STUDIES ON THE SONIC TREATMENT OF TOBACCO MOSAIC VIRUS*

By GERALD OSTER

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

PLATE 4

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INTRODUCTION

Takahashi and Christensen (1) found that the juice of plants suffering from tobacco mosaic virus disease was rendered non-infectious when subjected to intense sonic vibrations. Stanley (2) found that the biological activity of purified tobacco mosaic virus is reduced by sonic treatment and demonstrated that the sound has little or no effect on the activity of the virus if cavitation, normally associated with strong vibrations in liquids, is supressed by a lowering of the atmospheric pressure above the liquid. Kausche, Pfankuch, and Ruska (3) found with the electron microscope that sonic treated tobacco mosaic virus samples contained more short rod-like particles than are observed in untreated samples. The sonic treatment apparently results in a breakage into shorter fragments of the long rod-like particles associated with the tobacco mosaic virus disease and offers a convenient method of studying the relation between the size of the particles and their biological activity.

In experiments described in this paper, samples of centrifugally purified tobacco mosaic virus were subjected to strong sound vibrations for varying lengths of time. The physicochemical properties of the sonic treated material were determined and the material was tested for biological activity. The virus particles as well as the fragments produced by sonic treatment were made to aggregate end-to-end and the properties of the aggregates were studied.

EXPERIMENTAL METHODS AND RESULTS

Sonic Treatment.—Solutions of tobacco mosaic virus purified by differential centrifugation by the method of Stanley (4) were sonic treated in a magneto-striction sound generator operating at 9,000 cycles per second and producing approximately 100 watts of acoustic energy. The apparatus (type R-22-1 oscillator developed by the Submarine Signal Co.) contains an effective water-cooling system so that at no time did the temperature rise above 17°C. The author is indebted to Dr. Thomas Anderson of the Johnson Foundation for the use of this apparatus and also to Dr.

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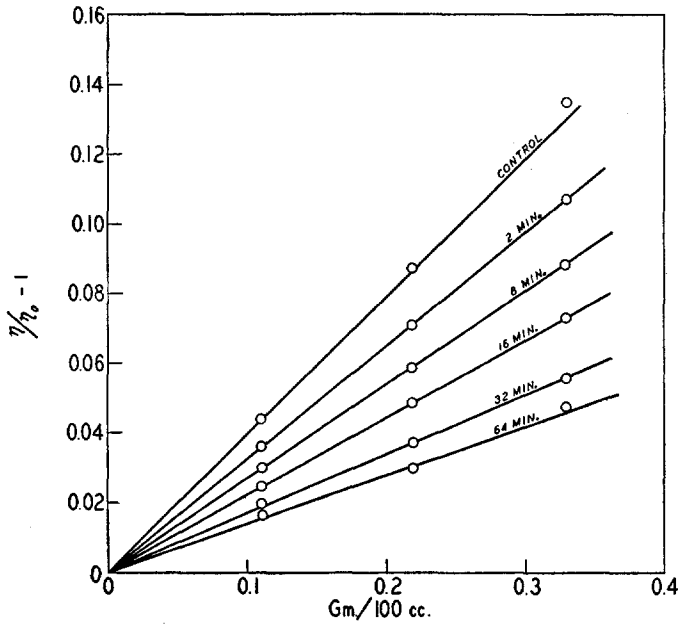
L. A. Chambers, formerly of the Johnson Foundation, for the use in preliminary experiments of the magneto-striction sonic generator developed by Chambers and Flosdorf (5). It was found that both sonic generators, when tuned to maximum efficiency, delivered approximately equal energy to the liquid.

When the virus solutions were subjected to the sound vibrations, violent swirling was observed. The swirling is associated with cavitation in the liquid, which is caused by the strong sound vibrations. When cavitation is suppressed by lowering the atmospheric pressure above the liquid, the swirling disappears.

Stream Birefringence and Viscosity.—The stream birefringence of the solutions of purified tobacco mosaic virus was observed by inverting a test tube of the material



TEXT-FIG. 1. Specific viscosity as a function of concentration for purified tobacco mosaic virus sonic treated for 0, 2, 8, 16, 32, and 64 minutes.

between crossed polaroids. It was found that the intensity of stream birefringence decreased roughly exponentially with time of sonic treatment.

The viscosities of solutions of tobacco mosaic virus also decrease as sonic treatment proceeds. Solutions of centrifugally purified virus (3.28 mg./cc. in 0.1 M phosphate buffer at pH 7.0) were subjected to sonic treatment for 2, 8, 16, 32, and 64 minutes. Viscosity measurements of various dilutions of the solutions were made in an Ostwald viscometer kept at constant temperature in a water bath at 27 ± 0.005°C. The specific viscosity, $\eta/\eta_0 - 1$, where η is the viscosity of the solution, and η_0 the viscosity of the solvent, was found to be linear with concentration (Text-fig. 1).

Electron Microscope Studies.—Electron micrographs were taken of samples of purified tobacco mosaic virus subjected to sonic treatment for varying lengths of

time. Small amounts of the samples were diluted to a concentration of 0.1 mg./cc. and transferred to electron microscope screens. The screens were allowed to dry and then dipped into distilled water to remove soluble salts. In order to obtain greater photographic contrast the screens were gold shadow cast according to the method of Williams and Wyckoff (6). The samples were observed in an RCA Console Model (type EMC-1) electron microscope having a magnification of 5,800.

Typical micrographs of the control material and of material sonic treated for 16 minutes are illustrated in Figs. 1 and 2. It is apparent from the micrographs that as a result of the sonic treatment the rod-like particles of the tobacco mosaic virus are broken up into fragments which have the same circular cross-section with a diameter of 15 mμ as have the original particles.

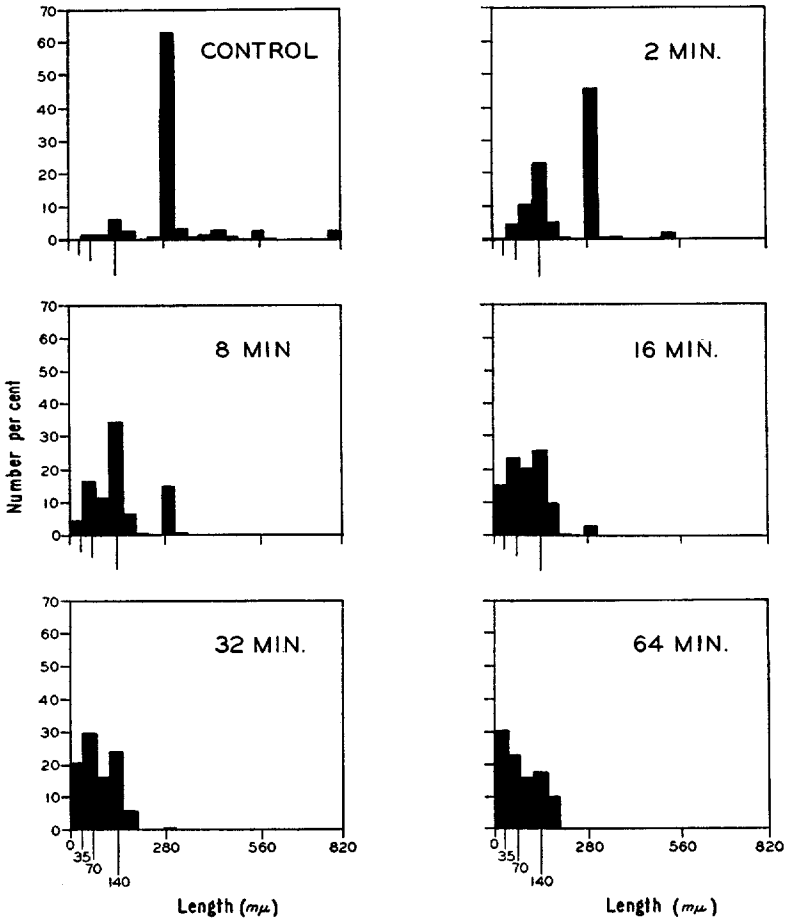
Representative electron micrographs were enlarged photographically and the lengths of all the particles in each picture were measured in intervals corresponding to 38 mμ with a variation within 19 mμ from the mean value of the interval. This spread in measurement was taken in an attempt to overcome all the errors involved in measuring particles of these sizes. The errors have been estimated to be about 10 mμ (7). The spread taken here is sufficiently narrow to show the essential features of the results of sonic treatment. About 300 particles from each sample were counted.

Text-fig. 2 gives the results of the size distribution measurements from electron micrographs of the control material and material sonic treated for 2, 8, 16, 32, and 64 minutes. Sixty-two per cent of the particles in the untreated sample are about 280 mμ in length. The control material was obtained from Turkish tobacco plants which had been infected for 25 days with tobacco mosaic virus and was purified within a week after harvesting the plants. The sample gives a size distribution of the particles approximately that found by Oster and Stanley (8) in the contents of hair cells from diseased plants. Large deviations from this size distribution for a purified sample can usually be attributed to the method of preparation and age of the sample. Thus Sigurgeirsson and Stanley (9) found a large number of aggregates of the 280 mμ in length particles in samples which had been allowed to stand for long periods of time.

After a sonic treatment of only 2 minutes the number of rods about 280 mμ in length is considerably reduced and the number of particles about half this length is increased. On further sonic treatment the number of particles 280 mμ in length decreased exponentially with time. The number of half lengths (about 140 mμ) increased and then decreased with time and the number of one-fourth lengths subsequently increased and then decreased with time. Because of their small size, the exact number of particles of one-fourth length and shorter is difficult to determine. It is clear, however, that practically no particles are produced having lengths between 280 mμ and 140 mμ, and not many particles are produced having lengths between 140 mμ and 70 mμ.

Chemical Effects of Sonic Treatment.—It has been observed (10) that thermal

denaturation of tobacco mosaic virus results in the production of insoluble protein and the release of nucleic acid. Prolonged sonic treatment, however, evidently produced no appreciable amount of insoluble protein since there



TEXT-FIG. 2. Size distributions from representative electron micrographs of purified tobacco mosaic virus sonic treated for 0, 2, 8, 16, 32, and 64 minutes (300 particles counted for each sample).

was no increase in the turbidity of the virus solutions. An attempt was made to determine whether nucleic acid was released on breakage of the particles by sonic treatment. An aqueous solution of purified tobacco mosaic virus, which had been sonic treated for 32 minutes, was brought to a concentration of 0.1 N with respect to sodium chloride. The solution was then brought to

its isoelectric point with dilute acetic acid and the precipitated material was spun for 15 minutes at 5,000 R.P.M. in an angle centrifuge. Since no nitrogen was detectable in the clear supernatant it may be concluded that no nucleic acid or other nitrogen-containing substances soluble at this pH and salt concentration was released.

The isoelectric points of many proteins are known to change when the protein is denatured. It was found, however, that the isoelectric point, as determined by a turbidimetric method (11), of sonic treated tobacco mosaic virus in distilled water was pH 3.92, which is the same as that of the untreated sample.

The immunological properties of sonic treated tobacco mosaic virus have been investigated by Dr. S. Malkiel of this laboratory (12). He found that sonic treated virus precipitated more antibody per unit weight of antigen,

TABLE I

Biological Activity of Sonic Treated Tobacco Mosaic Virus

Time of treatment	Dilution	No. of lesions (treated/control)	Relative activity
min.			*per cent*
0	1:500	—	(100.0)
2	1:200	1791/865	82.8
8	1:100	1265/739	34.3
16	1:10	1476/324	9.13
32	1:5	1169/743	1.57
64	1:1	450/1041	0.086

than did the untreated virus. This increase is attributed to the increased surface area of the broken-up virus particles.

It may be concluded from these observations that the chemical properties of the particles are not changed appreciably by sonic treatment.

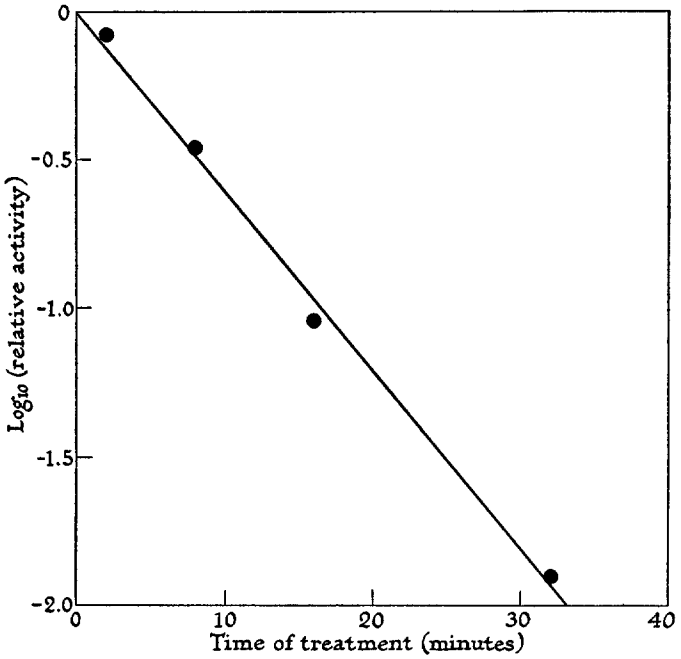
Biological Activity.—The biological activity of the sonic treated tobacco mosaic virus was determined from the number of lesions produced by it on *N. glutinosa* according to the local lesion method of Holmes and others (13). Preliminary tests were made to determine the order of magnitude of the biological activity of the treated and of the untreated virus. The samples were diluted in phosphate buffer so that each sample would give a number of lesions on one-half of the leaf comparable to the number given by the control material, diluted 1:500, on the other half. In Table I are given the results of these tests.

In order to determine whether the shorter rods have an inhibitory effect on the larger ones, mixtures of the control and the 64 minute treated sample were made and applied to the plants. It was found that there was no appre-

ciable difference in activity which could not be accounted for on the basis of the relative activities of the constituents of the mixtures.

In Text-fig. 3 the logarithm (base 10) of the relative activity is plotted as a function of time of sonic treatment. It is seen that the activity decreases exponentially with a rate constant given approximately by $k = 0.13$ min.$^{-1}$

Aggregation of the Particles.—It is well known that various chemical agents can cause end-to-end aggregation of tobacco mosaic virus particles. The electron micrograph studies of Sigurgeirsson and Stanley (9) showed that there



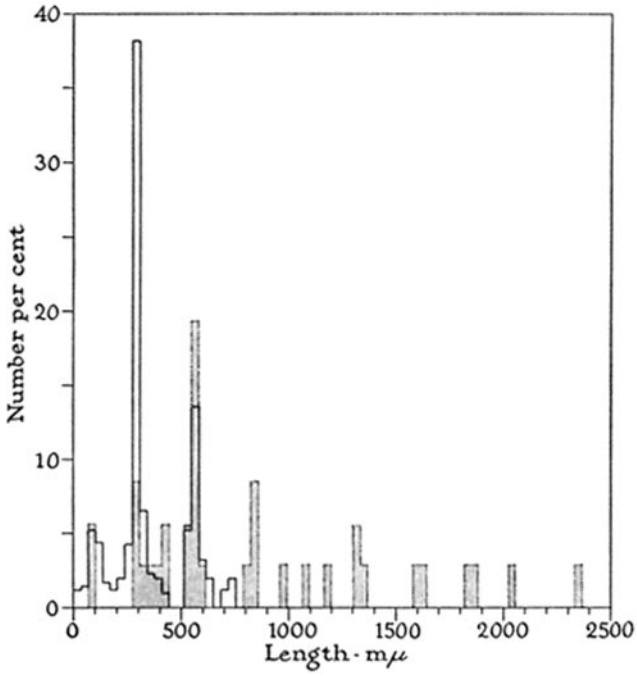
TEXT-FIG. 3. Biological activity of purified tobacco mosaic virus as a function of time of sonic treatment.

is considerable particle aggregation in expressed juice of diseased plants allowed to stand for long periods of time. Bawden and Pirie (14) have also shown that incubation of tobacco mosaic virus with trypsin or snail enzymes can cause aggregation of the particles.

A simple method for causing aggregation which does not require the introduction of extraneous protein material is the following: A solution of purified virus suspended in distilled water is precipitated by being brought to its isoelectric point (pH 3.92 in water) with 0.001 N hydrochloric acid. The sample is then incubated for 3 days at 37°C. The clear supernatant liquid is removed and the precipitate is dissolved in 0.1 M phosphate buffer at pH 7.0. This solution shows the same stream birefring-

ence as the original solution; but, if this material is now incubated for an additional 4 hours, it shows greatly increased stream birefringence.

When untreated virus particles are aggregated by the isoelectric method, the solution is highly thixotropic and the viscosity cannot be measured in an Ostwald viscometer. An electron micrograph of this material is shown in Fig. 3. The small beading along the rods and the granular background are artifacts caused by excessive exposure of the gold shadow cast film to the elec-

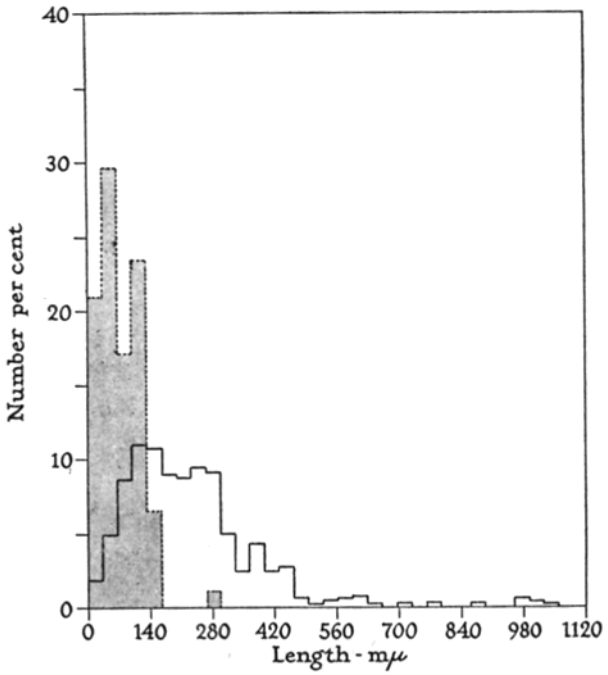


TEXT-FIG. 4. Shaded area, size distribution of aggregated tobacco mosaic virus particles (36 particles counted). Unshaded area, size distribution of the same material after strong stirring (265 particles counted).

tron beam in the microscope (15). A size distribution of the particles is shown in the shaded area of Text-fig. 4. Since the particles are very long and only a few appear on each micrograph, a size distribution was made of only 36 particles taken from a composite of four micrographs. This size distribution cannot, therefore, be regarded as having great statistical significance, but it shows that the predominant size corresponds to dimers of the 280 mμ length. The aggregated untreated virus was tested for biological activity by the lesion method and was found to be 48 per cent as active as the unaggregated material.

The long aggregated particles were broken up when the sample was subjected to strong stirring with a propeller motor-driven at about 6,000 R.P.M. The propeller

blade was 1 cm. in length and the sample was placed in a test tube 2.5 cm. in diameter kept in a cooling bath to prevent heating by the stirring. After the aggregated material had been stirred for 15 minutes, it showed a marked decrease in stream birefringence and in viscosity, and its biological activity was 92 per cent of that which it was before aggregation. The size distribution of 265 particles observed in electron micrographs of the stirred sample is shown in the unshaded area of Text-fig. 4. Thirty eight per cent of the particles are about 280 mμ in length and only 13 per cent are dimers.



TEXT-FIG. 5. Shaded area, size distribution of particles of tobacco mosaic virus sonic treated for 32 minutes (300 particles counted). Unshaded area, size distribution of the same material after aggregation (300 particles counted).

The small particles produced by sonic treatment can also be aggregated in the manner described above. An aqueous solution of 32 minute sonic treated material was made to aggregate by this method and was found to exhibit considerable stream birefringence although the original material had shown very little. The viscosity of solutions of this aggregated material was measured and it was found that the specific viscosity was twice that observed for the unaggregated sonic treated virus at the same concentrations. An electron micrograph of the aggregated material is shown in Fig. 4. There are many more curved particles in these samples than are usually observed

in normal samples of tobacco mosaic virus. The size distribution of the aggregated 32 minute sonic treated particles is shown in the unshaded area of Text-fig. 5. The size distribution is much broader than that for the unaggregated 32 minute sonic treated material shown in Text-fig. 2 and in the shaded area of Text-fig. 5. The biological activity of the aggregated sonic treated material was essentially the same as that of the unaggregated sonic treated material (3251 lesions for the former and 3169 for the latter).

The particles aggregate end-to-end more rapidly when the incubation temperature is increased. If a solution of sonic treated material in 0.1 M phosphate buffer at pH 7.0 is heated for 2 hours at its isoelectric point (pH 3.5 in buffer) at 60°C., and the pH is then brought back to 7.0, and is heated again at 60°C. for only 10 minutes the particles aggregate and the solution exhibits intense stream birefringence. Fig. 5 shows an electron micrograph, taken under low intensity, of 16 minute sonic treated material aggregated by this method. The points of junction of the particles are clearly visible.

DISCUSSION

Lauffer (16, 17) has shown that the stream birefringence and the high viscosity of solutions of purified tobacco mosaic virus are due to the elongated form of the particles. In the present work the decrease in intensity of stream birefringence and in viscosity with sonic treatment indicates a shortening of the particles. This conclusion is confirmed by direct observation in the electron microscope.

Sollner (18) found that vanadium pentoxide sols, known to contain highly asymmetric particles, show a marked decrease of stream birefringence on short sonic treatment. It is probable that the mechanism of breakage of these colloidal particles is similar to that operating in the breakage of the particles in samples of purified tobacco mosaic virus. There is no destruction of the vanadium pentoxide colloidal particles when cavitation, normally accompanying intense sound waves, is suppressed. This observation is in agreement with the observations of Stanley (2) that the biological activity of the tobacco mosaic virus samples is unchanged when cavitation is suppressed. Since cavitation is necessary for breakage of the particles and is accompanied by violent swirling, it is probable that the particles are broken by the strong macroscopic shearing stresses in the swirling set up in the liquid. The strong shearing forces accompanying cavitation might be expected to break elongated particles of colloidal dimensions in two since particles subject to Brownian movement but having superimposed random shearing forces should, from hydrodynamical considerations, show the greatest stress at the middle of the particles.

From the size distributions (Text-fig. 2) it appears that as a result of the sonic treatment the rod-like particles are broken in two, these halves broken

in two, and so on down to at least one-eighth the length of the 280 mμ rods. The various size distributions may be accounted for by the following approximate theory: Assume that any particle which is broken is broken in half and that every particle has an equal chance of being broken. (The latter assumption will be inaccurate for very short particles.) Assume also, for mathematical simplicity, that all the particles are initially of one length, L (nearly 280 mμ in our case). Then the following differential equations are simultaneously satisfied.

$$\frac{dN_L}{dt} = -kN_L \tag{1 a}$$

$$\frac{dN_{L/2}}{dt} = 2kN_L - kN_{L/2} \tag{1 b}$$

$$\frac{dN_{L/4}}{dt} = 2kN_{L/2} - kN_{L/4} \tag{1 c}$$

$$\frac{dN_{L/8}}{dt} = 2kN_{L/4} - kN_{L/8} \tag{1 d}$$

Equation 1 *a* expresses the rate of disappearance of particles which were initially all of the same length L, where k is the rate constant. Equation 1 *b* expresses the rate of appearance and disappearance of particles of length $L/2$. The first term on the right states that two particles are produced for every particle of length L which is broken. The second term expresses the disappearance of particles of length $L/2$ into smaller fragments. Equations 1 *c* and 1 *d* are the corresponding expressions for the rate of appearance and disappearance of particles of lengths $L/4$ and $L/8$ respectively.

Since we assume an homogeneous size L initially present, the above equations are accompanied by the boundary conditions: At time $t = 0$; $N_L = N_L(0)$, the number of particles of length L initially present, and $N_{L/2} = N_{L/4} = N_{L/8} = 0$.

Equations 1 *a*, 1 *b*, 1 *c*, and 1 *d* together with the boundary conditions can be shown to yield the solutions

$$N_L = N_L(0)e^{-kt}, \qquad N_{L/2} = N_L(0)e^{-kt}(2kt)$$
$$N_{L/4} = N_L(0)e^{-kt}\frac{(2kt)^2}{2!}, \qquad N_{L/8} = N_L(0)e^{-kt}\frac{(2kt)^3}{3!} \tag{2}$$

Thus, the number of particles of length L decreases exponentially with time; but the numbers of the smaller sizes go through maxima—the smaller the particles, the later the time at which the maximum occurs.

It is, of course, not possible to determine from the electron microscope the total number of particles in a sample but only the number per cent having certain lengths. Therefore, the number of particles of certain lengths at

time t given by Equations 2 must be divided by the total number of particles at time t. It can be shown mathematically that the total number of particles increases monotonically with time so that the form of the expressions for the number per cent of particles of given lengths as a function of time is that given by Equations 2. The size distributions given in Text-fig. 2 are seen to have the same functional relationship to time as those expressed by Equations 2. This similarity suggests that the theory is probably correct.

In the general case in which the particles are eventually broken into infinitely short pieces, the total number of particles of all possible sizes at a given time t is the summation of equations of the form of Equations 2 and is equal to $N_L(0)e^{kt}$, so that the number per cent of particles of length L at a given time is given by $100e^{-2kt}$; *i.e.*, the number per cent of particles of lenght L decreases at twice the rate at which the total number of particles of this size is decreasing. It is unlikely, however, that the particles are broken much below one-eighth the size of the 280 mμ in length particles. Therefore, the actual total number of particles is somewhat lower than that given for the general case, and the rate constant for the decrease in number per cent of particles of length L is greater than k but less than $2k$, where k is the rate constant for the decrease in total number of particles of length L.

Since the biological activity decreased exponentially with time of treatment and since the number of particles 280 mμ in length decreased exponentially with time while the numbers of other size particles go through maxima, it is highly likely that *only the particles of length 280 mμ are the biologically active units*. The theory is substantiated by the fact that the rate constant calculated from Text-fig. 2 for the decrease in number per cent of particles 280 mμ in length is about 0.19 min.$^{-1}$, which is greater than the value for the rate constant for the decrease in biological activity (k = 0.13 min.$^{-1}$) but less than twice its value, $2k$. A more detailed correlation would require an elaborate numerical analysis.

The results of these experiments showing that only particles 280 mμ in length are biologically active is in complete agreement with the findings of Stanley and his coworkers (19), but is at variance with the suggestion of Bawden (20) that the 280 by 15 mμ rods are aggregates and that the primary virus particle is much smaller and not greatly elongated.

In the discussion above it is shown that the decrease in biological activity with sonic treatment is due to, or at least associated with, the mechanical destruction of the virus particles 280 mμ in length. The destructive effects of sonic treatment on certain proteins such as egg albumin have been attributed to the action of hydrogen peroxide (21), which is known to be produced in minute traces in water when cavitation is present (22). Sollner (23), however, in referring to experiments on the destruction of proteins by sonic treatment, states that "in many cases it seems likely that there is a kind of surface de-

naturation." As shown in the earlier part of this paper, the physicochemical evidence indicates that the tobacco mosaic virus was not denatured by the sonic treatment.

The results of studies of the effects of sound waves on other viruses are quite varied. Scherp and Chambers (24) have found that the pathogenicity of poliomyelitis, human influenza, and swine influenza viruses is not affected by sonic treatment; but Rivers, Smadel, and Chambers (25) found that the activity of elementary bodies of vaccinia is decreased by sonic treatment. The latter workers attributed the decrease of activity to the chemical effects of the hydrogen peroxide produced. Krueger, Brown, and Scribner (26) found that the biological activity of bacteriophage decreased on sonic treatment. More recently, Anderson (27) has observed, in the electron microscope, that numerous ghosts are found in samples of sonic treated bacteriophage. Probably in the case of bacteriophage the elaborate structure of the particles, as revealed in the electron microscope, is mechanically weak, while in the case of tobacco mosaic virus, the ease of mechanical destruction is probably associated with the highly elongated form of the particles.

The nature of the forces operating on the ends of the particles to bring about aggregation in samples of tobacco mosaic virus is, at the present time, not understood. The spaces, some as great as 30 mμ, at the junctions of the particles as seen in Fig. 5 suggest that the forces may act over distances much greater than do ordinary molecular forces. Examination with an electron microscope possessing a resolution higher than that of the instrument used in these studies may, however, reveal the presence of connecting links. The presence of numerous curved particles of the aggregated 32 minute sonic treated material shown in Fig. 4 suggests that there may be free rotation about the bonds connecting the particles.

The biological activity of normal virus samples decreases when the particles are aggregated because fewer active units are available to infect the susceptible points on the surface of the leaves. Text-fig. 4 shows that rapid stirring tends to break the aggregates into the monomeric units 280 mμ in length. Evidently the junctions of the virus units in the aggregates are mechanically weak points. The stirring is accompanied by an almost complete restoration of the activity of that for the normal virus sample.

The fact that the biological activity of the 32 minute sonic treated material did not change when the particles were aggregated indicates that it is not possible by this aggregation method to regenerate active particles by polymerization of their fragments, either because the proper type of bond is not formed between the fragments or because the fragments were brought together in a different sequence from that in which they occur in the normal particles or for both these reasons. It is highly unlikely that the fragments would aggregate in the proper sequence and be pointing in the correct direction with respect to one another. The activity of the 32 minute sonic treated sample

was not reduced by aggregation as it was when normal size particles were aggregated, probably because the number of normal size particles in the 32 minute sonic treated sample is relatively small. If random polymerization of the particles is taking place as indicated by the broad size distribution (Text-fig. 5), it is highly improbable that one normal size particle would aggregate with another of the same size.

SUMMARY

Centrifugally purified samples of tobacco mosaic virus were subjected to intense sound vibrations of 9,000 cycles per second for 0, 2, 8, 16, 32, and 64 minutes. The viscosity and stream birefringence of the samples decreased with time of sonic treatment, but no chemical changes were found. Electron micrographs of the samples show that the particles are broken perpendicular to their long axis. In the untreated sample 62 per cent of the particles are about 280 mμ in length. As sonic treatment continued, the number of particles of this length decreased exponentially with time, the number half this length increased and then decreased, and the number of quarter length particles subsequently increased and then decreased. The biological activity of the samples, as determined by the half leaf lesion method, decreased exponentially with time of sonic treatment with a rate constant given by $k = 0.13$ min.$^{-1}$. A correlation exists between the size distributions and biological activity and shows that only the particles of length 280 mμ are the biologically active units.

Tobacco mosaic virus particles can be made to aggregate end-to-end when the material is heated at its isoelectric point and reheated after being brought back to pH 7. Material which was not sonic treated and was made to aggregate showed reduced biological activity, but the activity was increased when the aggregated material was subjected to strong mechanical stirring. Material which was sonic treated for 32 minutes and which was made to aggregate showed the same biological activity as the material which was sonic treated but not aggregated.

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EXPLANATION OF PLATE 4

Electron micrographs of tobacco mosaic virus treated by various methods taken on RCA Console Model (type EMC-1) electron microscope. Magnification 22,000 and gold shadow cast.

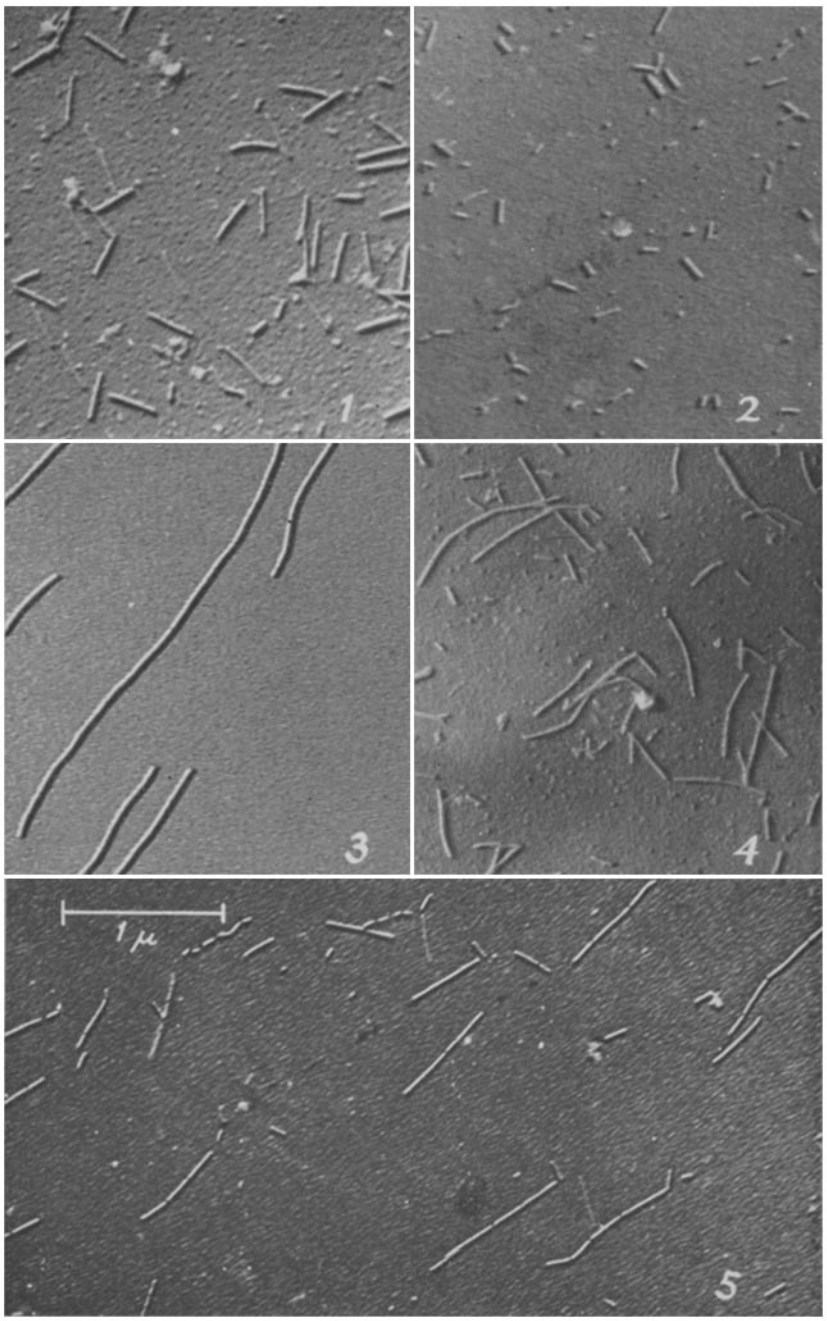
FIG. 1. Centrifugally purified tobacco mosaic virus.

FIG. 2. Tobacco mosaic virus sonic treated for 16 minutes.

FIG. 3. Tobacco mosaic virus particles aggregated by the isoelectric heat treatment at a temperature of 37°C.

FIG. 4. Tobacco mosaic virus sonic treated for 32 minutes and aggregated by the isoelectric heat treatment at a temperature of 37°C.

FIG. 5. Tobacco mosaic virus sonic treated for 16 minutes and aggregated by the isoelectric heat treatment at a temperature of 60°C.



(Oster: Sonic treatment of tobacco mosaic virus)