Reconstitution of Tobacco Mosaic Virus: Calorimetric and Related Studies*

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ABSTRACT: Previous investigations have shown that tobacco mosaic virus (TMV) can be reconstituted in its infective form from TMV protein (TMVP) and TMV RNA. In the present investigations, the heat of reconstitution of TMV in 0.1 M pyrophosphate buffer has been measured by a microcalorimeter.

The reconstituted TMV was found to be similar to TMV in most respects. The pH was found to increase during the initial stages of reconstitution and then decrease during the later stages almost to its original value. CsCl equilibrium density gradient studies showed that the supernatant fluid remaining after pelleting the reconstituted TMV contained only unreconstituted protein and nucleic acid and no unsedimentable nucleoprotein particles. Calorimetric studies on the enthalpies arising from change of pH of TMV and TMVP in 0.01 ionic strength phosphate buffer indicated that the virus and the protein have comparable surface features in the pH range 4–5. The heat for a hypothetical reaction between polymerized TMVP and RNA in 0.01 ionic strength phosphate buffer at pH 5.0 was calculated by an application of Hess's law as the difference between the heat of reconstitution and the heat of polymerization, the actual reconstitution having been carried out in 0.1 M pyrophosphate buffer. The heat thus calculated turns out to be −0.485 cal/g of TMVP. This corresponds to −2800 cal/mole of phosphate groups in the RNA chain.

Tobacco mosaic virus (TMV) has been reconstituted in infective form from TMV protein (TMVP) and TMV RNA (Fraenkel-Conrat and Williams, 1955). Optimum conditions for reconstitution are room temperature in pH 7.3, 0.1 M pyrophosphate buffer (Fraenkel-Conrat and Singer, 1959). It has been shown that TMV RNA could be substituted by polyadenylic acid (Fraenkel-Conrat and Singer, 1964) or by the viral RNA derived from a spherical bacteriophage, MS2 (Sugiyama, 1966), or by turnip yellow mosaic virus RNA (Matthews, 1966) in the reconstitution reaction. Furthermore, it has been shown that infective particles could be obtained by reconstituting TMV RNA with coat proteins from spherical viruses (Verduin and Bancroft, 1969). It has also been shown (Hirai and Wildman, 1967) that, in the plant cell, the assembly of TMV RNA and TMVP into nucleoprotein particles occurs in the region of the cell devoid of organelles visible in the light microscope.

It has also been shown (Franklin, 1955) that the X-ray diffraction diagram of repolymerized TMVP resembles that of TMV so closely that there can be no doubt that the structural arrangement of the protein characteristic of the virus is present under some conditions in the repolymerized protein. However, all these studies throw little, if any, light on the nature of the interactions between the protein and the RNA in the TMV.

As contrasted to this lack of thermodynamic information concerning the virus, there is considerable thermodynamic information relating to TMVP. Lauffer *et al.* (1958) found that the process of formation of rods from TMVP subunits is an endothermic reaction. It was predicted (Lauffer *et al.*, 1958) and subsequently verified experimentally (Stevens and Lauffer, 1965) that water is released during the formation of protein rods and subsequent tightening up of the structure. Indirect determinations of the enthalpies based on the dependence of the equilibrium constant on temperature (Smith and Lauffer, 1967; Banerjee and Lauffer, 1966) were made. More recently, calorimetric determinations of the enthalpies of the polymerization reaction (Stauffer *et al.*, 1970) have been made, confirming the prediction made earlier regarding the different modes of polymerization leading to the formation of protein rods (Lauffer *et al.*, 1967).

The aim of the present investigation is to make use of the information available on the polymerization of protein to determine from calorimetric measurements the enthalpy of association of protein and RNA in TMV particles. A great advantage of calorimetric determination over equilibrium studies is that the results are independent of any model for the polymerization process.

Some of the results presented here have already been reported in an abbreviated form (Srinivasan and Lauffer, 1969).

Materials and Methods

Purification of TMV. TMV was isolated from systemically infected tobacco plants using a method essentially the same as that of Boedtker and Simmons (1958), involving alternate high- and low-speed centrifugation. It was depigmented (Ginoza *et al.*, 1954) at the first high-speed pellet stage.

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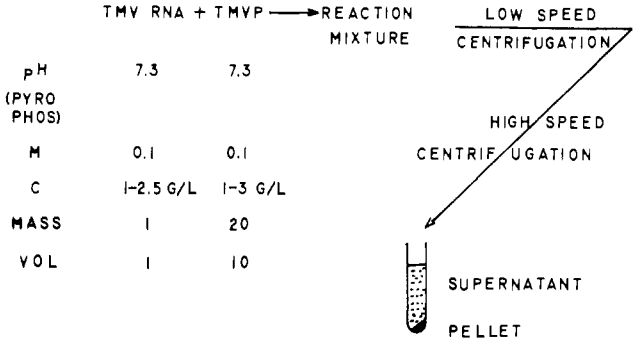

FIGURE 1: Reaction scheme.

Preparation of TMVP. TMVP was extracted from the virus by the acetic acid method of Fraenkel-Conrat (1957).

Preparation of TMV RNA. TMV RNA was extracted from TMV using the bentonite–phenol method of Fraenkel-Conrat *et al.* (1961) with slight modifications. TMV, water-saturated phenol, bentonite, and 0.1 M Versene were mixed in the ratio of 1 g:50 ml:1 g:0.5 ml, respectively, and stirred vigorously. This mixture was then centrifuged at 10,000 rpm for 15 min, when the aqueous and phenolic phases separated. The aqueous extract was then mixed with water-saturated phenol and recentrifuged as before. Traces of phenol in the aqueous phase were removed by shaking with three times its volume of ether. This procedure was repeated twice. Nitrogen gas was bubbled through the aqueous phase so obtained to get rid of any traces of ether. RNA was then precipitated by adding ethanol (2.5 times the volume of the aqueous phase) and 1 drop of 3 M sodium acetate for each ml of the aqueous phase. RNA was pelleted by centrifuging at 10,000 rpm for 10 min. This was dissolved in distilled water and centrifuged at 40,000 rpm for 2 hr when the bentonite was pelleted out. RNA was once again precipitated, using ethanol and 3 M sodium acetate, and centrifuged. The RNA pellet so obtained was dissolved in the pyrophosphate buffer and dialyzed against the same buffer for at least 3 days with daily change of buffer. The entire extraction and purification of RNA were done at 4°.

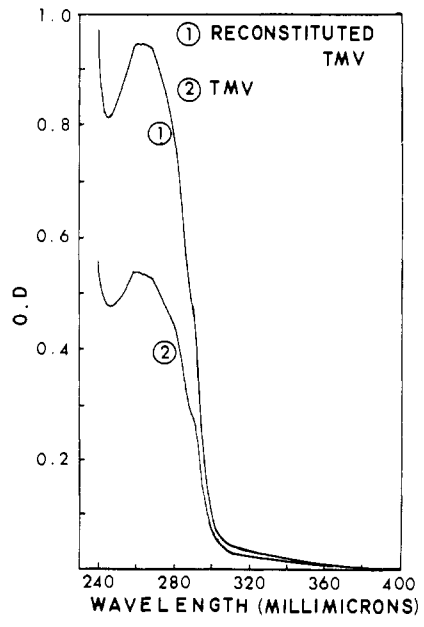

FIGURE 2: Ultraviolet absorption spectra of reconstituted TMV and TMV.

The pyrophosphate buffer used in the experiments was made by dissolving 44.6 g of sodium pyrophosphate in 800 ml of distilled water and then adding to this 1 N HCl to lower the pH to 7.3.

Concentration Determination. Concentration of TMV, TMVP, and TMV RNA solutions were determined by using a Cary spectrophotometer. Ultraviolet absorption curves were obtained from 400 to 220 mμ. When necessary, these spectra were corrected for scattering according to Englander and Epstein (1957). Concentrations of TMV were calculated from the optical density at 260 mμ using an extinction coefficient of 26 $(g/100\ ml)^{-1}$ (Fraenkel-Conrat and Williams, 1955). Protein solution concentrations were similarly obtained by using an extinction coefficient of 13 $(g/100\ ml)^{-1}$ at 280 mμ (Stevens and Lauffer, 1965). The concentration of RNA was obtained by using an extinction coefficient of 258.5 $(g/100\ ml)^{-1}$ at 258 mμ (Northrop and Sinsheimer, 1954).

Calorimeter. The calorimeter[1] used in these studies is a static conduction twin calorimeter. A blank is placed in one calorimeter, the solution under study in the other. The difference in the electrical potential between the two calorimeters is recorded by a potentiometer strip chart recorder. The instrument and details of operation are described by Benzinger (1965) and by Stauffer *et al.* (1970).

Experimental Procedures and Results

All experiments except those relating to the CsCl equilibrium density gradient centrifugation were done at room temperature. The CsCl centrifugation experiments were done at 4° to obviate denaturation of TMVP during the long hours of centrifugation.

The reaction scheme is shown in Figure 1. In all reconstitution experiments, the protein:RNA was 20:1, by weight. The resulting virus pellet was suspended in distilled water and the ultraviolet absorption spectrum was obtained. The mass of virus reconstituted was calculated from the optical density at the maximum using an extinction coefficient of 26 $(g/100\ ml)^{-1}$. Percentage reconstitution was calculated in each case.

1. Experiments to Show That Reconstituted TMV Is the Same As TMV. I. Ultraviolet absorption studies. The ultraviolet absorption spectra of the reconstituted TMV and TMV are shown in Figure 2. The two were identical in all the reconstituted TMV samples. Furthermore, the ratio of the OD_{max} to OD_{min}, both corrected for scattering, was found to be the same for the reconstituted TMV and TMV.

II. Infectivity tests. By infecting *Nicotiana glutinosa* plants with the reconstituted TMV, it was demonstrated that the reconstituted TMV is infective.

III. Buoyant characteristics. The reconstituted TMV was found to have the same buoyant density as TMV as

[1] Manufactured by the Spinco Division of Beckman Instruments.

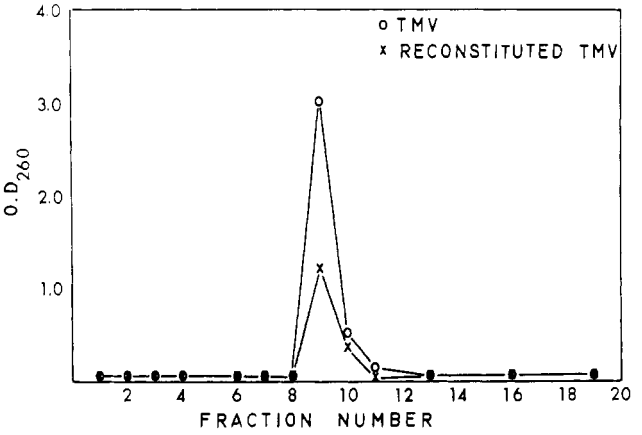

FIGURE 3: CsCl equilibrium centrifugation study of reconstituted TMV and TMV.

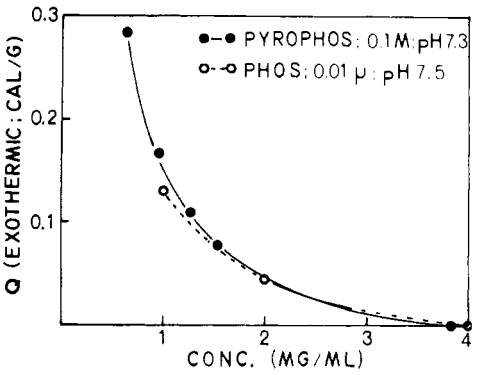

FIGURE 4: Plot of Q *vs.* concentration of TMVP.

shown in Figure 3. The centrifugation was done at 4°, for 21 hr at 23,000 rpm.

IV. ELECTRON MICROSCOPY. Electron micrographs of the reconstituted TMV were obtained using RCA electron microscope EMU-3F employing uranium shadowing. Except for a highly variable length, the reconstituted TMV rods were indistinguishable in appearance from TMV rods.

2. Auxiliary Experiments. I. HEAT OF DILUTION OF TMVP IN 0.1 M PYROPHOSPHATE BUFFER (pH 7.3). Starting with TMVP solution in 0.1 M pyrophosphate buffer at pH 7.3 at an initial concentration of 3.83 mg/ml, the heats arising from dilution were measured calorimetrically. The bicompartmental vessel was used for these experiments. Known volumes of protein and its dialysate were taken in the two compartments of the reaction vessel and the heat arising from dilution of the protein was measured. The final concentration of the protein was calculated. The control vessel contained the buffer solutions to account for heat of mixing. The heats thus obtained from dilution studies are shown in Figure 4.

II. HEAT OF DILUTION OF TMVP IN 0.01 IONIC STRENGTH PHOSPHATE BUFFER (pH 7.5). Dilution data for TMVP solution in 0.01 ionic strength phosphate buffer at pH 7.5 were obtained in a manner similar to that in I above and these are also shown in Figure 4.

III. HEAT OF DILUTION OF POLYMERIZED TMVP. The heat of dilution of TMVP in 0.01 ionic strength phosphate buffer at pH 6.0 was determined exactly as outlined in I above. The dilution of polymerized TMVP in 0.01 ionic strength phosphate buffer at pH 6.0 did not result in any change in heat content.

IV. HEAT ARISING FROM TRANSFERRING TMVP FROM PHOSPHATE BUFFER (pH 7.5, 0.01 IONIC STRENGTH) TO PYROPHOSPHATE BUFFER (pH 7.3, 0.1 M). TMVP in 0.01 ionic strength phosphate buffer at pH 7.5 was mixed with 0.3 M pyrophosphate buffer at pH 7.3. The relative volumes of the two reactants were so chosen that the final solvent of protein was 0.1 M pyrophosphate buffer at pH 7.3. The reaction resulted in considerable endothermic heat. The heat so measured had to be corrected for the heat of dilution of the protein from an initial concentration of 2.5 mg/ml to a final concentration of 1.7 mg/ml. This correction factor was obtained from Figure 4. In this experiment, the control vessel in the calorimeter contained the buffers of the two reactants in the reaction vessel. This automatically corrected for the heat arising from change of the solvent medium from phosphate buffer pH 7.5, 0.01 ionic strength to pyrophosphate buffer pH 7.3, 0.1 M. Thus, the heat obtained for transferring TMVP from phosphate buffer to pyrophosphate buffer, corrected for the dilution of protein, was found to be +0.785 cal/g of TMVP.

V. HEAT ARISING FROM TRANSFERRING TMV RNA FROM PHOSPHATE BUFFER (pH 7.5, 0.01 IONIC STRENGTH) TO PYROPHOSPHATE BUFFER (pH 7.3, 0.1 M). This quantity of heat was obtained by doing an experiment exactly like that in IV above, but using RNA instead of protein. A correction for dilution of RNA during the reaction was unnecessary because it was found by separate experiments that dilution of RNA in the concentration range 0.1–2.5 mg/ml did not result in any change in heat content. The heat obtained for transferring TMV RNA from phosphate buffer to pyrophosphate buffer was −4.9 cal/g of TMV RNA.

VI. HEAT ARISING FROM TRANSFERRING TMV FROM PHOSPHATE BUFFER (pH 7.5, 0.01 IONIC STRENGTH) TO PYROPHOSPHATE BUFFER (pH 7.3, 0.1 M). The experimental procedure for this was exactly the same as that for protein and RNA outlined in IV and V above. The heat of dilution of TMV was

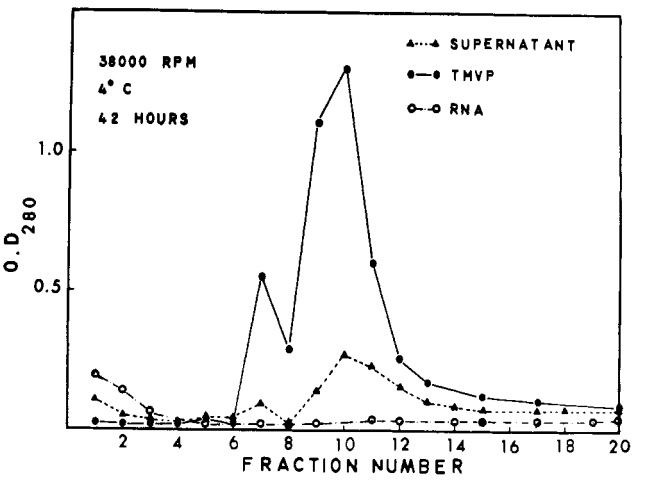

FIGURE 5: CsCl centrifugation study of the supernatant fluid. Included in the figure are also control TMVP and TMV RNA.

TABLE I: Calorimetric Data Relating to Reconstitution Reaction.

RNA Mass (mg) 1	Protein Concn (mg/ml) 2	Protein Mass (mg) 3	Reconstituted Virus (%) 4	ΔQ (mcal) 5	Q (cal/g) 6	Q Cor for Initial Concn of Protein = 2.5 mg/ml (cal/g) 7
0.71	1.05	14.28	69.1	2.914	0.295	0.182
1.31	2.90	26.10	73.0	3.565	0.187	0.198
1.38	2.90	27.55	78.0	4.665	0.217	0.228
1.10	2.32	22.04	72.1	2.632	0.166	0.160
1.43	3.00	28.50	62.0	3.520	0.199	0.212
1.49	3.15	29.93	83.5	4.785	0.192	0.208
						Av 0.198

found to be zero, thus obviating the necessity for dilution correction. The heat resulting from transferring TMV from phosphate buffer to pyrophosphate buffer was found to be zero.

VII. CsCl CENTRIFUGATION STUDIES ON THE SUPERNATANT FLUID. The supernatant fluid remaining after pelleting out the virus from the reaction mixture was subjected to CsCl density gradient studies. Fractions were collected after centrifuging for 42 hr at 4° at 38,000 rpm. The data obtained are shown in Figure 5. The figure also contains data for known amounts of control TMVP and TMV RNA.

The absence of peak in the supernatant fluid corresponding to TMV indicates the absence of reconstituted TMV particles. This was further confirmed by the following procedure. The area under the supernatant fluid curve from fractions 6 to 16 was measured. Similarly, the area under the reference protein curve was measured. From these two areas and a knowledge of the mass of the control protein, the mass of protein in the supernatant fluid was calculated. This corresponded well with the amount of protein that would be expected had there been only protein and RNA in the supernatant fluid and no small reconstituted TMV particles.

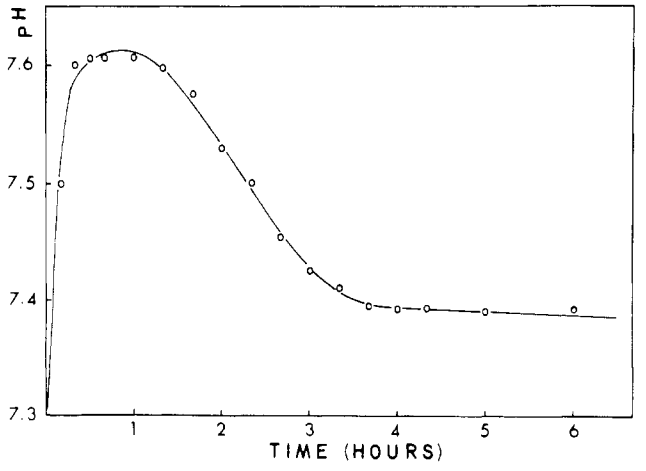

FIGURE 6: Variation of pH with time during reconstitution reaction.

VIII. CHANGE OF pH DURING RECONSTITUTION. These studies were carried out by reconstituting TMV using unbuffered TMVP (pH 7.3, 0.15 M KCl, c = 2.81 mg/ml) and unbuffered TMV RNA (pH 7.3, 0.15 M KCl, c = 0.0855 mg/ml). The pH was measured as a function of time after the two reactants were mixed. The experiment was performed in a nitrogen atmosphere to avoid interference from atmospheric carbon dioxide. The results are shown in Figure 6. The pH is seen to increase in the first hour of the reaction after which it returned to almost the starting pH of 7.3.

3. Calorimetric Study of the Change of Q with pH of TMVP and TMV. I. TMVP IN 0.01 IONIC STRENGTH PHOSPHATE BUFFER. TMVP in 0.01 ionic strength phosphate buffer at pH 7.5 was taken in the reaction vessel and was mixed with phosphate buffer of the same ionic strength but of a lower or higher pH. The control vessel contained the buffers. This automatically corrected for the heat arising from change of pH of the solvent. After the reaction was over, the pH of the contents of the reaction and the control vessels was measured. In most of the cases, these were found to be the same. When they were not, the Q values measured for the protein were corrected. These correction factors were obtained from measured heat values for change of pH of buffer.

While doing these experiments with TMVP, different concentrations ranging from 1.0 to 3.0 mg per ml were used. The Q values obtained experimentally were corrected to what they would have been had the initial concentration of protein been 2.5 mg/ml. The necessary correction factors were obtained from the dashed line of Figure 4. The Q values plotted in Figures 7 and 8 are these corrected values.

II. TMVP IN 0.1 IONIC STRENGTH PHOSPHATE BUFFER. The experimental procedure is exactly like that outlined in I above. Figure 8 shows the heat per gram of protein in 0.1 ionic strength phosphate buffer plotted as a function of pH, in the pH range 5.0–7.5. The figure also contains part of the data obtained with TMVP in 0.01 ionic strength phosphate buffer outlined in I above.

III. TMV IN 0.01 IONIC STRENGTH PHOSPHATE BUFFER. The calorimetric measurements of the heat arising from change of pH of TMV in 0.01 ionic strength phosphate buffer were done in a manner exactly like that of protein outlined in I above. The values obtained per gram of virus were multiplied by 1/0.95. The resulting Q values corre-

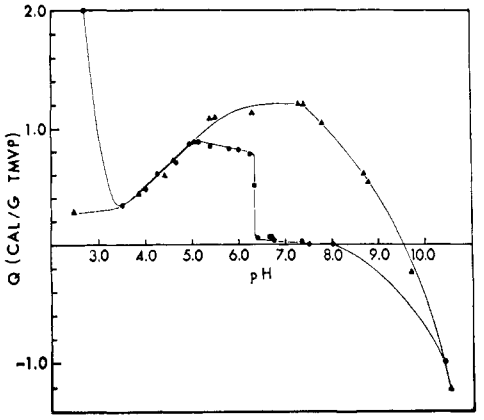

FIGURE 7: Plot of Q (cal/g of TMVP) *vs.* pH: (▲———▲) TMV in 0.01 ionic strength phosphate buffer. (●———●) TMVP in 0.01 ionic strength phosphate buffer.

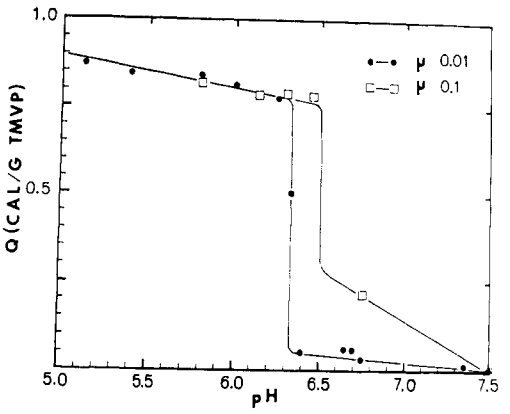

FIGURE 8: Plot of Q (cal/g of TMVP) *vs.* pH: (□———□) TMVP in 0.1 ionic strength phosphate buffer. (●———●) TMVP in 0.01 ionic strength phosphate buffer.

sponded to values per gram of protein in the TMV. Figure 7 contains these converted Q values plotted as a function of pH.

4. Calorimetric Measurements of the Heat of Reconstitution. For measuring this quantity, the "dimple vessel" of the calorimeter was found to be suitable. TMVP and TMV RNA, both in 0.1 M pyrophosphate buffer at pH 7.3, were placed in the ratio of 20:1 by weight in the reaction vessel of the calorimeter. The control vessel contained the buffer. This automatically corrected for the heat of mixing. A thorough mixing of the reactants was ensured all the time. The highest sensitivity setting in the calorimeter was employed. It took about 2–3 hr for the reaction to reach completion. The experiment was repeated and the data relating to these experiments are given in Table I. The heat of reaction per gram of protein, Q, in column 6 of Table I was calculated using

$$Q\ (\text{cal/g}) = \frac{\Delta Q\ (\text{in mcal})}{(\text{fraction reconstituted})(\text{total mass, in mg of TMVP used in the experiment})}$$

As can be seen from column 2 of Table I, the concentration of protein was not the same in all the experiments. The Q values in column 6 were therefore corrected to what they would have been had the initial concentration of protein been 2.5 mg/ml. The necessary correction factors were obtained from Figure 4. The corrected Q values are given in column 7 of Table I.

Discussion

TMV is a rodlike particle with a molecular weight of 40×10^6 (Lauffer, 1938a,b). As shown by X-ray diffraction (Bernal and Fankuchen, 1941), the virus particles are built up of subunits arranged in a regular way. The repeating unit of TMV is 69 Å and the protein subunits are arranged in a left-handed helical array, 16.33/turn, giving a pitch of 23 Å (Klug and Caspar, 1960). According to these measurements each TMV particle contains 2130 protein subunits. A single RNA strand is wound into a helix of the same pitch.

The data of Figures 7 and 8 show that the amount of heat absorbed in the major transition from largely unpolymerized to largely polymerized protein in the region between pH 7.5 and 6.2 is independent of ionic strength. However, at 0.1 ionic strength polymerization occurs at a slightly higher pH value than at 0.01 ionic strength. The data show further that in the pH region between about 5.0 and 6.2, heat is absorbed by protein as pH is lowered. The protein is largely polymerized in this entire range and this heat must therefore be related to structural changes in the polymerized protein. Between pH 6.2 and 5, there is a decrease in Q for TMV of 0.3 cal/g of protein. As will be shown below, this is probably related to hydrogen ion binding. Since TMVP binds roughly the same number of hydrogen ions in the same pH region, the increase of Q amounting to 0.1 cal/g must be the result of an increase of about 0.4 cal/g attributable to structural changes and a decrease of 0.3 cal/g attributable to hydrogen ion binding. Since the Q associated with polymerization when pH is lowered from 7.3 to 6.2 is about 0.8 cal/g, the ratio of heat absorbed in the primary polymerization step to that in the structural change of already polymerized protein is about 2:1. This must be compared with the finding of Jaenicke and Lauffer (1969) that there is a volume increase of 0.00157 ml/g attributable to effects other than electrostriction when TMVP is titrated from pH 6.8 to 5.5, and a volume increase of 0.0049 ml/g associated with the protein when titrated from pH 7.5 to 6.8, the region in which the major polymerization reaction occurs at 0.1 ionic strength, as used by Jaenicke and Lauffer. In this case, the ratio is about 3:1. The interpretation advanced to explain the volume changes was that as pH is lowered, the net negative charge on the TMV-like rod is lowered and this permits subunits to pack more tightly than previously and, therefore, to become more fully isolated from solvent. It is the removal from solvent that produces the volume change. The same explanation applies to the present finding.

This explanation is strengthened by the observation that, as shown by the data of Figure 7 in the pH region between approximately 3.5 and 5.0, the heat changes on polymerized protein are superimposable with those obtained with TMV. As shown by Scheele and Lauffer (1967), the hydrogen ion binding is very nearly the same for virus and

		cal/g
TMVP (phosphate), pH 5.0, 0.01 μ	$\xrightarrow{Q_1}$ TMVP (phosphate), pH 7.5, 0.01 μ	−0.880
TMVP (phosphate), pH 7.5, 0.01 μ	$\xrightarrow{Q_2}$ TMVP (pyrophosphate), pH 7.3, 0.1 M	+0.785
TMV RNA (phosphate), pH 5.0, 0.01 μ	$\xrightarrow{Q_3}$ TMV RNA (phosphate), pH 7.5, 0.01 μ	0
TMV RNA (phosphate), pH 7.5, 0.01 μ	$\xrightarrow{Q_4}$ TMV RNA (pyrophosphate), pH 7.3, 0.1 M	−0.258
TMVP (pyrophosphate), pH 7.3, 0.1 M + TMV RNA (pyrophosphate), pH 7.3, 0.1 M	$\xrightarrow{Q_5}$ TMV (pyrophosphate), pH 7.3, 0.1 M	+0.198
TMV (pyrophosphate), pH 7.3, 0.1 M	$\xrightarrow{Q_6}$ TMV (phosphate), pH 7.5, 0.01 μ	0
TMV (phosphate), pH 7.5, 0.01 μ	$\xrightarrow{Q_7}$ TMV (phosphate), pH 5.0, 0.01 μ	−0.330
TMVP (phosphate), pH 5.0, 0.01 μ + TMV RNA (phosphate), pH 5.0, 0.01 μ	$\xrightarrow{Q}$ TMV (phosphate), pH 5.0, 0.01 μ	−0.485

FIGURE 9: Calculation of the heat of reconstitution.

protein in this region. Tanford *et al.* (1955) found that ΔH for ionization of carboxyl groups when bovine serum albumin is titrated lies between +1500 and +2000 cal per mole. Between pH 3.5 and 7.5, TMV dissociates about 6 hydrogen ions per mole of protein monomer (Scheele and Lauffer, 1967). If ΔH of ionization is +2000 cal/mole, this would be 12,000 cal/mole or +0.69 cal/g of protein as the expected heat absorbed when TMV is titrated from pH 3.5 to 7.5. As shown in Figure 7, the experimental value is about +0.86 cal/g of protein. Thus, it is reasonable to assume that the thermal effect of changing pH with TMV is largely, if not entirely, attributable to ionization of carboxyl groups. As a consequence, it would appear that in the region between pH 3.5 and 5.0, the packing of the protein in polymerized rods is as tight as in TMV, because here the thermal effects coincide, eliminating the possibility of additional effects attributable to packing changes in the protein.

At pH values below 3.5 for protein and above 9 for both protein and virus, there are large thermal effects probably associated with denaturation.

From a thermodynamic point of view, the enthalpy associated with the formation of bonds between TMVP and TMV RNA could be thought of as the difference between the enthalpy for the process of reconstitution and the enthalpy of polymerization of TMVP. Thus, if TMV were reconstituted from polymerized TMVP having structure exactly comparable to that in the virus, then the heat associated with such a reaction would correspond to the heat of bonding between the protein and the RNA.

Q for the hypothetical reaction, TMVP (polymerized) + RNA = TMV in 0.01 ionic strength phosphate buffer at pH 5.0, can be calculated by applying Hess's law. At pH 5.0, as shown above, the protein is probably in the same condition of packing as is found in TMV. The first step is to determine Q per gram for bringing protein from pH 5.0 to 7.3 in 0.01 ionic strength phosphate buffer. This can be read directly from Figure 7. The same must be done for RNA. The Q for this turned out to be zero. Then both must be transferred to 0.1 M pyrophosphate buffer at pH 7.3. The reason is that the experiments of Fraenkel-Conrat and Singer (1959, 1964) show that the most efficient condition for reconstitution is 0.1 M pyrophosphate buffer at pH 7.3. Furthermore, attempts by us to bring about reconstitution in 0.01 ionic strength phosphate buffer were largely unsuccessful. Therefore, we measured the heat associated with transferring TMVP and also TMV RNA from 0.01 ionic strength phosphate buffer at pH 7.5 to 0.1 M pyrophosphate buffer at pH 7.3, then the heat of reconstitution in pyrophosphate buffer, then the heat associated with transferring virus back from 0.1 M pyrophosphate buffer (pH 7.3) to 0.01 ionic strength phosphate buffer (which turned out to be immeasurably small), and finally using the data shown in Figure 7, the heat associated with changing virus to pH 5.0 in 0.01 ionic strength phosphate buffer. All these were calculated to the basis of 1 g of protein. This means that in the case of TMV, heats are recorded on the basis of the amount of protein in the virus rather than on the basis of total weight of virus. Similarly in the case of RNA, heats are calculated on the basis of the amount of RNA which will combine with 1 g of protein in a reconstitution experiment. When all of these calculations are made, as shown in Figure 9, a value of −0.485 cal/g of TMVP is obtained as the heat of bonding between protein and RNA. This corresponds to −8500 cal/mole of TMVP monomer, which in turn corresponds to −2800 cal/mole of phosphate group in the RNA chain. Since Hess's law can be deduced on purely thermodynamic assumptions, this procedure is certainly valid. The only difficulty is that the total error involved in this sequence of measurements is bound to be greater than the error involved in a single direct measurement. We estimate, however, that our final result is probably not in error by as much as 0.1 cal/g.

The enthalpy found in the present study for the interaction between RNA and TMVP of approximately −2800 cal/mole of RNA phosphate is more than adequate to account for the enhanced stability of TMV over that of the polymerized protein. This point is made clear when one considers that when protein alone polymerized in 0.1 ionic strength phosphate buffer at pH 6.5, $\Delta H°$ is +206,000 cal/mole and $\Delta S°$ is +739 eu (Shalaby and Lauffer, 1967). Therefore, at 23° $\Delta F°$ is −12,500 cal/mole. If one considers the polymerizing unit to be the cyclical trimer, this would be associated with nine RNA phosphates in TMV which should contribute $9 \times (-2800)$ or −25,000 cal/mole. Even if partially compensated by $T\Delta S°$, this would enormously increase stability.

The calculations above permit further analysis. If the initial stage of "high-temperature" polymerization of TMVP involves linear polymerization of trimer to form open helices, as proposed by Lauffer *et al.* (1967), each "bond" involves the formation of three new contacts between protein monomers or

three peptide–peptide bonds. $\Delta F°$ for each of these would be $-12{,}500/3$ or -4170 cal/mole. Thus the enthalpy per mole for an RNA–peptide bond, -2800 cal, is of the same order of magnitude as the standard free energy per mole for the peptide–peptide bond. It is interesting to note that Caspar (1963) assumed plausibly that the free energy for the RNA–peptide bond was of the same order of magnitude as that for the peptide–peptide bond, which he estimated on the basis of his model to be -2700 cal/mole at 25–30°. Since our measurements provide no information about the entropy contribution to the free energy of the RNA–peptide bond, we cannot here claim to have validated Caspar's assumption.

It is rather generally thought that the bonding between RNA and protein in TMV is a salt linkage, presumably between negative phosphate and positive amino groups. Our present finding gives at least superficial support to this idea. According to Kauzmann (1959), in proteins, ΔH for salt linkages can be somewhat negative and ΔS can be somewhat less positive than for small ions. However, there are some difficulties with this theory. In the first place there are only two lysine amino groups per protein monomer. If there are in fact three salt linkages per monomer, at least one of them must be with an arginine amino group. Furthermore, Scheele and Lauffer (1967) have shown that between pH 6.5 and pH 1 two hydrogen ions are bound per monomer beyond the 15 hydroxyl groups present. This was interpreted to mean that the two lysine amino groups bind hydrogen ions at pH values far below their normal range, at least below pH 7. This result correlates with the well established inaccessibility of the lysine amino groups to a variety of reagents. Scheele and Lauffer (1969) advanced two theories to explain the titration of lysine amino groups at abnormally low pH values. One of them involved the postulate that in unpolymerized protein each of the two amino groups shares a single hydrogen ion with a phenolic hydroxide group, so that only one hydrogen ion is bound per pair instead of the normal two. Upon polymerization, these hydrogen ion sharing complexes are assumed to be disrupted and each complex then binds a hydrogen ion. If this also happens in copolymerization with RNA, then two sites for salt bonding with RNA would be made available. Figure 6 shows that there is actually an increase in pH when TMVP and TMV RNA are mixed in an unbuffered solution containing 0.15 M KCl. Isolation of the products of the reaction indicated that about 30% of the protein combined with RNA to form TMV in this experiment. This initial behavior, then, supports the idea that hydrogen ions are bound during reconstitution. The subsequent decrease in pH with time is difficult to explain. However, at 0.15 M, potassium chloride solution also exhibited a decrease in pH over the same time period, but of lesser magnitude.

A second difficulty with the idea that the bonding between the RNA and the protein is a salt linkage extends from the general experience that reconstitution is most successful at high values of ionic strength. High ionic strength weakens salt bonds and should, therefore, oppose reconstitution if salt bonds are primarily responsible. This argument is not necessarily conclusive because we have not been able to carry out reconstitution under equilibrium conditions. Therefore, the diminished yield obtained with low ionic strengths does not necessarily mean decrease of bond strength but might be attributable to some other factor.

There is some evidence to support the idea of salt bonds between lysine amino groups and RNA in TMV. The amino groups in TMV also have restricted accessibility to reagents, but this is not true for the lysine amino groups in polymerized protein. However, a difficulty still remains. The electrophoretic mobilities of TMV and polymerized TMVP are indistinguishable below pH 6.5 (Kramer and Wittmann, 1958). If three positive ions per protein monomer are involved in salt bonds in TMV, then the net negative charge on TMV should be 3/protein monomer greater than on polymerized protein. This should practically double the electrophoretic mobility at pH 6.5. One must conclude, therefore, that if, indeed, TMV binds RNA by salt linkages, some additional hypothesis must be found to account for the indistinguishable electrophoretic mobilities of TMV and polymerized TMVP at pH 6.5. Positive ion binding by TMV is a possibility, and there is some experimental support for this, for example, the evidence for hydrogen ion binding during reconstitution as discussed above.

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Structure of *Klebsiella aerogenes* Type 8 Polysaccharide*

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ABSTRACT: The capsular polysaccharide has been prepared from a strain of *Klebsiella aerogenes* type 8. It contained D-glucose, D-galactose, and D-glucuronic acid in the molar proportions 1:2:1. Periodate oxidation destroyed the glucuronic acid but not the neutral sugars. Carboxyl-reduced polysaccharide containing equimolar amounts of glucose and galactose was prepared. The glucose formed from glucuronic acid was identified using sodium borotritide as reductant. Partial acid hydrolysis was used to obtain oligosaccharides from the original polysaccharide, the oxidized polymer, and the carboxyl-reduced polysaccharide. The structures of several of these oligosaccharides have been determined, the largest fragment from any preparation being a tetrasaccharide. On the basis of the oligosaccharide structures and other information, a tetrasaccharide repeating unit is proposed with the structure

$$\begin{array}{l}\alpha\text{-GlcUA} \\ \quad 1\downarrow\uparrow 4 \quad \beta \qquad\quad \alpha \qquad\quad \beta \\ \text{-(}\rightarrow\text{3-Gal-1}\rightarrow\text{3-Gal-1}\rightarrow\text{3-Glc-1}\rightarrow\text{)-}\end{array}$$

Despite improved analytical methods now available, the number of bacterial exopolysaccharides whose structure is known is exceedingly limited. This is particularly true of gram-negative bacteria such as *Klebsiella aerogenes* in which most strains secrete large amounts of capsular or slime material of differing chemotype. Such results as have been published on the structure of exopolysaccharides from *K. aerogenes* types 2 and 54 (Gahan *et al.*, 1967; Conrad *et al.*, 1966) and from various *Escherichia coli* serotypes (*e.g.*, Jann *et al.*, 1965, 1968) indicate that, as in the polysaccharides of gram-positive bacteria, a repeating unit of varying complexity is normally found. This unit may be as simple as the trisaccharide types found in the exopolysaccharides of *E. coli* K30 and K42 (Hungerer *et al.*, 1967; Jann *et al.*, 1965) or it may be rather more complex such as the acetylated and pyruvylated hexasaccharide composed of four different sugars postulated for "colanic acid" in several species of Enterobacteriaceae (Sutherland, 1969; Lawson *et al.*, 1969). The disadvantage of many of the earlier, purely chemical, studies has been that labile noncarbohydrate components such as acetyl or pyruvyl groups have frequently been overlooked. The importance of thorough examination for such substituents can be seen in the increasing number of bacterial exopolysaccharides now known to contain such substituents. These include the capsular polysaccharides of many *Rhizobium* strains and species (Dudman and Heidelberger, 1969) and of *K. aerogenes* types 1 to 6 (Luderitz *et al.*, 1968).

An examination of the literature for a suitable organism to use in biosynthetic studies indicated that *K. aerogenes* type 8 (strain A4) produced a capsular polysaccharide containing three sugars, glucose, glucuronic acid, and galactose, in the approximate molar ratio 1:1:2 (Dudman and Wilkinson, 1956). Thus all the component sugars are readily determinable and the presumed nucleotide sugar precursors are available. However, no data on the structure of this polymer appear to have been published. A reexamination of the polysaccharide and a possible structure are now reported.

Materials

The K. aerogenes type 8 strain A4 was obtained from the departmental collection. It produced an exopolysaccharide capsule as determined by the India Ink technique (Duguid,

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