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RNA:PROTEIN AND PROTEIN:PROTEIN INTERACTIONS IN THE VIRIONS OF SOUTHERN BEAN MOSAIC VIRUS

(isometric plant viruses, stabilizing interactions, capsid structure)

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SUMMARY

Virions of southern bean mosaic virus (SBMV), a small RNA-containing isometric plant virus, are stabilized with strong linkages between the coat protein subunits mediated through divalent metal ions (magnesium, calcium) and hydrophobic interactions. Virion treatments interfering with these inter-subunit linkages markedly alter the conformation and stability of the SBMV capsid. The virion capsid is composed of the entire complement of the viral protein, including a small proportion of stable coat protein dimers formed with covalent linkages between the epsilon-amino groups of the lysyl residues. The viral RNA is located within the capsid but 'loosely' contacts only one-third of the total coat protein. A tentative structural model of SBMV virions based upon physico-chemical and morphological considerations is proposed.

INTRODUCTION

Plant virions are aptly suitable and convenient models for examining macro-molecular associations and for elucidating forces governing conformation and stability of biocopolymers. Most plant virions are simple ribonucleoproteins where the principal function of the viral protein is to physically encase and protect the viral genome. A cooperative association of the many intra- and intermolecular forces between the viral RNA and protein is essential for virion construction and stability. In broad terms, such forces are those governing the protein: protein and protein: RNA interactions in the stabilization of virion quaternary structure. Various intramolecular linkages also exist for maintaining the secondary and tertiary structures of the two individual viral components. Primarily, non-covalent linkages, viz., hydrogen bonding, hydrophobic interactions, salt linkages, and a variety of electrostatic interactions are involved in stabilizing plant virions (CASPAR 1963, KAPER 1973). Recent studies indicate that stable covalent linkages other than the disulfide bonds may exist between some of the viral

coat protein subunits, but their identity or significance is unknown (RICE 1974).

Information concerning stabilizing interactions and macromolecular architecture of plant virions has been derived largely from studies with tobacco mosaic virus (TMV). TMV virions are anisometric (15 x 300 nm) and contain a single RNA molecule comprised of 6400 nucleotides. This, in turn, is enclosed by a capsid composed of 2130 identical coat protein subunits. TMV-RNA is embedded tightly in and between the helically oriented coat protein structural units. Approximately 49 protein subunits are arranged per three turns of the helix, each subunit interacting with three nucleotides in tandem (CASPAR 1963). The predominant interactions between TMV-RNA and the capsid protein are salt linkages between the ionized RNA phosphates and the basic protein groups (probably arginine and lysine residues). Additionally, some interaction also exists between the amino groups of viral nucleotides and the coat protein (SCHUSTER and WILHELM 1963). Under appropriate *in vitro* conditions, isolated TMV-RNA and coat protein copolymerize to form virions. This association between TMV-RNA and the coat protein subunits for virion assembly is not a random process but is governed by a specific recognition phenomenon whereby the protein subunits first interact with certain reconstitution initiating sites on the viral genome (ATABEKOV 1973). Such sites contain unique nontranslatable nucleotide sequences which can be recognized by the protein subunits for interaction, but their exact size in terms of the nucleotide number or chemical identity is unknown. Limited evidence indicates that these recognition sites are located towards the 5'-terminus of TMV-RNA, outside the coat protein gene. ATABEKOV (1973) suggested that the genetic information contained in these nucleotide sequences on TMV-RNA for protein recognition differs from that governing the primary structure of polypeptides. Additional studies employing diverse model systems are needed to assess the significance and universality of such a recognition phenomenon between proteins and nucleic acid molecules.

As indicated earlier, virion stability is dependent upon a variety of molecular forces, and probably different virions are stabilized with different sets of interactions. An analysis of the types of stabilizing forces in isometric plant virions (KAPER 1973) indicates that such viruses may be classified into two categories. I. Virions stabilized with strong RNA:protein linkages, and II. Virions stabilized predominantly with strong protein:protein interactions. This classification is based on several physico-chemical virion characteristics of which the sensitivity to sodium dodecyl sulfate (SDS) is most striking. Generally, virions stabilized with RNA:protein linkages dissociate easily with low SDS concentrations (0.005 - 0.05%), while capsid-stabilized virions are stable even in much higher (1 - 5%) SDS concentrations. The following hypothesis was proposed (KAPER 1972) to explain the marked SDS sensitivity of RNA:protein stabilized virions. Consequential to the binding of SDS molecules to proteins through apolar residues, the subunit surface becomes more negative due to the anionic nature of SDS sulfate groups. If sufficient SDS molecules bind to

sites close to cationic amino acid residues attached to RNA through salt linkages, then neutralization of the cationic charge will repel the RNA. Consequently, such virions will disassemble if protein:protein interactions are not strong enough to stabilize the capsid. The use of SDS affords a simple and effective method for a gross analysis of the types of stabilizing interactions essential for virion integrity. As a corollary to this hypothesis, increased SDS sensitivity of the capsid stabilized virions following various treatments may reflect destabilization of the interprotein linkages (SEHGAL and DAS 1975).

Southern bean mosaic virus (SBMV) is a stable, RNA-containing, isometric (diameter 30 nm) plant virus. During the past several years, we have used SBMV as a model for examining the virion-stabilizing interactions. SBMV virions (mW 6.6 million) contain a single RNA molecule (mW 1.4 million) and *ca.* 180 protein subunits (SHEPHERD 1971). In isopynic CsCl gradients, the buoyant density of SBMV virions is 1.36 g/ml, indicating a RNA:protein ratio of *ca.* 1:4 (SEHGAL *et al.* 1970). Upon rate zonal sucrose density-gradient sedimentation, SBMV virions sediment sharply at 115 S, while SBMV-RNA sediment homogeneously at 25 S. Preliminary X-ray diffraction studies suggest that SBMV-RNA is located within the capsid (MAGDOFF 1960). 'Native' SBMV virions are refractory to proteases, nucleases or several protein denaturants, including SDS, suggesting a highly compact and ordered particle configuration. Our studies concerning SBMV structure involved use of selected chemical or physical agents to examine the *in situ* sensitivity of SBMV-RNA, perturbation in the virion stability, and characterization of the partially disassembled viral intermediates. In most studies, the bean strain of SBMV was used, but preliminary investigations with the cowpea strain showed that the dominant stabilizing interactions in these two SBMV strains were identical. Additionally, TMV strain U1 was used in some experiments for comparison.

RNA:PROTEIN INTERACTIONS IN SBMV VIRIONS

Considerable information on the *in situ* spatial orientation of the viral genome and RNA:coat protein interactions in virions can be obtained by comparing the sensitivity of encapsidated *versus* 'free' RNA to selected physical or chemical agents. Several prerequisites exist for such studies: (I) such agents should effectively penetrate through the capsid; (II) the structural integrity of virions or viral components must be fully retained during such treatments; and (III) whenever possible only those conditions should be employed where the 'free' RNA structure mimics that of the encapsidated RNA. Several chemicals (nitrous acid, hydroxylamine, sodium bisulfite) as well as ultraviolet (u.v.) light penetrate through the viral capsid without affecting the virion integrity. Furthermore, spectrophotometric and spectropolarimetric studies reveal that the structure of single-stranded RNA isolated from virions does not essentially differ from *in situ* RNA (TIKOCHONENKO 1969). Our assessment of the extent of RNA:protein linkages in SBMV and TMV virions is based on the biological express-

ivity and chemical modification of the viral RNA's following exposure to selected chemicals or u.v. light.

Nitrous Acid Treatment

Nitrous acid is a strong deaminating reagent for the aminoribonucleotides, converting adenine to hypoxanthine, guanine to xanthine and cytosine to uracil without affecting the phosphodiester linkages in a polynucleotide (SCHUSTER and SCHRAMM 1958, SINGER and FRAENKEL CONRAT 1969). It is also a potent inactivating and mutagenic for intact TMV or its isolated RNA (SCHUSTER and SCHRAMM 1958, SEHGAL and KRAUSE 1968). Considerable evidence exists indicating that deamination of a single aminoribonucleotide of the TMV genome results either in a lethal event or mutagenic conversion (SIEGEL 1965). The chemical modification of guanine possibly is lethal, while adenine or cytosine deaminations are preponderantly mutagenic (SINGER and FRAENKEL-CONRAT 1969).

When SBMV and TMV virions are reacted with nitrous acid (0.25 M sodium nitrite, 0.25 M acetate buffer, pH 4.2, 25°C), inactivation of SBMV is 12-15 times faster than TMV (FIGURE 1).

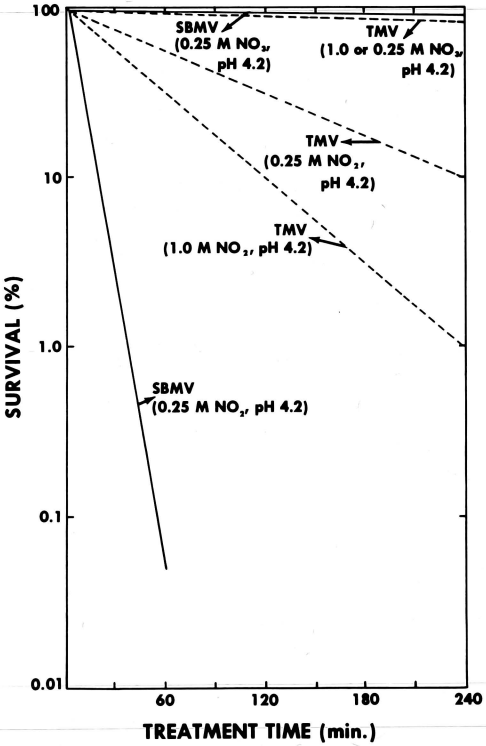

FIGURE 1. Inactivation kinetics of the virions of southern bean mosaic virus (SBMV) and tobacco mosaic virus (TMV) following treatment with nitrous acid (from SEHGAL and SOONG 1972).

The inactivation rate of intact SBMV approximates that of 'free' TMV-RNA reacted with nitrous acid (SEHGAL 1973a, SEHGAL and KRAUSE 1968). TMV inactivation proceeds at slower rate than SBMV even when exposed to fourfold higher concentration of nitrous acid. Infectivity of SBMV or TMV decreases exponentially in relation to treatment time, indicating that virion inactivation results from single hit events. Apparently, more sites (bases) for the deamination reaction are available for nitrous acid reaction in SBMV than in TMV. No decline in infectivity occurred for these viruses in the control experiments where sodium nitrate was substituted for sodium nitrite.

To assess the qualitative or quantitative differences in the chemical reactivity of *in situ* RNAs of SBMV and TMV, a prolonged period (7 days) of exposure to nitrous acid is necessary. This treatment causes no alteration in the serologic properties, sedimentation rate or buoyant density in CsCl of SBMV or TMV virions. Base composition analysis revealed (TABLE 1)

TABLE 1. Base composition of the nucleic acids after treatment (25°C, 168 hr) of the virions of southern bean mosaic and tobacco mosaic with sodium nitrate or nitrous acid at pH 4.2. Base composition was determined with two dimensional thin layer chromatography (from SEHGAL and SOONG 1972)

Bases or	*Untreated*		*Nitrate Treated*		*Nitrous Acid Treated*		*% Deamination with Nitrous Acid*	
Nucleotides	SBMV	TMV	SBMV	TMV	SBMV	TMV	SBMV	TMV
Adenine	26.1	29.8	25.9	29.5	6.7	24.8	74.0	16.9
Hypoxanthine	0.0	0.0	0.0	0.0	19.6	5.0		
Guanine	24.0	24.0	24.1	24.1	7.4	23.4	67.9	2.1
Xanthine	0.0	0.0	0.0	0.0	16.3	0.5		
Cytidylic Acid	22.5	17.5	22.5	17.5	10.2	11.2	54.6	36.0
Uridylic Acid	27.4	28.8	27.5	28.9	39.7	35.2		

that deamination of adenine, guanine and cytosine residues of encapsidated SBMV-RNA was, respectively, 4, 32 and 1.5 times faster compared to *in situ* TMV-RNA (SEHGAL and SOONG 1972). Two possibilities may explain these differences. SBMV coat protein subunits are bound 'loosely' compared to TMV, permitting thereby a more rapid diffusion of nitrous acid molecules through the capsid. Or most of the reactive amino groups of *in situ* SBMV-RNA are 'free' and accessible for nitrous acid reaction,

whereas such groups are intimately linked with the coat protein in TMV or are otherwise protected from a similar reaction. In intact TMV, cytosine deamination was twice compared to adenine, while guanine deamination was negligible (SEHGAL and SOONG 1972). Furthermore, other studies (CASPAR 1963, SINGER and FRAENKEL-CONRAT 1969) suggest that purine-containing sequences on TMV-RNA are preferentially linked to the coat protein. Apparently, availability of reactive sites rather than the diffusion of nitrous acid molecules through the viral capsid is the primary factor contributing to RNA deamination. Consequently, the increased *in situ* reactivity of SBMV-RNA with nitrous acid is due to a greater availability of the amino groups of viral ribonucleotides. This indirectly implies that a lesser degree of RNA:protein copolymerization exists through the amino groups of the viral ribonucleotides in SBMV compared to TMV.

A direct comparison of the nitrous acid reaction between the encapsidated and 'free' SBMV-RNA revealed, unexpectedly, that SBMV virions were inactivated twice as fast as 'free' RNA although the extent of the base deamination was identical (SEHGAL 1973a). Nitrous acid treatment results in an increased binding of the coat protein subunits to SBMV-RNA. Consequently, inactivation of SBMV virions with nitrous acid results from lethal deamination and inability of some of the treated virions to uncoat on the leaf surface to initiate infection, while SBMV-RNA inactivation is caused primarily by lethal deamination. Intact TMV is inactivated 5 - 6 times slower with nitrous acid compared to TMV-RNA which is due to protection by the coat protein of some of the sites on the viral genome involved in lethal deamination (SIEGEL 1965, SEHGAL and KRAUSE 1968). When attempts were made to isolate SBMV-RNA from nitrous acid-reacted SBMV with phenol and SDS followed by ethanol precipitation, highly fragmented or polydispersed RNA was obtained, while the same procedure yielded high molecular weight (25 S) SBMV-RNA from the untreated virions. In these experiments, RNA breakage occurred upon treating the nitrous acid-reacted SBMV with phenol and SDS, prior to the precipitation of RNA with ethanol (JEAN and SEHGAL 1976). Macromolecular SBMV-RNA (25 S) was isolated easily, however, from untreated or nitrous acid-reacted SBMV with the virion dissociative medium (SEHGAL 1973b). For nitrous acid-reacted SBMV, RNA cleavage may occur at sites where it is cross-linked with the viral protein (TIKOCHONENKO 1969). Had only the phenol-SDS method been used to isolate RNA from nitrous acid-reacted SBMV, we would have wrongly concluded that nitrous acid causes *in situ* depolymerization of SBMV-RNA. These results demonstrate that reliance on a single procedure for isolating RNA from 'native' virions and after exposure to agents inducing increased RNA:protein cross-linking can lead to erroneous interpretations.

Reaction with Sodium Bisulfite or Hydroxylamine

In contrast to nitrous acid, a non-specific deaminating reagent for aminoribonucleotides, sodium bisulfite deaminates only cytosine residues in the polynucleotides (SHAPIRO, COHEN and SERVIS 1970). Similarly, hydroxylamine at pH 6.1, specifi-

cally reacts with cytosine, while at pH 9.1, it reacts only with uracil (SCHUSTER and WITTMANN 1963). The inactivation kinetics of encapsidated and 'free' RNAs of SBMV and TMV upon treatment with these two chemicals are shown in FIGURE 2.

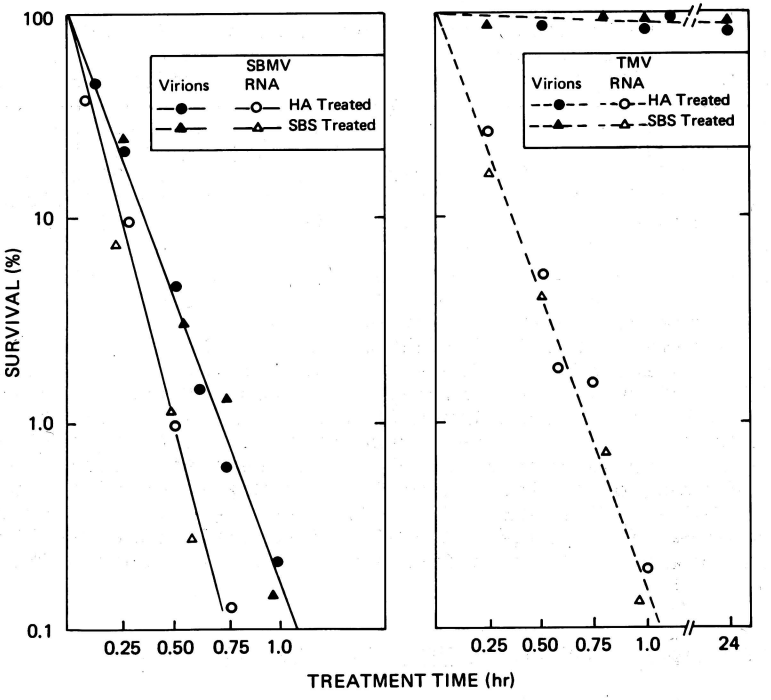

FIGURE 2. Inactivation of SBMV, TMV or their isolated RNAs following treatment (25°C) with hydroxylamine-HCl (HA) or sodium bisulfite (SBS). The reaction of virions or viral RNAs with HA (1.0 M) was performed at pH 6.1, while with SBS (1.0 M) was done at pH 5.1. (from DAS 1974)

Intact SBMV and isolated TMV-RNA are inactivated identically with sodium bisulfite or hydroxylamine. The inactivation rate for 'free' SBMV-RNA was only slightly faster than that of the intact SBMV. The exponential decline in the infectivity of SBMV, SBMV-RNA and TMV-RNA in relation to dose (treatment period) indicates that inactivation occurs with single hit events. TMV virions were essentially resistant to these treatments. The data on the reactivity of *in situ* or isolated RNAs of SBMV and TMV with sodium bisulfite showed (DAS 1974) that cytidine residues of encapsidated or 'free' SBMV-RNA and 'free' TMV-RNA were deaminated 2.5 - 3 times faster compared to RNA within TMV virions. These observations indicate a greater accessibility of the cytosine residues within SBMV virions for reac-

tion with sodium bisulfite or hydroxylamine compared to intact TMV and are consistent with the results obtained with nitrous acid treatment.

Ultraviolet Light Irradiation

Some measure of the extent of protection conferred by the capsid to the viral genome can be ascertained by examining the relative sensitivity of 'isolated' and encapsidated RNA to u.v. irradiation at 254 nm. For example, TMV-U1 virions are inactivated five times slower with u.v. light than TMV-U1 RNA, TMV-U2 RNA or TMV-U2 virions. It has been suggested (SIEGEL, WILDMAN and GINOZA 1956) that, whereas the types of RNA:protein linkages in TMV-U1 protect virions from u.v. irradiation, they do not protect intact TMV-U2. It is commonly believed that u.v. sensitivity differences between TMV-U1 and TMV-U2 are not due to a shielding effect *per se* of their respective proteins but signifies a more efficient energy transfer mechanism in TMV-U1 where the u.v. energy absorbed by RNA is transmitted rapidly to the capsid protein (STREETER and GORDON 1968). U. v. irradiation of intact SBMV or SBMV-RNA caused identical inactivations (SEHGAL 1973a). Furthermore, the extent of photoreactivation estimated for the irradiated SBMV or SBMV-RNA at two survival levels was identical. These observations are consistent with similar studies on isometric RNA phages where coat protein conferred no protection against absorption or migration of the u.v. energy (FURUSE and WATANABE 1971). Apparently, the type and extent of RNA:protein linkages in SBMV and several isometric RNA phages are markedly different from that of the rod-shaped TMV virions.

PROTEIN:PROTEIN INTERACTIONS IN SBMV

Observations presented in the preceeding section indicate that a majority of the amino groups of SBMV-RNA are not linked to the viral protein or, if such bonds do exist, these are of a 'labile' nature. Alternatively, the RNA:protein interactions in SBMV may be in the form of salt-linkages between the ionized RNA phosphates and cationic centers of the viral protein (TIKOCHONENKO 1969). In view of the proposed mechanism of neutralization of such RNA:protein linkages with SDS (KAPER 1972), it appears unlikely that such interactions are involved preponderantly in the 'native' virions since SBMV is markedly resistant to SDS. Consequently, the role of protein:protein interactions in SBMV stability was investigated.

SBMV Disassembly *in vitro*

An experimental system was developed to disassemble SBMV virions and to standardize procedures for separating the two viral components. This involved exposure (5°C, 1 hr) of the virions to a dissociative medium comprising of 0.1 M phosphate buffer, pH 7.5, 1 mM ethylenediamine tetraacetic acid (EDTA), 50µg/ml bentonite and 1% SDS, followed by rate zonal sucrose density-gradient sedimentation (FIGURE 3). An analysis of the factors contributing to SBMV dissociation revealed

that alkaline buffers (pH 7.5-9.1) of moderate ionic strength (0.1 M) in presence of other additives were most suitable (SEHGAL 1973b). When SDS was eliminated from the virion dissociative medium, SBMV was degraded into a 47-50 S subviral entity sedimenting faster than SBMV-RNA but much slower than the intact SBMV. Subsequently, an analysis of the virion disassembly process was made under more controlled experimental conditions.

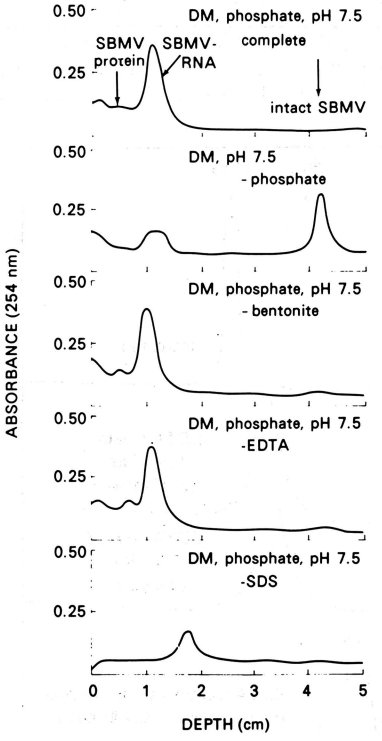

FIGURE 3. Effect of various components of the dissociative medium (DM) on *in vitro* disassembly of SBMV virions. The components of the complete DM included 0.1 M phosphate, pH 7.5, 1 mM EDTA, 50 µg/ml bentonite and 1% SDS. The treated virions (1 hr, 5°C) were analyzed with rate zonal sucrose density-gradient (5-35%) sedimentation and monitored with an ISCO fractionator at 254 nm (from SEHGAL 1973b).

Partial Virion Disassembly and Subviral Intermediates

Exposing SBMV virions to 10 mM EDTA in 0.1 M phosphate, pH 7.5, caused it to change rapidly from a sharply sedimenting 115 S entity to a broadly but homogeneously sedimenting 100 S form (FIGURE 4). Colorimetric RNA and protein determinations

or u.v. light absorption spectrophotometry revealed no differences between the EDTA-treated (100 S-SBMV) or untreated (115 S) virions (HSU, SEHGAL and PICKETT 1976). Apparently, the reduction in the sedimentation rate for EDTA-treated SBMV is caused by a conformational change -- a slight 'swelling' or relaxation of the virion capsid. In this 'relaxed' state SBMV virions are rendered sensitive to proteases, ribonuclease and SDS. This conformational change represents the first step preparatory to virion disassembly. Upon exposure to 0.4 M NaCl or 100µg/ml bentonite, the EDTA-treated virions (100 S-SBMV) readily degrade, yielding the 47-50 S subviral entity and a considerable amount of the viral protein remaining at the meniscus in the sucrose density-gradient column.

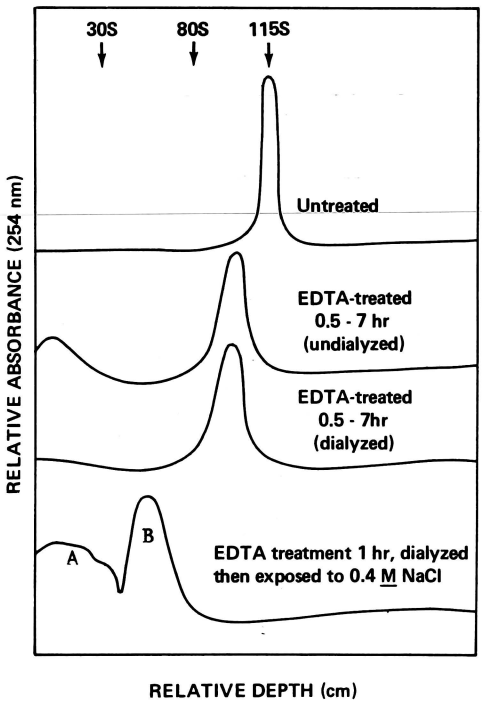

FIGURE 4. Sucrose density-gradient sedimentation profiles of the variously treated SBMV virions. The top profile shows the sedimentation behavior of untreated SBMV (115 S). The next two profiles are for EDTA-treated virions (100 S) before and after dialysis against 0.02 M phosphate buffer, pH 7.5. The bottom profile shows degradation of EDTA-treated SBMV into RNA 'free' protein (A) and the 47-50 S subviral entity (B).

Chemical analyses revealed that the 47-50 S subviral entity was a nucleoproteinaceous complex of intact SBMV-RNA and

approximately one-third of total virion protein. Exposure to 0.1% SDS, poly (G) or Proteinase K dissociates SVE (subviral entity) into SBMV-RNA and the residual protein. From these observations we proposed (SEHGAL and SINHA 1974) that SBMV protein subunits are not linked identically to the viral genome with *ca.*, two-thirds of the total protein showing none or very little interaction with SBMV-RNA.

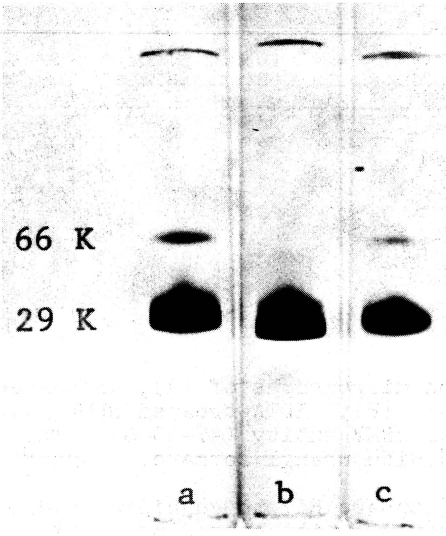

FIGURE 5. Electrophoresis of SBMV protein in 5% polyacrylamide gels containing 0.1% SDS. (a) Virions dissociated with heating (100°C, 5 min) in 1% SDS and 0.1% beta-mercaptoethanol; (b) protein sample from position A of the bottom profile in FIGURE 4; (c) subviral SBMV entity (47-50 S), sample from position B of the bottom profile in FIGURE 4.

Polyacrylamide gel electrophoretic patterns of viral protein isolated from intact SBMV or virions after various degradative treatments are shown in FIGURE 5. Intact SBMV dissociated with heating (100°C, 5 min) in SDS-beta mercaptoethanol or EDTA-treated SBMV (100 S) exposed (5°C, 1 hr) to SDS-mercaptoethanol yielded two protein bands, a minor protein (mW 66,000) and a major protein (mW 29,000). Quantitative determinations showed that in virions the minor protein was present in one-tenth the amount of the major protein. Both proteins also were associated with the 47-50 S SBMV entity. However, the bulk of SBMV protein released from EDTA-treated SBMV (100 S) following exposure to 0.4 M NaCl showed presence of only the major protein (mW 29,000). These results indicate that the minor protein and a small proportion of major protein subunits are linked to SBMV-RNA.

Striking morphological alterations were also observed in SBMV upon EDTA treatment and exposure of the EDTA-treated

virions to 0.4 M NaCl or bentonite to yield the 47-50 S subviral entity (FIGURE 6). Untreated SBMV virions possessed a regular or discrete structure and prevented the negative strain from penetrating the core. EDTA-treated SBMV virions (100 S) were spherical and of uniform size but showed a marked penetration and accumulation of the negative stain within the virion, indicative of physical alterations in the SBMV capsid.

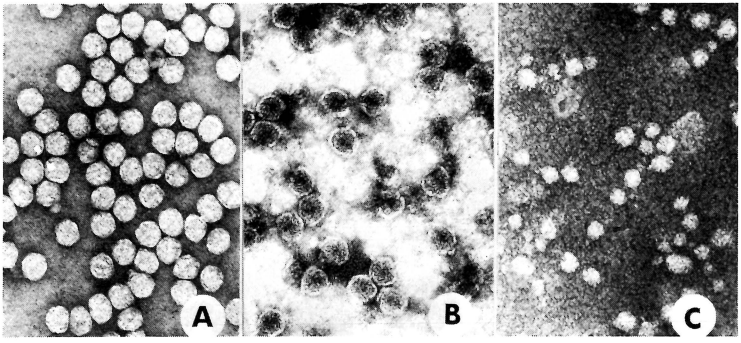

FIGURE 6. Electron micrographs of (A), untreated SBMV virions (115 S); (B), EDTA-treated SBMV (100 S); and (C), subviral SBMV entity (47-50 S). The samples were stained with uranyl formate. Magnification, 110,000

Exposure of bacteriophage R17 to guanidine hydrochloride causes similar changes in its capsid, permitting a penetration of the negative stain within the virion core (O'CALLAGHAN *et al.* 1973). Particles of the 47-50 S subviral entity were of variable size (diameter 15-25 nm), appeared 'loose' or deformed and lacked a discrete outline. These morphological alterations in the virion conformation and structure substantiate results obtained with the physico-chemical analysis.

Role of Divalent Metal Ions in Virion Conformation and Capsid Stability

The striking effect of EDTA causing conformational alterations (100 S → 115 S) and reduction in the capsid stability suggests that some metal ions are essential for SBMV structure. Therefore, the effect of exogenous addition of metal ions on the sedimentation rate and stability of EDTA-treated SBMV was examined. Additionally, the metal ion content of SBMV before and after EDTA treatment was determined with atomic absorption spectrophotometry.

Marked alterations occurred in the sedimentation rate of EDTA-treated SBMV following exposure to selected divalent cations (FIGURE 7). Although the divalent metal ions magnesium, calcium, manganese caused only a slight increase in the sedimentation rate of EDTA-treated SBMV (100 S→105 S), exposure to a mixture of these cations in appropriate amounts resulted in a complete 100 S → 115 S transformation. Such

virions also became resistant to proteases, SDS and ribonuclease (HSU, SEHGAL and PICKETT 1976). Exposure to spermine or spermidine also caused the 100 S →115 S change in EDTA-treated SBMV, but these virions remained sensitive to the enzymic attack or SDS. Apparently, the mechanism of interaction of spermine or spermidine with EDTA-treated SBMV is basically different from that of the metal ions.

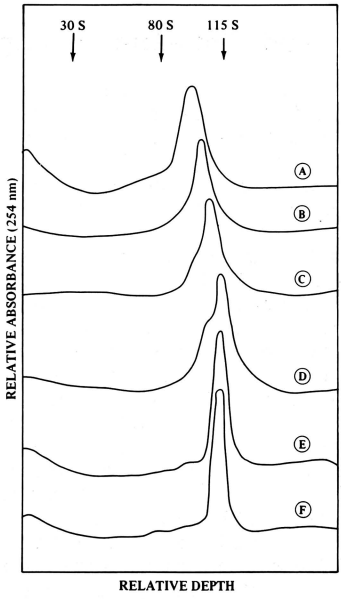

FIGURE 7. Sucrose density-gradient sedimentation profiles of EDTA-treated SBMV (100 S) following exposure to metal ions or polymines. (A) 100 S-SBMV. (B), 100 S-SBMV plus magnesium, 12 hr (C) 100 S-SBMV plus calcium, 12 hr (D) 100 S-SBMV plus magnesium and calcium, 12 hr (E) 100 S-SBMV plus magnesium, calcium and manganese, 12 hr or magnesium and calcium, 4 days (F) 100 S-SBMV plus spermine or spermidine, 12 hr (from HSU, SEHGAL and PICKETT 1976).

Metal ion analysis show (HSU, SEHGAL and PICKETT, 1976) that magnesium and calcium are the principal divalent cations associated with SBMV virions along with minor amounts of copper and zinc. Other divalent metal ions, including lead, iron, cadmium were absent. Approximately 120 magnesium and 80 calcium ions were bound firmly with each virion. EDTA

treatment removed essentially all divalent metal ions associated with SBMV, but exposure of EDTA-treated SBMV to the metal ions mixture with excess of the ions removed with dialysis resulted in a binding of 120 magnesium and 60 calcium ions to each virion. The mechanism by which divalent metals confer stability to SBMV capsid is unknown, but our data suggests involvement of these cations in the protein quaternary (protein:protein interactions) structure. This suggestion is in accord with the hypothesis (BANCROFT 1970) that divalent cations form bridges between the carboxylate ions of adjoining protein subunits to maintain compactness of small isometric viruses.

Effect of Freezing and Thawing on Capsid Stability

Sensitivity of virions to freezing and thawing in water affords another approach in examining the extent of protein:protein interaction. This treatment degrades capsid stabilized viruses into protein and RNA, while virions stabilized with RNA:protein linkages remain stable (KAPER 1973). Irreversible alterations occurred in virion conformation and capsid stability when SBMV was frozen-thawed in water. Such virions sedimented at 100 S and became sensitive to proteases, ribonuclease and SDS (SEHGAL and DAS 1975). Virions frozen in presence of several cryoprotectants e.g., magnesium ions, sucrose, sodium chloride, etc., showed no freezing damage, but addition of these chemicals after the freeze:thaw treatment had no protective effect. There is considerable evidence that freezing and dehydration affects biomolecules similarly (DARBYSHIRE 1974). Removal of 'bound' water from SBMV virions seemingly causes irreparable damage to the virion capsid with a concomitant reduction in the extent of protein:protein interactions. Apparently, freezing and thawing in water abolishes or weakens some of the linkages involved in protein:protein interactions, but complete dissolution of such bonds does not occur since this would have caused complete virion dissociation. The affects of freeze:thaw and EDTA treatments on the conformation and stability of SBMV virions are summarized in FIGURE 8.

Effect of 2-Chloroethanol on SBMV Capsid Stability

Hydrophobic interactions between the protein subunits of f2 bacteriophage play a dominant role in stabilizing its capsid (MATHEWS and COLE 1972). Since 2-chloroethanol preferentially destabilizes the hydrophobic interactions in proteins without affecting the alpha-helical structures (WEBER and TANFORD 1959, TIMASHEFF 1970), this chemical was employed to assess the role of the hydrophobic interactions in stabilizing the SBMV capsid. Exposure to 5-10% chloroethanol in 0.02 M phosphate buffer, pH 7.0, causes no change in the serologic properties, sedimentation rate or infectivity of SBMV virions. The chloroethanol-treated virions, however, are rapidly degraded, yielding the major and minor protein when electrophoresed in 5% polyacrylamide gels containing 0.1% SDS (FIGURE 9), while untreated SBMV or virions exposed

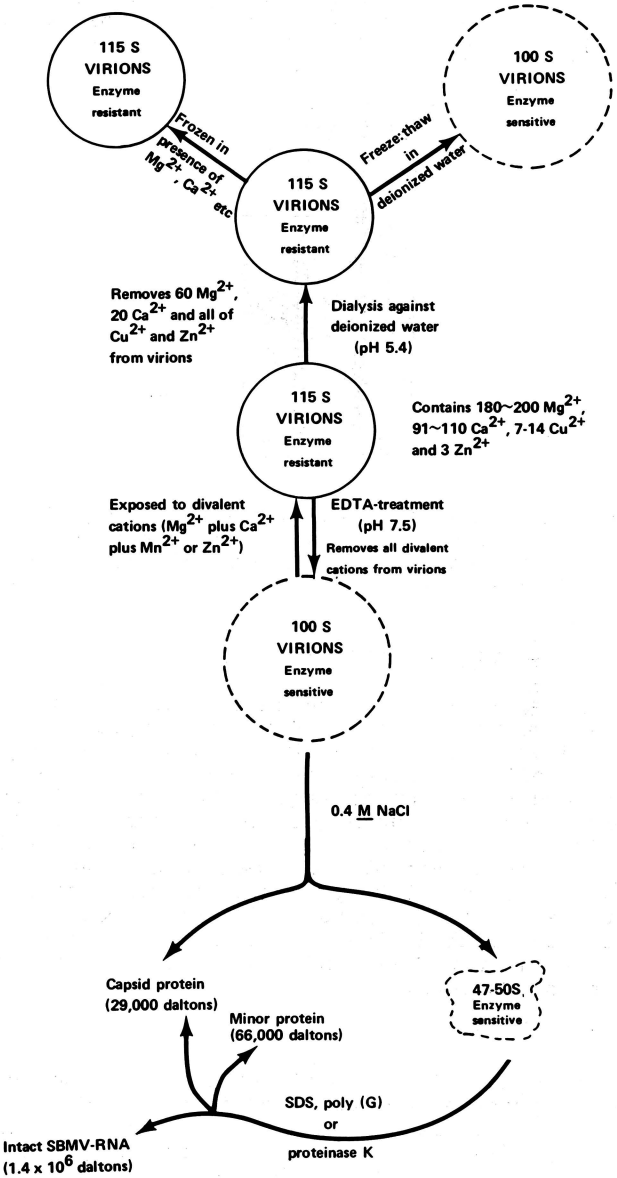

FIGURE 8. A schematic representation of the effects of freezing and thawing and EDTA treatments on the SBMV virions.

to 0.1% SDS remain intact at the origin. This destabilization of the SBMV capsid integrity with 2-chloroethanol resembled that of the virions treated with EDTA or frozen-thawed in deionized water.

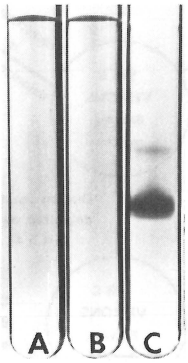

FIGURE 9. Electrophoresis of (A) untreated SBMV, (B) Virions exposed (1 hr, 5°C) to 0.1% SDS, and (C) 2-chloroethanol (10%) treated SBMV in 5% polyacrylamide gels containing 0.1% SDS.

THE VIRAL PROTEIN

Polyacrylamide gel electrophoretic analysis of SBMV protein prepared with a variety of isolation procedures invariably showed presence of the minor (mW 66,000) and major (mW 29,000) proteins. Furthermore, succinylation or carboxymethylation and reduction of intact SBMV or its isolated protein yielded two bands upon gel electrophoresis. The minor protein remains fully stable in the presence of several reducing agents (dithiothreitol, beta-mercapthoethanol, glutathione) or upon heating (100°C, 15 min) in SDS-urea solution containing these reducing agents, indicating that it was not a simple disulfide-linked dimer of the major protein. The identity of the minor protein and its location in the virions was determined as follows.

Serology

Polyacrylamide gel sections containing the separated minor and major protein were embedded in agar and reacted against antiserum prepared against the major protein and the normal serum (FIGURE 10). Strong and confluent precipitin bands developed upon reaction of the minor and major proteins with the antiserum, while a faint reaction developed against the normal serum; the latter was probably due to the presence of residual SDS in the gel sections containing the two antigens. These observations indicate existence of antigenic similarities between the minor and the major protein.

Amino Acid Composition

The data on amino acid composition of the electrophoretically separated minor and major SBMV protein determined with automated cation exchange chromatography (TABLE 2) showed that the two proteins were chemically similar.

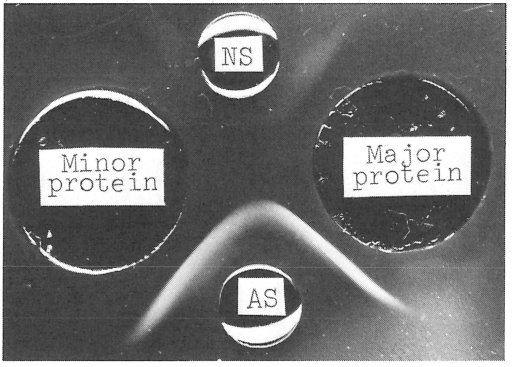

FIGURE 10. Immunodiffusion patterns employing polyacrylamide gel sections containing the minor and major proteins upon reaction with normal serum (NS) and antiserum (AS) prepared against the major protein.

Reaction with Dimethyl Adipimidate

Another approach concerning identification of the minor SBMV protein was based on the induced polymerization of the virion or the isolated major protein with dimethyl adipimidate and analysis of the products with gel electrophoresis (FIGURE 11). Dimethyl adipimidate introduces covalent cross-links between the epsilon-amino groups of lysyl residues (HARTMAN and WOLD 1967). Dimethyl adipimidate-treated virions following dissociation with heating (100°C, 5 min) in SDS-beta mercaptoethanol showed presence of several high molecular weight protein polymers along with a slight increase in the amount of the minor protein (mW 66,000). Similar treatment of the major protein monomers showed appearance of a protein migrating to the position of the minor protein, but other high molecular weight protein polymers were absent. Possibly, alterations in the conformation of the major protein following isolation from virions caused masking of some of the lysyl residues which were otherwise exposed on the virion surface. Consequently, high molecular weight protein polymers were not engendered when isolated protein monomers were treated with dimethyl adipimidate. The protein polymers resulting with dimethyl adipimidate treatment of virions or the minor protein fraction formed with similar treatment of the major protein monomers were fully stable in presence of reducing agents or heating in SDS-urea solution containing these reducing agents. These results suggest that the minor protein and other protein polymers are formed with cross-linking of the major protein monomers. Conformational changes due to this cross-linking may be responsible for the observed discrepancy in the molecular weight of

the minor protein which is slightly more than that expected for a major protein dimer. Results similar to dimethyl adipimidate treatment were obtained when virions or the major protein monomers were exposed to formaldehyde or glutaraldehyde.

TABLE 2. Amino acid composition of major and minor protein components of southern bean mosaic virus. The proteins were separated with preparative gel electrophoresis. Tryptophan was not determined.

Amino acid	Mole %	
	Minor protein	Major protein
Aspartic acid	7.9	8.5
Thereonine	11.4	10.2
Serine	8.6	9.6
Glutamic acid	8.4	8.8
Proline	4.7	4.4
Glycine	4.6	5.6
Alanine	6.7	6.7
Cystine	1.4	1.5
Valine	6.6	6.2
Methionine	3.0	2.6
Isoleucine	3.5	3.6
Leucine	11.0	10.3
Tyrosine	5.6	5.5
Phenylalanine	2.2	2.5
Histidine	1.0	1.5
Lysine	3.6	3.8
Arginine	9.9	8.8

The data from serologic studies, chemical composition and induced polymerization with dimethyl adipimidate suggest that the minor SBMV protein is a *stable* dimer of the major protein subunits.

Location of the Minor Protein in Virions

Virions of SBMV were radioiodinated using NaI^{125}, passed through an anion exchange column and then sedimented with sucrose density-gradient sedimentation. The virion integrity

was retained fully during these treatments. Following dissociation of the iodinated virions and polyacrylamide gel electrophoresis, it was observed that the radioactivity was incorporated into the minor protein (8,000 cpm/min) and the major protein (64,000 cpm/min) fractions. The relative proportion of radioactivity incorporated into the minor compared to the major protein (1:8) compares favorably with the quantitative estimation based on the staining intensity of these two protein fractions. These results indicate that the minor and major SBMV protein are apparently present on the virion surface.

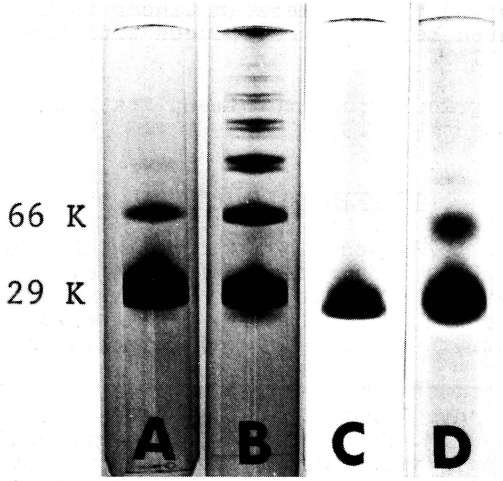

FIGURE 11. Polyacrylamide gel electrophoretic analysis of SBMV virions or major protein following treatment with dimethyl adipimidate (DMA). (A), Untreated virions, dissociated with heating (100°C, 5 min) in 1% SDS and 0.1% beta-mercaptoethanol; (B) DMA-treated virions, dissociated; (C) Untreated, major protein; (D) DMA-treated major protein.

A TENTATIVE STRUCTURAL MODEL FOR SBMV VIRIONS

We propose the following structural model for SBMV virions. The SBMV capsid is composed of the entire complement of the viral protein, including about 160 coat protein monomers and *ca.* ten to twelve *stable* dimers. Intersubunit linkages mediated with divalent metals (magnesium, calcium) and hydrophobic interactions between the neighboring protein subunits are responsible for virion stability and compactness. The viral genome is protected efficiently and located within the SBMV capsid. SBMV-RNA is linked 'loosely' with a few coat protein monomers and all of the dimers, with little or no association existing between SBMV-RNA and the bulk of the capsid protein. Based on the icosahedral symmetry of SBMV particles, it is tempting to propose that the protein subunits to which SBMV-RNA is linked may be present as pentamers at the twelve vertices of

the virion capsid. The remainder of the SBMV coat protein is arranged as clusters of hexamers at the twenty faces of the icosahedron. Such a structural SBMV model is consistent with the basic geometrical design necessary for the construction and stability of isometric virions.

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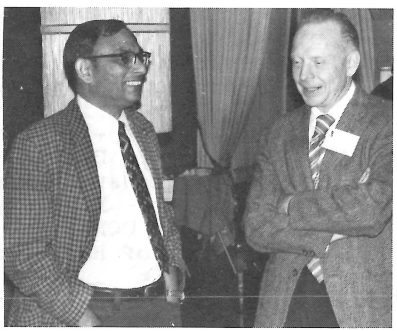

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