ACTION OF UREA ON TOBACCO MOSAIC VIRUS

II. The Bonds between Protein Subunits

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ABSTRACT The rate of urea degradation is not uniform over the tobacco mosaic virus (TMV) rod, as might be expected since the protein subunits appear to be chemically identical. Only two stable intermediates are formed; the four components with sedimentation constants, s_w^{20}, about 80, 120, 140, and 165 correspond to the two intermediates with and without RNA tails. Analysis of the reaction kinetics for the disappearance of whole virus and of the first intermediate was made in terms of a new hypothesis concerning protein bonding.

INTRODUCTION

The TMV rod appears to be a uniform helical assembly of 2130 protein subunits built about a single RNA spiral that lies embedded 40 Å from the rod axis or 20 Å from the hollow core (Franklin, Klug, and Holmes, 1957). The subunits must be very similar in amino acid sequence since tryptic digestion produces the predicted small number of N-terminal amino acids (Schramm and Anderer, 1955; Gish, Ramachandran, and Stanley, 1958) and carboxypeptidase action releases a single C-terminal amino acid, threonine (Harris and Knight, 1955). Nevertheless, neither alkali nor urea degradation proceeds uniformly along the rod. In alkali a succession of stable intermediates appear (Harrington and Schachman, 1956) and the final product comprises from 4 to 5 subunits (Schramm, Schumacher and Zillig, 1955). In urea, although the final product is the single subunit, two intermediates appear and, as reported in Paper I (Buzzell, 1960), the loci of stability vary among the particles. These data all suggest chemical variation among the subunits, as do the chemical aspects of the urea degradation kinetics reported here.

EXPERIMENTAL

Virus. The common strain of TMV was grown on Turkish tobacco plants and isolated by Simmons' technique, as described before (Buzzell, 1960). The isolated virus was stored as a 1.4 per cent suspension in .001 M pH 7.2 versene solution.

Monomer fractions for the three stocks of virus used were estimated from centrifuge data to be 61, 63, and 66 per cent with an uncertainty of about three per cent (Buzzell, 1960). The fractions of fragments larger than monomer were estimated to be 28, 23, and 21 per cent, respectively.

Other materials: The crystalline pancreatic RNase used was from Nutritional Biochemicals Corp. The urea was Fisher certified reagent grade.

Ultracentrifugation. The runs were made, mostly at 24,630 RPM, with the Spinco Model E ultracentrifuge equipped with optics suitable for ultraviolet absorption. As before, film optical density over the range required varied linearly with solution optical density. Photodensitometric traces made with a modified Spinco analytrol are shown in Fig 1. The drop in trace height from air space to solu-

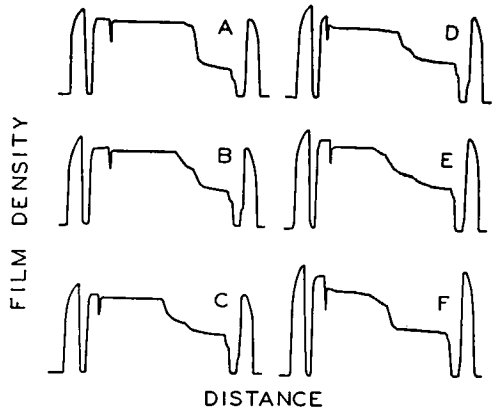

FIGURE 1 Typical absorption profiles for sedimentation runs. *A*, control TMV; *B*, partial degradation of monomer in urea buffered with phosphate—monomer and first intermediate coexist; *C*, maximum concentration of first intermediate achieved; *D*, solution *C* after RNase action; *E*, partial degradation of first intermediate—first and second intermediate coexist; *F*, partial degradation of first intermediate in cacodylate buffer—monomer and free RNA coexist and first intermediate concentration is low. For all traces the two outer peaks correspond to reference holes. Within the cell the sharp dip corresponds to the meniscus. Higher portions of the curve represent lower concentrations of virus material. Sedimentation direction is from left to right.

tion is a measure of non-sedimenting ultraviolet-absorbing material (protein in traces *B*, *C*, and *E;* protein plus degraded RNA in traces *D* and *F*). The trace height corresponding to total concentration is proportional to the difference between the height of the buffer plateau and the uniform plateau in a photograph taken during rotor acceleration. The concentration of a sedimenting component is proportional to the height of the strictly linear portion of the rise in the trace corresponding to the boundary of that component. Three estimates of concentration made for each run were averaged after appropriate correction for dilution during sedimentation.

Electron microscopy. An RCA model EMU-3 and a platinum shadow, carbon replica technique were used (Bradley, 1954; Williams and Wyckoff, 1946). The virus preparations, containing polystyrene latex particles (PSL) of 132 mμ diameter, were sprayed on mica with a low pressure nebulizer and were shadowed with platinum at an angle of 9 to 1.

Procedure for the kinetic experiments. All reactions were at 0°. For the reactions in phosphate and cacodylate buffers only the pH of the buffer is listed. For both buffers at .02 M the apparent pH in 6 M urea, measured with a Beckman glass electrode is 0.4 unit higher, in exact agreement with Burk and Greenberg's value for phosphate measured with the hydrogen electrode (Burk and Greenberg, 1930). For reactions in versene, citrate, and succinate solution the pH of the mixture, adjusted to 7.6, dropped somewhat during the reactions.

To start the reaction virus stock solution was pipetted into the buffered urea solution to give a concentration of 0.2 per cent. To stop the reaction 0.02 M pH 7.2 phosphate buffer was added in the ratio 6:1. Lest the exposed RNA fiber "tails" on the partially degraded virus rods be broken, the diluent was run down the test tube and the layers mixed by gentle twitching. A sample was immediately centrifuged at 4°. In RNase experiments 0.4 μg/liter was allowed to react at 10° for 1½ hours. Solutions to be examined in the electron microscope were dialyzed 1 to 2 hours at 4° against 0.002 M pH 7.2 phosphate buffer and diluted tenfold in distilled water. Stirring was needed to minimize dialysis time, but vigorous stirring led to substantial decrease in the s_w^{20} 130 component. Sedimentation boundaries of dialyzed material were not quite as sharp as controls.

Miscellaneous calculations. Corrections, previously described in detail (Buzzell, 1960), are needed to convert the observed fraction of the various components into mutually commensurate fractions, *i.e.* fractions of original monomer virus degraded. In brief the idea is this. As particles degrade, material is lost from the virus rods and light-scattering decreases. Hence, the optical density of the nonsedimenting free protein and the 40 per cent increase in optical density that would be produced if this material were assembled into virus rods (Fraenkel-Conrat and Williams, 1955) must be added to the observed optical density of the intermediate reaction products, appropriately pro-rated. Corrections also are needed for the contribution of fragment degradation. Virus, fragmented during stock preparation, appears to degrade in urea about as the first intermediate does (Buzzell, 1960). The simplest explanation, and that adopted here, is that unstable regions exposed by a break have already degraded, probably during early stages of the isolation procedure when plant enzymes are present (Kretovitch, 1958).

RESULTS

A. *The RNA Tail.* Because of reports of DNA breakage by shear (Cavalieri and Rosenberg, 1959; Davison, 1959) the handling procedures adopted for the

present experiments were gentler than the previous ones. Possibly as a result[1] a striking change was observed in the sedimentation patterns. The first intermediate of urea action now had a sedimentation constant, s_{w}^{20}, between 130 and 140 rather than 160 and 165 (Buzzell, 1960).[2] The new constant equals that for the first product of alkali degradation at 0°, and the previous equals that for a product appearing much later in alkali (Harrington and Schachman, 1956). Harrington and Schachman concluded that the second product arose from dimerization of the degraded rods since the constants correspond to rods one-third and two-thirds the virus length. This relationship appears fortuitous. RNase consistently and quantitatively converts the slower component (130 to 140 S) into the faster (160 to 165 S) as shown in a typical pair of traces in Fig. 1C and D. RNA degradation is indicated by the increment of expected size in the ultraviolet absorption for the non-sedimenting material and the equal decrement for the first intermediate. Calculations described before (Buzzell, 1960) show that the changes in ultraviolet absorption are those expected for substantially complete removal of the RNA tail from the first intermediate. Also, the same RNase treatment degrades free RNA since the boundary for intact RNA (Buzzell, 1960) disappears completely. RNase has no effect on virus when intact or in the early stages of degradation.

The RNase probably increases the sedimentation rate of the first intermediate by eliminating a viscous drag exerted by the RNA tail (Hart, 1958). A similar viscous drag is exerted by fibers on the bacteriophage T_2 (Bendet, Allison and Lauffer, 1958) and by single-stranded DNA extruded from partly disrupted bacteriophage ϕX174 (Guthrie and Sinsheimer, 1960). No appreciable decrease in solution viscosity can have occurred since the sedimentation rate of residual monomer is unchanged. Dimerization of the first intermediate particles, contingent on removal of their RNA tails, is conceivable but unlikely since the monomer did not aggregate. Furthermore in electron micrographs, as described below, the predominating length for particles with tails is two-thirds, not one-third the monomer length.

B. *The Second Intermediate.* When the first intermediate degrades a second boundary appears with sedimentation constant 80 to 100. After RNase action this boundary becomes very diffuse with a median constant of 120. To decide whether or not the second intermediate arises through degradation from the second end of the rod an electron microscopic search was begun for two-tailed particles. None were found. Far fewer tails than expected ($\sim$ 5 per cent of the rods had tails rather than $\sim$ 50 per cent, the number fraction of unbroken, readily degraded rods originally present) were observed, but tails are made visible only by the metal shadow and probably were present more often than seen. The tails were oriented

[1] It is also possible that the virus stocks used earlier contained RNase.

[2] At a total virus concentration of .03% the sedimentation rates of the various components are independent of one another and of the residual monomer present (Harrington and Schachman, 1953).

TABLE I

ELECTRON MICROSCOPE DATA

DISTRIBUTION OF SIZES OF SINGLE-TAILED PARTICLES

Reaction in 5M urea .02M pH 7.2 phosphate buffer, .0014M $MgCl_2$

Rod length		Reaction time: 9 min. (Number of particles)	Reaction time: 18 min. (Number of particles)
	($m\mu$)		
Long (L)	130–140	3	2
	140–	9	3
	150–	9	3
	160–	10	2
	170–	2	1
	180–	1	1
Short (S)	40–60	2	1
	60–80	2	6
	80–100	0	3
Ratio Short:Long (S/L)		0.12	0.8
Ratio calculated from centrifuge data (S_0/L_0)		0.25	1.0

randomly, so systematic masking of one tail seems unlikely. Also, the length distribution of tailed particles (Table I), which tallies with sedimentation data suggests that destruction had not been extensive.[3] Therefore the second intermediate boundary, reproducibly distinguishable from the first and from RNA, suggests a second stable region in the virus with locus varying from particle to particle.

C. *Kinetic Experiments.* Although obviously more complicated the reaction can be treated as the sequence A → B → C, where A is monomer and B and C, intermediates. This is justified because there appears to be a rate determining step, followed by more rapid degradation, for both monomer and first intermediate. If degradation proceeded uniformly, the population should remain quite homogeneous. According to the Poisson distribution, the probability would be negligible for deviation from the mean size by fifty subunits, a difference undetectable in the ultracentrifuge or electron microscope. The population instead becomes heterogenous, monomer and first intermediate coexisting, and later first and second intermediate (Fig. 1).

Rate constant calculations are illustrated in Table II. Since $A = A_0 e^{-k_A t}$, k_A can

[3] For random breakage the survival of single tailed long particles, L/L_0, is e^{-x}, where x is the average number of tail-breaking events per tail. The survival of one- and two-tailed short particles, S/S_0, is $1 - (1 - e^{-x})^2 \cong 2\ e^{-x}$ for $e^{-x} << 1$. Then $S/L = 2\ S_0/L_0$. S_0/L_0 could be estimated from centrifuge data, since no tail breakage occurred prior to observation, and was ¼ and 1 for the reaction times 9 and 18 minutes, respectively. For these times L, from table I, is 34 and 12, respectively. Therefore S should have been 17 and 24 instead of 4 and 10.

TABLE II

CALCULATION OF RATE CONSTANTS

Reaction in 6 M urea, .033M pH 7.2 cacodylate buffer

Time	Total material				/k_B§	0.07	0.08
	≧A	A	A/A_0 (obs.)	k_A‡	B/A_0 (obs.)	B/A_0 (calc.)	
min.	*per cent*	*per cent*					
0	86	63	1.00		0	0	0
10	55	39*	0.62	0.048	0.26	0.34	0.31
10	48	32	0.51	0.068	0.38	0.34	0.31
15	35	21	0.33	0.074	0.35	0.35	0.32
20	35	22	0.35	0.053	0.30	0.34	0.30
40	10	1	0.02	——	0.14	0.19	0.14
				$\bar{k}_A = 0.061 \pm 0.012$			

A = monomer; B = first intermediate

* The correction for degradation of fragments larger than monomer is described in the Experimental section.

‡ Close agreement for the mean of slopes, $\bar{k}_A$, and the slope of the least squares line was always obtained. This verifies the initial monomer fraction (63 per cent in this case), and also confirms the exponential rate equation.

§ Even if k_A differed from $\bar{k}_A$ by one standard deviation k_B would still lie between 0.07 and 0.08.

be obtained directly. For $k_A \neq k_B$,

$$B/A_0 = \frac{k_A}{k_B - k_A}\left[e^{-k_A t} - e^{-k_B t}\right] \qquad \text{(Moelwyn-Hughes, 1957)}$$

If k_A approximates k_B an estimate of k_B can be obtained from the relation

$$B_{max}/A_0 = \frac{k_A}{k_B} e^{-k_A t}$$

The estimate can then be refined by trial and error until good agreement is achieved between the calculated and observed B/A_0.

Rate constants for the reaction in various media are listed in Tables III and IV. The following relationships are noteworthy.

(*a*) Change from phosphate to cacodylate buffer decreases k_A drastically down to approximate equality with k_B.

(*b*) In cacodylate buffer divalent cation affects k_B alone and strongly. In phosphate buffer increase in divalent cation slowly decreases both constants.

(*c*) With increase in urea concentration from 4 to 5 M, k_A increases rapidly, about as the sixth power of the urea concentration. From 5 to 6 M k_A increases only about as the third power. Up to 6 M urea k_B increases little. However from 6 to 8 M k_B probably increases as the eighth power of urea concentration. Such dependence was found by Lauffer (1943) for virus degradation measured by light-scattering. Consideration of reaction conditions shows that degradation of the first intermediate must have been rate-limiting. Also, as Lauffer pointed out, a rapid initial decline in light-scattering occurred as if a stable intermediate had formed.

TABLE III
ANION EFFECT ON RATE CONSTANTS

Urea concentration	Anion	Anion concentration	pH	k_A‡	k_B‡
4M	phosphate	0.02 M	7.2	0.42 ± 0.14	0.08
5M	phosphate	0.002 M	7.2	0.71 ± 0.28	0.10
		0.02 M	7.2	1.48 ± 0.24	0.10–0.11
		0.02 M	7.2	1.97 ± 0.27	0.15–0.25
		0.02 M	6.7	0.32 ± 0.04	≦0.01
6M	phosphate	0.002 M	7.2	1.27 ± 0.45	0.06–0.08
		0.02 M	7.2	2.75 ± 0.45	0.25–0.35
5M	citrate	0.033 M	*	0.50	0.15
	succinate	0.033 M	*	1.0	0.15
6M	versene	0.001 M	*	0.54 ± 0.14	0.06–0.09
		0.00014 M	*	0.07	≦0.01
6M	cacodylate	0.033 M	7.2	0.061 ± 0.012	0.07–0.08

* Not buffered. pH in urea adjusted initially to 7.6 drops during reaction.
‡ Range of values have same significance as in Table 2.

TABLE IV
CATION EFFECT ON RATE CONSTANTS

Urea concentration	Cation	Cation concentration	k_A	k_B
	A. In .02 M pH 7.2 phosphate buffer			
5M	Mg^{++}	0.00014	1.25 ± 0.50	0.10 –0.20
	Mg^{++}	0.00028	1.23 ± 0.23	0.10 –0.20
		0.0028	0.36 ± 0.15	0.05 –0.07
		0.01	0.34 ± 0.14	0.08 –0.10
	Cu^{++}	0.00024	0.35	———*
6M	Mg^{++}	0.001	2.03 ± 0.42	0.20 –0.30
	B. In .033 M pH 7.2 cacodylate buffer			
6M	Ca^{++}	0.000087	0.040 ± 0.010	0.03 –0.04
	Ca^{++}	0.00035	0.030 ± 0.010	0.006–0.007

* Cu^{++} ion in urea solution has a high OD at 260 mμ so estimates of k_B are unreliable.

DISCUSSION

The observed characteristics of protein dissociation can be accounted for if the following assumptions are made:

(1) A system of paired glutamyl and aspartyl residues provide a large fraction of the binding between protein subunits.

(2) Acid amide pairs are linked by hydrogen bonds.

(3) Carboxyl pairs are linked either by hydrogen bonds or by chelating divalent cations.

(4) The protein subunits vary to some extent in acid amide content.

Acid amide pairs, being planar, can form two N-H—O bonds (Cochran and Penfold, 1952). Carboxyl pairs can form two very strong O-H—O bonds if unionized (Pauling, 1960), and can chelate divalent cations if ionized (Martell and Calvin, 1952).

Urea, being planar and a double acid amide (Vaughan and Donohue, 1952) should rapidly pry apart acid amide pairs. Urea could use both of its amide groups to bind to an ionized carboxyl, since both lengths and angles (Pauling, 1960; Vaughan and Donohue, 1952) are compatible with linearity of the N-H—O bonds (Pauling, 1960). If all N-H—O bonds are equivalent, urea should be half as effective for dissociating carboxyl pairs as for acid amide. Therefore, since saturation for the effect of urea concentration on k_A is approached at 6 M while k_B increases rapidly from 6 to 8 M, an excess of amide pairs may occur at the end of the rod and an excess of carboxyl pairs near the middle.

In absence of adsorbed anion, carboxyl-rich subunits would carry the greater charge density, and greater repulsion would compensate for the lower effectiveness of urea in carboxyl pair dissociation. Phosphate which binds to amide groups (Ubbelohde, 1939) and cationic groups should tend to equalize the charge density. So $k_A \cong k_B$ in cacodylate buffer and $k_A >> k_B$ in phosphate. Also divalent cations, most effective in cacodylate buffer, strongly decrease k_B only. Finally, lowering the the pH should and does drastically decrease k_B. The small decrease of k_A may reflect the increase of $H_2PO_4^-$ relative to $HPO_4^=$.

The characteristics of two other dissociation reactions have particular relevance for the proposed hypothesis. Similar to urea, concentrated acetic acid degrades TMV rapidly at 0°. The acid should act in a fashion analogous to urea but preferably pry the carboxylic pairs apart. Structural considerations indicate that hydrogen bond pairs between amide and unionized carboxyl groups also should be possible, although probably weaker than those between like groups (Cochran and Penfold, 1952; Pauling, 1960; and Vaughan and Donohue, 1952).

Protein dissociation occurs in alkaline solution at pH 10. This dissociation, which is slow, may involve some amide hydrolysis (Leach and Lindley, 1953; Stepka and Takahashi, 1950). As with urea, dissociation appears to start at one end of the virus (Harrington and Schachman, 1956). Initial production of a component with s_w^{20} 133 and subsequent slow conversion of it to one with s_w^{20} 170 resembles formation of the first intermediate in urea with slow hydrolysis of the RNA tail (Bawden and Pirie, 1957). In the final product, "A protein," the 4 to 5 subunits might be held together by chelation, since in very dilute solution A protein will dissociate further (Ansevin and Lauffer, 1959). Furthermore, magnesium ion prevents dissociation of the virus in alkali (Fu-Chuan Chao, 1958). Amides will also hydrolyze at low pH (Stepka and Takahashi, 1950) but unionized carboxyl pairs would form hydrogen bonds and the virus should and does not dissociate (Fraenkel-Conrat and Ramachandran, 1959).

Data on the constitution of the virus are compatible with the hypothesis. Glutamyl and aspartyl residues are quite uniformly distributed along the protein (Anderer, Uhlig, Weber and Schramm, 1960; Tsugita, Gish, Young and Fraenkel-Conrat, 1960). The average amide content is 20 to 22 (Ramachandran and Narita, 1958) or a possible 10 to 11 pairs. Of the average content of 16 carboxyl groups, 10 would be required to counteract the 10 positive groups not neutralized by RNA. The remaining 6 carboxyl groups could be paired. The two hydrogen ions per subunit released when the virus dissociates (Fraenkel-Conrat and Ramachandran, 1959) could correspond to one hydrogen bonded carboxyl pair. Two more carboxyl pairs are suggested by x-ray diffraction data which show that lead ions are bound near both surfaces of the hollow cylindrical rod (Caspar, 1956). Virus as normally prepared binds about one divalent cation per subunit (Loring and Waritz, 1957). Hence, on dissociation the net charge per subunit will increase only from 2 to 3 and a threefold increase in surface area can account for the twofold decrease in electrophoretic mobility (Knight and Lauffer, 1942).

The locus of urea-resistant bonding appears to vary from rod to rod. Particle lengths for the first intermediate product of urea action range from one-half to two-thirds the original monomer length (Buzzell, 1960). If all rods were identical in subunit arrangement, the variation in the number of subunits removed by urea action would, according to the Poisson distribution, be much less than the observed 400 subunits (approximately one-sixth the virus length). Less than 1 per cent of the rods should lose 100 subunits more or less than the average loss of 800 subunits. Furthermore, the resistant section appears to be short since the relative frequencies of particle lengths for the first intermediate do not change appreciably even after the second stage of the dissociation reaction gets underway (Buzzell, 1960). Therefore heterogeneity in amide content may occur in only a small fraction of the subunits and may not be readily observed. Numerous minor products are produced during trypsin digestion of the subunits (Gish, Ramachandran and Stanley, 1958; Woody and Knight, 1959; Ramachandran and Gish, 1959), but some of these products are artifacts and others may be (Gish, 1960; Gish, 1961).

To facilitate discussion the data have been analyzed only in terms of the amide-carboxyl binding hypothesis. The possibility also exists that the anomalies in the urea degradation arise from variation in the RNA base sequence. However, the sequence would have to vary from rod to rod, since the locus of urea resistance is variable. Variability in amino acid sequence for a small number of subunits also is conceivable. Nevertheless these two types of variability, although not ruled out, are not particularly favored by the data, and they are not in good accord with current ideas of genetics.

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