Polymerization-Depolymerization of Tobacco Mosaic Virus Protein. III. Changes in Ionization and in Electrophoretic Mobility*

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When unbuffered tobacco mosaic virus (TMV) protein at *p*H 6.4 and room temperature is depolymerized by lowering the temperature to 4°, there is a large drop in *p*H. This can be explained by the assumption that undissociated carboxyl groups, protected either fully or partially from the aqueous medium while within the polymerized protein, are exposed and ionized upon depolymerization. Differences between the titration curves for TMV protein and for the intact virus yield results consistent with this interpretation and show that up to 7 equivalents of hydrogen ion are dissociated per 10^5 g of protein during depolymerization to the level of "A protein." It can be shown that the entropy change attributable to the corresponding binding of hydrogen ions during polymerization is negative and that, therefore, this is not the source of the positive entropy change which must be responsible for the total polymerization reaction. The electrophoretic mobility observed for depolymerized TMV protein at *p*H 6.5 is approximately one-third that of the polymerized protein at the same *p*H value. This result is entirely consistent with the increase in negative charge per unit mass of protein caused by the exposure of ionizable carboxyl groups upon depolymerization. Electrophoretic mobility depends on both net charge and surface area per particle. Lack of coincidence between the electrophoretic-mobility curve and the titration curve of the virus can most reasonably be explained by the assumption that some of the carboxyl groups are situated within the helical grooves of the virus. This, in turn, suggests that the *p*H change that accompanies polymerization or depolymerization of the protein at *p*H 6.5 arises mostly from carboxyl groups located within the grooves on the assembled rod and is caused by alterations in the space-average dielectric constant of their environment. Anomalies in the virus titration curve between *p*H 7 and 8 indicate that the nucleic acid on the interior is in a slowly achieved reversible equilibrium with hydrogen or hydroxyl ions of the electrolyte medium.

It was reported by Lauffer *et al.* (1958) that the nucleic acid–free protein prepared from tobacco mosaic virus (TMV), the material designated "A protein" by Schramm (1947), could be polymerized and depolymerized when dissolved in 0.1 ionic strength phosphate buffer at *p*H 6.5 by merely changing the temperature. Since the polymerized state predominates at room temperature but is absent in the cold, polymerization is an endothermic process accompanied by an increase in entropy. The authors speculated that this entropy increase probably stems from a release by the protein of some element of the solvent, presumably water, during the polymerization process.

The polymerization reaction is *p*H dependent (Lauffer *et al.*, 1958). At *p*H 5 the material is in the polymerized state, while at *p*H 7.7 it is in the depolymerized state at all temperatures investigated. The purpose of the present communication is to report studies on the effect of charge on the polymerization-depolymerization of TMV protein, and to present evidence that the entropy changes associated with the change in charge are in the wrong direction to serve as the primary explanation for the endothermic polymerization, thereby supporting the initial assumption of change in water binding.

Three experimental approaches have been followed: measurement of *p*H changes in unbuffered protein solutions as subunits were polymerized into rods, comparison of acid-base titration curves for TMV protein and the whole virus, and measurement of electrophoretic mobility at *p*H 6.5 for both depolymerized and polymerized protein. A report that both *p*H changes and electrophoretic mobility changes accompanied the temperature reversal of polymerization was presented in abbreviated form at the Pittsburgh meeting of the Biophysical Society (Ansevin and Lauffer, 1959b). Other electrophoretic studies on TMV protein were published by Kramer and Wittmann (1958). They found that at *p*H values higher than about 6 the mobility of the depolymerized protein was about one-third that of the intact virus at the same *p*H values; however, at somewhat lower *p*H values the protein, then in the polymerized state, had a mobility indistinguishable from that of the intact virus. At *p*H values below the isoelectric point the protein migrated more rapidly than the intact virus. They interpreted their finding to mean that, when polymerized TMV protein depolymerizes, previously concealed positively ionizing groups are exposed. While our electrophoretic-mobility observations are entirely consistent with those of Kramer and Wittmann, our findings with respect to acid-base binding and *p*H changes during depolymerization, together with a detailed analysis of factors influencing electrophoretic mobility, lead to the conclusion that negatively ionizing, presumably carboxyl groups are exposed when TMV protein is depolymerized and, further, that the net effect of all ionization changes at *p*H 6.5 is to increase the magnitude of the negative charge per unit mass of protein.

MATERIALS AND METHODS

Tobacco Mosaic Virus.—Stock preparations of TMV were isolated by a method involving differential centrifugation and Versene treatment (Boedtker and Sim-

* Papers I and II of this series are Ansevin and Lauffer (1963) and Lauffer (1964). The data presented in this paper are taken in part from a dissertation submitted to the University of Pittsburgh by Allen T. Ansevin in partial fulfillment of the requirements for the degree of Doctor of Philosophy, January 10, 1962, and in part from a dissertation submitted to the University of Pittsburgh by Charles L. Stevens in partial fulfillment of the requirements for the degree of Doctor of Philosophy, April 18, 1962.

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mons, 1958; Englander *et al.*, 1960) from the frozen pulp of Turkish tobacco plants infected with the common strain of the virus.

TMV Protein.—The protein preparations (Ansevin and Lauffer, 1963) used in the electrophoretic-mobility studies, in the studies on *p*H changes during polymerization, and in some of the titration experiments were obtained by the alkaline-degradation method (Schramm and Zillig, 1955). Some of the protein preparations used in titration experiments were prepared by acetic acid extraction of the virus (Fraenkel-Conrat, 1957b).

*p*H *Measurements and Temperature Control.*—The *p*H measurements were made with a Beckman Model G *p*H meter equipped with Beckman calomel and shielded-glass microelectrodes. The temperature-compensation dial was adjusted in all cases for the temperature of the solution being measured. The *p*H values reported in experiments on the change of *p*H with temperature were the readings observed after temperature equilibrium had been achieved in the 1-ml sample compartment of the jacketed apparatus. Any *p*H meter-reading errors owing to thermal gradients in the electrodes were considered to be unimportant since all calculations were based on differences between readings for sample and for control virus treated in an identical manner. During an experiment, a thin stream of nitrogen was directed into the apparatus above the surface of the liquid to exclude carbon dioxide. In acid-base-titration experiments, temperature was controlled either by putting the cell and entire measuring apparatus into a thermostated cold room (3–5°) or by carrying out the titration in a jacketed cell maintained at 20°.

Electrophoretic-Mobility Determination.—Electrophoretic-mobility experiments were carried out in a Spinco Model H electrophoresis apparatus equipped with a microcell. Both schlieren and interference optical systems were employed. Mobility measurements were performed at 2° (depolymerized protein) and at 24.9° (polymerized protein). Mobilities were corrected to water at 20° by multiplying the observed mobilities by the ratio of the viscosity of the protein solution at the temperature of the run to the viscosity of water at 20°. The protein-solution viscosities at 2° and at 25° were 0.0174 and 0.0096 poise, respectively. At 2°, the mobilities observed in the ascending and descending limbs were the same and, therefore, average values are quoted. At the higher temperature, only mobilities obtained in the descending channel are used because only in this limb was reversible migration observed.

Acid-Base Titrations.—The titrated solutions were well stirred during accurately measured additions of standard acid or alkali and were swept constantly with nitrogen. All solutions titrated were 0.1 M in NaCl. The titration of protein at 4° was carried out with 19 mg of material at a concentration of 25 mg/ml. The titration of virus at 4° was made with 51 mg of TMV (48.5 mg of protein) at a concentration of 23 mg/ml, while that at 20° was performed with 171 mg of TMV (162 mg of protein) at a concentration of 11 mg/ml.

Experimental and Results

*p*H *Shifts During Polymerization.*—Measurements of *p*H were obtained as a function of the degree of protein polymerization by adjusting the temperature of the solution to various points between 4 and 30°. The protein used in this experiment was first extensively dialyzed against distilled water, then electrodialyzed for several hours in a field of about 50 v/cm. At the completion of electrodialysis, the *p*H was 4.3; the *p*H was subsequently raised to 6.4 by the addition of 0.01 N sodium hydroxide at room temperature. Sufficient sodium chloride was then added to bring the ionic strength of the protein solution to 0.1. The *p*H of this solution was observed as a function of temperature, as described under Materials and Methods. Control experiments were carried out with electrodialyzed intact virus, with Beckman standard buffer at *p*H 7, and with 0.1 ionic strength sodium chloride solution. The results obtained with TMV protein at a concentration of 5 mg/ml and with TMV at the same concentration, used as a control, are shown in Figure 1.

The change in *p*H meter reading with change in temperature was completely reversible for unbuffered protein and for TMV, sodium chloride, and sodium phosphate buffer controls; readings fell on the same curve whether the temperature was approached from above or from below. Results obtained with the phosphate buffer control agreed within ±0.01 *p*H unit with the published values for that buffer at 23 and 30° and were 0.05 *p*H unit too high at 7°. Therefore errors attributable to the effect of temperature change on the electrode assembly probably do not exceed +0.05 *p*H unit even at the lowest temperature used. Since this error is common to the data obtained on protein and on virus control, the difference between the *p*H-meter readings for the nucleic acid–free protein and the virus at a given temperature is therefore a measure of the actual *p*H change attributable to depolymerization of protein. The *p*H of the TMV protein is significantly lower in the depolymerized state than in the polymerized state. It is apparent, therefore, that when polymerized TMV protein depolymerizes either hydrogen ions are released or hydroxyl ions are bound.

Titration Experiments.—The results of the titration experiments on TMV protein and on an intact virus control are shown in Figure 2. A region of buffering appearing in the protein curve, but not in the virus curve, permits one to determine the amount of sodium hydroxide that must be added to neutralize the acid released during depolymerization in the preceding experiment. The titration results for protein solution at a temperature of 4° can be viewed as follows. As sodium hydroxide is added to TMV protein initially at a *p*H value near 5.7, at which the protein is polymerized, it begins to depolymerize and liberates hydrogen ions which neutralize the added base (or it binds hydroxyl ions) without appreciable change in *p*H; depolymerization to A protein must be considered complete by the time the *p*H has been raised to somewhere between 6 and 6.5 (Lauffer *et al.*, 1958). The difference between the titration curves for TMV, which does not depolymerize, and TMV protein, which does, is taken to mean that the depolymerization of polymer at *p*H 6 is accompanied by the liberation of 7 equivalents of hydrogen ion per 10^5 g of protein. This is the maximum difference between the curves at any *p*H value. When polymer at *p*H 6.5 is depolymerized to A protein at *p*H 6.5, the number of hydrogen ions exposed is about five per 10^5 g. If the nucleic acid on the interior of the virus makes any contribution to the virus titration curve between *p*H 5.5 and 6.5, which is not expected, then this figure could be slightly higher.

The buffer action for TMV protein in this region cannot be attributed to histidine because the protein does not contain this amino acid (Anderer *et al.*, 1960; Tsugita *et al.*, 1960), nor can it be ascribed to an N-terminal amino acid since in TMV protein this group exists in an acylated form (Fraenkel-Conrat and Ramachandran, 1959). The simplest interpretation of the result is that, when the polymerized protein depolymerizes, hydrogen ions are released from aspartic or glutamic acid residues, which normally would have a

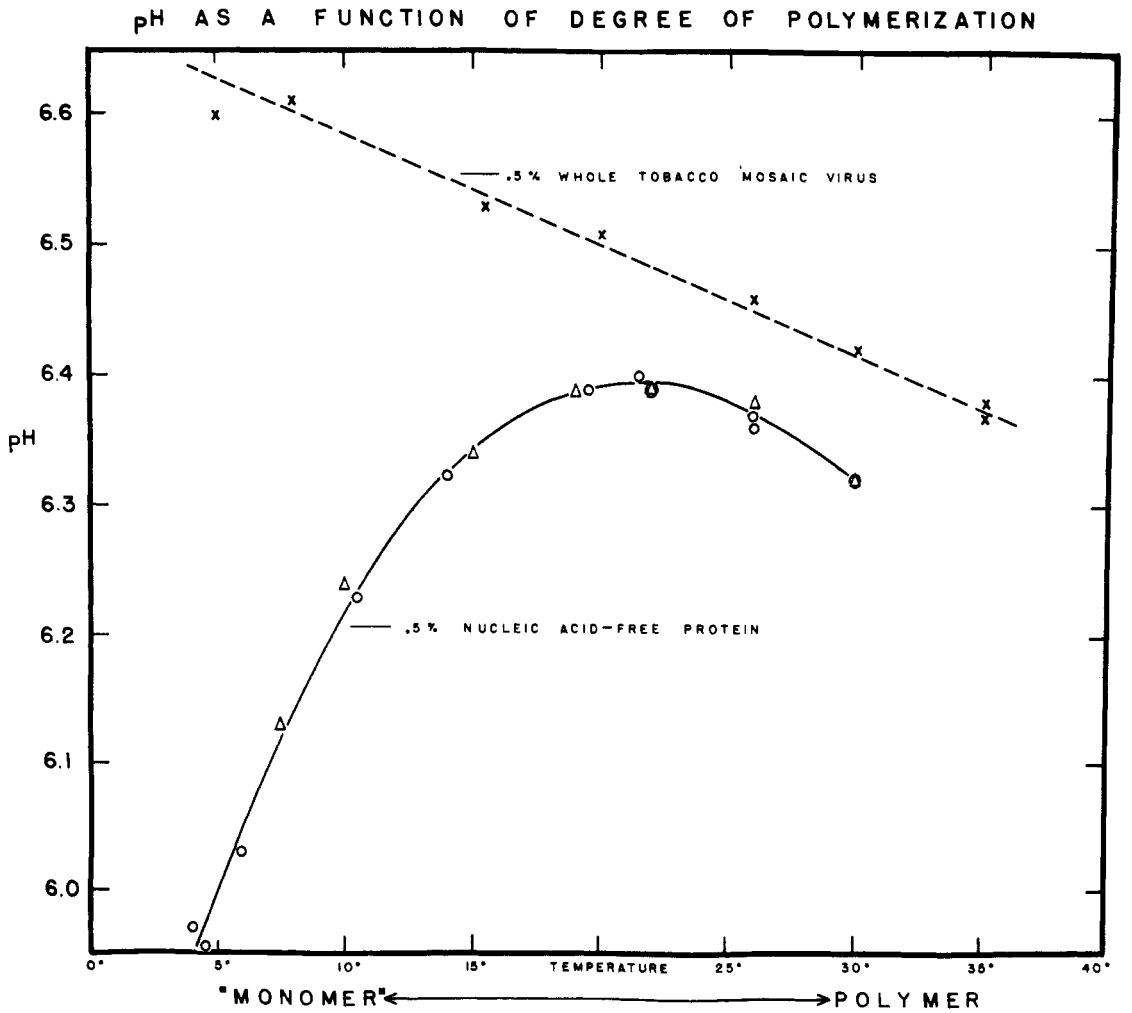

FIG. 1.—*p*H-meter reading of unbuffered TMV protein and unbuffered intact virus in 0.1 ionic strength NaCl, as a function of temperature and extent of protein polymerization (where monomer is A protein and polymer is protein rods). Circles, descending half of temperature cycle; triangles, ascending half of temperature cycle; crosses, tobacco mosaic virus control.

pK_a around 4.7 when present in proteins (Tanford and Epstein, 1954; Tanford and Hauenstein, 1956).

Data in qualitative agreement with these were obtained by Koshland *et al.* (1958) when TMV was degraded in 30% xylenesulfonate at 25° and by Fraenkel-Conrat (1957a) when the virus was degraded in 1% sodium dodecylsulfate. Furthermore, Fraenkel-Conrat and Ramachandran (1959) state on the basis of unpublished experiments that the disaggregation of virus by a variety of methods is associated with the release of about two hydrogen ions per peptide chain and that in the reaggregation of the protein, with or without the incorporation of the RNA, hydrogen ions are consumed.

A second titration curve was obtained for the intact virus at 20° over the *p*H range from the isoelectric point to *p*H 8 (Fig. 3). The curve at 20° is very similar to that for the virus at 4° and significantly different from the titration curve for protein alone. The information that can be obtained from the TMV-titration curves will be considered in the discussion of electrophoretic results.

Electrophoresis of Polymerized and Unpolymerized Protein.—The results of the electrophoretic-mobility studies carried out on depolymerized TMV protein at 2° and on extensively polymerized protein at 24.9° are shown in Table I. In both cases, all boundaries migrated toward the positive electrode. It can be seen that the mobility of the depolymerized protein is approximately one-third that of the polymerized protein. It can also be seen that a small amount of residual depolymerized protein remains at 25° and that there is a still smaller amount of a third component with an intermediate mobility. Experimentally, this result is in accord with the finding of Kramer and Wittmann (1958) that between *p*H 3 and 6, where TMV protein is

TABLE I

ELECTROPHORETIC MOBILITIES OF POLYMERIZED AND UNPOLYMERIZED TMV PROTEIN (1.5 MG/ML) AT *p*H 6.5 IN 0.05 IONIC STRENGTH SODIUM PHOSPHATE BUFFER

Polymerization State	Temp (°C)	Mobility ± Estimated $\sigma_x/\bar{x}$ (corrected to 20°), $\frac{\text{cm/sec}}{\text{v/cm}}$	Fraction of Total in Peak
Rods	25	-20.2×10^{-5} [a] ± 2%	0.75
Intermediate	25	-15.9×10^{-5} [a] ± 9%	0.10
A protein	25	-8.5×10^{-5} [a] ± 7%	0.15
A protein	2	-7.4×10^{-5} ± 7%	1.0

[a] Descending channel only.

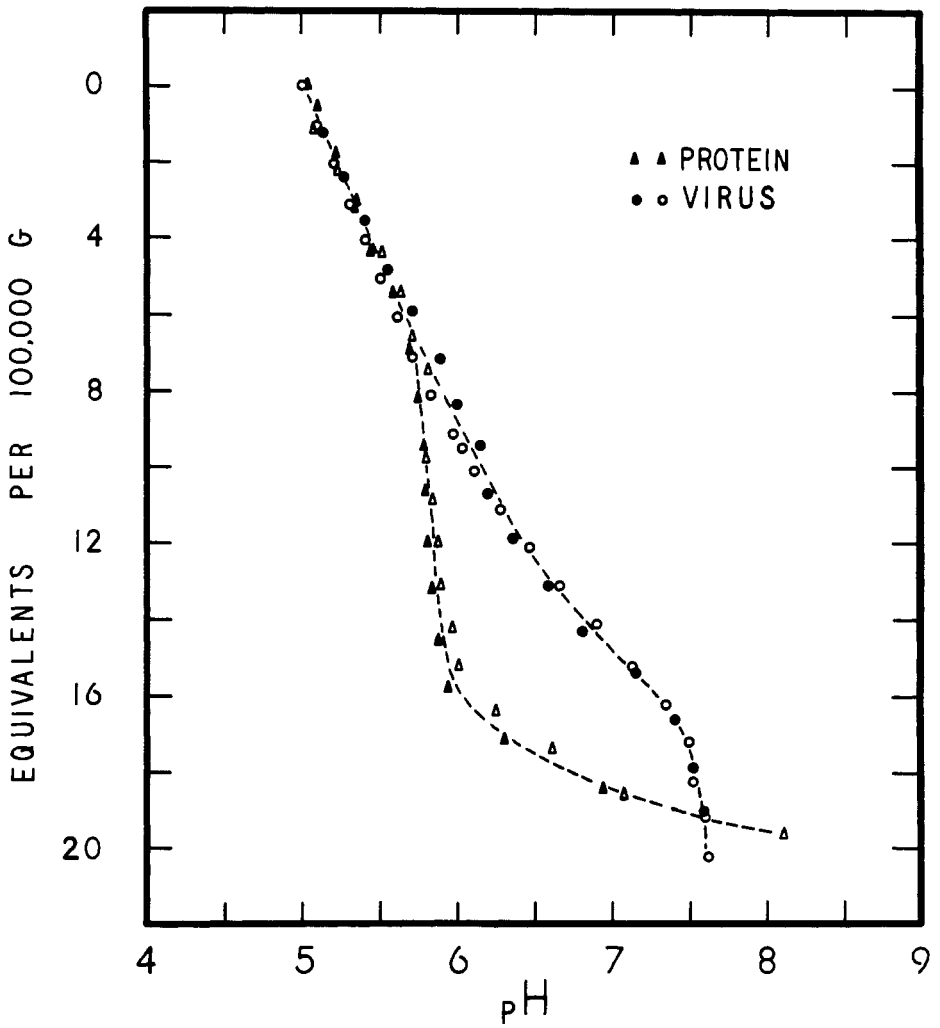

FIG. 2.—Titration curves of unbuffered TMV protein (triangles) and unbuffered intact virus (circles) in 0.1 ionic strength NaCl at 4°. Open symbols, addition of base; solid symbols, subsequent addition of acid. Titration regarded as beginning at *p*H 5.0 for purposes of calculation.

aggregated, it has an electrophoretic mobility comparable to that of the virus itself; but that at *p*H values above 6, where the protein is depolymerized, it has a mobility approximately one-third that of the intact virus. The experimental result is also consistent with the finding of Kleczkowski (1959) that the A protein of TMV had a much lower electrophoretic mobility than the product formed by polymerizing this material by ultraviolet irradiation into rodlike particles. However, as will be developed in detail in the discussion, the correct interpretation of this result must include an analysis of factors additional to those considered by Kramer and Wittmann (1958) or by Kleczkowski and Van Kammen (1961).

DISCUSSION

The Entropy Change.—It can be reasoned from the data published by Lauffer *et al.* (1958) that the enthalpy change for polymerization of TMV protein is not only positive but must have a high numerical value because the material changes from an almost fully depolymerized state to an almost fully polymerized state for an approximately 10° increase in temperature. Quantitative studies to be reported in a subsequent communication (C. E. Smith and M. A. Lauffer, data to be published) show that the standard enthalpy increase for polymerization is of the order of magnitude of 100,000 calories per mole of bonds formed between A protein units and that the standard entropy increase is of the order of magnitude of several hundred entropy units. The standard state is taken to be 1 mole/liter in an aqueous medium of *p*H 6.5 and 0.1 ionic strength at a temperature of 25°, where the monomer unit is considered to be A protein having the assigned particle weight of 100,000. Since it has been established in the present communication that 5 moles of hydrogen ion are liberated for each mole of A protein formed by depolymerization at *p*H 6.5 or, conversely, that 5 moles of hydrogen ion are bound for each mole of A protein polymerized, it is necessary to inquire into the contribution this ionization makes to the total entropy change for the polymerization reaction. This can be estimated to a first order of approximation by assuming that the groups deionizing during polymerization of TMV protein are comparable to gamma carboxylate ions of independent aspartic acid molecules. The model can be made slightly more realistic, however, by assuming that the pK_a of the group concerned is 4.7, the pK_a observed by Tanford and his collaborators (Tanford and Epstein, 1954; Tanford and Hauenstein, 1956) for side-chain-carboxyl deionizations in the two proteins, ribonuclease and insulin. Taking K for deionization as $10^{+4.7}$, one finds ΔF_0 for this reaction to be -6450 cal/mole at 25°. In the experimental medium used for reversible polymerization, phosphate buffer at *p*H 6.5, a second ionization change accompanied the neutralization of carboxylate ions: dihydrogen phos-

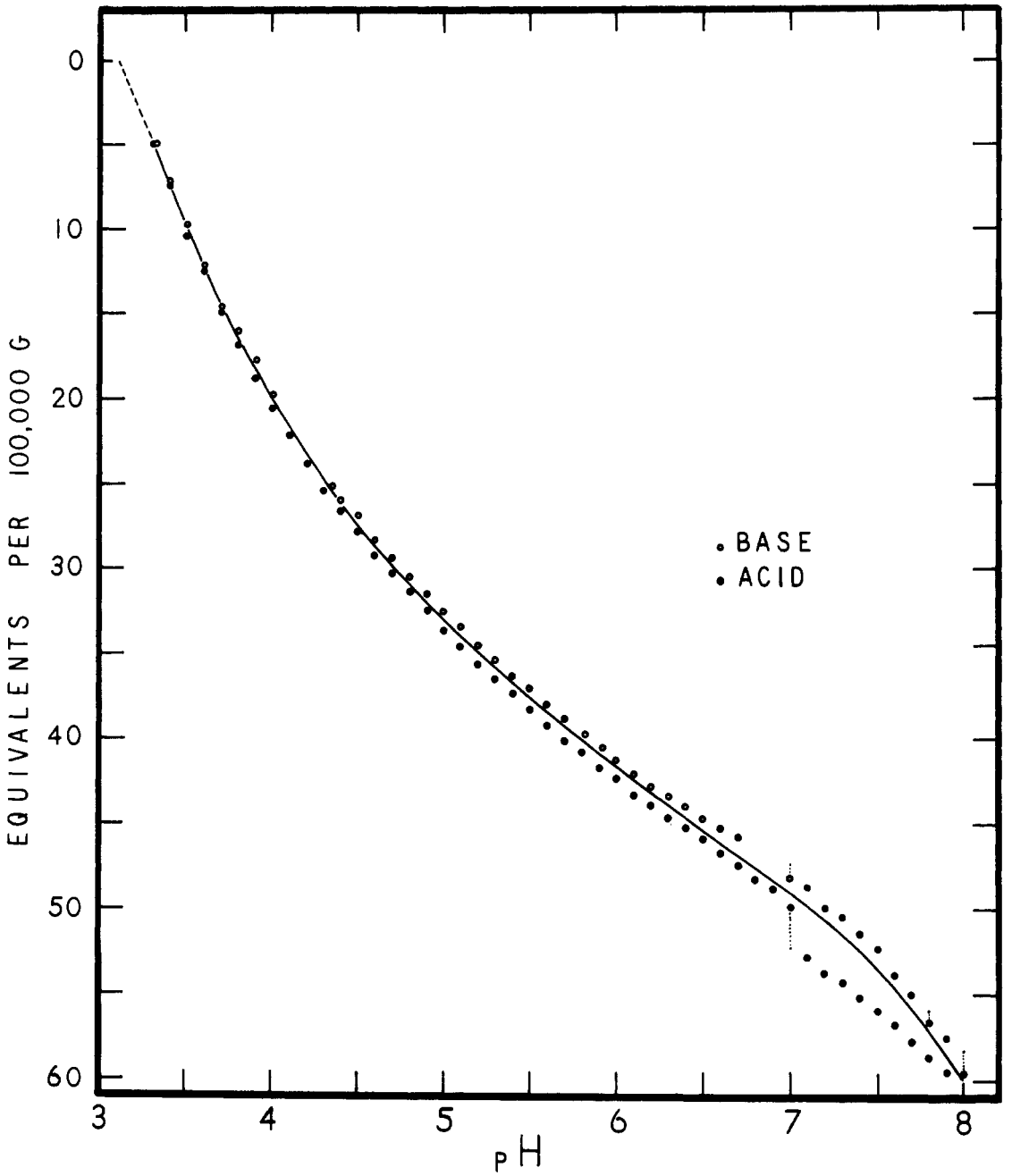

FIG. 3. Titration curve of unbuffered tobacco mosaic virus in 0.1 ionic strength NaCl at 20° from the estimated isoelectric point of 3.05 to *p*H 8.0. Solid symbols, addition of acid; open symbols, subsequent addition of base; dotted lines, extent of observed time dependence.

phate dissociated to hydrogen ion and monohydrogen phosphate. Since the protein is in equilibrium with the buffer, the net result can be written as the sum of two coupled reactions:

$$\text{protein-COO}^- + \text{H}^+ \longrightarrow \text{protein-COOH} \qquad \text{I}$$

$$\text{H}_2\text{PO}_4^- \longrightarrow \text{H}^+ + \text{HPO}_4^{2-} \qquad \text{II}$$

$$\text{protein-COO}^- + \text{H}_2\text{PO}_4^- \longrightarrow \text{protein-COOH} + \text{HPO}_4^{2-} \qquad \text{III}$$

ΔF_0 (cal/mole)	ΔH_0 (cal/mole)	Reaction
−6450	−1110	I
+9823	+987	II
+3373	−123	III

The ΔH_0 employed here for the protein deionization is that corresponding to the deionization of the gamma carboxyl of aspartic acid (Smith and Smith, 1942), and the standard free energy and enthalpy of the second reaction are those for the second ionization of phosphoric acid (Bates and Acree, 1943). Since $\Delta H - \Delta F = T\Delta S$, the standard entropy change for the summed ionizations is readily computed to be about −12 cal/mole degree at 25°. Considering that the polymerization of each mole of A protein at *p*H 6.5 involves the uptake of approximately five hydrogen ions, the net standard entropy change for ionization reactions during polymerization is about −60 cal/mole degree of A protein. Actually, polymerization experiments to be reported later (C. E. Smith and M. A. Lauffer, data to be published) are carried out in phosphate buffer solutions at *p*H 6.5, the *p*H of the standard state. Under this condition, the entropy at unit activity of R and R^- attributable to protonation of anionic groups on the protein is −8.5 cal/mole degree or −44 cal/degree for the protonation of 5 moles of groups. Since the polymerization of A protein is accompanied by a large increase of entropy, it is clear that the binding of H^+ by ionized carboxyl

groups, which involves a negative entropy change under the conditions employed, does not provide the entropy change for polymerization.

pH Shifts during Polymerization.—Observations of the *p*H of unbuffered protein as a function of temperature presented in Figure 1 cannot be used in a direct manner to give values for the number of hydrogen ions released during depolymerization. It can be shown, however, that these data are reasonably consistent with the findings from the titration curves of Figure 2. The data shown in Figure 1 demonstrate that it is possible to convert polymer at a meter reading of *p*H 6.4 and 20° to A protein at a meter reading of *p*H 5.95 and 4° without adding acid to the solution. Judging from the change of *p*H with temperature for the TMV control in Figure 1, polymer at 4° should show a *p*H reading 0.13 unit higher than at 20°. Thus, it should be possible to convert a hypothetical solution of unbuffered protein polymer at 4° with a *p*H of 6.40 + 0.13 = 6.53 into "A protein"[1] at 4° and *p*H 5.95 without adding any acid to the solution. From Figure 2 we find that the horizontal line which cuts the TMV titration curve at *p*H 6.53 cuts the "A protein" curve at *p*H 5.85. In other words, it can be deduced from the titration curves of Figure 2 that it should be possible to convert a hypothetical protein polymer at *p*H 6.53 and 4° into "A protein" at *p*H 5.85 and 4° without adding acid to the solution. Therefore, within an accuracy of 0.1 *p*H unit, the two experiments yield the same result.

Electrophoretic Mobility.—Although the electrophoretic-mobility curves of Kramer and Wittmann (1958) show very little change in velocity of negative migration as the *p*H of the virus is raised above 5, the titration curve of Figure 3 demonstrates that there is a steady increase in the number of equivalents of base bound or neutralized per particle from the isoelectric point up to at least *p*H 8, the limit of the titration data. The lack of correspondence between the mobility curve and the titration curve is in marked contrast to the behavior of serum albumins (Moyer, 1938; Abramson, 1934), egg albumin (Abramson, 1934), and gelatin (Abramson, 1934), where the two curves can be made to coincide. It is clear that above *p*H 5 base is bound without proportional increase of electrophoretically effective charge. This can be viewed as the binding of positive ions to balance electrically, in large measure, at least, the negative ions produced by the titration.

A possible mechanism may be found in the manner in which electrophoretic motion is produced and in the distinctive character of the surface of the virus rod. From the studies of Franklin and Klug (1956), it seems probable that the surface of the rod is not at all smooth but is furrowed by a prominent helical groove and by periodic radial indentations, both about 30 A deep and both a consequence of a helical stacking of subunits roughly ellipsoidal in shape. In the electrophoretic experiments of Kramer and Wittmann (1958), the value of the Debye-Hückel $1/\kappa$ was 11 A. According to electrophoretic theory, almost all the oppositely charged ions are closer than $3 \times 1/\kappa$ from the charged surface. The plane of shear must be located near the outside surface of the particle. Charges located within the 30 A helical groove should contribute very little to the mobility, because they are not shorn away and are therefore, in a sense, bound. Furthermore, it can be shown that the majority (over 65%) of the peripherally situated carboxyls would have ionized by the time the *p*H had been raised to 5.0; carboxyls located in cracks, however, could be expected to ionize only at higher *p*H values, because the space average of the dielectric constant of the medium surrounding carboxyls located within grooves is lower than that for those located on the protuberances. All this means that up to a *p*H of about 5, there should be a normal coincidence between the mobility curve and the titration curve of the virus, but significant discrepancies between the two curves should arise above *p*H 5 as the centrally situated carboxyls begin to ionize. Qualitatively, this is just what was observed.

All who have measured the electrophoretic mobility of TMV protein at *p*H values above the isoelectric point agree that the mobility of the depolymerized protein is only about one-third of that for the polymerized protein. It might, therefore, seem plausible to conclude that the net negative charge is less in the depolymerized than in the polymerized state. This could occur by the exposure of positively ionizing groups upon depolymerization. However, any conclusion that the net negative charge per unit mass in A protein should be about one-third that for the polymerized rod is both unjustified by electrophoretic theory and contrary to the experimental observations reported in this communication. As was mentioned earlier, the titration curves for TMV protein indicated that there was an increase of five negative charges for each A protein unit depolymerized from a polymerized rod at *p*H 6.5. From this figure and the value of the net negative charge per 100,000 g of protein in a polymerized rod at the same *p*H, as determined from a titration curve, a fractional increase of charge during depolymerization can be calculated. For the purposes of this calculation, it is supposed that the titration curve for polymerized protein, could this be obtained, would be the same as that for intact virus and that the net exposed charge per A protein unit within a polymerized rod is given by the number of moles of hydroxyl ions bound per 100,000 g of virus between the isoelectric point of the virus and *p*H 6.5. The isoelectric point appropriate to the titration experiment at 0.1 ionic strength can be estimated by extrapolation from the isoelectric values of *p*H 3.49 in 0.02 ionic strength buffer (Eriksson-Quensel and Svedberg, 1936) and *p*H 3.2 in 0.075 ionic strength buffer (Kramer and Wittmann, 1958; Kramer, 1957), assuming that there are no significant differences in ion-binding tendency. The isoelectric point for polymerized protein so obtained is *p*H 3.05. The charge per 100,000 g of polymerized protein is computed to be −40 faradays.[2] The fractional increase of charge per unit weight when protein depolymerizes is thus around 13%.[3]

Electrophoresis theory has not been notably successful in relating quantitatively net charge from titration to mobility. Nevertheless, the theoretical pro-

[1] Technically, this could not be pure A protein at *p*H 5.76 or 5.85, since complete depolymerization is not expected below *p*H 6, at least; thus, this protein solution must consist of an equilibrium mixture of A protein and polymers of unspecified length.

[2] This value must be considered a better estimate of the charge than that (−4.5 faradays/100,000 g) which can be calculated on the basis of electrophoretic theory (Watanabe and Ui, 1956). This should be true despite any contribution the nucleic acid might reasonably be considered to make to the titration curve of the virus.

[3] When the data of Kramer and Wittmann (1958) are interpreted as in the preceding paragraph, one can estimate that only about two-thirds of the charge determined by titration is electrophoretically effective at *p*H 6.5 in TMV and, therefore, presumably in polymerized A protein. If some of these "hidden" charges are exposed on depolymerization to become electrophoretically effective, then the fractional increase of effective negative charge per unit weight could be higher than 13%, but not more than about 70%.

portionality of mobility to average net charge per unit of surface area is probably approximately valid. When rods depolymerize, there is an increase in surface per unit weight of severalfold, the exact magnitude depending on the size and shape of A protein. Since the increase in net charge per unit weight is probably less than 2-fold, the mobility of the depolymerized protein should be considerably less than that of polymer. Calculations based on the theory of Gorin (1942) for cylinders and the theory of Henry (1931) for small spheres yield the same result.

Titration Experiments.—As mentioned earlier in the experimental section, the difference in Figure 2 between the titration curve for the purified protein and that for the virus can be interpreted as the result of "exposing" negatively ionizing side chains when the polymer depolymerizes. The maximum difference between the two curves at any one *p*H is 7 equivalents per 100,000 g of protein. The virus curves of both Figures 2 and 3 show buffer capacity extending far beyond the expected *pK* (~4.7) for carboxyls. The abnormality of the virus curve is most obvious in Figure 2 where it is seen finally to cross the protein curve at *p*H 7.6 with a new region of even greater capacity for OH^-/H^+ ion uptake. The same behavior is apparent in the curve of Figure 3 at 20°.

A second unusual feature of the virus curve is to be seen in Figure 3, namely, time dependence of the titration. Although such behavior is usually explained by denaturation (Tanford, 1962), there is no evidence of denaturation of either protein or virus between *p*H 6.5 and 8.0. A slow conformational change within the protein subunits of the virus can be excluded as a source of time dependence because the curve for pure protein does not show anomalies in the region *p*H 6.5–8.0. This leads to the conclusion that both the time dependence and the abnormal binding of hydroxyl ions above *p*H 7 are caused by a titration of nucleic acid on the interior of the virus structure. In this case, the dependence, which involves no lack of reversibility of the titration, is attributed to the time required for diffusion equilibrium between hydrogen or hydroxyl ions in the bulk electrolyte and the ionizable groups buried within the tight coil of the virus.

In view of the complication just discussed, it follows that previous attempts (Koshland *et al.*, 1958; Fraenkel-Conrat, 1957a; Fraenkel-Conrat and Ramachandran, 1959) to ascribe all OH^- ion binding during degradation of whole virus to groups on the protein, presumed to be involved in subunit bonding, must be regarded with caution. It appears that such information may be obtained only from experiments on the purified protein. Although the experiments herein reported were performed with protein free of nucleic acid, it should be pointed out that the values so far obtained apply to depolymerization to the level of A protein and thus do not necessarily indicate the maximum that could be obtained were depolymerization to be carried all the way to the fundamental subunit (Ansevin and Lauffer, 1959a).

So far, the exact interpretation of the hydrogen-ion uptake accompanying polymerization has been left open. Three possibilities can be considered. The simplest is that the formation of protein-protein bonds requires the presence of uncharged carboxyls in certain critical spots of contact, and that when the protein is depolymerized, bonds previously made to these carboxyls are broken so that the carboxyls are then free to ionize. Arguments against this view were presented in paper I of this series (Ansevin and Lauffer, 1963).

A second possibility is that ionization of carboxyls is suppressed in the polymerized state because of electrostatic interaction, but not in the depolymerized state where, on the average, charges are farther apart. Tanford (1962) has suggested that this type of electrostatic interaction shows up when insulin polymerizes. It can be anticipated, however, that the influence of this interaction for a monomer having the relatively large dimensions of A protein should be less than for insulin.

A third proposal for explaining the *p*H change during TMV-protein polymerization is that the involved carboxyls are located on the mating faces of the subunits and that the process of polymerization thrusts these carboxylate ions into a medium of low dielectric constant in which the ionized state is unstable. Closely related to this is the case where the carboxyls are presumed to be situated within the helical grooves of the virus or protein polymer and are kept in an un-ionized state at *p*H 6–6.5 by the low space-average dielectric constant of their environment. This mechanism was invoked above in explaining the unusual electrophoretic behavior of the virus.

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