The basic solution after extraction was acidified with hydrochloric acid and again extracted with ether. After drying over magnesium sulfate, the ether was removed from the extracts to give 3.4 g. (84% based on reacted VI) of IX, melting at 205–207°. Recrystallization from dioxane increased the melting point to 255–258°. Mixture with VIII depressed the melting point to 224–231°. Titration of an ethanolic solution of IX with 0.01 *N* sodium hydroxide solution gave a neutral equivalent of 329 (IX requires 331.1).

Anal. Calcd. for $C_{13}H_8O_6Cl_2$: C, 47.15; H, 2.44. Found: C, 47.08; H, 2.51.

Absorption Spectra.—Absorption spectra were measured using freshly prepared 1×10^{-4} molar solutions. Solvent for compounds VI, VII and XI was Eastman spectro grade 2,2,4-trimethylpentane; that for compounds V and X was heptane purified according to the procedure of Weissberger.[14]

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[CONTRIBUTION FROM THE VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA]

Ultracentrifuge Studies on Tobacco Mosaic Virus

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In view of the recent progress in the theoretical and technical aspects of ultracentrifugation it seemed of interest to re-examine solutions of purified tobacco mosaic virus in order to obtain information about the uniformity of the virus particles. Experiments are described which demonstrate the existence of boundary sharpening for solutions containing rod-like particles. The importance of this effect in the analysis of the shape of boundaries is discussed in relation to the determination of homogeneity. Evidence from boundary spreading experiments is presented to show that the virus preparations examined in this work are more homogeneous than those examined earlier. The existence of an anomaly in the centrifugation of mixtures is described, and its use in the detection of small amounts of trailing components is demonstrated. The virus preparations used in the present study are shown to contain not more than about 1% of monodisperse $^8/_4$ length particles. The results of this work are discussed in relation to the recent electron microscope studies of Williams and co-workers which indicate that the virus particles in solution are uniform with respect to length.

Introduction

The uniformity of tobacco mosaic virus particles has been the subject of much study and controversy. In addition, considerable attention has been directed toward the establishment of the identity of virus activity with the rod-like particles isolated from virus diseased tobacco plants. To some extent, experimental data relating to these problems have been conflicting; and, further, even the same data have been variously interpreted by different investigators. Some investigators have suggested that the virus particles have different lengths, varying from about 35 to 1000 mμ, any one of which or most of which possess biological activity.[1,2,3] Other research workers contend that there is a minimum length particle of about 300 mμ which possesses biological activity.[4–8]

The first point of view is based primarily upon the observation that particles of widely varying length are seen in electron micrographs of presumably purified virus. In addition, different results have been obtained from time to time by different investigators and even by the same workers when different virus preparations are studied in the ultracentrifuge, the diffusion cell, viscometers of different types, and in apparatus used for measurements of double refraction of flow.[3,9] Most of these inconsistent physical chemical data were obtained in the period of years immediately following the isolation and crystallization of tobacco mosaic virus by Stanley.[10]

On the other hand, more recent studies on different methods of isolation accompanied by viscosity and sedimentation analyses of the purified virus have yielded consistent and reproducible results suggesting uniformity of the virus particles with respect to length. The problem of aggregation has been studied by viscosity techniques. Although there has been little progress in developing a theoretical explanation for this characteristic end-to-end aggregation, empirical methods have been found which appear to reduce substantially the tendency of the virus particles to aggregate.[11,12]

Despite the progress in virus research there still appears to be a discrepancy between the sharp boundaries obtained in the ultracentrifuge for purified virus and the rather broad distribution of particle lengths as observed in the electron microscope. In view of the development of experimental techniques for the purification and study of the virus as well as the increase in knowledge with regard to the theoretical interpretation of ultracentrifuge and other physical chemical data, re-examination of this problem seemed advisable. This paper presents the results of an attempt to obtain more quantitative information about the uniformity of the rod-like particles and the results are discussed in the light of the electron microscope studies of Williams and co-workers.[13,14]

Materials and Methods

The tobacco mosaic virus preparations used in this study were obtained from juice extracted from diseased Turkish

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tobacco plants. The virus was purified by a series of cycles of alternate high- and low-speed centrifugation according to the method of Stanley.[15] The pellets obtained from the first two high speed centrifugation cycles were redissolved by soaking overnight in 0.1 *M* phosphate buffer at *p*H 7.0. Distilled water was used to dissolve the pellets from the 3rd and 4th centrifugation cycles and the final solution was stored in distilled water. The southern bean mosaic virus used in these studies was purified by a similar method and was placed at the disposal of the author through the kindness of Dr. Max A. Lauffer of the University of Pittsburgh. The degraded virus used in these studies was obtained by reacting tobacco mosaic virus with sodium dodecyl sulfate. Details of the preparations of this material will be the subject of another communication from this Laboratory. All ultracentrifuge studies were performed in a Spinco ultracentrifuge equipped with a Philpot–Svensson optical system.

Boundary Spreading Experiments

It has been pointed out by Lauffer[16] and others that the sedimentation constant of rod-like particles with axial ratios greater than 10 is relatively insensitive to length. Therefore, sharp boundaries in the ultracentrifuge would be observed even if there were slight variations in the lengths of the particles. From calculations involving the Perrin equation and the sedimentation equation,[17] the sedimentation constant can be related to length.

In addition to this insensitivity of sedimentation constant to length, it is well known that anisometric particles like tobacco mosaic virus show a marked dependence of sedimentation constant on concentration. This dependence must be considered in any quantitative analysis of a sedimenting boundary where the concentration changes from zero to the concentration of the solution being studied. Since the sedimentation constant, s, decreases with increasing concentration, C, and the solvent side of the boundary is at a lower concentration than the solution side, the trailing particles will have higher sedimentation rates than the leading particles and there will be a pronounced sharpening effect.[18,19] This effect results in a sharpening of the boundary as the particles move down the cell. Figure 1 shows corresponding pictures from two different studies of the sedimentation of tobacco mosaic virus in 0.01 *M* phosphate buffer at *p*H 7.0. In the first run the ultracentrifuge was operating at 5435 r.p.m. and boundary spreading, primarily due to diffusion, is clearly evident. In the second run, another sample of the same virus solution was run first at 5435 r.p.m. to allow the boundary to spread due to diffusion and then the machine was accelerated to 23,150 r.p.m. to increase the rate of sedimentation. The second, third and fourth pictures show clearly how the boundary sharpens progressively as it moves across the cell. This progressive sharpening is not recognized if the ultracentrifuge operates at 23,150 r.p.m. from the start since the boundary is exceedingly sharp throughout the run. It is imperative, therefore, that these facts be considered in any attempt to use the classical method of Svedberg and Pedersen[17] to account for boundary spreading in terms of the diffusion coefficient of the sedimenting particles. This method should be used only when s and D, the diffusion coefficient, are dependent on C or when the functions relating s and D to C are thoroughly established. Spreading of the boundary due to diffusion is proportional to $\sqrt{t}$ where t is the time after reaching speed. On the other hand, sharpening of the boundary due to the variation of s with C is a function of the distance travelled which, in turn, is directly related to the square of the angular rotation of the centrifuge and time, t. Therefore, different results would be expected at different rotor speeds and extrapolation to zero centrifugal field would be necessary to eliminate completely the effect of boundary sharpening. Another means of correcting for the sharpening effect is to study the boundary spreading as a function of concentration and extrapolate the results to infinite dilution. This becomes complicated because of the change in D as a function of C. In view of the absence of reliable data relating D to C for tobacco mosaic virus, and because of the difficulty of completely eliminating vibrations and precessional motion of the rotor at the low speeds required for these studies, this approach has been abandoned temporarily.

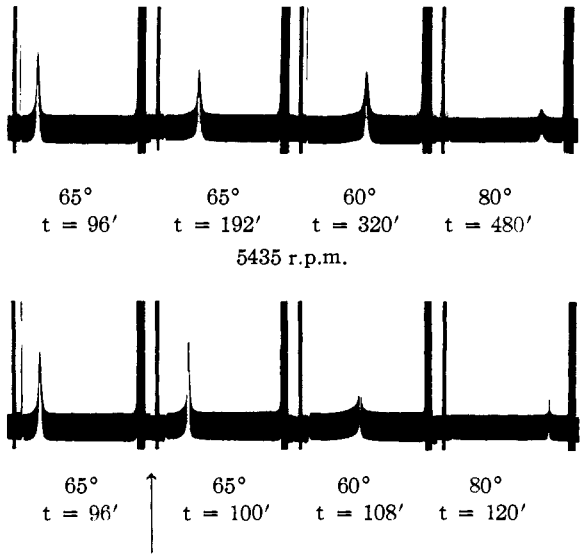

Fig. 1.—Boundary sharpening in the ultracentrifuge. Ultracentrifuge patterns of two different runs of TMV in 0.01 *M* phosphate buffer at *p*H 7.0. Concn. 0.4 g./100 cc.

In order to determine the possibility of changes in orientation of the rod-like particles and consequent changes in the frictional resistance of the particles at different speeds, the sedimentation constant was determined at different centrifugal fields. The results shown in Table I, indicate that no change in orientation of the particles occurs in the range of centrifugal fields examined.

TABLE I

SEDIMENTATION CONSTANT OF TOBACCO MOSAIC VIRUS AS A FUNCTION OF CENTRIFUGAL FIELD

concn. = 0.4 g./100 cc., 0.01 *M* phosphate at *p*H 7.0

Average centrifugal field no. of gravities × 10^{-3}	s^0_{20}, Svedbergs
2.1	163
5.0	169
11.3	171
22.9	170
38.2	171
54.6	169
79.5	170

It is important to note that the preparations of purified virus examined in this study show far less boundary spreading than those examined by Lauffer,[16] and Schramm and Bergold.[20] The apparent diffusion coefficients obtained by these workers from the sedimentation diagrams were greater than 4.5 × 10^{-8} cm.²/sec. whereas the results from the present study were much less than that value. Lauffer's measurements were performed at a centrifugal field about 3900 times gravity and the studies of Schramm and Bergold were made at a field about 29,000 times gravity. In the present study, the apparent diffusion coefficients were calculated from sedimentation experiments at a centrifugal field about 2100 times gravity. Since this centrifugal field was less than that employed by Lauffer and by Schramm and Bergold, the sharpening of the boundary should be less in this work and the apparent diffusion coefficient should be higher than that observed by the previous workers. That the apparent diffusion coefficient was much lower indicates that our virus preparations were more homogeneous than

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those studied earlier. This enhanced homogeneity is due presumably to the solvent treatment during the isolation and the salt concentration used during the study.

Since the diffusion coefficients calculated from the sedimentation diagrams decreased as the boundary moved through the cell, as shown in Table II, the sharpening effect was important even at the low centrifugal field used in this study. Pickels[19] suggested decelerating the rotor to very low speeds after the boundary had been brought into view at a higher speed. Then the diffusion process could be studied with appreciable migration of the boundary. The speed required for such an experiment on tobacco mosaic virus is so low that practical difficulties prevented the study.

TABLE II

APPARENT DIFFUSION COEFFICIENT OF TOBACCO MOSAIC VIRUS CALCULATED FROM ULTRACENTRIFUGE PATTERNS

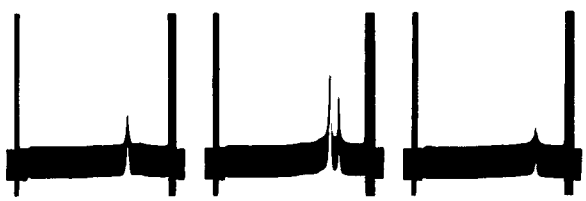
concn. = 0.22 g./100 cc., 0.01 *M* phosphate at *p*H 7.0

Distance of boundary from axis of rotation, mm.	Time, sec. × 10^{-4}	D^0_{20} max. ord. area, cm.²/sec. × 10^{13}	D^0_{20} infl. point, cm.²/sec. × 10^{13}
61.0	0.768	4.1	3.9
63.2	1.344	3.1	3.3
65.5	2.112	2.5	2.0
67.8	2.500	1.6	2.0

If the ultracentrifuge curve were more diffuse than that expected from independent diffusion data, we could state unambiguously that the virus is inhomogeneous. However, the apparent diffusion coefficient calculated from the sedimentation diagrams was lower than the reported diffusion coefficients.[16,20] Since some boundary sharpening occurs we cannot, from this type of study, attach limits to the distribution of particle lengths in the virus solution.

Detection of Other Components in Mixtures Containing Tobacco Mosaic Virus

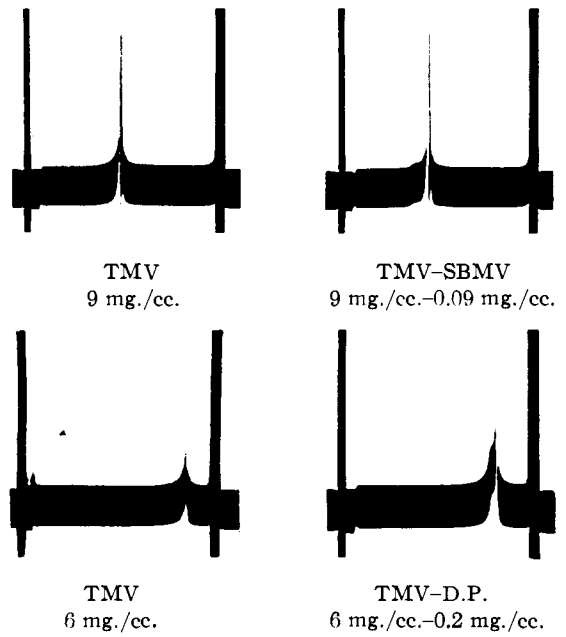
In the course of a detailed study of the analysis of mixtures in the ultracentrifuge it became clear that some information could be obtained regarding the presence in our solutions of small amounts of particles shorter than the main component. Degradation of tobacco mosaic virus by a variety of chemicals such as urea, mild alkali, and detergents has been shown to produce such particles. Particles of different sizes can be obtained from reactions at *p*H 9.5 to 10.0, but it is very difficult to obtain fractions composed mainly of particles of a single size. In sodium dodecyl sulfate solutions, however, particles were obtained most of which were of two distinct sizes which could then be separated into two well-defined fractions. With the appropriate ratio of detergent to virus, the virus particles were degraded to particles with sedimentation constants about 85% and 1.5% that of the virus. These were separated and the fraction composed of the larger particles was studied in the ultracentrifuge, viscometer, and electron microscope. Calculations from the data and observation in the electron microscope indicate that the particles are fairly uniform with a length about 70% of the most commonly observed intact virus particle. Figure 2 shows an ultracentrifuge pattern of the larger product of degradation (D.P.) and the virus from which it was obtained. These were both at the same concentration. In addition there is the pattern of the mixture of the two at equal concentration. It is apparent that the area under the curve corresponding to the D.P. boundary is larger than that of the virus boundary. Mixtures containing varying amounts of each component are being studied in detail[21] in the ultracentrifuge and viscometer to see whether the discrepancy between expected and observed areas in the ultracentrifuge patterns can be explained quantitatively by the theory of Johnston and Ogston.[22] According to these workers a D.P. particle in front of the virus boundary is sedimenting in a region of higher viscosity than a D.P. particle behind the boundary. Because of this difference in viscosity, the D.P. particle will sediment at a slower rate in front of the virus boundary than behind it. This results in a build-up of the D.P. concentration behind the virus boundary leading to the enhanced "apparent concentration." Since this effect begins as soon as the boundaries leave the meniscus and it continues during the migration of the particles throughout the run, the region between the two boundaries at any time will have a uniform concentration of D.P. particles which is higher than the concentration of similar particles in front of the virus boundary. This change in D.P. concentration at the virus boundary leads to a negative refractive index gradient which partially cancels the gradient due to the virus. One area is therefore enlarged and the other diminished. Thus a mixture known to contain equal parts of D.P. and virus might appear to be 3:1 mixture of D.P. and virus, respectively. Since it is the trailing component that is always magnified in appearance, experiments were designed to attach a lower limit to the amount of a trailing component that would be detectible.



D.P. 4.5 mg./cc. D.P.—TMV 4.5 mg./cc.–4.5 mg./cc. TMV 4.5 mg./cc.

Fig. 2.—Analysis of mixtures in the ultracentrifuge. Ultracentrifuge patterns of D.P., D.P. and TMV, and TMV in 0.01 *M* phosphate buffer at *p*H 7.0. Concn. of each substance 0.45 g./100 cc.

Figure 3 shows the controls and mixtures of solutions containing one part of southern bean mosaic virus with 99 parts of tobacco mosaic virus and two parts of 3/4 length particles with 60 parts of virus. It is clear from the patterns that the small amounts of the trailing components are readily discernible. Ultracentrifuge studies of the trailing components alone at comparable concentrations failed to show definite boundaries because the refractive index gradients were too small to be observed by the optical system. The ability to detect the smaller particles is due to the enhancement of the concentration of the trailing component caused by the tobacco mosaic virus particles.



TMV 9 mg./cc. TMV–SBMV 9 mg./cc.–0.09 mg./cc.

TMV 6 mg./cc. TMV–D.P. 6 mg./cc.–0.2 mg./cc.

Fig. 3.—Detection of small amounts of added impurity in the ultracentrifuge. Ultracentrifuge patterns on TMV and TMV–SBMV mixture and TMV and TMV–D.P. mixtures in 0.01 *M* phosphate buffer at *p*H 7.0.

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(22) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

Discussion

It is well known that the process of differential centrifugation is inefficient as a method of fractionating particles of different sedimentation rates. It is to be expected, therefore, that the final solution of purified virus would contain a distribution of particles which is related to the distribution in the original juice expressed from the plants. Differences in distribution of particle lengths between the original and final solutions would arise due to alterations of the particles caused by aggregation and rupture, or denaturation and insolubilization of the particles during the isolation procedure. Particles having sedimentation rates much larger and much smaller than those of the principal components would be virtually eliminated in the course of the four cycles of alternate high- and low-speed centrifugation.

If we were to assume that the final preparation contained particles of different sizes, it would be important to determine the sedimentation rate of the infectious principle in an effort to correlate this biological activity with a specific particle. Lauffer,[5] using the separation cell in the ultracentrifuge, demonstrated that the infectious agent had a sedimentation constant approximately equal to that observed for the principal component composed to particles with lengths calculated to be about 270 mμ.

Another approach to the problem of the uniformity of the virus particles would be the use of the ultracentrifuge in an attempt to determine the limits of the variation in particle lengths in solutions of purified virus. Sedimentation velocity experiments using solutions of tobacco mosaic virus indicate that there is a marked boundary sharpening effect. In view of this effect and because of the unreliability of existing diffusion data on virus solutions, no unequivocal statement can be made now from ultracentrifuge patterns in regard to the uniformity of virus particles. However, the electron microscope investigations of Williams and co-workers[13,14] provide useful information about the limits in the variations of particle lengths. Nearly all electron micrographs of purified tobacco mosaic virus show some particles which are shorter than 300 mμ. The results of Williams and co-workers using unpurified material indicate that these short particles probably arise from breakage of uniform virus particles during the process of drying the specimen for examination in the electron microscope. Our ultracentrifuge studies on mixtures of virus and degraded particles support the findings of Williams, *et al.* If $^3/_4$ length particles are mixed with virus then two boundaries will be observed in the ultracentrifuge. Even when $^3/_4$ length particles are present to the extent of only 2 parts to 98 parts of virus, the ultracentrifuge can be used to detect the shorter particles. These studies indicate that our recent preparations do not have monodisperse $^3/_4$ length particles present in amounts greater than about 1% of the total. Similarly, the experiments using southern bean mosaic virus as the added "impurity" indicate that mixtures containing 1 part of it with 99 parts of tobacco mosaic virus are readily differentiated from solutions containing only tobacco mosaic virus. The spherically-shaped southern bean mosaic virus particles have a sedimentation constant of about 115 *S*, which is approximately that calculated for a rod $^1/_5$ the length of tobacco mosaic virus. It is hoped that further degradation experiments on tobacco mosaic virus will yield particles of that length in reasonably pure form, so that further mixture studies can be performed.

At the present time we do not have a collection of particles ranging in length from about $^1/_{10}$ to $^3/_4$ that of tobacco mosaic virus so that the ability of the ultracentrifuge to detect a broad distribution of short particles has not yet been tested. Using β-lactoglobulin, kindly supplied by Dr. T. L. McMeekin, the optical system of the centrifuge has been checked for agreement between the observed area under the boundary and that determined from the protein concentration and the refractive index increment. Preliminary experiments at several different virus concentrations indicated that the area under the curve accounted for about 95% of the material as determined by Kjeldahl analysis.

It is important to note that other workers[8,23,24] obtain greater yields of virus due to additional treatment of the pulp remaining after expressing the plant juice. Studies of the type described here must be performed on the virus solutions prepared by these other methods before a detailed statement can be made about the uniformity of such virus preparations.

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