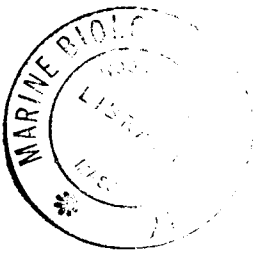

POLYMERIZATION-DEPOLYMERIZATION OF TOBACCO MOSAIC VIRUS PROTEIN

I. KINETICS

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ABSTRACT It was shown that a reversible endothermic association of TMV protein subunits (A protein) can take place at pH values below the isoelectric point as well as at pH 6.5. The polymerization occurring below the isoelectric point was found to be more complex than that at pH 6.5 probably because products other than the usual TMV-like rods were formed in addition to those rods and also because side-to-side aggregation of the rods took place readily. Kinetic studies indicated that polymerization can be treated as a second-order linear condensation. The rate of polymerization was found to be a critical function of pH, having a maximum value near pH 4.3. This behavior is at variance with the hypothesis that hydrogen-bonded carboxyl pairs play a dominant rate-determining role in the association of subunits. The dependence of the rate on pH was interpreted to indicate that electrostatic forces between subunits are a significant controlling factor in the polymerization of TMV protein.

INTRODUCTION

In 1958, Lauffer *et al.* reported that tobacco mosaic virus (TMV) protein obtained by mild alkaline digestion of TMV could be polymerized and depolymerized in 0.1 ionic strength phosphate buffers at pH 6.5 by merely changing the temperature. At room temperature, the polymerized state predominates; since the reaction is endothermic, it must involve an increase in entropy, ΔS. It was postulated that this positive ΔS is derived from the release of solvent molecules, presumably water, from the subunits during the polymerization process. It was further reported that at pH values above 7, the TMV protein remains in the dissociated state at all temperatures investigated, and at pH values in the neighborhood of 5 it remains polymerized at all temperatures investigated. The purpose of the present communication is to report studies on reversible polymerization at pH values below the isoelectric point (approximately pH 3.5) of the protein and kinetic studies on the polymerization reaction at several pH values. Brief mention of some of these results has been made previously (Lauffer, 1962).

MATERIALS AND METHODS

Low Molecular Weight Protein. Low molecular weight TMV protein (A protein) was separated electrophoretically from nucleic acid and other alkaline degradation products by a method similar to that of Schramm and Zillig (1955). 5 ml volumes of the final protein solution at a concentration of 10 mg/ml were rapidly frozen in a dry ice bath and stored at −25°C until needed, at which time they were rapidly thawed. The ultraviolet absorption spectrum of the protein showed a maximum at 281 mμ and a minimum at 251 mμ; the ratio of maximum to minimum in the spectrum was 2.55, indicating a high degree of freedom from nucleic acid. Sedimentation velocity determinations gave values consistent with the accepted sedimentation coefficients of A protein. Protein concentrations were estimated by measuring the ultraviolet spectrum and using the factor 0.82 mg/ml/OD, a factor given by Fraenkel-Conrat and Williams (1955).

Particle Size Estimates. In the experiments reported in this publication, polymerization was followed by determining optical density attributable to light scattering. Some of the experiments were carried out in a Beckman DU spectrophotometer at a wavelength of 320 mμ. The spectrophotometer was equipped with Beckman thermospacers which allowed the sample compartment to be maintained at any temperature from 0 to 30°C. A Cary model 14M spectrophotometer was used for experiments in which the kinetics of rapid polymerizations was studied. Turbidity measurements were made at a wavelength of 320 mμ with a 10 cm cylindrical cell of silica surrounded by a thermostated jacket. Filling of the cell, actuating the pen circuit, etc., usually required about 13 seconds; the recording was started at 15 seconds. Accordingly, in many instances the reaction had proceeded to a considerable extent before the first readings could be taken.

EXPERIMENTAL RESULTS

Occurrence of Temperature-Reversible Aggregation Below the Isoelectric Point

Experiments to investigate polymerization of TMV protein at pH values below the isoelectric point were carried out with acetate, phosphate, and Michaelis buffers at several ionic strengths (μ). At no time was any temperature reversal of the polymerization seen when the protein was in an acetate buffer, whether the ionic strength was 0.075, 0.1, or 0.6 μ. The behavior of the protein in either Michaelis buffer or phosphate buffer of 0.1 μ presented certain similarities. At a pH of 2.6 or below, the solutions were clear (except after long standing) both in the cold and at room temperature, while at pH values above 3.0 the solutions were quite turbid, whether at room temperature or at 5°C. However, with these buffers temperature-dependent aggregations were discovered in very narrow intermediate pH ranges.

Illustrative of the results obtained are those shown in Table I. In this table, optical densities, measured at 320 mμ with a Beckman spectrophotometer equipped with thermospacers, are listed in the sequence of their observation at 25°C and at 2°C for TMV protein at a concentration of 0.5 mg/ml in 0.1 μ Michaelis buffer at pH 2.75. It can be seen that polymerized material at 25°C depolymerizes when

TABLE I

OPTICAL DENSITY AT TWO TEMPERATURES FOR TMV PROTEIN (0.5 mg/ml) IN pH 2.75 MICHAELIS BUFFER, 0.1 μ

Temperature	Optical density at 320 mμ
°C	
25	0.700
2	0.275
2	0.272
25	0.425
25	0.450
2	0.485
2	0.520

the temperature is dropped to 2°C and repolymerizes in part when the temperature is returned to 25°C. At this stage, the polymerization has ceased to be reversible. Rather rapid loss of reversibility characterized the systems investigated at pH values below the isoelectric point.

Another aspect of the complexity of the process at low pH values is illustrated by the results obtained when polymerization was initiated by the rapid addition of sodium phosphate buffer to unpolymerized protein at 25°C to give a final protein concentration of 2 mg/ml, an ionic strength of 0.1, and a pH value of 2.9. During the first few minutes, the material became quite turbid, but then decreased in turbidity. About 8 minutes after mixing, the optical density was approximately 0.6. By the end of 15 minutes, it had decreased to about half that value, and then it gradually increased to a value of about 0.7 by the end of 100 minutes. After 250 minutes, most of this material was still able to reverse its polymerization because, when the temperature was reduced to 7°C, an optical density reading of 0.09 was obtained.

The pH value for temperature reversal of aggregation varied with the type of buffer used and with ionic strength as is illustrated in Table II.

Side-by-side association of polymerized rods occurred very readily as judged from electron micrographs. Aggregation products of three different types were

TABLE II

pH OF TEMPERATURE REVERSAL OF TMV PROTEIN POLYMERIZATION

Ionic strength	Michaelis buffer	Phosphate buffer
0.17	2.85	3.0
0.10	2.75	2.9
0.01	—	3.3

detected on visual inspection. Though the molecular organization of these three forms is not known, their macroscopic appearance can be described as (*a*) very fine haze with blue-white color, characteristic of TMV rods or gradually polymerized protein at pH 6.5; not temperature-reversible in low pH region; (*b*) macroscopically visible lint-like particles showing a greyish-colored turbidity in aggregate, largely temperature-reversible, partly convertible to type (*a*); (*c*) large gelatinous particles, also giving greyish-colored turbidity.

The size of the aggregated protein in the pH region of temperature reversibility was investigated in several experiments. In a number of cases no high molecular weight peaks were observed in the centrifuge because all the material responsible for turbidity seen in a test tube solution had gone to the bottom of the cell by the time the rotor had reached 5000 RPM. The sizes of these particles generally were too varied to show much of a peak but their disappearance from the cell could be observed as a clearing of the solution. The low molecular weight protein, which usually accounted for most of the protein, customarily was found to have a sedimentation coefficient of about 7 S. The electron microscope revealed that solutions with very fast settling particles contained many packets of polymer rods associated in a side-by-side manner.

Time Course of Polymerization Reaction

1. *General.* While the stoichiometry of the polymerization of nucleic acid–free protein of TMV has not been established experimentally, certain reasonable guesses about the reaction can be made. The simplest proposal is that the protein associates by the mechanism of condensation polymerization. Provided that the functional groups on the two ends of a polymer retain the same degree of reactivity as the functional groups on monomers, the instantaneous rate for a simple condensation polymerization can be expressed in terms of the concentrations of unreacted functional groups:

$$\text{rate} = k_2(1 - p)^2c^2$$

where k_2 = a second-order rate constant,
c = initial concentration of monomer,
p = fraction linked of originally free ends.

Although this type of rate equation can be justified in a number of cases for polymerization reactions which might be compared to the present one (Flory, 1953), there exist enough irregular features[1] about the polymerization of TMV protein to warrant an experimental examination of the reaction. An investigation of

[1] Some of these are the presence of stable intermediates, the possible involvement of ions from the solution, the formation of non-linear polymers, and the existence of subunit associations in at least two different directions.

the time course was undertaken with the hope that the order of the reaction as well as some of the details of the pH dependence could be determined.

2. *Order of the Reaction.* It is possible to derive a generalized rate equation for determining the order of a polymerization reaction which is applicable to experiments followed by means of turbidity measurements. The following equation applies to the case where the rate of depolymerization is very small compared to the rate of polymerization:

$$\log t_{p'} = (1 - n) \log c + \alpha$$

where $t_{p'}$ = time to reach p' fractional completion of the reaction,
α = a constant at a given pH,
p' = an arbitrary fraction which can be chosen in terms of a reference optical density,
n = order of the reaction.

A convenient way to detect the accumulation of polymer molecules is to observe the increase of turbidity as a function of time. If the reaction proceeds along similar sequences of polymer products, regardless of the initial concentration of monomer, then any chosen value for the ratio, elevation in turbidity above the level at zero time divided by initial concentration of protein, should represent a particular fractional completion of the reaction. It should be noted that these equations ought to be valid even when turbidity measurements deviate from ideality in the late stages of the reaction as a result of the lengthening of polymer molecules beyond approximately 1/20 the wavelength of the illuminating light.

If one plots log $t_{p'}$ against log c for a series of experiments with different initial concentrations, c, of monomer, a straight line should result with a slope of $(1 - n)$. The time $t_{p'}$ is evaluated as follows: a particular optical density, $OD_{p'}$, is chosen arbitrarily on a reference run made with an initial concentration, c_0. The same degree of polymerization, p', on other runs made at concentration, c, will occur at OD values equal to (c/c_0) $(OD_{p'})$ because optical density is directly proportional to concentration. The times required to reach these optical densities are the required values of $t_{p'}$. Polymerizations were carried out at different protein concentrations, ranging from 0.2 to 0.03 mg/ml, all in 0.1 μ potassium acetate buffer of pH 5.4. The samples were contained in a 10 cm cell thermostated at 20°C and kinetic tracings of turbidity at 320 mμ were recorded automatically. The polymerization products formed under these conditions, when examined in the electron microscope, showed every evidence of being comparable to the TMV-like, rod-shaped polymers seen in other polymerization experiments.

Reproductions of actual spectrophotometer tracings (solid lines) for five different concentrations of protein are presented in Fig. 1. For each concentration, the turbidity of the unpolymerized protein solution, which was small, was first adjusted to give a chart reading of zero, shown in the figure as a horizontal base

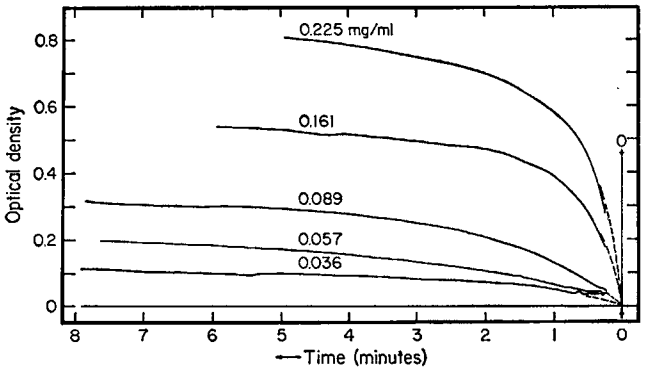

FIGURE 1 Concentration dependence of kinetic tracings of scattering optical density at 320 mμ for polymerization in 0.1μ ammonium acetate buffer of pH 5.28.

line. Each tracing was analyzed at four different points, corresponding to four values of p'. The results of this analysis were combined with similar data from a second experiment and the log-log plots of $t_{p'}$ *versus* concentration were drawn as shown in Fig. 2.

A polymerization conforming to the kinetics of a second-order reaction process, which includes the postulated condensation mechanism, should have points in the above mentioned log-log plot which fall along a straight line having the slope, -1. This follows from the previously derived expression that $n = 1$-(slope). As can be in Fig. 2, there is a considerable scatter of the points, but, in spite of the fact that the data from two different experiments were combined, a simple straight-line dependence is exhibited for three of the four cases. The average slope for the five lines, with weighting proportional to the number of points determining each line, is -1.23. For a simple reaction taking place by a single mechanism, the order of the reaction should be a small integer. The observed value of the slope is close to -1, and certainly is distant from -2, so that the best estimate of the order of the polymerization reaction from these experiments is that $n = 2$, a finding which is consistent with the postulate that the reaction mechanism is one of condensation polymerization.

An alternative analysis of the order can be attempted on the basis of estimated initial slopes. For this, an assumption is made that the early parts of the polymerization are unperturbed by nonidealities or significant back reactions. It follows from these assumptions and elementary light scattering theory that if a plot of experimental values for limiting initial slopes on a log scale *versus* log of respective protein concentrations gives a straight line with a slope of 2.0, a second-order process is indicated (Smith and Lauffer). Since, as shown in Fig. 3, the points obtained from estimates of initial rates approximate a straight line having the expected slope, the postulate of a second-order mechanism again seems tenable.

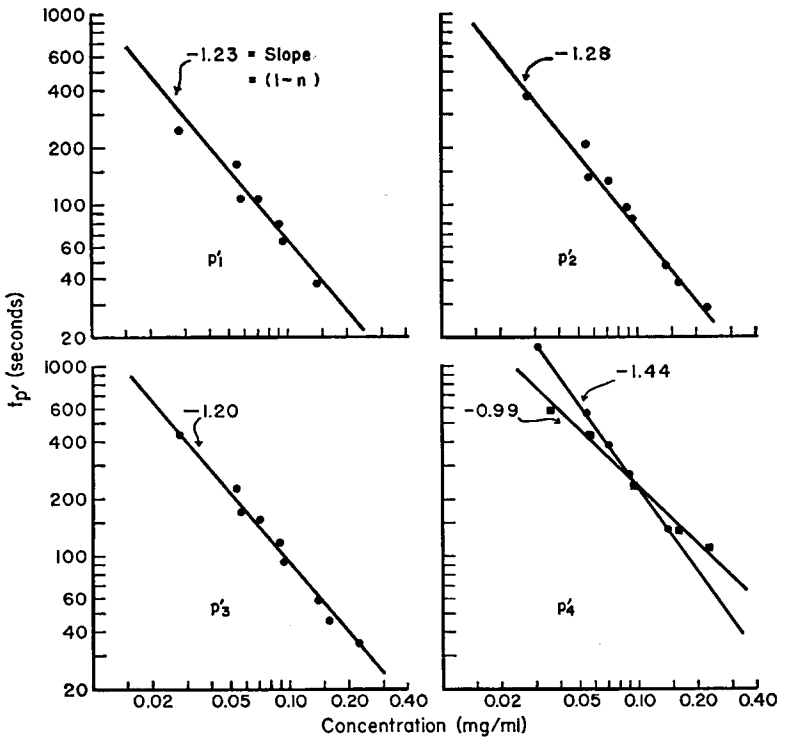

FIGURE 2 Graphs of log $t_{p'}$ *versus* log protein concentration for determining the order of the polymerization reaction. (Slope for second order = −1).

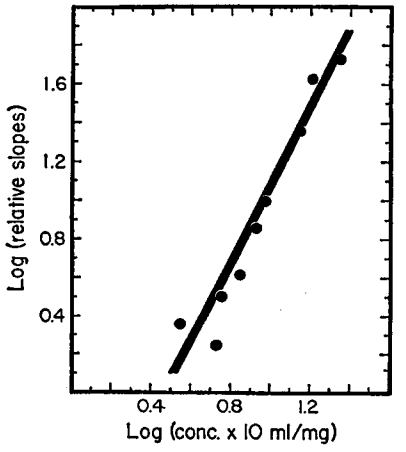

FIGURE 3 Test of fit of rate of polymerization (points), as judged by estimated initial slopes of turbidity tracings, to the slope for a theoretical log-log relationship for second-order kinetics (solid line).

3. *pH Optimum.* A second series of kinetic experiments was undertaken to determine the influence of pH on the rate of polymerization. In these investigations, the concentration of protein was kept constant within an experiment while the pH of the buffer was varied. For reasons that were not fully understood, the level of turbidity was not always the same from one day to another at the same pH and with the same concentration of protein, but within a single experiment a

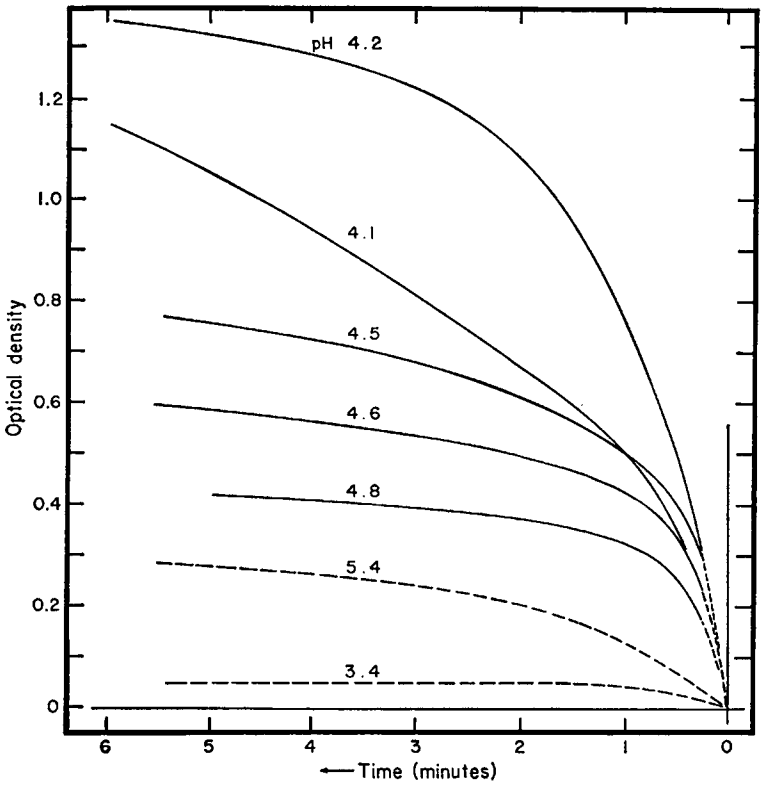

FIGURE 4 pH dependence of kinetic tracings of scattering optical density at 320 mμ for protein solutions at a concentration of 0.125 mg/ml polymerizing at 20°C in 0.033 μ ammonium acetate buffers (solid lines) and proportional kinetic tracings at two pH values from a second experiment (dashed lines).

general consistency was observed which allowed the comparison of rates and final polymer sizes at different pH values. Fig. 4 contains selected curves from one experiment performed at 20°C in 0.033 ionic strength ammonium acetate buffers of various pH values with protein at a concentration of 0.125 mg/ml. These spectrophotometer tracings illustrate the general trend of the pH dependence. Two curves from another experiment were inserted (dashed lines) on a proportional scale to show the relative rates of polymerizations at higher and lower pH values.

Fig. 5 shows the time course of polymerization observed at a higher pH range in an earlier experiment carried out under conditions of temperature, concentration, and ionic strength which were more favorable to polymer formation than those for the experiment of Fig. 4; in this experiment, readings were made with a Beckman DU spectrophotometer using a cell of 1 cm path length.

The tracings of Figs. 4 and 5 have two noteworthy features: first, the shape

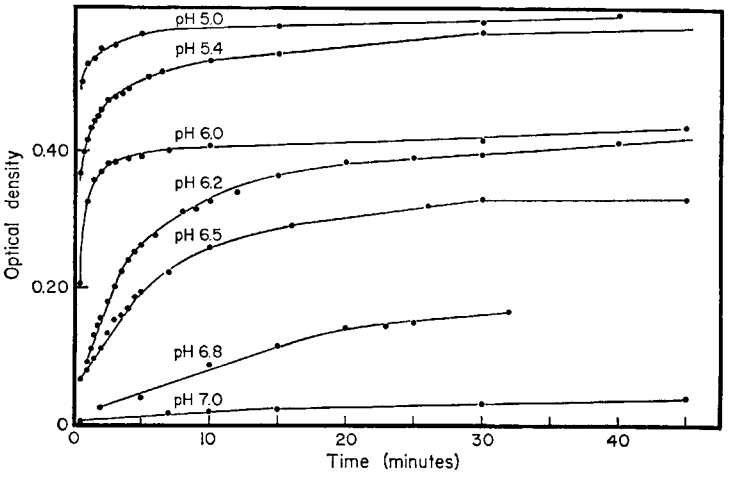

FIGURE 5 Dependence of scattering optical density per mg/ml at 320 mμ on pH and time for protein solutions at an approximate concentration of 0.25 mg/ml polymerizing at 30°C in 0.1 ionic strength sodium phosphate buffers; observed with a 1 cm cell.

of the kinetic curves changes radically below pH 5, and second, there is a maximum at pH 4.2 or 4.3 (the variability from one experiment to another does not allow this maximum to be located precisely), where the initial rate of polymerization appears to be fastest and the weight average size of the polymer is greatest. The analytic method used to determine the order of the reaction at pH 5.4 was not applied here because the character of the kinetic tracings obviously varied from one pH value to another. Probably the best available measure of the relative rates at different pH values is the slope of the initial portion of the tracing as estimated by visual inspection. These estimates are presented in graphical form in Fig. 6 where the dependence of rate of polymerization in 0.033 μ potassium acetate buffer on hydrogen ion concentration is quite obviously seen to be a bell-shaped one.

Polymerizations carried out at pH values close to the isoelectric point of the virus showed a most unusual pattern indicating a rather slow initial formation of polymer followed by what appeared to be an extremely rapid aggregation of the first products to particles of very large size—so large, in fact, that they settled out of solution on standing. Examples of these anomalous curves are given in Fig. 7 along with comparison curves from higher and lower pH values.

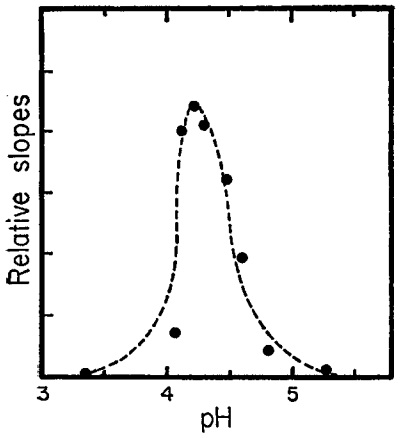

FIGURE 6 Dependence of rate of polymerization, as judged by estimated initial slopes of turbidity tracings, on pH of the medium for protein solutions at a concentration of 0.125 mg/ml polymerizing at 20°C in 0.033 μ potassium acetate buffers.

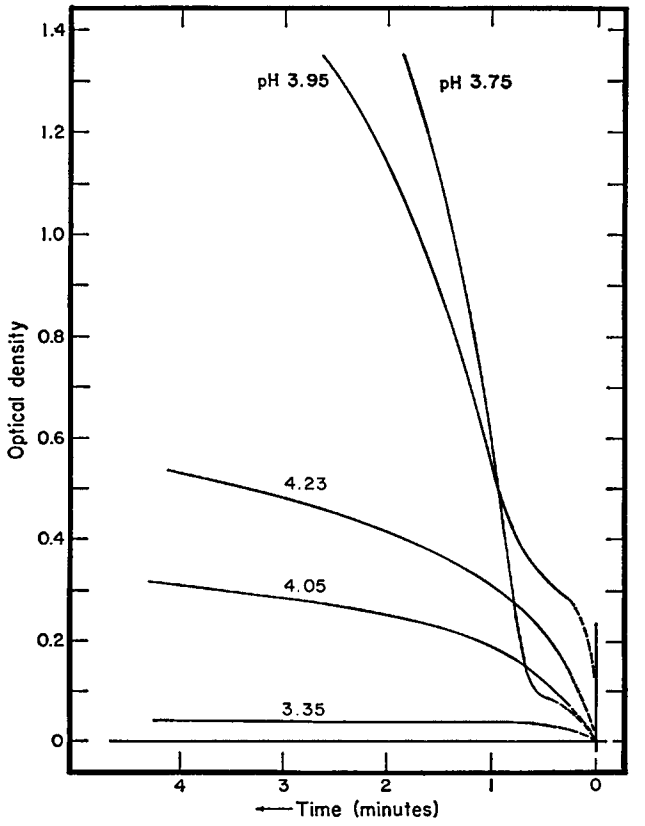

FIGURE 7 Kinetic tracings of scattering optical density at 320 mμ comparing anomalous polymerizations at pH 3.95 and pH 3.75 to normal polymerization curves at pH 4.05 and pH 4.23; polymerized at 20°C in 0.033 μ potassium acetate buffers with protein concentration at 0.084 mg/ml.

DISCUSSION

The results of this study established that a reversible endothermic association of TMV protein takes place at pH values below the isoelectric point as well as at values above it. In the lower pH range, however, the occurrence of the endothermic association was found to be correlated with the presence of particle masses so large they could be seen by eye. It was also found that the temperature-reversible product is not the most stable association form in the pH region near 3. Consequently, there is considerable doubt whether the particular polymeric product which shows temperature reversal of association in the low pH region is identical to the virus-like rods observed both in this region and at pH values above the isoelectric point. The possibility of finding alternative modes of association as the charge distribution on subunits is changed does not seem too surprising, particularly if one accepts the thesis that reversible endothermic association need not involve a mechanism requiring highly specific bonding sites.

Studies on the pH dependence of the polymerization rate provide information pertinent to an elucidation of the inter-subunit bonding "forces" that are responsible for the beautifully ordered macrohelix of the virus. While the findings so far reported are insufficient to identify these "forces," they do indicate that certain association mechanisms can be excluded as the rate-controlling step. Since, as was apparent from previous observations, the polymerization of TMV protein is endothermic and easily reversed by small temperature or pH changes, it must be that ordinary covalent linkages are not involved. The current finding that the rate of polymerization has a striking maximum, as well as the fact that the polymerization is strongly endothermic, provides evidence against another possibility: association by means of H-bonds between pairs of glutamyl or aspartyl residues (Buzzell, 1962; Fraenkel-Conrat and Ramachandran, 1959). The formation of hydrogen bonds of the above type in solutions uncontaminated by divalent metal ions (see comment by Buzzell, 1962) should depend on the availability of free acid groups. Thus a reaction controlled primarily by the formation of H-bonds ought to exhibit a rate which increases in proportion to the probability of finding at least one pair, and more likely several pairs, in the unionized state; furthermore, the rate should reach a plateau at a pH well below the pK_a (probably about 4.5) of the carboxyls concerned. Clearly, this behavior was not found. Instead, polymerization initiated at a series of pH values was found to be a critical function of the departure of the pH from approximately 4.3. This observation, together with the knowledge that polymerization is favored by increased ionic strength (Schramm and Zillig, 1955; Smith and Lauffer), can be interpreted as reflecting an important role played by electrostatic forces between reacting monomers.

The above comments about rate experiments must be qualified in part by pointing out that the interpretation of the kinetic data requires that certain assumptions be made. One of them is that the polymerization observed was a relatively simple

one in which the kinetics of turbidity development recorded in the earlier portions of the tracings was the same as that taking place in the first 15 seconds of the reaction. In defense of this assumption, it can be noted that the turbidity records seldom gave an indication that anything but a nearly straight line could be drawn between the origin at zero time and the first recorded turbidity. A second assumption made was that rates of reaction could be estimated on the basis of the initial slopes of turbidity curves. Following the reasoning of Oster (1947), this seems justified if the polymerization occurred by the mechanism of linear condensation rather than that of nucleated linear addition. His development indicated that a plot of turbidity *versus* time should be a straight line with slope proportional to the rate constant for the case of ideal linear condensation, or in the practical case where the products formed eventually become long compared to $\lambda/20$, a line bowed downward as time increases. On the other hand, the plot for nucleated linear addition should be a line bowed upward since, in this case, turbidity rises according to a term quadratic in time. In the current experiments, the shape of turbidity curves was that expected for linear condensation and different from that predicted for linear addition.

Since the final product, the virus-like rod, has a helical arrangement of subunits, it is necessary to examine the applicability of the theory of helical polymerization. In a recent discussion of this theory, Oosawa and Kasai (1962) presented an equation which is analogous to the one used in this study for examining the order of the reaction. Their development assumed that helical polymerization is initiated by the transformation of rapidly polymerized linear chains into one turn of the final helix, that further helical polymerization proceeds by the addition of monomer units at the screw dislocation edge, and that linear aggregation is reversible but that depolymerization of helical polymers is negligible. Their equation for the time t_p to reach p fractional extent of completion of the reaction can be reduced to the following:

$$\ln t_p = \frac{-n}{2} \ln c_0 + \ln K_p$$

where K_p = a constant at a given value of p,
c_0 = initial monomer concentration,
n = number of monomers per turn of the helix.

Since the highly diluted starting material used in the kinetic experiments should have had a size averaging between one and two units of the fundamental polypeptide, the slope expected by the helical mechanism should have been between -8 and -4. Instead, the value found was -1.2. Thus, within the limitations of the current experiments, it appears that neither internally initiated helical addition nor nucleated linear addition is a major mechanism for the polymerization of TMV protein.

Although an establishment of the details of the polymerization mechanism must await more refined experiments, our analysis of the kinetic data indicates that the reaction is controlled by a second-order process. This result has important implications for the formulation of an equilibrium constant for polymerization. Numerous studies show that the denaturation of TMV by heat, by urea, and by high pressure, all of which involve dissociation of the virus into small protein fragments followed by denaturation, is invariably a reaction of the first order (Lauffer and Price, 1940; Lauffer and Dow, 1941; Lauffer, 1943). It is therefore reasonable to conclude that depolymerization is a first-order process. The equilibrium constant for the polymerization of TMV protein can, therefore, be formulated in terms of the ratio of a second-order rate constant for polymerization to a first-order rate constant for dissociation. The success of this type of formulation will be demonstrated in a subsequent publication (Smith and Lauffer).

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