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Acid–Base Titrations of Tobacco Mosaic Virus and Tobacco Mosaic Virus Protein*

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ABSTRACT: Tobacco mosaic virus (TMV) is reversibly titrated between pH 2.5 and 9.5, and nucleic acid free TMV protein is reversibly titrated between pH 2.7 and 11. Neither an acid nor an alkaline end point is reached within the ranges of reversible titration of either the virus or the protein. The number of groups titrated within the reversible portion of the protein titration indicates that some functional groups of the protein are not exposed to the solvent. Comparison of the protein titration curves at 4 and 20° in the pH range where the protein is known to undergo endothermic polymerization indicates that about one hydrogen ion is bound per protein monomer as a result of polymerization.

Comparison of the protein titrations and the virus titration shows that under conditions permitting polymerization the ionization behavior of the protein resembles that of the virus.

Acid–base titration curves of tobacco mosaic virus (TMV) and TMV protein over a limited range of pH have been published previously (Ansevin *et al.*, 1964). The present study is an extension of the range of pH for the titration curves of both TMV and TMV protein and an extension of the range of temperature for the protein titration.

A unique feature of TMV is that it buffers more strongly in the range pH 7–9 than would be expected on the basis of amino acid content alone, histidine and free α-amino groups being absent (Anderer *et al.*, 1965). Furthermore, TMV protein at 4° exhibits a very strong buffering at about pH 6, caused by the binding of protons when the molecules polymerize into virus-like rods (Ansevin *et al.*, 1964). Estimates of the number of protons bound per monomer of the polymerizing protein have been made by several investigators. In this work a new estimate of this number is made from specific features of the protein titration curves.

Materials and Methods

Virus Preparation. Stock solutions of TMV were prepared by a method essentially the same as that of Boedtker and Simons (1958), involving centrifugation and treatment with EDTA of juice squeezed from Turkish tobacco plants systemically infected with the common strain of TMV.

Protein Preparation. Individual samples of TMV protein were prepared for titration from 2% TMV by a variation of the acetic acid method of Fraenkel-Conrat (1957). RNA was separated from the protein–67% acetic acid mixture by centrifugation at 4–6° for 10 min at 10,000 rpm. The protein was then separated from the concentrated acetic acid on a column of G-25 Sephadex in 0.1% HAc (pH 3.2) (C. L. Stevens, private communication). Effluent samples of about 15 ml were collected in 1 ml of 0.1 M phosphate buffer at pH 7.5, in which the protein precipitates. The tubes containing protein are then easily located and the protein is sedimented at 3000 rpm for 30 min. This step permits gentle packing of the protein and allows the solvent to be poured away. The protein is resuspended in 0.1 M KCl with dropwise addition of 0.1 M KOH to about pH 9. The preparation is then dialyzed against 0.1 M KCl, centrifuged at 40,000 rpm for 1.5 hr to remove aggregated protein, and dialyzed again.

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The ultraviolet absorption spectrum of such preparations routinely has a maximum:minimum ratio of 2.5, indicating a high degree of purity.

Concentration Determination. The concentrations of TMV and TMV protein solutions used for titration were determined in a Brice-Phoenix differential refractometer at 20°. The specific refractive increment used was 0.1852×10^{-3} $(mg/ml)^{-1}$. This value was calculated from the specific refractive increment of 0.1856×10^{-3} $(mg/gm)^{-1}$ determined by Stevens (1962) and the density of the solutions.

Titrants and Buffers. HCl at a concentration near 0.1 N was titrated to the sodium carbonate end point in the presence of methyl orange indicator against a freshly prepared 0.1000 N sodium carbonate solution made from oven-dried Na_2CO_3 and deionized water. KOH near 0.1 N was made from carbonate-free, concentrated Acculute standard volumetric solution (Anachemia, Ltd., Montreal, Canada) and was titrated with phenolphthalein indicator against the standardized HCl. HCl of 0.5 N concentration was standardized in the same manner as was the 0.1 N HCl and used in the determination of the acid end point in the protein titration. Standard buffers for calibration of the pH meter were made according to Bates (1962) and Bates *et al.* (1956).

Titration Procedure. The pH meter used was the Beckman Research pH meter calibrated immediately before titration at the temperature of titration, either 4 or 20°, with the standard buffers. The meter was checked against the standard after each titration. Drift of the calibration was negligible.

For titration 20-ml samples of 0.3–1.2% virus or protein, dialyzed against 0.10 M KCl, were added to a specially designed titration cell, and a small portion of each sample was set aside for concentration determination.

The cell consisted of a water-jacketed glass beaker with the top edge ground plane and a triangle of 0.25-in. rigid sheet plastic through which the electrodes and a thermometer protruded. The electrodes were a Beckman glass electrode and a Beckman calomel electrode with a saturated KCl bridge and fritted-glass junction. The electrodes and a thermometer were sealed permanently in place with a silicone rubber caulking compound. Two small holes were drilled through the triangle, one to admit nitrogen, the other to permit the escape of nitrogen and to admit a capillary through which titrants were added. The entire assembly was clamped together by means of three bolts fixed to a second plastic triangle under the beaker. A thin Teflon ring gasket with silicone grease sealed the joint between the beaker and the electrode unit.

When the cell had been filled, stirring was begun with a small, Teflon-coated magnetic stirrer. Then a moderate flow of N_2 washed in 0.01 M phosphate solutions was admitted through one of the small ports for 15–30 min. The nitrogen flow was then stopped and a microburet (Manostat, N. Y.) fitted with a capillary nozzle and filled with titrant was introduced with the nozzle tip just under the surface of the solution. The nitrogen admitting port and the opening around the nozzle were sealed with silicone grease. Titrant was added in small increments to the extreme of titration reversibility and the pH was recorded to the nearest 0.01 pH unit with each increment. Reverse titrations were performed immediately on the same sample by addition of the other titrant. Reverse titrations were performed only on the virus and on the protein at 4°. Forward and reverse solvent corrections (control curves) were determined for each experiment by titration of the dialysate with the same total amounts of titrants.

The time between addition of titrant and recording of pH was usually of the order of a few minutes but this practice was not followed until after it had been shown that longer periods did not result in further change of pH reading. Stabilization of the readings generally took longer in pH regions where the protein exhibited no buffering, sometimes as long as 10 min.

Molecular Weight. The value of molecular weight of the TMV protein monomer used for this study was 17.53×10^3 determined from the amino acid composition (Anderer, 1963). For calculating the protein content of a virus solution, a factor of 0.95 (Knight and Woody, 1958) was applied to the virus concentration.

Results

The titration curves were calculated from the experimental data as follows. (1) The difference in volumes of titrant added between the forward[1] virus or protein curve and the forward control curve was determined graphically at intervals of 0.2 pH unit or less. (2) The amount of titrant bound by the total amount of protein present was calculated with algebraic corrections for the difference between the volumes of sample and control. (3) These values were then normalized by division by the number of moles of 17.53×10^3 molecular weight protein subunits determined refractometrically to be present in 20 ml of the starting solution. (4) The resulting values were then plotted as a function of pH and the value at a calculated isoionic point was determined by graphical interpolation. (5) The value at the calculated isoionic point was subtracted from all the values and the results were replotted as a function of pH. (6) The same steps were followed for the reverse curves. Portions of some of the curves between pH 6 and 7 have been obtained for both acid and alkaline range titrations. A smooth transition between the ranges can therefore be plotted. The procedure for calculating the isoionic point is described in the discussion.

The titrations were repeated on several different samples and were found to be reproducible. Virus concentrations from 0.3 to 1.2% and protein concen-

[1] Here a forward curve is defined as the plot of pH *vs.* volume of the first titrant added, either HCl or KOH. A reverse curve is that obtained by subsequent addition of the other titrant to the sample.

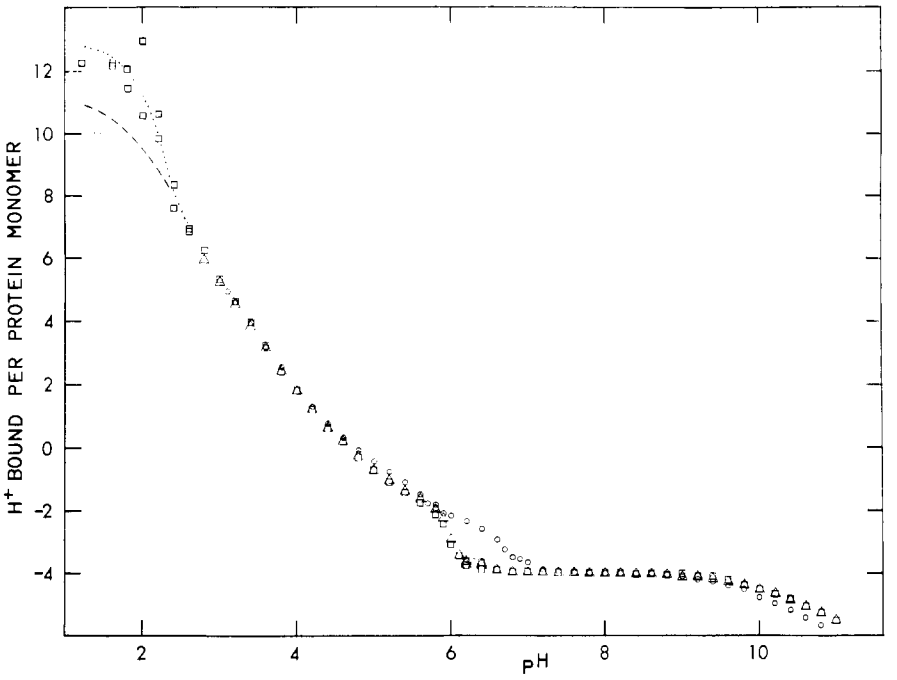

FIGURE 1: Acid–base titration on curves of TMV protein at 4 and 20°. HCl (□) at 4 and (○) 20°; KOH (△) at 4 and (○) 20°.

trations from 0.3 to 0.7% were titrated. No concentration effects were observed.

To examine the question of concentration effects a little more thoroughly, some concentrated protein samples at about pH 6.5 were diluted with preequilibrated 0.1 M KCl. The pH of each sample was monitored during dilution. No change in pH greater than 0.02 pH unit was observed upon dilution over the concentration range from 1.2 to 0.3%. The same result was obtained at 4 and 24°.

The reversible and irreversible portions of the protein titration curves are plotted in Figure 1. The virus titration curve is plotted in Figure 2.

Discussion

The titration data presented in Figures 1 and 2 are in general agreement with those published by Ansevin *et al.* (1964), with the following exceptions. The data of Ansevin *et al.* are simple titration curves uncorrected for the amount of titrant required to produce the same pH values in solvent alone. Such a correction makes no substantial difference except at pH values below 5 or above 9, and was negligible over the range (pH 5–8) studied by Ansevin *et al.* In the present study there was no time dependence observed in the alkaline range titration of the virus as was reported by Ansevin *et al.* Occasionally, however, some alkaline range results had to be discarded if the titration cell was not well sealed, since poor sealing gave rise to long equilibration times and irreversibility, presumably due to absorption of atmospheric carbon dioxide. Accordingly, there are slight differences between the present results and those of Ansevin *et al.*

The virus titration is reversible over the range shown in Figure 2. The limit of reversibility in the alkaline range was not determined exactly, but titrations were routinely brought to pH 9.5. Titration to pH 10.5 is not reversible.

In the case of the protein, the lower limit of reversibility is also not sharp but appears to be at about pH 2.7. The ionization of the protein is also reversible up to pH 11 but not as far as pH 12. Reversible alkaline range titrations were routinely brought only to pH 11.

In order to determine a reference point for plotting the titration curves it is convenient to know the numbers of amino acids with functional groups in the structure of TMV protein. In Table I the functional groups are listed with the frequency and pK values of each as observed in a characteristic protein.

Several interesting features of the curves are noted in comparison with the amino acid content. First, the absence of α-amino groups and histidine is reflected in the complete lack of buffering by the protein at 4° from a little below pH 7 to about 9. Second, the number of groups titrated on the protein between pH 2.7 and 7 is less than might be expected on the basis of amino acid content. There are only about 11 groups titrating in this range, of a complement of 15 carboxyls in the structure. It appears, then, that several of the carboxyl groups titrate at lower pH values than Table I predicts.

But the most interesting feature of these curves is the sharp rise in the titration curve of the protein at 4° as the pH is lowered from 6.1 to 5.9. One or more

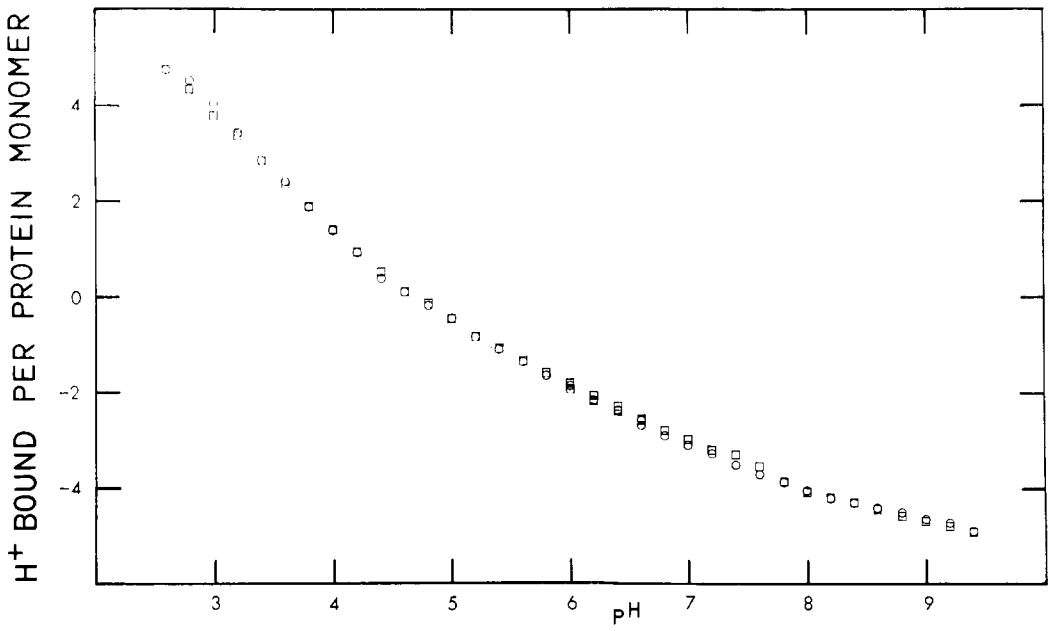

FIGURE 2: Acid–base titration curve of TMV at 4°. (○) HCl; (□) KOH.

groups bind protons much more strongly than expected. It has been shown by Lauffer *et al.* (1958) that it is in the range of pH 5–7 that TMV protein undergoes polymerization. The strong buffering has been explained by Ansevin *et al.* (1964) as follows. Some carboxylate groups situated on the polymerization-reactive faces of the protein subunit become buried in an organic medium of low dielectric constant as the polymerization proceeds. The groups therefore bind protons at a much higher pH than their ordinary pK. As shown by Ansevin *et al.* (1964), the binding of protons at pH 6.5 is accompanied by an increase in free energy.

It is at this point that the results of the dilution experiment are seen to be significant. Banerjee and Lauffer (1966) observed in osmotic pressure studies that polymerization of TMV protein is enhanced by increasing concentration. Ansevin *et al.* (1964) observed that upon depolymerization of unbuffered protein by decreased temperature there is a significant drop in pH, indicating that hydrogen ions were being released. The absence of a pH change upon dilution at 4°, then, indicates that the polymerization associated with changing temperature and the polymerization associated with changing concentration at low temperature are distinctly different processes.

Now returning to the problem of determining the reference point for plotting the titration curves, one notes that if the acid end point were known that point might provide a convenient reference. But, as Figure 1 shows, even taking the pH as low as 1.1 does not give a clean-cut end point. A calculated isoionic point has been chosen as the reference instead. R. Shalaby, K. Banerjee, and M. A. Lauffer (submitted for publication) have found by deionization with mixed-bed ion-exchange resin that the isoionic points of both TMV and TMV protein lie between pH 4.3 and 4.6. A hypothesis which fits the titration data may then be built according to the following reasoning. First assume that all of the functional groups are exposed to the solvent. It can be shown that then the isoionic point of the protein would be near pH 5.75, and that the slope of the titration curve near pH 10 would be more negative than it is. The simplest modification of the assumption which fits the titration data requires two steps. The first step is the assumption that three of the four tyrosine groups are buried in the organic portion of the molecule or are otherwise masked. Spectrophotometric titration by Chien *et al.* (1965) has shown this to be the case. This step reduces the expected negative

TABLE I: Characteristic pK's of the Functional Groups and Functional Group Content of TMV Protein.

Group	Frequency[a]	pK[b]
α-COOH	1	<4
Asp-COOH	6	4.7
Glu-COOH	8	4.7
His-NH	0	6.5
α-NH_2	0	7.8
Tyr-OH	4	9.95
Cys-SH	1	9.1–10.8[c]
Lys-NH_2	2	10.2
Arg-NH_2	11	>12

[a] Anderer *et al.* (1965). [b] Tanford (1961). [c] Cohn and Edsall (1943).

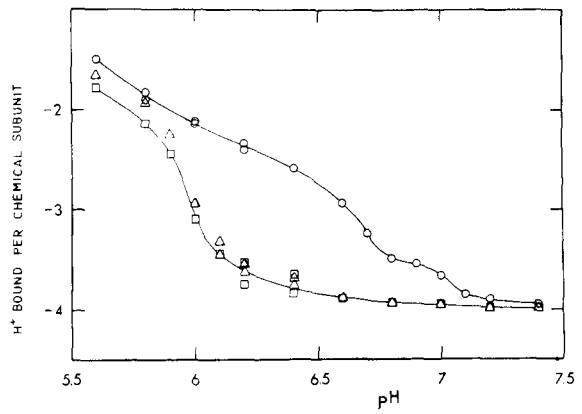

FIGURE 3: Enlarged view of the central portion of Figure 1. Symbols have the same significance as in Figure 1.

value of the slope near pH 10 somewhat, but does not affect the expected value of the isoionic point. If, in addition, two positive groups were also buried and either dissociated to their nonionic form or neutralized by an anion, the isoionic point would be at pH 4.65 which is quite close to the experimentally observed range.

The above argument can be easily followed if one observes that at pH 8 all exposed groups can be unambiguously assigned a charge value. If all groups are exposed to the solvent, there will be a contribution by the carboxylate groups of 15 negative charges; by the tyrosine groups and the cysteine, no contribution; and by the lysine and arginine groups, 13 positive charges. The net charge at pH 8 will be -2/protein monomer if all the groups are exposed. Counting[2] up the titration curve it is seen that the net charge will be zero at pH 5.75. But this point cannot be the isoionic point since it conflicts with the experimental findings. Making the two modifications suggested above, however, and following the argument again develops a hypothesis which fits the titration data. The protein curves, therefore, have been plotted with reference to the calculated isoionic points; pH 4.65 in the case of the protein titration at 4° and pH 4.75 in the case of protein at 20°. The virus curve has been plotted with reference to the calculated isoionic point for protein at 20°.

It is interesting to note that, if the two buried positive groups were arginine guanidino groups, the slope of the protein titration curve at pH 10 would still be expected to be more negative than observed because of buffering by the lysine amino groups. But if the two buried groups were the lysine amino groups the expected slope would be very close to that observed.

[2] Counting along the curve is justified in this case since R. Shalaby, K. Banerjee, and M. A. Lauffer (submitted for publication) found that neither potassium nor chloride is bound in this pH range, leaving hydrogen ions as the only contribution to charge between the isoionic point and pH 8.

An alternative to the hypothesis that the two lysine groups are buried in the NH_2 configuration is that these groups are in the NH_3^+ form and are electrostatically bonded to negatively charged groups, the configuration being stabilized by an environment of low dielectric constant. It must be pointed out, however, that the negative groups need not be side-chain carboxylate groups, for if this were the case, the isoionic point of the protein would not be at pH 4.65 but at about pH 5.75. Any model of the masking must include a means of neutralizing two more positive charges than negative charges by burying them either in the NH_3^+ state neutralized with negative ions or in the NH_2 state.

Since only 11 of the 15 carboxyl groups of TMV protein titrate between pH 2.5 and 8 it appears that at least some of the carboxyl groups titrate at pH values far below the expected intrinsic pK. Attempts to apply the customary Linderstrøm-Lang treatment to determine the intrinsic pK give some curious results. Our titration data and the ion binding data of R. Shalaby, K. Banerjee, and M. A. Lauffer, (submitted for publication) combine to give a net charge of zero over much of the acid range. Clearly the intrinsic pK cannot be unambiguously determined. Without further experimentation, then, all that can be said is that several carboxyl groups might be involved in salt linkages with positive groups in addition to the two already discussed and that these might become fully available for titration only at low values of pH where the protein is depolymerized or even denatured.

Now, attention is drawn to the dotted portion of the protein curve in Figure 1, between pH 1.1 and 2.7. This portion is not reversible and, as the scattering of points shows, not very reproducible. But it is believed that an end point has been reached, and the path of the data is interpreted as reflecting an irreversible destruction of the protein conformation below pH 2.7. The dashed line represents the expected path of titration in the absence of conformational change.

The difference in levels of the dotted and dashed lines should be noted. The dotted line is the expected result if there is an irreversible destruction of conformation. Such a disruption would change the environment of the two buried, normally positive groups, to an aqueous one and would permit them to ionize. The dotted and dashed lines, then, are separated by a difference of two protons bound per monomer at the acid end, the dashed line accounting for the titration of 15 carboxyl groups below pH 7 and the dotted line accounting for the titration of 15 carboxyl groups and two other groups.

Next, the titration of protein at 20° is considered. Except for the region above pH 9 and near pH 6 this curve is very similar to the curve for protein at 4°. Again, it is the region near pH 6 which is of greatest interest. Figure 3 is an expanded view of this region. The polymerization of TMV protein has been shown to be endothermic by Lauffer *et al.* (1958). At 20° the polymerization is substantially complete. The difference between the two curves at constant pH, then,

gives a measure of the number of hydrogen ions bound as a result of the temperature-mediated polymerization. The difference between the two curves at pH 6.5 is very close to one proton per monomer, the maximum being about 1.25 at about pH 6.25. The simplest interpretation of this result seems to be to assign a difference of about one proton per monomer as resulting purely from polymerization and the rest as due to charge effects.

But it must be pointed out that the total difference between the two protein titration curves in the region near pH 6 cannot possibly be attributed solely or even largely to charge effects. Two independent lines of reasoning lead to this conclusion. In the first, it is assumed that the potential in the neighborhood of ionized carboxyl groups does not change when the protein depolymerizes, as will obtain if they do not migrate upon depolymerization. Then, there will be no change in ionization attributable to charge effect and the observed decrease in electrophoretic mobility accompanying depolymerization (Kramer and Wittmann, 1958) is explained in terms of increase in surface area which decreases average surface potential even though it does not change the potential at a given point. The second line of reasoning, probably less sound, is to assume that the ionization is responsive to the average surface potential calculated from electrophoretic mobility results of Frist (1965) and Kramer and Wittmann (1958) for both polymerized and unpolymerized protein. When such a calculation is made, the difference between the hydrogen ion binding of polymerized and unpolymerized protein at pH 6.5 is only about 0.1 proton/monomer. This is an order of magnitude less than the observed effect.

Finally, the path of the virus titration curve deserves some comment. Although the virus and protein titration curves do not follow the same path, it is thought that roughly the same functional groups ionize on the virus as on the protein between any two widely spaced pH values. It is for this reason that Ansevin *et al.* (1964) assumed the two curves to be identical below pH 5. But this assumption requires the additional assumption that all surface features of the virus and protein are identical. The difference in path between the virus and protein curves can be attributed to an environment of low dielectric constant for several carboxyl groups in the virus structure. Since TMV is known to have a deeply grooved surface (Franklin and Klug, 1956), it is proposed that some carboxyl groups in the virus or polymerized protein are near the bottom of grooves in a region which has a dielectric constant intermediate between that of an aqueous environment and that of an organic environment. If these regions in the virus are sterically more stable than the corresponding regions in the polymerized protein, the virus regions would be expected to have a lower average dielectric constant than the protein regions which may have a fundamentally aqueous environment. The pK value of these carboxyl groups would then be high in the case of the virus and more nearly normal in the case of the protein.

Some evidence has been reported by Hulett (1965), who titrated metal-free and divalent metal-substituted TMV, that some of the carboxyl groups associated with the polymerization of the protein have pK values about 7.5–8 when in the stable virus structure. While the present study does not directly confirm this observation, the features of the virus titration curve could be so explained. Such a high pK value for a carboxyl group may be due to an environment of appropriately low dielectric constant. On the other hand, if RNA is responsible for the buffering near pH 8, it may be incorrect to plot the virus curve with reference to the point used for protein. But since the results of R. Shalaby, K. Banerjee, and M. A. Lauffer (submitted for publication) indicate that the isoionic points of TMV and TMV protein are essentially the same, titration by RNA is discounted and the procedure followed here in plotting the curve is believed to be correct.

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