion constant, the frictional ratio was calculated from sedimentation, diffusion, and specific volume data as described elsewhere.[1a] Then the axial ratio of the tobacco mosaic virus of Preparation A was calculated by means of equation 2 to be 18.6. This would correspond to a particle with a diameter of 13.8 and a length of 256 mμ.

The molecular weight can also be computed from viscosity and diffusion data in a manner described elsewhere.[1a] A value of 3.60×10^7 is obtained. This corresponds to a rod-shaped particle 14 mμ in diameter and 283 mμ in length.

VIII. Discussion

In previous publications,[1a,7] it was pointed out that the size and shape of tobacco mosaic virus protein particles could be determined by a variety of indirect methods and that, in spite of considerable variation, the results obtained were in fair agreement with each other and with the results obtained by direct examination in the electron microscope. The discrepancies observed were attributed to the fact that the data employed were assembled from the results of several laboratories using different virus preparations possibly in different states of aggregation. In the present study various measurements were made on the same virus sample in the same laboratory. The results of the various methods of determining the dimensions of the particles of virus Preparation A are summarized in Table IV. It can be seen that the agreement between the various methods is indeed excellent. This fact means not only that the size and the shape of the predominating particles in a tobacco mosaic virus preparation can

(23) H. Neurath, *Science*, **93**, 431 (1941).
(24) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).
(25) H. Neurath, *Chem. Rev.*, **30**, 357 (1942).
(26) H. Neurath and A. M. Saum, *J. Biol. Chem.*, **126**, 435 (1938).
(27) V. L. Frampton and A. M. Saum, *Science*, **89**, 84 (1939).
(28) V. L. Frampton and H. Neurath, *ibid.*, **87**, 468 (1938).
(29) W. M. Stanley and M. A. Lauffer, *Science*, **89**, 345 (1939).
(30) M. A. Lauffer and W. M. Stanley, *Arch. Biochem.*, **2**, 418 (1943).

be regarded as definitely known within a small limit of error, but also that the various indirect methods available for determining the size and shape of colloidal particles are reliable to a degree not hitherto believed probable.

TABLE IV

THE DIMENSIONS OF TOBACCO MOSAIC VIRUS PARTICLES

Methods	Diameter, mμ	Length, mμ	Mol. wt. ($\times 10^{-7}$)
Sedimentation and viscosity	13.6	276	3.32
Sedimentation and diffusion	13.8	256	3.16
Viscosity and diffusion	14.0	283	3.60
Electron microscope and X-ray	15.2	270	4.0

It has been shown elsewhere that the infectivity of tobacco mosaic virus is associated with the virus nucleoprotein particles.[31] Whether or not these particles are in a molecular state of dispersion is another question. In view of the fact that Preparation A has been shown to be at least nearly homogeneous, this question has little bearing upon the accuracy with which the average particle dimensions can be determined and upon the goodness of the data here presented as a test of indirect physico-chemical procedures. Nevertheless, the question is of considerable interest in its own right. Unless they can be shown to be artefacts, the distribution of lengths found in the electron micrographs and the spreading of the boundary observed by means of the ultracentrifuge would seem to indicate that even the most nearly homogeneous preparations of tobacco mosaic virus yet obtained are not strictly monodisperse. This would rule out the possibility that the virus preparations are in a molecular state of dispersion analogous to that of a solution of a simple organic compound, but it would not rule out the possibility that the tobacco mosaic virus preparations represent mixtures of very similar molecules analogous to the situation encountered in preparations of many natural and synthetic polymers. All of the chemical evidence relative to tobacco mosaic virus is consistent with the assumption of molecular dispersion. It seems to the author that as yet no really convincing experiment has been reported which shows that the primary tobacco mosaic virus particles, those about 270 mμ long, are not in a molecular state of dispersion, but neither has it been possible to obtain satisfactory evidence that they are. In the case of tomato bushy stunt virus, however, a strong defense can be made for the assumption that the nucleoprotein particles are molecularly dispersed.[22] The terminology used in this paper is that of the chemist. If it is eventually satisfactorily demonstrated that tobacco mosaic virus is not molecularly dispersed, the term "molecular weight" as here used will have to be reinterpreted slightly to mean merely the average ratio of the weight of tobacco mosaic particles to that of the hydrogen atom.

(31) M. A. Lauffer, *J. Biol. Chem.*, **151**, 627 (1943).

IX. Summary

Two essentially monodisperse preparations of tobacco mosaic virus were found to have intrinsic viscosities of 39.0, sedimentation constants corrected to water at 20° of about 185 Svedberg units, and one was found to have a diffusion constant corrected to water at 20° of 5.3×10^{-8} cm.^{2}/sec. The partial specific volume was determined on two chemically purified preparations to be 0.73. The size and shape of the predominating particles were calculated from various combinations of the above constants. The results of all possible combinations were found to be in excellent agreement and to be in accord with direct measurements from an electron micrograph of the preparation.

A partially aggregated preparation of virus was found to have a bimodal distribution of particle sizes, as determined from an electron micrograph, and an intrinsic viscosity of 80.7, and to have two boundaries in the ultracentrifuge with sedimentation constants of 187 and 216*s*. It was shown that the sedimentation, viscosity, and electron micrograph data were mutually consistent when interpreted in terms of the theories under examination in this study. A highly aggregated virus preparation was found to have a very high sedimentation rate and intrinsic viscosity.

The excellent agreement found in this study between the results of indirect physico-chemical procedures and direct observation with the electron microscope affords strong evidence of the reliability of the methods of determining the size and shape of particles within the colloidal range based upon combinations of viscosity and sedimentation, viscosity and diffusion, and sedimentation and diffusion studies.

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