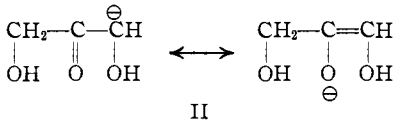
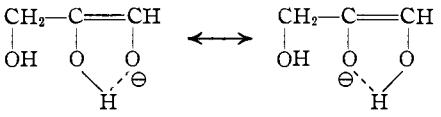


II

alternative, less likely, possibility is that both I and II are resonance forms of the same substance due to hydrogen bonding, *e.g.*



If the rate-determining step involves a neutral molecule, as suggested by the primary salt effect data, this step may be the attack of a hydroxyl ion on either the glyceraldehyde molecule or the neutral enediol.

It should be noted that, although the starting materials were dimeric D,L-glyceraldehyde and dihydroxyacetone, the rates of depolymerization should not have to be taken into account. The rate of depolymerization of dimeric dihydroxyacetone has been found to be[20]

$$k = 0.00255 + 4.03 \times 10^{7}[OH^{-}]\text{min.}^{-1}$$

and that of dimeric glycolaldehyde[21]

$$k = 0.0073 + 3.15 \times 10^{6}\,[OH^{-}]\text{min.}^{-1}$$

Furthermore, our experiments showed no difference in reaction rate between freshly prepared solutions of glyceraldehyde and those which had stood at room temperature for two weeks.

Acknowledgments.—We wish to express our appreciation to Dr. G. Forrest Woods, of the University of Maryland, for his interest in this work, and to Mr. M. L. Peller for the preparation of the anthrone.

(20) R. P. Bell and E. C. Baughan, *J. Chem. Soc.*, 1947 (1937).
(21) R. P. Bell and J. P. H. Hirst, *ibid.*, 1777 (1939).

SILVER SPRING, MARYLAND RECEIVED OCTOBER 2, 1950

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICS, UNIVERSITY OF MICHIGAN]

Electron Microscopic Observations on the Unit of Length of the Particles of Tobacco Mosaic Virus[1]

BY ROBLEY C. WILLIAMS[2a] AND RUSSELL L. STEERE[2b]

The electron microscope is used to gain evidence of the degree of uniformity of length of the rod-like particles of tobacco mosaic virus. Small droplet patterns, obtained by spraying the virus suspension upon specimen screens, are photographed and the lengths measured of all virus rods in the patterns. Most of the lengths are uniform as measured. The lengths of the few non-uniform rods in each pattern are summed, and the sum divided by an integer. It is found that the division can be performed without remainder in almost all cases, and it is concluded that in the partially purified suspensions examined over 96% of the virus particles exist as either monomers of very uniform length, or as dimers. The length of the monomer of tobacco mosaic virus is found to be 298 ± 1 mμ.

Introduction

In recent years many investigators have examined the distribution in length of the particles of tobacco mosaic virus (TMV) by the aid of the electron microscope. A review of the literature up to the close of 1945 is included in a paper by Sigurgeirsson and Stanley[3] on the distribution in length of the virus particles in unpurified, fresh suspensions and in unpurified, stored suspensions. Since 1945 several papers[4,5] have appeared in which the distribution in length of the TMV particles has been studied as a function of the age of the virus infection, the method of extraction from the plant cells, the *p*H and ionic strength of the extraction and suspending media, and the method of purification of the virus particles.

While the details of the reported observations appear to be somewhat contradictory and confusing, certain generalizations can be made: (1) one type of particle (to be here called the monomer), about 290 mμ in length, is usually found in greatest abundance, with a secondary abundance maximum at twice this most common length; (2) some particles considerably shorter than the monomeric length are always found; (3) any post-extraction treatment of the virus suspensions, such as purification or severe changes in *p*H, causes the distribution curve of lengths to broaden; (4) even for the monomer a fairly broad distribution of lengths is reported, of the order of 100 mμ at the foot of the distribution curve of these particles.

In the investigations concerning the lengths of the TMV particles, as measured on electron micrographs, there is seen a tendency to identify the lengths as measured with those that are presumed to exist in the virus suspensions themselves. It is to be anticipated, however, that in the preparation of electron microscopic specimens the effects of the rapidly varying interfacial surface tensions, which must accompany the drying of a large drop of aqueous material, could radically change the distribution in length of particles as elongated as those of TMV. Such a change might result from the breakage or the aggregation of the particles, or even from multiple events such as aggregation followed by breakage. The net result of these changes *must always be to broaden* the distribution

(1) This research has been supported in large part by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

(2) (a) The Virus Laboratory, University of California, Berkeley, California; (b) Rockefeller Institute for Medical Research, New York City, New York.

(3) Sigurgeirsson and Stanley, *Phytopath.*, **37**, 20 (1947).

(4) Crook and Sheffield, *Brit. J. Exp. Path.*, **27**, 328 (1946); Oster and Stanley, *ibid.*, **27**, 261 (1946).

(5) Takahashi, Rawlins and collaborators, *Am. J. Bot.*, **33**, 356 (1946); *Phytopath.*, **37**, 73 (1947); **38**, 279 (1948); **39**, 672 (1949); *Am. J. Bot.*, **36**, 642 (1949); *J. Bact.*, **57**, 131 (1949).

of lengths, and hence it can be concluded that the electron micrographic measurements have consistently presented a conservative picture of the degree of uniformity existing in the virus suspensions themselves.

Another source of uncertainty in the evaluation of the distribution of lengths as measured on electron micrographs is the difficulty of choosing "representative" fields to photograph and measure. Even the most carefully prepared microscope specimen screens will have gross variations in particulate morphology from one area to another, and it can only be hoped that the fields chosen for measurement are typical.[6] A partial solution to the problem of uncertainty of representation is to photograph many fields at "random." But true selection is not possible; the areas under the wires of the specimen grid are unobservable, and owing to different conditions of drying may contain particulate patterns quite unlike those in the accessible parts of the specimen. It is frequently observed that a microscope specimen of a fairly concentrated suspension of purified TMV is more densely populated with particles near the grid wires than near the centers of the grid openings, and that the peripheral areas of a large aqueous drop, upon drying, are grossly overpopulated with virus particles. Since it remains to be demonstrated that the *length distribution* of TMV particles is independent of their distribution in *numbers* on the specimen screens, it is apparent that, in studies of length distribution, true randomness of selection may be impossible.

A preparative method which eliminates some of the difficulties associated with non-representative fields has recently been reported by Backus and Williams.[7] In this technique the microscope specimens are prepared by spraying minute droplets of suspension on the specimen screens, with resultant dried droplet patterns small enough to be photographed in their entirety. It has recently been suggested to us by Dr. S. E. Luria that this technique might be adapted in some cases to a study of the degree of identity between the size and shape of particles as they exist in an original suspension and as they appear in electron micrographs from the same suspension. The essence of the suggestion is this: A droplet of virus suspension leaving the orifice of the spray gun might be expected to contain virus particles with a distribution of shape and size identical with that in the suspension as a whole. After the droplet has dried on the specimen screen the distribution in size and shape of the virus particles may have been altered by forces of surface tension and convection. But if particles of only one size are present in the droplets as they leave the spray gun it should be possible to fit together all the fragments as observed in a micrograph of any one droplet pattern in a way such as to yield an integral number of particles of uniform size. If, on the other hand, a precise fitting of the fragments cannot be made, without remainder, the evidence is good that the particles in the droplets, and in the original suspension, are relatively non-uniform.

Certain requirements must be met in order that this method of analysis can be safely applied to a study on uniformity of length of TMV particles. In the first place all the particles in any one droplet pattern must be measured, and all droplet patterns which are photographed must be analyzed (if they are of adequate technical quality); that is, no selection can be exercised as to which particles or which droplet patterns shall be measured. Secondly, dilutions must be so adjusted that the number of particles in each droplet pattern is small, in order to reduce the number of possible ways in which non-uniform lengths can be combined. In the third place, a rigorous limit must be set upon the failure of fitting: the amount by which the sum of the lengths of a number of non-monomers can differ from that of an integral number of mean lengths of the monomers. A safe working limit is established by a requirement that the distribution curve of monomer lengths obtained by fitting be no broader than the distribution curve of the measured lengths of the monomers that are observed to require no fitting.

There are severe limitations upon the type of information to be gained by the application of the "fitting" method described above. If the measurement yields only a small percentage of precise fits, with many particle fragments unaccounted for, it is safe to conclude that there is polydispersity in the original suspension, or at least in the droplet issuing from the spray gun. If the degree of fitting is very good, with very few particles left over, there is still ambiguity in those cases where frequently it is found that the sum of the fragment lengths in any given droplet pattern is equal to two or more monomeric lengths. It can be concluded that in this case there are only monomers, dimers, ... etc., in the suspension (*i.e.*, that the suspension is not randomly polydisperse), but there seems to be no way of determining the relative percentage of particles of each length. This uncertainty is due to lack of knowledge as to whether the disturbing forces on the particles during droplet desiccation are mainly aggregative, disruptive, or both.

Experimental

The Virus Suspensions.—Young White Burley tobacco plants were inoculated with crude juice pressed from plants infected with a wild strain of tobacco mosaic virus, and small sections of the leaves of several plants were harvested at the end of 3 days and 5 days following inoculation. The leaf sections were immediately homogenized in a mechanical, glass homogenizer, and the resulting pulp and juice diluted tenfold with distilled water. The material was then heated to 50° for 5 minutes, cooled to room temperature, and centrifuged for 5 minutes at about 3000 g. The relatively clear supernatant liquid was then diluted for spraying upon the microscope specimen screens. In the preparation to be called A a further dilution of about 400:1 was made with a diluent of normal tobacco plant juice which had been previously clarified and diluted 160:1 from its concentration as expressed from the leaves. In Preparation B the dilution was 64:1 with distilled water. The large difference in the dilution factors for Preparation A and B is due to the circumstance that A was harvested 5 days after inoculation

(6) An example of the difficulty of finding truly representative fields can be found in the paper by Oster and Stanley.[4] Their nomograph, based on about 300 particles, shows that only about 9% are shorter than 200 mμ. Yet, in the accompanying photograph of a field containing about 110 particles, it is readily determinable that about 25% are shorter than this length.

(7) Backus and Williams, *J. Appl. Phys.*, **21**, 11 (1950).

and B was harvested 3 days after inoculation. All specimen screens were shadowed with about 10 Å. of uranium at a 3:1 angle.

Measurements of Electron Micrographs.—The lengths of the virus particles were determined by measuring the original micrographs with the aid of a × 15 microscope fitted with an ocular scale. The coincidence of the real image of the photographic film with the ocular scale was frequently tested by searching for a parallax displacement between image and scale. The scale was calibrated by the measurement of micrographs containing unshadowed polystyrene latex particles[8] of known size. Only those latex particles near the center of the micrograph field were measured for purposes of calibration. The photographs of the virus and of the latex particles were obtained at nearly the same values of the objective and projector currents in the microscope, and the effect on magnification of small variations in these currents, from photograph to photograph, was corrected for. The mean magnification of the micrographs was × 2500, and the over-all magnification as seen through the measuring microscope was × 37,500. The only selection of the micrographs to be measured was the rejection of a few of inferior technical quality.

A correction factor for field distortion[9] in the micrographs was established and applied to each measurement of length of the virus particles. This correction is necessary for micrographs of such low magnification and in which measurements must be made as far as 2 cm. from the magnetic center of the electron image. In the present case involving the measurement of rod-shaped objects the correction is particularly laborious, since the values for tangential and radial distortion are different and must be separately corrected for. The corrections were made in the following way: Curves for radial and for tangential distortion of the electron microscope image were constructed according to the method of Hillier.[9] The length of the image of a linear object not oriented either radially or tangentially is affected by both types of distortion, and one must take the components of length of the particle in these two orthogonal directions and apply the corrections separately. The undistorted length of the particle can then be obtained by taking the vector resultant of the two orthogonal components as corrected. For each TMV particle observations had to be made of its length, radial distance from the magnetic center of the micrograph, and its orientation with respect to the radius vector to the particle. It was found that the corrections could be made from a family of curves in which the net correction for distortion in length was plotted as a function of radial distance for 10 values of orientation between 0 and 90°. The greatest correction was 20%, with most of the corrections lying between 3 and 7%.

TABLE I

LENGTHS OF TOBACCO MOSAIC VIRUS PARTICLES AS MEASURED ON ELECTRON MICROGRAPHS OF SPRAY-DROPLET PATTERNS. PREPARATION A—DILUTION WITH PLANT JUICE

(1) Micrograph no.	(2) No. of particles with lengths between 7.3 and 7.9 scale units	(3) Lengths of all particles not included in Col. (2)			(4) Sum of lengths in Col. (3) ÷ an integer	(5) Lengths not accounted for in Col. (4)
1775a	1	10.8	10.3	1.5	7.54 (3)	
b	12	9.2	3.7	1.8	7.35 (2)	
		6.0	8.5		7.25 (2)	
c	3	15.0			7.50 (2)	
		10.9	10.1	1.7	7.57 (3)	
1776a	7	10.5	5.0		7.75 (2)	
		4.3				4.3
b	2	15.1			7.55 (2)	
c	2	14.6			7.30 (2)	
1777a	1	3.7	10.3	0.9	7.45 (2)	
b	12	4.7	2.9		7.60 (1)	
c	3	3.9				3.9
11070a	5	10.5	4.5		7.50 (2)	
b	11	15.5			7.75 (2)	
		12.6	3.3		7.95 (2)	
11071a	5	14.8			7.40 (2)	
		14.7			7.35 (2)	
b	9	14.0	9.3		7.78 (3)	
c	7	10.1	2.6	2.5	7.60 (2)	
Sums of numbers of particles	80		35		36	2

(8) Backus and Williams, *J. Appl. Phys.*, **20**, 224 (1949).
(9) Hillier, *ibid.*, **17**, 411 (1946).

Results

The results of the corrected measurements of the lengths of the TMV particles are presented in Tables I and II, and in the accompaying distribution curves of lengths. Table I lists results of the measurement of all particles observed on micrographs of Preparation A. The lengths are here expressed in terms of divisions on the ocular scale of the measuring microscope, and can be changed to millimicrons on the specimen by multiplying by 38.8. Column (2) of the tables lists the number of particles in each droplet pattern within the range of lengths indicated (an 8% spread). These particles are considered to be the monomers. Column (3) gives the individual lengths of all particles not falling within the range shown in Column (2).

TABLE II

LENGTHS OF TOBACCO MOSAIC VIRUS PARTICLES AS MEASURED ON ELECTRON MICROGRAPHS OF SPRAY-DROPLET PATTERNS. PREPARATION B—DILUTION WITH DISTILLED WATER

(1) Micrograph no.	(2) No. of particles with lengths between 7.4 and 8.0 scale units	(3) Lengths of all particles not included in Col. (2)		(4) Sum of lengths in Col. (3) ÷ an integer	(5) Lengths not accounted for in Col. (4)
1787a	2	15.5		7.75 (2)	
		8.7	6.6	7.65 (2)	
		15.5		7.75 (2)	
b	13	3.3			3.3
		15.4		7.70 (2)	
		15.7		7.85 (2)	
		15.5		7.75 (2)	
1788a	4	15.1		7.55 (2)	
		12.9	2.6	7.80 (2)	
b	7	16.1		8.05 (2)	
		15.5		7.75 (2)	
		15.1		7.55 (2)	
c	10	15.6		7.80 (2)	
		12.8	10.0	7.60 (3)	
		15.0		7.50 (2)	
		15.0		7.50 (2)	
		8.9			8.9
1789a	12	15.4		7.70 (2)	
		10.7			10.7
		15.4		7.70 (2)	
		15.6		7.80 (2)	
b	9	9.8			9.8
		10.7			10.7
c	7	15.0		7.50 (2)	
		9.6			9.6
1790a	7	9.4	6.2	7.80 (2)	
		15.7		7.85 (2)	
		15.5		7.75 (2)	
Sums of numbers of particles	71	32		45	6

In Column (4) are listed the lengths resulting from dividing the sum of the lengths in Column (3) by the integer whose value is shown in parentheses. The last column lists those lengths for which no fit is found. At the bottom of the table are summed the numbers of particles in each column, except for Column (4), where the number of equivalent particles of monomeric length is shown. Table II is constructed similarly for the measurements on Preparation B.

Figures 1a and 2a are nomographs constructed from the measurements of lengths of the TMV particles on micrographs of Preparation A. The particle lengths in Fig. 1a are shown directly as measured and corrected for distortion. In Fig. 2a the monomeric particle lengths and the lengths in Col. (5) of Table I are shown as they are in Fig. 1a, but the non-monomeric particles which fit the scheme exhibited in Column (4) of Table I are now shown in terms of their equivalent monomeric lengths. The purpose of plotting the results as in Fig. 2a is to exhibit how few particles lie outside this scheme of fitting, and to show how closely the sums of the lengths of the fragments fit integral numbers of monomeric lengths. In Figs. 1b and 2b are plotted similar distribution curves for Preparation B.

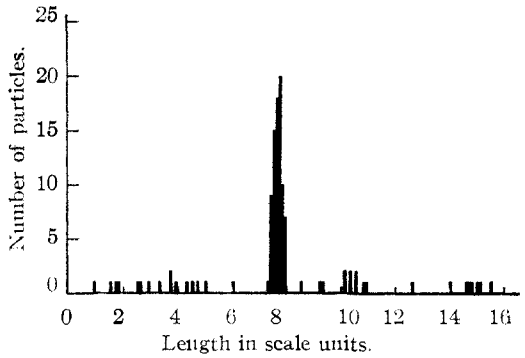


Fig. 1a.—Distribution of particle lengths of TMV as measured on electron micrographs of droplet patterns from Preparation A. One abscissa scale unit = 38.8 mμ.

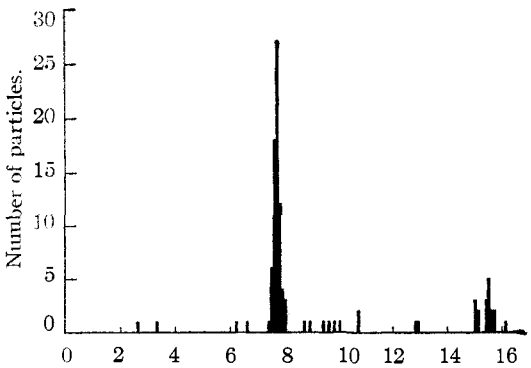


Fig. 1b.—Preparation B.

The arithmetic mean of the lengths of the single TMV particles falling in the monomeric range (from 7.3 to 8.0 scale units) is 7.65 scale units for Preparation A, and 7.70 scale units for Preparation B. The calibration of the eye-piece scale is based upon measurements made at this Laboratory and elsewhere[10] of the mean diameter of unshadowed polystyrene latex particles. The adopted value of 259 mμ for this diameter yields a scale calibration of 38.8 mμ/scale division. The mean lengths of the TMV rods are found to be 297 mμ in Preparation A and 299 mμ in Preparation B. The probable error of a *single* measurement of length is ± 10 mμ, and the probable error of the *mean length* of the 150 monomer particles measured is ± 1 mμ.

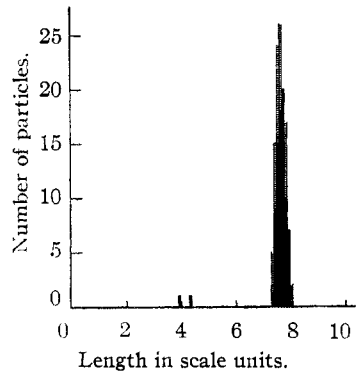


Fig. 2a.—Distribution of lengths of TMV particles from Preparation A, obtained by summing the lengths of the fragments in each droplet pattern and dividing by an integer. Monomeric particle lengths are shown in solid black. One abscissa scale unit = 38.8 mμ.

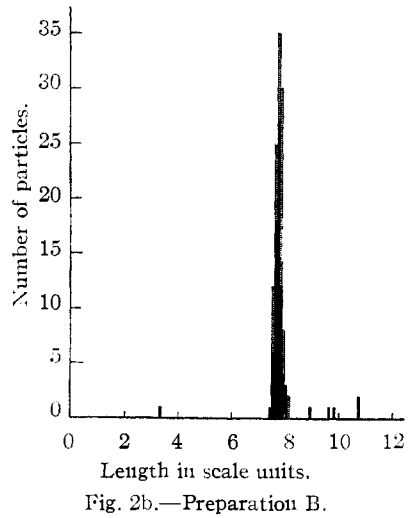


Fig. 2b.—Preparation B.

Discussion

The major results of this investigation are the evidences that: (1) the particles of TMV in our unpurified suspensions exist almost wholly in the form of intact monomers or dimers; (2) the particles which appear as monomers in the electron micrographs have an extremely narrow length distribution, only slightly greater than the estimated errors of measurement.

The first result, of course, has been intimated for years by less precise and unambiguous measurements involving sedimentation, diffusion, viscosity and electron microscopy. There has never been general agreement, however, that the sedimentation

(10) Gerould, *J. Appl. Phys.*, **21**, 183 (1950).

and diffusion experiments are decisive on the matter of uniformity of length, partly because of differences of preparative procedures between laboratories, and partly because the sedimentation diagrams cannot be quantitatively interpreted with precision and confidence. There has been ample evidence of polydispersity in preparations examined at various times, but a clear demonstration of monodispersity or paucidispersity is much more difficult to substantiate. Previous electron microscopic measurements, particularly those made on unpurified suspensions by Sigurgeirsson and Stanley,[3] and by Oster and Stanley,[4] have given results fairly similar to those shown in Figs. 1a and 1b of this paper, but the reasons for the existence of particle lengths which are neither monomeric nor dimeric have been obscure.

The paucidispersity of the virus suspensions yielding the data in Tables I and II seems to be well established, if one grants that enough samples have been measured to afford statistical reliability, and that the closeness of fit of the summed lengths of the non-uniform particles has not come about largely by accident. A total of only 218 particles has been measured, but the measurements have been made on 23 independent samples (droplet patterns) each believed to be highly representative of the suspensions.[8,11] In only one case (micrograph 1775b) has there been any doubt as to the way in which to combine the fragments. As can be seen from Figs. 2a and 2b the spread in lengths of the summed fragments, divided by a small integer, is barely greater than the spread in the lengths of the obviously uniform particles. In Preparation B the indications of duodispersity in the virus suspension are particularly impressive, since 90% of the particles as directly measured in the micrographs and corrected for distortion have either monomeric or dimeric lengths. In Preparation A some 75% are in these categories.

It is to be emphasized that Figs. 2a and 2b are not intended to represent the true distribution of lengths of the virus particles in the suspensions. The existence of dimers, as well as monomers, is clearly indicated by the measurements, but there appears to be no way to determine their relative abundance in the suspension by electron microscopy alone.[12] There does appear to be a clear difference between Preparations A and B, and one might conclude that the effect of dilution with distilled water is to increase the proportion of dimers in Preparation B. But the difference can also be explained by merely assuming that forces causing aggregation during the drying of the droplet are relatively enhanced in the case of the distilled water suspension. Previous investigators[5,13] have observed that aggregation appears to increase with preparative procedures involving suspension of the virus particles in distilled water.

The high degree of uniformity of measured length of the monomeric particles is impressive. An error of at least 5 mμ for a single observation of length is to be expected, owing to small errors of measurement and to the slightly varying amounts of uranium piled up on the ends of the rods. In consideration of the likely errors of the measurements themselves it appears that in our two TMV preparations there exists at least a majority of particles whose average variation in true length is in the neighborhood of only 5 mμ, or somewhat less than 2% of the length of the virus particles.

The foregoing results have an indirect bearing on three interesting and important questions: (1) the degree of monodispersity of highly purified TMV suspensions which are commonly observed to yield fairly sharp, single-component sedimentation boundaries, (2) the degree of precise monodispersity that infectious virus particles must have, and (3) the length of the infectious unit as it exists in the plant cell. The results suggest that almost complete monodispersity is possible in even purified suspensions, and demonstrate that it may now be possible to reconcile sharp sedimentation boundaries with the previously observed polydispersity in electron microscope specimens.

It should also be possible to re-examine more precisely the correlation of infectivity with strict monodispersity in relatively unpurified suspensions. The question as to the length of the infectious unit as it exists in the plant cell is so far unanswerable by any direct approach. Williams and Steere[14] have examined electron micrographs of wholly crude, undiluted juice suspensions of tobacco mosaic virus, and have found bundles of elongated, parallel particles, but these bundles may or may not portray the virus as it exists in the cells. It must be concluded from the results of others, and particularly from the results of the present investigation, that almost all the virus particles readily extractable from the cells by homogenization possess highly uniform lengths of great structural integrity: great enough to withstand the disruptive effects of the homogenization, partial purification, and high dilution. From this, one is led to conclude that the readily extractable virus particles in the cells possess a distinctive, fundamental length (probably close to 300 mμ, but possibly twice this length), although at any one instant a large fraction of them might be found associated together in groups of several unit lengths.

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(11) Williams and Backus, THIS JOURNAL, **71**, 4052 (1949).

(12) Sedimentation studies of others have indicated strongly that, in freshly purified suspensions, only a small fraction of the particles are present as uniform-sized dimers.

(13) Schachman and Kauzmann, *J. Phys. Coll. Chem.*, **53**, 150 (1949).

(14) Williams and Steere, *Science*, **109**, 308 (1949).