STUDIES ON THE AGGREGATION REACTIONS AND BASIC DYE BINDING OF TOBACCO MOSAIC VIRUS*

I. Variation of pH, Particle Asymmetry, Acid and Base Titration Results, Irreversible Binding of Methylene Blue, Ultraviolet Absorption, and Extent of Heat Denaturation in Tobacco Mosaic Virus Solutions with Time of Standing

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INTRODUCTION

The occurrence of end-to-end aggregation of tobacco mosaic virus (TMV), on standing in either the purified or crude state, has been known for some time (1–3). It has been recently observed (4) that the results of acid and base titrations are strongly time-dependent and that on standing for a certain period of time a slow increase in pH occurs in aqueous solutions of TMV. The possibility that the reaction which produces the pH increase is the end-to-end aggregation comes to mind at once. Moreover, the mechanism of the reaction which becomes evident is that acid groups are destroyed in the formation of linkages joining the ends of two TMV monomers together. It would also seem likely that the acid groups involved are the nucleotide phosphates and that the same groups are the ones active in the synthesis or self-duplication of virus within the living host cell. In order to confirm these concepts and to work out a more precise mechanism of aggregation, the present series of investigations were undertaken.

Indication that the nucleic acid is involved in virus activity has been provided by the findings of Ryzhkov, Smirnova, and Gorodskaya (5). These workers showed that practically all virus activity was destroyed when any one of several basic dyes, including methylene blue, was added to infected plant juice to a concentration of 2.5 per cent. This loss of activity, furthermore, was found to be irreversible, since dialysis did not restore virus activity for all the basic dyes studied except safranine. Further activity experiments carried out at

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Stanford, using a dialyzed solution of purified TMV plus methylene blue at high concentration, confirmed this result. In addition to failing to inactivate the virus irreversibly, safranine has also been observed not to bind with TMV irreversibly (4) in the same manner as methylene blue. Michaelis (6) states that such basic dyes as methylene blue and crystal violet have been considered to bind with nucleic acids, and he shows by analysis of the absorption spectra that the combination of basic dye with nucleic acid is a specific one. Thus since the negatively charged nucleic acid groups bind basic dyes with loss of activity, the nucleic acid is apparently required for virus reproduction.

In order to establish the relation between the same groups and the process of end-to-end dimerization, and to determine quantitatively the number of groups involved, it was decided to study the binding of methylene blue with tobacco mosaic virus by the method of equilibrium dialysis. The fact that this basic dye does bind to the nucleotide phosphate groups would at first sight seem to be contradictory to the findings of Oster and Grimsson (7). They showed that when safranine is bound to TMV the absorption spectrum of the dye is not altered, whereas when it is combined with the undegraded nucleic acid separated from TMV by heat denaturation, the resulting absorption maximum shifts to shorter wave lengths. However, in the case of methylene blue, these studies show that binding of an irreversible type to the nucleic acid groups does occur. The inconsistency with the results of Oster and Grimsson can be explained when it is considered that methylene blue is a polymerizing dye, whereas safranine is not.

EXPERIMENTAL

Nine preparations (numbers 5B, 6, 7, 8, 12, 13, 17, 18, 19) and a pooled solution of TMV were used in this series of studies. The TMV was in each case purified by the general method described by Stanley (8). Table I summarizes the media employed and the number of cycles of differential centrifugation required, in the purification of TMV in the various preparations. When TMV in the most monomeric state possible was desired, 0.01 M phosphate was used as the washing medium, in the manner suggested by Schachman and Kauzmann (9).

The pH measurements and titrations were made using the Beckman pH meter with the standard 1190 electrode and the 1190E electrode for base titrations. Blank titrations, using solvents corresponding to those used for the TMV titrations, were carried out in order that corrections might be made for the buffering action of impurities or the phosphate added. Between measurements all solutions were stored at 1–2°C., and just prior to measurement the solutions were warmed and agitated to dispel excess carbon dioxide. A small crystal of phenylurethane was added to each sample to inhibit the growth of bacteria, for preparations 6 through 12, and for the remaining ones, small amounts of carbon tetrachloride were added.

At first, the viscosity measurements were made using Ostwald viscometers having flow times of 80 to 100 seconds, with an average velocity gradient of 2000 $sec.^{-1}$ (capillary radius 0.03 cm.). For TMV preparation 12, capillary viscometers of special

design permitting serial dilutions of a single aliquot of the original protein solution, with velocity gradients of 186 sec.$^{-1}$ and 85 sec.$^{-1}$ described by Desreux and Bischoff (10) were used.

Since the total binding of methylene blue with tobacco mosaic virus was found to consist of both an irreversible and a reversible part, the former was at first studied in detail. The occurrence of irreversible binding or staining of the virus with dye was demonstrated by the technique of repeatedly washing the complex obtained by adding the dye in excess to the virus, measuring the amounts of dye removed in all washes. Also this technique was used to determine quantitatively ratios of irreversible binding. Two methods of washing were employed: (1) repeated equilibrium dialyses against fresh portions of buffer, and (2) repeated high speed centrifugations.

TABLE I

Descriptions of the TMV Preparations Studied

Preparation No.	Total No. cycles	Solvents used to suspend pellets at indicated cycle Nos.			Concentration stock solution	Initial pH
		0.1 M phosphate	0.01 M phosphate	Water		
					per cent	
5A	8	1–4		5–8		5.10
5B	8	1–5	6–7	8	2.01	5.28
6	4		1–3	4	5.10	7.11
7	4		1–3	4	2.96	6.80
8	4		1–3	4	3.08	
12	4		1–3	4	3.64	6.97
13	4		1	2–5	1.15	6.85
17B	5		1	2–5	2.33	5.90
18	5		1	2–5*	0.70	5.90
19	5			1–5	1.28	6.53

* Conductivity water used as the wash medium here.

The method of equilibrium dialysis employed at first, for TMV preparations 5B and 6, involved the use of strips of cellophane arranged in a V-shape according to the procedure described by Teresi (11) for the study of anionic dye binding; but since considerable experimental difficulty arose from the use of it when the ion studied is methylene blue, the technique was further refined for later studies. It was found that the methylene blue was highly adsorbed onto the cellophane, especially if the strips had not been previously boiled. Therefore, a slightly more standardized setup was used for some of the studies on preparation 7, consisting of one-quarter inch strips of cellophane mounted on short pieces of glass tubing which were inserted into rubber stoppers. The free ends of cellophane were closed off by tying tight knots and entire units were fitted into small test tubes so that the knots just came to the bottoms of the tubes. Separate control curves had to be worked out for each one of these units as varying amounts of dye became absorbed into the different knots. For the remaining studies on preparations 7 and 8, precision-made, two compartment dialysis cells were used. They consisted of two hollowed out pieces of lucite, greased at the joining sur

faces, and clamped together tightly after inserting a piece of boiled cellophane between the two pieces.

To calculate the amount of dye bound to protein, it is necessary to know the amount initially entered, the amount adsorbed to the cellophane and other parts of the cell, and the amount which remains free in solution. The concentration of free dye is determined by measuring the light absorption at 620 millimicrons in the Beckmann spectrophotometer of the solution obtained by the appropriate dilution of the dialysate at equilibrium. The molar extinction determined for this wave length resulting from measurements on standard solutions was 4.60×10^4, agreeing well with the value obtained from the plot given in the article by Michaelis (6). A straight line was obtained, the slope of which yielded the extinction coefficient, up to a dye concentration of 5×10^{-5}M.

For each set of determinations, generally four samples were set up in duplicate with the virus to which the dye was added at four different total concentrations, and corresponding controls were set up at the same time, in which buffer was substituted for the virus solutions inside the dialysis bags. The buffer medium used in all solutions, at pH 7.3, was phosphate of 0.02 ionic strength. For the irreversible or firm binding studies the dye was added directly to the virus side of the membrane, and for the reversible binding studies it was added to the opposite side.

The amounts of dye adsorbed, for a single equilibrium determination, will not be the same for the TMV samples as for the corresponding controls, since the virus removes some dye from solution by irreversible binding thus lowering the equilibrium concentration of free dye. The position of the corrected control curve, drawn parallel to the one measured, is determined by the point defined by the initial equilibrium molarity of the TMV sample and the corresponding millimols adsorbed as read off the control curve for the initial single equilibrium dialyses.

For firm binding studies (I) two samples were set up in duplicate, by adding methylene blue to 2 ml. of solution on the protein side at two fairly high, initial concentrations and washing seven times by successive equilibrium dialyses, using 4 ml. of buffer solution each time. After the third washing dialysis, the inside solution of one of each pair of duplicates was saved in the cold for later viscosity and sedimentation studies. After the seventh dialysis the two remaining single samples were saved for light absorption spectra studies in the visible region.

A number of firm binding studies were made on TMV preparation 7 at various times of standing and under varying conditions of washing. For studies III the method of washing used was that of repeated ultracentrifugations. The procedure was carried out in such a way as to approximate as closely as possible the conditions of studies (V) in the lucite cells, adding reagents to make the same final concentrations. The solutions were made up in the small plastic tubes for rotor 40 of the Spinco model L preparative centrifuge, and were centrifuged in the Spinco L, refrigeration on, at 30,000 R.P.M. for 42 minutes. The supernatants were at once poured off into tared test tubes, so that the supernatant weights could be taken and their volumes calculated, for the samples not completely drained. The entire operation was repeated in subsequent centrifugal washings. The concentrations of methylene blue in all supernatants were determined by measuring in the Beckmann, after the proper dilutions had been made.

Further studies (VI) were carried out in which dye was added at two concentrations to 1 ml. of virus solution inside the dialysis bag, using the stopper-mounted tubes. The contents of the dialysis bags were then dialyzed successively three times against 3 ml. portions of buffer. The intrinsic viscosity of the inside solution was measured as well as the dye concentration outside after each dialysis. The measurements were made in the Ostwald viscometers, and the fluid used to dilute each inside solution was buffered methylene blue at a concentration equivalent to the measured equilibrium molarity at that washing. For the final dialysis, instead of washing, the samples were equilibrated against a solution of methylene blue at 3.2×10^{-4} molar.

Finally, irreversible binding determinations (studies VIII) were made, using the lucite cells, on a preparation (No. 8) made up to be as nearly monomeric as possible, in order that comparisons could be made between different TMV solutions having varying aggregation states over a fairly wide range.

The concentrations of TMV in all stock solutions used were determined by means of the micro Kjeldahl method of nitrogen analysis assuming a factor of 6.02 for milligrams of protein/milligrams of N (12), corresponding to 16.6 per cent nitrogen. For some of the later preparations, concentrations were determined by means of colorimetric phosphorus analysis (13) assuming a phosphorus composition of 0.51 per cent (12).

For some of the studies, it was thought possible to determine protein concentration by measuring the ultraviolet absorption at 260 millimicrons in the Beckmann spectrophotometer, after determining the extinction coefficient or using that observed by Takahashi (14). However, on making repeated determinations on different preparations, it became evident that the values were far from constant, although the mean was not far from Takahashi's value of 3.24 (optical density/milligram/cubic centimeter). The extinction coefficient appeared to vary with the method of preparation of TMV, the concentration of the stored solution and the suspending medium, and its time of storage. Therefore, it became desirable to study also the ultraviolet absorption of various TMV solutions as a function of time of standing, and to see whether the results could be correlated with the other measured quantities found to vary with time.

In some independent studies carried out by Cooper and Loring (15), another property of the virus was found to vary with time, and that is the extent to which nucleic acid is split off by heat denaturations under standardized conditions. The procedure used for the heat denaturation was somewhat modified from that described by Markham and Smith (16). Two sets of determinations were carried out by Cooper and Loring on two solutions of a TMV preparation (No. 13), at a concentration of 0.37 mg./cc., one in water and the other in 10^{-6} M phosphate buffer (pH 7.3), using the following procedure:

(1) Pipette 10 ml. TMV solution in 100 ml. beaker, add NaCl to give concentration of 0.01 M, and adjust to pH 7.1.

(2) Immerse beaker in boiling water bath for 10 minutes and cool.

(3) Adjust pH to 7.1.

(4) Centrifuge down the coagulated precipitate.

(5) Wash twice with 0.01 M NaCl and add washings to supernatant.

(6) Measure phosphorus content of supernatant.

In some determinations carried out by me on the later preparations to confirm their results, the above procedure was used with a change in the washing procedure: the precipitate was washed once with water instead of with 0.01 M NaCl. This resulted in proportionately higher phosphorus values, which could be corrected by multiplying by a constant factor to give parallel results (see Fig. 3 *b*).

RESULTS AND CALCULATIONS

pH Changes

The pH changes occurring on standing were studied for all the preparations of TMV. In the purification of the TMV in preparation 5, an aliquot of the

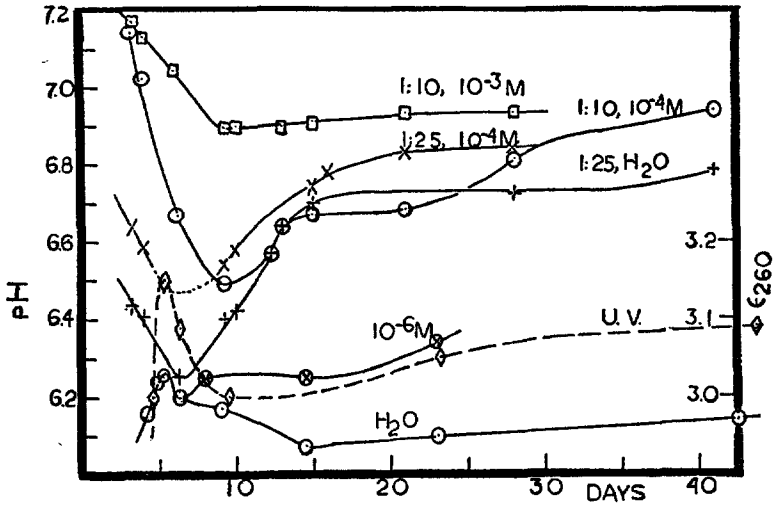

FIG. 1. Changes in pH with time for TMV preparation 7 in various media (upper curves) and the pooled recyclized solutions washed once with 0.1 M phosphate; ultraviolet absorption changes for the latter (dashed line).

total material was washed with water for the last four cycles and called No. 5A, the remaining material with phosphate and water, referred to as No. 5B. The pH of the final solution of No. 5A in water was noted to have remained practically unchanged in a period of 3 months (from 5.1 to 5.2), whereas the pH of the solution of No. 5B increased in pH by more than one unit in the same same time (see Table III).

To determine whether trace amounts of phosphate are needed to catalyze the second reaction, some studies were made on preparation 7, in which both the TMV concentration was varied and the phosphate concentration varied from zero to 0.001 M. Some of the results of these studies are shown in Fig. 1, and those for preparations 5 and 12 in Fig. 2 *a*. It was noted, however, that for some preparations of TMV such as No. 5B there was no initial dip in

the curve; *i.e.*, the pH began to rise after the first measurement. These particular preparations were the ones requiring more cycles of differential cen-

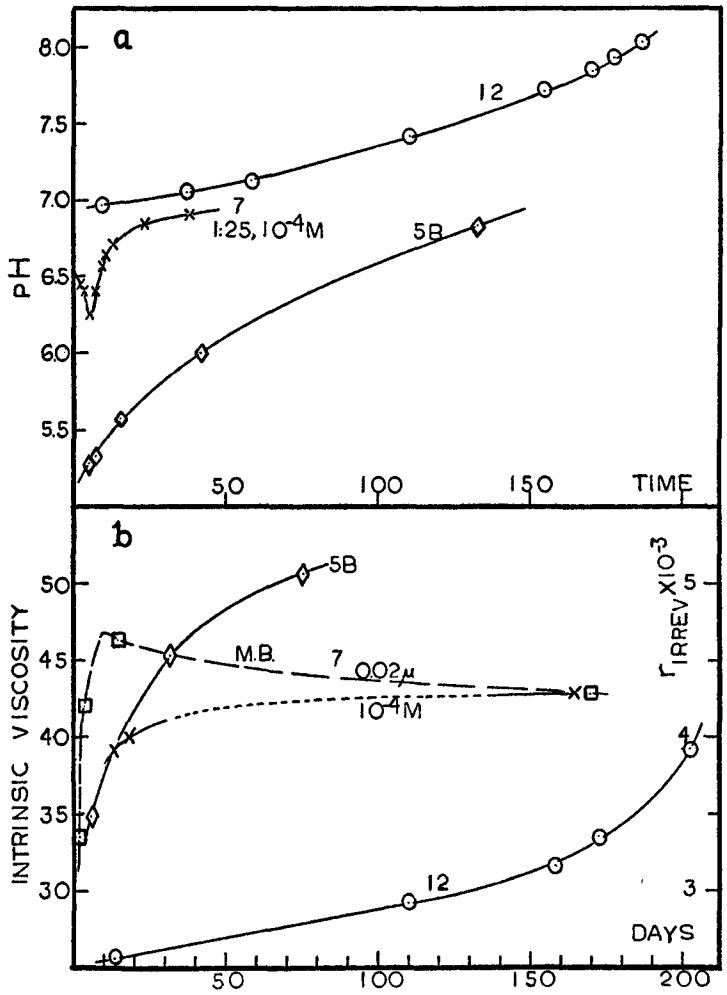

FIG. 2 *a*. Variation of pH with time for preparations 5B, 7, and 12. TMV 7 diluted 1:25 in 10^{-4}M phosphate.

FIG. 2 *b*. Parallel variation of intrinsic viscosity for preparations 5B, 7, and 12; variation of irreversible binding of methylene blue for TMV 7 (dashed line).

trifugation, so that a longer time necessarily elapsed in their purification. For the plots, in order that the minimum be reached at the same time, the zero time of the reaction was taken as the time the virus is last in phosphate buffer. This should be valid at least for the preparations with only one or two cycles

in phosphate. For TMV 5B, however, the suspending medium was phosphate for so many cycles, that by the time the first pH measurement had been taken, the time for the pH to reach a minimum had probably already passed. A corresponding zero time, in this case, would more likely be at the end of the second cycle in phosphate rather than the last one. The average position of the minima in the curves shown appears to be at 7 days from the time the virus was last in phosphate. Thus, in general, the virus undergoes an initial reaction for 7 days in which the pH decreases, which is followed by a reaction involving a slower, similar increase in pH.

The rate of the first reaction does not appear from these pH-time curves to have been increased by the addition of phosphate, for the minimum position does not shift to the left, but rather shifts to the right, if at all. However, the extent of both reactions, as judged by the magnitudes of the vertical displacements, appears to be increased by the addition of phosphate. The second reaction is noted to have been increased in 10^{-4} M phosphate for both TMV 7 and the pooled solution (Fig. 1). The effect of increasing the TMV concentration appears to favor the second reaction involving the pH increase as does the addition of phosphate to 0.0001 M. If, however, the phosphate is present at a concentration of 0.001 M, this reaction is inhibited, especially at the higher TMV concentration of 0.30 per cent.

A consideration of Table I reveals another interesting phenomenon. It will be noted that initial pH's are always lower if the wash phosphate was at a higher concentration or was used more frequently, or if the initial TMV concentration was either quite low (less than 1 per cent) or quite high (more than 2 per cent). If only water was used for at least the last three cycles, and if the solution percentage is of the order of 1 per cent, the initial pH is quite high, and, further, pH change studies reveal that these solutions undergo much greater initial pH drops.

The initial disaggregation reaction which results in the dip in pH can be induced usually at any time of standing by dilution of a concentrated stock solution. A glance at Fig. 3 *b* reveals that a pH curve is repeated by a similar second dilution of the same preparation. Similar studies on TMV 7 gave the same results, the secondary drop being even greater than the first.

To determine the effect of pretreatment, of varying phosphate concentration in the suspending media used in the preparation, a pooled solution of TMV (Nos. 11 to 14) was centrifuged down, and was suspended in successive aliquots in water, 0.01 M, and 0.1 M phosphate, and further washed by water. The pH and ultraviolet absorption changes in water and in some cases 10^{-4} M phosphate were then studied for each aliquot. For the water and 0.01 M phosphate-washed fractions, both pH and ultraviolet were seen to increase slightly and continuously, whereas for the 0.1 M phosphate-treated aliquot there is a sharp, initial increase followed by a dip, the effect being accentuated by placing in dilute phosphate.

Viscosity Changes

Intrinsic viscosity measurements were made concurrently with pH measurements on two preparations of TMV (Nos. 5B and 12), in order that the asymmetry increase could also be studied as a function of time and correlated with the pH increase. The results of viscosity increase for these preparations are

TABLE II

Variation of Intrinsic Viscosity of TMV Preparation No. 12 with Time of Standing

Velocity gradient sec.$^{-1}$	Time of standing	TMV concentration	$\eta\ sp/c$	Standard deviation	$\eta\ sp/c$ corrected to 186 sec.$^{-1}$	Intrinsic viscosity
	days	*mg./cc.*				
186	13	0.807	44.1			25.54
		0.660	40.2	±2.7		
		0.346	34.3			
85	110*	3.64	23.7		22.7	29.1
		2.18	26.3		25.2	
		1.21	29.3		28.2	
186	159	3.28	41.7			31.2
		2.18	37.7			
		1.64	37.5			
		0.66	33.4			
186	173	3.64	36.8			33.5
		1.91	36.2			
		1.29	34.9			
		0.78	34.0			
186	203	0.887	77.5			39.1
186		0.596	71.5			
186		0.450	63.0	±2.0		
85		0.225	48.8	±2.9	47.6	
85		0.150	46.1		44.9	

* This sample had been diluted 1:10 in phosphate of 0.02 ionic strength and allowed to stand 12 days before measurement.

shown in Fig. 2 *b*, and are to be compared with the corresponding pH changes shown in Fig. 2 *a*. The corresponding curves for a given preparation are clearly seen to have similar shapes. It will also be noted that no pH measurement was made at a sufficiently early time to show the initial pH drop. The viscosity data required for the determination of intrinsic viscosities of TMV 12 have been presented in Table II. In the extrapolations required for the two most critical intrinsic viscosities, the first and the last, the best straight lines were fitted to the data by the method of least squares. For both preparations the undiluted solutions in water were allowed to stand at 1–2°C., the required

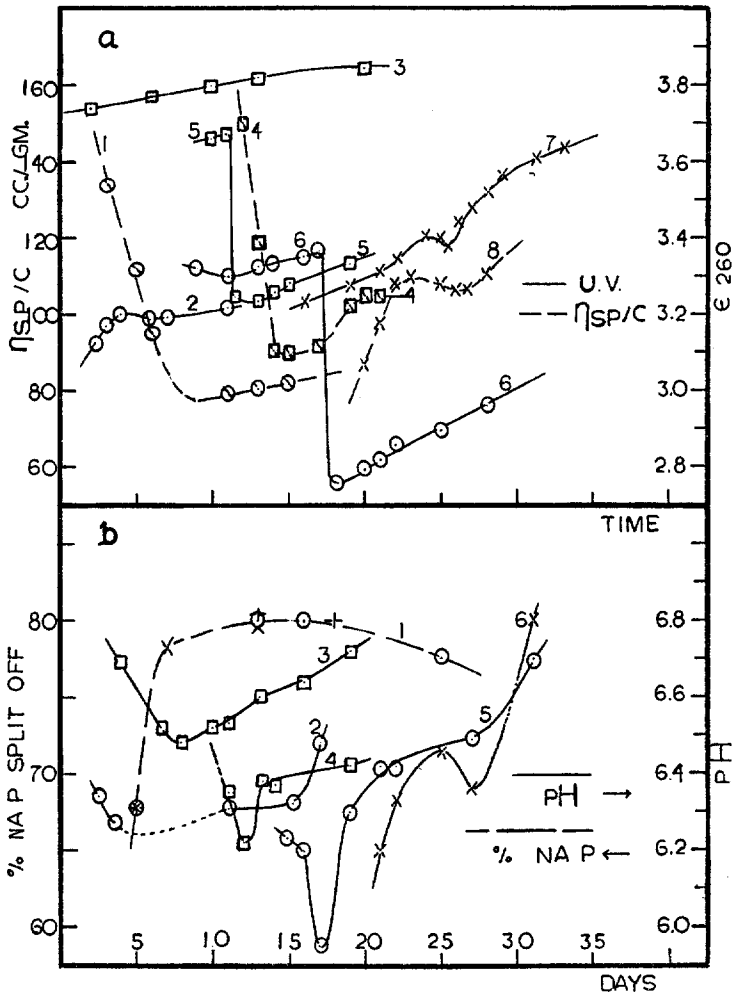

FIG. 3 *a*. Changes in specific viscosity/*c* (dashed lines) and ultraviolet absorption (solid lines) with time for preparations 18 and 19. Curves 1 and 2, TMV 18, 3:50 dilution 1 in water; curve 3, TMV 19, 1:25 dilution 1 in water; curves 4 and 5, TMV 19, 1:25 dilution 2 in water; curve 6, TMV 18, 3:50 dilution 2 in water; curves 7 and 8, TMV 18, 3:50 dilution 3 in 10^{-6}M phosphate.

FIG. 3 *b*. Changes in pH and extent of heat denaturation with time for preparations 13, 18, and 19. Curve 1, percentage nucleic acid phosphorus for TMV 13 (results of W. D. Cooper), 0.04 per cent solution standing in water (plus signs), same in 10^{-6}M phosphate (crosses), and for TMV 18, 3:50 in water (open circles); curve 2, pH for TMV 18, dilution 1; curve 3, TMV 19, dilution 1; curve 4, TMV 19, dilution 2; curve 5, TMV 18, dilution 2 in water; curve 6, TMV 18, dilution 3 in 10^{-6}M phosphate.

dilutions made, and phosphate added to 0.02 ionic strength, just before the viscosity measurements, each time the intrinsic viscosity was determined.

Before intrinsic viscosities can be converted into corresponding axial ratios (17) by use of the Simha equation, the viscosity lowering effect of increasing velocity gradient resulting from the orientation of asymmetric particles along the lines of flow must be studied. This has been done[1] by intrinsic viscosity measurements, at four or five velocity gradients, permitting extrapolations to zero, on a preparation of monomeric TMV, TMV dimerized by irreversible combination with methylene blue, and a preparation of fibrous thymus desoxyribonucleoprotein (DNP). These proteins have been characterized by viscosity, diffusion, and analytical ultracentrifuge studies, to establish homogeneity and determine molecular weights, in this laboratory[1] and as described in the literature. The corrections thus obtained have been used for the evaluation of axial ratios of non-fibrous DNP in various media (18).

The complex nature of the various initial changes observed to occur suggests that end-to-end and side-to-side aggregation and disaggregation reactions must take place in varying combinations depending on the conditions. To define the nature of these initial reactions more clearly, the viscosity of various TMV solutions was studied as a function of time, each sample separately, for preparations 17, 18, and 19, and the results correlated with pH, ultraviolet absorption, and heat denaturation changes observed concurrently for the same samples. The correlated results are shown plotted together in Figs. 3 *a* and 3 *b*. It can be seen that for all solutions showing an initial pH drop, a corresponding steep viscosity drop also occurs, only 3 to 4 days later in time. Corresponding rises can be compared in a similar manner.

To settle the question as to whether addition of phosphate furthers the extent of the second slow reaction involving a pH increase and a parallel viscosity increase, intrinsic viscosity determinations were made on solutions of TMV 7 standing in water and 10^{-4} M phosphate. If no phosphate was added the water solution showed no increase, whereas the 10^{-4} M phosphate solution, by undergoing irreversible end-to-end aggregation showed an increase from 39.1 to 42.7 in about 150 days.

Acid and Base Titrations

The results of pH changes and of acid and base titrations were used together to calculate the corresponding numbers of hydrogen ions reacting per TMV monomer, as will be described.

The titration curves for preparation 7 at two protein concentrations in 0.0001 M phosphate and those for a preparation of TMV (No. 6) are shown

[1] Results to be published.

in Fig. 4.[2] For the latter titrations, a higher protein concentration, which more closely approached that of the undiluted solution of TMV 12, was used.

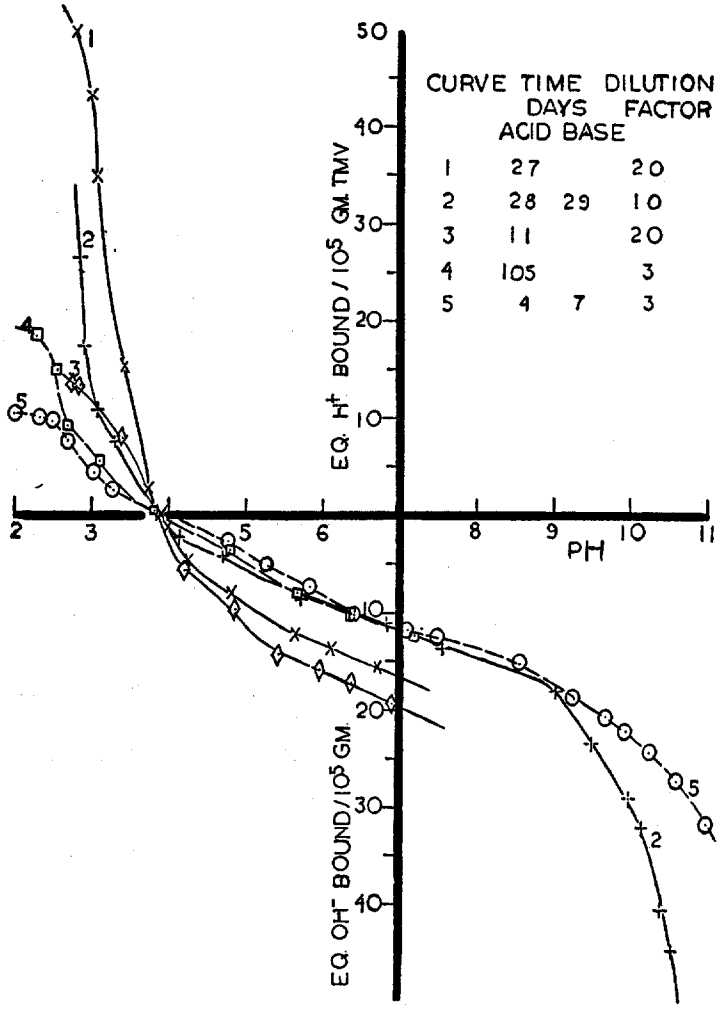

FIG. 4. Results of acid base titrations for TMV 6 (1.70 per cent) in water (dashed lines) and TMV 7 at different times, at two dilutions, in 10^{-4}M phosphate (solid lines). Threefold dilutions of TMV 6 made just before the measurements, and all dilutions of TMV 7 made after 8 days of standing.

A striking difference in the maximum acid binding is at once noted, depending on the time of standing and also on the TMV concentration. The more aged virus consistently shows greater values of maximum acid binding, whereas the

[2] In the calculation of the amount of proton binding, the hydrogen ion activity coefficients used were based on the total hydrochloric acid concentrations. Calculated

least aged virus exhibits a much lower value than would be expected from the appearance of the base-binding curve. When the TMV concentration is increased tenfold, as for TMV 6, the resulting maximum acid binding is decreased to roughly one-half of that found for the corresponding ages at the lower concentration. Moreover, the steep rise in the acid-binding curve begins to occur at about 0.5 pH unit lower for the 1:10 dilution of TMV 7 than for the 1:20 dilution. It will be further noted that by either increasing the TMV concentration or the time of standing (curves for TMV 7), the curves are displaced upward in the middle pH region, decreasing OH^- binding. Tentative explanations for these facts will be offered in the Discussion, upon consideration of reversible aggregation effects and of the initial reaction TMV monomers can apparently undergo.

Change in Ultraviolet Absorption and Extent of Heat Denaturation

It can be seen in Fig. 3 that for most of the samples showing a large initial pH and viscosity drop, there also occurred an appreciable and much more sudden drop in the light absorption at 260 millimicrons, expressed as optical density/c in which c is concentration in milligrams per cubic centimeter. In addition to the results shown, a few determinations were made on another preparation (No. 13), the one used by Cooper and Loring (15) for heat denaturation studies. In this case, for the stored solution, diluted 3.2 to 100 (0.37 mg./cc.), a still much larger drop in ultraviolet absorption occurred (from 4.36 to 2.98 at respectively 7 days to 46 days) along with a much larger pH drop (from 6.85 to 5.23 at corresponding times). It is interesting to note that the extinction coefficient of the undiluted stock solution did not fall at all in the same time, but rather increased to 4.63. However, there is one anomaly: one sample (TMV 19, dilution 1) did not show this correspondence at all, as the viscosity and pH dropped but the ultraviolet absorption did not. This behavior was noted also in studies not shown on other preparations.

Plots for the extent of heat denaturation show a close time correspondence to the changes in pH and viscosity, only the direction of change is reversed. This is to be expected in the light of the fact, to be discussed, that initial disaggregation reactions which liberate terminal nucleotide phosphoric acid for dissociation must also make this nucleic acid more labile for splitting by heat denaturation. Two additional determinations of the TMV 19 (second dilution)

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equivalents of hydrogen bound to protein were corrected for the apparent equivalents bound by the corresponding controls run in parallel using the same volumes.

A constant increment was in each case finally added to or subtracted from the calculated value of equivalents of hydrogen ion bound per 10^5 gm. of TMV, in order that each of the resulting curves should cross the axis at the isoelectric point, pH 3.9, as determined by Oster (3). The base-binding calculations were carried out in a parallel manner.

sample showed an increase of from 68.0 to 71.8 per cent at corresponding ages of 12 and 14 days, as expected from the pH decrease.

Hydrogen Ion Uptake in Dimerization

Since corresponding curves for pH and viscosity increase have the same shape, it is reasonable to conclude that a common reaction, that of end-to-end dimerization, is responsible for both changes. It is of interest, then, to calculate the number of hydrogen ions taken up per TMV monomer in this reaction. This may be obtained from the number of hydrogen ions reacting per TMV monomer in going from one aggregation state to another; *i.e.*, in a given time interval. The intrinsic viscosity values can be converted to average axial ratios by making use of the experimentally determined corrections to the Simha equation for the given velocity gradient, as described. After making the assumption that the average axial ratios observed result from definite mixtures of monomers and dimers only, it is possible to calculate the fraction of dimers present corresponding to these average axial ratios; the error resulting from making this assumption has been stated to be small (1). The calculation is made assuming an axial ratio for TMV monomers (P_1) of 19.7, the value obtained from the length of 300 mμ determined by Williams *et al.* (20) from electron micrographs, and the width of 15.2 mμ determined from results of light scattering, x-ray diffraction, and electron microscope studies (21), so that the following equation applies:

$$\bar{P}^2 = xP_2^2 + (1 - x)P_1^2 = (3x + 1)19.7^2$$

in which $\bar{P}$ is the weighted square average axial ratio observed, x is the fraction of dimers.

Next the measured pH increase occurring in the given time interval must be converted into a corresponding number of protons reacting per TMV monomer. By multiplying the measured pH increase by the slope of the titration curve drawn for the same or similar solution of TMV, at the mean of the pH range involved, the number of equivalents of hydrogen ion reacting per 10^5 gm. of TMV is obtained. To calculate the number of equivalents per mole of TMV, the latter product is multiplied by 400, assuming a molecular weight for TMV of 4×10^7, the value calculated from the particle size and from light-scattering measurements (20). The magnitudes of the slopes are found to vary only slightly with TMV concentration, the values at pH 6.5 being 3.01, 2.80, and 2.70 for the corresponding TMV percentages of 0.149, 0.298, and 1.70. Since both preparations 5B and 12 were studied at a higher concentration than the highest one of 1.70 per cent used for the titration studies (TMV 6, diluted 1:3), the values of slope for preparation 6 were used and considered to correspond closely enough. The values used, at the respective pH means, are presented in Table III.

Finally, it is of interest to convert the calculated number of hydrogen ions per TMV monomer taken up in going from one known aggregation state to another to the maximum number of protons reacting per monomer for 100 per cent conversion of monomers into dimers. Before this can be done, the mechanics of the reaction must be considered. Let us assume that in the net dimerization reaction y protons are taken up at the reactive end of each monomer, so that in the formation of the junction the total number of ions reacting is $2y$. Let it be further assumed that in the over-all reaction the average state of ionization of the free ends of the particles is the same before as after dimerization, so that there is no net uptake of hydrogen ions at the free ends in the reaction. Next, assume a mixture of monomers and dimers in which x is the

TABLE III

Calculation of Maximum Hydrogen Ion Binding Ratio in the Spontaneous Dimerization Reaction

TMV preparation No.	C	Velocity gradient sec.$^{-1}$	Time of standing	Intrinsic viscosity	ν^*	b/a	x	pH	Δ pH	Slope	Δ H^+	y	Average y
	mg./cc.		*days*	*cc./gm.*									
5B	20.1	2000	6	34.8	48.0	26.2	0.211	5.28	1.10	3.29	1445	3260	3305
			76	50.6	70.0	34.1	0.637	6.38					
12	36.4	186	13	25.9	35.8	20.15	0.015	6.97	1.25	2.70	1340	3350	
			200	38.5	53.2	26.4	0.283	8.22					

* The values of viscosity increment were calculated assuming a partial specific volume of 0.724, according to Lauffer (35).

fraction of dimers. Let z be the average number of protons per TMV monomer already reacted in the formation of the dimers present from all monomers. It can be shown that the following equation will then apply:

$$z = \frac{1 - x}{1 + x} y$$

Then, for the reaction between any two aggregation states, the number of protons reacting in going from state 1 to state 2 (Δ H^+) is equal to

$$\left(\frac{1 - x_1}{1 + x_1} - \frac{1 - x_2}{1 + x_2}\right) y$$

Solving for y, y = maximum number of protons for 100 per cent dimerization =

$$\frac{(1 + x_1)(1 + x_2)}{2(x_2 - x_1)} \Delta H^+.$$

The x's are calculated from viscosity results as described, and Δ H^+ is the observed number of hydrogen ions taken up in the chosen time interval. The values of all intermediate quantities calculated and the final value of y com-

puted using the above equation are presented in Table III. The average of two values determined for the maximum number of protons reacting per TMV monomer is found to be 3305. It will be shown that this result checks very closely with the irreversible binding ratio found for the binding of dimeric methylene blue with monomeric TMV, resulting also in 100 per cent dimerization of the virus.

Polymerization of TMV Resulting from Complex Formation as a Function of the Methylene Blue Concentration

When methylene blue is added directly to the virus inside the dialysis bag to an initial concentration which is greater than a certain threshold value, a considerable amount of dye binds irreversibly to the virus. This was first noted to be true when one of the virus-dye complexes thus formed was centrifuged, and the resulting pellet was observed to be colored much more intensely blue than the supernatant. It will be shown that threshold concentration required for stable complex formation is roughly the minimum molarity at which the dye itself is dimeric. The amount of irreversible binding resulting is greater when the methylene blue is added directly to the virus, than when added on the other side of the dialysis membrane, even though the amounts added are the same, giving the same final total concentration, in both cases. The explanation for this is that when the dye is added directly to the virus the initial dye concentration interacting with TMV is greater, meaning that the former in this case is more highly polymerized.

Individual total dye-binding ratios and the irreversible binding ratios were calculated for all the firm binding studies, as follows: for the first washing dialysis, the total number of millimols free in solution and the millimols adsorbed, obtained by interpolating off the corrected control curve, were both subtracted from the number of millimols entered into the cell, leaving the amount of dye bound to protein. This value was converted into a binding ratio by dividing by the number of millimols of protein present which was obtained from the volume and concentration, assuming a molecular weight of 4×10^7. For each subsequent washing, the value for total amount present before dialysis was obtained by subtracting from the initial total the entire amount removed in all the previous washes, and the value for millimols adsorbed was taken off the same control curve at the point corresponding to the respective equilibrium molarity. To obtain finally the irreversible binding ratio, the curve obtained by plotting binding ratios as a function of equilibrium molarity was extrapolated to zero concentration, for all of the reversibly bound dye will have been removed only when the dye concentration in the dialysate has reached zero.

The two binding curves obtained for studies I, for two different amounts of dye added initially inside, are shown plotted in Fig. 5, and the calculated

quantities are presented in Table IV. It will be at once noted that the irreversible dye to protein binding ratios for the two samples differ almost exactly by a

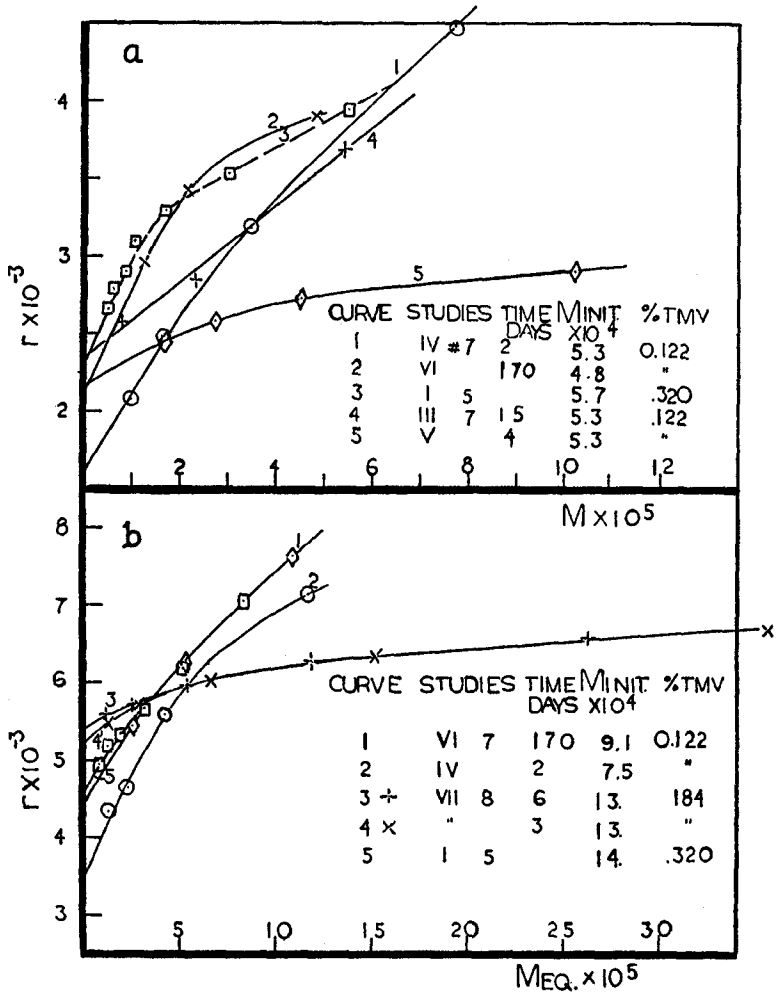

FIG. 5 *a*. Results of irreversible binding of methylene blue in dimeric state for TMV preparations 5B and 8 and for No. 7 at several times of standing.

FIG. 5 *b*. Results of irreversible binding of methylene blue in the tetrameric state for TMV preparations 5B, 7, and 8.

factor of two, the values being 2280 and 4590. Intrinsic viscosity determinations were also made on samples later set up to reproduce those obtained in studies I at the third washing, in which the diluting fluids were buffered methylene blue solutions at the measured equilibrium dye concentrations. The values obtained for these samples, at a velocity gradient of 186 sec.$^{-1}$, were

112 and 197 respectively, corresponding to axial ratios of 50 and 70. But dilution of the complexes, as well as washing off free methylene blue, causes breakdown of higher TMV polymers, as is seen from the viscosity curves, so that the average axial ratios of the complexes in the original undiluted solutions are roughly 20 per cent higher. TMV in sample 1, in which the dye most likely binds as dimers (see below), is seen to be on the average in a higher state of

TABLE IV

Studies I on the Irreversible Binding of Methylene Blue with Tobacco Mosaic Virus, Preparation 5B, pH 7.3

Dialysis No.	M at equilibrium $\times 10^6$	Millimols free $\times 10^4$	Millimols adsorbed $\times 10^4$	Total initial millimols $\times 10^4$	Millimols washed off $\times 10^4$	Millimols bound $\times 10^4$		r
Sample 1. 0.02 cc. of 0.0576 M methylene blue added								
A	5.51	3.32	2.39	12.02	2.20	6.31		3940
B	3.04	1.84	2.34	9.82	1.22	5.64		3530
C	1.72	1.032	2.32	8.60	0.69	5.25		3290
D	1.065	0.640	2.31	7.91	0.43	4.97		3100
E	0.876	0.527	2.30	7.48	0.35	4.65		2920
F	0.630	0.379	2.30	7.13		4.45		2790
Sample 2. 0.05 cc. of 0.0576 M methylene blue added								
A.1	19.02	11.51	5.00	28.35	7.61	11.89		7350
2	18.87	11.40			7.55			
B.1	8.50	5.14	4.43	20.74	3.40	11.17	11.28	7050
2	8.25	4.50	4.42	20.80	3.30	11.38		
C.1	5.19	3.14	4.31	17.34	2.08	9.89	9.92	6180
2	5.34	3.23	4.32	17.50	2.14	9.95		
D	3.16	1.91	4.25	15.26	1.26	9.10		5670
E	1.96	1.187	4.23	14.00	0.80	8.58		5350
F	1.31	0.792	4.21	13.20	0.52	8.20		5100
G	0.873	0.528	4.21	12.68		7.94		4950

polymerization than the dimeric state, and in sample 2, in which the dye binds in a still more polymerized condition, the TMV exists roughly in an end-to-end tetrameric state.

On still other samples, in which the initial methylene blue concentration was considerably lower, no polymerization of the TMV resulted and no irreversible binding was observed for these samples. To determine the initial dye molarity at which irreversible dimerization first occurs and to study the effect of further increasing the dye concentration, intrinsic viscosity of a whole series of samples was related to both the total initial dye concentration and the equilibrium molarity. The results are shown in Fig. 6, the lower curve representing the

results for several samples of preparations 6 and 7, plotted as a function of the calculated initial dye molarity on the protein side of the dialysis membrane. It will be noted that a very steep rise in viscosity occurs at a concentration of 1.9×10^{-4} M. The fact that this rise to a viscosity corresponding roughly to TMV dimers is so steep and that at higher concentrations is gradual would suggest that the formation of TMV dimers is an irreversible process and that the formation of higher polymers is reversible. Direct confirmation of this is provided by the experiments on TMV 7 (studies VI) in which intrinsic viscosity

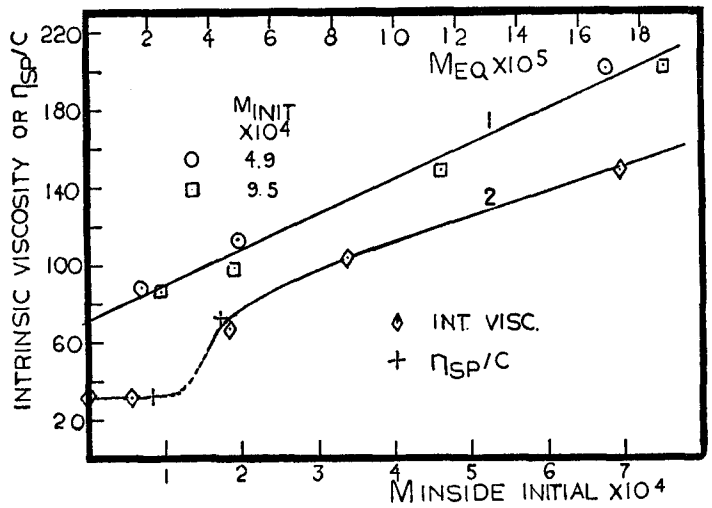

FIG. 6. Variation of intrinsic viscosity or $\eta sp/c$ with initial or free methylene blue concentration in various equilibrium dialysis samples. Curve 1 (use scale at top), firm binding (studies VI), intrinsic viscosity as a function of equilibrium molarity times 10^5; curve 2 (use scale at bottom), intrinsic viscosity (studies V and VI) or $\eta sp/c$ (binding studies on TMV 6, 1:25) as a function of the initial dye molarity times 10^4 on the protein side of the membrane.

determinations were made after each of three successive washings, and once more after methylene blue was put back into the system in sufficient concentration to form TMV tetramers, by dialyzing against 3.2×10^{-4} M dye. If the higher polymer formation is a reversible reaction, one would expect the viscosity to drop with successive washes and to increase again on the addition of dye. Not only was this found to be the case, but also the intrinsic viscosity was found to vary linearly with the equilibrium molarity, the point for the final dialysis against dye being included. The straight line relation obtained for both samples is shown at the top of Fig. 6. On extrapolating the line to zero concentration, a value of 70 is obtained for the intrinsic viscosity, which should be that of the TMV complex formed irreversibly, for no further break-

down can occur when all free dye has been removed. The axial ratio corresponding to this intrinsic viscosity is found to be 41.0 which agrees within 4 per cent with the theoretical value for dimeric TMV. Thus final proof of the irreversible

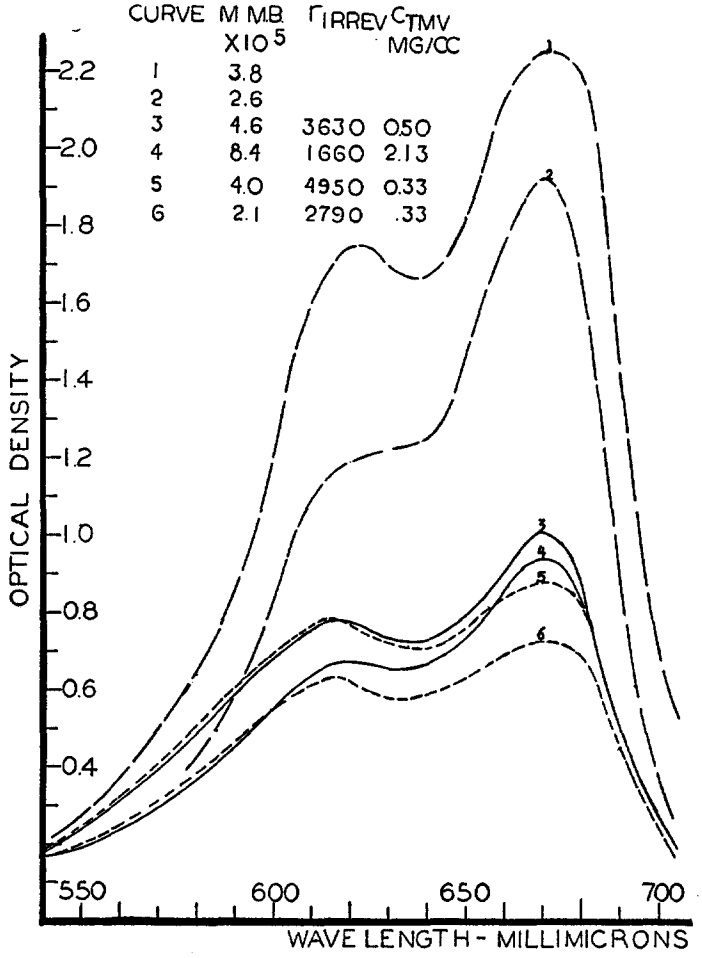

FIG. 7. Light absorption curves for various methylene blue–TMV complexes and dye solutions in phosphate buffer of 0.02 ionic strength.

formation of TMV dimers is provided. So far, the only experimental means presented to define the extent of polymerization has been viscosity. In addition, confirmatory results of both sedimentation studies, permitting molecular weight calculations, and electron micrographs[1] have been obtained. The formation of an appreciable fraction of TMV dimers when TMV is combined with methylene blue has also been confirmed in the laboratory of O'Kon-

ski, employing the method (21) of measuring birefringence and relaxation time in a sinusoidal electric field, for studying electrical orientation and relaxation effects.

A consideration of the initial dye concentration at which TMV dimers are first formed, in terms of the most probable polymeric state of the methylene blue present in solution, is of interest. An estimation of the polymeric state for a given molarity may be made from a study of the light absorption spectrum in the region of 600 to 670 millimicrons, as described by Michaelis and Granick (22). If the dye concentration is sufficiently low, there is a single peak (α) at 665 millimicrons, but between the concentrations of 1.27×10^{-4} M and 5.97×10^{-4} M, a second peak (β) begins to appear at a wave length of approximately 615 millimicrons. According to Michaelis, the α-peak represents dye monomers and the β-peak, dimers. Thus at the threshold concentration for TMV dimer formation of 1.9×10^{-4} M, some methylene blue dimers must be initially present in solution, suggesting that the dye which binds irreversibly must also be dimeric. Absorption spectrum studies made on the TMV-dye complexes, the results of which are shown in Fig. 7, reveal this to be the case, for the curves showed pronounced β-peaks, with the position of maximum slightly shifted to the left (to 618) relative to the control solution of dye in the dimeric state, indicating some higher polymer formation, for the samples having irreversible binding ratios of 3000 or less. For the sake of simplicity, we shall say such samples bind methylene blue, on the average, in the dimeric state. The position shifts still further, to 615 (to correspond more nearly to a γ-peak) for the sample having a binding ratio close to 5000, in which the methylene blue can be said, for simplification, to bind as tetramers, on the average. When the terms "dimeric" and "tetrameric," as applied to bound methylene blue, are used in this paper, the meaning intended is that they represent only very rough approximations.

Correlation of the Irreversible Binding Ratios for Different TMV Preparations in Different Aggregation States

Since methylene blue must bind irreversibly in the polymeric state to convert TMV irreversibly to end-to-end dimers, as described above, it is logical to conclude that the irreversibly bound dye is that which goes into the junction between two joining monomers, thus serving to cement the monomers together. For a preparation of TMV more highly aggregated at the time of the binding experiment one would then expect to find less irreversible binding than for the less aggregated ones, since the esterified nucleotide phosphate groups within the junction of naturally formed TMV dimers, being uncharged, could not be expected to react with methylene blue. Exactly this was found to be the case. The most convincing evidence is obtained by the direct correlation of pH and methylene blue irreversible binding in a single sample, TMV 7. The cor-

responding plots are shown in Fig. 2, from which we can see that the time changes are exactly parallel only reversed in direction, as expected. Also on comparing the irreversible binding ratios, as summarized in Table V of three preparations of TMV (Nos. 5B, 7, and 8) having different average axial ratios, it will be noted that preparation 8, the least aggregated, has the highest ratio, and No. 5B, the most aggregated, has the lowest ratio.

In the same way in which calculations could be made to determine the maximum hydrogen binding involved in dimerization, as described, we are now in a position to calculate the maximum ratio of irreversible binding of methylene blue which effects 100 per cent dimerization of the virus. Another equation was

TABLE V

Summary of Ratios for the Irreversible Binding of Methylene Blue with Tobacco Mosaic Virus

TMV preparation No.	Studies No.	Age of preparation at time of experiment	r_0 Dimeric	$2 \times r_0$ Dimeric	r_0 Tetrameric	Average r_0 Tetrameric
		days				
5B	I (cellophane bags)		2275	4550	4590	4570
7	III (ultracentrifugation)	15	2315	4630		4630
	IV (mounted tubes)	2	1765	3530	3570	3550
	V (lucite cells)	4	2100	4200		4200
	VI (mounted tubes)	170	2145	4290	4270	4280
8	VII (lucite cells) 1	3			5190	
	2	6			5310	

developed which defines the maximum dye binding in terms of the observable quantities, the actual irreversible binding ratio in a mixture and the corresponding average axial ratio determined from the intrinsic viscosity at the time of the binding experiment. If the latter was not determined at that time, it was necessary to interpolate off a curve obtained by plotting intrinsic viscosity as a function of time of standing. The curve used for TMV 5B was that given in Fig. 2 *b*, and a similar curve was drawn for TMV 7 from the viscosity measurements made on this preparation.

Let x be the fraction of dimers initially present in a given preparation, and let $2y$ be the maximum number of methylene blue molecules, or y the number of dimers, which can bind at the end of a TMV monomer to form the junction. It can then be shown that z, the observed average number of dye molecules bound per TMV monomer, for a given mixture, is given by the equation:

$$z = \frac{2y}{1 + x}$$

Solving for y, $y = (z/2)(1 + x)$. This equation, of course, describes the way in

which only end-to-end aggregation lowers the observed irreversible binding. There are, in addition, other configurations, such as those existing in monomers initially in which nucleic acid groups are not appreciably ionized, which result in lowered irreversible binding in an entirely independent manner.

The results of a number of firm binding determinations on preparation 7, in which the dye was added in such concentration originally that it was bound irreversibly in the dimeric form, are depicted in Fig. 5 *a*. In Fig. 5 *b* are shown the results of firm binding determinations on preparations 7 and 8 in which the dye is bound irreversibly in the tetrameric form. All the values of r_0, the irreversible binding ratio, obtained from these curves and from those for TMV 5B by extrapolation to zero concentration, have been summarized in Table V.

TABLE VI

Calculation of the Maximum Binding of Tetrameric Methylene Blue in the Dimerization of TMV

Preparation No.	C	Time of standing	Intrinsic viscosity	ν at 2000 sec.$^{-1}$	b/a	x	Methylene blue irreversible binding ratio	Maximum binding ratio	Average
	mg./cc.	*days*							
5B	3.20	16	40.0	55.3	29.2	0.400	4560	6390	6465
7	1.18	15	40.5	56.0	29.5	0.413	4630	6540	
		170	45.1	62.4	31.8	0.533	4270	6550	
8 (No. 2)	1.77	6	29.25	40.4	23.1	0.123	5310	5960	

Since the results for tetrameric binding, as explained, are always in exact correspondence to those for dimeric binding by a factor of two, all the dimeric irreversible binding ratios were multiplied by two to convert to a common basis of binding in the tetrameric form. When the above equation is then applied to the results of preparations 5B, 7 (studies VI), and 8 (nearly monomeric), the maximum binding ratios calculated for tetrameric binding are found to be 6390, 6545, 5960 respectively. The two values for TMV 7 at different times are seen to agree almost exactly. The values of intermediate quantities involved in the calculation of the above are summarized in Table VI. Thus the average maximum irreversible binding ratio for TMV 5B and 7 for tetrameric binding is 6465, or 3230 for dimeric binding, which agrees very closely with the maximum average proton binding of 3300 involved in dimerization, calculated as described. TMV 8 exhibited an appreciably lower irreversible maximum binding, presumably because its time of storage was so short (6 days) that its state of reversible initial aggregation must be considered to be still high, thus resulting in a lessened availability of dissociated nucleotides for irreversible binding. For this reason, this value was not included in the average.

Changes in the Irreversible Binding Ratio for a Given TMV Preparation Occurring with the Time of Standing

A number of firm binding studies were carried out on the single preparation, TMV 7, and the conditions of the determinations were varied for two of them, *i.e.* for studies V the equilibrium dialyses were carried out at room temperature and for III the washings were effected by repeated ultracentrifugations. Changing of the conditions would be expected to change the results for the reversible portion of the binding, but the irreversible binding ratios should not be altered as long as irreversible binding occurs. It was found, for example, that for studies III the threshold dye concentration necessary for irreversible binding and for TMV dimer formation was higher than for the other studies. Sample 1, which was set up using concentrations previously found to give the dimeric complex, showed no irreversible binding, and had an intrinsic viscosity corresponding to a mixture of mainly monomers. The irreversible binding ratios obtained by extrapolation were 0, 830, and 2340 and the corresponding intrinsic viscosities at 2000 sec.$^{-1}$ measured at the fourth washing were found to be 49, 58, and 81 respectively. Thus only in sample 3, set up to be tetrameric, was the dimeric complex formed. The most likely explanation for these unexpected results is that the formation of TMV dimers, irreversibly at least, is a slower reaction than at first thought, taking place in a day or more, which was confirmed in some separate studies. In the centrifugation washing method, the time of contact of the virus with the dye at its initial concentration is less than an hour, whereas in the dialysis method, an average concentration almost as high exists for a day or more.

On considering, then, the irreversible binding ratios (as given in Table V) for all the studies made at different times on TMV 7 where such binding occurs, we find that the values at first increase sharply, reaching a maximum after about 15 days of standing, after which the irreversible binding must fall off gradually. As already mentioned, exactly parallel sequences occur in other series of measurements, all of which indicate an initial rapid increase in dissociation of nucleic acid groups, followed by a slow decrease in their dissociation, which occurs concurrently with end-to-end aggregation.

Binding Studies at a Higher Concentration of TMV

In all the studies reported so far, the TMV concentrations used in the binding experiments were in the range of 0.1 to 0.2 per cent. In addition, three firm binding samples were set up using TMV preparation 6 at a concentration of 1.01 per cent. The methylene blue concentrations used were those calculated to be in proportion to the higher TMV concentration in order to form the dimeric and tetrameric complexes in samples 2 and 3. However, an inspection of the binding curves[1] reveals that not even the dimeric complex could have been formed in any sample. Instead, the irreversible binding ratios were found to be

520, 600, and 725 respectively, which are about one-quarter of that normally expected for the formation of the dimeric complex. These results would suggest that the virus at this higher concentration is reversibly aggregated, in such a way that the nucleic acid groups are tied up.

DISCUSSION

Results of all studies carried out show that aqueous solutions of TMV generally exhibit on standing five types of changes which occur in a parallel manner as a function of time of standing. These variables measured as a function of time are pH, specific viscosity divided by concentration, the absorption of ultraviolet light, the extent of nucleic acid splitting by heat denaturation, and the irreversible binding of methylene blue. As the pH decreases in the initial reaction, viscosity and sometimes the ultraviolet absorption decrease, whereas the dye binding and apparently also the extent of nucleic acid splitting increase in magnitude. As the pH increases in the subsequent irreversible end-to-end aggregation, all the above change in the reverse direction as expected. The fact that these very separate types of changes can thus be correlated provides good evidence that active groups in the TMV rods undergo two successive types of reactions, occurring at the particle ends and involving opposite changes in ionization. Evidence that the groups involved which produce the pH changes are actually the nucleotide phosphates is provided both by the experiments on methylene blue binding and by the fact that the extent to which nucleic acid is split off by enzymatic hydrolysis with intestinal nucleophosphatase (23)[3] and by heat denaturation varies with the age of the preparation, the conditions employed each time being the same.

In the first reaction, in the gross, a dissociation of phosphoric acid groups occurs, resulting in both an increase in the number of negative charges on the polynucleotide chains and in the lability of these chains, confirmed by the greater ease of nucleic acid splitting. A detailed consideration of this initial reaction reveals that the manner in which the disaggregation occurs is highly complex and variable. In the second reaction, along with the pH increase, and

[3] Cohen and Stanley (23) have attempted to reproduce the results of Schramm, showing that nucleic acid could be split off quantitatively from a 2.54 per cent solution of TMV, but were unable to do so. Further confirmation of the hypothesis presented in this paper would be provided if it explained this discrepancy: Stanley's preparation might have been a fresh one, with nucleic acid undissociated, whereas the nucleic acid of Schramm's TMV preparation might have undergone extensive hydrolysis. However, conditions used were not the same, so this comparison cannot be made. Stanley and Cohen, however, provided confirmation by presenting data showing differences in TMV phosphorus recovery after enzyme action between two preparations of different ages under the same conditions. For the more aged preparation the virus recovery was about 15 per cent lower after reacting with the enzyme than for the fresh preparation.

reversed state of ionization of phosphates, an increase in the number of end-to-end polymers occurs, as shown by viscosity results. This would suggest that the nucleotide phosphates which are involved in the end-to-end dimerization are also the groups which are reacting with hydrogen ions, meaning that the groups concentrated at an end of one TMV rod may interact with those at the end of another to form a junction.

A plausible reaction explaining all these effects would be the formation of phosphotriester linkages between two adjacent intermeshed chains projecting from the ends of the two joining particles. The manner in which a set of chains from the end of one particle can intermesh with the set from the other particle will be discussed. Since normal chain linkages are 3′–5′ diester linkages in ribonucleic acid, as Brown and Todd (24) point out, triester cross-linkages between parallel chains would be between the 2′ and 5′ positions. Since ribonucleic acid has been shown to have about one triester linkage per four nucleotides, by studies on ribonuclease action and by the results of electrometric titrations (24), the possibility of a great number of such linkages being present in TMV nucleic acid is to be expected.

Since the formation or hydrolysis of internucleotide ester linkages is only known to occur enzymatically in the presence of ribonuclease, we must conclude that because TMV appears to undergo such changes on standing, the TMV particle must have its own ribonuclease content built in. As will be discussed, all the observed facts are consistent with this picture. For the enzymatic formation of each phosphotriester cross-linkage, then, the net reaction involves the uptake of one hydrogen ion and the release of one molecule of water.

As to the detailed nature of the initial, reversible disaggregation reactions, resulting in increased availability of nucleic acid, which occur, some interesting deductions may be made by comparison of the variables measured. In cases in which the pH drops, that is for the concentrated water solutions of TMV, purified with the minimum of phosphate washing, the specific viscosity/c is seen to drop also, meaning that the virus is undergoing end-to-end disaggregation. This appears to occur, to a greater or lesser extent, over the entire TMV concentration range. In the cases in which there is an initial steep rise in both pH and viscosity and also ultraviolet absorption (*e.g.* TMV preparation 18 in 10^{-6} M phosphate), too rapid to be the final irreversible aggregation, the reaction must be the breakup of reversible side-to-side aggregates already formed. This is seen to be the initial reaction when the virus has already undergone prolonged standing in phosphate (*e.g.* TMV 5B), causing an extensive breakup of end-to-end and formation of side-to-side aggregates and thus a low initial pH. The evidence for the conversion of aggregates to the side-to-side type by phosphate is provided by the fact that the $\eta sp/c$ *versus* c in Table II for the second intrinsic viscosity determination, in which case only the solution

had been standing in phosphate of 0.02 ionic strength, has a negative slope, whereas all the others have strongly positive slopes. Moreover, intrinsic viscosity measurements on TMV 7 in 0.0001 M phosphate yield increasingly negative slopes of $\eta sp/c$ *versus* c with longer times of standing up to 170 days.

Further evidence that addition of phosphate at high TMV concentrations results in the most complete destruction of end-to-end aggregates with retention or formation of the side-to-side type is provided by viscosity-time results on preparations 17B and 18 (dilution 3). It will be noted that for TMV preparation 18 in 10^{-6} M phosphate, the initial nsp/c is much lower than the first value measured for dilution 1 (see Fig. 3*a*). Initial $\eta sp/c$ values for TMV 17B, diluted 1:50, were 81 in 0.0001 M phosphate and 135 in water for respectively 1 day of standing (one-half day after dilution) and 7 days of standing ($3\frac{1}{2}$ days after dilution). In the former case viscosity increased rapidly with time, whereas in the latter case it dipped slightly. The corresponding changes for ultraviolet absorption and pH for TMV 18 in 10^{-6} M phosphate are seen to be nearly parallel.

The results of ultraviolet absorption and pH measurements made on the recyclized pooled solution, washed once with water, 0.01 M and 0.1 M phosphate show that previous phosphate addition in the purification gives the same effect. Whereas the solutions washed with water or 0.01 M phosphate simply showed slow, continual increases, that treated with 0.1 M phosphate (Fig. 1) gave a lower initial ultraviolet absorption value and sharp, initial rises for both quantities, indicating side-to-side aggregate breakup, followed by slight decreases.

The fact that the change in ultraviolet absorption is apparently associated with side-to-side aggregation reactions, is deduced from the following observations: When an initially end-to-end aggregated preparation is diluted too soon so that the initial pH drop is only slight (*e.g.* TMV 18 and 19, dilution 1), the viscosity drop is seen to be less steep and is not followed by an appreciable rise (No. 18), and, further, there is no ultraviolet absorption drop (Nos. 18 and 19). However, for a later dilution or a higher TMV concentration after dilution, the viscosity drop is steeper followed by a rise (TMV 19, dilution 2); there is an almost vertical drop in the ultraviolet absorption (TMV 18 and 19); and there is a corresponding deeper and sharper dip in pH, as for TMV preparations 7, diluted 1/10, 13, and 19. It would appear that the side-to-side aggregate formation is favored by conditions sufficient to break down the most easily ruptured end-to-end aggregates but which will not destroy reversible aggregates altogether, that is by too great a dilution. By maintaining a higher concentration of TMV for a longer time, more extensive aggregation of the end-to-end type in particular results, so that on dilution a breakdown of these aggregates occurs to leave more of the side-to-side type.

Some interesting relationships observed in the acid base titration curves may

be explained in terms of these effects. First, an over-all comparison of the set of curves for TMV 6, at 1.70 per cent, with those for TMV 7, at concentrations of 0.15 or 0.30 per cent, reveals that at the much higher concentration in the former case, the extent of the maximum acid binding is always much lower, because of the greater initial reversible aggregation already demonstrated. No conclusion can be drawn as to the maximum base binding, because in strongly alkaline solutions the rate of degradation by hydrolysis is high enough to change the pH appreciably as the measurement is being taken. With longer times of standing the increased maximum acid binding is explained by the ability of the acid to destroy side-to-side aggregates, thus liberating carboxyl groups which are titrated. Along these lines, it will be noted that the tenfold dilution of preparation 7 in 0.0001 M phosphate, which had stood for 27 days, under which conditions side-to-side aggregation is more extensive as discussed, showed still less acid binding until the pH was decreased below 3. Base binding in the region of 4 to 8 is seen to decrease successively by parallel displacements upward by increasing the time of storage at the same concentration and further by increasing both the time and concentration after dilution. In addition to aggregation effects on carboxyl titration, the low maximum acid binding observed for the fresh solutions could be explained by the greater release of dissociable nucleotide acid groups than for the aged solutions, there being a greater extent of esterification in the folded chains (to be described) in the fresh TMV. Thus, on decreasing the pH below 3, there would presumably be a more extensive hydrolysis in the fresh solutions of ester cross-linkages, resulting in a greater release of hydrogen ions. This hydrolysis can be expected from the fact that triester linkages are very sensitive to both acid and alkali (see (24), page 416).

Theoretical: TMV Structure

To explain the initial reactions increasing the availability of nucleic acid and those reactions of the extended polynucleotide chains which follow causing end-to-end aggregation, an attempt will be made to evolve a mechanism and a structural scheme. As a model for structural considerations, the results of x-ray diffraction analysis made by Watson (26) and Franklin (27) may be used. Watson has suggested a structure for TMV consisting of a central thin core of nucleic acid to which is attached two-chained units of globular protein of molecular weight approximately 35,000, arranged in a helix about the core. By treatment with dilute alkali, Schramm (28) obtained nucleic acid–free fragments containing four such units, but by treatment with sodium dodecyl sulfate, Fraenkel-Conrat and Singer (29) have broken down TMV to pure protein units of about one-half the molecular weight of the two-chained ones, that is to single chains having the minimum molecular weight calculated from amino acid analyses (28). Franklin (27) also points out that there is a cylindrical shell of

higher density with a radius of 55 A, which could be attributed to the nucleic acid itself or to a non-hydrated region surrounding the heavily hydrated central core containing the nucleic acid. The latter possibility would seem to be more likely, and we shall accept it as a model.

Cohen and Stanley (30) found that the particles of nucleic acid split off by heat denaturation from the fresh virus had a molecular weight of approximately 300,000. This would correspond roughly to a single polynucleotide chain extending the entire length of a TMV rod. However, to explain the initial reactions, we shall postulate a folded configuration such that the polynucleotide chain folds back on itself, lengthwise to the TMV particle, at least once.

The arrangement of material in the hydrated core can be reasonably considered to be very loose, such that there would be room for a few extending polypeptide chains from adjoining TMV particles to be inserted into the core. In order that this loose structure may have stability, the existence of numerous triester cross-linkages as well as hydrogen bonds between neighboring polynucleotide chains (or between the two parts of a doubled chain) can be postulated. One may consider, then, that the inserted polypeptide chains from adjoining particles can have an enzymatic action to hydrolyze the triester cross-linkages, starting most likely with those holding in the shorter folded ends of polynucleotide chains, near the ends of TMV rods, and working inward.

The initial pH decreases in all cases can be explained by the enzymatic hydrolysis of triester linkages, which can vary in extent depending on how long the concentrated solutions stand before dilution to maintain the necessary aggregates intact. If the solution is diluted too early, the end-to-end disaggregation which occurs may be simply explained by the pulling out of inserted polypeptide chains from the end-adjoining particle, since they are attached by electrostatic attraction only. If, however, the enzyme action is allowed to continue by longer standing before dilution, until hydrolysis of cross-linkages proceeds right down to the chain end, there is no longer a firm anchoring of this chain end within the core. On diluting at this stage, as the reversibly joined particles are pulled apart, the freed polynucleotide chain end would be expected to be pulled out by electrostatic attraction to the inserted polypeptide chain as it is being withdrawn.

At this stage, a general loosening of the nucleic acid structure is seen to occur, with a number of polynucleotide chains extending outward from the ends of TMV rods. These chains are now free to interact with those extending from side-adjoining particles to effect the initial side-to-side aggregation or more slowly with those from end-adjoining particles to cause the irreversible end-to-end aggregation. The sudden drop in ultraviolet absorption which now occurs is most easily explained by the greatly increased possibility for the interaction of extended polynucleotide chains with basic groups on side-adjoining particles. This would be expected from the results of Blout and Asadourian

(31), which show a lowering of the extinction at 260 millimicrons of DNA solutions by the addition of certain proteins. That the initial small increases in absorption at 260 mμ always observed can arise from ribonuclease action on cross-linkages is suggested by the results of Kunitz' studies (32) on the effect of ribonuclease on the ultraviolet absorption of yeast nucleic acid. The apparent catalytic effect of phosphate in causing extension of polynucleotide chains permitting side-to-side aggregation most likely results from a disrupting or repelling effect on compact nucleic acid structure, opposite to the stablizing effect observed for cations, in particular the divalent ones (see (24), pages 526–527).

Such an extension of a few single polynucleotide chains would explain the finding of Oster (3) that, by heating to 60°C. at the isoelectric point, loose end-to-end aggregates are formed, in which gaps up to 30 millimicrons appear where the particles join as seen in the electron microscope. At these gaps there is most likely an electrostatic interaction between a few projecting polynucleotide chains and polypeptide chains from the ends of joining particles. Also, it was observed that heating at the isoelectric point (unpublished results) causes the release of a dialyzable fraction which has the characteristic absorption spectrum of nucleic acid, implying that there is not only an extension but a splitting off of a few short chains, possibly branches or folded-in ends.

Precipitation of TMV with hot trichloracetic acid by the Schneider method was shown by Waritz (33) to cause also the splitting off of small nucleotide fragments. He detected the presence of adenine and cytodylic acid, resulting from hydrolysis of the fragments, in the supernatant from the precipitation. Fraenkel-Conrat and Singer (29) have also observed that under similar conditions a great number of N-terminal proline amino and some ε-amino lysine groups are released, which do not appear under other conditions effecting removal of nucleic acid, suggesting that these groups are peptide-bonded to ω-carboxyl groups to form peptide cycles. The effect of hot trichloracetic acid, then, would be to hydrolyze not only tri- and some diester linkages to loosen the nucleic acid structure and split off fragments but also various peptide bonds in the cycles to release amino groups.

It would be of great interest to derive, now, a mechanism describing the irreversible dimerizations occurring after the extension of polynucleotide chains by hydrolysis, either by the slow spontaneous reaction or by combination with polymeric methylene blue. The two types of dimerization were shown to be competitive and in fact equivalent as to the number of charges neutralized. Since by combination with methylene blue the irreversible end-to-end dimerization occurs only when the dye binds irreversibly, it is logical to conclude that all the dye thus bound goes into the junction. The number of molecules which would have to be fitted in to the junction space is about 6400 for dimeric binding, as we have seen. This is far in excess, however, of the number which can be

fitted in the closest packed configuration in a single layer between the ends of two particles, which is calculated to be roughly 1000. To reconcile this discrepancy, a junction configuration must be conceived which permits multilayering of the dye particles. One such configuration would be an intermeshing of a set of polynucleotide chains extending from the end of one particle to those from another, the whole being maintained as a stable lattice of opposing charges by the insertion of bridging dye polymers oriented perpendicularly to the chains.

However, the assumption that such a configuration depends on electrostatic forces only to hold it together, would not adequately explain the great stability of the dimeric complexes, which are unaffected by removal of free dye and long standing. Therefore, instead of the shallow intermeshing of the extended portions of chains, which probably occurs in the reversible end-to-end polymerization above dimers, we shall postulate a deep intermeshing, in which the extended chains from one particle are inserted well into the core of another. With this configuration, the full extent of hydrogen bonding in the initial, folded arrangement of chains in the monomer is made possible, along with a consequent, great increase in stability.

Furthermore, the same type of intermeshing provides a more satisfying explanation for the slow irreversible, end-to-end dimerization occurring in TMV solutions on standing. In addition to permitting many more nucleotide sites to react, this configuration is seen to correspond to the initial arrangement of chains in monomers before unfolding by hydrolysis has taken place, so that the environment is right for the required reversed enzymatic reactions to take place. As described earlier, esterification to form cross-linkages between the two sets of chains of two joining monomers must now occur within the cores. As before, a few inserted polypeptide chains will act enzymatically to catalyze the reverse reaction. Energy could be conceivably provided for the reaction by the hydrolysis of a few pyrophosphate groups located on nucleotide phosphates, formed originally in the hydrolysis reaction.

Since there has been shown to be an equivalence between the number of hydrogen ions taken up in the spontaneous dimerization and the number of methylene blue molecules combining in dimeric binding, it is of interest to consider the number of phosphate sites required for both reactions in comparison with the total number available. Since, in esterification, the number of hydrogen ions taken up is the number of triester linkages formed, the number of sites reacting is about 3200 per monomer or 6400 per dimer formed. One may also presume that for all cases of irreversible binding with methylene blue there is an exact neutralization of charge of the phosphate sites involved in the formation of the dimer. In this way, the binding of a dimeric unit of dye, with a positive charge at each end to effect bridging, is equivalent to the formation of a pair of triester cross-linkages. Taking into account the fact that there is bridg-

ing to the chains on opposite sides of a given chain, there is seen to be neutralization of the 3200 sites per monomer when all the irreversibly bound 3200 molecules of dye go into the intermeshing space in the core. For tetrameric binding, rather than assume simply the same number of binding sites to give twice the binding, one must assume that one-half as many polymeric units must now go into the intermeshed core, and the remaining half of the irreversibly bound dye would have to combine with the nucleic acid in the outer portions of the TMV dimer. The retention of irreversibly bound dye at the outside ends of dimers in the case of tetrameric, but not dimeric, dye binding can be conceived to occur by presuming that the folded nucleic acid configuration is retained in the former case but not in the latter. Thus, although methylene blue concentration is being continually lowered by irreversible combination with the virus, the concentration still remains sufficiently high in the tetrameric binding case so that there are enough dye polymers at the ends to combine with hydrolyzed nucleotide phosphates to replace any polypeptide chains, initially combined with the nucleic acid, from the same or from some adjacent TMV particle.

There appear, then, to be a total of 6400 nucleotide phosphate sites per TMV monomer, assuming a symmetrical arrangement. The total number of nucleotides in a TMV molecule, assuming a molecular weight of forty million and a 1:1 ratio between P atoms and nucleotide units, is calculated from the phosphorus composition (12) to be 6700. This means that nearly all the nucleic acid in the reacting half of a TMV particle is used up in the dimerization, implying that after intermeshing all sites of a given chain are quite close to those of some adjacent chain.

It should be especially noted that only the dye polymer has the proper arrangement of positive charges, *i.e.* one or more at each end, to effect the cross-bridging. The single positive charge of methylene blue is normally distributed by resonance between the two ends and the central sulfur atom, but in the field of negative charges one would expect in the resulting stable state that the charge would be drawn to one end or the other, so that the monomer could not make an effective bridge. This is in accordance with the experimental findings.

At first sight, one would expect methyl green to show the same kind of irreversible binding behavior at all concentrations, since, unlike methylene blue, it is dicationic without polymerization. Kurnick (34) has studied the specific combination of this dye with cellular desoxyribonucleic acids. However, both dialysis and activity experiments show this to be not the case, as methyl green does not irreversibly bind to TMV and deactivate it. To study only the reversible binding to the other acid groups of the virus, the carboxyl groups, it is necessary to use a non-polymerizing, singly charged dye such as safranine. With safranine some irreversible binding was found to occur, although not of the nucleic acid but as a result of precipitation.

The fact that such basic dyes as methylene blue cause complete irreversible inactivation of the virus suggests a means for either prevention or cure of the disease in tobacco plants, provided that a practical means were found to put the dye into the plant without harming it.

SUMMARY

1. Aqueous solutions of tobacco mosaic virus were found to undergo a number of spontaneous changes on standing in the cold. The results of pH measurements, acid and base titrations, intrinsic viscosity determinations, studies on the irreversible binding of methylene blue with the virus, ultraviolet absorption, and the extent of nucleic acid splitting by heat denaturation indicated the occurrence of two successive reactions, the first one causing the release of hydrogen ions and a greater lability of the nucleic acid, and the second one, which involved end-to-end dimerization and which took place after 8 days of standing, requiring hydrogen ions.

2. The first over-all reaction was found to be a mixture of various types of reversible disaggregation and aggregation reactions, the nature of which depended on the pretreatment, the TMV concentration, the time of standing, and the phosphate concentration. For longer times of standing at high protein concentration a sudden drop in ultraviolet absorption is noted after dilution; also the drops in viscosity and pH are largest with a steep rise following, indicating the greatest breakup of end-to-end aggregates with formation of the side-to-side type. For concentrated solutions of TMV in water which have not stood long no drop in ultraviolet absorption is noted on dilution; the decrease in the other quantities is less, indicating that only a less extensive breakdown of end-to-end aggregates occurs. Addition of phosphate to concentrated solutions of TMV causes formation of side-to-side aggregates which break up on dilution.

3. Using the results for the pH increase and the viscosity increase in a given time interval for a given TMV preparation and also the slope of the corresponding titration curve at the pH mean, a value for the number of hydrogen ions taken up per TMV monomer in the formation of the end-to-end dimer was finally calculated. The average result obtained for two preparations was 3300.

4. Methylene blue, in the polymeric form, was demonstrated to cause complete irreversible conversion of TMV monomers to end-to-end dimers. At dye concentrations above 10^{-4} M, higher TMV polymers are formed, but these are broken down to dimers on removal of free dye by dialysis. The irreversible binding ratios were shown to be decreased in accordance with the extent of the end-to-end aggregation of the preparation at the time of the experiment, which is in agreement with the concept that the irreversibly bound dye polymers go into the junction formed between two interacting TMV monomers. On the basis that only the monomers initially present in solution can react, max-

imum binding ratios corresponding to complete conversion of monomers to dimers were calculated from the observed irreversible binding ratios and from the fraction of dimers initially present which was obtained from viscosity data. The average result for three preparations in different states of aggregation was calculated to be 6565 for tetrameric binding or 3230 for dimeric binding, which agrees closely with the result obtained for the uptake of hydrogen ions per TMV monomer in the spontaneous dimerization.

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