Margison, G. P., and O'Connor, P. J. (1973), *Biochim. Biophys. Acta 331,* 349-356.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372-1379.
Masamune, Y., Fleischman, R. A., and Richardson, C. C. (1971), *J. Biol. Chem. 246,* 2680-2691.
Papirmeister, B., Dorsey, J. K., Davison, C. L., and Gross, C. L. (1970), *Fed. Proc., FED Am. Soc. Exp. Biol. 29,* 727.
Prakash, L., and Strauss, B. (1970), *J. Bacteriol. 102,* 760-766.
Rayman, M. P., and Dipple, A. (1973), *Biochemistry 12,* 1202-1207.
Reiter, H., Strauss, B., Robbins, M., and Marone, R. (1967), *J. Bacteriol. 93,* 1056-1062.
Riazuddin, S., and Grossman, L. (1977), *J. Biol. Chem. 252,* 6280-6286.
Schaffner, W., and Weissmann, C. (1973), *Anal. Biochem. 56,* 502-514.
Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112,* 346-362.
Strauss, B. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 1670-1675.
Strauss, B., and Robbins, M. (1968), *Biochim. Biophys. Acta 161,* 68-75.
Strauss, B., Scudiero, D., and Henderson, E. (1975), Molecular Mechanisms for Repair of DNA, Part A, Hanawalt, P. C., and Setlow, R. B., Ed., New York, N.Y., Plenum Press, pp 13-24.
Sun, L., and Singer, B. (1975), *Biochemistry 14,* 1795-1802.
Venitt, S., and Tarmy, E. M. (1972), *Biochim. Biophys. Acta 287,* 38-51.
Verly, W. G., Paquette, Y., and Thibodeau, L. (1973), *Nature (London), New Biol. 244,* 67-69.
Weiss, B. (1976), *J. Biol. Chem. 251,* 1896-1901.
Wood, W. I. (1976), *Anal. Biochem. 73,* 250-257.

Studies of Virus Structure by Laser-Raman Spectroscopy. Turnip Yellow Mosaic Virus and Capsids†

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ABSTRACT: Laser-Raman spectroscopy of the turnip yellow mosaic virus (TYMV) and its capsid indicate the following features of the structure and assembly of the virion. The secondary structure of coat-protein molecules in TYMV is comprised of 9 ± 5% α-helix, 43 ± 6% β-sheet, and 48 ± 6% irregular conformation and is not altered by the removal of the RNA from the capsid. Introduction of as many as 200 chain scissions per RNA molecule also does not affect the overall secondary structure of the encapsulated RNA, which is 77 ± 5% in the A-helix form. Tryptophan and cysteine residues of the coat protein appear to be in contact with the solvent, while only one of three tyrosines per coat protein is available for hydrogen bonding of its *p*-hydroxyl group with H_2O molecules. Both cytosine and adenine residues of TYMV RNA are protonated in substantial numbers near pH 4.5, suggesting elevation of their respective pK_a values within the virion. The Raman data are consistent with chemical evidence favoring interaction between protonated bases of RNA and amino acid side chains of coat protein in TYMV.

TYMV[1] is composed of 180 identical coat-protein molecules which reside in an icosahedral shell or capsid. The capsid contains one molecule of RNA which appears to be in contact with the protein. The RNA is rich in cytosine bases (22.4% A, 17.2% G, 38.3% C, and 22.1% U) and tends to slowly degrade in the isolated virion. RNA-free capsids can be produced by treatment of TYMV with alkali (Kaper, 1975).

The coat-protein molecule contains 189 amino acid residues in known sequence (Kaper, 1975). Empirical methods predict a secondary structure which contains 16% α-helix, 41% β-sheet, and 43% irregular structure (Turano et al., 1976). The distribution of amino acid residues along the chain partially segregates into hydrophobic and hydrophilic regions. The stability of the capsid and protein structure has been analyzed in terms of hydrophobic interactions and specific interactions involving the 13 acidic residues (Glu plus Asp), the 10 basic residues (Lys plus Arg), and the 4 Cys, 3 Tyr, and 2 Trp residues. The stabilizing interactions in the virion may include hydrogen bonding between the OH groups of Tyr and $-CO_2^-$ groups of Glu or Asp and, at lower pH values, specific hydrogen bonds between $-CO_2H$ and protonated cytosine residues (Kaper, 1975).

The Raman spectrum of a virus contains bands or "lines" due to light scattered in transitions involving the vibrations of molecular subgroups. The frequencies and intensities of these lines are influenced by the secondary structure of the macromolecule, hydrogen-bonding interactions, and the environment of the molecular subgroups in question. Therefore, the Raman spectrum potentially contains considerable information about structure and interactions (Thomas et al., 1976; Hartman et

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[1] Abbreviations used are: TYMV, turnip yellow mosaic virus; rRNA, ribonucleic acid from ribosomes; $\Delta\sigma$, spectral slit width.

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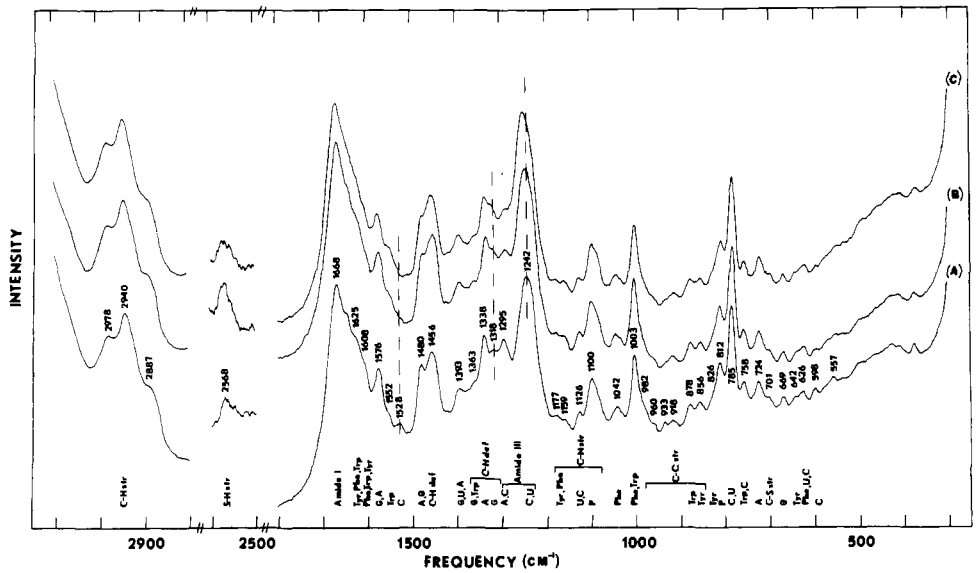

FIGURE 1: Raman spectra at 32 °C of TYMV in 0.3 M KCl solutions at (A) pH 7.5, 62 μg/μL; (B) pH 4.8, 75 μg/μL; and (C) pH 4.1, 71 μg/μL. Conditions: excitation wavelength, 488.0 nm; slit width, 10 cm^{-1}; scan speed, 25 cm^{-1}/min; rise time, 10 s; amplification, $A = 1$ (300–1800 cm^{-1}), $A = 3$ (2500–2600 cm^{-1}), and $A = \frac{1}{3}$ (2800–3100 cm^{-1}). Broken vertical lines show frequency shifts. Abbreviations: Standard one-letter symbols are used for the RNA bases and three-letter symbols for the amino acids. Also, P = phosphate, str = stretching, def = deformation, and C-S, C-C, C-N, C-H, and S-H denote their respective functional groups.

al., 1973). In particular the ionization of a conjugate acid (e.g., $-CO_2H$) or the protonation of a conjugate base (e.g., $-CO_2^-$) may make major changes in the spectrum which allow the measurement of spectroscopic titration curves for one kind of subgroup in the virus. Such titration measurements are far more informative than the measurement of the total uptake of protons measured in potentiometric titrations. A clear example of the effectiveness of this method is the recent study of the protonation of polycytidylic acid by Raman spectroscopy (Chou & Thomas, 1977) which is particularly relevant since the RNA of TYMV contains nearly 40% cytosine residues.

In a previous publication, Raman spectroscopy was used to study the structure and interactions of TYMV (Turano et al., 1976), but the TYMV samples contained highly degraded RNA. Spectra of the capsid were not presented, and the influence of pH was not observed. Here we present Raman spectra of RNA-free capsids and of TYMV containing RNA which is much less degraded than previously. The spectrum of rRNA is also presented to show the changes involved in protonation of adenine and cytosine residues (spectra of TYMV RNA could not be obtained for this purpose). Information is presented concerning the protonation of adenine and cytosine residues, the environment of Tyr, Trp, and Cys residues, and the secondary structure of the coat-protein molecules in the capsid.

Materials and Methods

The materials and methods used in this work have been described in previous publications (Turano et al., 1976; Thomas et al., 1976). Stock solutions of TYMV and capsid were prepared at the Agricultural Research Center, Beltsville, Md., by methods previously described (Kaper, 1975).

To obtain virus or capsid at a given pH value, a portion of the stock solution was diluted to 5 mL with H_2O, titrated with KOH or HCl to the desired pH, pelleted in a Beckman SW 50.1 rotor,[2] and dissolved in 50 μL of H_2O to obtain a final concentration of ca. 50 mg/mL. The buffering capacity of the virus or capsid was more than adequate to buffer the final solution, as was checked by occasionally diluting the final solution and measuring the pH.

Ribosomal RNA was extracted from *Escherichia coli* and purified by standard methods (Hartman et al., 1970), titrated to the desired pH, precipitated with ethanol, dried, and dissolved in a small volume of H_2O.

Results and Conclusions

Integrity of Samples

The integrity of the virus and capsid used in this study was checked by sucrose gradient centrifugation both before and after exposure to the laser beam. Narrow bands were obtained in these sedimentation profiles which were at the correct positions for virus and capsid. Spectra of virus and capsid were also recorded in the ultraviolet region and were appropriate for the respective samples.

RNA extracted from virus which had been exposed to the laser was examined by polyacrylamide gel electrophoresis. The electrophoresis profiles showed one sharp peak at the migration distance expected for high-molecular-weight RNA (Hartman et al., 1970) which contained about 95% of the ultraviolet absorbance on the gel. The remaining 5% was contained in a region on the low-molecular-weight side of the main band. Heating the RNA revealed the existence of many hidden breaks. The RNA is, however, much less degraded than that used in previous work (Turano et al., 1976).

Raman Spectra

Raman spectra of TYMV in H_2O containing 0.3 M KCl were recorded at pH 7.5, 4.8, and 4.1. Representative spectra are shown in Figure 1. Assignments of the prominent lines to specific groups of RNA and capsid have been made previously (Turano et al., 1976) and are given in Figure 1. Interpretation of specific lines will be given below. Spectra of TYMV were also recorded as a function of time of exposure to D_2O to allow measurement of the rate of exchange of cysteinyl SH to SD.

Spectra of RNA-free capsids in H_2O solution (0.3 M KCl)

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

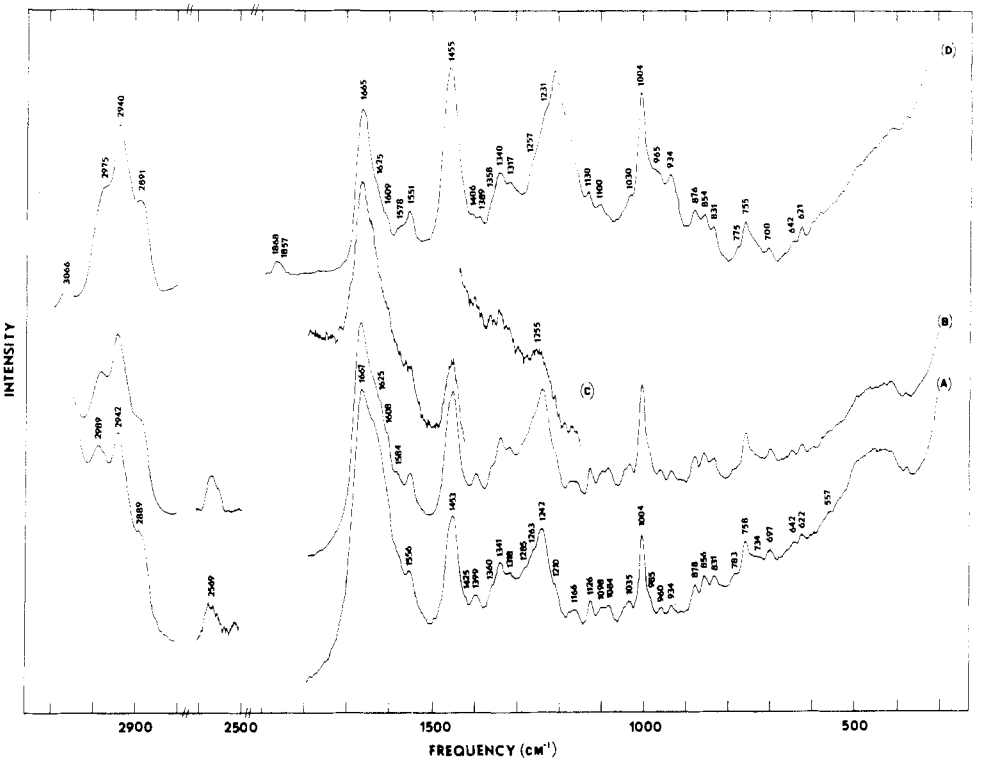

FIGURE 2: Raman spectra at 32 °C of TYMV capsids in H_2O solutions (0.3 M KCl) at (A) pH 7.5, 35 $\mu g/\mu L$; (B) pH 4.8, 76 $\mu g/\mu L$; and (C) pH 7.5 (the 1150–1800-cm^{-1} region only), precipitated from solution by denaturation at 70 °C; and in D_2O solutions (0.3 M KCl) at (D) pD 7.8, 68 $\mu g/\mu L$. Other conditions are as in Figure 1.

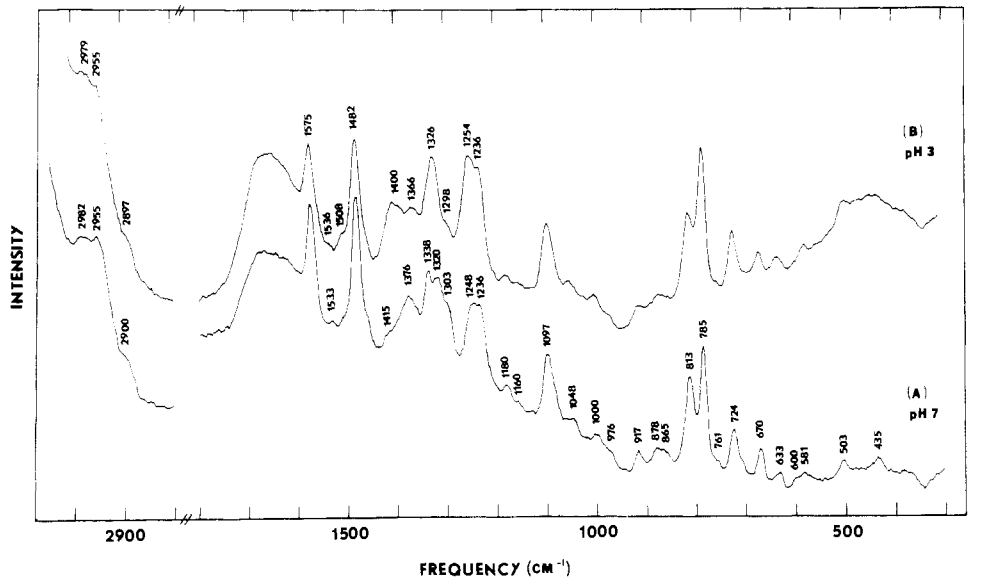

FIGURE 3: Raman spectra at 32 °C of rRNA from *E. coli* in H_2O solutions at (A) pH 7 and (B) pH 3. Other conditions as in Figure 1, with the exception that $A = 1$ (300–1800 and 2800–3100 cm^{-1}).

were recorded at temperatures of 0 to 50 °C at pH 7.5. Spectra recorded at 32 °C, for samples of TYMV which had previously been heated to 70 °C, differed in the amide III region when compared with spectra of unheated TYMV (Figure 2A,C). Spectra of capsids in H_2O solution (0.3 M KCl) at pH 4.8 and in D_2O solution (0.3 M KCl) at pD 7.8 were also recorded (Figure 2B,D).

Raman spectra of H_2O solutions of rRNA at neutral and low pH are shown in Figure 3. These are used in the discussion which follows.

Interpretation of the Spectra

Secondary Structure of the Coat-Protein Molecules. The secondary structure of the coat-protein molecules in the capsid is estimated from the positions and intensities of the amide I, amide I′, and amide III Raman lines (Lippert et al., 1976). The spectra indicate that the protein chains contain 9 ± 5% α-helix, 43 ± 6% β-sheet, and 48 ± 6% irregular structure. The cited limits of error reflect uncertainties in the Raman line intensities and do not include errors inherent in the method of Lippert et

al. (1976), which we estimate to be within ±10%. This is in excellent agreement with the predicted secondary structure (Turano et al., 1976). As temperature is increased above 50 °C, Raman scattering in the amide III region becomes weak and broad with a band maximum near 1255 cm^{-1} (Figure 2C). This indicates that much of the β-sheet structure is lost above 50 °C. At lower temperatures, at both pH 7.5 and 4.8, no change in secondary structure is observed.

Since the amide III region is obscured by Raman lines due to cytosine and uracil residues of TYMV RNA, the secondary structure of the coat-protein molecules in the virion must be estimated mainly from the amide I line which remains at the same frequency as for capsids and does not change vs. pH at room temperature. We conclude that the presence of RNA in the capsid makes little or no change in the secondary structure of the coat-protein molecules.

Environment of the Tyrosine Residues. Tyrosine residues in proteins give rise to Raman lines near 855 and 825 cm^{-1} which have been correlated with the environment and state of hydrogen bonding of the OH group of Tyr. If these OH groups are strongly bound to negative acceptors (e.g., $-CO_2^-$), R_{Tyr} ($= I_{855}/I_{825}$) is near 0.3. If these OH groups form moderately strong hydrogen bonds to H_2O, R_{Tyr} is about 1.25. If these OH groups accept a strong hydrogen bond, R_{Tyr} is near 2.5 (Siamwiza et al., 1975). In a globular protein, different Tyr groups could exist in any of the above situations so that the actual value of R_{Tyr} may reflect the average environment of several groups.

The value of R_{Tyr} for TYMV capsids (see Figure 2) is found to be 1.25 ± 0.1 at pH 7.5 and 4.8. We conclude that either (a) all three Tyr residues form moderate hydrogen bonds to the solvent water (i.e., are exposed to solvent H_2O molecules) or (b) one Tyr forms a strong hydrogen bond to a $-CO_2^-$ group, a second Tyr forms a moderate hydrogen bond to H_2O, and a third Tyr accepts a strong hydrogen bond from a positive donor group such as the $-NH_3^+$ group of a Lys residue. These alternatives will be further discussed below.

The measurement of the true value of R_{Tyr} for the virus (Figure 1) is made difficult by the following factor. The relatively strong line near 815 cm^{-1} (from the RNA backbone) overlaps the 825 cm^{-1} line from Tyr and the position of the latter line tends to shift with varying environment. (Note that this line is near 832 cm^{-1} in the capsid.) It is clear, however, that R_{Tyr} for the virus is much larger than reported previously by Turano et al. (1976), since scattering near 815 cm^{-1} was not compensated. We estimate that the true value of R_{Tyr} for TYMV should be near 1.2. This suggests that the inclusion of RNA in the capsid does not greatly change the environment of the Tyr residues.

Environment of Tryptophan Residues. The Trp residue gives rise to a number of strong lines in the spectrum of proteins (Yu et al., 1975; Chen et al., 1974). A strong line near 760 cm^{-1} indicates the presence of Trp and is seen in the spectra of capsid and virus. A line from Trp near 1360 cm^{-1} is sensitive to the environment of the indole ring and becomes sharp and relatively strong if the indole residue is surrounded by other hydrophobic groups (Chen et al., 1974). In the spectra of both capsid and virus no sharp line appeared when we examined the 1360-cm^{-1} region at high resolution ($\Delta\sigma = 5$ cm^{-1}). Therefore, we confirm the conclusion of Turano et al. (1976) that the two Trp residues per coat-protein molecule are exposed to solvent.

Location of SH Groups. The interaction of the SH groups of the Cys residues of TYMV with various reagents has received considerable attention (Kaper, 1975). We previously attempted to estimate the extent to which these groups were in contact with solvent D_2O molecules by examining the rate of deuterium exchange of SH groups (Turano et al., 1976). Refinement of the earlier experiments indicates that SH to SD exchange is complete for all four Cys residues within 1 h after exposure to D_2O at 32 °C. The exchange is temperature dependent, since little or no exchange occurs at 0 °C after several hours in D_2O. We conclude that at physiological temperatures all SH groups are in contact with solvent water molecules.

Protonation of Carboxyl Groups. In previous work, the Raman spectrum of TYMV was obtained only at pH 7 (Turano et al., 1976). We have now examined the spectra of both virus and capsid at lower pH values as well as the spectra of β-lactoglobulin at neutral and low pH (Frushour & Koenig, 1975). The spectra of the capsid (Figure 2) show no significant change in intensity of the line near 1400 cm^{-1} between pH 7.5 and 4.8. The spectra of β-lactoglobulin exhibit a similar line which remains unchanged as pH is lowered to 3.5. Since β-lactoglobulin contains about 50 carboxyls per molecule, the above data suggest that the 1400-cm^{-1} line is not due only to $-CO_2^-$ groups, many of which should protonate by pH 3.5. Raman scattering of $-CO_2^-$ groups expected near 1400 cm^{-1} is probably very weak in the present case and cannot be used to accurately measure protonation of $-CO_2^-$ in TYMV.

Protonation might also be detected by infrared spectroscopy, which permits measurement of the absorption band at 1710 cm^{-1} due to the C=O stretching vibration of $-CO_2H$ groups (Hartman & McDonald-Ordzie, in preparation). We are now applying this method to TYMV.

Protonation of Cytosine Residues. One proposed mechanism for the stabilization of the TYMV structure at pH less than 6 is specific hydrogen-bond formation between protonated cytosine residues and $-CO_2H$ groups (Kaper, 1975). Since protonated cytosine residues give a Raman spectrum which differs significantly from that of cytosine, we looked for these changes in the spectra of rRNA (Figure 3) and TYMV (Figure 1).

As cytosine residues in RNA become protonated, we expect to observe major changes in the Raman spectra of H_2O solutions (Chou & Thomas, 1977; Lord & Thomas, 1967). These include losses in intensity (I) of the strong lines near 1245, 1295 and 1525 cm^{-1} from neutral cytosines and increases of I of lines near 1255 and 1545 cm^{-1} from protonated cytosines. Since complete protonation will not be achieved for rRNA or TYMV and since uridine residues contribute a strong line near 1230 cm^{-1}, significant protonation will appear as a slight decrease of I near 1525 cm^{-1}, and an increase of I near 1550, a marked decrease of I near 1295 cm^{-1}, and an increase of I near 1245 cm^{-1} (accompanied by a shift to higher frequency). These changes are clearly seen for rRNA and to a lesser extent for TYMV in Figure 4, where the 1150–1600-cm^{-1} region is redrawn over a flat baseline. Spectra of TYMV were normalized to the intensity of the line at 1003 cm^{-1} (Phe + Trp) and spectra of rRNA to the intensity of the line at 1097 cm^{-1} (PO_2^-). Recalling that TYMV is about 60% protein (which contributes considerably to I in the 1200- to 1300-cm^{-1} region as seen in Figures 1 and 2), the above intensity changes are understandably less dramatic in TYMV than in rRNA. Note also that the strong line near 780 cm^{-1} from cytosine and uridine residues is not greatly affected by the protonation (Lord and Thomas, 1967).

Since the Raman lines mentioned above may be further complicated by the phenomenon of Raman hypochromism or hyperchromism (Small & Peticolas, 1971; Lafleur et al., 1972), quantitative estimates of protonation have not been attempted from these data. We may conclude, however, that by pH 4.8 a considerable fraction of the cytosine residues in

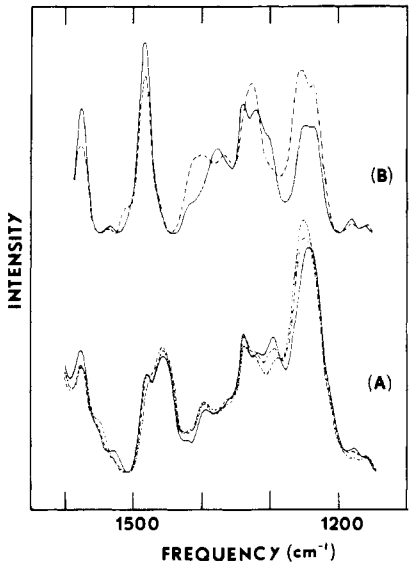

FIGURE 4: Redrawn Raman spectra at 32 °C of (A) TYMV (1150–1600 cm^{-1}) in 0.3 M KCl solutions at pH 7.5 (—), pH 4.8 (- - -), and pH 4.1 (- · -) and of (B) rRNA from *E. coli* (1150–1600 cm^{-1}) in H_2O solutions at pH 7 (—) and pH 3 (- - -). Note: No major changes occur below 1200 cm^{-1} in spectra of TYMV at various pHs, whereas several significant changes occur below 1200 cm^{-1} in spectra of rRNA. See Figure 3.

TYMV are protonated. This fraction increases as pH is lowered to 4.1. Since the normal pK_a of cytidine is 4.2, we can estimate that about one-half of all cytosine residues should be protonated at pH 4.1. This is consistent with the observed moderate increase in I at 1250 cm^{-1} which is less than would be expected if all residues were protonated (Lord & Thomas, 1967).

Protonation of Adenine Residues. Adenine residues with a normal pK_a = 3.7 may also protonate at lower pH values. However, unless the pK_a is increased by the molecular environment or by a specific interaction with an adjacent group, fewer adenines than cytosines will be protonated at a given pH. The observation of protonated adenine is also rendered more difficult by spectral interference from other bases as well as amino acid residues. The most useful lines are those near 1480 and 1575 cm^{-1} which are much stronger for neutral than for protonated adenine (Figures 3 and 4B). The spectra of TYMV (Figures 1 and 4A) also show an intensity decrease in both of these lines as pH decreases. We conclude that protonation of adenine occurs by pH 4.8 and is present in significant quantity by pH 4.1.

Secondary Structure of RNA. Two prominent Raman lines arise from the phosphodiester linkages of RNA. The weaker of these, near 1100 cm^{-1}, is insensitive to changes in the conformation of the RNA chain. The stronger line, near 815 cm^{-1}, arises from the -COPOC- linkages, and the intensity of this line depends on conformation. The ratio of the intensities of these lines R_p (= I_{815}/I_{1100}) has been used to measure the fraction of phosphodiester linkages in an ordered configuration, which is believed to be of the DNA A-helix type (Lafleur et al., 1972; Erfurth et al., 1972; Brown et al., 1972; Thomas & Hartman, 1973).

In the previous Raman study (Turano et al., 1976), the TYMV contained highly degraded RNA and the quantity R_P was measured from the spectra uncorrected for scattering due to the capsid. In the present work, the TYMV contains relatively nondegraded RNA and the same uncorrected value of R_p (i.e., 1.0) is obtained. We conclude that the fraction of phosphodiester linkages in ordered configurations is largely unchanged by the introduction of 100 to 200 chain scissions in each RNA molecule.

The available spectra of TYMV capsids now allow further a correction to I_{1100} for interfering Raman scattering from protein subgroups. This is accomplished by using the intensity of the protein line at 1004 cm^{-1} as an internal standard. The corrected value of R_p is 1.26 which implies that about 77% of the phosphodiester linkages are in an ordered state. This is in much closer agreement with values found for other RNA species but is still significantly lower than expected for a protein-free RNA molecule in aqueous solution. Further discussion of this point is given elsewhere (Turano et al., 1976; Thomas & Hartman, 1973).

Discussion

From Raman spectra, we have deduced two alternative possible bonding schemes for the Tyr OH groups in TYMV capsid. We now mention other data which will eliminate one of these possibilities.

Ionization of the Tyr OH group has been measured by ultraviolet absorption spectroscopy. Results show that one Tyr is completely inaccessible in nondegraded capsids and the remaining two residues have pK_a values perturbed to higher than normal values (Re & Kaper, 1975). It is also known that one Tyr reacts rapidly with tetranitromethane after which the capsid degrades (Re & Kaper, 1975). These data suggest that at least one Tyr OH group is strongly bonded to either $-CO_2^-$ or $-NH_3^+$ and is not accessible to solvent water. Therefore, the alternative stated under "Environment of the Tyrosine Residue", that one OH group is strongly hydrogen bonded to a $-CO_2^-$ group, a second OH group is strongly bonded to an $-NH_3^+$ group, and a third OH group is in contact with solvent water, is favored by chemical studies. The pK_a of the third OH group could still be increased if this Tyr side group resided in a hydrophobic cleft of the coat-protein molecule. That the inaccessible Tyr OH is bonded to $-NH_3^+$ of Lys is reinforced by the fact that two Lys groups (those at positions 142 and 152) do not react with nitrobenzenesulfonic acid (R. Teter & H. Duranton, private communication).

On the basis of the nonreactivity of Trp residues 75 and 95 with *N*-bromosuccinimide, it has been proposed that the middle third of the coat-protein molecule is not on the surface of the molecule (Kaper, 1975). On the other hand, our interpretation of the Raman spectra of both TYMV and its capsid shows the indole side groups to be on a surface that is accessible to solvent H_2O molecules. Therefore, hydrophobic regions may contain fissures or clefts which will admit H_2O but which will sterically exclude larger reagents such as *N*-bromosuccinimide.

Our present results confirm the previous conclusion that the SH groups of all four Cys residues in TYMV exchange rapidly with D_2O at 32 °C (Turano et al., 1976). Again this result is only partially in agreement with the results of studies of the chemical reactivity of SH groups with mercurials in which one SH group reacts rapidly with *p*-mercuribenzoate (*p*MB) after which the capsid degrades (Kaper, 1975). Apparently, D_2O molecules can reach the SH groups, whereas the larger *p*MB molecule is excluded. The existence of a path for solvent penetration is confirmed by the fact that small aliphatic mercurials react with TYMV more rapidly than *p*MB and produce rapid degradation of the virion (Kaper, 1975).

Previous studies have shown that the stability of the TYMV structure is increased when the pH of the solution is lowered from 7 to below 6 (Kaper, 1975). The stabilizing interactions have been shown to involve RNA and protein and it has been

postulated that a specific interaction may occur by hydrogen bonding between $-CO_2H$ groups of the protein and protonated cytosine residues of the RNA (Kaper, 1975). Raman spectra of TYMV clearly show that a significant number of cytosine residues are protonated at pH 4.8 and more become protonated at pH 4.1. Since the pK_a of cytosine is 4.2, without perturbing interactions we would expect only 20% of the cytosine residues to be protonated at pH 4.8. The very significant changes seen in the spectra (Figure 4) suggest a larger fraction of protonated cytosine which requires the existence of a significant number of perturbed cytosine residues with pK_a values higher than 4.2.

This conclusion is in complete harmony with the proposed interaction but, of course, does not prove that such specific interactions (which would increase the normal pK_a values for both cytosine and $-CO_2H$) indeed take place. We have not been able to observe the protonation of $-CO_2^-$ groups at pH 4.8 and 4.1 with Raman spectra, although such protonation has been observed in tobacco mosaic virus by infrared spectroscopy (Hartman & McDonald-Ordzie, in preparation). We are currently studying the infrared spectra of TYMV in order to identify possible $-CO_2H$ groups.

Although no specific interactions have been postulated for protonated adenine residues of TYMV RNA, our data suggest that protonated adenine exists also at pH 4.8. Since this is 1.1 pH units above the normal pK_a for adenine residues (at which less than one in ten should be protonated), we conclude that normal pK_a values of adenine have been perturbed to substantially higher values in TYMV. This suggests that specific interactions involving protonated adenine residues may also exist in TYMV.

References

Brown, K. G., Kiser, E. J., and Peticolas, W. L. (1972), *Biopolymers 11,* 1855–1869.

Chen, M. C., Lord, R. C., and Mendelsohn, R. (1974), *J. Am. Chem. Soc. 96,* 3038–3042.

Chou, C. H., and Thomas, G. J., Jr. (1977), *Biopolymers 16,* 765–790.

Erfurth, S. C., Kiser, E. J., and Peticolas, W. L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 938–941.

Frushour, B. G., and Koenig, J. L. (1975), Advanced Infrared Raman Spectroscopy, Clark, R. J. H., and Hester, R. E., Ed., London, Heydon and Son, Ltd.

Hartman, K. A., Amaya, J., and Schachter, E. (1970), *Science 170,* 171–173.

Hartman, K. A., Lord, R. C., and Thomas, G. J., Jr. (1973), *Phys. Chem. Prop. Nucleic Acids 2,* 1–89.

Kaper, J. M. (1975), The Chemical Basis of Virus Structure, Dissociation & Reassembly, New York, N.Y., North Holland Publishing Co., American Elsevier Publishing Co.

Lafleur, L., Rice, J., and Thomas, G. J., Jr. (1972), *Biopolymers 11,* 2423–2437.

Lippert, J. L., Tyminski, D., and Desmeulles, P. J. (1976), *J. Am. Chem. Soc. 98,* 7075–7080.

Lord, R. C., and Thomas, G. J., Jr. (1967), *Spectrochim. Acta, Part A, 23,* 2551–2591.

Re, G. G., and Kaper, J. M. (1975), *Biochemistry 14,* 4492–4497.

Siamwiza, M. N., Lord, R. C., Chen, M. C., Takamatsu, T., Harada, I., Matsuura, H., and Shimanouchi, T. (1975), *Biochemistry 14,* 4870–4876.

Small, W. E., and Peticolas, W. L. (1971), *Biopolymers 10,* 69–88.

Thomas, G. J., Jr., and Hartman, K. A. (1973), *Biochim. Biophys. Acta, 312,* 311–322.

Thomas, G. J., Jr., Prescott, B., McDonald-Ordzie, P. E., and Hartman, K. A. (1976), *J. Mol. Biol. 102,* 103–124.

Turano, T. A., Hartman, K. A., and Thomas, G. J., Jr. (1976), *J. Phys. Chem. 80,* 1157–1163.

Yu, N.-T., Lin, T.-S., and Tu, A. T. (1975), *J. Biol. Chem. 250,* 1782–1785.