Alkaline Degradation of Turnip Yellow Mosaic Virus. II. *In Situ* Breakage of the Ribonucleic Acid*

J. M. Kaper† and Jane E. Halperin†

ABSTRACT: In short alkaline treatments (5–10 min at pH 11.50–11.55), under conditions of high ionic strength (1.0 M KCl) at 30°, turnip yellow mosaic virus (TYMV) degrades only partially into capsids (ATC) and small ribonucleic acid (RNA). The residual virus isolated from such reaction mixtures was found to be inactivated, and its RNA was broken down to segments with an $s_{20,w}$ of about 5 S. From this it was concluded that an essential step in the alkaline degradation of TYMV was the *in situ* breakage of the RNA, but that it was not the specific mechanism causing its ultimate release from the capsid. The limiting conditions for *in situ* breakage of the RNA, in reactions of 8-min duration, were found to be pH 9.6 for minimal and pH 10.95 for practically complete degradation. At pH 10.35, the degradation of the RNA inside TYMV followed first-order kinetics with respect to the concentration of unattacked RNA encapsuled in the virus, although initially there was a much faster reaction rate. In contrast to alkaline degradation of isolated TYMV RNA performed under otherwise identical conditions, the sole end product of the *in situ* degradation of TYMV RNA was 5 S RNA. This specificity of attack suggested an arrangement of the RNA inside TYMV, in which only a limited number of phosphodiester linkages would be susceptible to attack. Such an arrangement would be provided if large portions of TYMV RNA are intertwined between the protein subunits in a regular pattern.

In the previous publication of this series (Kaper, 1964), the conditions for controlled alkaline degradation of turnip yellow mosaic virus (TYMV)[1] were described. At pH 11.55 and at 30° with a relatively high ionic strength (1 M KCl), it was shown that the principal degradation products resulting from the alkaline degradation reaction were artificial top component (ATC, empty virus protein shells or capsids) and low molecular weight ribonucleic acid (RNA). The uptake of alkali accompanying this degradation and measured as a function of time showed a very rapid consumption in the first few minutes of the reaction, after which a plateau was reached. This suggested the occurrence of a rapid ionic event, *i.e.*, a titration of the available (basic) groups on the viral protein and RNA. However, no direct correlation was found between KOH consumption and the extent of degradation of TYMV into ATC and RNA. At pH 11.55 it could, for instance, be shown that at those stages of the reaction where KOH consumption had practically ceased considerable amounts of physically apparently undegraded TYMV were still present in the reaction mixture. The release of RNA from the virus, although directly associated with the alkaline conditions of the reaction, had, therefore, to be considered as a separate phase in the reaction following the titration of the ionic groups.

The most unusual aspect of the alkaline degradation of TYMV has always been the fact that the protein shell remains intact in the process. In this way it distinguishes itself clearly from the situation as it is found with TMV (Anderer, 1963; Caspar, 1963), for instance. Undoubtedly, the demonstrated stability of TYMV's empty protein shell (Kaper, 1964) is largely responsible for this behavior, the icosahedral arrangement of its subunits apparently providing for a stable minimum energy state (Caspar and Klug, 1962). Given this particular situation, a number of problems remain to be settled. One of these, the mechanism of the release of RNA, is the most intriguing one and could be of further importance to our understanding of the more detailed structural relationships that presumably exist between the RNA and the protein of TYMV (Klug and Finch, 1960).

When the alkaline degradation is executed under the high ionic strength conditions, we encounter a situation where all the RNA of each virus particle is found either inside or outside the protein shell. When it is found outside the shell, it is probably degraded and has a sedimentation rate of 4–5 Svedbergs. The questions that arise immediately are: (1) is the viral RNA first degraded inside the protein and then released? (2) is the RNA degradation the very mechanism of its escape? and (3) is the RNA released in its original mo-

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† Mailing address: Plant Virology Laboratory, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

[1] Abbreviations used: TYMV, turnip yellow mosaic virus; ATC, artificial top component (empty protein shells or capsids); TY-A, alkali-treated turnip yellow mosaic virus; TY-A(BR), alkali-treated turnip yellow mosaic virus with all particles containing broken (5 S) RNA.

lecular dimension (molecular weight about 2 million) and degraded outside the viral capsid?

The present contribution deals with these and related questions through a study of some properties of residual, apparently intact, TYMV which has survived various alkaline treatments and the RNA isolated therefrom.

Experimental Section

Materials and Apparatus. Chemicals, purified TYMV, and ATC were obtained as described earlier (Kaper, 1964). Also, the same equipment was used for ultraviolet spectrophotometry, reactions at constant pH, and preparative and analytical ultracentrifugation. The latter apparatus, however, was now also equipped with an ultraviolet optical system. Photographic exposures from these analyses were scanned with the Beckman Model RB Analytrol supplemented with a film densitometer attachment.

Methods

pH-Stat Reactions. All physical manipulations during alkaline treatments were performed, as described in the previous publication of this series (Kaper, 1964). All reactions were carried out at 30° and in 1.0 M KCl; pH and length of treatments were varied. The virus concentration during the reactions was 0.3%, but the total amount of material varied with the purpose of the reaction. When the pH was the experimental variable, each reaction was carried out separately; the strength of titrant (KOH in 1.0 M KCl) was chosen to suit the particular reaction conditions, and the reaction was terminated by neutralization with HCl. When the reaction of TYMV under alkaline conditions was studied as a function of the length of the treatment, the reaction was usually stopped in the same manner; however, when only small samples of reaction mixture were required for further study, aliquots of known volume were withdrawn from the reaction vessel and neutralized with a pH 7 buffer of sufficient strength. All reaction mixtures were submitted to an overnight dialysis at 4° against a 0.1 ionic strength buffer of pH 7.

Analytical Ultracentrifugation. Reaction mixtures and RNA preparations were routinely examined in the analytical ultracentrifuge, and (in most cases) the ultraviolet optical system was employed to record their sedimentation patterns. The solutions were adjusted, with the appropriate solvents, until they possessed an absorbancy at 260 mμ of about 1.0. Unless mentioned otherwise, reaction mixtures were ultracentrifuged at 20° in a 0.1 ionic strength buffer of pH 7; RNA preparations were centrifuged at temperatures of about 5° in a 0.02 M KH_2PO_4–NaOH buffer of pH 7.2. Sedimentation coefficients are reported as $s_{20,w}$. Cells used were the standard aluminum-type or the capillary-type synthetic boundary cell.

Analysis of Sedimentation Patterns. The densitometer tracings of the ultracentrifuge patterns provided a direct proportionality between the densitometer's pen deflection and the optical density at 260 mμ up to a value of 1.2 because of a preselected combination of light-source intensity, exposure time, and a standardized photographic development procedure. Assuming that the absorption coefficients at 260 mμ of the different components would not differ significantly from one another, and after correcting for radial dilution, a direct estimate could be made of the relative concentrations of components with different sedimentation rates in the RNA preparations. In the same manner, the reaction mixtures were analyzed for the amounts of residual alkali-treated TYMV (TY-A), which was a direct measure of the RNA still enclosed by the viral capsid. The relative amount of RNA released from the capsid could then be calculated.

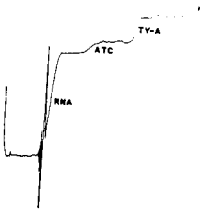

FIGURE 1: Densitometer tracing representing sedimentation pattern of alkaline degradation reaction mixture of TYMV (reaction conditions: 8 min, pH 11.52, 30°, 1.0 M KCl). Sedimentation from left to right. Picture taken 10 min after centrifuge reached speed of 33450 rpm.

Isolation of RNA. RNA was extracted from alkali-treated virus preparations (TY-A) by means of phenol extraction (Gierer and Schramm, 1956). Ether extraction or ethanol precipitation of the RNA was employed to remove excess phenol. The final RNA solutions were stored frozen.

Infectivity Assay. Virus and RNA preparations were diluted for infectivity assay in 0.02 M KH_2PO_4–NaOH buffer of pH 7.2. The solutions to be assayed were compared with the appropriate standards on 6–12 half-leaves of Chinese cabbage (*Brassica pekinensis* Rupr., cv. Pe-tsai). The plants were preconditioned and grown under controlled conditions, as described by Diener and Jenifer (1964).

Results

Isolation and Some Properties of TY-A. Alkaline treatment of TYMV at about pH 11.55 in 1.0 M KCl at 30° during 5–10 min resulted in a mixture composed of TY-A, ATC, and degraded RNA (Kaper, 1964). Figure 1 shows a densitometer tracing of the sedimentation pattern of such a reaction mixture (exact reaction conditions: 8 min at pH 11.52) after overnight dialysis against a 0.1 μ buffer of pH 7. TY-A can be identified readily in this tracing as the fastest sedimenting component, and its concentration was determined to be 20% of the total amount of ultraviolet-absorbing material present. Other components visible in Figure 1

TABLE I: Infectivities of Structurally Intact, Residual TYMV Isolated from Alkaline Degradation Reaction Mixtures[a] and of RNA Extracted from TY-A (TY-A RNA).

Material	Concn (μg/ml)	Av No. of Lesions per Half-Leaf	Relative Infectivity: No. of Lesions A / No. of Lesions B $\times$ 100%
A. TY-A	10	0.4	2
B. TYMV	10	22.8	
A. TY-A RNA	250	3.6	3
B. TYMV RNA	250	119	

[a] Reaction conditions: 8 min, pH 11.52, 30°, 1.0 M KCl.

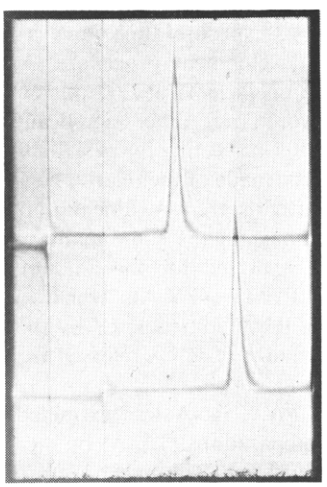

FIGURE 2: Sedimentation properties of TY-A compared with those of untreated TYMV. Sedimentation from left to right. Picture taken at 50° Schlieren angle, 24 min after centrifuge attained speed of 23150 rpm. Wedge cell (upper pattern): TY-A at 0.3% concentration. Standard cell (lower pattern): TY-A and TYMV mixed in equal proportions at 0.3% total concentration.

are an unidentified small amount of material with sedimentation rate intermediate between TY-A and ATC which is also present in the original virus preparation (see also Kaper, 1964), ATC, and degraded RNA.

TY-A was isolated from such reaction mixtures by several cycles of differential centrifugation. It could not be distinguished from TYMV in its ultraviolet spectrum or in its sedimentation properties. Figure 2 shows a sedimentation diagram of isolated TY-A (upper pattern) and of TY-A and TYMV mixed in equal proportions (lower pattern). However, the infectivity of TY-A, in comparison with that of the TYMV it was derived from, was almost entirely abolished (Table I) despite the fact that the latter had undergone the same series of treatments with omission of alkali.

RNA prepared from TY-A by means of phenol extraction (TY-A RNA) was tested for infectivity. It was also found to be essentially uninfectious in comparison with RNA extracted from untreated virus (Table I). This loss of infectivity was readily explained after examination of the sedimentation patterns of the respective RNA samples (Figure 3). While the RNA from the control exhibited the usual major component with a sedimentation rate of approximately 30 S and some 30% of trailing material with a continuous distribution of sedimentation rates (Haselkorn, 1962), TY-A RNA seemed to be a much smaller entity (sedimentation rate about 5 S). It was, therefore, concluded that under the conditions of alkaline treatment described above the RNA inside TYMV was degraded prior to its release into the reaction mixture with concomitant formation of ATC.[2]

Limiting pH Conditions for the Degradation of RNA inside TYMV. The above findings seemed to warrant a careful analysis of the upper and lower limits of pH under which degradation of the RNA inside TYMV could be achieved. To this end TYMV was submitted to a series of alkaline treatments of 8-min duration, with pH values varying between 9.6 and 10.95. In each reaction mixture, the relative amount of TY-A was determined from a sedimentation pattern densitometer tracing, and, therefore, a direct estimate of the amount of RNA released from the virus could also be made. TY-A was subsequently isolated from the different reaction mixtures, and the RNA from each of these preparations extracted as described. The TY-A RNA preparations were then ultracentrifuged in a synthetic boundary cell, and their sedimentation patterns recorded with the ultraviolet optical system. Figure 4 represents the densitometer tracings of a selection of these RNA preparations. It can be seen that the pH 9.8 treatment yielded virtually undegraded RNA, while 8 min at pH

[2] Such TY-A preparations in which all particles contain degraded RNA will be abbreviated TY-A(BR) throughout the rest of this paper.

TABLE II: Conversion of RNA Inside TYMV into 5 S RNA, at Different pH Values but Otherwise Identical Conditions.[a]

Sedimentation Diagram	pH	RNA Released Into Reaction Mixture[b] (%)	Amount of 5 S RNA in TY-A (%)	Total Amount of 5 S RNA Formed[b] (%)	Total Amount of RNA Inside TYMV Left Unattacked[b] (%)
...	9.61	0	11	11	89
Figure 4a	9.81	0	15	15	85
...	9.88	0	17	17	83
...	10.14	0	44	44	56
Figure 4b	10.40	6	55	57	43
...	10.69	7	68	70	30
Figure 4c	10.95	15	92	93	7

[a] Conditions were: 8 min, 30°, 1.0 M KCl. [b] Expressed as a percentage of the amount of RNA initially present inside TYMV during the reaction.

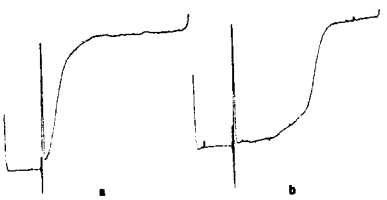

FIGURE 3: Densitometer tracings representing sedimentation patterns of the RNA samples obtained from TY-A and from untreated virus. Sedimentation from left to right. Pictures taken 20 min after centrifuge reached speed of 59,780 rpm. a, RNA from TY-A (8 min at pH 11.52, 30°, 1.0 M KCl); b, RNA from untreated TYMV.

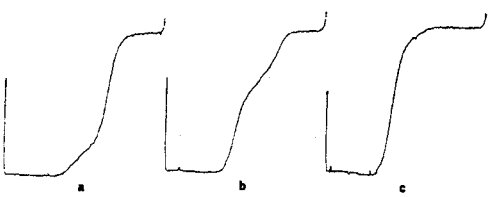

FIGURE 4: Densitometer tracings representing sedimentation patterns of the RNA samples extracted from TY-A preparations resulting from alkaline treatments of TYMV (8 min, 30°, 1.0 M KCl) at different pH values. Sedimentation from left to right. Pictures taken about 24 min after centrifuge reached speed of 42,040 rpm. Cell, synthetic boundary; a, RNA from TY-A (pH 9.81); b, RNA from TY-A (pH 10.40); c, RNA from TY-A (pH 10.95).

10.95 was sufficient to destroy all of the 30 S RNA inside TYMV.

The most significant feature of the sedimentation patterns of Figure 4, however, is the fact that they imply that each RNA preparation analyzed (except for the two extreme pH values used) was a mixture predominantly composed of two components, the native (presumably intact) 30 S TYMV RNA and its *in situ* degradation product, RNA fragments sedimenting with approximately 5 S. As far as could be judged from the patterns obtained, there was no polydispersity at the trailing edge of the 5 S component, but some could definitely be detected between the 30 S and 5 S components. Although this material could be degradation products of intermediate size, recent data have suggested that it represents 5 S RNA in aggregated state (see later in this section). In spite of this difficulty, the relative amounts of 5 S RNA were determined from the corresponding densitometer tracings in each of the TY-A RNA preparations. After correction for the RNA already released from the capsid (see above), the total amount of 5 S RNA formed in the reaction could be calculated. On the assumption that 5 S RNA was the ultimate degradation product of TYMV RNA treated under these conditions, and neglecting the possibility that the relatively small amount of the polydisperse material sedimenting between 5 S and 30 S RNA was a degradation product of intermediate size, the amount of 5 S RNA formed was taken as an inverse measure of RNA as yet unattacked.[3] Table II presents the relevant data of these calculations.

Kinetics of the Breakage of RNA inside TYMV. The breakage of RNA inside TYMV as a function of time was studied at pH 10.35. After reactions of 2–24-min

[3] In our hands, RNA extracted from untreated TYMV contained on the average 30% of slower material with a continuous distribution of sedimentation rates. Since this material, as well as 30 S RNA, presumably participates in the alkaline reaction, the residual 30 S RNA detected in TY-A preparations could not be taken as a direct measure for the RNA left unattacked inside the virus.

TABLE III: Conversion of RNA inside TYMV into 5 S RNA[a] as a Function of the Time of Reaction.

Sedimentation Diagram	Length of Treatment (min)	RNA Released into Reaction Mixture[b] (%)	Amount of 5 S RNA in TY-A (%)	Total Amount of 5 S RNA Formed[b] (%)	Total Amount of RNA inside TYMV Left Unattacked (%)
Figure 5a	2	0	17	17	83
...	4	0	47	47	53
...	6	0	54	54	46
Figure 5b	8	0	58	58	42
...	12	5	67	68	32
Figure 5c	16	7	74	76	24
...	24	7	84	85	15

[a] Reaction conditions: pH 10.35, 30°, 1.0 M KCl. [b] Expressed as a percentage of the amount of RNA initially present inside TYMV during the reaction.

FIGURE 5: Densitometer tracings representing sedimentation patterns of RNA samples extracted from TY-A preparations resulting from alkaline degradations of TYMV (pH 10.35, 30°, 1.0 M KCl) at different lengths of time. Sedimentation from left to right. Pictures taken about 20 min after centrifuge reached speed of 42,040 rpm. Cell, synthetic boundary; a, RNA from TY-A (2 min); b, RNA from TY-A (8 min); c, RNA from TY-A (16 min).

duration, TY-A was isolated from each reaction mixture, and RNA was extracted from it. The RNA samples were ultracentrifuged and the relative amounts of RNA converted to 5 S RNA inside the virus were determined, as described above. The data are given in Table III; Figure 5 shows a representative selection of the corresponding sedimentation patterns. As with the series of reactions at different pH values, a conversion of 30 S RNA into 5 S RNA seemed to be the reaction that predominated. In Figure 6, the data of this reaction are presented as a first-order plot.

Sedimentation Behavior of 5 S TYMV RNA. As part of a more detailed study of 5 S TYMV RNA to be reported elsewhere, this material was extracted from TY-A(BR) preparations isolated after alkaline treatments of TYMV for 8 min at pH 11.00. Under these conditions all of the RNA had been degraded *in situ*. The resulting 5 S RNA preparations showed a pronounced tendency to aggregate. This could be made visible in ultracentrifugations of longer duration, and could be tested quantitatively by applying the radial dilution law to the sedimentation patterns obtained at regular time intervals.

An effective way to deaggregate ribonucleic acids and to expose possible "hidden breaks" in the phosphodiester-sugar backbone without affecting the covalent structure appreciably is to apply short treatments at moderately elevated temperature (Haselkorn, 1962; Huff *et al.*, 1964; Kaper *et al.*, 1965). 5 S TYMV RNA preparations in 0.02 M KH_2PO_4–NaOH buffer of pH 7.2, at concentrations corresponding to an OD_{260} of 1.0, were therefore exposed to 60° in a water bath for periods of time ranging from 5 to 20 min. The solutions were rapidly cooled and subsequently ultracentrifuged for periods of at least 90 min. The synthetic boundary cell was used in order to examine the boundaries also for heterogeneity at the trailing edges. Although no conservative boundary analyses (Schachman, 1959) were made at this stage, visual inspection of densitometer tracings of such sedimentation patterns (Figure 7) revealed little if any sedimentation heterogeneity at either edge of the boundary. The radial dilution test, moreover, indicated that deaggregation of 5 S RNA could be accomplished at even the mildest heat treatment applied (5 min at 60°).

Alkaline Treatment of Isolated TYMV RNA. TYMV RNA was isolated from untreated virus, and an amount equivalent to that which would normally be used as intact virus was submitted to alkaline treatment under the usual conditions of salt strength and temperature, at a pH of 10.5 during 8 min. Immediately following, a control reaction with TYMV was performed under identical conditions. The latter reaction mixture was processed as usual, and RNA was extracted from the residual virus. The RNA from the first reaction (iso-

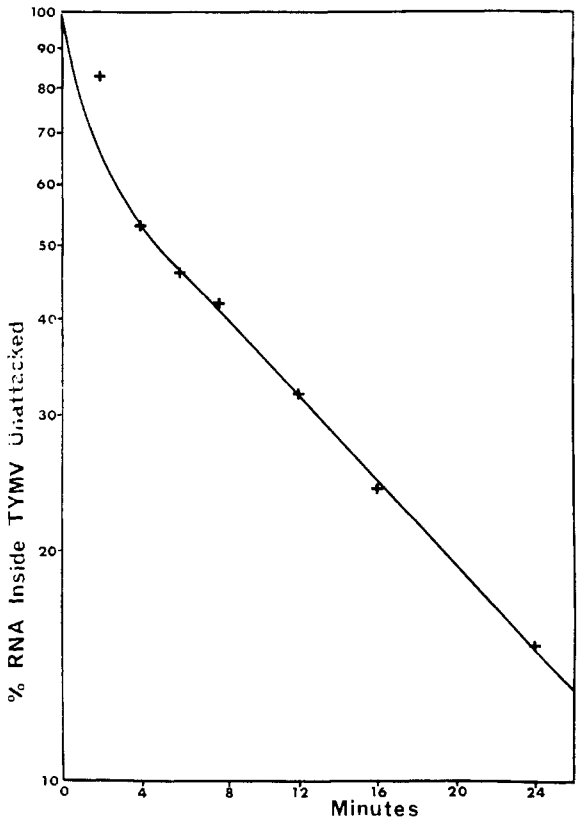

FIGURE 6: First-order plot of the *in situ* degradation kinetics of TYMV RNA at pH 10.35, 30°, in 1.0 M KCl.

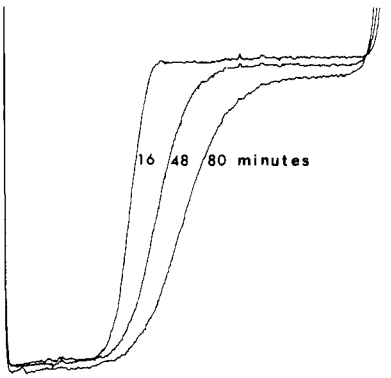

FIGURE 7: Densitometer tracings of sedimentation patterns of 5 S TYMV RNA extracted from TY-A(BR) obtained in an alkaline treatment of TYMV (8 min, pH 11.00, 30°, 1.0 M KCl). The 5 S RNA was heated for 5 min at 60° prior to ultracentrifugation. Sedimentation from left to right. Photographs were taken at 16, 48, and 80 min after a speed of 42,040 rpm was attained. Cell, synthetic boundary.

$$1\ \text{TYMV} \xrightarrow[\text{breakage}]{\text{RNA}}$$

(containing one 30 S RNA)

$$1\ \text{TY-A(BR)} \dashrightarrow \xrightarrow[\text{detachment}]{\text{RNA}} \xrightarrow[\text{release}]{\text{RNA}}$$

(containing n 5 S RNA)

$$1\ \text{ATC} + x\ \text{small RNA}$$

(derived from n 5 S RNA)

lated RNA) was isolated by precipitation with ethanol followed by two cycles of washing and reprecipitation. Finally, the two RNA preparations were ultracentrifuged using the ultraviolet optics. Figure 8 shows the densitometer tracings of the respective sedimentation patterns (Figure 8b and c) plus that of an RNA preparation subjected to similar incubation conditions, but where alkali was omitted (Figure 8a). The sedimentation pattern of RNA resulting from alkaline treatment of isolated TYMV RNA exhibited a continuous distribution of sedimentation rates in contrast to that of the RNA control (predominantly 30 S RNA), and to the RNA isolated from the alkali-treated virus (mixture of 5 S RNA and 30 S RNA). As a final check, isolated TYMV RNA was also alkali-treated in the presence of a stoichiometric amount of ATC. Its sedimentation behavior after treatment exhibited pronounced polydispersity similar to that shown in Figure 8b.

Discussion

At the present stage a faithful representation of what is known of the alkaline degradation reaction of TYMV could be given by the following equation. Apparently, TYMV RNA prior to its release from the capsid is already split *in situ* into an as yet not exactly known number of smaller segments of similar sedimentation rates. Such apparently intact virus [TY-A(BR)], devoid of infectivity and containing only small 5 S RNA segments, but in many other respects such as sedimentation behavior, chemical composition, and kinetics of release of RNA, behaving substantially similar to untreated TYMV (J. M. Kaper and J. E. Halperin, unpublished work), can actually be isolated and studied. It can therefore be concluded that just the *in situ* degradation of the RNA is not the primary mechanism of its release from the capsid. This, however, would not imply that the described RNA breakage is unessential to its ultimate release. In fact, it would be difficult to visualize how an RNA strand of about 6000 nucleotides would have to work its way out of the viral capsid without damaging the latter substantially. From the previous publication of this series (Kaper, 1964), it could be inferred that the alkaline degradation reaction was at least of a "two-step" nature, namely, a detachment step and a release step. To this an RNA-breakage step could now be added, although at this stage it is not possible to indicate with certainty in what sequence these steps would take place. It would actually be quite likely that at relatively high pH values (11.0–11.5) the RNA breakage and detachment from the protein would take

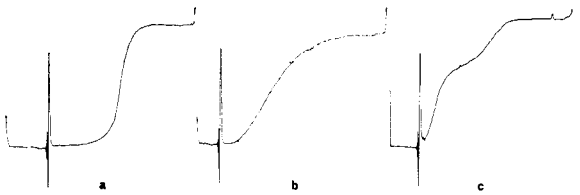

FIGURE 8: Densitometer tracings representing sedimentation patterns of different TYMV RNA preparations. Sedimentation from left to right. Pictures taken about 16 min after centrifuge attained speed of 59,780 rpm. a, isolated TYMV RNA treated for 8 min at 30°, 1.0 M KCl, but omitting alkali; b, isolated TYMV RNA treated for 8 min at pH 10.50, 30°, 1.0 M KCl; c, RNA extracted from TY-A resulting from alkaline treatment of TYMV for 8 min at pH 10.50, 30°, 1.0 M KCl.

place simultaneously. No direct experimental evidence has been obtained yet as to the exact nature of the RNA-release step, but in view of the discussion to follow, a mechanism involving a temporary expansion or swelling of the capsid would seem the most logical.

Clearly, the most important aspect of the *in situ* degradation of TYMV RNA is that it gives rise to a degradation product, a major proportion of which apparently possesses uniform sedimentation characteristics (Figure 7). Within fairly narrow limits, this material sediments with an $s_{20,w}$ of 4.6 S (and is therefore called 5 S RNA), and behaves as a homogeneous component of considerably smaller molecular weight (50,000–60,000) than the full RNA complement of TYMV. (A more detailed study of 5 S RNA is in progress.) In addition, the apparent specificity of the distribution of sedimentation rates, in patterns obtained from the reactions at different pH values and at all times during the treatment (Figures 4, 5, and 8c), suggests that the *in situ* degradation of TYMV RNA to 5 S RNA is an all or nothing phenomenon. It is, therefore, in sharp contrast with a similar treatment of isolated TYMV RNA, as exemplified in Figure 8b, which result is typical for a random attack on the RNA in which all susceptible phosphodiester bonds stand an equal chance of being hit. A very similar pattern of degradation was produced, for instance, by thermal treatment of isolated bacteriophage R 17 RNA (Gesteland and Boedtker, 1964).

The kinetics of the *in situ* degradation of TYMV RNA (Figure 6) show that the reaction, at least in its later stages, is first order with respect to the concentration of unattacked RNA encapsuled in the protein. In the underlying case, this would normally be taken to represent a successive breakage of the $n - 1$ specifically susceptible regions of the phosphodiester backbone at random, and should produce a sedimentation distribution pattern characteristic for n different RNA segments of molecular weights ranging between that of intact TYMV RNA and 5 S RNA (n = number of 5 S TYMV RNA segments per virus particle). Such a sedimentation pattern should be very similar to the one representing the alkaline treatment of isolated TYMV RNA (Figure 8b, polydispersity!), except that it would have to show an accumulation of 5 S RNA with time of treatment. Instead, a much more specific degradation pattern was found (Figure 8c) suggestive of a highly cooperative nature of the *in situ* RNA-breakage process. This apparent paradox could be explained if a complete virus particle is the initial reactant, so that its concentration limits the rate of reaction. It could for instance be conceived that the penetration of OH^- ions to the susceptible regions of the RNA backbone is rate determining, and/or, on the other hand, that the actual reaction with the RNA is extremely rapid because of perhaps a catalytic action of traces of metals (see Huff *et al.*, 1964). The initial, faster rate of degradation, which is apparent from Figure 6, is also as yet not understood. An explanation could possibly be found in the fact that routinely 30% of the RNA extracted from TYMV is partially degraded [according to Haselkorn (1962) even much more]. If such a situation is representative for the original population of virus particles, it could perhaps be that the virus particles containing partially broken RNA are also in general more vulnerable to alkaline attack. This situation would tend to favor a more rapid formation of 5 S RNA in the beginning.

The implication of a highly specific degradation pattern obtained from an attack on TYMV RNA *in situ* is that there is only limited access to the phosphodiester linkages. Such a situation would arise if major portions of the RNA are locked in the substructure of the viral capsid. This wonld not be too surprising if it is considered that with TMV (the only virus where the exact location of the RNA is known) the RNA is also deeply buried between the protein subunits (Franklin *et al.* 1959). An alternative explanation for a nonrandom attack on the RNA would be that specific regions of otherwise more or less centrally located RNA, possibly because of some form of interaction with the protein, would be more susceptible than others. In either case, however, it follows that if the resulting RNA segments are of approximately equal molecular weights the vulnerable regions of the RNA strand must be distributed in some regular pattern throughout the viral capsid. It should be pointed out that Klug and Finch (1960) already suggested a regularity of the RNA folding inside TYMV on the basis of an X-ray diffraction study of the virus and its capsid (top component).

Of the two alternative arrangements proposed for the RNA inside TYMV, we prefer the first one, *i.e.*, where relatively large regions of the RNA are intertwined between the subunits in a regular pattern. The molecular weight studies on 5 S RNA and the observation of a great similarity in the properties of TY-A and TYMV, which we briefly mentioned above, seem to support this idea. In addition, it has recently been found that *p*-mercuribenzoate, which reacts with great ease with the sulfhydryl groups of TYMV protein (Kaper and Houwing, 1962a,b), will also inactivate and degrade TYMV RNA *in situ* (Kaper and Jenifer, 1965). In this

case again, the implication was that the RNA and protein components of TYMV are in close structural association.[4]

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[4] Recently completed X-ray diffraction studies by Klug and associates (A. Klug, personal communication) on TYMV and its empty capsids seem to suggest that a major portion of the RNA is distributed in 32 "packets" each associated with "rings of 5 and 6 protein subunits" which together make up the 32 capsomeres in a TYMV capsid. This concept is in complete agreement with the ideas resulting from the studies reported in this publication.

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