Alkaline Degradation of Turnip Yellow Mosaic Virus.* I. The Controlled Formation of Empty Protein Shells

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Turnip yellow mosaic virus (TYMV) degrades rapidly at alkaline *p*H values (*p*H 11–12). The degradation products identified so far are empty protein shells (ATC), small protein fragments, and small ribonucleic acid (RNA). Degradation reactions were carried out under controlled conditions. The influence of *p*H, ionic strength, and time of reaction on the particular nature of the protein reaction products formed was systematically investigated. At high ionic strength, the formation of ATC was favored; at low ionic strength, predominantly small protein fragments were formed. Alkali-consumption patterns registered in these reactions indicated that an ionic event takes place, but there was no simple and direct correlation between alkali consumption and the amount of virus degraded. It indicated the possible participation of a second event in the reaction effecting the release of RNA. Similar alkaline reactions were carried out with empty protein shells of natural origin (NTC) and those produced from whole virus by alkaline degradation (ATC). In contrast to whole virus, empty protein shells did not degrade into small protein fragments when treated at low ionic strength. Thus, under these conditions, TYMV protein shells seem to be more stable than whole virus. This is in striking contrast to tobacco mosaic virus (TMV) where the reaggregated protein denatures under milder conditions than whole virus.

The stability of the physical structure of a virus like turnip yellow mosaic virus (TYMV)[1] is brought about by a balance of forces, chemical as well as physical in nature. Covalent bonds are responsible for the primary structure of the nucleic acid and the protein. However, the specific spatial configurations of these components, and the way in which certain numbers of identical subunits may assemble themselves into a stable shell, seem to be largely dependent on the interplay of a variety of different forces such as electrostatic interactions, hydrogen bonding, van der Waals interactions, and hydrophobic bonds. Caspar and Klug (1962) have pointed out that the assembly of a virus protein shell is driven by the energy obtained from the formation of the intersubunit bonds. The stable virus structure is given by the lowest energy state of a particular arrangement of the protein subunits, and the main argument of their paper is that there are only a limited number of designs to do this. The icosahedral shell provides the minimum energy state for isometric viruses such as TYMV.

While the detailed three-dimensional structure of "simple" viruses can be elucidated, in principle, by means of X-ray diffraction (Klug and Caspar, 1960), it will be the combination of results from this technique with those from chemical studies on the degradation of virus into, and/or reaggregation from, its components which could possibly shed light on the processes of dissociation and assembly of virus components that presumably occur at certain stages of the infection.

The stepwise degradation of TMV into its protein subunits and their reconstitution with or without RNA to particles resembling the original virus in almost every detail has been much studied. (Schramm *et al.*, 1955; Schramm and Zillig, 1955; Fraenkel-Conrat and Williams, 1955; Harrington and Schachman, 1956; Fraenkel-Conrat, 1957; Lauffer *et al.*, 1958; Fraenkel-Conrat and Ramachandran, 1959; Anderer, 1959a,b; Ansevin and Lauffer, 1963; and others). Analogous studies on the alkaline degradations of TYMV were started (Kaper and Steere, 1959). When they were pursued in somewhat more detail, they led to the surprising finding that one of the main products of the degradation reaction was the empty protein shell of the virus (Kaper, 1960a). This protein shell was identical in all of its investigated properties (Kaper, 1960b; Finch and Klug, 1960; Kaper and Houwing, 1962a and b) with the protein component found by Markham (1951; Markham and Smith, 1949) in TYMV-infected plants. In analogy with the "naturally found top component" (NTC), the artificially produced shells were named "artificial top component" (ATC). In addition to these empty protein shells, the alkaline reaction mixtures contained virus protein smaller in size than the complete shells. Since TYMV protein has been shown to consist of an assembly of protein subunits of approximately 20,000 mw (Harris and Hindley, 1961; Symons *et al.*, 1963) arranged in a specific geometrical pattern forming an icosahedral particle (Huxley and Zubay, 1960; Nixon and Gibbs, 1960; Klug and Finch, 1960), it seems reasonable to assume that the smaller protein found in these reaction mixtures consisted of aggregates of the protein subunits that were probably denatured.

Although it seems most logical that nucleic acid–protein interactions could also contribute to a particular minimum energy state of viruses, the possibility of the existence of empty, or partially empty, protein shells in the diseased host has been amply demonstrated in a number of cases (Markham and Smith, 1949; Matthews, 1960; Yamazaki and Kaesberg, 1961; Mazzone *et al.*, 1962; Bancroft, 1962; Kelley and Kaesberg, 1962; Sinsheimer, 1959; Eigner *et al.*, 1963). Also, the release of RNA from TYMV with preservation of the quaternary structure of the protein shell seems to indicate that in certain cases the interaction between nucleic acid and protein provides only a secondary contribution to the minimum-energy state. This contribution should not be neglected entirely, however. Reaggregation of TMV protein, under

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[1] Abbreviations used in this work: TYMV, turnip yellow mosaic virus; ATC, artificial top component; NTC, naturally found top component.

certain conditions, gives rise to the so-called "stacked-disk" structure (Franklin and Commoner, 1955; Markham *et al.*, 1963), which is slightly different from the normal configuration.

So far, there have been indications in the literature that at least a number of negatively charged primary phosphoric acid groups of the RNA of TYMV are involved in some type of salt linkage with the basic groups of the viral protein and also with a polyamine recently detected in the virus (Markham 1959; Johnson and Markham, 1962). The concept of interactions of nucleic acids with polyamines is not a new one but has now also been demonstrated experimentally for isolated TYMV-RNA (Mitra and Kaesberg, 1963).

These considerations, and the finding of the peculiar mode of RNA release from TYMV mentioned above, prompted me to further investigate this reaction. In order to study the RNA-protein interactions in TYMV, it seemed of great importance, however, to prevent breakage of other linkages (such as interprotein subunit bonds). The alkaline degradation system in the form utilized previously (Kaper, 1960a,b) was therefore not particularly suitable. The present publication presents the reaction conditions under which TYMV can be induced to release its RNA, leaving most of the protein as empty (but intact) shells, and those under which TYMV will be degraded to predominantly "small protein."

Experimental

Materials

All *chemicals* used were of analytical quality and were purchased from commercial drug houses.

Virus was obtained from infected Chinese cabbage plants that were grown in a growth room with artificial light and constant temperature. It was isolated and purified according to well-established methods. If virus alone was the desired end product, Steere's (1956) butanol-chloroform procedure was used. If NTC was desired as a by-product, the virus was obtained according to Markham and Smith (1949). TYMV and NTC were then separated by a differential centrifugation procedure.

ATC was prepared by the alkaline degradation methods at high ionic strengths, to be described later in this publication. It was isolated from the reaction mixtures by differential centrifugation.

The sedimentation diagrams shown in Figure 1 are representative for the quality of the virus materials used throughout the experimentation.

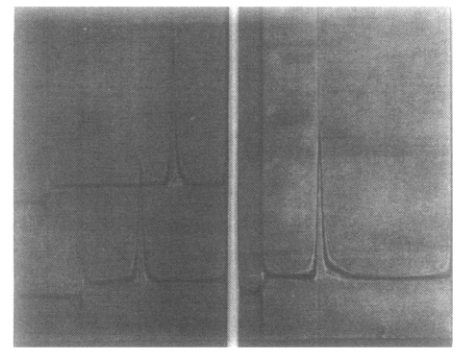
Apparatus

Reactions at constant pH were carried out in an all-glass thermostated reaction vessel. The cover of this vessel provided holes for glass and calomel electrodes, a mechanical stirrer, a titrant tube, and gas inlets and outlets. This reaction vessel (TTA3 titration assembly from Radiometer, Copenhagen), in combination with the TTT1 automatic titrator and the SBR2/SBU1 titrigraph recorder from the same manufacturer, was used as a *p*H-stat. Water was pumped into the jacket of the reaction vessel from a Wilkins-Anderson Co. constant-temperature bath.

Preparative ultracentrifugation was performed with a Spinco Model L ultracentrifuge.

Analytical ultracentrifugation was carried out with the Spinco Model E ultracentrifuge equipped with schlieren optics. All cells used had an optical path of 1.2 cm.

Ultraviolet spectra were made with a Cary Model 14 automatic recording spectrophotometer.



a b

FIG. 1.—Sedimentation diagrams of starting preparations used in alkaline degradation reactions. (a) Wedge cell (upper pattern): TYMV, concentration 0.3%. Standard cell (lower pattern): ATC, concentration 0.3%. Picture taken at 55° schlieren angle 15 minutes after reaching speed of 33,450 rpm. (b) NTC, concentration 0.3%. Picture taken at 55° schlieren angle 13 minutes after reaching speed of 33,450 rpm.

Planimetry of enlarged tracings of ultracentrifuge schlieren patterns was performed with a compensating polar planimeter, calibrated in the metric system, from Keufel and Esser Co.

Methods

*p*H-*Stat Reactions.*—Prior to reaction, TYMV, ATC, and NTC were concentrated to levels where addition of a small volume (0.5–0.6 ml) to the mixture in the reaction vessel would result in a concentration equivalent to about 0.35% of virus. These concentrated preparations were first thoroughly dialyzed against glass-distilled water; subsequently their concentrations were established. For TYMV the optical density at 260 mμ was determined. The concentration was obtained from a recently redetermined optical density–concentration relationship (10 mg/ml TYMV = 85 OD units). With ATC and NTC the concentration was established by means of nitrogen determinations with the micro-Kjeldahl method. NTC and ATC were taken to have 15% of nitrogen (unpublished work with D. W. Kupke, Dept. of Biochemistry, University of Virginia). All reactions were carried out at constant temperature (30°) at well-defined KCl concentrations, at constant *p*H, and for specific lengths of time. In order to do this, 7 ml of a glass-distilled water solution of KCl was pipetted in the reaction vessel. The concentration of KCl was such that, upon mixing with the protein samples, the necessary KCl concentration would be reached. The KCl solution was flushed with a stream of nitrogen gas to free it from dissolved CO_2. The nitrogen stream was maintained throughout the length of the reaction. Prior to the addition of the protein or virus samples, the KCl solution was brought to the *p*H of the reaction with 0.5 N KOH in a KCl solution of the same concentration to be used in the reaction. The KOH was added from a 0.5-ml syringe buret driven under control of the *p*H-stat; the amount of alkali added was registered on the recorder. After stabilization of the system, the virus or protein solution was added; the *p*H-stat was kept in operation for the duration of the reaction. The reaction was terminated by neutralization of the mixture to a *p*H of about 8. This was done manually by careful addition of 1.0 N HCl solution. To get an impression of the "background" alkali uptake, control samples of dialysate were run under identical conditions, and their alkali uptake was

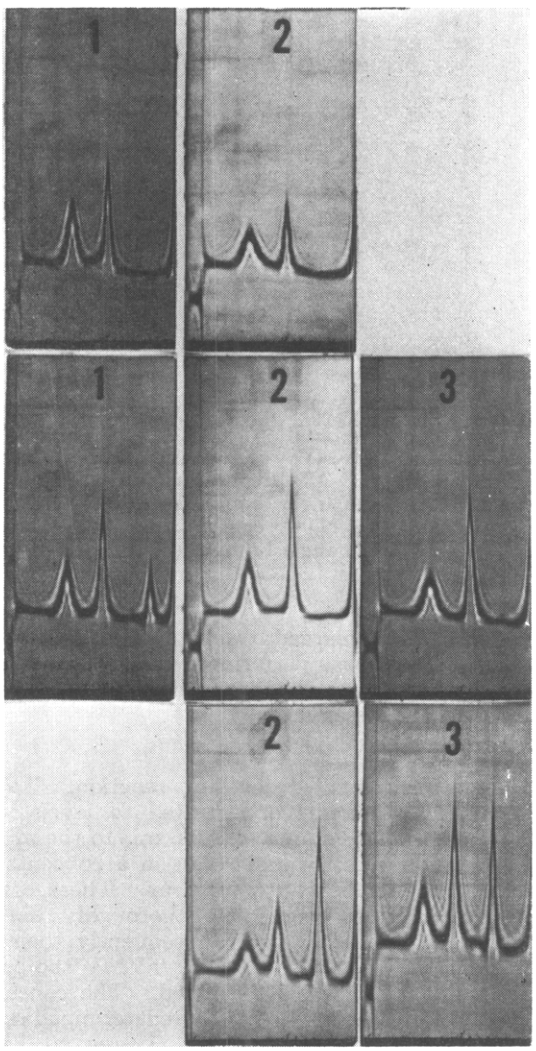


FIG. 2.—Sedimentation diagrams of alkaline degradation reaction mixtures of TYMV. All reactions were carried out at 30° and 1.0 M KCl concentrations; *p*H and length of reaction were varied. Pictures were all taken at 30° or 40° schlieren angle and about 7 minutes after reaching speed of 33,450 rpm. Pictures (a)–(c): influence of *p*H. Pictures (1)–(3): influence of reaction time. (a) *p*H 12.05: (1), 2 minutes; (2), 30 minutes. (b) *p*H 11.55: (1), 10 minutes; (2), 30 minutes; (3), 60 minutes. (c) *p*H 11.00: (2), 30 minutes; (3), 90 minutes.

registered. The neutralized reaction mixtures were subsequently dialyzed overnight in the cold against a large volume of a *p*H 7 buffer of 0.1 ionic strength. At this point, a few remarks must be made with regard to some limitations encountered with the *p*H-stat in studying the alkaline degradation reaction of TYMV.

(1) Occasionally some difficulties were encountered in obtaining reliable quantitative estimates of alkali uptake at the relatively high *p*H values used (*p*H 11.0, 11.55, 12.05). This is probably because at these *p*H values the virus showed some buffering effect, and KOH itself has a considerable buffering capacity. Small deviations in *p*H standardization, caused by external disturbances, and "sluggishness" of glass electrodes at these high *p*H values may cause relatively large errors in alkali uptake.

(2) In extremely rapid ionic reactions, such as the one that presumably takes place after addition of virus to the KCl solution, the addition of titrant lags behind with respect to the alkali consumption capacity of the system. Had the virus solution been "titrated" from neutrality, the alkali consumption registered in the initial stages would not have been a true measure of the consumption potential at the particular *p*H set for the reaction. To make reactions of relatively short duration possible, the virus was added after the KCl solution was brought to the desired *p*H. In this way the above effect could be reduced.

Ultracentrifuge Analyses.—To obtain an impression of the extent and nature of the alkaline degradation reactions, the dialyzed mixtures were ultracentrifuged at 20°. The components observed could be identified by means of their sedimentation rates. In the "results" section, it can be seen that generally four different components could be recognized. These will be denoted as TYMV, ATC, small protein, and RNA.[2] It must be stressed, however, that particularly small protein and RNA should not be regarded as monodisperse molecular species. The sedimentation analyses were generally carried out using a synthetic-boundary cell. This had the advantage of providing a better overall picture of the sedimenting mixture because the slower components, RNA and small protein, could then be resolved away from the meniscus; and at the same time ATC and TYMV, if present, had not yet sedimented to the bottom of the cell. These patterns, in some instances, were also used for the determination of the concentration of the various components relative to each other by means of area analysis of the enlarged patterns. While the use of a double-sector cell would have had the distinct advantage of a "built-in" baseline, the difficulty of resolving slowly sedimenting components would introduce an uncertainty in evaluating their concentration.

Determination of the Concentrations of Reaction Products.—Areas under the enlarged tracings were integrated. The radial dilution was taken into account in determining the concentration, but no corrections were applied for the Johnston-Ogston effect (Johnson and Ogston, 1946). Specific refractive increments for ATC and NTC were determined by Dr. D. W. Kupke, Department of Biochemistry, University of Virginia Medical School, with a Bryce-Phoenix differential refractometer at a wavelength of 546 mμ, and were found to be 0.190 and 0.191 ml/g, respectively. The Δn of RNA was taken to be 0.188 ml/g (Boedtker, 1959), Δn of TYMV was assumed to be 0.190 ml/g. Concentrations of the individual reaction products were expressed as percentages of the amount of degraded virus or, therefore, as a percentage of the sum of the concentrations of the reaction products. In this way errors caused by volume changes in the reaction mixture during dialysis were eliminated.

RESULTS

In the preliminary studies leading to the systematic series of experiments described in this section, our main goal was to replace the alkaline degradation conditions, as previously described (Kaper 1960a,b), by a system that would allow better outside control. The *p*H-stat system described in the experimental section provided constancy of *p*H, temperature, and environment (nitrogen). Reaction time could be controlled, and the virus concentration could be selected high enough to provide a clearly visible pattern with the schlieren optics of the analytical ultracentrifuge. In our previous system, a 0.14 M phosphate buffer was employed to maintain the *p*H. Here, use was made

[2] A very small amount of a fifth component, sedimenting between TYMV and ATC (sedimentation coefficient about 82), is often observed. This material is already present in the starting virus preparations. Its identity is not known.

TABLE I

RELATIVE AMOUNTS OF ATC AND OTHER REACTION PRODUCTS FORMED IN ALKALINE DEGRADATION REACTIONS OF TYMV AT *p*H 11.55 AND 30°. THE INFLUENCE OF VARYING REACTION TIME AND OF DIFFERENT IONIC STRENGTHS

Number of Corresponding Figure	Time of Treatment (min)	KCl (molarity)	ATC Formed[a] (%)	RNA + Small Protein Formed[a] (%)	TYMV Undegraded[b] (%)
2b (1)	10	1.00	61	39	21
2b (2)	30	1.00	57	43	Negligible
2b (3)	60	1.00	60	40	Negligible
3b	30	0.50	46	54	Negligible
3d (2)	30	0.05	18	82	19

[a] Expressed as percentage of the total amount of degradation products detected in centrifugation diagrams of the reaction mixtures. [b] Expressed as percentage of the total amount of material detected in centrifugation diagrams of the mixtures.

of the third ionization step of phosphoric acid, and, consequently, the ionic strength of the solution was between 3- and 6-fold of its molarity. This led to a number of experiments in which different salts at relatively high ionic strengths were tried. The type of salt proved to be relatively insignificant; consequently, a simple neutral salt (KCl) was selected for the series of reactions to be described later. The ionic strength of the reaction medium, on the other hand, turned out to be decisive in determining the nature of the protein reaction products.

Alkaline Degradations of TYMV at pH 12.05, 11.55, and 11.00.—Reactions were carried out at the *p*H values mentioned during 30 minutes and in the presence of 1.0 M KCl. The composition of the reaction mixtures after neutralization and dialysis is shown in the centrifuge diagrams of Figures 2a(2), 2b(2), and 2c(2). Values of *p*H 12.05 and 11.55 resulted in total degradation of the virus, but 30 minutes at *p*H 11.00 was clearly insufficient to accomplish total degradation of TYMV. In the sedimentation pattern (Fig. 2c[2]), the components were identified in order of decreasing sedimentation rates as TYMV, ATC, and RNA, respectively (cf. Kaper, 1960a). The *p*H 11.55 reaction is the best of those investigated in terms of completeness of reaction and homogeneity of reaction products. Area analysis of the gradients showed that the ATC peak comprised 58% of the total material, thus indicating about a 92% yield of shells if the RNA content of the virus is taken to be 37%. (In the following paragraphs such a reaction is occasionally referred to as "clean.") Although no special efforts have been made so far to study the RNA produced in reactions of this kind, it should be pointed out that it is probably low molecular since the sedimentation rate observed in these mixtures was in the order of 3 Svedbergs.

The *p*H 12.05 system suffers from losses of material during neutralization (some precipitation was occasionally observed), and aggregation and polydispersity during ultracentrifugation were observed rather frequently.

The reactions at the above *p*H values were subsequently repeated, varying the reaction time. At *p*H 12.05 and 11.55, where complete degradation had been accomplished in 30 minutes, the reaction time was cut down to 2 minutes (Fig. 2a[1]) and 10 minutes (Fig. 2b[1]), respectively, to see whether an intermediate stage between virus and shells could be detected. (Partially filled protein shells, such as described by Matthews, 1960, could, for instance, be intermediates in the alkaline degradation leading to ATC.) Two minutes at *p*H 12.05 produced complete degradation, however, and less material had been lost as a result of aggregation. Ten minutes at *p*H 11.55, on the other hand, resulted in incomplete degradation, judging from the substantial amount of virus that was left in the reaction mixture. No intermediate stages were detected. In this reaction, the amount of ATC found was close to that expected for a "clean" degradation (61% of the degraded virus). The amount of undegraded TYMV was 21% of the total material present.

Similar to the reactions with reduced reaction times, the effect of prolonged reaction time was investigated at *p*H 11.55 and 11.00. The times of treatment were prolonged to 60 and 90 minutes, respectively. At *p*H 11.55 (Fig. 2b[3]) no appreciable changes from the 30-minute treatment (Fig. 2b[2]) were observed in the reaction mixture. ATC concentration was estimated to be 60%. The 90-minute treatment at *p*H 11.00 resulted in somewhat more degradation of TYMV into ATC and RNA (Fig. 2c[3]) as compared with the 30-minute treatment.

Alkaline Degradation of TYMV at Various Salt Concentrations.—To get an impression of the influence of ionic strength on the nature of the alkaline degradation reaction, a series of reactions was carried out with diminishing salt concentrations. The result of this reaction series is given in Figure 3a–e. Figure 2b(2) should also be included in this series because this reaction was carried out under identical conditions at 1.0 M KCl concentration.

With diminishing KCl concentration, two effects can be observed: The most significant effect is that below 0.75 M KCl there is evidence for the formation of an increasing amount of small protein fragments in addition to ATC. As the salt concentration is further diminished to 0.05 M KCl, only relatively small amounts of ATC are formed, and the bulk of the virus is then degraded to small protein and RNA fragments. These two components overlap in the sedimentation diagrams shown, but they can be partially resolved when the centrifugation is continued at higher speed and for a longer period of time (Fig. 4b). An illustration of the changed pattern of TYMV degradation is given in Table I where, among others, the concentrations of the components obtained from area analysis of the patterns of the reactions at 1.0 M KCl, 0.5 M KCl, and 0.05 M KCl are given. The second effect observed is that at lower salt concentration TYMV becomes gradually somewhat more resistant to alkaline degradation. This effect becomes apparent at concentrations below 0.5 M KCl but is most significant at a KCl strength of 0.01 M (Fig. 3e).

Here again, to obtain an impression of the sequence of events in the formation of small protein, the reaction in the presence of 0.05 M KCl was performed at varying reaction times. Figure 3d(1, 2, and 3), representing reactions at *p*H 11.55 and 30° for 10, 30, and 60 minutes, respectively, must be examined in sequence. It will then be observed that with increasing reaction time, both small protein fragments and ATC are formed at the expense of the virus. There are no intermediate

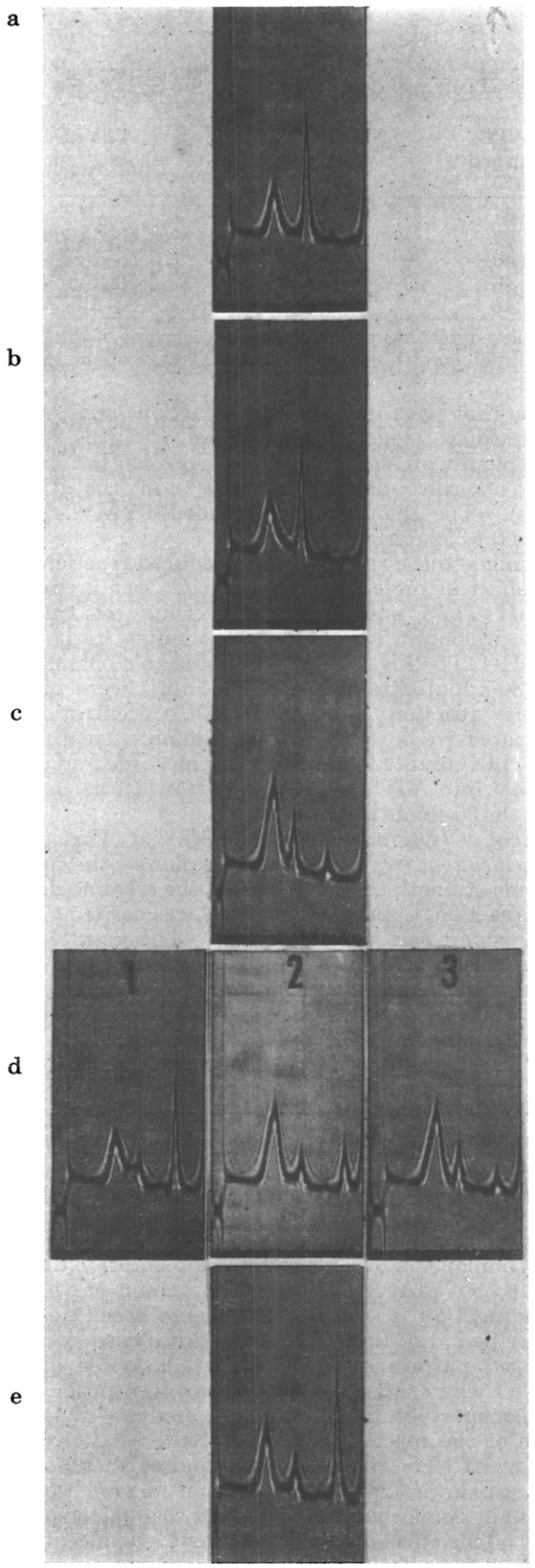


FIG. 3.—Sedimentation diagrams of alkaline degradation reaction mixtures of TYMV. All reactions were carried out at 30° and *p*H 11.55; KCl concentration and length of reaction were varied. Pictures were all taken at 30° or 40° schlieren angle and about 7 minutes after reaching speed of 33,450 rpm. Pictures (a)–(e): influence of ionic strength. Pictures (1)–(3): influence of reaction time. (a) 0.75 M KCl and 30 minutes. (b) 0.50 M KCl and 30 minutes. (c) 0.15 M KCl and 30 minutes. (d) 0.05 M KCl: (1), 10 minutes; (2), 30 minutes; (3), 60 minutes. (e) 0.01 M KCl and 30 minutes.

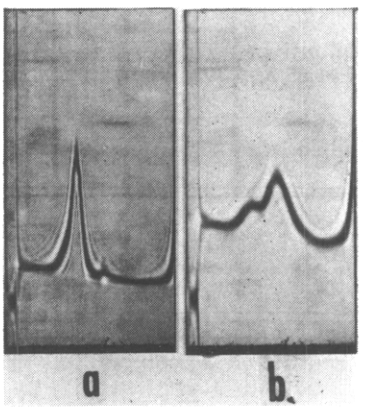


FIG. 4.—Sedimentation diagram of alkaline degradation reaction mixture of TYMV. Reaction was carried out at 30°, *p*H 12.05, 0.05 M KCl concentration and length of reaction was 30 minutes. (a) Picture taken 8 minutes after reaching a speed of 33,450 rpm; schlieren angle 40°. (b) Same mixture but picture taken 8 minutes after centrifuge had been accelerated to 42,040 rpm; schlieren angle 30°.

stages between a full virus complement and ATC. In the 10-minute reaction, some evidence can be found for the presence of aggregates of a size intermediate between ATC and small protein fragments.

To obtain complete conversion into small protein fragments, it was necessary to go to a more extreme *p*H. Figure 4a shows a reaction mixture resulting from a degradation of TYMV at *p*H 12.05 for 30 minutes at low KCl concentration (0.05 M). This centrifugation pattern can be directly compared with those of Figure 3 to show the virtual absence of ATC. In Figure 4b the same reaction mixture is shown 8 minutes after the centrifuge speed was increased to 42,040 rpm. Here it can be seen that the broad peak observed in the previous picture (Fig. 4a) consists of a mixture of two polydisperse components, the leading one being small protein fragments and the trailing material being RNA.

Alkaline Treatment of ATC and NTC.—The influence of salt concentration on the direction of the alkaline degradation reaction with TYMV, as described in the previous paragraph, was rather striking. It therefore seemed of some interest to investigate whether these same conditions applied in the absence of RNA from inside the protein shell. For this purpose ATC was isolated and concentrated from reaction mixtures where alkaline degradation had been performed at high salt concentrations. The other components of the reactions were eliminated by means of differential centrifugation. The essential purity of the resulting ATC has already been shown in the sedimentation diagram represented by Figure 1a.

This material, at a concentration of about 0.2%, was treated at 30° and *p*H 11.55 for 30 minutes in the presence of 0.75 M KCl and 0.05 M KCl, respectively. The results are shown in Figure 5a,b. At 0.75 M KCl no degradation took place; but, unexpectedly, 0.05 M KCL conditions also showed only negligible degradation of the shells into small protein fragments. This was in striking contrast with the situation observed with the RNA-containing shells (i.e., whole virus), as can be seen from Figure 3a and 3d(2), respectively. To make certain that this contradicting situation was not caused by the fact that the protein shells used were artificially formed (ATC), the reactions were repeated under approximately identical conditions with NTC. It can be seen from Figure 6a,b that

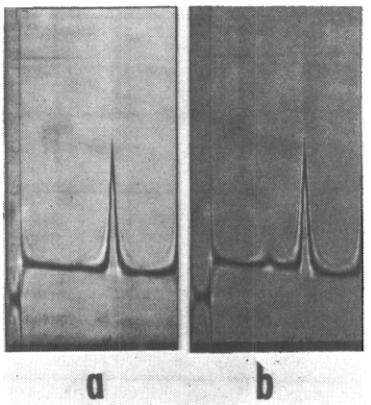


FIG. 5.—Sedimentation diagrams o alkaline degradation reaction mixtures of ATC. Reactions were carried out at 30°, *p*H 11.55 for 30 m nutes. KCl concentration was varied. Pictures were taken at 40° schlieren angles about 8 minutes after reaching a speed of 33,450 rpm. (a) 0.75 M KCl. (b) 0.05 M KCl.

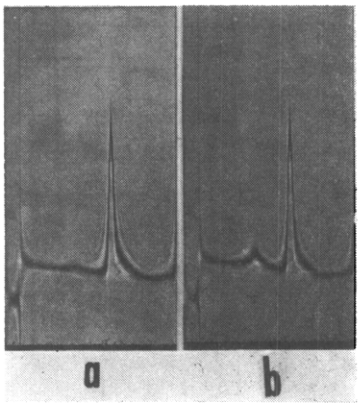


FIG. 6.—Sedimentation diagrams of alkaline degradation reaction mixtures of NTC. Reactions were carried out at 30°, *p*H 11.55 for 30 minutes. KCl concentration was varied. Pictures were taken at 40° schlieren angles about 8 minutes after reaching speed of 33,450 rpm. (a) 0.75 M KCl. (b) 0.05 M KCl.

the use of NTC instead of ATC did not alter the situation. In fact, it would have been difficult to distinguish the NTC and ATC reaction mixtures from each other if the NTC preparation had not contained a heavier impurity on the leading side of the main component (see also starting preparation, Fig. 1b).

Alkali Consumption during Degradation Reactions.—In this paragraph the results of the alkali consumption taking place in a number of the reactions described above, after suitable correction for "background" consumption, will be given. Immediately after the addition of virus to the KCl solution (which had been brought to the *p*H of the reaction prior to virus addition), a drop of a few tenths of a *p*H-unit was observed. The *p*H-stat reacts to this condition by immediately delivering KOH at maximal capacity. Figure 7 demonstrates this effect with the steep portion of the curves registered. At high KCl concentration this extremely rapid uptake of OH^- ions stops only shortly before the full equivalent of KOH has been added to the system. During the remaining portion of the reaction time only minor amounts of KOH are consumed. It is interesting to note in this respect that a virtual completion of the ionic reaction does not necessarily imply that all the TYMV has been converted into ATC. Figure 2b(1) shows the reaction mixture after 10 minutes at *p*H 11.55 in 1.0 M KCl. The degradation was incomplete; 21% TYMV was still physically intact after this treatment. Yet Figure 7 (curve 1 or 2) shows that the ionic reaction at this point was virtually complete.

The effect of decreasing KCl concentrations on the uptake of KOH by the alkaline-degradation systems is shown in the bundle of curves of Figure 7. It will be noted that at the same time a significant decrease in the initial steep portion of the curves takes place. However, after the break in the curve, KOH consumption continues at a slower, but in comparison with the high ionic strength reactions, significant rate. Although in Figure 7 the impression is created that all the reactions with TYMV have the same total KOH uptake, it will be shown in Figure 8 that this is not the case. Here the KOH consumption curves for reactions at *p*H 11.55 and 30° are given. The reaction times were extended to 60 minutes, however. One of the two curves represents the reaction at 1.0 M KCl concentration, the other at 0.05 M KCl. The corresponding sedimentation diagrams of these mixtures are represented by Figures 2b(3) and 3d(3). The KOH consumption of the reaction in the presence of 0.05 M KCl clearly exceeded that of the 1.0 M KCl medium.

Finally, in Figure 7, the KOH consumption of the treatments of ATC at *p*H 11.55 and 30° for 30 minutes is also shown. At both 0.75 M and 0.05 M KCl concentrations (see also Fig. 5a,b), the systems behaved similarly. Total KOH uptake was small and reached a plateau shortly after addition of the protein.

DISCUSSION

The alkaline degradation studies described in this paper represent the initial stage of an attempt to investigate the problems of the interactions of the RNA and protein of TYMV and of its intersubunit bonds. As has been stated in the introduction, these studies were a direct consequence of our previous finding that treatment of TYMV at *p*H 11–12 could lead to the formation of empty protein shells. If this reaction could be "cleaned up" sufficiently so that protein shells were the predominant reaction product, it might become feasible to study the processes of detachment and release of nucleic acid in somewhat more detail.

The limited aim of obtaining a "clean" alkaline degradation reaction has been achieved to at least some degree of satisfaction. Under the experimental conditions of the degradation experiments represented by Figure 2b, a smooth release of RNA apparently takes place with time and is terminated after about 30 minutes. The reaction conditions can be maintained for another 30 minutes without any significant effect on the products of the reaction previously obtained. The RNA released is apparently quite small, and one must, therefore, assume that not only an RNA-protein dissociation takes place but considerable RNA degradation as well. It is not known what causes this extensive degradation. Although alkaline hydrolysis would be an obvious candidate, it was pointed out earlier (Kaper, 1960b) that under the conditions of the reaction it does not seem likely that hydrolysis would take place at such a rapid rate.

It is of some interest to study the alkali consumption patterns registered by the *p*H-stat in the reactions at high ionic strength which favor the formation of ATC. Curves 1 and 2 of Figure 7 are representative. The initial steep portion of the curves seems to indicate a rapid ionic reaction. After a short lapse of time (8–15 minutes), during which moderate consumption

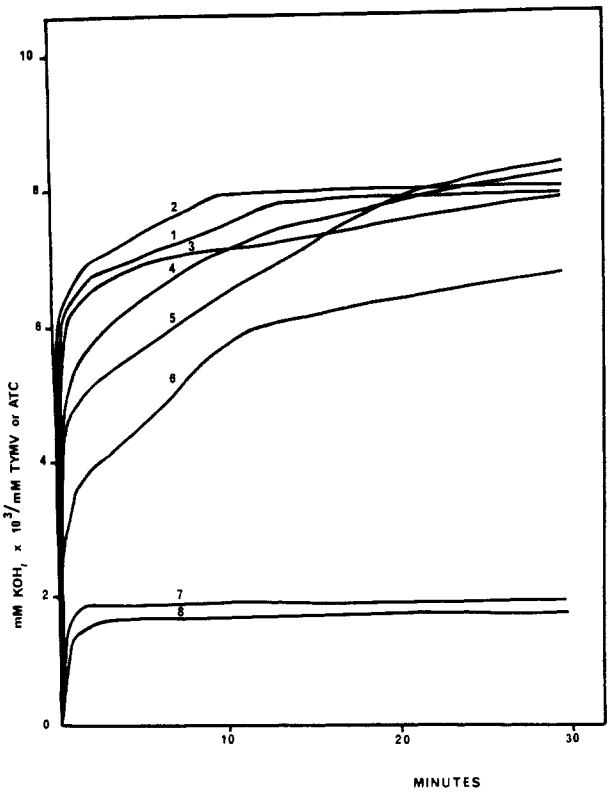


FIG. 7.—Alkali consumption patterns registered by a *p*H-stat during alkaline degradation reactions of TYMV and ATC. All reactions were carried out at 30°, *p*H 11.55, during 30 minutes. The ionic strength was varied. Curve 1, TYMV, 1.0 M KCl; curve 2, TYMV, 0.75 M KCl; curve 3, TYMV, 0.50 M KCl; curve 4, TYMV, 0.15 M KCl; curve 5, TYMV, 0.05 M KCl; curve 6, TYMV, 0.01 M KCl; curve 7, ATC, 0.75 M KCl; curve 8, ATC, 0.05 M KCl.

takes place, a plateau is reached. This aspect of the reaction contains a number of interesting points worth discussing. It will be noted from Figure 2b(1) that a considerable portion of the virus (21%) is still physically intact, while curves 1 and 2 (and also the actual alkali consumption diagram of this 10-minute experiment, which is not given here) show that the KOH consumption has reached a plateau. A number of explanations can be offered for this phenomenon. In the first place, it could be argued, of course, that no salt linkages of the type suggested in the literature are involved; and, consequently, that the ionic reaction, ase videnced by the rapid KOH uptake, has nothing to do with the actual detachment and release of RNA. This does not seem likely to us although only a relatively small fraction of the total amount of KOH consumed can be accounted for by the potential interaction sites for the RNA (unpublished results). Another explanation for the presence of this residual amount of virus could be that the RNA inside the protein is already detached, but a second nonionic event effecting the release of the RNA has not taken place yet for a number of particles in the population. This second event could be the removal of a few protein subunits from the shell, a change in configuration of the protein shell, or it could be (instantaneous?) degradation of RNA into small pieces. Whatever the nature of this second step, however, it must allow instantaneous escape of the RNA because the absence, in the reaction mixtures of short-duration experiments, of particles intermediate in size between whole virus and ATC indicates that it is an all-or-nothing process. Some

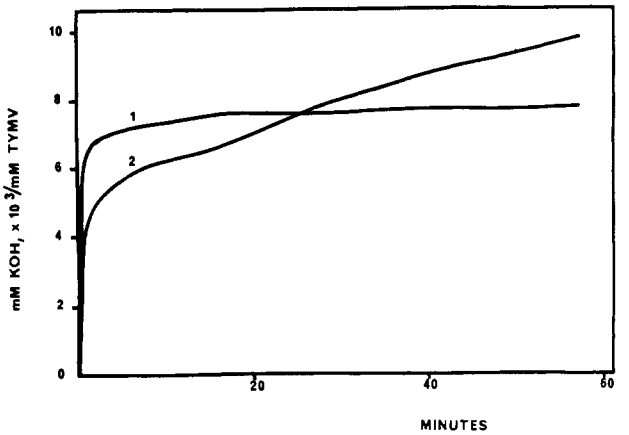


FIG. 8.—Alkali consumption diagrams registered by a *p*H-stat during alkaline degradations of TYMV. All reactions were carried out at 30°, *p*H 11.55, during 60 minutes. The ionic strength was varied. Curve 1, 1.0 M KCl; curve 2, 0.05 M KCl.

insight into this problem could conceivably be gained from a study of the biological properties of undegraded virus isolated from these short duration reaction mixtures and from the physicochemical and biological properties of RNA isolated therefrom. These studies have recently been initiated in this laboratory.

The effect of ionic strength on the direction of the alkaline degradation reaction seems to confirm a role of electrostatic interactions in the maintenance of the physical integrity of TYMV. Alkaline conditions as employed in the above reactions will weaken or break salt linkages such as those proposed for the interaction of RNA and protein. On the other hand it would also enhance electrostatic repulsions. In general, there is a tendency for all the molecules to expand and to break existing linkages. For TYMV this is excellently demonstrated in the reactions where the KCl concentration was 0.05 M (Figs. 3d and 4). The presence of a large amount of neutral salt, such as in the high ionic strength reactions, swamps the charged groups with counterions and, consequently, weakens salt links even more, and also electrostatic repulsions. As a result of this there is a tendency to dissociate the RNA from the protein, but apparently there is not enough electrostatic repulsion to disrupt the protein. The picture given above is undoubtedly an oversimplification. This is already demonstrated in the alkaline reactions with ATC and NTC (Figs. 5 and 6). Even though some of the protein degrades at low KCl concentration and *p*H 11.5, this amount is negligible in comparison with the corresponding reaction with complete virus. RNA apparently helps somehow to disrupt the protein shell under alkaline conditions. One possibility to consider would be an expansion of the RNA inside the virus, which would impose a mechanical stress upon the shell from the inside. It is worth noting that this greater stability of the protein shell of TYMV, in comparison with the virus, is contrary to the situation found in TMV, where the reaggregated protein denatures under far milder conditions than the complete virus.

The alkali consumption diagrams of the low ionic strength reactions (Fig. 7, curves 4 and 5) are more difficult to understand. The decreasing steep portion of the curves is probably indicative of a decrease in titratability of the appropriate groups inside the virus. Unfortunately, as in the experiments with a high KCl concentration, the amount of undegraded virus that can be demonstrated in the sedimentation diagrams

of the reaction mixtures (Fig. 3d) cannot be related directly to the amount of alkali consumed. Presumably, in this particular version of the alkaline degradation also, there is a second step involved to release the RNA. Most likely this is the disruption of the protein shell. Figure 8 shows a gradual further uptake of KOH in these reactions following the steep section of the curve. This further uptake exceeds the levels that can be attained with, for instance, 1.0 M KCl. Curve 2 is the reaction at 0.05 M KCl concentration conducted for 60 minutes and resulting in the formation of predominantly small protein fragments (Fig. 3d[3]). Curve 1 is a reaction under the same conditions but with 1.0 M KCl leading to ATC (Fig. 2b[3]). It seems quite feasible that the gradual KOH uptake noticed in the reactions at low ionic strength is caused by a liberation of masked groups of the protein and the nucleic acid following disruption of the protein shell.

These problems, and several others we have touched upon, require more detailed investigation. These studies are in progress and will be communicated at a later date.

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