Nucleic Acid–Protein Interactions in Turnip Yellow Mosaic Virus

Abstract. *Mild alkaline treatment induces the formation of a specific RNA complex inside turnip yellow mosaic virus. Dialysis at* pH *4.5 destroys this complex inside the capsid, but does not destroy complexes that have been removed from their capsids. These facts were interpreted in terms of a breakage and subsequent re-formation of RNA-protein links. In combination with supporting potentiometric data, they suggest histidinyl-phosphate salt links or carboxylate-amino hydrogen bonds, or both, as the principal means of association of turnip yellow mosaic virus protein and RNA.*

In contrast to the fairly detailed knowledge of the gross geometric disposition of the ribonucleic acid (RNA) and protein constituents in small isometric viruses, virtually nothing is known about the mutual interactions of these components [for a review, see (*1*)]. In order to gain some understanding of these interactions, turnip yellow mosaic virus (TYMV) has been studied in the past by means of the following approaches. (i) Through a reaction with the organic mercurial *p*-mercuribenzoate, TYMV was converted into a form in which the physical stability of the virus was determined solely by the existing RNA-protein interactions (*2*). Dissociation of this derivative was brought about by upward titration to the *p*H range of 6 to 7. This indicated a possible participation of relatively low *pK* proton-donating groups in the RNA-protein association. (ii) An equally important but separate development has been the controlled degradation studies of TYMV, which effected the dissociation of the virus into empty capsids and RNA (*3*, *4*). Here, the alkaline degradation method (*3*, *5*) has proved the most useful, because it was recently realized that it would permit an analysis of the dissociation event without actually having to release the RNA of all the particles into the reaction environment. Thus, if alkali treatment of TYMV is limited to moderate *p*H values (*p*H 10.85 or lower), the principal events taking place are a limited *in situ* fragmentation of the RNA followed, probably almost simultaneously, but an aggregation or complexing of these fragments (*5*, *6*). The RNA from alkali-treated particles is then obtained, by standard isolation methods (*7*, *8*), as an apparently homogeneous complex whose most striking characteristic is the considerably higher sedimentation rate as compared with that of native, untreated TYMV-RNA. Hence, according to the dissociation properties of the mercurial-TYMV derivatives mentioned earlier (*2*), complexing of the RNA fragments inside the viral capsid at mildly alkaline *p*H could be interpreted as a rupture of RNA-protein interactions followed by an internal rearrangement of the RNA and the formation of secondary RNA-RNA links between the fragments.

These studies have now been developed further, and in this report it will be shown that at *p*H 4.5 it is impossible to obtain an RNA complex from alkali-treated TYMV (TY-A). This is interpreted to mean that under these conditions of *p*H RNA-protein links are restored, possibly at the expense of the RNA-RNA interactions of the complex, which by themselves were proved to be insusceptible to *p*H 4.5. From these experiments, and a separate titration study of the virus (*8*), conclusions are drawn regarding the specific nature of the RNA-protein interactions in TYMV.

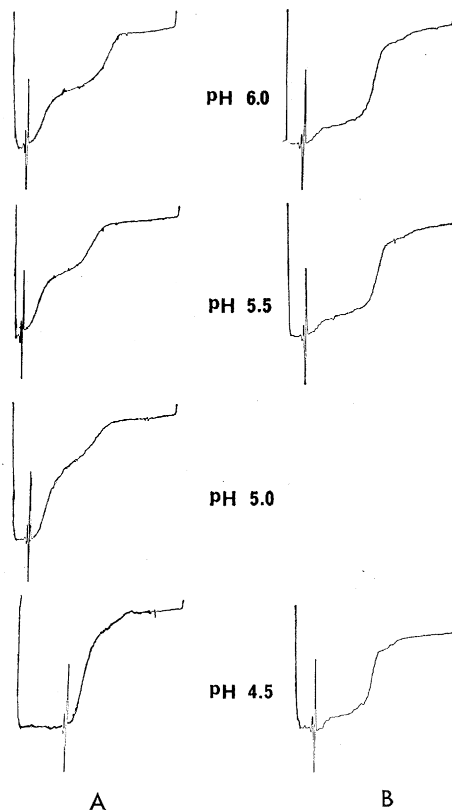

Fig. 1. Densitometer tracing of sedimentation patterns of RNA from turnip yellow mosaic virus, registered with ultraviolet optics. Sedimentation from left to right. All exposures shown were taken approximately 15 minutes after centrifuge had attained a speed of 59,780 rev/min; temperature of run, 5°C. (A) RNA complexes were isolated at *p*H specified, ultracentrifuged in 0.1*M* Na acetate of *p*H 6.0. (B) RNA complexes were isolated at *p*H 6.0 from purified alkali-treated TYMV, ultracentrifuged in 0.1 ionic strength buffers of *p*H specified.

Turnip yellow mosaic virus was isolated according to Dunn and Hitchborn (*9*) from fresh leaves of Chinese cabbage plants, infected 21 days, that were grown in a light room at 20°C. Alkali treatments of TYMV were in 1.0*M* KCl at *p*H 10.85 and 30°C for 8 minutes in a *p*H-stat (*3*). Neutralized reaction mixtures were dialyzed overnight at 4°C against buffers of specified *p*H's. They were subsequently extracted with buffer-saturated phenol in order to examine the TY-A particles in the reaction mixture for the presence of the RNA complex. On occasion, TY-A was first isolated from the reaction mixtures by differential ultracentrifugation prior to phenol extraction. This removed the RNA released into the reaction mixture during alkali treatment, and thus gave a better estimate of RNA complexing. Preparations of RNA were analyzed by means of analytical ultracentrifugation with ultraviolet optics. The presence of the RNA complex could be spotted immediately from the observation of a fast-sedimenting boundary with a sedimentation coefficient exceeding that of the native TYMV-RNA, and from the fact that it could be dissociated into its constituent fragments by a short heat pulse of 5 minutes at 65° to 70°C (*5*).

In a typical experiment, TYMV was treated with alkali under standard conditions, as described above; and portions of the reaction mixture were dialyzed in 0.1*M* Na acetate buffers of *p*H 6.0, 5.5, 5.0, and 4.5, respectively. After phenol extraction of the dialyzed reaction mixtures, the RNA samples were diluted for ultracentrifugation in 0.1*M* Na acetate buffer of *p*H 6.0. Figure 1A shows the series of ultracentrifuge patterns obtained. Each of the sedimentation patterns, except the one derived from RNA isolated from TY-A dialyzed at *p*H 4.5, exhibited a fast-sedimenting component, the sedimentation coefficients of which are

listed in Table 1. It is, therefore, evident that when TY-A (which does contain the complex) is brought back to *p*H 4.5, the possibility of isolating this complex is lost. This can be interpreted in two ways: Either the forces between the RNA fragments are broken as a consequence of the low *p*H per se, or the balance of forces between protein and RNA on the one hand and RNA fragments on the other is shifted at the latter's expense, inducing the RNA fragments to reassociate with the protein capsid, and at *p*H 4.5 these forces could be stronger than the interfragment bonds. In the latter case, breakage of the interfragment bonds could have taken place *in situ* simultaneously with the renewed RNA-protein association, or both sets of interactions could have been broken upon disruption of the capsid structure with phenol. The possibility that the complex is destroyed because of this realignment of competing intercomponent interactions, rather than plain breakage of RNA-RNA interactions due to *p*H 4.5 conditions alone, is favored by a separate experiment in which a study was made of the *p*H-dependence of the sedimentation behavior of the RNA complex and of a control sample of native TYMV-RNA. The RNA complex was prepared from isolated TY-A particles, derived from TYMV that was treated with alkali under standard conditions and dialyzed at *p*H 6.0, while the control RNA came from TYMV treated identically, except for omission of the alkaline conditions. The relevant results of this experiment are presented in Fig. 1B and Table 1. At *p*H 4.5 there is a fast-sedimenting major component, in contrast to the RNA that was extracted from TY-A dialyzed at *p*H 4.5. When the *p*H goes down from 9.0 to 4.5, there is a tendency for the RNA complex to sediment faster and to form faster-sedimenting aspecific aggregates, but no dissociation into its constituent fragments is apparent. The slight increase in sedimentation rate with decreasing *p*H is also seen in the native RNA used as control and can probably be attributed to shrinkage of the particle as a result of increased intraparticle interactions (*10*). In the sedimentation analysis of the RNA complexes isolated at different *p*H's, analytical ultracentrifugation of all the samples was performed in a single solvent (0.1*M* Na acetate, *p*H 6.0). Thus, it was possible to judge the "rigidity" of the complex from its sedimentation rate without interference from the "natural" *p*H-dependence of TYMV-RNA sedimentation shown above. The data in Table 1 show that as the *p*H of TY-A dialysis and RNA extraction was lowered, the sedimentation coefficients decreased. Probably the balance of forces inside the virus gradually shifted in favor of the RNA-protein association, resulting in an increasingly "loose" structure of the complex, ending eventually in its destruction at *p*H 4.5.

Table 1. Sedimentation coefficients of RNA isolated from turnip yellow mosaic virus. Except for the native RNA-control in the last column, all RNA preparations were obtained from TYMV that was first treated with alkali and then dialyzed at lower *p*H. The *p*H column indicates *p*H of RNA extraction in the first $s_{20,w}$ column and the *p*H of ultracentrifugation solvent in the second $s_{20,w}$ column. Ionic strength of ultracentrifugation solvents was 0.1.

*p*H	$s_{20,w}$ in Svedberg units		
	RNA complex isolated at *p*H given, ultracentrifuged at *p*H 6.0	RNA complex isolated at *p*H 6.0, ultracentrifuged at *p*H given	Control: native RNA isolated at *p*H 6.0, ultracentrifuged at *p*H given
9.0		38.9	31.6
7.2		39.4	32.2
6.0	38.3	40.5	33.6
5.5	35.8	42.1	33.6
5.0	31.4		
4.5	No complex	45.2	37.5

The above *p*H-induced internal rearrangements of the RNA fragments point to some form of electrostatic interaction between TYMV-RNA and its protein capsid. However, it is unlikely that salt links between negative RNA phosphate groups and positive amino acid residues on the protein are the primary mode of interaction, as they are with tobacco mosaic virus (*11*). This is in view of the above-mentioned dissociation of the mercurial-TYMV derivative at *p*H 6 to 7 (*2*), and the dissociation of TYMV into intact RNA and empty capsids with 8*M* urea or 12*M* formamide (*4*), or by heating at 60°C and *p*H 6, as has been done recently in this laboratory. The above conclusions are amplified by potentiometric titrations of TYMV and its components (*8*), which have revealed the presence of buried proton-donating groups, titrating from *p*H 3.8 to 6.0. These groups might be directly involved in the RNA-protein association.

In the *p*H range of 6.0 to 3.8, and one or two *p*H units further downward (*12*), the following residues could qualify for prototropic activity—on the protein: histidinyl- with a *pK* of about 6, and glutamyl- /aspartyl- with *pK*'s around 4.5 (*13*); on the RNA: cytidine-phosphate- with amino-*pK* of 4.2, adenosine-phosphate- with amino-*pK* of 3.7, and guanosine-phosphate- with amino-*pK* of 2.4 (*14*). Histidinyl residues could interact via a classic salt link with the negative phosphate residues of the RNA. This would explain most of the dissociation behavior of the virus at both high and neutral *p*H's and high ionic strength, as well as the dissociation of the mercurial-TYMV derivative between *p*H 6 and 7. It would also account for the results of the present study, except that a slightly lower histidinyl-*pK* would have to be assumed.

However, it seems more likely that the proton-donating protein carboxyl groups as well as nucleotide amino groups are involved in an interaction, which at the same time functions as the principal means of association of the two components. Pairs of juxtaposed protein carboxylate and nucleotide amino groups of similar *pK* could each bind one proton stronger than would normally be expected on the basis of the *pK*'s of the participating groups individually. This type of "hydrogen-bonding" was also proposed for juxtaposed carboxylate-pairs to explain the abnormal proton binding in tobacco mosaic virus protein (*11*). Like the carboxylate-pairs of tobacco mosaic virus protein, the carboxylate-amino pairs in TYMV would not yield their hydrogen-bonded proton until a *p*H around neutrality was reached. This is in full agreement with the dissociation behavior of the mercurial derivative of TYMV mentioned above. On the other hand, re-formation of this "hydrogen-bond" cannot be expected until a hydrogen-ion concentration is reached sufficient to reassociate protons with half of the carboxylate-amino pairs. This is at the highest *pK* of either of the groups participating in the interaction; hence,

at *p*H 4.5. The experimental results shown in Fig. 1 and Table 1 are in agreement with this proposition, since this is also the approximate *p*H at which it becomes impossible to isolate the RNA-complex previously formed inside TYMV at alkaline *p*H. Hence it is tempting to see the alkaline treatment of TYMV and its subsequent reversion to *p*H 4.5 as a model of the dissociation and reassociation of the RNA and protein components of this virus. At first there would be a breakage of carboxylate-amino links due to removal of protons at a *p*H exceeding neutrality and a subsequent formation of bonds between RNA fragments leading to complex formation, after which, at *p*H 4.5, the linkage with protein is restored at the expense of the interfragment interactions and leading to the destruction of the complex.

J. M. KAPER

Plant Virology Laboratory, Crops Research Division, Agricultural Research Service, Beltsville, Maryland 20705

References and Notes

1. J. M. Kaper, in *Molecular Basis of Virology*, H. F. Fraenkel-Conrat, Ed. (Reinhold, New York, 1968), p. 1.
2. —— and F. G. Jenifer, *Biochemistry* **6**, 440 (1967). A mercurial derivative of TYMV can be prepared by reacting *p*-mercuribenzoate with the capsid sulfhydryl groups at *p*H 4.6. A similar reaction with the capsids alone leads to their disintegration. Hence, the stability and physical existence of the mercurial-substituted virus particle is determined solely by the RNA-protein interactions.
3. J. M. Kaper, *Nature* **186**, 219 (1960); *J. Mol. Biol.* **2**, 425 (1960); *Biochemistry* **3**, 486 (1964); W. Longley and R. Leberman, *J. Mol. Biol.* **19**, 223 (1966).
4. G. Jonard, D. Ralijaona, L. Hirth, *C. R. Hebd. Seances Acad. Sci. Paris* **264D**, 2694 (1967); J. P. Bouley and L. Hirth, *ibid.* **266D**, 430 (1967).
5. J. M. Kaper and J. E. Halperin, *Biochemistry* **4**, 2434 (1967); L. Bosch, E. M. Bonnet-Smits, J. van Duin, *Virology* **31**, 453 (1967).
6. The formation, quality, and subsequent detection of the specific RNA-complex is dependent, to some extent, on the method of virus isolation, the *p*H of the alkali treatment, and the method of RNA isolation. For example, virus isolated from infected tissue frozen for storage (as contrasted to fresh storage) yields less or no complex at all upon alkali treatment, for reasons as yet not understood. Treatment at higher *p*H will give better *in situ* fragmentation, but less of and a slower-sedimenting complex. Finally, RNA isolation by methods milder than phenol extraction (*7*) does yield an RNA complex from alkali-treated virus from frozen tissue, although the quality is inferior.
7. A. Gierer and G. Schramm, *Z. Naturforsch.* **11b**, 138 (1956).
8. J. M. Kaper and R. Alting Siberg, *Virology* **38**, 407 (1969). Comparative potentiometric titrations of mercurial-substituted TYMV, TYMV, and freeze-degraded TYMV in the *p*H range of 3.8 to 10.5 have exposed the presence of proton-donating groups, titrating from *p*H 3.8 to 6.0, which are not normally apparent in the titration of the intact virus. The titration of TYMV protein capsids, freeze-degraded capsids, and TYMV-RNA by themselves suggests, on the other hand, that these groups are not buried in the tertiary and quaternary structure of these components individually. Hence the conclusion that these groups, whose location could be on the protein as well as on the RNA, are participating in the integration or are hidden because of the integration of the viral components into the complete virion.
9. D. B. Dunn and J. H. Hitchborn, *Virology* **25**, 171 (1965).
10. S. Mitra and P. Kaesberg, *J. Mol. Biol.* **14**, 558 (1965).
11. D. L. D. Caspar, *Advan. Protein Chem.* **18**, 37 (1963).
12. Although no titrations were performed below *p*H 3.8 because of difficulties with irreversible precipitation of the virus, it has been experimentally determined that there are buried proton-donating groups with *pK*'s below 3.8 (*2*).
13. C. Tanford, *Advan. Protein Chem.* **17**, 69 (1962).
14. C. Scholtissek, *Protoplasmatologia* **5**, No. 3a (1966).
15. Supported in part by PHS grant AI-04322. R. Alting Siberg assisted in some of this work.

16 July 1969

Keratohyalin: Extraction and in vitro Aggregation

Abstract. *Keratohyalin can be extracted from epidermis by 1.0 molar phosphate buffer* (*p*H *7.0*), *as demonstrated histochemically and ultrastructurally. If the extracted material is dialyzed against water, it aggregates to form granules that are similar to keratohyalin granules in size, shape, histochemical staining, and electron opacity.*

Several lines of evidence suggest that keratohyalin is a structural protein or protein complex of the epidermis (*1*). This unique substance gradually accumulates in cells just beneath the outermost layer (stratum corneum) of the skin as discrete cytoplasmic granules without a limiting membrane (*2*). As cells containing keratohyalin granules mature and enter the stratum corneum, these discrete granules lose their identity, but little is known about the eventual function of keratohyalin (*3*). Furthermore, keratohyalin granules have not been isolated in sufficient quantity to permit direct physical and chemical studies (*4*).

Studies of nonepidermal tissues have demonstrated that relatively insoluble proteins and protein complexes (such as collagen and myosin) can be partially solubilized by salt solutions or buffers and then aggregated by manipulating the ionic strength of the extracting solution (*5*). Such aggregates may resemble the protein or protein complex in the natural state both morphologically and biologically; study of these aggregates has resulted in considerable data on structure and function.

Using previous extraction and aggregation studies as a model, I have developed a technique (extraction by phosphate buffer and aggregation by dialysis against water) for obtaining granules from the epidermis, which are morphologically and histochemically similar to keratohyalin granules.

Cattle-hoof epidermis was employed in these studies because of the large amount of keratohyalin present. Fresh tissue (1 g, wet weight) obtained from

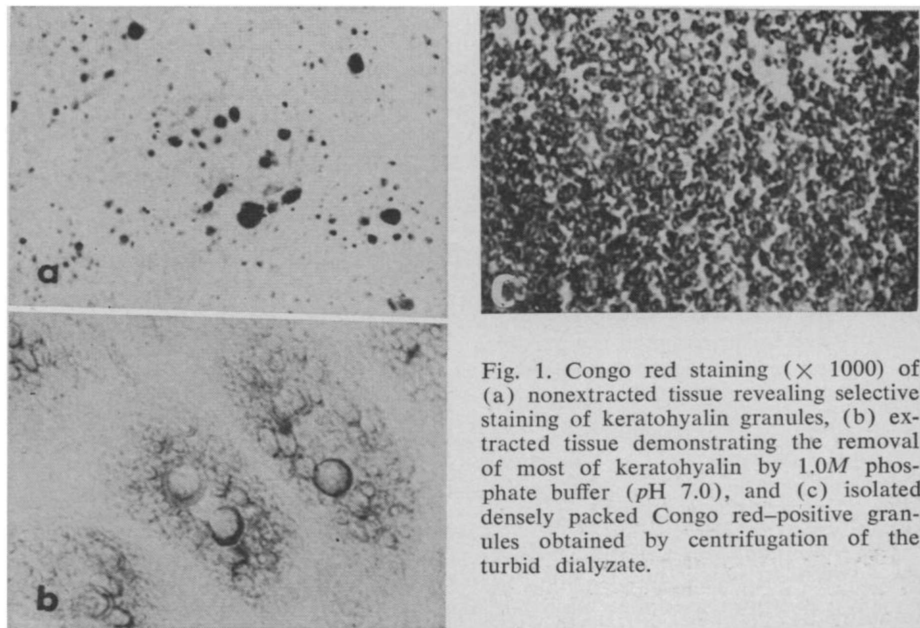

Fig. 1. Congo red staining (× 1000) of (a) nonextracted tissue revealing selective staining of keratohyalin granules, (b) extracted tissue demonstrating the removal of most of keratohyalin by 1.0*M* phosphate buffer (*p*H 7.0), and (c) isolated densely packed Congo red–positive granules obtained by centrifugation of the turbid dialyzate.