Hydrogen Exchange as a Probe of Conformation of Tobacco Mosaic Virus and Its Coat Protein*

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ABSTRACT: The kinetics of H–D exchange of TMV and of TMV protein at pD 7 were measured by infrared spectrophotometry. In the virus about 85 hydrogen atoms/protein monomer (containing 291 exchangeable hydrogen atoms) exchange extremely slowly ($T_{1/2}$ = 25,000 min) whereas in the protein about 53 exchange slowly with a rate comparable with that of other native proteins. The exchangeable hydrogen atoms of TMV protein were replaced by tritium during 1-week's exposure to [^{3}H]H_2O at pH 8–9 at 4°, and the exchange-out was studied over a wide pH range. Around pH 4.5 where the protein is predominantly aggregated the exchange rate was minimal; in acid it was faster than at alkaline pH. The extrapolated number of very hard to exchange hydrogen atoms was about 96/monomer between pH 2.6 and 5.

TMV reconstituted from tritiated protein lost the isotope at pH 5.0 at a very slow rate which was of the same order of magnitude as that of the protein aggregated to rods at that pH. Again 85 and 95 residues per monomer were almost nonexchangeable, respectively. It seems that about 5 moles of water/monomer can be bound by the rods made of protein alone. While the gross structure of the protein coat in TMV and TMV protein rods is similar, native and reconstituted virus is less accessible to hydrogen exchange than the protein aggregated in the absence of RNA. When virus or protein rods were exposed to [^{3}H]H_2O for 1 week at pH 5 most of the bound isotope was readily exchanged out, the kinetics resembling those for detergent-denatured protein. It appears that only the surfaces of the rods become labeled under these conditions, and that there the conformation is largely random. In TMV, the kinetics of exchange-in (D_2O) are similar to those of exchange-out ([^{3}H]H_2O). The similarity suggests that the slowly exchanging hydrogen atoms are most likely peptide hydrogen atoms. Sixty per cent of them are very hard to exchange (30% in α helices, the other 30% presumably engaged in subunit binding). Thirty to forty per cent of the peptide bonds are in randomly coiled conformation located near the surfaces of the rod. It appears that no water is bound by TMV and TMV protein particles in a manner to depress its exchange rate noticeably.

Tobacco mosaic virus protein may exist in several reversible states of aggregation. Only under particular conditions does the protein occur in monomeric form partially or completely deformed or unfolded (Anderer *et al.*, 1964). Usually it forms a trimer which, through a number of steps is transformed into more complex aggregates and finally by helical arrangement of the subunits to hollow rods (Caspar, 1963; Lauffer and Stevens, 1968).

Optical rotatory dispersion data revealed the presence of approximately 30% of α helix (Simmons and Blout, 1960). This value has been confirmed by circular dichroism measurements which, moreover, showed that the content of α helix increases in 67% acetic acid to 42% (Schubert and Krafczyk, 1969). Based on information derived from the chemical modifications of amino acid residues, immunochemical properties, and amino acid replacement data, an attempt was made to suggest a specific folding of the polypeptide chain in order to explain exposed and buried regions in TMV protein (Fraenkel-Conrat, 1969).

Yet another approach to understanding the conformation of a polypeptide chain is by hydrogen exchange which in theory demonstrates the presence of rigid structures in a macromolecule. To date, however, this technique has not been applied to reversibly polymerizing systems. Preliminary experiments (Fraenkel-Conrat, 1965) showed that when TMV protein, which had been reversibly denatured in urea or acetic acid, was subjected to renaturation in the presence of tritiated water, it bound about 35 tritium atoms. Furthermore, twice this number of tritium atoms were immobilized in TMV obtained by reconstitution from the protein and RNA in [^{3}H]H_2O containing water, and about 100 tritium atoms were bound firmly in TMV if both renaturation and reconstitution were performed in presence of [^{3}H]H_2O. The great pH dependence of both the polymerization equilibrium and the exchange kinetics suggested an investigation of the relationship between the hydrogen exchange in TMV protein and the concentration of hydrogen ions. Analyses of the exchange reaction of TMV protein at its various stages of aggregation, as well as of native and reconstituted TMV are here reported. Furthermore, the hydrogen exchange in the virus rods has been compared with that of rods composed of protein alone.

Materials and Methods

The wild-type strain of TMV was used in all experiments. TMV protein was obtained from the virus by treatment with 67% acetic acid according to the method of Fraenkel-Conrat (1957). For some experiments acetic acid was removed by dialysis against distilled water and the precipitated protein was dissolved by cautious addition of 1 M NaOH up to pH 9.

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TMV RNA was obtained by the phenol method according to Fraenkel-Conrat *et al.* (1961).

Deuterium oxide (99.90%) was obtained from Bio-Rad Laboratories, Richmond, Calif. Tritiated water (1 Ci/g) was a preparation of New England Nuclear Corp., Boston, Mass. SDS[1] was from Mann Research Laboratories, N. Y. Other chemicals were of analytical reagent grade.

TMV and TMV protein concentrations were determined by ultraviolet spectrophotometry using a Cary Model 15, recording spectrophotometer and absorption coefficients $A_{260}^{1\%}$ 27 and $A_{281}^{1\%}$ 13, respectively.

Tritium radioactivity was measured by mixing 200 μl of sample with 10 ml of Bray's scintillation solution (Bray, 1960) and counting in a Packard Tri-Carb liquid scintillation spectrometer. The radioactivity was measured to a statistical counting error between 1 and 2%.

H–D exchange in TMV and TMV protein was measured by means of infrared spectrophotometry essentially according to the technique of Hvidt (1963). A Perkin-Elmer Model 421 double-beam grating infrared spectrophotometer equipped with calcium fluoride cells of 0.1-mm path length was employed.

To remove water from the samples freeze-drying techniques could not be used since both TMV and TMV protein became denatured and insoluble in water. Water, therefore, was removed and replaced by deuterium oxide using Sephadex G-25 fine, previously dried over P_2O_5 under vacuum and swollen in D_2O. The sample was passed through a 0.9 × 8 cm column and only the leading shoulder and peak of the preparation were collected.

Part of the eluate was introduced into a measurement cell for infrared spectrophotometry. The remainder was used to determine the pH and absorbance. Readings were transformed into pD value by means of the equation pD = pH(app) + 0.4 (Glasoe and Long, 1960).

The rate of H–D exchange was determined by repetitive scanning of the spectral range 1900–1300 cm^{-1}. A scan speed of about 1 cm sec^{-1} was used in the region of the amide I (1650 cm^{-1}) and amide II (1540 cm^{-1}) bands and of about 4 cm sec^{-1} through the remainder of the spectrum. The times indicated in the text and figures refer to the moment at which the maximum of the amide II band was passed. The spectra were recorded directly on a logarithmic absorbancy scale. The zero absorbancy was adjusted without cells in the beams and rechecked with both cells filled with D_2O. All measurements were carried out at a constant room temperature 21 ± 1°. Between scans the cells were kept at 21 ± 1° in a desiccator to minimize the heating effect of the beam of radiation.

Calculation of Hydrogen–Deuterium Exchange. Infrared data were treated under assumption of a linear relationship between the decrease of the amide II band and the number of exchanged peptide hydrogen atoms.

The number of unexchanged peptide hydrogen atoms, n, was calculated in a manner similar to that suggested by Willumsen (1967) from

$$n = \left[\left(\frac{A_{1540}}{A_{1650}}\right)_N - \left(\frac{A_{1540}}{A_{1650}}\right)_D\right]\frac{150}{\Delta R}$$

where A_{1540} and A_{1650} are absorbancies read at the respective wave numbers and the subscripts refer to the sample in native (N) and denatured (D) forms. The denatured sample was obtained from the native protein by heating in 1% SDS at 60° for 18 hr. The difference of absorbancy (ΔR) between the completely denatured sample (D) and the completely protonated (H) (the latter measured as a thin-film cast from water solution on a calcium fluoride plate) was determined from the relationship

$$\Delta R = \left(\frac{A_{1540}}{A_{1650}}\right)_H - \left(\frac{A_{1540}}{A_{1650}}\right)_D$$

This value was found to be 0.60 ± 0.03 for TMV and 0.57 ± 0.05 for TMV protein. Both values are greater than those published for some globular proteins (Blout *et al.*, 1961; Hvidt, 1963; Willumsen, 1966) but comparable with that of cytochrome *c* (Ulmer and Kägi, 1968). The numbers of unexchanged peptide hydrogen atoms were calculated relative to 150 peptide bonds present in TMV protein monomer.

Hydrogen–Tritium Exchange. The exchange of tritium into TMV and TMV protein was usually performed at pH between 8 and 9 using 3 ml of salt-free solution containing 15 mg of the virus or protein and 6 mCi of tritiated water. Solutions were left at 4° for 1 week or more. These preparations will be referred to as [^{3}H]TMV and [^{3}H]TMV protein, respectively.

Reconstitution of labeled virus was performed according to the method of Fraenkel-Conrat and Singer (1959) in 0.1 M sodium pyrophosphate buffer (pH 7.2) at room temperature overnight using [^{3}H]TMV protein. Tritiated water was added to the TMV RNA solution to give a specific radioactivity of 2 mCi/ml prior to the reconstitution.

Labeled protein rods were obtained by addition of 13.8 mg of sodium dihydrogen phosphate dried over P_2O_5 under vacuum to 1 ml of [^{3}H]TMV protein. The pH of this solution was about 4.9 and it was 0.1 M in phosphate.

Denatured labeled TMV protein was prepared from labeled protein rods (see above) by adding 1% of dry SDS and leaving at room temperature overnight.

The sequence of these procedures is shown in Figure 6.

The H–T-exchange experiments conducted for several hours were measured by the two-column-type gel filtration technique of Englander (1963). Generally, Sephadex G-25 fine grade was used.

By applying 0.8 ml of [^{3}H]TMV or [^{3}H]TMV protein (5 mg/ml) to the "large" jacketed column, 2.5 cm in diameter and 5 cm high, the tritiated preparation was rapidly separated from the excess of radioactive water. The effluent containing the first radioactive peak was collected and at different time intervals aliquots of 0.4 ml (about 1 mg/ml) were put on a "small" jacketed column 0.9 cm in diameter and 7 cm high. Three fractions were taken from the peak and their absorbancy and radioactivity were measured.

[1] Abbreviations used in this work are: SDS, sodium dodecyl sulfate; H–D exchange, hydrogen–deuterium exchange; H–T exchange, hydrogen–tritium exchange; [^{3}H]TMV and [^{3}H]TMV protein, virus, and protein, respectively, labeled by incubation with tritiated water at pH between 8 and 9 for a week; exchangeable hydrogen atoms, the term used in this paper indicates collectively all hydrogen atoms in the TMV protein molecule which can be substituted by hydrogen isotope atoms, *i.e.*, peptide hydrogen atoms and side-chain "active" hydrogen atoms (NH_2, >NH, OH, and SH).

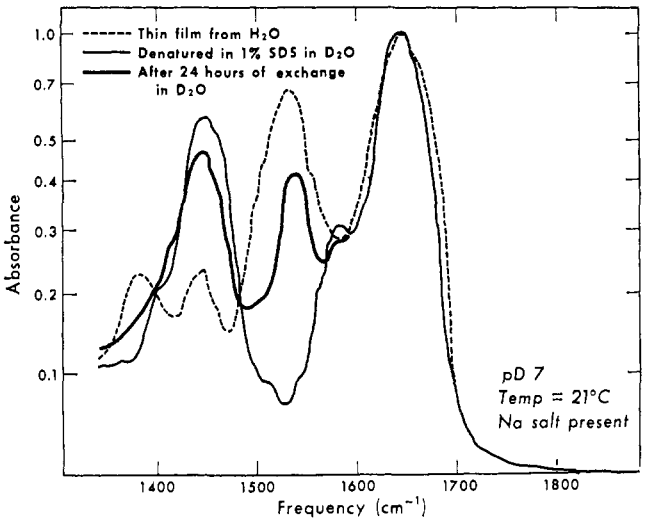

FIGURE 1: Infrared absorption spectra of TMV. The spectra are so adjusted that the absorbance of the amide I band (at 1650 cm^{-1}) amounts to 1.0. The experiments were performed at pD 7, at 21°, at protein concentration between 10 and 20 mg per ml and in the absence of salts. The 24-hr D_2O-exchange curve coincides with the thin-film curve above 1600 cm^{-1}. For experimental details, see Methods.

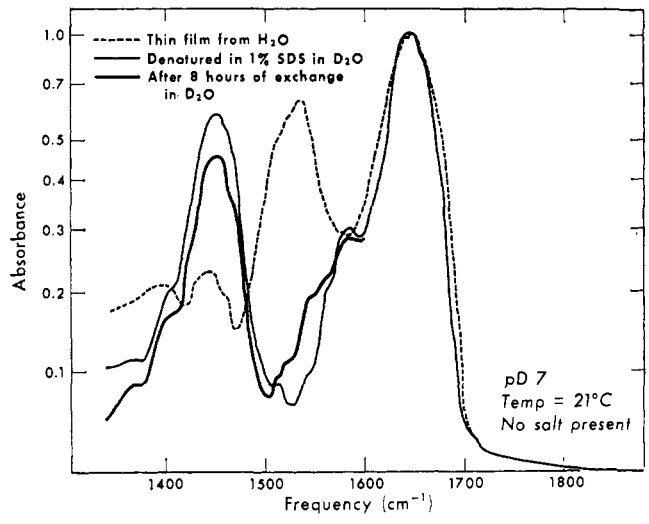

FIGURE 2: Infrared absorption spectra of TMV protein. The adjustment and conditions are the same as given in Figure 1. The 8-hr D_2O-exchange curve coincides with the thin-film curve above 1600 cm^{-1}.

Since TMV protein solutions become opaque at room temperature between pH 4 and 6, 5 μl of 20% SDS solution was added to 250 μl of the sample prior to ultraviolet spectrophotometry.

The exchange experiments performed with the virus and polymerized protein for several days were done using a dialysis device (Englander and Crowe, 1965). Usually 5 ml of the preparation containing 0.3–0.5 mg/ml was placed in a dialysis bag and immersed in an appropriate buffer. At the beginning of each experiment the buffer was changed frequently. At different time intervals 250-μl samples were taken from both the dialysis bag and the buffer, the radioactivity of the latter being considered as background value for the first sample. Absorbancy was measured in the first sample only.

Calculation of Hydrogen–Tritium Exchange. The number of unexchanged hydrogen atoms, n, was calculated in a manner similar to that suggested by Englander (1963). The equation $n = (C/A)((111 \times E)/C_0)$ was used where C and A are radioactivity (in counts per minute) and the absorption of the sample, respectively, and 111 is the gram-atom concentration of hydrogen in water. Molar absorption coefficient amounting to $E_{260} = 49.9 \times 10^3$ for virus and $E_{281} = 22.8 \times 10^3$ for TMV protein were used. The initial radioactivity in the equilibration solution is C_0 (in counts per minute).

Results

Hydrogen–Deuterium Exchange in TMV and TMV Protein. In order to follow the replacement of peptide hydrogen atoms by deuterium atoms, the kinetics of exchange-in was measured, *i.e.*, the exchange of D in solution with H in the peptide. Infrared spectra of TMV and TMV protein at different stages of exchange, recorded over the range 1340–1880 cm^{-1} are shown in Figures 1 and 2. The strong absorption band at 1650 cm^{-1} (amide I band) corresponds to the C=O stretching frequency of the peptide bond (Schellman and Schellman, 1964). During the exchange reaction with either native virus or protein the amide I band does not change in height; after denaturation with SDS, however, a small displacement toward the lower wave numbers occurs. This would suggest to us that some conformational charge has

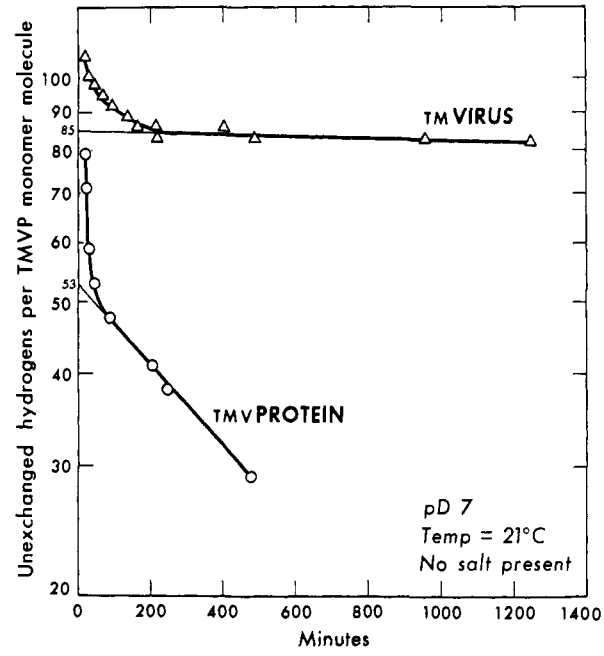

FIGURE 3: Time course of exchange of peptide hydrogen atoms for deuterium atoms in TMV and TMV protein. The experiments were performed at pD 7, 21°, and in the absence of salts. The number of unexchanged hydrogen atoms is plotted on a log scale *vs.* time. The straight-line part of each curve extrapolated to the ordinate indicates the number of peptide hydrogen atoms exchanging with the slowest rate.

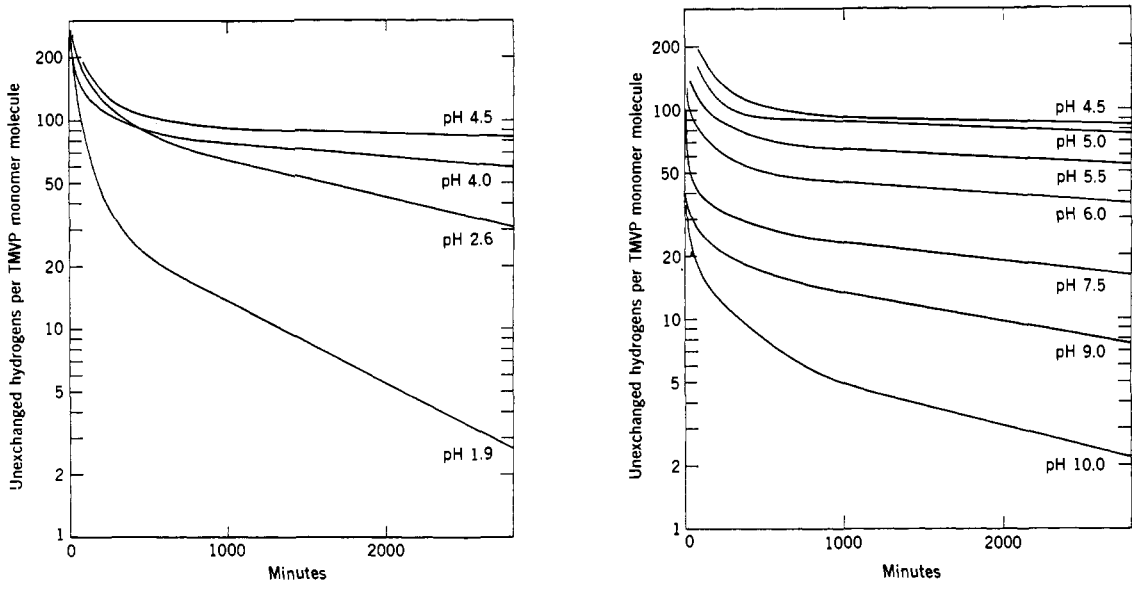

FIGURE 4: Hydrogen–tritium exchange in TMV protein at various pH values. Temperature 4 ± 0.05° was applied to decrease the rate of hydrogen exchange and to minimize aggregation near pH 5. The pH of the protein dissolved in 67% acetic acid, 1.9, was calculated by linear extrapolation of a plot: logarithm of acetic acid concentration *vs.* pH value. (a, left) pH 1.9, 67% acetic acid; pH 2.6, 0.1 M acetic acid; pH 4.0, 0.1 M sodium acetate, pH 4.5, 0.1 M sodium acetate. TMV protein (5 mg/ml) in 67% acetic acid incubated with tritiated water at 4° for 1 week. Final specific radioactivity 2 mCi/ml. The solution (0.8 ml) was applied to the "large" column containing Sephadex G-25 equilibrated with the appropriate concentration of acetic acid. (b, right) pH 4.5, 0.1 M sodium acetate; pH 5.0, 0.1 M sodium acetate, and pH 5.5, 0.1 M sodium acetate. To 0.5 ml of [^{3}H]TMV protein 13.8 mg of $NaH_2PO_4 \cdot H_2O$ was added and left overnight at room temperature. Then 4.5 ml of the appropriate buffer was added and the opaque solution was introduced into a dialysis bag. pH 6.0, 0.1 M sodium phosphate; pH 7.5, 0.1 M sodium phosphate; pH 9.0, 0.1 M sodium carbonate; and pH 10.0, 0.1 M sodium carbonate. [^{3}H]TMV protein (0.8 ml) was applied to the "large" column containing Sephadex G-25 equilibrated with the appropriate buffer.

occurred. Detection of the adjacent small band at 1575 cm^{-1} which is due to the ionization of carboxyl groups of the protein side chains and which overlaps partially the amide II band indicates that a satisfactory level of resolution has been obtained.

The band at 1540 cm^{-1} (amide II band) arises from the coupled C–N vibration and N–H deformation frequency (Miyazawa *et al.*, 1958). Its absorption decreases as the exchange proceeds in D_2O and hydrogen atoms on peptide nitrogens are replaced by deuterium atoms. At the same time, the band at 1450 cm^{-1} increases. This increase is caused by the deuterated peptide groups and the DHO formed during the exchange reaction.

The infrared spectra of TMV and TMV protein are very similar both in the protonated and deuterated forms (Figures 1 and 2). This is not surprising since the virus contains only 5% RNA which contributes negligibly to the infrared spectrum. As would be expected the amide II band disappears after complete deuteration of either preparation.

In the absence of salts, at pD 7 and at 21°, the exchange was found to be much slower in the virus than in the protein alone. After 24-hr reaction with D_2O a strong absorption band at 1540 cm^{-1} was still present in TMV (Figure 1) and 82 hydrogen atoms/TMV protein monomer remained unexchanged. In contrast, in TMV protein only a small absorption corresponding to 29 unexchanged hydrogen atoms persisted around the amide II band after 8-hr reaction with D_2O (Figure 2).

A plot of the logarithm of unexchanged peptide hydrogen atoms *vs.* time is shown in Figure 3. Direct comparison of the two exchange curves reveals a striking difference in rate and number of hydrogen atoms involved. In TMV about one-third of the peptide hydrogen atoms exchanges rapidly. After approximately 200 min the curve becomes a straight line and gives a rate constant of 2.8×10^{-5} min^{-1} (half-life time 25,000 min). Such a slow rate of exchange seems never to have been observed in other proteins studied. Extrapolation from the linear portion of the graph to zero time indicates 85 very hard to exchange hydrogen atoms. In contrast, in TMV protein about two-thirds of the peptide hydrogen atoms exchange fast. After approximately 100 min the curve turns into a straight line and defines a rate constant of 1.3×10^{-3} min^{-1}; extrapolation yields 53 hard to exchange hydrogen atoms. This rate, which can be classified as slow, is still about 50 times faster than the rate of slow exchanging peptide hydrogen atoms in the virus.

pH Dependence of Hydrogen–Tritium Exchange in TMV Protein. Exchange experiments conducted at 10 pH values are shown in Figure 4a,b. The number of hydrogen atoms remaining per TMV protein monomer are plotted on a logarithmic scale *vs.* time. The exchange curves obtained below pH 4.5 (Figure 4a) can be extrapolated to about 300 hydrogen atoms unexchanged at the beginning of the experiment. This value is in accordance with the sum of 150 peptide hydrogen atoms and 141 side-chain-exchangeable hydrogen atoms present in the TMV protein monomer. It also furnishes evidence of the complete tritiation of all classes of exchangeable hydrogen atoms prior to the back-exchange.

The H–T exchange is fast with TMV protein completely disaggregated in 67% acetic acid. The exchange curve displays a straight-line part which indicates the presence of a

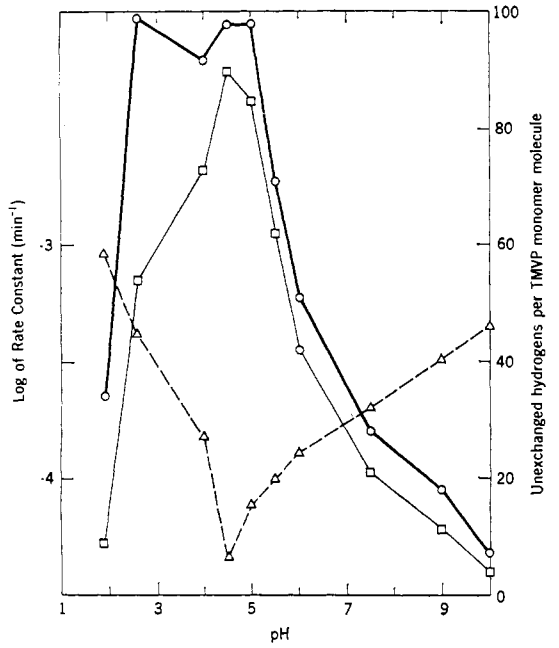

FIGURE 5: Effect of pH on hydrogen–tritium exchange in TMV protein; experimental conditions as in Figure 4. (□) Number of hydrogen atoms unexchanged after 24 hr (right ordinate); (○) number of hydrogen atoms extrapolated to zero time from linear part of the curves shown in Figure 4 (right ordinate). (Δ) Log of rate constant (left ordinate).

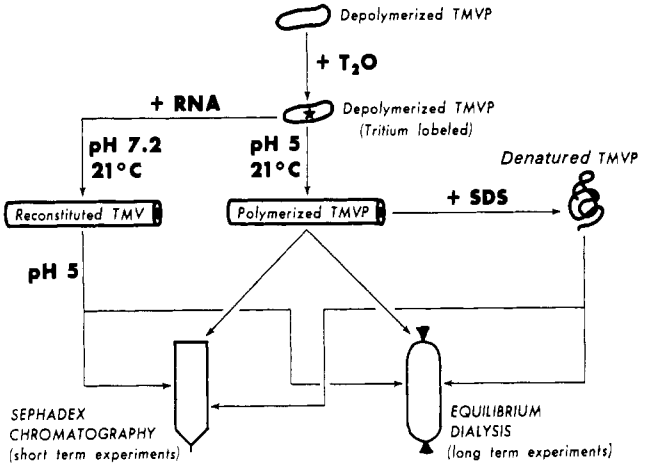

FIGURE 6: Scheme of tritium-labeling and exchange experiments at pH 5. Experimental details are given in Material and Methods. Hydrogen–tritium exchange. Exchange experiments employing either chromatography on Sephadex G-25 or equilibrium dialysis were conducted in 0.1 M sodium phosphate buffer (pH 5.0) at 21°.

group of 34 hydrogen atoms exchanging moderately slow with the rate constant of 9.1×10^{-4} min^{-1} (half-life time 760 min).

The form of the kinetic exchange curves observed at very acidic pH values (Figure 4a) differed markedly from those found in alkali (Figure 4b). For example, many hydrogen atoms did not exchange at pH 1.9 and 2.6 during the first 10 hr. In contrast, at pH 7.5, 9.0, and 10.0 the hydrogen atoms which exchange rapidly dominate the reaction.

In order to evaluate the significance of the H–T-exchange curves obtained at various pH values, three parameters were selected for further analysis: (1) number of hydrogen atoms unexchanged after 24 hr. This time has been chosen arbitrarily to be in the linear part of all kinetic curves; (2) number of hydrogen atoms obtained by extrapolation from the linear part of the curve to zero time. This value defined the group of hydrogen atoms exchanging out of the protein molecule with the lowest first-order rate constant; and (3) the rate constant for the above-mentioned group of hydrogen atoms. These three parameters which pertain only to slowly exchanging atoms are plotted as separate curves in Figure 5. It can be seen that the H–T exchange in TMV protein has a minimum rate of 4.6×10^{-5} min^{-1} (half-life time 15,000 min) (triangles), higher or lower pH increasing this rate. The maximum number of hydrogen atoms unexchanged after 24 hr (squares) also occurs at pH 4.5. Finally between pH 2.6 and 5.0, the number of hydrogen atoms extrapolated to zero time (circles) fluctuate around a value of 96 despite the considerable differences in rate constants within this pH range.

Exchange at pH 5 in the Virus and Protein. The following detailed exchange-out studies were performed with native and reconstituted virus as well as with reaggregated and denatured protein. The H–T experiments were done at pH 5, *i.e.*, in the region where the rate of hydrogen exchange was minimal. The scheme of these experiments is shown in Figure 6. No attempt was made to evaluate H–T exchange out of the RNA moiety. Since the exchange of nucleic acids is so rapid, its contribution to long-term exchange experiments is negligible.

A kinetic analysis of the H–T exchange during the first hour of reaction is presented in Figure 7. It can be seen that the exchange of tritium out of the virus and out of the protein rods proceed in a similar manner. The number of unexchanged hydrogen atoms is, however, somewhat greater in protein than in virus at all times.

TMV protein aggregated in 0.1 M sodium phosphate at pH 5 dissociates in the presence of SDS. This reaction is easy to observe since the opaque protein solution becomes clear within 1 min. In such denatured protein H–T exchange proceeds considerably faster than with either reconstituted virus or with the reaggregated protein.

The H–T exchange continuing over a period of 5 days is shown in Figure 8. The curves in the upper part of the graph indicate that after the release of fast and intermediate exchanging hydrogen atoms, both reconstituted virus and protein rods still retain a large number of very hard to exchange hydrogen atoms. A semilogarithmic plot of the data shows the exchange to be first order during the time period studied. In TMV the number of hydrogen atoms involved in this extremely slow reaction amounts to 85/protein monomer. TMV protein rods exhibit a slightly higher value amounting to 95 hydrogen atoms/monomer. More significant, however, appears the observation that the rate of exchange is three times slower in the virus ($k_3 = 2.3 \times 10^{-5}$ min^{-1}; half-life time 30,000 min) than in the aggregated protein ($k_2 = 6.9 \times 10^{-5}$ min^{-1}; half-life time 10,000 min). On the other hand, when the TMV protein was largely depolymerized by means of 1% SDS at the same pH, the exchange was fast with more than 90% of the peptide hydrogen atoms becoming replaced within a few hours. Strikingly

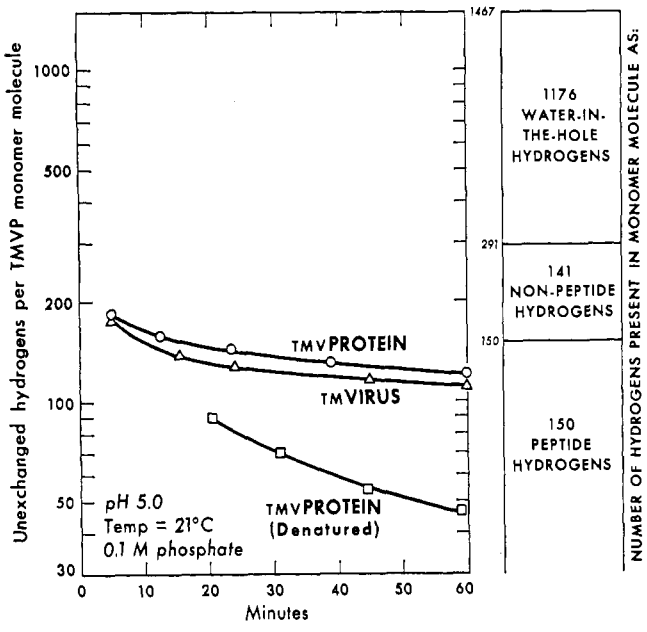

FIGURE 7: Hydrogen–tritium-exchange curves during the first 60 min at pH 5.0, 21°, and in 0.1 M sodium phosphate buffer. The number of unexchanged hydrogen atoms is plotted on a log scale *vs.* time. (○) Polymerized TMV protein, (△) reconstituted TMV, and (□) protein denatured in 1% SDS. On the right side of the graph the number of each type of potentially exchangeable hydrogen atoms per TMV protein monomer is indicated.

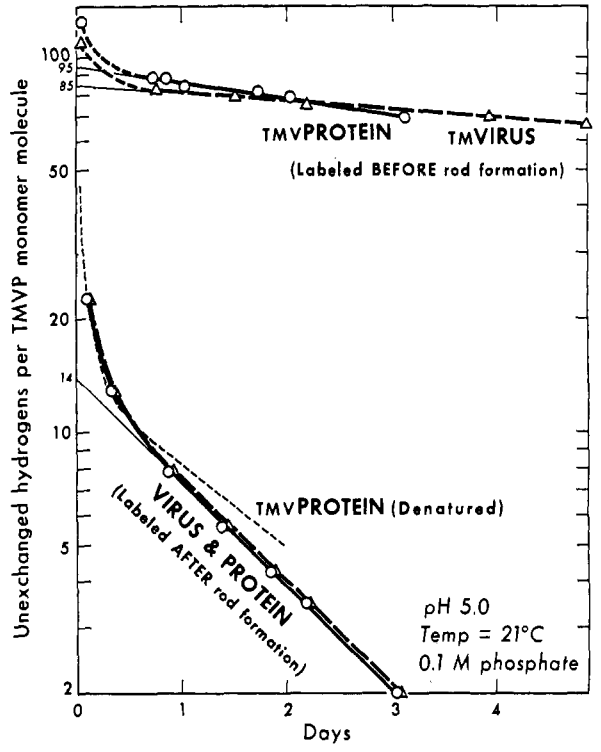

FIGURE 8: Long-term experiments on hydrogen–tritium exchange of TMV (△--△) and TMV protein (○—○). The experiments were performed at pH 5.0, 21°, and in 0.1 M sodium phosphate buffer. The two curves in the upper part of the graph represent reconstituted virus and polymerized protein obtained, respectively, from [^{3}H]TMV protein. In the lower part, the thin dashed line shows the exchange in TMV protein denatured in 1% SDS; the two remaining curves represent the exchange from virus and protein labeled after rod formation. These preparations were obtained by incubation of nonradioactive TMV or polymerized TMV protein with tritiated water at pH 5 in 0.1 M sodium phosphate buffer for 1 week.

similar fast-exchange curves were obtained with TMV and TMV protein which had been labeled after rod formation (*cf.* curves in the lower part of Figure 8). Native TMV was used for these experiments, although virus reconstituted in H_2O (rather than [^{3}H]H_2O) might also have been employed. Because the TMV and TMV protein rods were formed in the absence of tritiated water, penetration of the isotope atoms was from the outside. Tritium atoms were thus prevented from being trapped and rendered immobile within inaccessible parts of the protein coat. Experiments revealed no difference between the exchange in TMV and protein rods under these conditions. Most hydrogen atoms exchanged fast and only 14 hydrogen atoms exchanged with a rate constant of 1.2×10^{-3} min^{-1}.

Discussion

Experiments in D_2O. It has been demonstrated by Schramm and Zillig (1955), that TMV protein in phosphate buffer at neutral pH represents a mixture of polymers with molecular weights up to one million daltons at sufficiently high ionic strength. This polymerization occurs more readily in D_2O than in H_2O, and it is in both solvents similarly dependent on ionic strength (Khalil and Lauffer, 1967).

The infrared spectrophotometric procedure used in the present investigation detects the exchange of the peptide amide hydrogen atoms only. These hydrogen atoms are of particular importance in evaluating molecular conformation since they play a key role in maintaining the backbone of the protein. The observed difference in H–D exchange between native TMV and TMV protein is evident both in the number of very hard to exchange hydrogen atoms and in the rate constants of the reaction (Figure 3). These facts may result from a difference in quaternary structure between the virus and protein under the experimental conditions used (D_2O, pD 7, no salt). With TMV protein, the existence of small oligomers in equilibrium with larger ones facilitates the exchange of a great number of peptide hydrogen atoms. In the case of TMV, however, only limited substitution of hydrogen by deuterium atoms occurs. This suggests that such differences of exchange are probably due to differences in the degree of aggregation of the protein alone under the conditions used and in the virus.

Experiments with [^{3}H]H_2O. It is generally accepted that the pH dependence of the hydrogen-exchange reaction is due to acid and base catalysis. In the case of randomly coiled poly-D,L-alanine, the rate of the exchange has been described, at any given pH and temperature, by equations (Hivdt and Nielsen, 1966; Englander and Poulsen, 1969). The rate of the exchange for this polymer is minimal at pH 3 and increases in the same manner with acid or base catalysis.

In the case of TMV protein (Figures 4 and 5) the rate of H–T exchange is slowest at pH 4.5. Unexpectedly low values for the rate constants are obtained between pH 4 and 6. Since in this pH range the protein exists as large polymers,

the question arises whether such low values are a reflection of the rodlike quaternary structure. Comparison of rate–pH profiles of some model randomly coiled polymers like poly-D,L-alanine, poly-D,L-lysine (Englander and Poulsen, 1969), and polyisopropylacrylamide (Klotz and Mueller, 1969) reveals that near the minimum value the rate constants are somewhat greater than those predicted by theory. With TMV protein, the exact opposite effect is observed. The basis for this anomaly may lie in the fact that during polymerization TMV protein takes up hydrogen ions (Fraenkel-Conrat and Ramachandran, 1959), releases water, and probably undergoes a change in the dielectric constant around carboxyl groups (Lauffer and Stevens, 1968). Polymerization, in effect, transfers TMV protein from water solution to a more hydrophobic environment. This change in local environment could be the cause for the restrained reactivity of hydrogen atoms, thus rendering the rate constants lower than expected.

A somewhat unexpected observation is that the exchange curves obtained below pH 5.0 (Figure 4a,b) indicate that also some side-chain hydrogen atoms exchange at a sufficiently slow rate to be detected during the first 2-hr reaction by the techniques used. It should be noted that side-chain hydrogen atoms exchange fast in many globular proteins. Reasons indicated above pertain also to these hydrogen atoms, presumably limiting their rate of exchange from aggregated TMV protein.

The increased exchange rate in TMV protein both in more acidic and more alkaline solutions than pH 4.5 is probably due primarily to hydrogen and hydroxyl ion catalysis. Hence, the direct comparison of all data obtained at various pH values should permit identification of the probable kinetic mechanism of hydrogen exchange. This may be accomplished by graphical analysis of the exchange data shown in Figure 4, as suggested by Willumsen (1968).[2] Application of the original equation did not give satisfactory results. Experimental points were scattered and data obtained at pH 4.0, 2.6, and 1.9 deviated discontinuously downward a graph. When, however, the minimum rate of exchange in TMV protein was accepted to be at pH 4.5, the points fell remarkable well on a curve (Figure 9). All data obtained between pH 4.0 and 10.0 fit the continuous monotonic function; thus the EX_2 mechanism proposed by Hvidt and Neilsen (1966) suffices to explain the hydrogen exchange of both polymerized and dissociated states of TMV protein. This means that the protein displays high conformational motility as far as exposure of the peptide hydrogen atoms to exchange with solvent water is concerned.

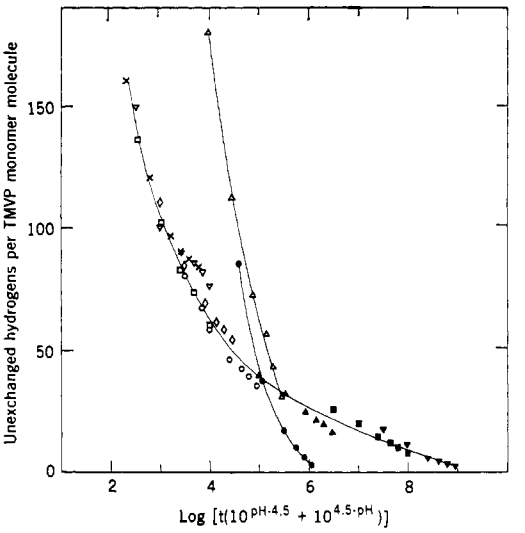

FIGURE 9: Hydrogen–tritium exchange in TMV protein as a function of time and pH. The number of unexchanged hydrogen atoms per TMV protein monomer is plotted *vs.* the logarithm of the expression: $t(10^{pH-4.5} + 10^{4.5-pH})$ *cf.* footnote 2. The data are taken from those shown in Figure 4 at 100, 300, 500, 800, 1400, and 2000 min. Symbols represent measurements at pH: (●) 1.9, (Δ) 2.6, (×) 4.0, (▽) 4.5, (□) 5.0, (◊) 5.5, (○) 6.0, (▲) 7.5, (■) 9.0, and (▼) 10.0.

Experiments performed at pH 1.9 and 2.6 evidently do not fit the curve on Figure 9. On the other hand, the points can be placed along another continuous monotonic function of different shape. The common feature in these experiments is the presence of acetic acid, although at very different concentrations (11 and 0.1 M, respectively). The presumption is that acetic acid may affect the acid-catalyzed acceleration of H–T exchange and/or the conformation of TMV protein.

Let us consider a plot of pH dependence of the rate constant (Figure 5). If a vertical line were drawn through the minimum the limbs of the plot would appear asymetric. The slopes of these limbs indicate that the acid-catalyzed exchange in TMV protein is accelerated by a factor of 1.5 as compared with that catalyzed by alkali.

Spectropolarimetric studies showed that the intrinsic Cotton effects of TMV protein vary little over a pH range 2.0–2.6 and 6.0–10.5 (Jaenicke and Lauffer, 1969; Schubert and Krafczyk, 1969). The most interesting range for protein aggregation between pH 4 and 6, however, has not been tested in detail. Presumably some other altered structural form of the protein appears at acidic pH which is not recognized by the optical techniques. This supposition can be

[2] The overall exchange equation is based on the motility model (Hvidt and Nielsen, 1966)

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} \text{exchange}$$

which considers that any peptide hydrogen atom is either in a totally shielded conformation, N, where no exchange can take place or in an open conformation, I, in which full exposition to solvent water occurs accompanied by exchange of hydrogen atoms with a first-order rate constant, k_3. Depending on the relative magnitude of the rate constants, two limiting cases can be derived referred to as EX_1 and EX_2 mechanisms. EX_1 predominates in proteins with low motility and it is determined when $k_3 \gg k_2$, then $k_m = k_1$. EX_2 was found in proteins of high motility when $k_2 \gg k_1, k_3$, then $k_m = (k_1/k_2)k_3$; in this case k_m is pH dependent even when k_1/k_2 is pH independent. Willumsen (1968) found for α-chymotrypsinogen and α-chymotrypsin that the degree, p, to which the whole molecule contains unexchanged peptide hydrogen atoms was a continuous function of $t(10^{0.3-pD} + 10^{pD-6.3})$, accepting that at pD 3.3 the rate of exchange exhibits a minimum. In the present work the minimum was found at pH 4.5 so that Willumsen's equation has been modified accordingly

$$p = \frac{1}{h}\sum_{m}^{h} e^{\frac{k_{1,m}}{k_{2,m}} c(10^{pH-4.5} + 10^{4.5-pH})_t}$$

where m is any peptide hydrogen atom, h their total number, k_1, m/k_2, m, the transconformational equilibrium constant, C is a pH-independent constant, and t time.

supported by data on the aggregation of TMV protein below pH 3.5, the kinetics of which were found complex. The aggregation was not found to be reversed by a lowering of the temperature, and electron microscopy revealed side-by-side aggregation of the rods (Ansevin and Lauffer, 1963).

TMV Protein in 67% Acetic Acid. It would be of interest to compare the hydrogen exchange of aggregated TMV protein with that of the native protein monomer. Such an approach would give more detailed insight into the number of hydrogen atoms involved in intersubunit binding. The only solvent in which TMV protein occurs as not completely unfolded monomer is concentrated acetic acid.

Anderer *et al.* (1964) demonstrated that TMV protein in 67% acetic acid exists virtually as a monomeric unit with a molecular weight of 17,500 daltons. These authors showed also that the monomer is slightly swollen, considerably elongated, and highly dehydrated as compared with those TMV protein molecules incorporated into the native rod. The content of α helix amounts to 42% (Schubert and Krafczyk, 1969). The H–T-exchange studies in the present work show that in 67% acetic acid there is a pronounced acid catalysis and fast exchange of many hydrogen atoms (Figures 4 and 5). Their exchange, however, is slower than that calculated for poly-D,L-alanine under the same conditions. This would suggest that the randomly coiled conformation does not prevail in the protein dissolved in 67% acetic acid. One has to remember, however, that 67% acetic acid represents instead of a true water solution, a solution of water in acetic acid which may disturb the exchange reaction.

The number of hard to exchange hydrogen atoms extrapolated from the linear part of the exchange curve amounts to 34, or to 23% of the total peptide hydrogen atoms. This value is lower than the reported content of α helix. Graphical analysis of the exchange curve indicates that the next class contains 100 intermediate to exchange hydrogen atoms, or 67% of the total. Hence, TMV protein in 67% acetic acid cannot be used as a reference for the exchange in the polymerized protein. The location of points in Figure 9, particularly those obtained at pH 2.6 and 1.9, may suggest that this plot is more sensitive to detect differences of measured rate constants and changes in catalysis of exchange, than alteration of protein conformation. As a matter of fact, TMV protein has a quite different conformation in 67% than in 0.1 M acetic acid.

Conformation of Virus and Protein. The numbers of unexchanged hydrogen atoms extrapolated to zero time do not vary considerably between pH 2.6 and 5.0 (Figure 5). These 96 hydrogen atoms, about 60% of the peptide hydrogen atoms, correspond well to the numbers extrapolated from data obtained under different experimental conditions: (a) 85 atoms in TMV at pD 7, at 21° in the absence of salt (Figure 3); (b) 85 atoms in TMV and 95 atoms in TMV protein both at pH 5.0, at 21° in 0.1 M acetate (Figure 8). These numbers are also similar to those reported earlier (Fraenkel-Conrat, 1965) for tritium binding of TMV reconstituted in [^{3}H]H_2O from protein renatured in [^{3}H]H_2O. It should be pointed out that these very hard to exchange peptide hydrogen atoms were found only in virus or virus-like protein rods, and are thus associated with the superhelical arrangement of the protein coat.

The form of the H–D- and H–T-exchange curves obtained with TMV was found to be very similar (Figures 3 and 8, respectively). This implies that the hydrogen atoms measured by these techniques are virtually all peptide hydrogen atoms. It should also be pointed out that the same number of extrapolated very hard to exchange hydrogen atoms were found in TMV both at pH 5 and at pD 7. Under these conditions little difference was observed in the rate constants of these hydrogen atoms. Apparently the assembly of the protein with RNA in the virus is so strong that it shields about 60% of the peptide bonds from exchange with solvent water even in spite of hydroxyl ion catalysis.

The results of the H–T-exchange experiments carried out with reconstituted virus and with renatured and reaggregated protein which were both labeled *before* rod formation, do not differ significantly (Figures 7 and 8). Both preparations exchange approximately 60% of their peptide hydrogen atoms with extremely slow rate. We assume this to mean that 60% of the peptide bonds are hidden well inside of the rod wall: half of them located within α helices and half (*i.e.*, 30% of all peptide hydrogen atoms) presumably engaged in intersubunit binding.

The close similarity of the exchange curves of TMV and TMV protein labeled *after* rod formation to that of the denatured protein indicates unequivocally that the surface of both the virus rods and the protein rods is predominantly formed from randomly coiled polypeptide chains. Probably some 30–40% of the peptide bonds are involved in this conformation. It remains to be resolved, whether the 10% peptide hydrogen atoms exchanging moderately slowly (*cf.* curves in the lower part of Figure 8) constitute an additional group displaying some bonding, since they were also observed after detergent treatment of the protein.

The question arises whether there is any difference between the virus and protein rods which can be detected by hydrogen-exchange techniques. While such a difference could exist, it need not necessarily be a proof for changes in the folding of the polypeptide chain. Changes in the hydrogen-exchange kinetics measure primarily the availability of a considerable part of the protein surface. Comparison of the rate constants of very hard to exchange hydrogen atoms in the virus and protein rod reveals about three times lower value in the case of virus. It could be suggested, therefore, that the conformation of the virus rod is somewhat more compact than that of the polymerized protein.

Attachment of H_2O to the Rods. The number of extrapolated very hard to exchange hydrogen atoms is slightly greater in TMV protein rods than in TMV (Figure 8). This does not seem plausible, for the virus would be expected to be of a more rigid and tight conformation than the rod composed only of protein. The data shown in Figure 7 furnish evidence that also in short-term experiments the reaggregated protein rod retains more unexchanged hydrogen atoms than does the reconstituted virus. The difference amounts to approximately 10 hydrogen atoms. It is tempting to speculate that these 10 additional hydrogen atoms originate from water molecules which have been trapped during the polymerization. If this is true, then one could expect that these five water molecules were clathrated in that area where RNA nucleotides can be bound, *i.e.*, in the hypothetical groove.[8]

[8] This is not in contradiction with observations on the release of water upon polymerization of TMV protein (Lauffer and Stevens, 1968). That release of water molecules pertains to the transition of

The authors are fully aware that the presented value is only approximate. As a matter of fact, this result depends on the accuracy of the molar absorption coefficients of TMV and TMV protein used in the calculation.

The experiments shown in Figure 7 furnish indirect evidence that in 0.1 M sodium phosphate at pH 5 and at 21° there is no firm binding of considerable amounts of water within the hole in the reconstituted virus or in the restored protein rod. This hole in TMV is 3000 Å long and of 20-Å radius. In this space a little more than 1.25×10^6 water molecules can be placed. This corresponds to approximately 588 water molecules or to 1176 water hydrogen atoms per protein monomer. The exchange curves do not suggest the presence of bound hydrogen atoms of that magnitude at the beginning of the experiment. This water appears to be motile and probably flows freely along the hole and perhaps also across the wall.

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hydrated monomer to less hydrated polymer. There is no evidence in the present work that water of hydration is not rapidly exchangeable.