its low protein content. The preference for and persistence of the drug in muscle after a single dose are not surprising in view of the growth depressing effect of *O*-EtThr in chicks. A lower metabolic activity of muscle tissue than of other tissues would account for a slower turnover of *O*-EtThr-^{14}C.

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Role of Cation and Anion in the Polymerization and Depolymerization of Tobacco Mosaic Virus Protein*

Krishnadas Banerjee† and Max A. Lauffer‡

ABSTRACT: Potassium and chloride ion binding by unpolymerized TMV protein (2–6°), by polymerized TMV protein (22–26.2°), and by TMV, all in buffers with pH values near 6.5, were measured. Potassium and chloride specific electrodes were used. No binding of either ion was detected for the virus or for the protein in both the polymerized and the unpolymerized states. The estimated error of measurement was such that the binding of one ion per protein monomer (molecular weight 17,500) should have been detected. Therefore, there is no appreciable change in the binding of potassium or chloride when TMV protein polymerizes at pH 6.5 and the large entropy increase which drives the polymerization must be associated entirely or almost entirely with the previously demonstrated release of water molecules.

The negative change in free energy necessary to drive the endothermic polymerization of tobacco mosaic virus (TMV) protein in 0.1 M phosphate buffer at pH 6.5, first observed in this laboratory, was assumed by Lauffer *et al.* (1958) to come from the release of water molecules, resulting in an increase in enthalpy and in entropy. Since then other aspects of this reversible polymerization have been studied in detail. From all these results, Lauffer (1966) proposed a model, subsequently modified (Lauffer *et al.*, 1967). But there was originally no experimental evidence to exclude the possibility of the role of ions in this reaction system. Even in the previous paper on ion binding by Shalaby *et al.* (1968), this point was not fully clarified because no experiments were done at pH 6.5 on both polymerized (23°) and unpolymerized (4°) protein. With the availability of new specific ion electrodes, the work was undertaken again to find out if ions, particularly potassium and chloride ions, participate in the polymerization reaction and contribute anything toward the thermodynamic parameters mentioned above.

Materials and Methods

TMV. The common strain of TMV was isolated by alternate high- and low-speed centrifugation with a step involving depigmentation with EDTA according to Boedtker and Simmons (1958).

TMV Protein. The protein was prepared from the virus by the acetic acid method of Fraenkel-Conrat (1957) with slight modification.

The aggregated protein obtained after removal by dialysis of the acetic acid was picked up in dilute KOH to a final pH near 7.5. It was then centrifuged at 40,000 rpm for 3 hr in the cold to remove any undegraded virus or denatured protein. The supernatant fluid was then exhaustively dialysed for 3 or 4 days against deionized water containing mixed-bed, ion-exchange resins (Bio-Rad Laboratories, Richmond, Calif., AG 501-X8 mesh). This step was necessary for obtaining native isoionic protein. It was observed that a large amount of material leaked out of the dialysis tubing and this material could not be redissolved even in 0.1 M KOH. If the ion-exchange resin was mixed directly with the protein, some of the protein denatured during stirring, and it was difficult to remove the denatured protein from the aggregated isoionic protein.

Isoionic TMV was also prepared in the same way by dialyzing against mixed-bed resin. For each experiment, freshly prepared isoionic TMV or TMV protein was used because,

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† Present address: Department of Radiation Therapy and Nuclear Medicine, St. Francis General Hospital, Pittsburgh, Pa. 15201.

‡ To whom correspondence should be addressed.

if large quantities of virus or protein were stored with alcohol or toluene to stop the growth of bacteria, the presence of the slightest amount of these organic solvents in the titrating sample poisoned the specific electrodes ultimately used, and erroneous results were obtained. Conversely, if the samples were frozen immediately for later use there was always some denaturation during the thawing procedure. The pH of the isoionic virus or protein varied from one preparation to another between 4.3 and 4.6.

Concentration Determination. Concentrations of TMV and TMV protein were determined before and after each experiment with a Cary spectrophotometer. In all experiments the ultraviolet absorption was measured from 400 mμ. Phosphate buffer (0.033 M, pH 7.5) was used as blank and solvent. The spectra were corrected according to the method of Englander and Epstein (1957). The extinction coefficients of virus (260 mμ) and protein (281 mμ) were taken as 0.27 and 0.13 for a concentration of 0.1 mg/ml for 1 cm path length cells (Fraenkel-Conrat and Williams, 1955). The maximum (281 mμ) to minimum (250 mμ) ratio, a criterion for the purity of the protein solution, varied from 2.4 to 2.6.

Chemicals. All chemicals were analytical reagent grade and were used without further purification. KOH solution was prepared from Acculute standard volumetric solution. Deionized distilled water was used for all experiments.

Experimental Procedure. pH measurements were carried out at the specific temperature with a Beckman Expandomatic pH meter using a Beckman glass electrode and a saturated calomel electrode. Standard buffer solutions were used for standardization of the pH meter. Ion binding was measured potentiometrically with either a potassium or chloride specific electrode (Orion Research, Cambridge, Mass.) and a Beckman saturated calomel electrode with fritted glass as the reference electrode, in connection with either a Beckman Research pH meter or a vibrating reed electrometer connected to a Leeds & Northrop Type K 3 universal potentiometer and a Honeywell recorder. The calomel electrode had a fritted junction. When our electrodes were calibrated with known concentrations of KCl, the electromagnetic force measured even at very low concentrations was steady after equilibrium was reached in 15–20 min. This indicates that flow of KCl through the fritted junction is negligible.

The cell consisted of a water-jacketed glass beaker as described by Scheele and Lauffer (1967). The specific ion electrode, a glass electrode, the reference electrode, and a thermometer were introduced into the beaker. A sample volume of 10 or 20 ml was used for all experiments. A small Teflon-coated magnetic bar was used to mix the reagents with the sample and also to facilitate temperature equilibration. The time between the addition of titrant and reading of electromagnetic force or pH was usually of the order of 15–20 min.

Just before doing experiments with the virus or protein, the specific electrodes were calibrated at the desired temperature. The Nernst slopes obtained were within 5% of the theoretically calculated values. The potassium specific-ion electrode was calibrated with potassium phosphate buffer and KCl and the chloride electrode with KCl. The reagents used for calibration of the electrodes were exactly the same as those used for titration of the virus or protein.

First the isoionic virus or protein was allowed to come to temperature equilibrium in the water-jacketed cell. In case of K^+ binding, the pH of the sample was slowly raised by the addition of small amounts of potassium phosphate buffer (pH 6.5–6.6). The potential was measured with the specific-ion electrode, and the pH of the sample was measured simultaneously with another pH meter. In this way the whole range from pH 4.3 to 6.6 was covered. The final molarity of K^+ varied from 0.04 to 0.1.

TABLE I: Chloride Ion Binding to TMV and TMV Protein at 2°.[a]

TMV (21 mg/ml)			TMV Protein (4 mg/ml)		
Anion Concn (M × 10^4)			Anion Concn (M × 10^4)		
Added (m_1)	Free (m_2)	pH[a]	Added (m_1)	Free (m_2)	pH[a]
1	1.30	6.53*	1	1.30	6.62*
2	2.10	6.46	2	2.10	6.65
3	3.05	6.44	3	2.90	6.63
4	4.10	6.43	4	4.00	6.64
6	6.10	6.44	6	5.90	6.63
8	8.00	6.44	8	7.95	6.55
10	10.30	6.42	10	10.00	6.55
20	20.00	6.36	20	20.00	6.33
30	31.00	6.34	30	30.00	6.32
40	40.20	5.32*	40	40.00	6.51*
60	60.00	6.61*	60	60.00	6.43
80	80.00	6.55	80	80.00	6.35
100	99.90	6.52	100	100.00	6.17
180	180.00	6.49	180	180.000	6.45*
260	260.00	6.49	260	260.00	6.36
340	340.00	6.48	340	340.00	6.34
420	420.00	6.44	420	420.00	6.30
620	620.00	6.41	260		
820	820.00				

[a] Asterisks indicate points where KOH was added to raise the pH.

While measuring the chloride ion binding, KOH was added slowly to raise the pH of the isoionic sample to around 6.5. Then KCl was added, and the potential across the chloride electrode and pH of the sample was measured. As more and more chloride was added to increase the ionic strength, the pH was dropped and more KOH was added to raise the pH again to about 6.5 as shown in Table I.

The number of moles of ions bound (ν) per mole of protein monomer (17,530) was calculated from the relation $\nu = (m_1 - m_2)/m_p$, where m_1 is the molarity of ion added, m_2 is the molarity of free ion, and m_p is the concentration of protein or virus.

It was assumed that the presence of protein did not affect the ionic species activity coefficient γ_+ and γ_- and that the ionic species activity coefficient was equal to the mean activity coefficient $\gamma_\pm$. It was also assumed that the salt was completely dissociated.

Results

A typical plot of electromotive force against cation concentration is shown in Figure 1. The extent of binding of chloride ion to TMV and TMV protein in one of the experiments is shown in detail in Table I. All other experiments showed the same kind of result. There was almost no binding of K^+ or Cl^- to TMV or TMV protein at room temperature (22–26.2°) or in the cold (2–6°).

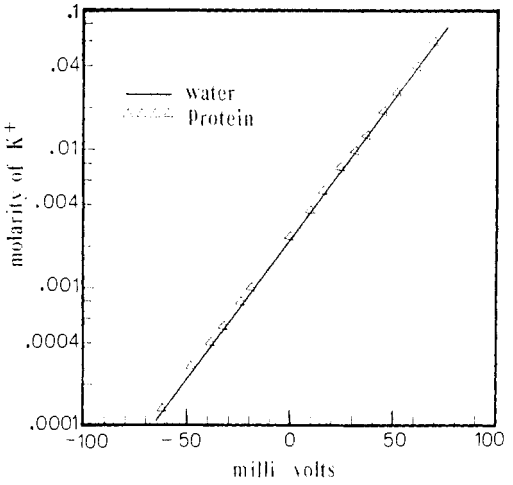

FIGURE 1: Plot of potential as a function of molarity of K^+ added to TMV protein (concentration = 10.64 mg/ml, temperature = 6°.

Discussion

Shalaby *et al.* (1968) showed that isoionic virus or protein did not bind any potassium or chloride ions. Around pH 7.3 less than 1 mole of ion was bound per mole of protein monomer. These results indicated that possibly the ions are not bound around pH 6.5. However, all the experiments were done at room temperature and one can never be sure what would actually happen at low temperature and at pH 6.5 where the protein is depolymerized. It was also crucial for us to know if there was any difference in the amount of ion bound by the protein at pH 6.5 at room temperature (polymerized) and in the cold (depolymerized).

The accuracy of potential measurements in each experiment was within 0.05 mV and the largest variation from day to day was within 0.5 mV. Even if we take the conservative view and regard 0.5 mV as the experimental error, we would have been able to detect the binding of one ion per protein monomer up to 0.01 M concentration of the electrolyte added. At this concentration of the ion, turbidity experiments show that at 23° the protein was of a molecular weight 8 to 12 times that of a trimer. Hence, we can say that up to this stage of polymerization of TMV protein the amount of ion bound was certainly less than one. Since the results indicated zero or negligibly small binding by both unpolymerized and polymerized protein, we can conclude that changes in potassium or chloride ion binding do not contribute to the enthalpy and entropy change in our polymerization reaction.

Scheele and Lauffer showed that when TMV protein is polymerized in 0.1 M phosphate buffer at pH 6.5, approximately one hydrogen ion is bound per mole of protein monomer. Ansevin *et al.* (1964) pointed out that the entropy change associated with hydrogen ion binding in such a buffer, on the assumption that the ionized and the un-ionized forms of the appropriate carboxyl groups on the protein are both at unit activity, was −8.5 cal/mole degree. Stevens and Lauffer (1965) and Jaenicke and Lauffer (1969) have shown that water is released when TMV protein polymerizes. Since there is little or no change in the binding of ions other than hydrogen and since the contribution of hydrogen ion binding to the entropy change is negative, the very large positive entropy change must come exclusively or very nearly exclusively from release of water.

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CORRECTIONS

In the paper "An Effect of Calcium Ions on the Activity, Heat Stability, and Structure of Trypsin," by Tibor Sipos and Joseph R. Merkel, Volume 9, No. 14, July 7, 1970, page 2766, the following correction should be made.

Figure 5a: the numbers on the "TAME Activity" scale should be doubled.

In the paper "IUPAC–IUB Commission on Biochemical Nomenclature. Abbreviations and Symbols for the Description of the Conformation of Polypeptide Chains. Tentative Rules (1969)," Volume 9, No. 18, September 1, 1970, page 3471, the following correction should be made.

Page 3478, footnote 6: "Weltaufer (1961)" should read "Wetlaufer (1961)."

In the paper "Inhibition of Hepatic Protein Synthesis by α-Methyl-DL-tryptophan *in Vivo*. Further Studies on the Glyconeogenic Action of α-Methyltryptophan," by Michael Oravec and T. L. Sourkes, Volume 9, No. 22, October 27, 1970, page 4458, the following corrections should be made.

Page 4458, column 1, first paragraph of text, lines 8 and 9: reference should be to Oravec and Sourkes, 1969.

Page 4458, column 2, last line of the introduction: reference should read Oravec and Sourkes, 1968.

Page 4459, column 2, line 23: Oravec and Sourkes, 1969.

Page 4463, column 1, first paragraph of Discussion, line 3: reference should be Oravec and Sourkes, 1967–1969.

Page 4464, column 2: reference to Oravec and Sourkes, 1968a, should read Oravec and Sourkes, 1969; reference to Oravec and Sourkes, 1968b, should read Oravec and Sourkes, 1968.