Characterization of cowpea chlorotic mottle virus and its assembly.

B.J.M. Verduin

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ERRATA

page 7,	line 34	*from isolated* should read	*assembled from isolated*
" 12,	" 20	recognizes " "	recognize
" 20,	" 12	creats " "	creates
" 24,	" 9	due a " "	due to
	" 12	scatering " "	scattering
" 27,	" 3	-42 to 84kJ/mol " "	-42 to -84 kJ/mol
		4 kJ/mol " "	-4 kJ/mol
	" 38	are " "	is
" 31,	" 3	method Lowry " "	method of Lowry
" 37,	" 13	CO^{2+} " "	Co^{2+}
" 37,	Table 3.1 line 16	completely RNA should read	completely degraded RNA
" 38,	Fig. 3.4, line 5	+ 10 µg should read	+ 5 µg
" 39,	line 37	B to F should read	D to F
" 40,	Fig. 3.5, line 9 + 12	60°C should read	60°
" 48,	line 2	nuclei acid should read	nucleic acid
" 56,	" 20	dln/c " "	dln c
" 57,	" 5 + 11	θ " "	(θ)
" 60,	Fig. 5.1, line 5	right " "	left
" 64,	line 9	this two type " "	these two types
" 67,	" 23	similar for to " "	similar to
" 74,	" 28	$^{35}S/^{32}P$ " "	$^{35}S/^{32}P$
" 93,	" 34	isolated gel-filtration should read	isolated by gelfiltration
" 97,	" 5	, should read	.
" 101,	" 11	ia " "	is
" 103,	" 16	5' " "	3'
" 107,	" 8-9	proteins " "	protons
" 107,	" 20	Nickersona " "	Nickerson

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Characterization of cowpea chlorotic mottle virus and its assembly

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 3 november 1978
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

ISN 104275-03

STELLINGEN

1. De wijze waarop Darwish en medewerkers de weerstand van tinsulfide films op tin meten is onjuist en hun interpretatie van de resultaten is twijfelachtig.

   Darwish, N.A., Khairy, E.M., Allan, A.M. en Mahgoub, A.E. (1977). Journal of Electroanalytical Chemistry 83, 99-109.

2. De procedure voor het hechten van nucleïnezuren aan geactiveerde polysacchariden zoals beschreven door Potuzak en Dean is niet geschikt voor ribonucleïnezuren.

   Potuzak, H. en Dean, P.D.G. (1978). Nucleic Acid Research 5, 297-303.

3. Met het meten van de polarisatiegraad als funktie van de tijd kan geen onderscheid gemaakt worden tussen de twee modellen voor tijdsafhankelijke depolymerisatie van de fluorescentie, die door Dale en medewerkers zijn voorgesteld.

   Dale, R.E., Chen, L.A. en Brand, L. (1977). Journal of Biological Chemistry 252, 7500-7510.

4. De bepaling van de kernspin relaxatietijden T_1 en T_2 van water protonen in weefsel is niet geschikt voor de diagnose van kankergezwellen en tumoren.

   Swartz, H.M. (1978). Journal of Magnetic Resonance 29, 393-396.

5. Het ontbreken van een verklaring voor het verschil tussen twee zuur-base titratie curven van *brome mosaic virus* in het artikel van Pfeiffer en Durham maakt de conclusies ongeloofwaardig.

   Pfeiffer, P. en Durham, A.C.H. (1977). Virology 81, 419-432.

6. Het gebruik van de extinctiecoefficiënt $E^{1\%}_{260\ nm} = 338{,}4$ voor de bepaling van de concentratie van intact *cowpea chlorotic mottle virus* ribonucleïnezuur is onjuist.

   Bancroft, J.B., Hiebert, E., Rees, M.W. en Markham, R. (1968). Virology 34, 224-239.
   Adolph, K.W. en Butler, P.J.G. (1977). Journal of Molecular Biology 109, 345-357.

7. De conclusie van Adolph en Butler dat bij hun methoden om *cowpea chlorotic mottle virus* te assembleren geen divalente ionen zijn vereist, in tegenstelling tot de methode van Bancroft en Hiebert, volgt niet uit hun resultaten.

   Bancroft, J.B. en Hiebert, E. (1967). Virology 32, 354-356.
   Adolph, K.W. en Butler, P.J.G. (1977). Journal of Molecular Biology 109, 345-357.

8. Het is niet alleen wenselijk de experimenten over de specificiteit van de reaktie tussen het RNA en het manteleiwit van *cowpea chlorotic mottle virus* te ondersteunen met kinetische gegevens van de associatiereaktie, doch ook gebruik te maken van de heterologe RNAs met eenzelfde moleculaire massa.

   Adolph, K.W. en Butler, P.J.G. (1977). Journal of Molecular Biology 109, 345-357.
   Driedonks, R.A. (1978). Proefschrift, Universiteit van Leiden.
   Herzog, M. en Hirth, L. (1978). Virology 86, 48-56.

9. Scheiding van de nucleoproteine deeltjes van komkommermozaïekvirus na evenwichtscentrifugering van 28 mg virus in 1,3 g RbCl opgelost in 2,2 ml 0,02 M natriumfosfaat, 0,01 M EDTA pH 7,0 moet worden betwijfeld.

   Lot, H. en Kaper, J.M. (1976). Virology 74, 223-226.

10. Onderzoek dient te worden beoordeeld op basis van de probleemstelling en de oplossing daarvan en niet op basis van het aantal publikaties dat jaarlijks verschijnt en het aantal malen dat deze publikaties door andere onderzoekers geciteerd worden.

11. Het verwisselen van de termen peuterspeelzaal en crèche dient te worden vermeden.

B.J.M. Verduin
Characterization of cowpea chlorotic mottle virus and its assembly
Wageningen, 3 november 1978.

Aan de nagedachtenis van mijn ouders

CONTENTS

Abbreviations and symbols.

AET	2 - aminoethylisothiouronium bromide hydrobromide
AMV	alfalfa mosaic virus
A-protein	Bacteriophage-attachment or maturation protein; structural component of bacteriophage, one copy per virus particle.
A-protein	TMV - alkali protein; a mixture of oligomers of the structural coat protein subunit (n = 1 to 7).
Assembly buffer	10 mM Tris-HCl, pH 7.4 10 mM KCl 5 mM $MgCl_2$ 1 mM DTT
B	second virial coefficient
BBMV	broad bean mottle virus
Bentonite	clay used to adsorb RNase
BMV	brome mosaic virus
BSMV	barley stripe mosaic virus
^{o}C	degree Celcius
c	concentration
cal	4.184 joules
CCMV	cowpea chlorotic mottle virus
CD	circular dichroism
CMI/AAB	Commonwealth Mycological Institute/Association of Applied Biologists
ΔD	optical density difference between the left and right circular polarized components of light
DEP	diethylpyrocarbonate
DIECA	diethyldithiocarbamate
Dissociation buffer	50 mM Tris-HCl, pH 7.4 1 M NaCl 1 mM DTT
DTT	dithiothreitol
$E^{1\%}_{260\ nm}$	extinction of a 1% solution at 260 nm and an optical pathlength of 1 cm
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis (2-aminoethylether) tetraacetic acid
Fluorinert	FC-48, trade mark of 3 M Co; a heavy chase solution (ρ = 1.93 g/cm^3) for use with ISCO density gradient fractionator
ΔG	free energy change
ΔH	enthalpy change
h	hour(s)

ISCO	Instrumentation Specialties Co.
^{o}K	degree Kelvin
kJ	kilojoules (1 joule = 0.239 cal)
l	optical path length
M	molecular weight
$\bar{M}_o$	mean residue weight
$\bar{M}_{w,app}$	apparent weight average molecular weight
$\bar{M}_{w,o}$	weight average molecular weight at c = o
Macaloid	clay used to adsorb RNase
mCi	milliCurie; 10^{-3} Curie. The Curie is the unit of radioactive decay: 3.7×10^{10} desintegrations per second
2-ME	2-mercaptoethanol
min	minute(s)
MSE	Measuring Scientific Equipment
MW	molecular weight
n	degree of polymerization
n_D^{25}	refractive index at 25°C and the sodium D line
ORD	optical rotatory dispersion
PEG	polyethylene glycol
poly (A)	polyadenylic acid
poly (U)	polyuridylic acid
PT	pseudo top component (52 S)
PT-D	pseudo top doubles (110 S)
PVX	potato virus X
r	radius of spherical particle or distance from axis of rotation
R	gas constant, 8.31439 joules $deg^{-1}mol^{-1}$
rev/min	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
s	sedimentation coefficient
$s^0_{20,w}$	s corrected for water as solvent at 20°C and extrapolated to zero concentration
S	Svedberg unit of sedimentation, 10^{-13} seconds
ΔS	entropy change
SDS	sodium dodecyl sulphate
sec	second(s)

SW	swinging bucket rotor
T	temperature, ^{o}K
T	triangulation number
TBSV	tomato bushy stunt virus
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus
TRV	tobacco rattle virus
Tris	tris(hydroxymethyl)-aminomethane
TYMV	turnip yellow mosaic virus
UV	ultraviolet
$\bar{v}$	partial specific volume
virus buffer	0.1 M sodium acetate, pH 5.0 1 mM sodium azide 1 mM EDTA
(θ)	mean residue molar ellipticity in degrees centimeter^2 per decimol
λ	wavelength
μ	ionic strength
μ	micro-
ρ	density of the solution
ρ_o	density of the solvent
ω	angular velocity, radians/sec
f_2)) f_r)) MS_2)) Qβ)) R_{17})) μ_2)	RNA bacteriophages belonging to the Leviviridae

1. INTRODUCTION

The smaller spherical viruses consist of one or a few nucleic acid molecules encapsulated by a protein coat, a capsid, built up of a distinct number of functionally equivalent structure units (Watson and Crick, 1956; Caspar and Klug, 1962). The structure unit may contain one or a few different polypeptide chains, but all structure units are similar. Larger spherical viruses may be surrounded by membranes and additional protein layers, but this survey is centered to the smaller, simple viruses.

Upon penetration of a new virus into a susceptible host cell, uncoating of the virus particle takes places i.e. the protein capsid dissociates and the viral nucleic acid is released. The nucleic acid, which constitutes the virus genome, directs in the cell the replication of viral nucleic acid and the synthesis of virus specific proteins including coat protein. Finally the newly synthesized nucleic acids are encapsulated by virus coat proteins, which forms a protective coat around the nucleic acid. This last step in the virus multiplication process is called the assembly.

For some plant viruses like the bromoviruses this assembly can be mimicked in vitro under appropriate solvent conditions, without any further external information. The design and stability of the structure is only determined by the specific bonding of its parts: coat protein and nucleic acid. This assembly process distinguishes itself from template assembly, e.g. the formation of a prohead of bacteriophage T_4, in which an internal protein core apparently plays a scaffolding role (Casjens and King, 1975), and enzymic assembly, e.g. maturation of the prohead by proteolytic cleavage of the major capsid protein (Casjens and King, 1975). The structures, formed during the assembly process, must be biologically active and have to retain the ability to dissociate and to release the nucleic acid, when infecting a new plant cell. Knowledge of the dissociation and assembly process of viruses in vitro may yield more understanding of how viruses perform their biological function in vivo.

Cowpea chlorotic mottle virus (CCMV) a bromovirus has been chosen as the

research object for the following reasons:

1. CCMV is a small icosahedral virus consisting of four nucleic acid molecules encapsidated by 180 identical polypeptide chains. The four RNA molecules have been numbered 1 through 4 in order of decreasing size. RNA-1 and RNA-2 are encapsulated separately in a protein coat, while RNA-3 and -4 form one nucleoprotein particle (Bancroft and Flack, 1972). The three largest RNA molecules are the minimum set required for infectivity (Bancroft, 1971b). The smallest RNA molecule, which contains the coat protein cistron and is presumably the messenger for in vivo coat protein synthesis, is not required for infectivity, but is regenerated upon infection. The capsid protein cistron is also in RNA-3, but it is not translated in vitro to produce capsid protein (Lane, 1974). So only four RNA molecules and one type of polypeptide chain are comprising purified and infective CCMV preparations.
2. CCMV can be assembled from its isolated RNA and coat protein to form infective nucleoprotein particles which are resistent to exonuclease (Bancroft and Hiebert, 1967). Also conditions have been described where isolated dissociated coat protein aggregates into an empty protein shell (Bancroft et al., 1968b).
3. The split-genome nature of CCMV gives rise to some interesting questions about regulation of assembly. How are RNA-3 and -4 encapsidated together into one nucleoprotein particle? Does the coat protein recognizes the four homologous RNA molecules?
4. CCMV can easily be purified in relatively high yield (0.4 mg nucleoprotein per gram of wet tissue) from infected plants.

The objective of this investigation was to study the assembly of a spherical plant virus and characterize the assembled nucleoprotein particles. Attention was focused on conformational changes accompanying dissociation and assembly of nucleoprotein particles and the function of RNA-4 in the nucleoprotein particle containing RNA-3.

For tobacco mosaic virus it has been demonstrated that, an aggregate of 34 coat protein subunits, called the disk, recognizes the homologous RNA and forms an initiation complex in the assembly process. The high affinity of the coat protein for the RNA makes it possible for the protein to select its RNA from a pool of different RNA molecules in the plant cell. In the case of spherical plant viruses the existence of an initiation complex acting as a starting mechanism in assembly has not been detected so far.

A brief outline of the principles governing the construction of viruses is

given in chapter 2. According to these principles only few types of subunit aggregation and intersubunit bond variation may occur. Furthermore a description of the assembly of tobamoviruses, Leviviridae and bromoviruses is given, and in particular protein-RNA recognition and interaction are compared. This chapter is ended with a listing of the possible interactions, which stabilise the nucleoprotein particles.

A prerequisite for studying the assembly of a virus is starting material of constant quality. Apart from this quality a thorough knowledge of the characteristics of virus, RNA and protein is required in order to characterise the assembled products. Chapter 3 describes the conditions for isolation and storage of intact virus and RNA, and their characterization. The use of reducing agents, which protect the virus and coat protein from being oxidised, caused in situ degradation of RNA. This degradation could effectively be prevented with chelating agents.

In chapter 4 some procedures for the isolation of CCMV coat protein are described. The $CaCl_2$ method yields high concentrations of RNA-free protein, even when isolation has to be carried out in the presence of in situ degraded RNA. This chapter also contains a description of the conditions for the formation of an empty protein shell.

In chapter 5 the partial specific volumes and the circular dichroism of CCMV, its protein and RNA are compared to obtain information about the conformation of the RNA and the protein in situ and in solution. The dissociated protein is characterized by sedimentation equilibrium experiments.

The several types of aggregate of the coat protein subunit, formed after changing pH and ionic strength of the solution, did not comprise an aggregate of size intermediate between the dimer and the capsid. This hypothetical intermediate, like the disk with TMV is presumed to be involved in the initiation process of assembly. Assuming an irreversible change in the isolated coat protein after dissociation at pH 7.5, whole virus was dissociated and analysed for the presence of an RNA-protein complex.

Chapter 6 describes the pH- and salt-dependent dissociation of CCMV and the occurrence of a RNA-protein complex containing 4 to 8 protein subunits per RNA molecule. The RNA-protein complex obtained after dissociation of CCMV was used for reassociation. The nucleoprotein particles obtained by this dissociation-association method have been compared with the nucleoproteins obtained by the known assembly procedures (Bancroft and Hiebert, 1967; Adolph and Butler, 1977). The results are described in chapter 7.

In chapter 8 the general importance of the in situ RNA degradation is

discussed. Next some structural and functional aspects of the coat protein dimer are compared with the present data about assembly. Furthermore suggestions for future research with regard to RNA-protein interactions and the function of RNA-3 and -4 in the assembly process are presented.

A part of the results presented in this thesis (Chapter 3 and 4) has already been published (Verduin, 1974; Verduin, 1978b). Chapter 6 will be submitted for publication with L. Visser and C.J.M. Saris as co-authors and Chapter 7 will be submitted with A.J.M. Stams as co-author. Therefore the chapters of this thesis are presented in the form of a paper with omission of acknowledgements and references, which are gathered at the end of this thesis.

2. LITERATURE REVIEW ON THE ASSEMBLY OF VIRUSES

The assembly of viruses has been reviewed in several publications: some of them dealing with single viruses or virus groups: spherical plant viruses (Bancroft, 1970); simple RNA bacteriophages (Hohn and Hohn, 1970); bromoviruses (Lane, 1974); tobacco mosaic virus (Butler and Durham, 1977); others covering the whole area of virus assembly (Leberman, 1968; Fraenkel-Conrat, 1970; Eiserling and Dickson, 1972; Hershko and Fry, 1975; Casjens and King, 1975; Bouck and Brown, 1976).

For a better understanding of the following chapters a brief review of literature pertaining to virus structure, the assembly of tobamoviruses, spherical RNA bacteriophages (Leviviridae), bromoviruses and factors influencing assembly in general is given. The three virus groups are compared regarding the occurrence of a starting mechanism for assembly by formation of a specific initiation complex of RNA and coat protein and the driving force of the assembly process. In several cases details of isolation or assembly conditions of the three virus groups are given in order to allow comparison with the present-day assembly conditions and to demonstrate similarities among the different groups.

Virus structure.

Protein subunits in the shells of simple viruses are arranged according to a few basic principles (Crick and Watson, 1956; Caspar and Klug, 1962). The first principle is specificity; the design and stability of the subunits enforce assembly into organized structures without a template or other specific external control. The second principle is economy: large structures are composed of many copies of a few kinds of subunit. In the case of small viruses, economy is enforced by the size of their nucleic acid, sufficient to encode only one or a few structural proteins (Watson and Crick, 1956). These principles together imply symmetry: specific, repeated bonding patterns for identical building blocks lead to a symmetrical final structure. The simplest viruses thus exhibit either helical symmetry, for rod-like structures, or icosahedral symmetry, for spherical structures.

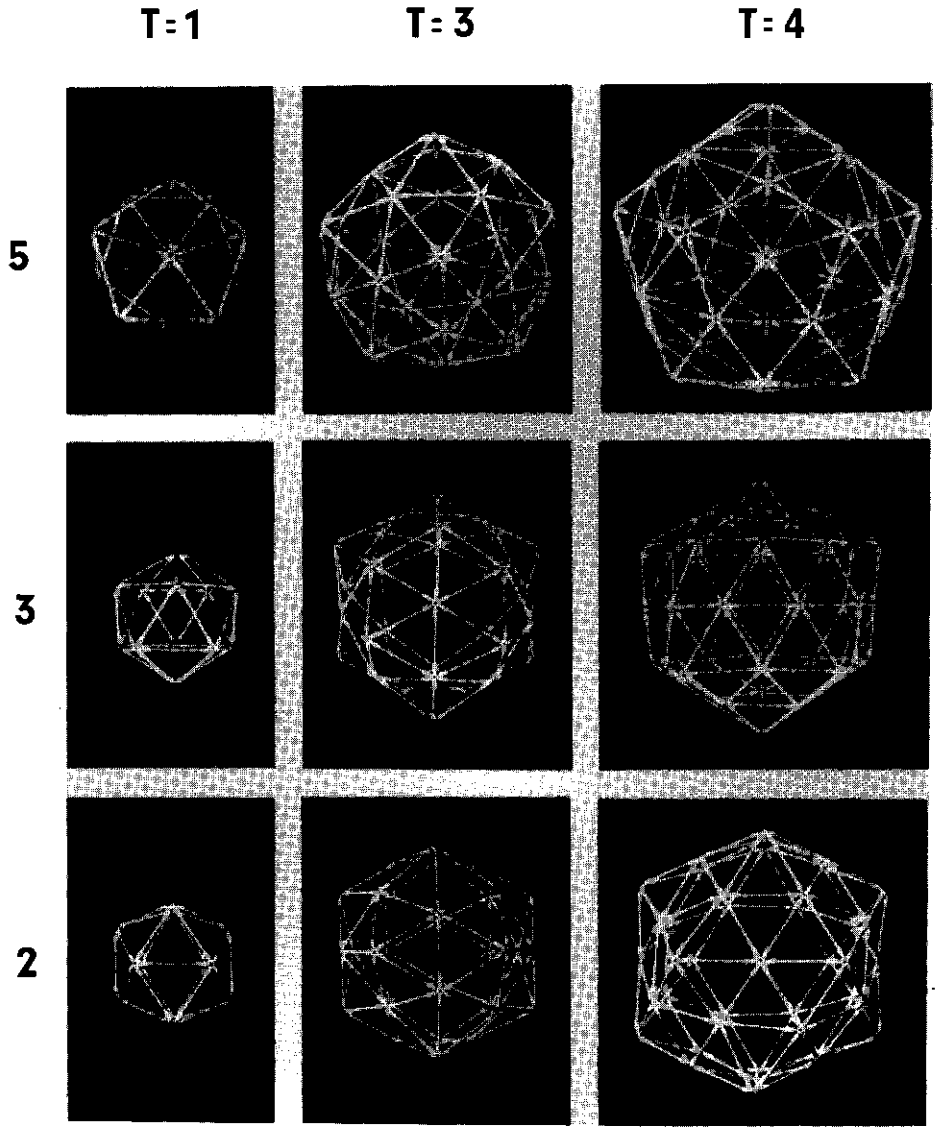


Fig. 2.1. Lattices of T = 1, 3 and 4 particles viewed along the 5, 3 and 2 fold icosahedral symmetry axes. The triangulation number T represents the mean number of triangles per icosahedral face. T = 1 represents the icosahedron.

Icosahedral symmetry implies exactly sixty equivalent locations visualized in the three corners of each of the 20 equilateral faces of an icosahedron (see Fig. 2.1, T = 1). All bonding angles between the protein subunits are identical. However a large number of viruses are built from multiples of sixty chemically-identical proteins. Thus, the construction of large, self-assembling structures requires a certain flexibility in the subunits to enable them alternative modes of bonding to their neighbours or of some degree of internal bending, visualized in the hexamer and pentamer clustering of the coat protein subunits (see Fig. 2.1, T = 3). This flexibility is called quasi-equivalence (Caspar and Klug, 1962).

By this principle of quasi-equivalence icosahedral lattices can be built in which the protein subunits are arranged according to the socalled triangulation number (Caspar and Klug, 1962). The triangulation number T represents the mean number of triangles per icosahedral face. Only a limited number of values for T are allowed in building a protein shell, like T = 1, 3, 4, 7, 9, etc. In Fig. 2.1 the lattices with triangulation numbers 1, 3 and 4 are shown, viewed along the characteristic icosahedral 5-, 3- and 2- fold symmetry axes. In each triangle 3 protein subunits may be located giving a total of subunits of 60, 180 and 240 for T = 1, 3 and 4 lattices respectively.

Different arrangements of the identical subunits in the icosahedral lattice are possible. Depending on their positions relative to the symmetry axes of the icosahedron, a dimer, trimer or a hexamer/pentamer clustering of structural subunits can be distinguished. These clusters are called morphological subunits (capsomers), due to their appearance in the electron microscope (Caspar and Klug, 1962).

In general, assembly of biological structures involving identical protein subunits and RNA are characterised by three steps: initiation, where protein and RNA recognize and form a complex, which starts further aggregation; elongation and termination, where either the length of the RNA molecule or the geometry of the assembled product limits further binding of protein. The last two steps are quite obvious for linear growth around a RNA molecule (see next section for assembly of tobacco mosaic virus), while the formation of a shell requires more imagination with regard to steps intermediate initiation and termination (Crane, 1950). Occurrence of half shells has not been demonstrated. Assembly into a sphere appears to be an all or nothing process, which is highly cooperative.

Tobamoviruses.

The in vitro reassembly of infective tobacco mosaic virus, particles (TMV,

type member of the tobamoviruses) first reported in 1955 by Fraenkel-Conrat and Williams, is the prime example of self-assembly of a biological system. Fraenkel-Conrat and Williams (1955) carried out their reconstitution experiments by mixing a 1% (w/v) protein solution with one tenth its weight of TMV-RNA at pH 6 in 0.03 M acetate at 3°C for at least 24 hours. Since then the assembly process has been investigated in numerous studies, which have been recently summarized by Butler and Durham (1977).

Only the essential aspects will be described for the type member of the tobamovirus group, unless stated otherwise. The TMV particle is a rigid hollow cylinder with a discrete length of about 300 nm and an inner diameter of 3.6 nm (Caspar, 1963). Its molecular weight is about 40×10^6, and it contains about 5% RNA (of molecular weight 2.1×10^6). The protein subunits (of molecular weight 17,400) are arranged in a single start helix with a pitch of 2.3 nm and 16.34 subunits per turn. The single stranded RNA molecule, which is in an open state and moderately extended, is intercalated between the turns of the protein helix at a radius of 4.0 nm, with three nucleotides per protein subunit. The RNA molecule determines the length of the virion, running for the whole length (with perhaps a ring of 17 protein subunits at each end to seal in and protect the ends of the RNA).

Protein obtained by either the acetic acid method (Fraenkel-Conrat, 1957) or the alkali method (Schramm et al., 1955, Fraenkel-Conrat and Williams, 1955) can reassociate into different oligomeric and polymeric aggregates of the coat protein subunit depending on protein concentration, pH, ionic strength or temperature of the solvent. Apart from the helical polymer (< 40 S, at low pH, high ionic strength and high temperatures) and the A-protein (4 S, at high pH, low ionic strength and low temperature) an oligomeric unit, called the disk (20 S, at neutral pH, moderate ionic strength and room temperature), containing 34 protein subunits has been observed. This protein oligomer occurs at physiological conditions as intermediate between A-protein and helix and appears to play a crucial role in the initiation of the assembly process of RNA and protein (Butler and Klug, 1971). The disk, comprising two rings of 17 subunits each, recognises a certain region of the RNA molecule. It appears probable that this specificity in vitro also occurs in vivo since less than 2% of the RNA in virus preparations comes from the host cell (Siegel and Huber, 1970; Siegel, 1971) and in mixed infections only very low concentrations of particles with masked genomes have been obtained: potato virus X - potato virus Y - tobacco mosaic virus (Goodman and Ross, 1974) and barley stripe mosaic virus - tobacco mosaic

virus (Dodds and Hamilton, 1974). Some genomic masking occurs if one of the virus coat proteins is inactive or absent (Atabekova et al., 1975). The probability of non-specific interaction is higher at the elongation (Talliansky, Atabekova and Atabekov, 1977), than at initiation of reconstitution.

At present the following sequence of assembly steps is hypothesised based on observation in the electron microscope (Otsuki et al., 1977; Ohno et al., 1977; Ohno et al., 1977; Butler et al., 1977; Lebeurier et al., 1977) and sequencing of encapsidated RNA regions (Zimmern and Wilson, 1976). A loop of RNA, located approximately 1000 nucleotides from the 3'-terminus, inserts into the central hole of the disk, rearranging the two layers of the disk into a starting helix. Subsequently elongation takes place in the direction of the 5'-end by addition of coat protein either in the form of disks or of A-protein. Hereby the unencapsidated RNA is supposed to be pulled through the central hole of the already formed rod before it is moulded into a helix. Partly reassembled TMV particles therefore should show in the electron microscope a rodlike appearance with two RNA strands protruding from the same end of the nucleated rod which has been demonstrated by Lebeurier et al. (1977) and Butler et al. (1977). After finishing assembly in the direction of the 5'-end as a result of the length of the RNA molecule, assembly proceeds towards the 3'-terminus making the virus particle RNase resistent (Ohno et al., 1977).

The driving force for protein-polymerization processes has been attributed to the increase in entropy of solvent molecules as a consequence of hydrophobic binding (Lauffer, 1975). Hydrophobic groups dissolved in water structurise the surrounding water molecules thereby diminishing the translational and rotational freedom of the water molecules i.e. the entropy of water. Clustering of these hydrophobic groups decreases their area of contact with water and consequently leaves more water molecules to move free. This increase in entropy of water ($\Delta S > 0$) causes hydrophobic groups to turn away from water spontaneously; they form a "hydrophobic bond" ($\Delta G < 0$ according to $\Delta G = \Delta H - T \Delta S$). Stevens and Lauffer (1965), demonstrated the release of approximately 30 water molecules per protein subunit upon polymerization of the coat protein into a helix. From calorimetric studies on this polymerization process positive values of ΔH were obtained (Stauffer et al., 1970; Srinivasan and Lauffer, 1970), indicating an endothermic polymerization. These results, together with similar results on other polymerizing systems, forced Lauffer (1975) to make the following statement: "All endothermic polymerization processes have to be water entropy-driven". This driving force does not exclude H-binding, ion-pair formation or Van der Waals interaction to take part in the interaction of protein with nucleic acid.

It is probable that these types of interaction are involved in the specificity of the reaction (Chothia and Janin, 1975).

From X-ray diffraction studies of crystals of the disk (Champness et al., 1976) and of fibers of the nucleoprotein (Stubbs et al., 1977; Holmes et al., 1975) the secondary and tertiary structure of the protein and the nucleic acid have been determined. In the disk the protein subunit consists of 4 radially oriented α-helices connected by random coil structures. The interior of the disk adjacent to the central hole could not be detected by X-ray diffraction analysis of crystals due to high internal mobility of this region. The binding of RNA to the disk causes a conformational transition in the polypeptide chain. One of the four radial α-helices is elongated and also a new helical stretch is formed. This α-helix orients parallel to the cylindrical axis and creats a rigid wall which separates the RNA from the central hole. This conformational change is also observed when the disk is polymerized into a helical rod by lowering the pH and is confirmed by circular dichroism measurements (Schubert and Krafczyk, 1969).

Formation of the α-helix parallel to the cylindrical axis is attended with the location of 4 acidic and 2 basic amino acid residues in an hydrophobic environment near the inner radius of the protein. This clustering of mainly carboxyl groups is called the "carboxyl cage" (Stubbs et al., 1977). The hydrophobic environment causes the carboxyl groups to deprotonate two pH units above their normal pK of 4.6. It explains the anomalous acid-base titration behaviour (Caspar, 1963), whereby near pH 7.0 several protons are released, which cannot be accounted for by histidine residues because histidine is absent in TMV. The delayed proton release of carboxyl groups has also been observed with several other plant viruses: CCMV (Jacrot, 1975), BMV (Pfeiffer and Durham, 1977), potato virus X (Shaw, 1997) and tobacco rattle virus (Durham and Abou Haidar, 1977).

The RNA molecule lies between subunits in successive turns of the virus helix so that the binding site is in two halves, between the top of one subunit and the bottom of the next. One half is the base binding site, located on one of the four radial helices. This helix has a large number of hydrophobic aliphatic residues, which form three faces to bind the three bases. The other half of the binding site is a helix providing three arginines to form ion pairs with the three phosphate groups.

The present view on assembly agrees well with the present knowledge of replication and translation of TMV-RNA. The region of the RNA which has a high affinity for the coat protein is located just left of the 5'-end of coat protein cistron. The binding of the protein may regulate the processing of the RNA to prevent formation of coat protein messenger. Such repression of viral non-coat

protein synthesis by coat protein has been demonstrated with RNA bacteriophages and will be described in the following section.

Leviviridae.

A second group of viruses of which assembly has been studied comprises the isometric ribophage group. These socalled Leviviridae are characterised by icosahedral particles with a diameter of about 25 nm (Fenner, 1976). Members of this family are f_r, f_2, MS_2 and Qβ. The genome consists of one molecule of singlestranded RNA with a molecular weight of about 1.2×10^6 and is surrounded by 180 identical protein subunits. A third phage structural component, the A-protein or maturation protein is necessary to obtain infectious virus. Only when one copy of A-protein per protein shell is present, properly sedimenting and RNase resistant particles are produced which can attach to their bacterial hosts.

The first succesful in vitro reaggregation of isolated RNA bacteriophage coat protein and RNA to form phage-like particles has been achieved by Sugiyama et al. (1967) with MS_2 and by Hohn (1967) with f_r. RNA isolated from the phage by phenol extraction and coat protein isolated by dissociation of the virus in 66% (v/v) acetic acid have been mixed and either dialysed against 0.15 M saline buffered at pH 7.2 with Tris at 4^oC (Hohn, 1967) or incubated in a similar buffer at 37^oC for a number of hours (Sugiyama et al., 1967). The reassembled particles sediment at about 70 S compared with 80 S for the virus, they contain less RNA and are biologically inactive. Since the discovery of the A-protein of R_{17} (Roberts and Steitz, 1967; Steitz, 1968 a,b,c), about one copy per virus particle, reconstitution experiments including RNA, coat protein and A-protein are possible. When partially purified A protein from R_{17} is added to an assembly mixture containing coat protein and RNA, the yield of infective particles increased several hundredfold over control experiments from which A-protein is omitted. Still the biological activity does not approach the activity of the original virus as shown for plant viruses.

When MS_2 RNA is assembled with low molar ratios of coat protein a nucleoprotein (complex I) with approximately 6 protein subunits per RNA molecule is formed. It has a sedimentation coefficient very close to that of free RNA. The formation of complex I seems to be a specific process, and MS_2 RNA can not be substituted by other RNAs (Sugiyama et al., 1967). Although recognition of protein by homologous RNA and subsequent complex formation occurs, there was no difference in physical and biological properties of phage-like nucleoprotein particles assembled from the complex and additional protein in comparison with particles

assembled directly from isolated RNA and protein. During in vitro protein synthesis with MS_2 RNA as messenger RNA a complex similar to complex I is formed. The formation of this complex then greatly reduces the synthesis of some non-coat proteins, whereas the synthesis of coat protein is almost unaffected. These results support the proposition that complex I is involved in the mechanism for the shut-off synthesis of non-coat proteins in MS_2 infected cells (Sugiyama and Nakada, 1968). Reinvestigation of the formation of complex I for f_2 in an ionic environment identical with that needed for in vitro protein synthesis reveals that repression of RNA polymerase synthesis is due to the attachment of six molecules of coat protein per RNA molecule (Zagórska et al., 1975; Chroboczek and Zagórksi, 1975).

The coat protein, can aggregate into well defined structures in the absence of RNA and A-protein. When bacteriophage f_r protein, isolated by the acetic acid method, in water is dialysed against 0.02 M phosphate buffer containing 0.1 M NaCl icosahedral shells resembling the phage coat are formed (Herrmann et al., 1968). Matthews and Cole (1972a) have demonstrated the need for low temperatures, high protein concentration or the presence of RNA to promote shell formation of f_2 coat protein. Chemical modification of amino acid of f_2 coat protein shows the necessity of arginines and C-terminal tyrosine for shell forming activity (Matthews and Cole, 1972b).

Small aggregates of the coat protein subunit with sedimentation coefficients of 5.5 S (9 subunits) and 11 S (18 subunits) have been demonstrated for R_{17} (O'Callaghan et al., 1973) and f_2 (Zelazo ans Haschemeyer, 1970). They only exist under strong denaturing conditions and their role as intermediates in phage assembly is speculative.

An extensive review of the structure and assembly of the simple RNA bacteriophages has been given by Hohn and Hohn (1970) and Knolle and Hohn (1975). The first assembly experiments of RNA bacteriophages ran parallel to those performed with spherical plant viruses to be described in the next section.

Bromoviruses

In 1967, the first spherical plant virus, CCMV has been reassembled from its isolated and separated components (Bancroft and Hiebert, 1967). The RNA has been obtained by phenol extraction and the protein by dissociating the virus in 1 M NaCl at pH 7.4 and removing the RNA by centrifugation. A typical assembly experiment proceeds as follows: 1 part by weight of RNA dissolved in 10 mM Tris-HCl pH 7.4, 10 mM KCl, 0.5 mM $MgCl_2$ is mixed with 3 parts by weight of protein dissol-

ved in 20 mM Tris-HCl pH 7.4, 1 M NaCl, 1 mM dithiotreitol (DTT) and dialysed for at least two hours at 4°C against 10 mM Tris-HCl pH 7.4, 10 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT (Bancroft and Hiebert, 1967).

Similar procedures could be used to reassemble brome mosaic virus (BMV) and broad bean mottle virus (BBMV), which together with CCMV make up the group of the bromoviruses (Hiebert et al., 1968; Harrison et al., 1971). This virus group is characterised by the multicomponent nature of the genome, divided over three different nucleoprotein particles (Bancroft, 1970a, 1971a, Gibbs, 1972, see for review on bromoviruses Lane 1974). The relationship between the RNA molecules and the three different nucleoprotein particles of CCMV is shown in Fig. 2.2. The RNA molecules are numbered according to decreasing molecular weights:

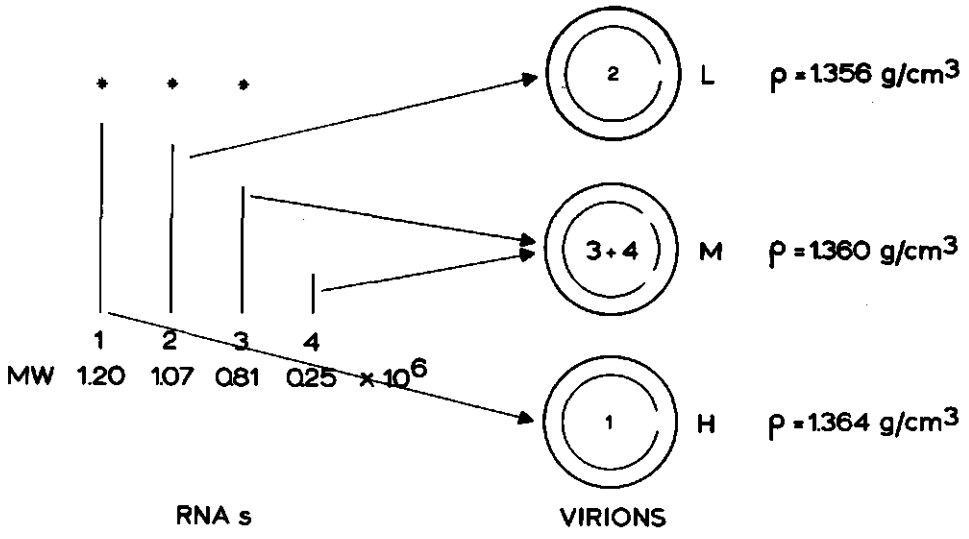


Fig. 2.2. Schematic representation of the distribution of CCMV RNA molecules over virions of different buoyant density. The RNAs marked with an asterisk constitute the genome of the virus, and RNA-4 arises during infection from RNA-3 and codes for the coat protein. Three types of nucleoprotein particle are present, called light (L), medium dense (M) and heavy (H) particles according to their density (ρ) in CsCl (Bancroft and Flack, 1972).

RNA -1, -2, -3 and -4 with molecular weights of 1.20, 1.07, 0.81 and 0.25 x 10^6 g/mol respectively (Reijnders et al., 1974).

The two largest RNA molecules are encapsidated separately in a heavy (H, RNA-1) and a light nucleoprotein particle (L, RNA-2). RNA-3 and -4 are encapsidated together in a medium dense (M, RNA-3+4) particle (Bancroft, 1971b, Bancroft and Flack, 1972). Biological activity of extracted RNA is only obtained when RNA-1,

-2 and -3 are present in the inoculum. A similar relation between RNA and nucleoprotein has been demonstated for BMV and BBMV (Lane and Kaesberg, 1971, Hull, 1972).

The bromoviruses are stable around pH 5.0 and sediment apparently homogeneous at 88 S. Increasing the pH to 7.5 at low ionic strength ($\mu < 0.2$) causes a drop in sedimentation coefficient to 78 S and makes the virus particle sensitive to RNase and protease (Incardona and Kaesberg, 1964; Bancroft et al., 1967; Bancroft et al., 1968b; Incardona, 1973; Adolph, 1975; Pfeiffer and Hirth, 1975; Pfeiffer, 1978). This phenomenon is called the swelling process. The effective hydrodynamic radius of the virus particle increases due a radial extension of RNA and protein within the nucleoprotein particle as demonstrated for BMV with intensity fluctuation spectroscopy (Zulauf, 1977) and neutron small-angle scatering (Jacrot et al., 1967; Jacrot et al., 1977; Chauvin et al., 1978). The swelling process appears to be irreversible as the sedimentation coefficient of the swollen particles backtitrated to pH 5.0 does not return to 88 S, but becomes 84 S. The radii of BMV nucleoprotein particles increase from 13.4 nm at pH 5.0 to 15.5 nm at pH 7.5 in the presence of 0.2 M KCl and 10 mM EDTA. Upon backtitration to pH 5.0 the radius becomes 13.7 nm. Reversibility can be obtained in the presence of 10 mM $MgCl_2$ but in this case the swelling at pH 7.5 does not proceed to its full extend ($r = 15.0$ nm, Chauvin et al., 1978).

Increasing the salt concentration ($\mu > 0.5$) at pH 7.5 causes the swollen particles to dissociate into RNA-protein complexes and protein subunits (Hiebert, 1969). This phenomenon has been used to isolate the protein from the mixture after sedimentation (Bancroft and Hiebert, 1967) or precipitation (Yamazaki and Kaesberg, 1963; Verduin, 1974: chapter 4) of the RNA. The precipitated RNA can be recovered by dissolving in EDTA and subsequent phenol extraction (Herzog et al., 1976; Verduin, 1978b: chapter 3). A schematic view of the swelling and dissociation of the bromovirus nucleoproteins is given in Fig. 2.3.

The isolated coat protein, free of nucleic acid, has the capacity to form several quaternary structures dependent on conditions of ionic strength, pH and temperature: sheets, tubes, empty protein shells, double shells and rosettes (Bancroft et al., 1968a; Bancroft et al., 1969a; Adolph and Butler, 1974; Pfeiffer and Hirth, 1974 a and b). The formation of empty protein shells is of great interest as it indicates that the protein structure holds the information for the formation of icosahedral particles (Bancroft et al., 1968a; Finch and Bancroft, 1968). The other structures are probably formed after small changes in the intersubunit bondangles as described by the quasi-equivalent theory of Caspar and Klug (1962). In contrast to natural protein capsids (top components), which do not contain RNA, such as occur with cowpea mosaic virus (CPMV) and turnip yellow

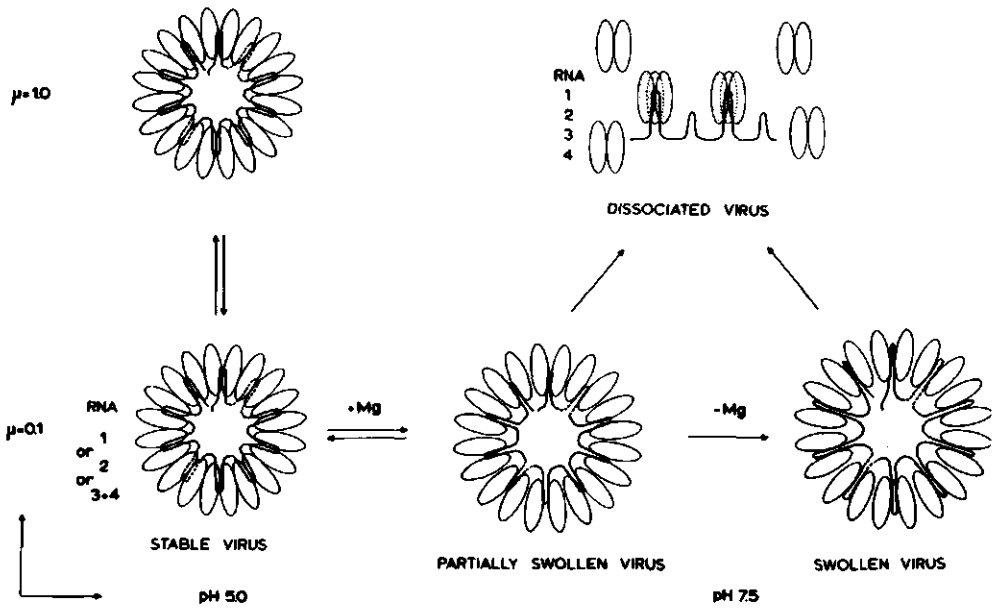


Fig. 2.3. A schematic representation of the swelling and dissociation of the bromoviruses. In vertical position ionic strength and in horizontal position the pH has been varied. At low ionic strength pH 7.5 both the presence and the absence of Mg^{++} has been shown. A virus particle is represented by a cross section of the icosahedral particle, through 4 three-fold and 4 five-fold symmetry axes. In this way 8 RNA loops, in interaction with hexamer and pentamer clusters of the coat protein are in the plane of the paper, while two are out (broken line). The strings represent the RNA molecules and the ellipses represent protein subunits. The dotted ellipses represent protein subunits with a high affinity for the RNA, observed with dissociated virus (Chapter 6). The location of RNA and protein is based on structural analysis of BMV and its similarity with CCMV (Jacrot et al., 1976, 1977).

mosaic virus (TYMV) (Semancik, 1966; Van Kammen, 1967; Markham and Smith, 1949; Kaper, 1960) these artificially made capsids have less electronegative charge than the intact nucleoprotein particles and are therefore called pseudo top components (Bancroft et al., 1968a). Similar capsids can be obtained after degradation of nucleoproteins in 0.5 M $CaCl_2$ at pH 5.6 or in 1 M KCl pH 12 (Pfeiffer and Hirth, 1974a; Incardona, 1968; Bancroft, 1970b).

The radial extension of RNA and protein within nucleoproteins during the swelling process is caused by repulsion between the negatively charged phosphate groups of the RNA and the negatively charged protein. The negative charge of the protein increases due to deprotonation in the pH range from 6.5 to 7.0. Titration of nucleoprotein solutions with alkali between pH 5.0 and 8.0 causes the release of approximately 7 protons per CCMV or BMV coat protein subunit in 0.2 M KCl (Jacrot, 1975; Pfeiffer and Durham, 1977). Upon backtitration with acid

to pH 5.0 approximately the same amount of protons were adsorbed although the titration curves obtained with alkali and acid did not coincide over the whole pH range from 5 to 8. A hysteresis loop was observed with all bromovirus (Jacrot, 1975; Pfeiffer and Durham, 1977). Maximum displacement of protons was found at pH 6.9 (5°C) or 6.8 (23°C) for CCMV in 0.2 M KCl and pH 6.4 (23°C) for BMV in 0.05 M KCl, which coincide with the pH values at which the structural changes of the nucleoproteins take place (Incardona and Kaesberg, 1964; Incardona et al., 1973; Adolph, 1975). At least for CCMV the structural changes in the nucleoprotein upon swelling is not due to conformational changes in the RNA and must therefore be attributed solely to the protein (Adolph, 1975b).

Bromovirus coat proteins can also encapsidate other RNA molecules (Bancroft et al., 1969b; Adolph and Butler, 1977). A typical nucleotide sequence is not obligatory necessary to protein shell formation and the only requirement for nucleation and shell formation at neutrality is the repeating phosphate backbone of the nucleic acids. Even polyanions, like polyvinyl sulphate and sodium dextran sulphate, other than polynucleotides may serve in the shell formation process. Using oligo (U) it was demonstrated that at least a minimum requirement of 23 nucleotides is needed to induce capsid formation (Bancroft et al., 1969b). The final product sedimented faster than 70 S indicating the presence of polyanions with a minimum molecular weight of 1×10^6.

The assembled products have been characterised by sedimentation analysis, infectivity tests and observations in the electron microscope. However, the assembled particles have not been analysed for RNA content, nucleoprotein composition or type of interaction between the protein subunits or the protein and the RNA.

The next section describes the possible interactions in macromolecules, their nature and conditions for stability.

Interactions

The strongest interactions between macromolecules are the covalent bonds involving the sharing of electrons. The potential energy has a sharply defined minimum of the order of -167 to -419 kJ/mol at the equilibrium bond length which is in the range 0.1 - 0.2 nm for the atoms of organic molecules. The activitation energy required for forming or breaking covalent bonds is also very high and in biological systems such bonds are formed or broken only by catalytic action of enzymes.

All non-covalent molecular interactions are electrostatic in nature and are the result of the forces between some combination of charge, dipole or induced

dipole, e.g. ionic bonds, hydrogen bonds, and Van der Waals bond. The energy of the attractive interactions at the equilibrium separation ranges from the order of -42 to 84 kJ/mol for ionic bonds to about 4 kJ/mol for the fluctuation dipole-induced dipole of London dispersion force which is the physical basis of the Van der Waals bond. The hydrogen bond, involved in base pairing of nucleic acid molecules, possesses free energy of interaction intermediate those of the ionic or Van der Waals bonds (10 kJ/mol base pairs, Tinoco et al., 1973). The bond distances range from about 0.2 to 0.5 nm, being longer for the weaker interactions.

In general, any non-covalent molecular bond results not from a single type of interaction, but rather from a combination of different types. The hydrogen bond, which is principally a dipole-dipole interaction, can also be described as partially ionic and does involve some sharing of electrons. Moreover, the strength of the various interactions may give a misleading impression of their significance in biological structures. Ionic bonds are not normally formed in an aqueous environment, although they are quite strong. The reason for this is that the formation about each ion of a hydration shell of water molecules held by charge-dipole interactions leads to a larger decrease of free energy than a single salt link. This charge-dipole interaction is also the driving mechanism of the ionization of acids.

The weakest bonds are, in a way, the most important in determining the structure and bonding properties of macromolecules. The selections of coordinated very weak forces in the design of biological macromolecules makes their stable conformations a sensitive function of their internal composition and of the interactions with their external environment. Thus, dynamic molecules can be selected for specific functions. This would not be possible if their design were determined by a few strong interactions. Nevertheless, the functional conformational changes which do take place are likely to be small. If very different folding or packing arrangements of macromolecules had comparable stability, the probability of achieving any unique structure would be small and, moreover, the activation energy required for transmissions between grossly different states would be very high (Caspar, 1966 a and b).

So far forces between molecules have been considered. However, the stability of any system depends both on the forces between its component parts and on the entropy. The entropic nature of hydrophobic interactions, as mentioned with TMV, and their significance in protein structure have been clearly described by Kauzmann (1959).

The influence of the external environment on electrostatic and hydrophobic binding are different. High temperatures and ionic strengths weaken or disrupt

electrostatic bonds but favour hydrophobic bonds. The importance of hydrophobic protein-protein interactions with TMV has clearly been demonstrated by Lauffer (1975). Structural studies elucidated also hydrophobic interactions between the bases of the RNA and hydrophobic amino acids in an α-helix of the coat protein subunit (Stubbs et al., 1977). Apart from the hydrophobic interactions both RNA and protein are involved in electrostatic interactions. These ionic bonds have been demonstrated between the phosphate groups of the nucleic acids and the basic amino acids of the protein or between the acidic and basic amino acids of different subunits. A combination of hydrophobic and electrostatic interactions in the protein is demonstrated with the "carboxyl cage" of TMV. The carboxyl groups in this cage release their protons at high pH values due to the hydrophobic environment and the sharing of the protons with other charged groups (Stubbs et al., 1977). Similar types of interaction, phosphate-basic amino acid interaction and abnormal-titrating carboxyl groups, have been demonstrated for the Leviviridae and the bromoviruses although they have not been investigated in detail as for TMV.

In conclusion TMV and the Leviviridae coat protein recognize their homologous RNas, which has not yet been demonstrated for the bromoviruses. Oligomeric units of coat protein subunits occur with both TMV and Leviviridae but with the latter no fundamental function during the assembly could be assigned. TMV has a typical entropy driven assembly due to hydrophobic interactions and the Leviviridae and the bromoviruses most likely follow the same principle, however with some more electrostatic contributions.

3. DEGRADATION OF COWPEA CHLOROTIC MOTTLE VIRUS RIBONUCLEIC ACID IN SITU

Immediately after purification of cowpea chlorotic mottle virus (CCMV) *in situ* degradation of RNA-2 into two distinct RNA fragments begins. Upon storage of purified virus, even at 4°C, all four viral RNAs degraded into small heterogeneous pieces. Addition of thiols accelerated degradation, as did higher temperatures. Addition of chelating agents in low concentrations prevented the *in situ* RNA degradation.

Isolated CCMV-RNA was not degraded in the presence of thiol compounds. If the RNA was, however, reassembled with isolated coat protein into virus-like particles, degradation of the RNA occurred. A mixture of empty protein capsids and RNA also caused degradation of RNA. In a mixture of isolated RNA and purified virus, both degradation of added and *in situ* RNA occurred, but not to the extent of the degradation of the same amount of RNA within virions.

Addition of radical scavengers to purified virus partially prevented the *in situ* degradation of CCMV-RNA, suggesting a radical mediated mechanism for the nucleic acid degradation.

INTRODUCTION

Cowpea chlorotic mottle virus (CCMV) is frequently used for the study of the assembly process of spherical viruses (Lane, 1974, for review). CCMV produces three types of spherical nucleoprotein particle, each with a diameter of 25 nm and a protein coat constructed of 180 identical structural subunits. The particles have similar sedimentation coefficients but can be distinguished by buoyant density differences in CsCl or RbCl. The particles contain four RNAs, RNA-1, -2, -3 and -4 with molecular weights of 1.20, 1.07, 0.81 and 0.25 x 10^6 respectively. The smallest two are encapsidated together, the other two separately (Bancroft and Flack, 1972).

If CCMV is kept in buffer solutions at pH 5.0, where the virus is presumed to be stable and resistant against nuclease (Bancroft and Hiebert, 1967) the RNA degrades (Bancroft and Flack, 1972; Verduin 1974, Chapter 4).

I report here on the *in situ* degradation of CCMV-RNA and the characterization of virus particles containing degraded RNA. The influence of thiol compounds, chelating agents and different quaternary structures of CCMV coat protein on the

degradation of RNA is described. Finally, a sequence of reactions involving a superoxide radical and causing the in situ RNA degradation is proposed.

MATERIALS AND METHODS

Isolation of CCMV. CCMV was purified from the leaves of 7-days-infected cowpea plants as described before (Verduin, 1974, Chapter 4). Once it was established that EDTA prevented RNA degradation, the leaves were homogenized in buffer containing 10 mM disodium EDTA and all other buffers, contained 1 mM disodium EDTA. Virus was stored in 0.1 M sodium acetate pH 5.0 containing 1 mM sodium azide and 1 mM disodium EDTA (virus buffer).

Preparation of washed and native coat protein. Coat protein was prepared according to the $CaCl_2$ method (Verduin, 1974, Chapter 4). Dissociated protein, free of RNA, was associated into spherical particles, called empty capsids or pseudo top components (PT) by dialysing overnight at 4°C against 1.0 M NaCl, containing 1 mM dithiothreitol (DTT) buffered with 50 mM sodium acetate at pH 5.0 (Bancroft et al., 1968a). The empty capsids formed were collected by centrifugation (3 h 180,000 g). The supernatant, containing dissociated protein, which did not reassociate into empty capsids, was discarded. The pellet was resuspended in 1.0 M NaCl, 1 mM DTT buffered with 50 mM Tris-HCl, pH 7.5 which dissociated the empty capsids again. The solution was then cleared from aggregated protein and undissociated capsids by centrifugation for 3 h at 180,000 g. Repeated association and dissociation of coat protein in the presence of thiol compounds may remove contaminating protein and selects viral protein which is still capable of capsid formation.

Extraction of RNA from CCMV. Purified virus was diluted to a final concentration of 0.5 - 1.0% (w/v) in a solution containing 0.1% (w/v) macaloid, 2 mM EDTA, 0,1 M NaCl, 1% (w/v) sodium dodecyl sulphate (SDS), buffered with 50 mM sodium acetate at pH 5.0, and stirred for 15 minutes at room temperature. One volume of a mixture of phenol (freshly distilled and water-saturated), m-cresol (distilled) and 8-hydroxyquinoline (v:v:w = 7.5:1:0.075) and one volume of chloroform were added and the emulsion was stirred for another 15 min at room temperature (Kirby, 1965; Perry et al., 1972). The phases were separated by centrifugation (5 min at 5,000 g) and the water phase was extracted twice more with phenol. Phenol was removed by extraction with ether and the ether was evaporated with nitrogen gas. RNA was precipitated from the aqueous solution with 2.5 volumes of cold absolute ethanol and one drop of 3.5 M sodium acetate pH 5.5 per ml of aqueous phase. Using this method more than 70% of the theoretical yield of RNA could be obtained, based on $A^{1\%}_{1\ cm}$ at 260 nm = 250 for RNA and an RNA content of 24% (Bancroft and Hiebert,

1967; Bancroft et al., 1968b). The absorbance ratios: $A_{260\ nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ were 2.2 and 2.5 respectively, and the RNA contained less than 0.1% (w/w) of protein as determined by the method Lowry et al. (1951).

Occasionally RNA was prepared from the nucleic acid precipitate, obtained when virus was dissociated in 0.5 M $CaCl_2$, for preparation of protein. The pellet, consisting of a complex between the phosphate groups of the RNA and Ca^{++}, was resuspended in 10 mM disodium EDTA and dialyzed for at least 48 h against successive changes of EDTA solution. Residual protein was removed by one extraction with the chloroform-phenol mixture and the RNA was further purified as described above. About 60% of the theoretical yield of RNA could be obtained. After extensive dialyses, to remove EDTA, the RNA had the same spectral properties mentioned above, but the RNA preparation contained less RNA-1 and -2 and was more degraded (Fig. 3.4A) than the RNA obtained by phenol extraction of virus at pH 5.0 (Fig. 3.4G).

Assembly of virus and empty capsids. Viral RNA, dissolved in 10 mM Tris-HCl pH 7.4 containing 10 mM KCl and 0.5 mM $MgCl_2$, was mixed with coat protein in the proportion 1:3 (w/w) and dialysed against 10 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT buffered with 10 mM Tris-HCl at pH 7.4 (assembly buffer) for two h at 4°C (Bancroft and Hiebert, 1967). The reassembled products were dialysed against virus buffer and used without further purification. Empty capsids for the examination of the effect of coat protein on RNA degradation were prepared from the washed dissociated protein by dialysing overnight at 4°C against 0.2 M NaCl, 1 mM DTT, buffered at pH 5.0 with 50 mM sodium acetate. A lower salt concentration was used to prevent an ionic strength effect on the RNA degradation, although no effect was observed between 0 and 0.2 M NaCl.

Polyacrylamide gel electrophoresis. Viral RNA was analysed by electrophoresis at 10°C or 60°C in 2.6% (w/v) polyacrylamide gels with the buffer system used by Loening (1967, 1969). Glycerol (final concentration 10% (v/v)) was added to RNA solutions before application to the gel. If RNA within nucleoprotein particles had to be analysed, the particles were dissociated at room temperature in a solution of 10% (v/v) glycerol and 2% (w/v) SDS in water, for at least five minutes, and 50 µl of the solution of the dissociated virus, equivalent to 10 µg of RNA, was subjected directly to electrophoresis for 4 h at 4 V per cm of gel. After electrophoresis the gels were kept overnight in water and scanned at 260 nm with a Beckman Model 25 spectrophotometer equipped with a gelscanner. As a control the gels were stained afterwards in 0.01% (w/v) Toluidine Blue in 40% (v/v) methoxyethanol to detect irregularities and extra bands not resolved with the gelscanner.

Evaluation and comparison of the state of the degraded RNA. For measuring in situ degradation of CCMV-RNA, virus at a concentration of 0.16% (w/v) in virus buffer, without EDTA, containing 1% (v/v) 2-mercaptoethanol (2-ME) was incubated for 2 h at 37°C, unless stated otherwise. The degradation was stopped by adding EDTA to a final concentration of 1 mM. A sample was analysed on 2.6% (w/v) polyacrylamide gels.

Scans were taken of gels containing RNA from virus incubated for 2 h at 37°C in the presence of EDTA (undegraded RNA, A), the absence of EDTA (completely degraded RNA, B) and in the presence of various compounds (test RNA, C) and total areas under the absorption curves were determined by weighing. The degree of degradation was expressed as $(A-C)/(A-B) \times 100\%$.

Electron microscopy. Electron micrographs of virus particles were obtained with a Siemens Elmiskop I. The nucleoprotein particles were negatively stained on a carbon coated grid with 2% (w/v) uranyl acetate in double distilled water.

Analytical ultracentrifugation. Sedimentation analysis and the determination of buoyant densities in RbCl were performed in a Beckman Model E analytical ultracentrafuge equipped with Schlieren optics. The densities of the nucleoprotein particles were determined by the method of Ifft et al. (1961) from Schlieren patterns obtained after centrifugation of 0.025% (w/v) CCMV and 38% (w/v) RbCl in virusbuffer for 40 h at 20°C and 44,770 rev/min. A density-weight percentage relationship for RbCl was obtained from International Critical Tables.

Rate zonal centrifugation. Virus (250 µg) incubated under different conditions was dissociated in 2% (w/v) SDS and layered on top of a 10-50% (w/v) linear sucrose density gradient and centrifuged for 15 h at 25,000 rev/min. and 20°C in an Beckman SW 41 rotor (Neal and Florini, 1972). The contents of the tube were fractionated by an ISCO density gradient fractionator Model 185 and the absorption at 281 and 257 nm was monitored with two LKB Uvicords II absorption-meters connected with two Servogor recorders.

Infectivity test. Virus infectivity was measured by local lesion assay on leaves of horticulturally soft Chenopodium hybridum L. (Rochow, 1969). The preparations to be tested were inoculated with a glass spatula onto one-half of 10 leaves, dusted with Carborundum. The opposite halves were inoculated with control virus, a standard virus preparation of 0.1 $A_{260\ nm}$ per ml producing about 40 lesions per half leaf. The necrotic lesions were counted after five days and the infectivity was expressed as: (total number of lesions on ten test halves/total number of lesions on the control halves) x 100.

Chemicals and enzymes. Thiol compounds were obtained from Boehringer. 2-ME, 2-aminoethylisothiouronium bromide hydrobromide (AET) and pancreatic ribonuclease

were obtained from Koch-Light, Calbiochem and Sigma, respectively. All other chemicals used were analytical grade products from Merck. Disodium EDTA was soluble up to 0.2 M in sodium acetate at pH 5.0. Superoxide dismutase isolated from bovine erythrocytes was a gift from Dr. S.G. Mayhew.

RESULTS

In vitro *degradation of CCMV-RNA* in situ

If CCMV was purified by the procedure of Bancroft et al. (1972) the RNA from the virus always showed some degradation. The electropherogram (Fig. 3.1B) showed in addition to the virus specific RNAs-1, -2, -3 and -4, two peaks which will be

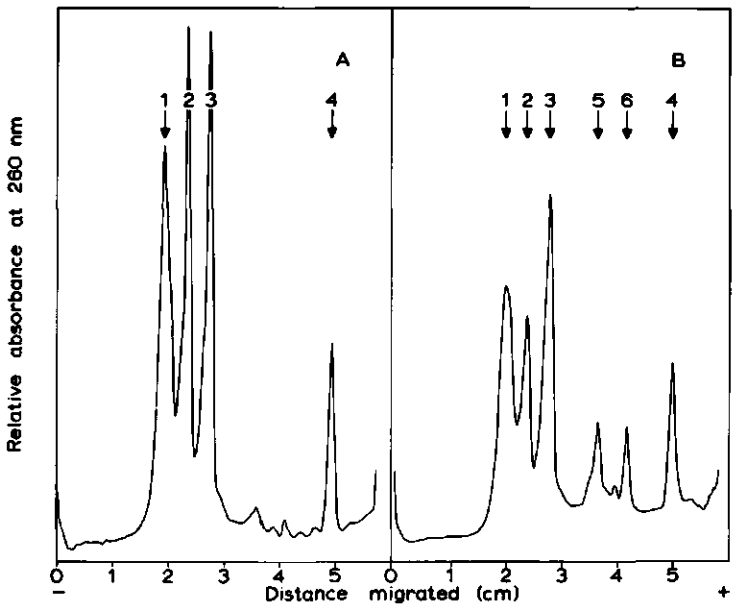


Fig. 3.1. Electrophoresis patterns of CCMV-RNA in 2.6% (w/v) polyacrylamide gels at 10°C in the buffer system described by Loening (1967, 1969). Virus, 3 days after starting purification, was dissociated in 10% (v/v) glycerol and 2% (w/v) SDS and, 40 µg of dissociated virus in 50 µl were applied onto the gels and electrophoresed for 4 h at 4 V/cm of gel. (A) Virus purified and kept with EDTA and, (B) virus purified and kept without EDTA. The arrows and the respective numbers indicate the distinct RNA species found with infectious CCMV at pH 5.0.

referred to as RNA-5 and -6, with molecular weights of 0.64 and 0.45 x 10^6 respectively. RNA-5 and -6 were observed within 48 to 72 h after starting the purification. In contrast, virus purified and stored in the presence of 1 mM EDTA,

had little degraded RNA (Fig. 3.1A).

If electrophoresed in polyacrylamide gels at low temperatures, CCMV-RNA showed occasionally more heterogeneity (Fowlks and Young, 1970; Bancroft, 1971). This heterogeneity, visualized as band broadening and overlapping of RNA-1 and -2 (Fig. 3.1B), could be eliminated by heating the sample and was therefore probably due to different secondary structures of the four RNA components. Electrophoresis of RNA from dissociated virus at 60°C gave highly reproducible RNA patterns, always with the same relative amounts of the four RNA species and was therefore used in all further experiments. With this procedure no difference could be found between RNA patterns of isolated RNA and RNA from dissociated virus put directly on gels. Variations in the relative amounts of the RNA species caused by the procedure for extracting the RNA (Bancroft and Flack, 1972) were eliminated using our methods. It should be emphasized that electrophoresis at lower temperatures (10 to 25°C) resulted in a better separation of the RNA species if the RNA had been isolated from the virus particles and precautions had been taken to eliminate secondary structure or residual protein (Bancroft, 1971). With the standard procedure at 60°C, virus preparations with and without RNA species 5 and 6 were compared. From the different peak heights of RNA-1 and -2 relative to RNA-3 (Fig. 3.2A and

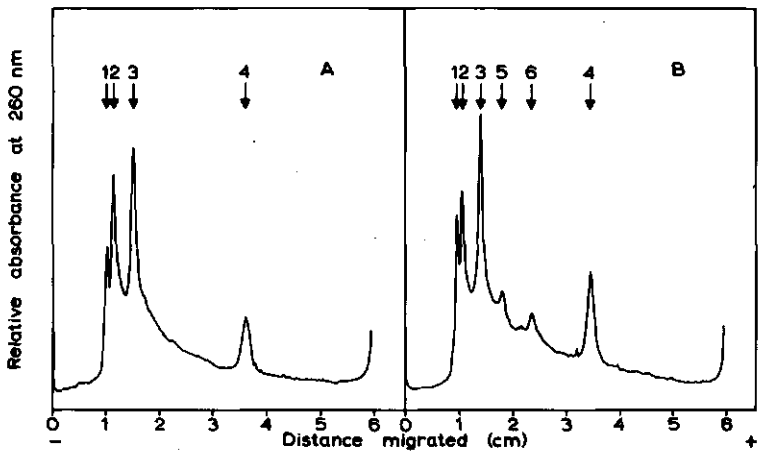


Fig. 3.2. Electrophoresis patterns of CCMV-RNA in 2.6% (w/v) polyacrylamide gels at 60°C in the buffer system described by Loening (1967, 1969). (A) Virus purified and kept with EDTA and, (B) virus purified and kept without EDTA. For details see Fig. 3.1.

B) it was concluded that the appearance of species 5 and 6 was accompanied by a loss of RNA-2. A similar conclusion for the origin of RNA-5 and -6 was drawn by Bancroft and Flack (1972).

Equilibrium density gradient analysis in RbCl of virus with degraded RNA, did not reveal any change in the nucleoprotein component ratio, nor in the density of the particles. By analysis on polyacrylamide gels or RNA from separated particle fractions, RNA-5 and -6 were detected in nucleoprotein particles with the same buoyant density as the particles containing RNA-2. This confirmed that soon after virus purification, some viral RNA-2 degraded into RNA-5 and -6, without any change in the buoyant density of the particle containing the degraded RNA.

Storage of virus

Besides specific degradation of RNA-2, into RNA-5 and -6, a random degradation of the other RNA components was observed, when virus was kept for 96 hours at 4°C in 0.1 M sodium acetate buffer pH 5.0 containing 1 mM sodium azide. First, degradation of RNA-1 occurred as indicated by the change in peak height relative to RNA-4 (Fig. 3.3B, D and F). Storing for longer periods resulted in the degradation of RNA-2, -3 and even RNA-4 (results not shown). The degradation of RNA-1 and -3 and the further degradation of RNA-2 did not result in distinct absorption peaks in 2.6% (w/v) polyacrylamide gels but rather an increase of absorbance in the area between RNA-3 and -4.

In the presence of 1% (v/v) 2-ME the in situ degradation at pH 5.0 and 4°C was much faster. Virus kept for 96 hours with 2-ME contained RNA that no longer produced a distinctive absorption pattern on 2.6% (w/v) polyacrylamide gels (Fig. 3.3E). The nucleic acid apparently had been degraded into fragments with molecular weights lower than 100,000 which had run off the gel. No in situ RNA degradation was ever observed, if the virus was preserved in buffer containing 1 mM EDTA, and the virus could be stored for 6 months and longer in the refrigerator at 4°C without any change in the RNA pattern. 2-ME did not cause significant in situ degradation of virus RNA in the presence of EDTA.

Ribonuclease contamination

Saha (1974) reported that ribosomal RNA could be specifically degraded by low concentrations of added RNase. Therefore the effect of various RNase inhibitors on the purification of CCMV was examined. Macaloid or diethylpyrocarbonate (DEP), with final concentrations in the leaf homogenate of 1% (w/v) or 3% (v/v) respectively, did not prevent the degradation. Using chloroform-butanol in the virus purification, a procedure which might denature enzymes adsorbed at the surface of virus particles (Geelen et al., 1972), had no effect on the RNA degrada-

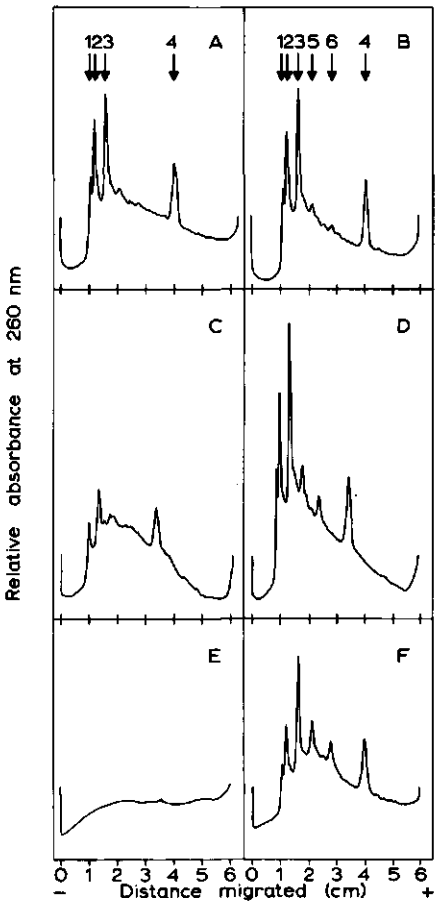


Fig. 3.3. Electrophoresis patterns of CCMV-RNA in 2.6% (w/v) polyacrylamide gels. The effect of incubation with and without 2-ME for various periods of time on the in situ degradation of CCMV-RNA. Virus, 30 h after the start of the purification without EDTA, was kept for (B) 2, (D) 24 and (F) 96 h at 4°C and pH 5.0 in 0.1 M sodium acetate buffer containing 1 mM sodium azide; A, C, and E show the patterns obtained when virus was kept for the same periods under identical conditions in the presence of 1% (v/v) 2-ME. Arrows and numbers indicate the distinct RNA species found with infectious CCMV. Electrophoresis was at 60°C. For further details see Fig. 3.1.

tion. Neither was the RNA degradation inhibited when virus was kept after purification in the presence of macaloid or DEP or was re-extracted with chloroform-butanol. From these results it appears unlikely that an RNase on the outside of the virus particle, was responsible for the degradation of the RNA in situ. This conclusion was supported by the finding that virus incubated for 30 minutes at 37°C in 0.1 M sodium acetate, pH 5.0 and 1 mM sodium azide with pancreatic RNase, even at an enzyme virus ratio of 1/10 on a weight basis, did not stimulate in situ RNA degradation.

Influence of chelating agents

EDTA prevents alfalfa mosaic virus (AMV) loosing infectivity (Van Vloten-Doting et al., 1970). It appeared that EDTA was also a very effective inhibitor

of the in situ degradation of CCMV-RNA. Addition of 10 mM EDTA during purification of virus prevented the appearance of significant amounts of RNA-5 and -6. To see whether the effect of EDTA was due to its chelating properties or to some other specific effect, three other chelating agents were tested at different concentrations. Ethyleneglycol-bis (2-aminoethylether) tetra-acetic acid (EGTA) prevented the in situ degradation of CCMV-RNA as effectively as EDTA at concentration ranging from 0.1 M to 0.5 mM. Citrate was less effective, but 1 mM citrate inhibited RNA degradation to some extent. Diethyldithiocarbamate (DIECA) affected the degradation of the RNA only at high concentrations. If virus was incubated with 0.1 M DIECA the RNA pattern on gels was similar to that of virus without degraded RNA. These results suggest that di- or tri-valent cations may be involved somehow in the in situ degradation of CCMV-RNA. However, addition of Mn^{2+}, Mg^{2+} or Co^{2+} in tenfold molar excess over 1 mM EDTA did not overcome the effect of EDTA.

The effect of thiol compounds

As 2-ME stimulated the in situ degradation of CCMV-RNA in the absence of EDTA, several thiol compounds were tested. CCMV was incubated at pH 5.0 with different concentrations of the compounds listed in Table 3.1 for 2 h at 37° and

Table 3.1. Stimulation of the in situ degradation of CCMV-RNA by thiol compounds.

	Thiol compounds					
-SH molarity	cysteine	DTT	glutathione	2-ME	thioglycollic acid	DIECA
0.1	++++[1)]	++++	+++	++++	+++	NT
0.5	++++	++++	+++	+++	++++	+
0.01	++++	++	+++	+	++++	++
0.005	NT[2)]	NT	NT	NT	+++	+
0.001	++	+	+	+	++	NT
0	-	-	-	-	-	-

CCMV (1.6 mg/ml) was incubated for 2 h at 37°C in 0.1 M sodium acetate buffer, pH 5.0, containing 1 mM sodium azide and the thiol compound in the desired concentration. The reaction was stopped by adding an equal volume of 20% (v/v) glycerol, 4% (w/v) SDS and 2 mM EDTA in water. An aliquot of 0.05 ml was analysed on 2.6% (w/v) polyacrylamide gels. The different degrees of degradation were determined after 4 h electrophoresis at 60°C with a voltage of 4 V/cm of gel.

1) +++++, indicates completely RNA, showing little or no absorbance on 2.6% (w/v) polyacrylamide gels; -, no degradation.
2) NT, not tested.

the degradation of the RNA was compared. All thiol compounds stimulated the in situ degradation of CCMV-RNA at pH 5.0. Of the different compounds DIECA was least effective. Increasing the thiol concentration stimulated degradation of the RNA. Cysteine, DTT and glutathione stimulated the degradation maximally at a concentration of 0.01 to 0.05 M while a further increase in concentration had no effect. Thioglycollic acid even decreased the amount of degraded RNA at concen-

trations of 0.05 to 0.1 M.

The pH of all thiol compound solutions were adjusted to 5, before adding to the incubation mixture. The adjustment of pH was necessary since the RNA degradation depended on pH. In the range from pH 4.5 to pH 6.5 the most rapid degradation was found at pH 5.0 (results not shown).

Isolated RNA did not degrade when incubated with the various thiol compounds at 37°C.

The effect of coat protein

If one volume of isolated RNA (0.4 mg/ml) was incubated at 37°C with one volume of CCMV (1.6 mg/ml) in the presence of 2-ME, less RNA degradation was observed than with virus alone (Fig. 3.4A, B and C). Apparently the added RNA had an effect similar to EDTA.

However, if one volume of isolated RNA (0.4 mg/ml) was mixed with one volume

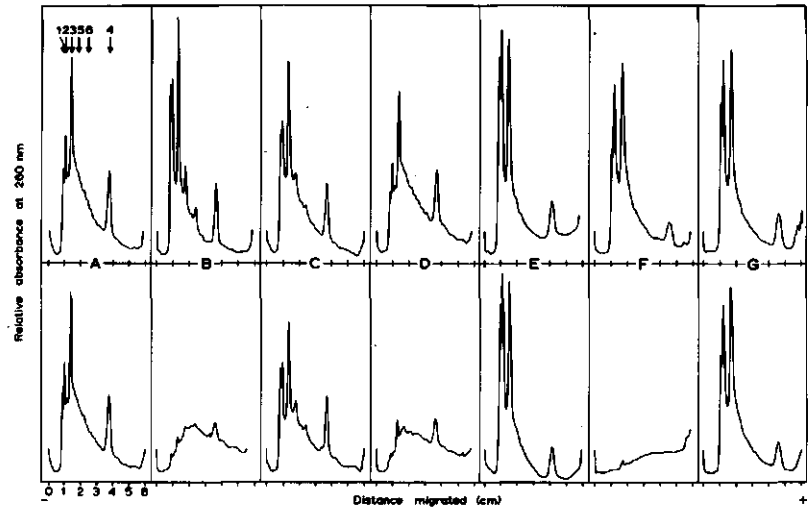


Fig. 3.4. Electrophoresis patterns of in situ degraded CCMV-RNA in 2.6% (w/v) polyacrylamide gels at 60°C; the effect of different states of aggregation of the coat protein. Lower diagrams: purified virus preparations were incubated in 0.1 M sodium acetate, pH 5.0, 1 mM sodium azide and 1% (v/v) 2-mercaptoethanol at 37°C for 2 h prior to electrophoresis. (A) 10 µg of isolated RNA, (B) 40 µg of virus, (C) 20 µg of virus + 10 µg of isolated RNA, (D) 30 µg of empty protein capsids + 10 µg of isolated RNA, (E) 40 µg of 'virus', reassembled in the presence of EDTA, (F) 40 µg of 'virus' reassembled in the absence of EDTA, (G) 10 µg of isolated RNA. Upper diagrams: as lower ones, except that all preparations were incubated at 37°C without 1% (v/v) 2-ME, prior to dissociation of the nucleoproteins with SDS and electrophoresis on gels. For details of electrophoresis see Fig. 3.1. Two different preparations of RNA were used: (A), (C) and (D), RNA obtained from a phenol extraction of the Ca-precipitated RNA during the protein preparation; (E), (F) and (G), RNA obtained from phenol extraction of virus at pH 5.0, as described in Materials and Methods.

empty protein capsids (1.2 mg/ml) in 0.2 M NaCl, 1 mM DTT and 50 mM sodium acetate buffer, pH 5.0 and incubated at 37°C in the presence of 2-ME, the RNA degraded (Fig. 3.4D). Probably the higher protein-RNA ratio is responsible for the degradation of the RNA because in situ degradation was independent of the presence or absence of 0.2 M NaCl (results not shown).

If virus particles were reassembled from isolated RNA and washed coat protein as described by Bancroft and Hiebert (1967) the RNA degraded in situ upon storage of the assembled particles in virus buffer without EDTA or after incubation with 2-ME at 37°C (Fig. 3.4F). This also demonstrated the need for high protein-RNA ratios to induce RNA degradation. Incorporation of 1 mM EDTA in the buffer solutions prevented degradation of RNA during assembly, and in situ RNA degradation of reassembled virus particles upon incubation with 2-ME at 37°C (Fig. 3.4E).

These results show that virus coat protein is involved in the in situ degradation of CCMV-RNA, although native virus structure is not essential for activity. Because the in situ degradation can be prevented by EDTA or other chelating agents it appears unlikely that the in situ degradation of the RNA is due to nucleases encapsidated by the virus.

Characterization of virus with in situ *degraded RNA*

Incubation of virus with 1% (v/v) 2-ME at 37°C for 4 h resulted in the degradation of its RNA into fragments with molecular weights of less than 100,000 (cf Fig. 3.5A and E). Virus particles containing degraded RNA sedimented at the same rate as virus with undegraded RNA (Fig. 3.5B and F). The buoyant density of these nucleoprotein particles in RbCl and the relative amounts of each of the components were not changed (Fig. 3.5C and G). No aggregation or disintegration of virus particles was apparent from the sedimentation behaviour and in the electron microscope the virus particles were similar to those of virus with undegraded RNA (Fig. 3.5D and H). From these results it may be concluded that in the in situ degradation of the RNA into low molecular weight fragments did not have an effect on the morphology, density and hydrodynamic properties of the virus. The degraded RNA did not show distinct bands after electrophoresis on 6 or 10% (w/v) polyacrylamide gels, indicating that the RNA had degraded to heterogeneous small fragments. On gels it was difficult to establish a quantitative relation between the loss of RNA-1, -2, -3 and -4 and the appearance of small heterogeneous fragments. Therefore virus was incubated for 0, 60 and 120 minutes at 37°C with 1% (v/v) 2-ME, dissociated with SDS and analysed on 10 to 50% (w/v) linear sucrose density gradients (Fig. 3.6, B to F). It was shown that the degradation of RNA-1, -2 and -3

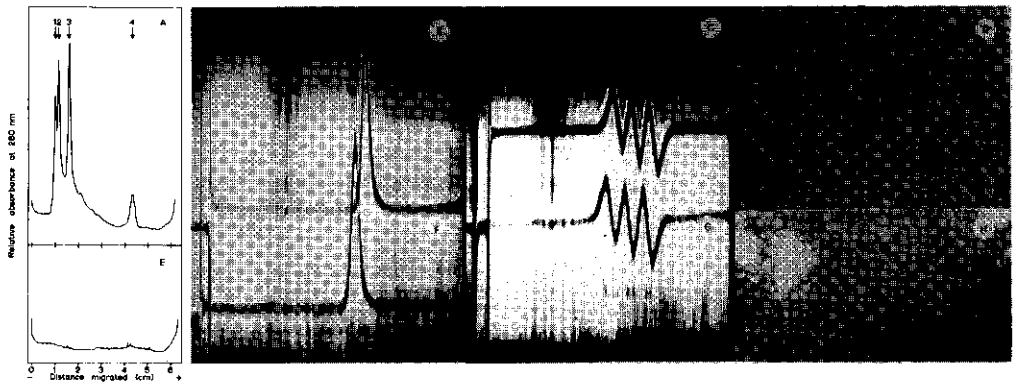


Fig. 3.5. Characteristics of CCMV, purified and kept in the presence (A to D) and absence (E to H) of EDTA, after incubation for 4 h at 37°C with 1% (v/v) 2-ME.

(A,E). Electrophoresis patterns of RNA in 2.6% (w/v) polyacrylamide gels at 60°C. Arrows and numbers indicate the distinct high molecular weight RNA species found in CCMV. For details of electrophoresis see Fig. 3.1.

(B,F) Schlieren pattern of CCMV (5 mg/ml) sedimenting in the analytical ultracentrifuge. The picture was taken 16 minutes after the rotor had reached a speed of 31,410 rev/min at 20°C. The sedimentation is to the right. Bar angle: 60°C.

(C,G) Schlieren pattern of the isopycnic centrifugation of CCMV in RbCl after 40 h at 44,770 rev/min at 20°C. Centrifugal force to the right; concentration: 0.25 mg/ml, bar angle: 60°C.

(D,H) Electron micrographs of CCMV on carbon coated grids, negatively stained with 2% (w/v) uranyl acetate in distilled water. The bar represents 100 nm.

was accompanied by increasing amounts of slowly sedimenting heterogenous material (compare Fig. 3.6, D to F). The areas under the absorbance patterns of the gradients were cut as indicated in the Fig. 3.6 and weighed. The absorption areas appeared to be the same within 10%, indicating that the different gradients contained the same amount of RNA. So the conclusion can be drawn that incubation of virus with 2-ME degraded its RNA into small heterogeneous fragments and that RNA was not lost due to aggregation of RNA or failure of virus to dissociate. Immediately after incubation, the same virus preparations were analysed on 2.6% (w/v) polyacrylamide gels for direct comparison of the degraded RNA patterns on gels and in sucrose gradients (compare Fig. 3.7 with Fig. 3.6). The decrease in the peak heights of RNA-1, -2 and -3 in the electropherogram corresponded with the increase of slowly heterogeneous sedimenting material in the sucrose gradients (Fig. 3.6 and 3.7, lower panels). Also some CCMV-RNA was degraded in situ when the virus was incubated for these long periods with 2-ME at 37°C even in the presence of EDTA (Fig. 3.6 and 3.7, upper panels).

Virus, purified and kept without EDTA, rapidly lost its infectivity, when incubated with 2-ME. Table 3.2 shows that after 90 minutes incubation about 10% of the infectivity was left, whereas CCMV, purified and kept with EDTA, then in-

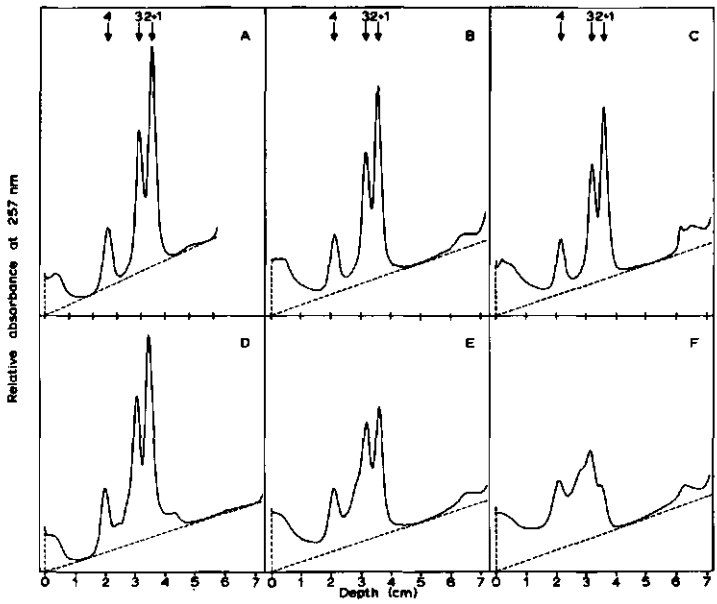


Fig. 3.6. Rate zonal sucrose density gradient centrifugation patterns of CCMV-RNA in different states of degradation. Virus (2.5 mg/ml), purified and kept without EDTA, incubated with 1% (v/v) 2-ME in 0.1 M sodium acetate, pH 5.0, containing 1 mM sodium azide at 37°C for (D) 0, (E) 60 and (F) 120 min, was dissociated with 2% (w/v) SDS and 0.25 mg of the dissociated product was analysed on 10 to 50% (w/v) linear sucrose density gradients in an SW 41 rotor for 15 h at 25,000 rev/min and 20°C. The contents of the tubes were monitored at 257 and 281 nm. As control, virus purified and kept with EDTA was incubated at 37°C with 2-ME in the presence of 1 mM EDTA, during (A) 0, (B) 60 and (C) 120 min. The absorbance at the top of the gradient, shown also with control virus, is due to the SDS-denatured coat protein from the dissociated virus. Near the bottom of the tube some absorbing material could be detected, which appeared to be SDS precipitated in about 50% (w/v) sucrose.

cubated with 2-ME in the presence of 1 mM EDTA, retained 65% of the infectivity of the control preparation, treated with 2-ME on ice. From these data the rate of inactivation can be calculated; $t_{\frac{1}{2}}$, incubation time after which 50% of the infectivity is lost, is 30 minutes. Table 3.2 further shows that virus, which was purified and kept without EDTA, contained some RNA-2 degraded into RNA-5 and -6 and had 20% lower infectivity, compared with virus that was purified and kept with EDTA.

The role of oxygen in the degradation

Murata et al. (1972b, 1973) reported in situ degradation of the ds-DNA of bacterophage J_1 by low concentrations of thiol compounds. These authors explained their results by the action of highly reactive superoxide radicals $O_2^{-\cdot}$, which could directly or indirectly inactivate the nucleic acid. To examine whether this

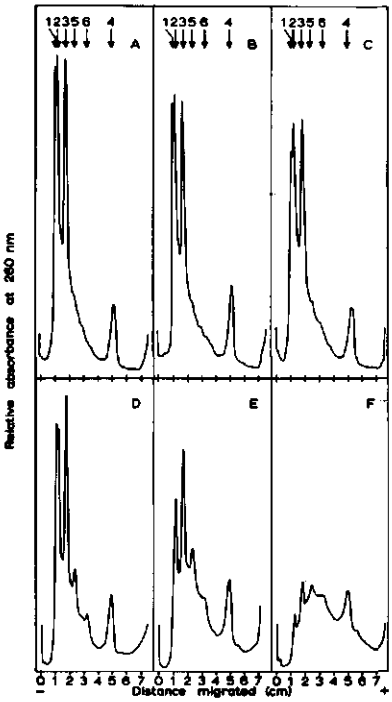


Fig. 3.7. Electrophoresis patterns of CCMV-RNA degraded to different degrees in 2.6% (w/v) polyacrylamide gels. A sample of 0.065 mg of the dissociated product described in Fig. 3.6. was analysed on polyacrylamide gels as described in Fig. 3.1. For explanation of symbols see Fig. 3.6.

Table 3.2. Infectivity of CCMV-preparations with in situ degraded RNA in Chenopodium hybridum L.

Incubation time with 2-ME (minutes)	Infectivity*)	
	Virus purified, kept and incubated with EDTA	Virus purified, kept and incubated without EDTA
0	100	78
30	109	52
60	70	13
90	65	10

Virus (1.6 mg/ml), with or without 1 mM EDTA, was incubated with 1% (v/v) 2-ME at 37°C in 0.1 M sodium acetate, pH 5.0, containing 1 mM sodium azide. After 0, 30, 60 and 90 minutes 1 mM EDTA was added and samples were removed from the incubation mixture and diluted to a virus concentration of 0.1 $A_{260\ nm}$/ml with 10 mM sodium phosphate, pH 6.0, containing 1 mM $MgCl_2$. Virus preparations, after incubation were tested on 10 half leaves of Chenopodium hybridum L. The opposite halves were inoculated with control virus: a standard virus preparation, producing about 40 lesions per half leaf.

*) Infectivity was expressed as (total number of lesions on ten test halves/ total number of lesions on the control halves) x 100. Numbers are averages of three experiments.

mechanism was responsible for the 2-ME induced degradation of CCMV-RNA, CCMV was incubated with 2-ME but without EDTA in a N_2 atmosphere, or in the presence of compounds known to inactivate superoxide radicals. Superoxide dismutase and 2-amino-ethylisothiouronium bromide hydrobromide (AET) both scavenge superoxide radicals (McCord and Fridovich, 1969; Murata and Kitagawa, 1973a and b). Bubbling nitrogen gas through a virus solution for 10 minutes prior to incubation with 2-ME under N_2 in a sealed tube reduced the in situ degradation of CCMV-RNA (Fig. 3.8D) compared with control virus without N_2 (Fig. 3.8C). Incubation of virus in

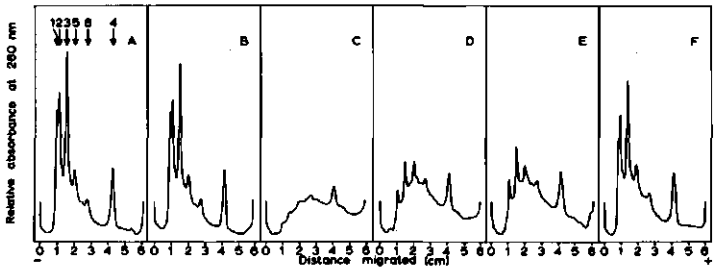


Fig. 3.8. Electrophoresis patterns of in situ degraded CCMV-RNA in 2.6% (w/v) polyacrylamide gels at 60°C; the effect of several radical scavengers on the RNA degradation.

(A) Virus in 0.1 M sodium acetate buffer, pH 5.0, + 1 mM sodium azide kept on ice.
(B) Virus in buffer without EDTA incubated for 2 h at 37°C.
(C) Virus in buffer without EDTA incubated for 2 h at 37°C with 1% (v/v) 2-mercaptoethanol.
(D) Virus in buffer without EDTA; the solution was bubbled with nitrogen gas for 10 min, sealed and incubated for 2 h at 37°C with 2-ME under N_2.
(E) Virus in buffer without EDTA incubated for 2 h at 37°C with 2-ME in the presence of 3.5×10^{-8} M superoxide dismutase.
(F) Virus in buffer without EDTA incubated for 2 h at 37°C with 2-ME in the presence of 3% (w/v) 2-aminoethylisothio-uronium bromide hydrobromide.

Before electrophoresis, viral preparations were dissociated in a 1 mM EDTA, 10% (v/v) glycerol and 2% (w/v) SDS mixture and 0.065 mg of nucleoprotein was applied to gels.

the presence of 0.35×10^{-8} M superoxide dismutase without nitrogen gas also decreased in situ degradation of CCMV-RNA (Fig. 3.8E). Both results demonstrate that the involvement of molecular oxygen can partially explain the process. The almost complete inhibition of the in situ CCMV-RNA degradation in the presence of 3% (w/v) AET, a radical scavenger (Freese and Freese, 1966), suggests that RNA degrades predominantly by a free radical mechanism (Fig. 3.8F).

With AET, lower concentrations were sufficient to prevent the in situ RNA degradation. With superoxide dismutase, however, complete inhibition could not be observed, even at a concentration of 10^{-5} M.

DISCUSSION

In purified CCMV, some RNA-2 degraded into distinct RNA fragments; RNA-5 and -6. Upon storage, RNA within purified virus degraded further *in situ*. 2-ME stimulated this degradation. The degraded RNA consisted of small heterogeneous RNA fragments. Low concentrations of EDTA and other chelating agents prevented the *in situ* RNA degradation.

I do not know of any described degradation or inactivation of a plant virus by reducing agents. A protective effect of EDTA was also demonstrated for AMV by Van Vloten-Doting *et al.*, 1970, who did not explain the effect. Complete inactivation of AMV occurred when the purified virus was kept for 14 days at $4^{o}C$. This inactivation was prevented by EDTA.

For bacterial viruses Murata *et al.* (1971, 1972a and b) have observed degradation of isolated and *in situ* nucleic acid by reducing agents which could be prevented by chelating agents. The rates of inactivation of bacteriophage J_i by 2-ME (Murata *et al.*, 1972a) are comparable with my data from infectivity tests, although it has to be borne in mind that the rate is strongly dependent on the concentration of the reducing agent and the pH of the incubation mixture (Murata *et al.*, 1971; Murata and Kitagawa, 1973b). Murata *et al.* (1973) explained their results by a radical mediated mechanism, without specifying the process.

Suppression of RNA degradation by radical scavengers also suggests the involvement of a radical. Cecil and McPhee (1959) suggested the following reactions for the oxidation of a thiol compound (RSH) by oxygen, catalysed by traces of contaminating metals, e.g. Fe^{++}:

$$Fe^{++}RS \text{ complex} + O_2 \longrightarrow Fe^{+++}RS \text{ complex} + O_2^{-\cdot},$$

$$O_2^{-\cdot} + RSH \longrightarrow HO_2^{-} + RS^{\cdot},$$

$$RSH + RS^{\cdot} + Fe^{+++}RS \text{ complex} \longrightarrow Fe^{++}RS \text{ complex} + RSSR + H^{+}.$$

I have found that, for the *in situ* degradation of CCMV-RNA, oxygen is required as well as free radicals. The effect of chelating agents can also be explained by assuming a metal mediated inactivation.

The fact that RNA-2 is split into two distinct fragments, while the other RNAs are not, suggests that RNA-2 is somewhat differently packed in the coat protein shell. A similar degradation mechanism during purification catalysed by ascorbic acid rather than 2-ME is feasible. The radical species responsible for the breaks in CCMV-RNA has not been determined.

Van Hemmen and Meuling (1975) demonstrated that the inactivation of DNA by radiolysis of oxygenated aqueous DNA solutions was strongly pH-dependent. Under

slightly acidic conditions the superoxide radical is in equilibrium with an hydroperoxyl radical ($HO_2^{\cdot}$) according to $HO_2^{\cdot} \rightleftharpoons O_2^{-\cdot} + H^+$ with a pK value of 4.9 (Van Hemmen and Meuling, 1975). These authors found that a decrease in pH of the incubation medium increased the rate of inactivation, suggesting an hydroperoxyl radical ($HO_2^{\cdot}$) as inactivating radical rather than the superoxide radical ($O_2^{-\cdot}$). Both $O_2^{-\cdot}$ and $HO_2^{\cdot}$ can react with H_2O_2, formed from $O_2^{-\cdot}$, $HO_2^{\cdot}$ and water, to produce an $OH^{\cdot}$ radical. This hydroxyl radical can react with nucleic acids by a mechanism similar to enzyme catalysed hydrolysis (Yagi, 1972). The in situ degradation of CCMV-RNA was maximal at pH 5.0 and decreased at both acidic and basic pH values; hence for CCMV, a hydroxyl radical might be the inactivating species.

Probably coat protein is a source of traces of metal ions. In virus purified using EDTA, RNA can degrade in situ after extensive dialysis of the virus against several changes of EDTA-free buffer. Apparently the coat protein collects trace amounts of metals from buffer and salt solutions of analytical grade chemicals during this procedure. The degradation of CCMV-RNA in situ varied among different virus preparations probably because of the varying amounts of contaminating metals. The resistance of isolated RNA to degradation by 2-ME was not absolute, but demonstrated the low free-metal content of isolated RNA. It will be necessary to analyze the metal content of CCMV preparations to test this hypothesis. A study of the influence of metals on the integrity of RNA and virus at pH 5.0 and 7.5 in the presence or absence of thiol compounds will be needed to discriminate between the complexity of either specific metal or oxygen effects. In preliminary experiments I have observed that Cu^{2+} prevented in situ degradation of CCMV-RNA. Here, a metal scavenged the superoxide radical and thus prevented in situ RNA degradation.

Some contribution to the in situ RNA degradation of semiquinones, generated by oxidation of polyphenols or the reduction of O-quinones, which are adsorbed to the coat protein, can not be excluded. However, extraction of virus from tissue in the presence of DIECA and ascorbic acid, known to prevent polyphenoloxidase activity and covalent linkage of quinones to virus, did not prevent in situ RNA degradation. Isolated CCMV-RNA can be inactivated by a mixture of chlorogenic acid and tyrosinase as was reported by Woods and Agrios (1974), although the inactivating species, a quinone, semiquinone or RNase was not unequivocally demonstrated. Bancroft (1971) extracted degraded RNA from virus, which was also incubated with chlorogenic acid and tyrosinase. The influence of quinones on CCMV in vivo and in vitro is now being investigated.

Dawson & Kuhn (1974) found low specific infectivities with CCMV from 10- to 20-days-infected cowpea plants. When this virus of low specific infectivity was

subjected to phenol extraction, over 85% of the RNA was lost and the remainder appeared degraded and was relatively noninfectious (Wyatt and Kuhn, 1975). I neither observed low specific infectivities of CCMV from 20-day-infected cowpea plants nor degraded RNA if the virus was purified in the presence of EDTA (results not shown).

I have observed that brome mosaic virus and tobacco mosaic virus ribonucleic acids were also degraded in situ, when virus was incubated with 2-ME (results not shown). The degradation could be prevented by chelating agents. At least for BMV, CCMV, TMV, AMV (Van Vloten-Doting et al., 1970) and tobacco streak virus (Fulton, 1975) addition of EDTA to homogenization and storage buffers greatly improves the preparation of high quality virus with increased stability. Furthermore, since sulphhydryl reducing agents are frequently included in enzyme reactions with viruses, addition of chelating agents to the reaction mixtures to improve virus stability is highly recommended.

4. THE PREPARATION OF CCMV-PROTEIN IN CONNECTION WITH ITS ASSOCIATION INTO A SPHERICAL PARTICLE

Three coat protein preparation methods are described for cowpea chlorotic mottle virus (CCMV). Only the use of 0.5 M $CaCl_2$ at pH 7.5 resulted in protein with less than 0.25% (w/w) residual RNA, independent of the state of degradation of RNA within the nucleoprotein particles. After dialysis of the isolated protein in 0.2 M NaCl at pH 5.0 and pH 5.7 pseudo top component (PT) and PT-doubles (PT-D) were formed respectively. The formation of PT-D could be suppressed by Ca^{++}. The pH dependent dissociation and association of the isolated coat protein showed a hysteresis of about 0.5 pH unit. The pH at which 50% of the protein was present as PT was about 5.5 in the association reaction and about 6.0 in the dissociation reaction.

INTRODUCTION

The self-assembly of a spherical plant virus was first described in 1967 for cowpea chlorotic mottle virus (CCMV) (Bancroft and Hiebert, 1967; for a recent review see Bancroft, 1970). CCMV consists of three nucleoprotein particles, each with a diameter of 25 nm and a protein coat constructed of 180 identical polypeptides. Four species of RNA of different size were found in those particles (Fig. 4.1A). The two smallest were associated with one particle; the other two were each associated with a different particle (Bancroft and Flack, 1972).

When the virus was dialysed against 1 M NaCl at pH 7 it dissociated into RNA and protein subunits. Isolated protein subunits themselves associated into icosahedral particles (pseudo top component, PT) when they were subsequently dialysed against 0.2 M NaCl at pH 5. The resulting protein particle had approximately the same geometric arrangement as in the native virus. However when a dialysis medium of pH 5.7 was used the protein particles which formed were a mixture of PT and PT surrounded by one or several layers of protein (pseudo top doubles and rosettes, PT-D) (Bancroft et al., 1968b; Bancroft et al. 1969a).

The virus protein association was influenced by nucleic acid; virus nucleic acid was found to cause the formation of an icosahedral particle even at low ionic strength and neutral pH, where the protein itself did not associate. The formation of an icosahedral particle under these conditions was already accomplished by a small polynucleotide e.g. poly U, n = 23 (Bancroft et al., 1969b).

Different methods to prepare viral protein were therefore compared to their degree of contamination with nuclei acid. The results show that the formation of PT-D was not due to nucleic acid contaminating the viral protein preparations. The formation of PT-D can be prevented in the presence of divalent metal ions, particularly Ca^{++}.

MATERIAL AND METHODS

CCMV, obtained from Dr. J.B. Bancroft, was grown in cowpea plants, Vigna unguiculata (L.) Walp. var. Blackeye Early Ramshorn, and purified by the polyethylene glycol method as described by Bancroft et al. (1972). The only modification was that the leaf homogenate was kept at 4°C and at pH 4.7 for one hour. The purified virus was stored at 4°C in 0.1 M sodium acetate pH 5.0 containing 1 mM sodium azide. Virus concentrations were determined with $E^{1\%}_{1\ cm}$ at 260 nm = 58.5 in 0.1 M sodium acetate buffer, pH 5.0 (Bancroft, personal communication).

The viral coat protein was prepared by the following methods:

Method A. A virus suspension (4 mg/ml) was dialysed overnight at 4°C against 1 M NaCl, 1 mM dithiothreitol (DTT) buffered with 20 mM Tris-HCl pH 7.4. The virus dissociated under these conditions and the RNA was sedimented by centrifugation for 16 h at 73,000 g. The top three-fourths of the supernatant was used as a viral protein solution (Bancroft and Hiebert, 1967).

Method B. This follows method A except that the separation of the RNA was done by zonal centrifugation. Sixty milligrams of virus (1 mg/ml) were dialysed overnight against the buffer mentioned above. The dialysed solution was centrifuged in a 10 to 50% (w/v) sucrose density gradient linear with the radius in dissociation buffer for 24 h at 30,000 rev/min in an MSE B XIV zonal rotor. After centrifugation, the contents of the rotor were fractionated and the pooled protein fractions concentrated by ammonium sulphate precipitation (0.6 saturation). After dialysing the protein against dissociation buffer, it was used for association studies.

Method C. Virus, 10 mg/ml, was dialysed against 0.5 M $CaCl_2$, 1 mM DDT buffered with 50 mM Tris-HCl pH 7.5. After dialysing for 6 to 20 h at 4°C a white precipitate of RNA was collected by low speed centrifugation. The supernatant was then centrifuged for 2 h at 115,000 g to remove residual undissociated virus. The protein solution obtained was either stored at 4°C and dialysed against 1 M NaCl pH 7.5 before being used for association studies, or dialysed and used immediately.

Protein concentrations were determined spectrophotometrically, assuming $E^{1\%}_{1\ cm}$ at 280 nm to be 12.7 in 1.0 M NaCl pH 7.4 (Bancroft, personal communication). RNA was determined by the orcinol method.

Viral RNA was analysed by electrophoresis in 2.6% (w/v) polyacrylamide gels according to Loening (1967, 1969) at 60°C (Reijnders et al., 1974). Virus was dissociated at room temperature in a solution of 10% (v/v) glycerol and 2% (w/v) sodium dodecyl sulphate (SDS) in water. The mixture was subjected directly to electrophoresis with a current of 3.5 mA per gel for 3.5 h. The gels were scanned at 260 nm or stained with 0.01% (w/v) Toluidine Blue in 40% (v/v) methoxyethanol in water. Gels were destained in water.

Electron micrographs of virus particles and protein aggregates were obtained with a Siemens Elmiskop 101. The material was negatively stained over holes in a carbon film with 1% (w/v) uranylacetate in double-distilled water.

Sedimentation analysis was performed in an MSE analytical ultracentrifuge equipped with Schlieren optics and an ultraviolet absorption scanner.

All buffer solutions were made at room temperature and contained 1 mM DTT. Solutions of pH 5.0 to 6.3 were buffered with 50 mM sodium acetate and above pH 6.3 the solution was buffered with 50 mM Tris-HCl, unless otherwise stated.

RESULTS AND DISCUSSION

The results of the three methods used to prepare coat protein are summarized in Table 4.1. Compared are percentages of theoretical yield and absorbance ratios. The percentage of theoretical yield was calculated by assuming that the protein content of the virus is 76 percent by weight (Bancroft et al., 1968a). The yield increased from about 50% in method A to more than 90% in method C. The absorbance ratio at 280 and 260 nm indicates the amount of residual RNA. This ratio varied from 1.3 for method A to 1.75 for method C, indicating a RNA con-

Table 4.1. Yield and spectral properties of virus coat protein preparations.

Method [1]	Yield[2]	A_{280}/A_{260} [3]
A	50%	1.40 ± 0.10
B	60-80%	1.55 ± 0.05
C	90%	1.70 ± 0.05

1) A, B and C refer to the respective methods described under materials and methods.

2) Percentage of theoretical yield based on a protein content of the virus of 76% (w/w) (Bancroft et al., 1968b).

3) The absorbance ratio of 280 and 260 nm indicates the presence of residual nucleic acid. A_{280}/A_{260} = 1.75 indicates less than 0.25% (w/w) residual RNA.

tamination of about 2.3% and less than 0.25% (w/w) respectively.

The contaminating RNA in protein prepared by method A or B is probably low molecular weight RNA, which is not precipitated by 1.0 M NaCl and subsequent centrifugation during 16 h at 73,000 g. Ammonium sulphate precipitation of the protein used in method B improved the absorbance ratio but still did not eliminate all of the RNA. $A_{280\ nm}/A_{260\ nm}$ increased from 1.35 to 1.50 in a typical experiment.

It was not possible to use ionic exchange chromatography to purify the protein because it is insoluble under the conditions of low ionic strength and neutral pH, necessary to bind the residual RNA to the column. We therefore used $CaCl_2$ (method C), which precipitates the RNA quantitatively as has been previously described for brome mosaic virus (Yamazaki and Kaesberg, 1963). The contaminating low molecular weight RNA in methods A and B is due to degradation of the RNA in the virus particles prior to dissociation in 1 M NaCl. Details of the RNA degradation process inside the virus will be published elsewhere (see Chapter 3).

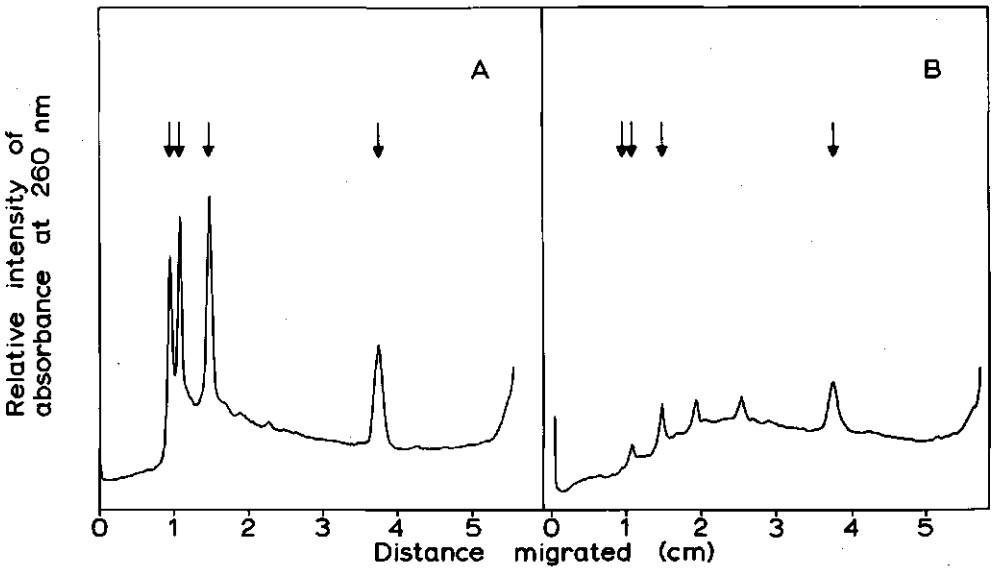


Fig. 4.1. Electrophoresis pattern of CCMV-RNA in 2.6% (w/v) polyacrylamide gels at 60°C. Virus was dissociated in 10% (v/v) glycerol and 2% (w/v) SDS and analysed directly on gels. (A) Virus with intact RNA; (B) Virus with degraded RNA. The arrows indicate the positions of the RNA components isolated from biological active virus. The anode is at the right.

Fig. 4.1A shows the electrophoresis pattern of intact CCMV-RNA and Fig. 4.1B the pattern of strongly degraded CCMV-RNA. Both were run at 60°C after application of about 12 micrograms of RNA. It is clear from Fig. 4.1 that large amounts of

degraded RNA can be present in purified virus and these may contaminate protein prepared by method A or B.

In contrast to what was observed by applying method A or B, almost no variation in spectral properties was found for protein prepared according to method C, irregardless of the degree of RNA degradation in the original virus preparation. This has been verified repeatedly by preparing protein from different batches of virus, each of which contained different amounts of low molecular weight RNA, as determined by polyacrylamide gel electrophoresis.

When protein in 1.0 M NaCl, pH 7.5, was dialysed against 0.2 M NaCl, pH 5.0, or pH 5.7, the protein associated into PT and into a mixture of PT and PT-D respectively. PT sedimented with $s_{20,W}$ of about 52 S and PT-D with $s_{20,W}$ of about 110 S. The PT-D preparation was more inhomogeneous than PT as shown in the electron micrographs in Fig. 4.2A and B. The two preparations differed in their

Fig. 4.2. Electron micrographs of protein aggregates obtained if CCMV-protein was dialysed from 1 M NaCl pH 7.5 to 0.2 M NaCl pH 5.7 (A) or pH 5.0 (B). The aggregates were negatively stained with 1% (v/v) uranylacetate in water. The bar represents 50 nm. The arrows point to PT-D particles.

stability. The PT-D preparation often precipitated during storage while PT at pH 5.0 remained soluble for months at 4°C. The association of the protein into PT and PT-D at pH 5.7 in 0.2 M NaCl was not appreciably influenced by the protein preparation method used as judged from sedimentation analysis. Contaminating RNA did not interfere with the formation of PT-D.

The formation of PT-D at pH 5.7 could be suppressed by the addition of Ca^{++} ions in the dialysis procedure. The sedimentation patterns of the association products in the presence of 0.2 M NaCl and 1 mM DTT at pH 5.7, with either 10 mM EDTA or 10 mM $CaCl_2$ are shown in Fig. 4.3A and B respectively. The addition of Ca^{++} ions probably induced a compact conformation of the protein at pH 7.5, necessary for the formation of an intact icosahedral particle like PT at pH 5.0 in 0.2 M NaCl.

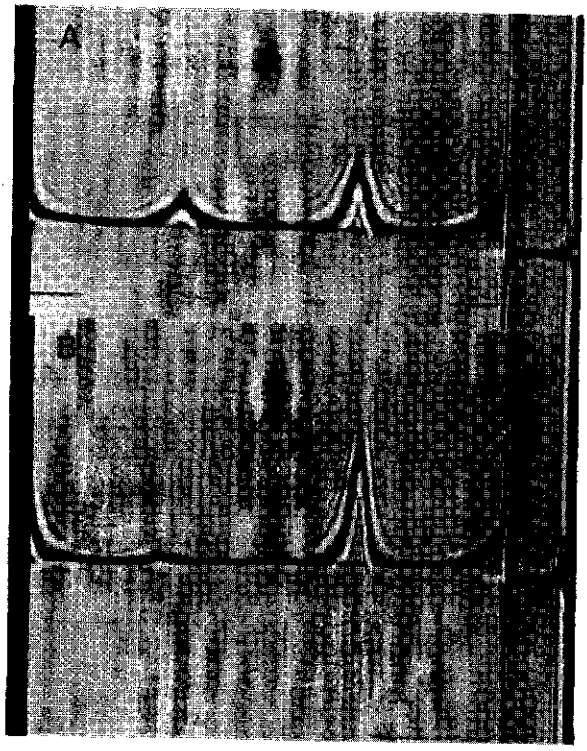


Fig. 4.3. Schlieren sedimentation patterns of CCMV-protein after dialysing from 1.0 M NaCl 7.5 to 0.2 M NaCl pH 5.7 in the presence of either 0.01 M EDTA (A) or 0.01 M $CaCl_2$ (B). Protein concentrations: 3.35 mg/ml. The picture was taken 14 min after the rotor reached a speed of 30,000 rev/min at 20.0°C. The sedimentation is from right to left.

The partition of protein among PT and dissociated protein in 0.2 M NaCl, 10 mM $CaCl_2$ from pH 7.5 to 5.0 depended on the pH and ionic strength of the protein solution from which the dialysis was started.

Fig. 4.4 shows how the partition of protein differed in the association and dissociation reaction. The pH at which 50% of the protein was present as PT was about 5.5 in the association reaction and about 6.0 in the dissociation reaction. PT as the percentage of total protein was determined from the areas in the ultraviolet absorption scans of dissociated protein and total protein. A similar hysteresis effect has been found in acid-base titration studies with CCMV (Johnson et al., 1973). This effect, as well as that of divalent ions, is in agreement with the model for the association and dissociation of CCMV as proposed by

Bancroft et al. (1973).

The control of the self-association and the dissociation of CCMV-protein offers good prospects for the study of the kinetic and thermodynamic aspects of these processes without any interference of byproducts like PT-D.

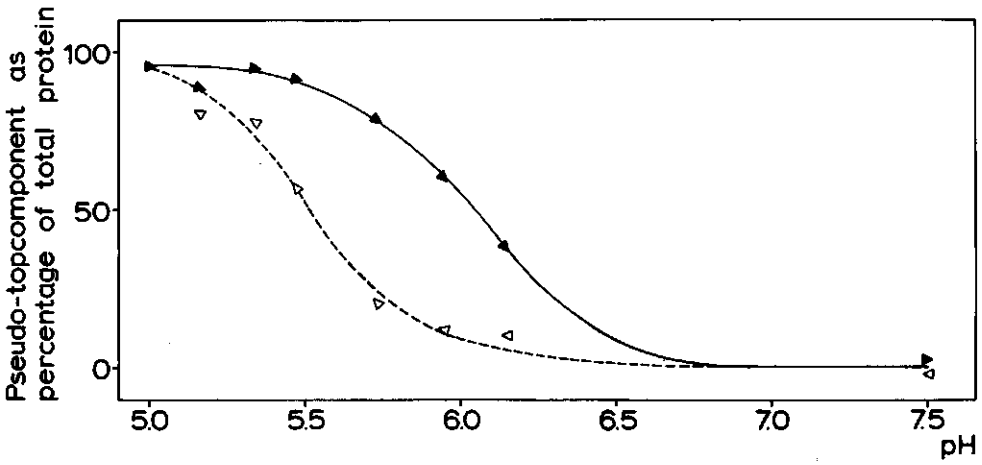


Fig. 4.4. The dissociation and association of CCMV-protein as a function of the pH in 0.2 M NaCl and 0.01 M $CaCl_2$, expressed as PT component as percentage of total protein. The percentage of PT was determined from the areas of PT and dissociated protein in the ultraviolet absorption scan. (Δ——————Δ) Dissociation behaviour of PT if the pH was increased from pH 5 to 7.5 by dialysis to a higher pH. (Δ----------Δ) Association of CCMV-protein if the pH was lowered from pH 7.5 to 5 by dialysis.

5. PARTIAL SPECIFIC VOLUME AND CIRCULAR DICHROISM OF CCMV AND SEDIMENTATION EQUILIBRIUM OF THE DISSOCIATED COAT PROTEINS OF THREE BROMOVIRUSES

When the pH and the ionic strength of cowpea chlorotic mottle virus (CCMV) coat protein solutions are changed so that the dissociated protein associates into empty spherical particles changes in the apparent partial specific volume are observed. From the apparent partial specific volume of the empty protein shells, 0.745 cm^3/g and of isolated CCMV-RNA, 0.476 cm^3/g both in 0.2 M NaCl at pH 5.0, an apparent partial specific volume of nucleoprotein, 0.680 cm^3/g can be calculated. This calculated value does not equal the value of 0.719 cm^3/g, determined experimentally for the virus at pH 5.0. The difference is caused by RNA-protein linkages in the nucleoprotein particle.

Sedimentation equilibrium of the dissociated protein of broad bean mottle virus (BBMV), brome mosaic virus (BMV) and CCMV shows non-ideal hydrodynamic behaviour and suggests that the coat protein subunits occur mainly as dimers at pH 7.5.

Circular dichroism measurements on CCMV nucleoprotein, its RNA and coat protein show only small variations in the secondary structure of both RNA and coat protein when association into nucleoproteins or empty protein shells occurs. The protein contains little if any ($< 1\%$) α - helical structures, both in solution and inside the virion. More than 60% of the bases of CCMV-RNA are paired or stacked and the amount of base pairing decreases when the RNA is encapsulated, both at pH 7.0 and 5.0. The difference in secondary structure between virus RNA in solution and inside virus particles is discussed in view of the types of interaction stabilizing the nucleoproteins and the penetration of the RNA into the protein capsid.

INTRODUCTION

Broad bean mottle virus (BBMV), brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) are three bromoviruses. Each bromovirus preparation contains four RNA molecules, numbered 1 through 4 in order of decreasing size. The two smallest RNA molecules are enclosed in a protein shell consisting of 180 copies of a single polypeptide (MW $\sim$ 20,000) arranged on the surface of an icosaeder. The other two RNA molecules form two seperate nucleoprotein particles with protein coats identical to those containing RNA-3 and -4. (Lane, 1974).

The three bromoviruses are stable around pH 5.0 and sediment around 90 S. Increasing the pH of the virus solution to pH 7.5 at low ionic strength causes a drop in the sedimentation coefficient to about 80 S e.g. the virus swells.

At high ionic strength and pH 7.5 the virus dissociates into protein and nucleic acid components (Bancroft, 1970 b).

In this chapter I would like to report about the determination of partial specific volumes and circular dichroism of CCMV and its isolated RNA and protein, and the variation of these properties during swelling and dissociation of CCMV. The dissociated protein of the three bromoviruses is characterized by sedimentation equilibrium.

MATERIALS AND METHODS

Purification of virus, RNA and protein. Bromovirus nucleoproteins, their RNAs and coat proteins were isolated by the same procedures and with the same buffers as described for CCMV (Verduin, 1974: Chapter 4; Verduin, 1978: Chapter 3). BBMV was grown in broad beans (Vicia faba cv. Driemaal Wit) and BMV in barley (Hordeum vulgare L. cv. Cambrinus). Virus was purified from 7-day-infected plants and finally kept in virus buffer: 0.1 M sodium acetate, pH 5.0, containing 1 mM sodium azide and 1 mM EDTA at 4°C. Protein and RNA were isolated from fresh or stored virus and both were used in the present experiments. The RNA was kept frozen at -20°C and the protein was stored in the pseudo top conformation (PT) at pH 5.0. For purified virus preparations extinction coefficients $E^{1\%}_{260\ nm}$ = 54.0 (BBMV), 50.0 (BMV) and 58.5 (CCMV) were taken, and for all RNAs $E^{1\%}_{260\ nm}$ = 250. Extinction coefficients $E^{1\%}_{280\ nm}$ = 2.3 (BBMV), 8.1 (BMV) and 12.7 (CCMV) were used for the isolated protein in dissociation buffer at pH 7.5 (Lane, 1974; Verduin, 1974: Chapter 4). Cowpea mosaic virus (CPMV), purified top, middle and bottom components were a gift from Dr. J.L.M.C. Geelen and had been prepared according to Geelen et al. (1972).

Density determinations. Densities of nucleoprotein, protein and RNA solutions were determined by measuring the vibration frequency of a glass tube filled with the sample in a DMA 02C apparatus of Anton Paar (Austria), equipped with a digital precision device for density measurements in liquids and gases. (Kratky et al., 1969). The apparatus has been calibrated with samples of known density: air and distilled water. In all cases the densities of the dialysis buffers were determined in parallel experiments.

Partial specific volumes. The partial specific volume ($\bar{v}$) can be calculated from the density increment of a solution of know concentration with the aid of the equation (Cassasa and Eisenberg, 1964):

$$\bar{v} = 1/\rho_o - 1/c \{ (\rho - \rho_o)/\rho_o \}$$

where ρ_o is the density of the solvent i.e. the dialysis buffer, ρ the density of the solution and c the concentration in g/cm^3. Concentrations were estimated from light absorbances of solutions at 260 and 280 nm using the extinction coefficient given above.

Analytical ultracentrifugation. A MSE Mark I analytical ultracentrifuge equipped with an ultraviolet scanning system, using monochromatic light of 280 nm wavelength was used for sedimentation equilibrium experiments according to the low speed sedimentation equilibrium principle (Schachman and Edelstein, 1966) with a combination of high and low speeds to attain equilibrium (Howlett and Nichol, 1972).

Filled Epon double sector cells with a light path of 20 mm were used. Column height was about 3.5 mm. Time required to reach equilibrium was determined by comparing absorption versus distance from the axis of rotation (r) tracings at various times during the run. After attainment of equilibrium the natural logarithm of the concentration (c) was plotted against the square of the distance from the axis of rotation (r). From the slope of these plots molecular weights were calculated by a modified equation of Svedberg and Pederson (1940), viz.

$$\bar{M}_w = \frac{2\ RT}{(1 - \bar{v}\rho)\omega^2} \quad \frac{d\ln/c}{dr^2}$$

where $\bar{M}_w$ is the weight averaged molecular weight, R the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume, ρ the density of the solution and ω the angular velocity. From two adjacent points in the ln c versus r^2 plot a $\bar{M}_w$ was calculated belonging to the average concentration of the two points. Data were processed without smoothing.

The analytical ultracentrifuge was checked once every six months, by determining the molecular weight of lysozyme (MW = 13,600) according to the described method of sedimentation equilibrium centrifugation.

Circular dichroism. CD measurements were done with a Roussel-Jouan dichrograph, model CD 185, calibrated with epiandrosterone. Spectra of nucleoprotein, RNA and protein solutions in cuvettes with different pathlengths (10, 5 and 2 mm) were recorded in the wavelength range of 325 to 210 nm. Constant nitrogen flushing was employed to prevent water condensation on the cuvettes, which were thermostatted at 5^o for virus and protein and 10^o C for RNA.

The results are reported in terms of mean residue molar ellipticity, (θ), given by the relation:

$$(\theta) = 3300 \frac{\Delta D. \bar{M}_o}{c.l}$$

where $\bar{M}_o$ is the average molecular weight of an amino acid residue or a nucleotide; 107 for CCMV protein and 323 for CCMV nucleic acid. θ for virus was calculated on the basis of nucleotide residue molar concentration. Δ D is the optical density difference between the left and right circularly polarized components of light obtained by multiplication of the full scale setting (1×10^{-5} OD/mm) and the peak height in mm. The symbol c denotes the concentration in mg per ml and l is the path length in cm. The units of θ are degrees cm^2 per decimol. No corrections for the refractive index of the medium was applied.

RESULTS

Partial specific volumes.

In table 5.1. the apparent partial specific volumes of CCMV protein under different conditions and for several concentrations of protein are shown. The apparent partial specific volumes were not extrapolated to zero concentration (Cassasa and Eisenberg, 1964; Lee and Timasheff, 1974), but for each buffer a mean value was calculated. The apparent partial specific volume of dissociated protein decreased from 0.737 cm^3/g in 0.5 M $CaCl_2$ pH 7.5 to 0.729 cm^3/g in 1 M NaCl pH 7.5. Formation of an empty protein capsid at 1 M NaCl pH 5.0 further decreased the apparent partial specific volume to 0.726 cm^3/g, which increased again to 0.728 cm^3/g when the NaCl concentration was lowered to 0.2 M. Strong variations in density measurements occurred with protein solutions dialysed for 48 h at 20°C. Probably this is caused by precipitation of the protein.

From the amino acid analysis of CCMV protein (Lane, 1974) and the specific volume of each amino acid residue (Cohn and Edsall, 1943) a partial specific volume of 0.742 cm^3/g was calculated. The values for BBMV- and BMV-protein are 0.745 and 0.742 cm^3/g respectively.

Table 5.1. Partial specific volumes of CCMV-coat protein in different buffer solutions at pH 5.0 and 7.5. *

$c(g/cm^3)$	$\bar{v}(cm^3/g)$	buffer
0.0102	0.738	0.5 M $CaCl_2$
0.0119	0.735	1 mM DTT
0.0124	0.737	50 mM Tris-HCl pH 7.5
0.0093	0.730	1.0 M NaCl
0.0098	0.723	1 mM DTT
0.0109	0.731	0.01 M $CaCl_2$
0.0107	0.731	50 mM Tris-HCl pH 7.5
0.0083	0.719	1.0 M NaCl
0.0082	0.721	0.01 M ME
0.0083	0.738	0.01 M $CaCl_2$ 50 mM sodium acetate pH 5.0
0.0065	0.724	0.2 M NaCl
0.0113	0.722	1 mM DTT
0.0130	0.738	0.01 M $CaCl_2$
0.0107	0.727	50 mM sodium acetate pH 5.0

* Solutions were dialysed for 2 days at 4°C against the indicated buffer solutions. Concentrations were determined with the extinction coefficient of CCMV-coat protein: $E^{1\%}_{280\ nm} = 12.7$, without correction for light scattering. Densities of solutions were determined with the density measuring apparatus of Anton Paar (DMA/02C) at 21.02 ± 0.01°C.

Table 5.2. Partial specific volumes of three bromovirus nucleoproteins at pH 5.0.*

	$c(g/cm^3)$	$\bar{v}(cm^3/g)$	buffer
BBMV	0.0165	0.714	0.2 M NaCl
	0.0196	0.730	1 mM DTT
	0.0129	0.685	1 mM EDTA
	0.0142	0.715	50 mM sodium acetate pH 5.0
BMV	0.0169	0.745	0.2 M NaCl
	0.0146	0.746	1 mM DTT
	0.0123	0.747	1 mM EDTA
	0.0100	0.751	50 mM sodium acetate pH 5.0
CCMV	0.0065	0.704	0.2 M NaCl
	0.0113	0.699	1 mM DTT
	0.0130	0.699	0.01 M $CaCl_2$
	0.0107	0.699	50 mM sodium acetate pH 5.0

* Solutions were dialysed for 2 days against the indicated buffer solutions, at 20°C for BBMV and BMV and 4°C for CCMV. Concentrations were determined with the extinction coefficients: $E^{1\%}_{260\ nm} = 54.0$ (BBMV), 50.0 (BMV) and 58.5 (CCMV), without correction for light scattering. Densities of solutions were determined with the density measuring apparatus of Anton Paar (DMA/02C) at 20.20 ± 0.01°C for BBMV and BMV and at 21.02 ± 0.01°C for CCMV.

The difference between the calculated and experimentally determined values must probably be attributed to the difference in solvent conditions. A value of 0.737 cm^3/g, determined for CCMV-protein in 0.5 M $CaCl_2$, pH 7.5, will be used in sedimentation equilibrium experiments for all three bromovirus proteins in 0.5 M $CaCl_2$, pH 7.5.

The apparent partial specific volume of the bromovirus nucleoproteins in 0.2 M NaCl pH 5.0 are shown in Table 5.2. For BBMV, BMV and CCMV respective values of 0.711, 0.747 and 0.700 cm^3/g were determined. A rather large difference in volume is found for the three bromoviruses, which would not be expected from their rather similar amino acid composition and RNA content.

Table 5.3 shows the apparent partial specific volumes of CCMV-nucleoprotein, RNA and protein, determined under the same conditions to allow direct comparisons. Using the values of 0.745 cm^3/g for the protein, 0.476 cm^3/g for the RNA and the data that the virus particles contain on the average 24% RNA an apparent partial specific volume of the nucleoprotein can be calculated: 0.680 cm^3/g. This value is lower than the experimentally determined value of CCMV nucleoprotein: 0.719 cm^3/g. This increase in volume upon assembly of CCMV from RNA and protein might be attributed to both ionic and hydrophobic interactions. Both interactions have positive volume effects opposite to hydrogen bonds which have negative ΔV values (Suzuki and Taniguchi, 1972). The striking difference between the apparent

Table 5.3. Partial specific volumes of CCMV nucleoprotein, its ribonucleic acid and coat protein at pH 5.0*

	$c(g/cm^3)$	$\bar{v}(cm^3/g)$
Nucleoprotein	0.0151	0.718
	0.0124	0.717
	0.0167	0.715
	0.0038	0.724
RNA	0.0030	0.476
	0.0030	0.477
	0.0036	0.474
Protein	0.0095	0.749
	0.0115	0.746
	0.0142	0.746
	0.0157	0.738

* Solutions were dialysed for 2 days at 20°C against 0.2 M NaCl, 1 mM DTT, 1 mM EDTA and 50 mM sodium acetate buffered at pH 5.0. Concentrations were determined with the extinction coefficients $E^{1\%}_{260\ nm}$ = 250 (RNA), 58.5 (nucleoprotein) and $E^{1\%}_{280\ nm}$ = 12.7 (protein), without correction for light scattering. Densities of solutions were determined with the density measuring apparatus of Anton Paar (DMA/OZC) at 20.20 ± 0.01°C.

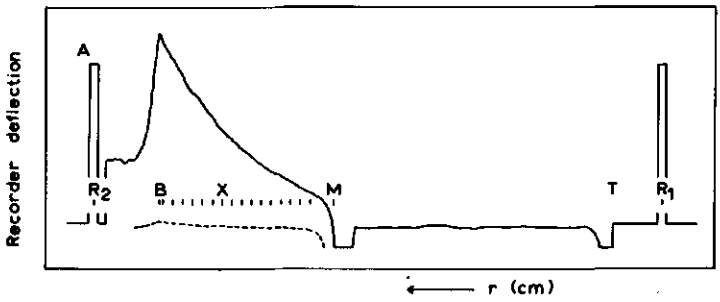
partial specific volume of CCMV-protein in 0.2 M NaCl pH 5.0 with either $CaCl_2$ (0.728 cm^3/g, Table 5.1) or EDTA (0.745 cm^3/g, Table 5.3), might be explained by calcium binding and/or presence of pseudo top doubles as described in chapter 4.

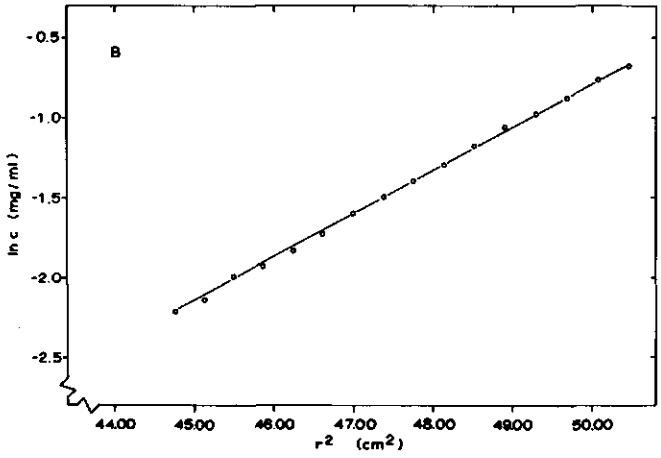
The difference between the apparent partial specific volumes of CCMV (Table 5.2 and Table 5.3) is due to binding of $CaCl_2$ (Noguchi et al.,1971) which decreases the volume. The value for BBMV (0.711) agrees well with the value determined pycnometrically (0.717) at pH 7.0, μ = 0.1 by Yamazaki et al. (1961) but contrasts with the value of 0.75 reported by Paul (1961).

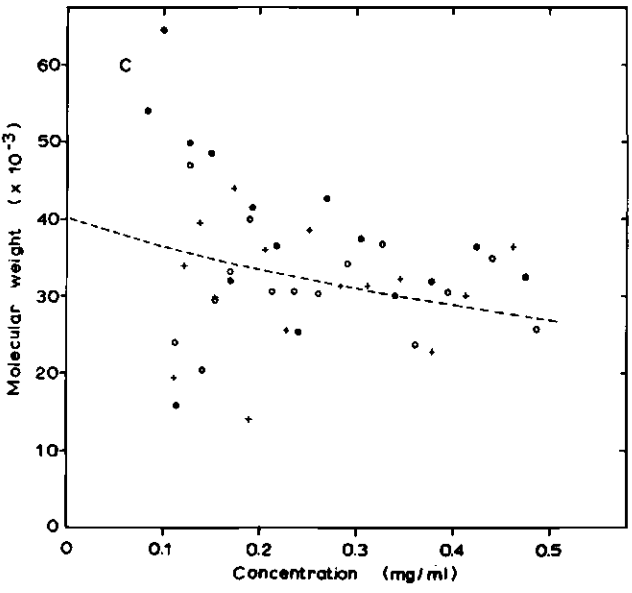
Sedimentation equilibrium of the bromovirus coat proteins.

An absorbance tracing of CCMV coat protein in 0.5 M $CaCl_2$ pH 7.5 after low speed sedimentation equilibrium at 12,580 rev/min and 10°C is shown in Fig. 5.1 A. The corresponding ln c vs r^2 plot from the data points (X) yields an almost straight line and is shown in Fig. 5.1 B. Using the experimentally determined partial specific volume of 0.737 cm^3/g (see Table 5.1) without correction for temperature (Hunter, 1966; Bull and Breese, 1968, 1973), an apparent molecular weight of 32,000 i.e. 1.7 times the molecular weight of the structural subunit of CCMV (MW = 19,400), was calculated. From the data points in Fig. 5.1 B, supplemented with data obtained from two parallel cells of the same centrifuge run a plot of $\bar{M}_w$ vs c was constructed (Fig. 5.1 C). The results reasonably well fit the non-ideality equation (Yphantis and Roark, 1971):

Fig. 5.1. Sedimentation equilibrium of CCMV-protein in 0.5 M $CaCl_2$ pH 7.5.
(A) Recorder trace illustrating sedimentation equilibrium of CCMV-protein in a 20 mm double-sector cell. The optical density (recorder deflection at 280 nm increases in a vertical direction. Increasing distance (r) from the axis of rotation is to the right. T and B indicate top and bottom of the cell, X represent the points from which the data were obtained. M indicates the air-solution meniscus of the sample. The broken line represents an absorbance trace of the same double-sector cell filled with buffer in both compartments. R_1 and R_2 indicate the reference holes in the counter balance at a distance of 5.700 and 7.299 cm from the rotor axis. CCMV-protein (0.2 mg/ml) dissolved in 0.5 M $CaCl_2$, 1 mM DTT and 50 mM Tris-HCl pH 7.5, was centrifuged for 74.5 h at 10,000 rev/min and 44 h at 13,000 rev/min and 10° C.
(B) Determination of the molecular weight of CCMV-protein in 0.5 M $CaCl_2$ pH 7.5 by sedimentation equilibrium. On the ordinate is the natural logarithm of the concentration in mg/ml and the abscissa represents the square of the distance (cm^2) from the axis of rotation. Points are obtained from the marked positions (X) in Fig. 5.1A. For further details see also Fig. 5.1A.
(C) Weight averaged molecular weight (M_w) of CCMV-protein in 0.5 M $CaCl_2$, pH 7.5 as a function of protein concentration, as determined by sedimentation equilibrium. Data were obtained from Fig. 5.1A and B (+) and from two parallel cells (0,0) of the same centrifuge run. For details of centrifugation see Fig. 5.1A.


A
R2
B
X
M
T
R1
U
Recorder deflection
r (cm)


B
-0.5
-1.0
-1.5
-2.0
-2.5
ln c (mg/ml)
44.00
45.00
46.00
47.00
48.00
49.00
50.00
r2 (cm2)


C
60
50
40
30
20
10
0
Molecular weight (x 10-3)
0
0.1
0.2
0.3
0.4
0.5
Concentration (mg/ml)

$$\frac{1}{\bar{M}_{w,app}} = \frac{1}{\bar{M}_{w,0}} + B\,c$$ using the values

$\bar{M}_{w,0}$ = 40,400 and B = 24.74 x 10^{-6} ml/mg of Adolph and Butler (1976). I don't have sufficient and accurrate data to exclude monomer-dimer or monomer-trimer associations (Adams, 1967; Van Holde et al., 1969).

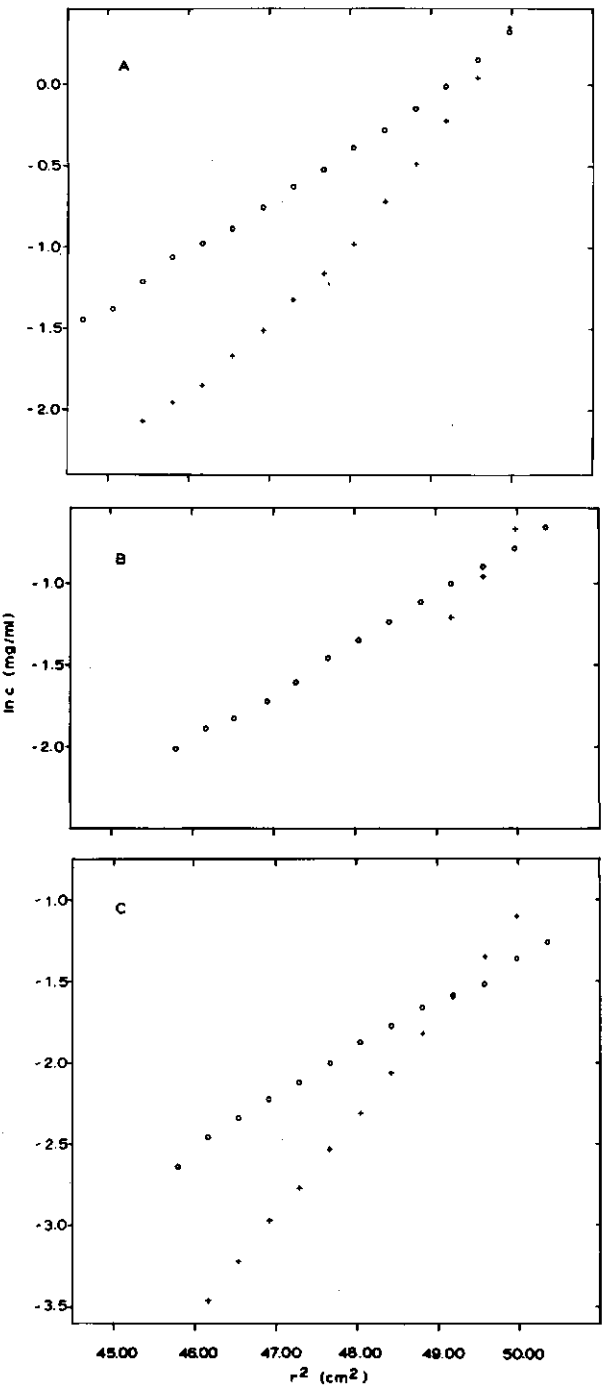
Comparison of the sedimentation equilibrium of the three bromovirus coat proteins at two different speeds showed a similar behaviour for BMV and CCMV (Fig. 5.2 B and C) but strong aggregation of BBMV (Fig. 5.2 A). At 13,000 rev/min for BBMV, BMV and CCMV an apparent weight average molecular weight throughout the cell of 34,000, 32,000 and 33,000 respectively was calculated. These values for $\bar{M}_{w,app}$ were lower after sedimentation equilibrium at 20,000 rev/min, respectively 31,000, 31,000 and 29,000 for BBMV, BMV and CCMV. The curvature in the line of the ln c vs r^2 plot of BBMV protein suggest a monomer-dimer or monomer-trimer equilibrium with non-ideality (Adams, 1967; Van Holde et al., 1969). The molecular weight of BMV-protein does not agree with the concentration independent value of 40,000 found by Paul and Buchta (1971).

Circular dichroism.

Circular dichroism was used for studying the conformation and conformational change of protein and RNA when CCMV dissociated into its components as has already been shown for RNA bacteriophages (Schubert, 1969; Oriel et al., 1971; Henkes and Middlebrook, 1973).

The CD spectra of two types of aggregation of the isolated coat protein: dimer and empty protein shell are shown in Fig. 5.3. Both curves coincide at several wavelengths and exhibit a negative minimum around 207.5 nm, indicating a high content of unordered conformation of the coat protein (Oriel et al., 1971; Chen et al., 1972; Jirgensons, 1973). Chen et al. (1972) have deduced a relationship for estimating the helical content of globular proteins from $(\theta)_{222\ nm}$.

Fig. 5.2. Determination of the molecular weight of (A) BBMV-, (B) BMV- and (C) CCMV-protein in 0.5 M $CaCl_2$ pH 7.5 by sedimentation equilibrium. On the ordinate is the natural logarithm of the concentration in mg/ml and the abscissa represents the square of the distance (cm^2) from the axis of rotation. BBMV-, BMV- and CCMV-protein (0.3 $A_{280\ nm}$/ml), dissolved in 0.5 M $CaCl_2$, 1 mM DDT and 50 mM Tris-HCl, pH 7.5, were centrifuged for 52 h at 13,000 rev/min (O) followed by 20 h at 20,000 rev/min (+). For BMV-protein at 20,000 rev/min only 3 data points are available.


A
B
C
0.0
-0.5
-1.0
-1.5
-2.0
-1.0
-1.5
-2.0
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
ln c (mg/ml)
45.00
46.00
47.00
48.00
49.00
50.00
r² (cm²)

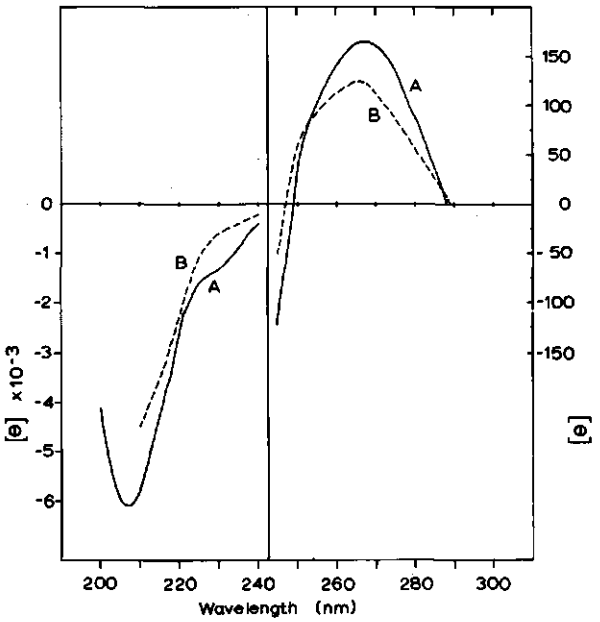


Fig. 5.3. Circular dichroism spectra of dissociated protein (A, ——) and empty protein shells (B, ---) of cowpea chlorotic mottle virus at 5°C. The dissociated protein was dissolved in 1 M NaCl, 50 mM Tris-HCl, pH 7.5 and the empty protein shells in 0.2 M NaCl pH 5.0, 0.1 M sodium acetate pH 5.0. (θ) is expressed in degrees cm²/decimol.

Applying this relationship to CCMV-protein, an helical content smaller than 1% has been estimated. Very low α-helix contents have also been found for cowpea mosaic virus protein (unpublished results) and alfalfa mosaic virus (Driedonks, 1978). The intensity of the negative band at 207.5 of dissociated protein at pH 7.5 is larger than that of protein capsids at pH 5.0. A similar decrease in intensity is observed for the weak positive maximum at 265 nm, which represents the circular dichroism of the aromatic amino acid residues (Schubert and Krafezyk, 1969; Budzynsky, 1970; Jirgensons, 1973). I have no explanation for these decreases in ellipticity. The two crossover points in the CD spectra of this two type of protein aggregate differ only slightly: 249 and 288 nm for dissociated protein at pH 7.5 and 247 and 289 nm for empty capsids at pH 5.0.

Curve C in Fig. 5.4 represents the circular dichroism of isolated RNA. One negative band at 207.5 nm and two positive ones at 222.5 (weak) and 265 nm (strong) are visible. Between these bands three crossover points are observed: at 217.5, 237 and 298 nm.

The CD spectrum of CCMV-RNA with the afore mentioned maxima resembles a

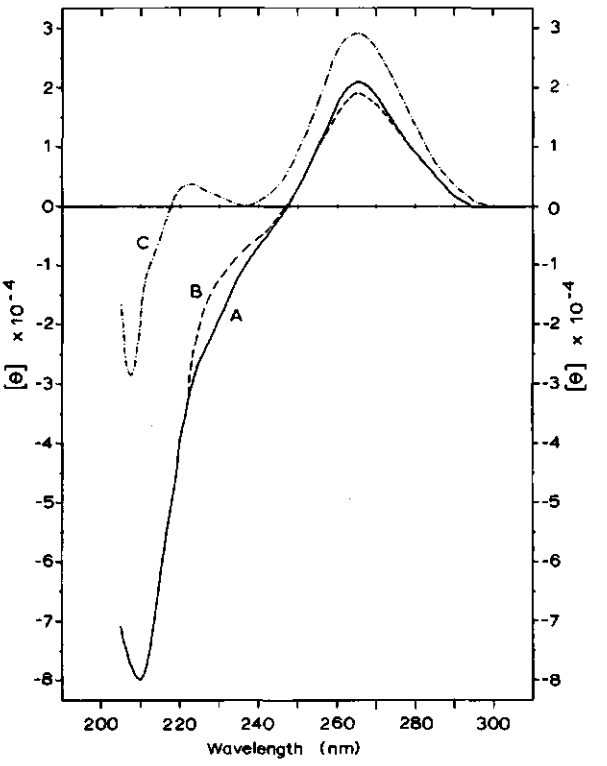


Fig. 5.4. Circular dichroism spectra of CCMV at pH 7.0, 0.1 M sodium phosphate (A, ——), CCMV at pH 5.0, 0.1 M sodium acetate (B, --) and CCMV-RNA at pH 7.5, 20 M Tris-HCl and 0.1 mM $MgCl_2$ (C, -·-). Spectra were determined at 5°C (virus) and 10°C (RNA). (θ) is expressed in degrees cm^2/decimol.

typical RNA spectrum (Cox et al., 1975) as observed also for RNA from viruses (Mengo virus: Kay et al., 1970; μ_2: Isenberg et al., 1971; R_{17}: Bobst et al., 1974) and from ribosomes (Adler et al., 1970). Analysis of the circular dichroism in terms of two conformations, (McMullen et al., 1967) double stranded with the spectral characteristies of RNA double helix, and single stranded with base stacking, according to the method of Gratzer and Richards (1971) shows that there is extensive (> 60%) base pairing in the CCMV-RNA molecule((θ) 265 mn = 22,000 deg cm^2 $dmol^{-1}$) at pH 7.0 in the presence of Mg^{2+}.

In Fig. 5.4 also the CD spectra of CCMV at pH 5.0 and 7.0 are shown. Both curves exhibit bands at 210 and 265 nm together with crossover points at 247 and 295 nm. The ellipticity around 210 nm is mainly due to the protein moiety of the virus. The intensity of the band at 265 nm, representing mainly the RNA, is due

to base pairing and base stacking (Samejima et al., 1968, Gratzer and Richards, 1971; Verma et al., 1971; Phillips and Bobst, 1972).

The difference in the intensity of the 265 nm band for the CCMV at pH 7.0 or 5.0 appears to indicate more ordered RNA in virus particles at pH 7.0 than a pH 5.0. The observed ellipticities of CCMV at pH 7.0 and 5.0 (20,500 and 19,000 deg cm^2 $dmol^{-1}$) are somewhat higher than the ellipticity found for BMV at pH 6.7, 0.2 M NaCl (17,000 deg cm^2 $dmol^{-1}$). Much higher ellipticities, and consequently more ordered RNA, have been observed for chicory yellow mottle virus ($(\theta)_{265\ nm}$= 26,000 deg cm^{-2} $dmol^{-1}$, Piazzolla et al., 1977) and bottom and middle component of cowpea mosaic virus, CPMV, $(\theta)_{265\ nm}$= 25,000 and 27,000 respectively, (unpublished results).

Since the molar ellipticities for both RNA and virus were calculated on the basis of nucleotide residue molar concentration and the contribution of the protein to the intensity of the 265 nm band has not been taken into account as it may be assumed to be neglicible, $(\theta)_{265\ nm}$ can be compared directly for isolated RNA and in situ RNA. The high intensity observed for isolated RNA indicates a highly ordered state of the RNA in 0.1 mM $MgCl_2$ at pH 7.5. The ordering decreases when the RNA is encapsidated at pH 7.0 and even more when encapsidated at pH 5.0.

Around 230 nm a shoulder in the CD spectra of both protein and nucleoprotein at pH 7.0 and 7.5 was observed. This shoulder was absent at pH 5.0. I'm inclined to attribute this change in intensity to variation in configuration of the aromatic acid residue tyrosine (Piazzolla et al., 1977).

DISCUSSION

The use of partial specific volumes to obtain information about protein-protein and protein-RNA interactions of CCMV appeared to be rather limited. Although the density measurements were very accurate and consistent I have not achieved the accuracy of measurement of apparent partial specific volumes for which I had hoped. Concentration determination appeared to be difficult due to variable light scattering of the concentrated protein and nucleoprotein preparations. To overcome this problem, dry weight determinations were performed. The limited amount of material, only 0.7 ml was necessary for determination of the density and the precence of high salt concentrations hampered the dry weight determinations. Dissolving freeze-dried protein in the appropiate buffers was not possible because freeze-dried coat protein was no longer soluble.

Interpretation of changes in partial specific volume on the basis of certain types of interactions (Suzuki and Taniguchi, 1972): ionic bond ($\Delta V>0$),

hydrophibic bond ($\Delta V>0$) and hydrogen bond ($\Delta V<0$) appeared to be difficult. Ionic bonds and protonation of carboxyl groups have been shown to result in a volume increase of the macromolecule solution (Katz and Miller, 1971, 1972; Noguchi et al., 1971). The volume increase observed after hydrophobic bond formation of small organic molecules does not agree with the volume increase observed after isoelectric heat or presure dependent denaturation of proteins (Brandts et al., 1970; Hvidt, 1971; Jaenick, 1971).

The hydrophobic interactions, which are presumed to be broken during protein denaturations, should cause a volume decrease (Suzuki and Taniguchi; 1972). Negative ΔV values have been shown upon denaturation of proteins in variable concentrations of guanidine hydrochloride (Lee and Timasheff, 1974) indicating also strong medium effects (Katz and Miller, 1972). So, explaining volume effects by types of interaction remains speculative; denaturation may cause aggregation with positive, or unfolding of the polypeptide chain with negative ΔV values.

The apparent partial specific volume of the RNA agrees well with the results obtained for alfalfa mosaic virus (0.46 cm^3/g, Heijtink et al., 1977).

The apparent molecular weight of dissociated CCMV coat protein varied between the monomer and dimer molecular weight. Although a reasonable agreement with a non-ideal dimer solution occurred (Adolph and Butler, 1976) the decrease in molecular weight found with increasing speed suggests a monomer-dimer equilibrium (Adams, 1967; Van Holde et al., 1969). A more precise analysis over a wide concentration range will be necessary to resolve this discrepancy. The sedimentation equilibrium of BMV is similar for to that of CCMV. The results obtained for BBMV suggest a monomer-trimer equilibrium or association of the coat protein subunit larger than the trimer. BBMV, unlike BMV and CCMV, does not form an empty protein shell, but a heterogeneous population of oligomers of the protein subunit, which sediment around 25 S (Bancroft, 1970 b).

The circular dichroism measurements of CCMV, its RNA and coat protein revealed a very low helical content (< 1%) of the protein. A highly unordered structure like the random coil would therefore appear most likely. Such dominating random coil structure contrasts with the situation observed with TMV coat protein, which has a high α-helix content (Schubert and Krafezyk, 1969; Budzynski, 1970) and with bacteriophage f_2 coat protein, which contains a high β-structure content (Henkes and Middlebrook, 1973). Extreme differences in structures of the capsid proteins are thus found with viruses, which have many dissociation and association features in common. A very low helical content

together with a highly unordered structure as deduced from the strong negative CD band in the range 195-210 nm, Jirginsons, 1973 has also been observed for the protein in the natural top component of cowpea mosaic virus (CPMV, unpublished observations). CPMV is quite different from CCMV with respect to virus particle stability and protein-protein interactions (Kaper, 1972). However the nature of the stabilising forces might not at all be related to the type of secondary structure of proteins.

The specific ellipticity of virus at 265 nm is smaller than the sum of the fractional ellipticities of the isolated RNA and protein. This is most easily explained by assuming some melting of the secondary structure of the RNA upon encapsidation. Dye binding and hypochroicity studies of CCMV and CCMV-RNA by Adolph (1975 a and b) do not exclude a more decreased secondary structure of the RNA in situ. However dye binding and hypochromicity are favored by base pairing while the CD band at 265 nm has also a strong contribution of base stacking and therefore both types of results do not contradiet each other.

For several single stranded and double stranded DNA viruses also a lower optical rotation for the nucleoprotein in comparison with the isolated components was observed (Maestre and Tinocco, 1967). This lower optical rotatory dispersion could be mimicked with DNA in 24% LiCl, indicating that the internal DNA is in more dehydrated state exhibiting less base stacking (Powell and Richards, 1972).

For RNA bacteriophages μ_2, f_2 and R_{17}, all belonging to the Leviviridae, the ellipticity of the phage equals the sum of the fractional ellipticities of RNA and protein.

Also viruses with a highly ordered structure of the RNA inside the virus particles are known. Chicory yellow mottle virus RNA is also in situ more structured than in solution (Piazzolla et al., 1977). The high ellipticities at 265 nm of 27,000 for middle component of CPMV and of 25,000 deg cm^2/dmol for bottom component of this virus also indicate more structure for the RNA in situ than for isolated RNA.

The ellipticity of isolated CPMV-RNA has been assumed to be similar to the ellipticity found for CCMV-RNA.

Kaper (1972) has suggested to classify the spherical RNA viruses according to their type of interaction, which stabilize the nucleoprotein particles. In this arrangement tymoviruses are predominantly stabilised by means of protein-protein linkages and cucumoviruses by means of protein-RNA linkages. Comoviruses, Leviviridae and bromoviruses, in this order occupy an intermediate position between

the tymo- and cucumoviruses where both types of interaction contribute more or less equally to their stability.

By small angle X-ray and neutron scattering experiments of several spherical RNA viruses it has been demonstrated that the measure of penetration of the RNA into the coat protein capsid correlated with the type of interaction within the nucleocapsid. The tymoviruses show little penetration, while the cucumoviruses have their RNA largely embedded in the coat protein capsid (Fischbach and Anderegg, 1976; Jacrot et al., 1977).

I have found that the observed differences in ellipticities at 265 nm of in situ or isolated CCMV-RNA, of CPMV-RNA and of RNA from Leviviridae (Schubert, 1969; Oriel et al., 1971; Henkes and Middlebrook, 1973) correlate well with the type of interaction stabilizing the nucleoprotein particles and the measure of penetration of the RNA in the coat protein capsid. Comoviruses, stabilised by protein-protein and protein-RNA linkages and with little penetration of its RNA into coat protein, contain RNA which is more structured than its isolated RNA. On the other hand, bromoviruses, which are mainly stabilised by protein-RNA linkages and which show moderate penetration of the RNA in the protein capsid contain RNA which is less ordered than its isolated RNA.

For rodlike viruses e.g. TMV, such correlation is not possible. The ORD of TMV RNA in situ is twice as large as for isolated RNA (Bush and Scheraga, 1967). However X-ray diffraction experiments have indicated that there is no base pairing and base stacking of in situ TMV-RNA (Stubbs et al., 1977). Probably the rigid helical array of TMV-RNA in the nucleoprotein particle causes the increase in optical rotation as compared to isolated RNA (Bush and Scheraga, 1967).

6. DISSOCIATION OF CCMV AT VARIOUS SALT CONCENTRATIONS AROUND NEUTRAL pH

Using rate zonal centrifugation the salt and pH dependent dissociation of CCMV was analysed. After 24 h incubation of CCMV at pH 7.50 in 1 M NaCl large amounts of coat protein dimers and four intact RNA components containing 4 to 8 bound coat protein subunits per RNA component were found.
Dissociation at pH values between 6.60 and 7.00 in 1 M NaCl also showed RNA-protein complexes, called dissociation intermediates, which contained almost 100% of the original protein coat and never significant amounts of RNA-4. In 1 M NaCl both increasing the pH in the incubation mixture between 6.60 and 7.00 or short incubation times (0.5 and 2 h) at pH 7.50 caused RNA-4 to be released from virus particles before any other RNA was released. At short incubation times prior to centrifugation also a protein aggregate sedimenting between the dimer and RNA-4 was observed.
At low salt concentrations (0.1 M NaCl, pH 7.50) the coat protein remained attached to the RNA, but the complexes had a much lower sedimentation coefficient than virus at pH 5.00. Increase of salt concentration up to 1.0 M NaCl caused a gradual release of coat protein dimers.
At high salt concentrations (2.0 M NaCl, pH 7.50) no protein dimers occurred in solution but an empty protein shell was observed in addition to the released RNA.

INTRODUCTION

Purified cowpea chlorotic mottle virus (CCMV) consists of three nucleoprotein particles with different buoyant densities but similar sedimentation coefficients. This small spherical plant virus contains four RNAs: RNA-1, -2, -3 and -4, with molecular weights of 1.20, 1.07, 0.81 and 0.25 x 10^6, respectively (Reijnders et al., 1974). The smallest two RNAs are encapsidated together, the other two separately. The protein coats of all three particles are identical, consisting of 180 copies of a single polypeptide, arranged on the surface of an icosahedron (Bancroft and Flack, 1972). CCMV belongs to the bromoviruses, which have the characteristic ability to dissociate at pH 7.50 and 1 M NaCl into protein dimers (Adolph and Butler, 1976) and nucleic acid (Bancroft, 1970b; see for review on bromoviruses, Lane, 1974). These components of CCMV can be reassembled into virions at the same pH by lowering the ionic strength to about 0.1.

The self-assembly of CCMV *in vitro* appears to be non-specific. Many polyanionic species can be encapsidated by CCMV protein (Bancroft *et al.*, 1969b). Moreover, the products formed upon assembly with RNA did not completely resemble the original virus. Very low concentrations of particles containing RNA-3 + 4 were found and all the reassembled nucleoproteins were less stable in CsCl or RbCl (Chapter 7). Varying the assembly conditions did not induce specificity nor did it improve the stability or the nucleoprotein distribution of the assembled products in RbCl and CsCl, which is characteristic for the bromoviruses (Adolph and Butler, 1975, 1976, 1977; Chapter 7). Specific interaction between RNA and protein has been shown for bacteriophage MS_2 and tobacco mosaic virus (TMV). Either an intermediate structure (complex I of MS_2) or a protein aggregate (disk of TMV) appeared to be involved in the specific assembly process (Sugiyama *et al.*, 1967; Chroboczek *et al.*, 1973; Butler and Klug, 1971).

A study of the association properties of the isolated coat proteins of CCMV and BMV (Adolph and Butler, 1974; Pfeiffer and Hirth, 1974b; Verduin, unpublished results) did not reveal any aggregate, which might recognize the RNA. However, at the same conditions of ionic strength and pH different conformations of the coat protein could be observed, depending how the protein had been isolated (acidic or alkali). These conformations were demonstrated in both association-dissociation studies (Verduin, 1974, chapter 4) and acid-base titration studies (Johnson *et al.*, 1973; Jacrot, 1975) of the isolated coat protein.

Therefore the dissociation behaviour of CCMV around neutral pH at various sodium chloride concentrations was analysed. Protein aggregates as well as protein-nucleic acid complexes, which might be necessary for specific assembly of CCMV, have been detected, when virus was kept at acidic conditions prior to dissociation.

MATERIALS AND METHODS

Preparation of virus, RNA and coat protein. CCMV, isolated RNA, dissociated coat protein and pseudo top component (PT) were prepared as described before (Verduin, 1974, chapter 4; Verduin, 1978, chapter 3).

Incubation of CCMV under varying salt conditions around neutral pH. CCMV (1 mg/ml) was incubated at 5°C for 24 h in buffered NaCl solutions containing 1 mM dithiothreitol (Cleland's reagent, DTT) unless stated otherwise. The NaCl concentration was varied between 0.2 and 2.0 M NaCl and the incubation mixtures were buffered with 50 mM Tris-HCl or 50 mM NaH_2PO_4-NaOH between pH 6.50 and 7.50. No difference in dissociation behaviour was found using either buffer. Tris-HCl was

used when RNA was precipitated with $CaCl_2$. pH was measured with a Radiometer pHM 26 at the desired temperature and an accuracy of 0.05. Measurement of pH in solutions containing sugar, high concentrations of NaCl or reducing agents was done one minute after immersing the electrode in the solution. Longer periods gave erratic results both with the buffered solutions or standard pH buffers. In between the measurements the electrode was immersed in distilled water.

Rate zonal sucrose density gradient centrifugation. At the end of the incubation, 0.25 ml (250 µg) of nucleoprotein solution was layered on a linear 10 to 50% (w/v) sucrose density gradient and centrifuged for 15 h in a Beckman SW 41 rotor at 30,000 rev/min and 5°C (Neal and Florini, 1972). Linear sucrose density gradients were prepared by pipetting layers of 2.5 ml 50%, and successively 2.2 ml of 40, 30, 20 and 10% in a Beckman SW 41 cellulose nitrate tube, followed by diffusion of the discontinuous gradient for 22–26 h at 5°C. The sucrose solutions had the same buffer and salt composition as the incubation mixture, which was applied. After centrifugation the tube was punctured at the bottom and the contents were displaced by 60% (w/w) sucrose solution or Fluorinert (ISCO: ρ = 1.94 g/cm^3), using a Model 183 or 185 ISCO density gradient fractionator. The absorbance was monitored at 257 and 281 nm with two LKB Uvicords II in series, with optical path lenghts of 3 mm. Five-drop fractions were collected, and analysed for RNA by polyacrylamide gel electrophoresis as described (Verduin, 1978, Chapter 3), for protein by gel electrophoresis as described by Laemmli (1970) or inspected by electron microscopy.

^{32}P- and ^{35}S-labelling of CCMV. Cowpea seeds (Vigna unguiculata (L.) Walp. var. Blackeye Early Ramshorn) were incubated for 48 h at 25°C in the dark in moistened vermiculite. The germinated seeds were transferred to Hoagland mineral salt solution, deficient in phosphate and sulphate, and placed in a climatic room. The growing conditions of this hydroponic culture were: illumination with fluorescent tubes (25 kWatt/m^2 at the height of the primary leaves) at 30°C and a relative humidity of 75% for 12 h and 30 min, followed by darkness for 11 h and 30 min at 22°C (Huxley and Summerfield, 1976). After five days the primary leaves were inoculated with CCMV (1 mg/ml). Twentyfour hours after inoculation the plants were divided in two groups and transferred to solutions containing either 5 mCi ^{35}S-sulphate or 5 mCi ^{32}P-phosphate. 70 h thereafter the leaves were harvested and virus was isolated as described by Verduin (1974, Chapter 4) omitting the rate zonal centrifugation step. Purity of the labelled virus (specific radioactivities one day after purification: ^{35}S-virus: 11.4 x 10^6 cpm/mg and ^{32}P-virus: 2 x 10^6 cpm/mg) was checked on sucrose gradients at pH 5.00. All radioactivity applied to the gradient sedimented with a coefficient of 88 S. Incubations of

labelled virus were performed in the presence of unlabelled virus at a final concentration of 1 mg nucleoprotein per ml. To avoid contamination of the gradient with ^{35}S from dissociated protein at the top, the contents of the tube were collected from the bottom in 10 drop fractions. Radioactivity was counted in a Packard Tricarb scintillation counter, using 7 ml of Insta-gel per ml of aqueous nucleoprotein solution in an appropriate dilution. In double-labelling experiments the ^{35}S-counts in the ^{3}H-channel were corrected for the spill-over of the ^{32}P-counts.

Chemicals. Dithiothreitol was obtained from Calbiochem. ^{32}P-phosphate and ^{35}S-sulphate were purchased from The Radiochemical Center, Amersham. Insta-gel was a product from Packard Instrument Co. All other chemicals used were pro analyse and were obtained from Merck & Co.

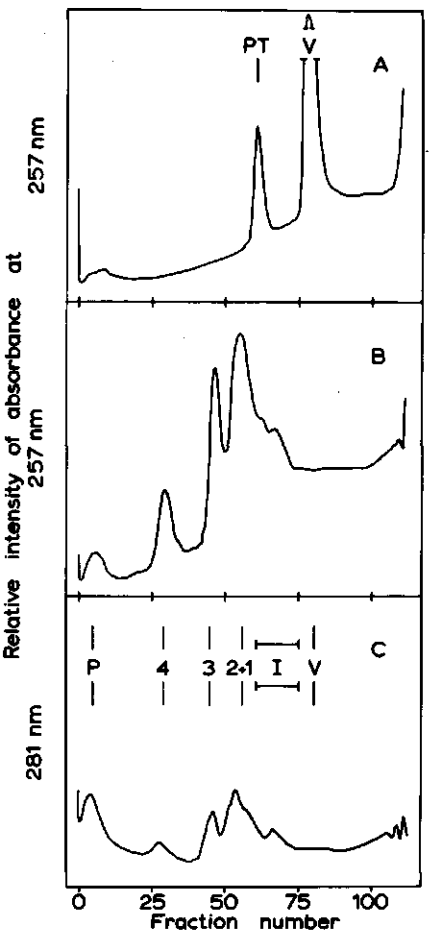


Fig. 6.1. Rate zonal sucrose density gradient centrifugation analysis of dissociated cowpea chlorotic mottle virus (CCMV). (A) 400 µg of pseudo top component (PT) and 250 µg of CCMV in 1.0 M NaCl, 0.05 M sodium acetate pH 5.00 and 1 mM dithiothreitol (DTT) were applied to a linear 10 to 50 (w/v) sucrose density gradient containing the same buffer and salt molarities. The gradient was run for 15 h at 30,000 rev/min and 5°C in a Beckman SW41 rotor and the contents of the tubes were analysed. (B) 250 µl of CCMV (1 mg/ml) in 1.0 M NaCl, 0.05 NaH_2PO_4-NaOH pH 7.50 and 1 mM DTT and incubated for 24 h at 5°C was layered on a 10 to 50% (w/v) sucrose density gradient, in the same buffer, centrifuged, and then analysed at 257 nm. (C) as (B) but analysed at 281 nm. Positions of virus, PT, RNA-1+2, -3 and -4 and dissociated protein (P) are indicated. "I" represents the region where "dissociation intermediates" were found, when CCMV was dissociated in 1 M NaCl but at lower pH values. Sedimentation is from left to right. Positions in the gradients are not always directly comparable for different tubes and incubations. Measurements with the LKB Uvicord II at high sensitivity resulted in peaks superimposed on sloping base lines, causing shifts of peak maxima.

RESULTS

Characterization of the dissociated products in 1 M NaCl at pH 7.50.

Virus incubated for 24 hours at 5°C in 1.0 M NaCl pH 7.50 was centrifuged in 10 to 50% (w/v) linear sucrose density gradients (Fig. 6.1). The dissociation appeared to be complete since no significant amount of absorbing material was found in fractions 80 to 75, where intact virus (88 S) and its swollen form (78 S) should be found (Fig. 6.1 B and C). Dissociated coat protein, showing one band with a molecular weight of 20,000 in polyacrylamide gels was found near the top of the gradient around fraction 5. Sedimentation equilibrium centrifugation experiments showed this protein to be a dimer of the structural subunit (Chapter 5). Analysis of fractions 30, 45 and 55 by polyacrylamide gel electrophoresis, revealed RNA-4, -3 and -2+1 respectively, as the main components. In these fractions RNA-2 and -1 were not separated on these sucrose gradients. As markers, intact virus (fraction 80) and PT (fraction 60, 52 S) were centrifuged simultaneously in similar gradients at pH 5.00 (Fig. 6.1A).

Between fraction 60 and 75 RNA-3 was found in addition to RNA-2 and -1. The unexpected position of this RNA is probably caused by aggregation in the high salt concentration. This aggregation has been detected to a much greater extent if isolated RNA was centrifuged under these conditions and therefore seems to be specific for protein-free RNA under high salt conditions. Using a mixture of ^{32}P- and ^{35}S-labelled virus, an attempt was made to detect residual protein on the RNA. Virus containing ^{32}P-labelled RNA was mixed with virus labelled in its protein coat with ^{35}S, and dissociated. The products were analyzed on sucrose density gradients (Fig. 6.2). Although most of the ^{35}S-counts were found near the top of the gradient some counts were found in the gradient at the positions of the different RNA components. The number of coat protein subunits per RNA molecule was calculated assuming the RNA content of a virus particle to be 1.11 x 10^6 g/mole and using the cpm ratio $^{35}S/^{32}P$ of the undissociated virus mixture as a standard. For example fraction 30 in Fig. 6.2, containing RNA-3 and some protein subunits, a value of 6.1 protein subunits per RNA molecule was calculated, viz.

$$\frac{\text{number of } ^{35}S \text{ counts (2222 cpm)}}{\text{number of } ^{32}P \text{ counts (122 cpm)}} \times \frac{\text{MW RNA-3 } (0.81 \times 10^6)}{\text{MW RNA average in CCMV } (1.11 \times 10^6)} \times \frac{\text{number of protein subunits per undissociated virus particle (180)}}{^{35}S/^{32}P \text{ ratio in unfractionated mixture of } ^{32}P\text{- and } ^{35}S\text{-labelled virus (392)}} = 6.1.$$

For the fractions containing RNA-4, -3 and -2+1 average values of 4, 6, and 8 protein subunits per RNA molecule, respectively, were calculated for one run with

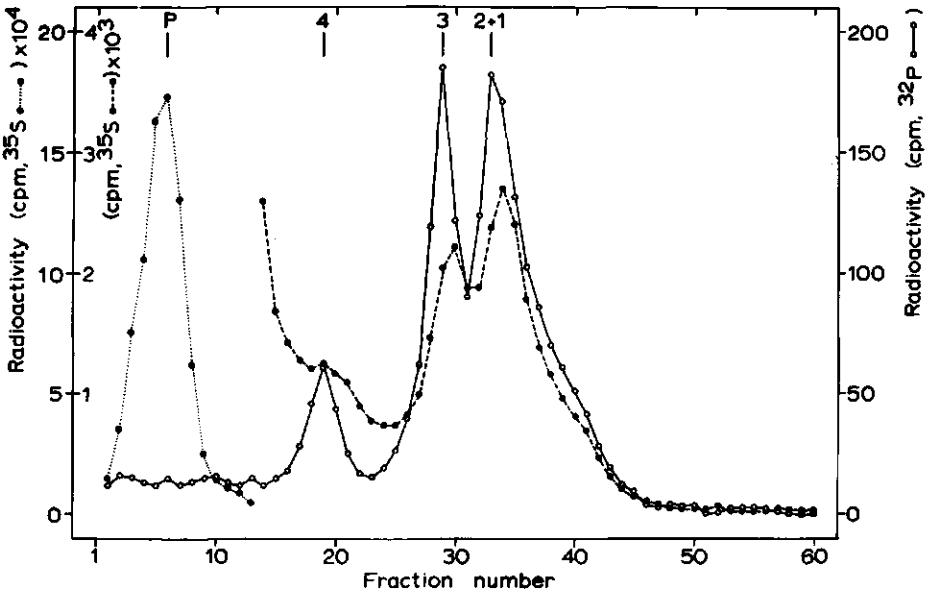


Fig. 6.2. Rate zonal sucrose density gradient centrifugation analysis of dissociated CCMV, with ^{35}S-labelled coat protein and ^{32}P-labelled RNA. CCMV was incubated in 1 M NaCl at pH 7.50 at 5°C for 24 h and centrifugated as described in Fig. 6.1. $^{35}S/^{32}P$ in virus = 392. Using this ratio and the datum that an RNA equivalent of 1.11×10^6 gram/mol is encapsidated by 180 protein subunits (MW = 19,400 g/mol), 4, 6, and 8 protein subunits bound per RNA-4, -3, and -2 or -1, respectively, can be calculated.

a variation of ± 2 in the four fractions determined for each RNA. Therefore these experiments could not discriminate between a constant number of subunits per nucleotide and per RNA molecule. These protein subunits were not bound to the RNA when virus was dissociated in 1% (w/v) sodium dodecylsulphate (SDS) (results not shown).

pH dependence of the dissociation in 1 M NaCl.

Below pH 6.50 the virus was stable and behaved as control virus at pH 5.00 sedimenting homogeneously.

Analysis of virus, incubated at different pH values between 6.60 and 7.00 showed more heterogeneity. Upon increasing the pH, more virus became dissociated and more released protein and RNA-4, -3 and -2+1 appeared (Fig. 6.3). Areas of the absorption peaks of the dissociation products in the sucrose density

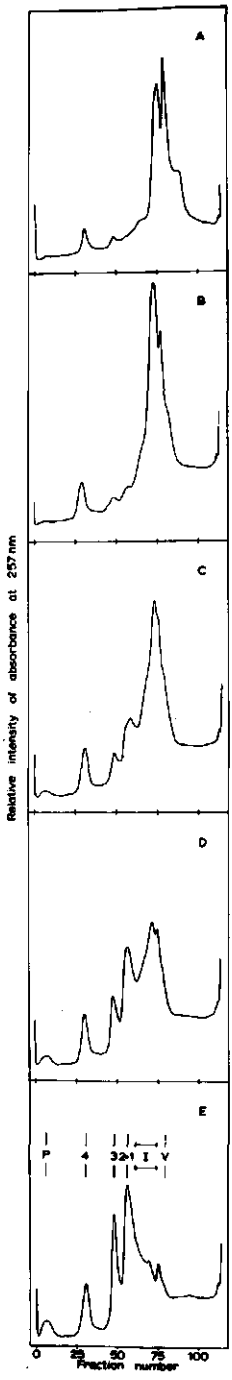


Fig. 6.3. Rate zonal sucrose density gradient centrifugation analysis of CCMV incubated in 1 M NaCl and at different pH values. CCMV (1 mg/ml) was incubated for 24 h at 5°C in 1 M NaCl (A) pH 6.60, (B) 6.70, (C) 6.80, (D) 6.90 and (E) 7.00 and analysed on linear 10 to 50% (w/v) sucrose density gradients, containing the respective buffer and salt molarities. Further centrifugation conditions as described in Fig. 6.1. Diagram A was obtained under slightly different centrifugation conditions as compared to the other diagrams.

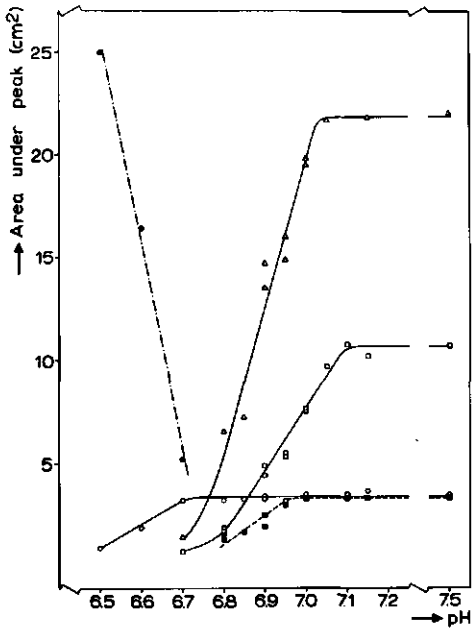


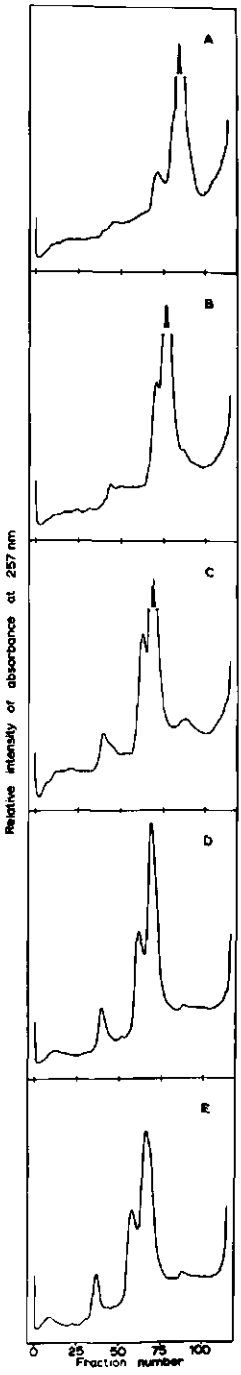
Fig. 6.4 pH-dependent release of RNA and protein components of CCMV in 1 M NaCl. CCMV (1 mg/ml) was incubated at 5°C and pH values between 6.50 and 7.50 in 1 M NaCl and analysed in the respective buffer as described in Fig. 6.1 and 6.3. The assignments of the regions of the gradient are as in Fig. 6.3 E. The areas under the absorption peaks at 257 nm or 281 nm for the different products were measured in cm^2 and plotted against the pH. (● –·–·– ●) virus, (■ ------- ■) dissociated protein, (○ ——— ○) RNA-4, (□ ——— □) RNA-3, and (Δ ——— Δ) RNA-1+2.

gradients were plotted as a function of pH, as is shown in Fig. 6.4. RNA-4 started to be released around pH 6.50 while no equivalent amounts of dissociated protein and RNA-3 were detected. Near pH 6.70 the maximum amount of RNA-4 could be detected, while at this pH the first release of RNA-3 and -2+1 occurred. The amount of RNA-4 released, as checked by polyacrylamide gel electrophoresis, remained constant upon increasing pH, while the amount of RNA-3 and -2+1 increased.

In addition to dissociated protein and RNA components, other stable protein-nucleic acid complexes were detected on sucrose density gradients (Fig. 6.3). Above pH 6.60 the amount of virus decreased and slower sedimenting material appeared (fractions 60 to 75). This material could be detected until pH 7.00, although the quantity decreased with increasing pH and the decrease correlated well with the appearance of RNA-3 and -2+1. A mixture of ^{32}P- and ^{35}S-labelled virus was incubated for 24 h at 5°C in 1 M NaCl pH 6.75 and analysed on sucrose density gradients. The contents of fractions 60 to 75 have $^{32}P/^{35}S$ ratios equal or slightly lower than the ratio determined with unfractionated virus. From these experiments it was concluded that most of the protein was still attached to RNA-3, -2 and -1. To explain the much lower sedimentation coefficient of the complexes a structure different to the sphere (either of intact virus, d = 28 nm or swollen, d = 31 nm) had to be assumed (McCammon et al., 1975). Negatively stained preparation of these fractions did not show as many spherical particles in the electron microscope as expected from the amount of nucleoprotein present, assuming a spherical appearance of the complex. No evidence was obtained for other defined structures after negative staining of these preparations. These complexes are referred to as dissociation intermediates.

Time dependence of the dissociation in 1 M NaCl at pH 7.50.

When shorter incubation times prior to centrifugation were chosen at pH 7.50 and 1 M NaCl (0.5 and 2 h compared to 24 h) analysis of the sucrose density gradients revealed a protein aggregate sedimenting faster than the peak of the dimer coat protein (Fig. 6.5). The maximum amount of this aggregate was found at 30 min, the shortest possible time tested, while with increasing incubation time less aggregation was found. These experiments also showed that most or all of the RNA-4 was released within 30 min from the start of the incubation since no increase in the amount of RNA-4 was found. Upon longer incubation times there was little release of RNA-3 and -2+1 at the early stages, as checked by polyacrylamide gel electrophoresis.



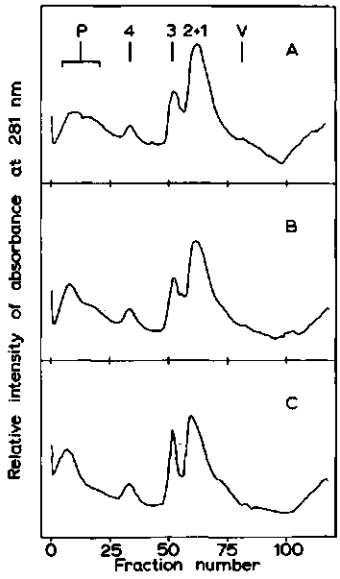


Fig. 6.5. Rate zonal sucrose gradient centrifugation analysis of CCMV after different incubation periods in 1 M NaCl at pH 7.50 and 5°C. CCMV (1 mg/ml) was incubated for (A) 30 min, (B) 2 h and (C) 24 h prior to centrifugation on linear 10 to 50% (w/v) sucrose density gradients as described in Fig. 6.1. Note the shoulder on the fast sedimenting site of the absorption peak of the dissociated protein.

Fig. 6.6. Rate zonal sucrose density gradient centrifugation analysis of CCMV after incubation for 24 hours at pH 7.50 in different salt concentrations. CCMV (1 mg/ml) was incubated for 24 h at pH 7.50 and 5°C in (A) 0.25 M, (B) 0.30 M, (C) 0.35 M, (D) 0.40 M, and (E) 0.45 M NaCl prior to centrifugation in sucrose density gradients, containing the respective buffer and salt molarities. Further centrifugation analysis as described in Fig. 6.1 No marker positions are indicated, because of difference in density and viscosity among the contents of the tubes. Nevertheless the patterns may be compared with Fig. 6.1 to assign the various peaks to the components of CCMV.

Ionic strength dependence of the dissociation around neutral pH.

Unlike at 1 M NaCl and pH 7.50, CCMV did not dissociate at low concentrations of NaCl at pH 7.50 after 24 h of incubation, but had a lower sedimentation coefficient than virus at pH 5.00 (Bancroft et al., 1968b; Adolph, 1975c). Increasing the salt concentration from 0.2 M to 0.45 M NaCl further decreased the sedimentation coefficient of the nucleoprotein particles, which gradually became more heterogeneous (Fig. 6.6). Inspection by electron microscopy of fractions containing this heterogeneous material revealed no significant amount of spherical particles nor any other distinct structure. Experiments with ^{35}S- and ^{32}P-labelled virus showed that the appearance of heterogeneity and slower sedimenting nucleoprotein complexes was accompanied by gradual release of coat proteins from the RNA. An average of about twice as much residual coat protein bound per RNA molecule in the complexes was found at 0.45 M NaCl compared to 1.0 M. RNA-4 was not released earlier than the other RNA components as it was when dissociation occurred in 1.0 M NaCl with increasing pH.

Increase in pH and in salt concentration separately have been shown to stimulate the dissociation of CCMV, although different intermediates were found. Therefore the sedimentation profiles of the dissociation products after incubation of virus at different salt concentrations (0.45 M, 1.0 M and 2.0 M) and pH values (6.75 and 6.95) were compared. The results are shown in Fig. 6.7. Viral preparations sedimented homogeneously at pH 6.75 in 0.45 M NaCl. At the same pH but in 1.0 M NaCl the preparation became heterogeneous; the virus became dissociated and a considerable amount of RNA-4 and other dissociation intermediates were observed. At 2.0 M NaCl more RNA molecules were released, including RNA-3 and -(2+1), but no dissociated protein was detected. The 281 nm absorption pattern (dotted line) of Fig. 6.7 C revealed a significant protein band near the position of RNA-2+1, which cosedimented with PT.

At pH 6.95 in 0.45 M NaCl, protein and RNA were released although material banding at the positions of both intact virus and swollen virus was still detected. In 1.0 M NaCl release of more RNA molecules was observed, with a relatively high proportion of RNA-4, while some dissociation intermediates were also present. The pH increase from 6.75 to 6.95 at 2.0 M NaCl had much less effect on the dissociation than at lower salt concentrations. This is probably because dissociation has proceeded further at this high NaCl concentration. Again protein was found near the position of RNA-(2+1), but not at the top of the gradient (Fig. 6.7 F dotted absorption pattern). Selective precipitation of RNA by $CaCl_2$ showed more fully the presence of this protein. The protein cosedimented

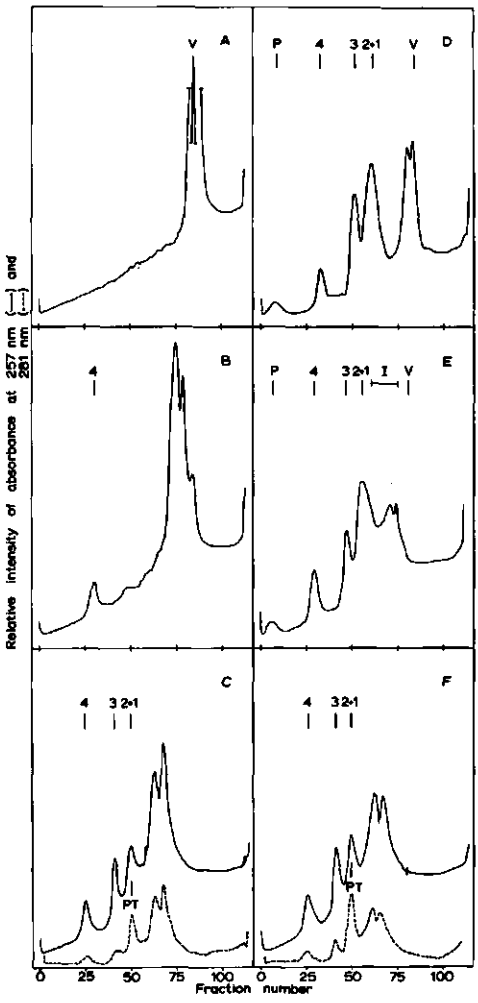


Fig. 6.7. Rate zonal density gradient centrifugation analysis of CCMV incubated at different salt concentrations and pH values. $CCMV_o$ (1 mg/ml) was incubated for 24 h at 5°C at pH 6.75 in (A) 0.45 M, (B) 1.0 M, and (C) 2.0 M NaCl, and at pH 6.95 in (D) 0.45, (E) 1.0 M, and (F) 2.0 M NaCl prior to centrifugation in sucrose density gradients, containing the respective buffer and salt molarities. Further centrifugation conditions as described in Fig. 6.1. For diagram C and F also the 281 nm absorption pattern has been included to show the strong protein absorbance of PT around fraction 50.

with PT (Fig. 6.8) and in the peak fraction high concentrations of spherical particles could be detected by electron microscopy. An empty protein shell was also found with brome mosaic virus (BMV), when the virus was dissociated in high salt concentrations around neutral pH (Pfeiffer and Hirth, 1974 a and b). Under these conditions no PT was formed from the dissociated protein at pH 7.50 by increasing the salt concentration, both for BMV and CCMV.

Herzog, Pfeiffer and Hirth (1976) found that BMV, which is related to CCMV, preferentially released RNA-1, -2 and -3 over RNA-4 in 1.5 M NaCl or 1.0 M $CaCl_2$. Either the high ionic strength, known to precipitate high molecular weight RNAs

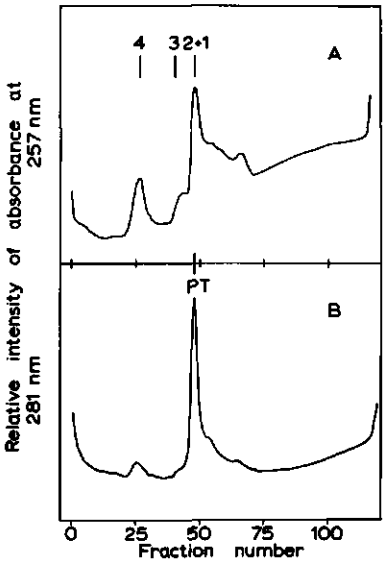


Fig. 6.8. Rate zonal sucrose density gradient centrifugation analysis of CCMV dissociated in 2.0 M NaCl at pH 6.90 in the presence of $CaCl_2$. CCMV (1 mg/ml) was incubated for 24 h at pH 6.90 and 5°C in 2.0 M NaCl and 0.1 M $CaCl_2$ prior to centrifugation in gradients, containing 0.05 M Tris-HCl pH 6.90 and the same salt molarities. Further centrifugation conditions as described in Fig. 6.1. Absorption measurements of the contents of the tube were done at (A) 257 nm and (B) 281 nm.

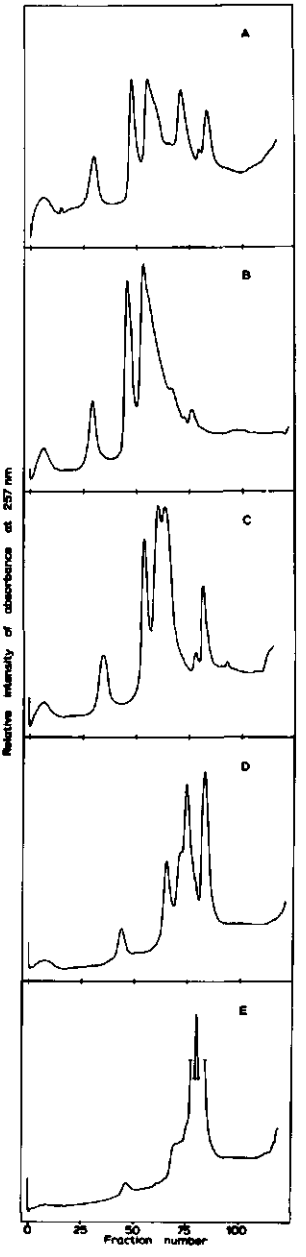


Fig. 6.9. Rate zonal sucrose density gradient centrifugation analysis of CCMV dissociated at pH 7.50 in the presence of 1 M of the different alkali chlorides. CCMV (1 mg/ml) was incubated for 24 h at 5°C and pH 7.50 in (A) 1 M LiCl, (B) 1 M NaCl, (C) 1 M KCl, (D) 1 M RbCl, and (E) 1 M CsCl prior to centrifugation in gradients, containing the respective buffer and salt molarities, as for incubation. Further centrifugation conditions as described in Fig. 6.1. No marker positions are indicated because of differences in density and viscosity among the contents of the tubes. Nevertheless the patterns may be compared with Fig. 6.1 to assign the various peaks to components of CCMV.

preferentially, explains the solubility of small RNAs (CCMV incubated in 2.0 M NaCl and 0.1 M $CaCl_2$ pH 6.90 contains a substantial amount of soluble RNA-4, Fig. 6.2A) or a different dissociation applies to BMV.

Cation dependence of the dissociation at pH 7.50.

Changing the type of cation, used in the incubation mixture, had a remarkable effect on the dissociation behaviour of CCMV at pH 7.50. From absorption patterns of the gradients as shown in Fig. 6.9, Na^+ appeared to be most effective in dissociating the virus into RNA and/or RNA-protein complexes and high concentrations of protein dimers. Li^+ seemed to stabilise protein-protein interactions, since less protein was found near the top of the gradient and intermediates and undissociated virus were present in larger amounts. K^+, Rb^+ and Cs^+ in this order were less able to release protein and RNA from the virus particle.

DISCUSSION

Using rate zonal centrifugation for analysis and fractionation of dissociation products it has been shown that dissociation of CCMV at pH 7.50 in 1.0 M NaCl resulted in protein dimers and nucleic acid (Verduin, 1974, Chapter 4). The four RNAs were intact and had 4 to 8 bound coat protein subunits per RNA molecule. These protein-nucleic acid complexes might be analogous to "complex I" found with bacteriophages (Sugiyama et al., 1967; Chroboczek et al., 1973; Chroboczek and Zagórsky, 1975; Zagórska, Chroboczek and Zagórski, 1975). Although the coat protein subunits seemed firmly bound to RNA molecules upon dissociation of virus, they have not been detected upon assembly of isolated RNA and protein dimers.

The protein aggregates and the protein-nucleic acid complexes have now been tested for their function in the self-assembly process of CCMV. Preliminary results showed that the RNA molecules, with 4 to 8 coat protein subunits per molecule, mixed with coat proteins reassemble into particles that are more homogeneous and stable in RbCl, as compared to assembly with phenol extracted RNA (chapter 7).

To facilitate the explanation of the results two protein conformations are assumed. The compact conformation refers to the tertiary structure found with protein subunits quasi-equivalently arranged in the shell of either virus or PT at pH 5.00. The loose conformation refers to the tertiary structure of protein dimers in solution, usually at pH 7.50. The transition from compact to loose is governed by deprotonation, is strongly time dependent and reversible (Johnson et al., 1973; Verduin, 1974, Chapter 4; Jacrot, 1975). The presence of dimers of

the coat protein subunit, as well as other factors (aggregation in high salt solutions, temperature dependence of association, calorimetry), suggest a strong hydrophobic interaction as the main interaction between the coat protein subunits.

Using the neutron scattering analysis data of BMV (Jacrot et al., 1976, 1977) for CCMV together with the assumed conformations a hypothetical model covering the data concerning the salt concentration dependent dissociation of CCMV around neutral pH is proposed (Fig. 6.10). In this model a fragment of the RNA molecule,

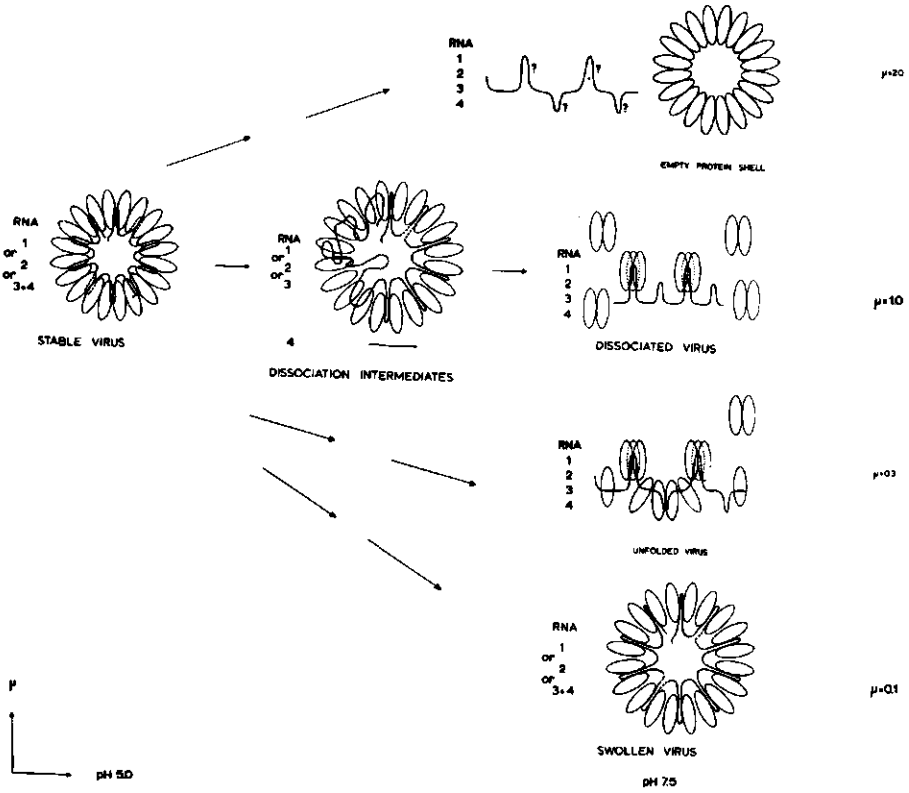


Fig. 6.10. Hypothetical model for the salt-dependent dissociation of CCMV around neutral pH. Horizontally virus is shown at μ = 1.0, pH 5.00: stable virus, pH 6.90: dissociation intermediates, and pH 7.50: dissociated virus. Vertically virus is shown at pH 7.50, varying the ionic strength from 0.1: swollen, 0.3: unfolded, 1.0: dissociated, to 2.0: empty protein shells. Virus is represented by a cross section of the icosahedral particle, cut through 4 three-fold and 4 five-fold symmetry axes. In this way 8 RNA loops, in interaction with hexamer and pentamer clusters of the coat protein subunits are in a plane, while two are out (broken line). The threads with loops represent fragments of the viral RNA molecules, numbered according to their increasing molecular weights. The ellipses represent the coat proteins (MW = 19,400), in solution always present as dimers. The dotted ellipses represent the protein subunits bound at the high affinity sites of the RNA. The location of RNA and protein is based on structural analysis of BMV (Jacrot et al., 1976, 1977) and its similarity with CCMV.

which binds the residual protein subunits after dissociation of the virus in 1 M NaCl, pH 7.5 (dissociated virus), is represented by a loop structure. These fragments might correspond with the 3'-terminal fragment, which is nearly identical for all four CCMV-RNA molecules (Bastin et al., 1976).

Incubation of virus at pH values between 6.50 and 7.00 in 1 M NaCl showed the appearance of dissociated intermediates in addition to RNA and coat proteins. Considering the low sedimentation coefficients and the high protein-RNA ratios, RNA loops protruding from the protein shell or a non-spherical structure had to be assumed (McCammon et al., 1975), stabilised by weak protein-protein and protein-RNA interactions. Probably RNA-4, which was negligible in the dissociation intermediates, was not involved in sufficient protein-RNA interactions, to stick to the RNA-3-protein complex. The weak protein-protein interaction between the dimers is probably due to a transient state between the compact and the loose conformation of the coat proteins. A similar time dependent transient state causes the faster sedimenting protein found after short incubation periods in 1 M NaCl at pH 7.50.

When the salt concentration was increased (2.0 M NaCl) before the pH was changed from 5.00 to 7.50, empty protein shells were found in addition to free RNA, so the protein-nucleic acid interactions were further weakened while the protein-protein interactions were reinforced. The protein probably retained the compact conformation since PT could not be formed by increasing the salt concentration of a dissociated protein solution at pH 7.50, where the coat protein is assumed to be in the loose conformation. For BMV (Pfeiffer and Hirth, 1974 a and b) strong hydrophobic interactions, known to be reinformed by high or very low salt concentrations, had to be considered to explain the protein-protein interactions. This is also true for CCMV, and may explain the effect of different alkali chlorides on the dissociation of CCMV at pH 7.50. The Li^+-ion is known to induce an ordered structure of water molecules and in this way competes with protein molecules for hydration water, thereby favouring hydrophobic interactions (Sun et al., 1974). The Cs^+-ion "breaks" the structure of water molecules, also those present around the protein molecules as hydration water (Lewin, 1974). Both ions at 1 M concentration probably stabilize the virus by strong protein-protein interactions, while in the case of Cs^+ the size of the hydrated ion may prevent effective competition with protein binding sites for RNA.

The swollen virus (0.1 M NaCl, pH 7.50) is mainly stabilised by hydrophobic protein-protein and electrostatic protein-RNA interactions. The protein-RNA interaction is essential since it prevents the protein from aggregation in very large irregular structures (Bancroft, 1970b; Verduin, unpublished results). In-

creasing the salt concentration (0.3 M NaCl) weakens the hydrophobic interactions between the coat proteins while electrostatic protein-RNA interactions are not yet broken. It is assumed that the viral RNA is unfolded, while the protein is bound to it. The complex must be mainly stabilised by protein-RNA interactions since the coat protein alone does not significantly aggregate under these conditions (Adolph and Butler, 1974; Verduin, unpublished results). Furthermore most protein is released from the RNA above an ionic strength of 0.3, which confirms the results obtained by Hiebert (1969).

7. RNA CONTENT AND ISOPYCNIC CENTRIFUGATION IN RbCl OF ASSEMBLED AND REASSOCIATED BROMOVIRUSES

Broad bean mottle virus (BBMV), brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) were assembled by mixing isolated viral RNA and coat protein under appropiate conditions. All three bromoviruses showed upon rate zonal centrifugation analysis of the assembled products a band sedimenting around 80 S besides variable amounts of nucleoprotein sedimenting between 70 and 80 S and > 110 S. The sedimentation pattern was similar for different assembly methods and for different preparation methods of the starting material, RNA and protein.

SDS polyacrylamide gel electrophoresis of the fractions of a sucrose gradient showed that the 70 to 80 S fraction contained mainly RNA-3, the > 110 S fraction RNA-3 and RNA-4, while the 80 S band contained RNA-1 and -2 and only small amounts of RNA-3 and -4.

Assembly experiments with the separate viral RNAs showed that the 70 to 80 S material was mainly obtained when RNA-3, -4 or fragmented RNA were encapsidated. Material sedimenting > 110 S was obtained with RNA-3, RNA-4 or a mixture of RNA-1 and -2.

Only 40% of the assembly products were stable in 35% RbCl and survived isopycmic centrifugation analysis. The stable particles contained mainly RNA-1 and -2.

If on the contrary the bromoviruses were dissociated into protein and RNA-protein complexes containing 4 to 8 protein subunits per RNA molecule and subsequently reassociated changing the ionic strength of the mixture, only minor amounts of 70 to 80 S and > 110 S sedimenting material were found and most reassociated nucleic protein sedimented at 80 S. Almost ninety percent of the products were stable in RbCl. This was the case with BMV and CCMV, but not with BBMV. The products which were not stable in RbCl contained one or several copies of RNA-3 and RNA-4.

INTRODUCTION

Broad bean mottle virus (BBMV), brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) constitute the group of the bromoviruses. Characteristically each bromovirus consists of three nucleoprotein particles, each carrying a portion of the genome. Two particles contain each one RNA molecule (CCMV-RNA-1: 1.20×10^6, CCMV-RNA-2: 1.07×10^6) and one particle contains two RNA molecules (CCMV-RNA-3: 0.81×10^6; CCMV-RNA-4: 0.25×10^6). All particles have an almost equal RNA content of 1.1×10^6 and are therefore difficult to separate by isopycnic density gradient centrifugation. The protein coats of all three particles are identical,

consisting of 180 copies of a single polypeptide (MW-CCMV-protein : 19,400) arranged on the surface of an icosaeder (Lane, 1974).

BBMV, BMV and CCMV have been assembled from isolated nucleic acid and protein components by mixing them under appropriate conditions (Bancroft and Hiebert, 1967; Hiebert and Bancroft, 1969; Adolph and Butler, 1977). The products of the assembly in vitro sediment around 80 S, between native (88S) and swollen (78S) virus. The RNase resistence of the biological activity indicates encapsidation of complete RNA genomes. Products sedimenting around 80 S have also been assembled from RNA fragments and small synthetic nucleotides (Hiebert et al., 1969; Adolph and Butler, 1977). Therefore the sedimentation coefficient does not appear to be a criterium for properly assembled products.

A better criterium would be the RNA content of the assembled products. The quality of the encapsidated RNA molecules can unambiguously be determined with polyacrylamide gel electrophoresis. The nucleoproteins of the bromoviruses can be separated in RbCl gradients after isopycnic centrifugation to allow an estimation of the relative proportions of the three nucleoprotein particles. By RNA analysis of the assembled products in RbCl the RNA content of the stable nucleoprotein can be determined. So another criterium would be the stability of the assembled products in RbCl gradients.

Therefore, in the experiments described in this chapter, BBMV, BMV and CCMV have been assembled according to the know assembly procedures (Bancroft and Hiebert, 1967; Adolph and Butler, 1977) and the assembled products have been analysed for RNA content and their stability in RbCl gradients. The assembled products have been compared with the nucleoproteins obtained after dissociation and reassociation of virus. During dissociation and reassociation of the virus the RNA and protein components have not been isolated separately (Chapter 6).

BBMV and BMV, were included in these investigations in order to detect similarities or differences within the bromovirus group as done in other studies (Verduin, 1978a; Krüse et al., manuscript in preparation).

MATERIALS AND METHODS

Bromovirus nucleoproteins, their RNA and coat proteins were isolated and kept as described in chapter 5, 4 and 3 (Verduin, 1974; Verduin, 1978b). Representative absorption spectra of the isolated coat proteins are shown in Fig. 7.1. For preparation of fresh CCMV-protein, freshly isolated CCMV (2 mg/ml) was incubated 2 h at room temperature in 1 M NaCl, 1 mM DTT buffered at pH 7.4 with 50 mM Tris-HCl and the protein was separated from the RNA and undissociated virus by pas-

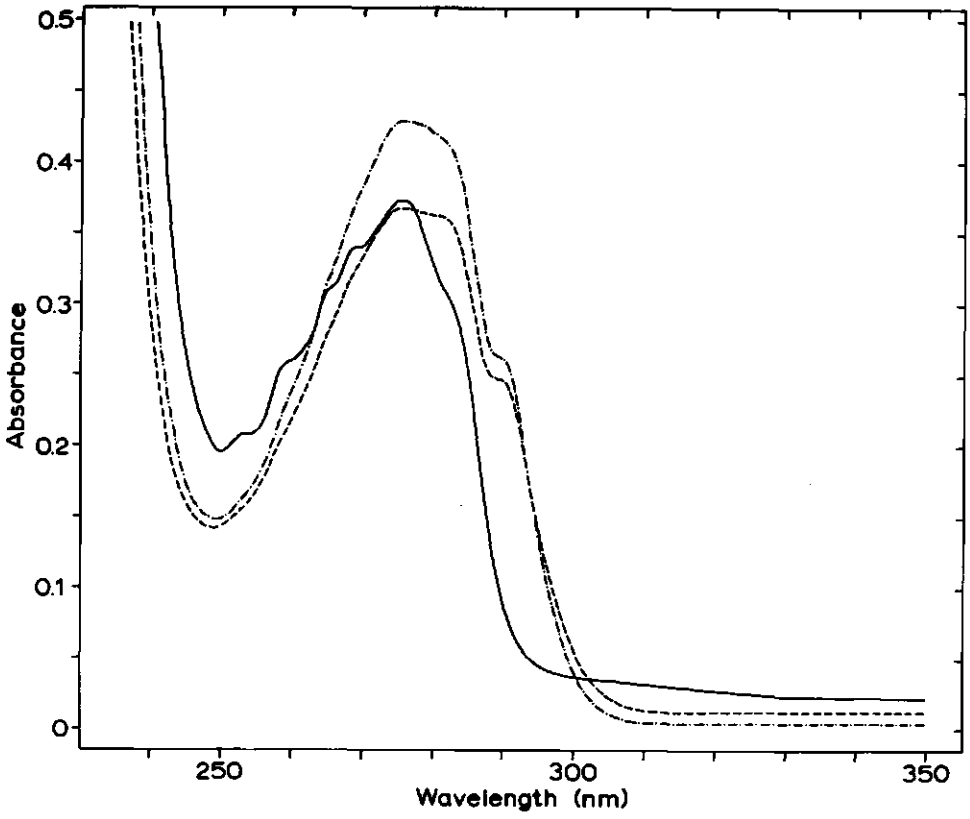


Fig. 7.1. UV absorption spectra of the isolated bromovirus coat proteins (——): BBMV, (. _ . _ .): BMV and (----): CCMV in 0.5 M $CaCl_2$, pH 7.4.

sage through Sephadex G-200, equilibrated with the same buffer.

SDS polyacrylamide gel electrophoresis, rate zonal centrifugation, isopycnic centrifugation in the analytical ultracentrifuge, electron microscopy and infectivity test were performed as described in chapter 3, (Verduin, 1978b). With rate zonal centrifugation the sedimentation coefficients of the assembled and reassociated nucleoproteins were calibrated with markers: (PT (52 S), unswollen virus (88 S), virus dimers (124 S) and trimer (152 S). Separate CCMV-RNA molecules were prepared by dissociation of virus with SDS, followed by rate zonal centrifugation and fractionation as described by Adolph (1975b).

Preparative isopycnic centrifugation in RbCl was performed in an MSE SW 60 rotor for at least 60 h at 39,000 rev/min and 5°C. RbCl (35% (w/w), n_D^{25} = 1.3721,

ρ^{25} = 1.3471 g/cm^3) was dissolved in virus buffer. The gradient covered a density range including virus and protein, but RNA sedimented to the bottom of the tube.

Assembly procedures. Method A based on descriptions of Adolph and Butler (1975, 1976, 1977): RNA was dialysed against 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and the protein was dialysed against 10 mM Tris-HCl, pH 7.5, containing 250 mM NaCl. After mixing the RNA and the protein solutions the mixture was dialysed against 50 mM sodium phosphate buffer, pH 6.0, containing 50 mM NaCl for 2 h at 20°C. A protein-to-RNA weight ratio of 3:1 and 4:1 was used.

Method B (Bancroft and Hiebert, 1967): RNA was dialysed against 10 mM Tris-HCl, pH 7.4, containing 10 mM KCl and 0.5 mM $MgCl_2$ and protein was dialysed against dissociation buffer: 50 mM Tris-HCl, pH 7.4, containing 1.0 M NaCl and 1 mM DTT. After mixing protein and RNA solutions (3:1 and 4:1) the mixture was dialysed against assembly buffer: 10 mM Tris-HCl, pH 7.4, containing 10 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT for 2 h at 4°C.

Method C: Virus was dialysed against dissociation buffer for 24 h at 4°C (chapter 6) followed by dialysis for 2 h against assembly buffer at 4°C.

General outline of sampling and identification of the nucleoproteins during the assembly procedures: RNA and protein solutions were dialysed overnight at 4°C, against buffered solutions described for Method A, B and C. RNA was added to the protein solution in the appropriate proportions: 1:3 and 1:4 corresponding to a nucleoprotein concentration of 1 mg/ml and incubated or dialysed against buffer used with the different assembly methods. Subsequently the solution was dialysed for at least 2 h at 4°C against virus buffer to stabilise the nucleoprotein particles and to make comparison in RbCl gradients possible. After assembly and after stabilization samples were taken for SDS polyacrylamide gel electrophoresis to analyse the RNA content and for rate zonal centrifugation.

RESULTS

Rate zonal centrifugation analysis of nucleoprotein particles assembled from isolated viral RNA and coat protein.

If isolated protein and nucleic acid of the bromoviruses were assembled the resulting nucleoprotein preparations always contained particles sedimenting around 80 S, independent of the assembly method. The rate zonal centrifugation analysis of the bromovirus nucleoproteins assembled at low ionic strength, pH 7.4, in the presence of Mg^{++} (Method B) and with RNA/protein weight ratios of 1:3 and 1:4 is shown in Fig. 7.2. Besides 80 S particles, slow (70 to 80 S) and fast (> 110 S) sedimenting nucleoproteins were observed. These slow and fast sedimenting products

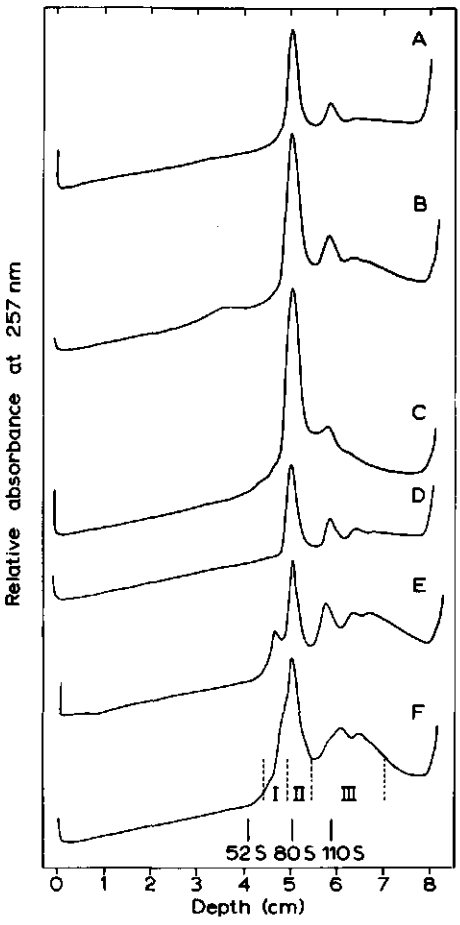


Fig. 7.2. Rate zonal density gradient centrifugation patterns of assembled bromoviruses. RNA and coat protein were mixed in weight ratios 1 : 3 (A) BBMV, (B) BMV, (C) CCMV and 1 : 4 (D) BBMV, (E) BMV, (F) CCMV corresponding to a nucleoprotein concentration of 1 mg per ml and dialysed for 2 h at 4°C against a low ionic strength buffer pH 7.4 with Mg^{2+} (assembly buffer, Method B), followed for at least 2 h at 4°C against virus buffer. 250 µl of the assembled nucleoprotein preparations was analysed on 10 to 50% (w/v) linear sucrose density gradients in an SW 41 rotor for 15 h at 25,000 rev/min and 5°C. The contents of the tubes were monitored at 257 and 281 nm. For CCMV (RNA/protein: 1/4) fractions I, II and III are indicated, which have been used for electron microscopy (Fig. 7.5).

could amount up to 50% of the 260 nm absorbing material on gradients but varied with different assembly experiments. When during assembly a RNA/protein ratio of 1:4 was used, which is lower than occurs in the native virus particles an increase in the proportions of 70 to 80 S and > 110 S nucleoproteins was observed (Fig. 7.2D, E and F). This heterogeneity was found with assembled products analysed both at pH 7.4 and 5.0. All three bromoviruses gave very similar sedimentation profiles. BBMV nucleoprotein had a strong tendency to aggregate, causing loss of material in the sedimentation profiles.

Care was taken to prevent degradation of the RNA by adding EDTA to buffers used in the purification and storage of the bromoviruses (Verduin, 1978b; Chapter 3). Representative UV absorption patterns of RNA, isolated from assembled

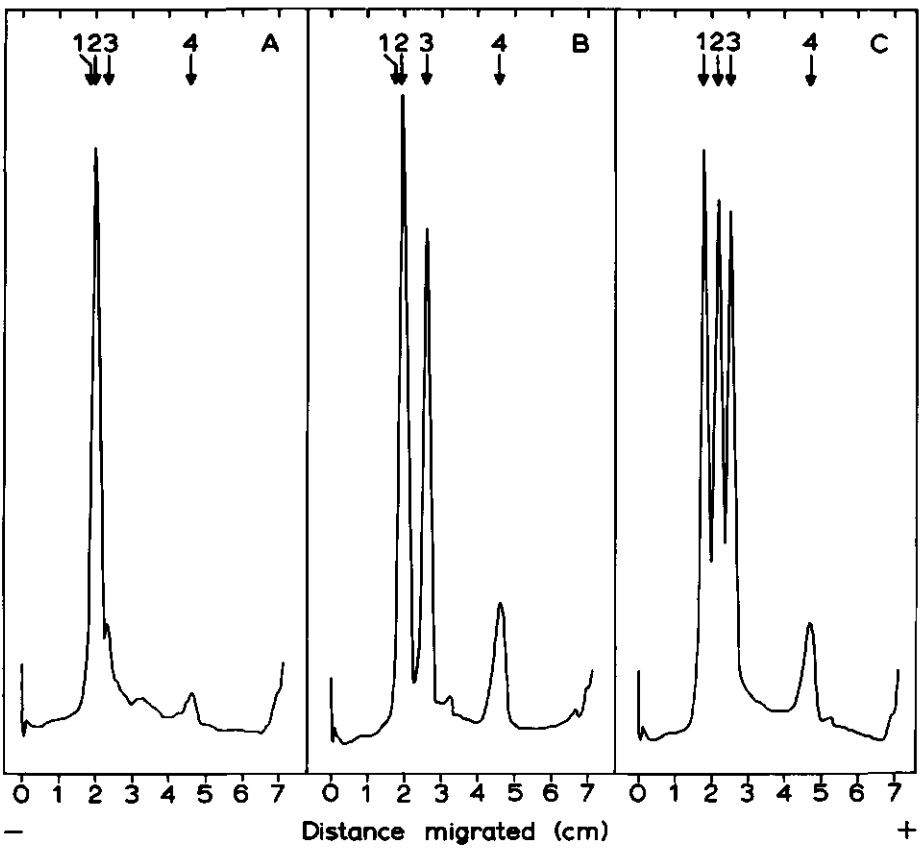


Fig. 7.3. Electrophoresis patterns of bromovirus RNAs from assembled nucleoprotein particles in 2.6% (w/v) polyacrylamide gels at 20°C; the effect of assembly on the integrity of the RNA. (A) BBMV, (B) BMV and (C) CCMV. Assembled nucleoprotein in (of which the rate zonal centrifugation analysis is shown in Fig. 7.2D to F) virusbuffer at pH 5.0 was dissociated in 10% (w/v) glycerol, 2% (w/v) SDS and 1 mM EDTA and 50 µl (40 µg) was applied to gels and electrophoresed for 4 h at 4 V/cm of gel. The arrows and the respective numbers indicate the distinct RNA species found with the bromoviruses.

nucleoprotein particles in virus buffer, in 2.6% (w/v) polyacrylamide gels are shown in Fig. 7.3. No RNA degradation had occured during the assembly procedure, which was confirmed by infectivity tests. The infectivity of the assembled nucleoprotein particles, was tested for BMV and CCMV and ranged between 50 and 150% of the infectivity of the original viruses.

Assembly of CCMV-protein and RNA at pH 6.0 and ionic strength 0.1 (Method A) resulted in more > 110 S particles (Fig. 7.4B) as compared to assembly at low ionic strength, pH 7.4, with Mg^{++} (Fig. 7.4A). This increased heterogeneity

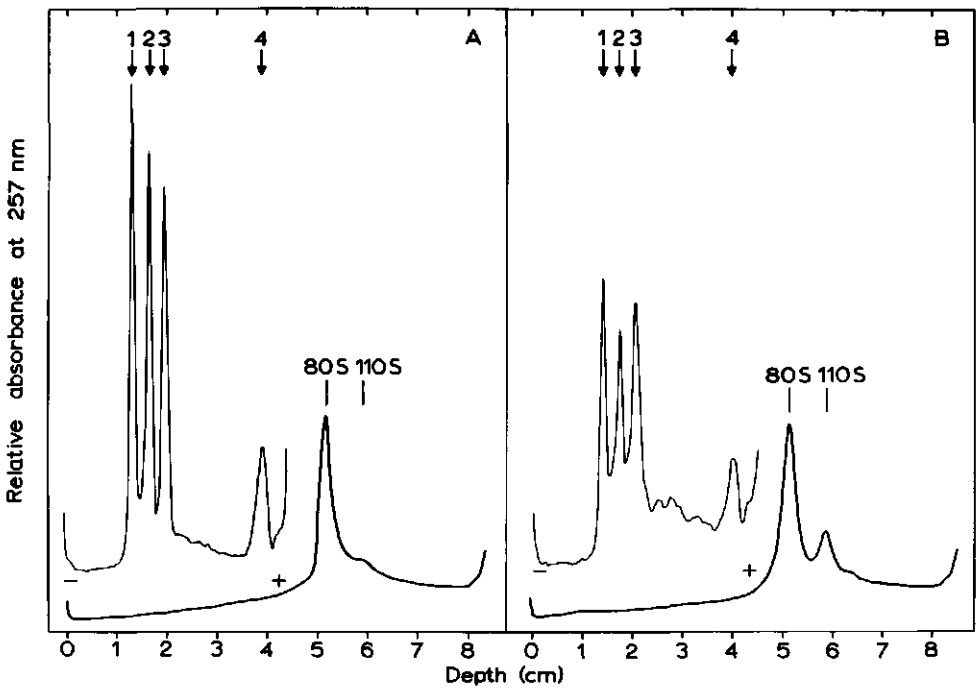


Fig. 7.4. Rate zonal centrifugation (lower diagrams) and SDS electrophoresis patterns (upper diagram) of CCMV-nucleoprotein particles assembled (A) at low ionic strength, pH 7.4, with Mg^{2+} (Method B) and (B) at ionic strength 0.1, pH 6.0 (Method A). For details of centrifugation see Fig. 7.2 and of electrophoresis Fig. 7.3.

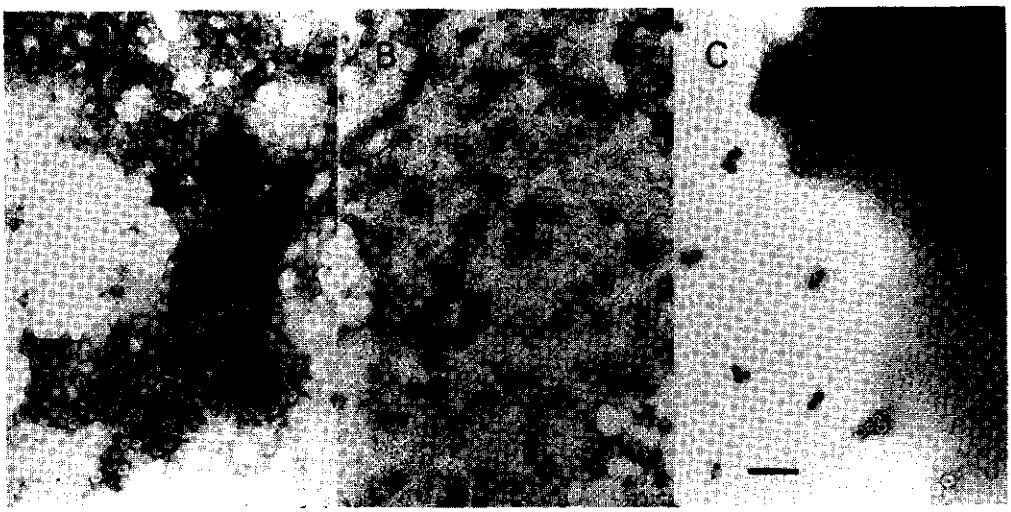


Fig. 7.5. Electron micrographs of assembled CCMV nucleoproteins, after rate zonal centrifugation and fractionation (A) 70 to 80 S particles, (B) 80 S particles and (C) > 110 S particles. The size of the fractions are indicated in Fig. 7.2F. The nucleoproteins were examined on carbon coated grids, negatively stained with 2% (w/v) uranyl acetate in distilled water. The bar represents 100 nm.

appears to be due to degraded RNA (Fig. 7.4B upper tracing) probably caused by the absence of Mg^{++} at neutral pH and low ionic strength during initial mixing. Similar results were obtained with BBMV and BMV.

For further characterization of the 70 to 80 S and > 110 S nucleoprotein particles the CCMV-material was examined in the electron microscope, and the influence of the previous history of virus and protein and of isolated and separated RNA components was tested. For this, CCMV nucleoprotein particles were only assembled at low ionic strength, pH 7.4, with Mg^{++} (Method B).

The particles sedimenting in sucrose density gradients between 70 and 80 S (Fraction I in Fig. 7.2F) appeared in the electron microscope as spherical particles of equal size and with equal stain penetration (Fig. 7.5A). The main band (80 S, Fraction II in Fig. 7.2F) showed variation in diameter, stain penetration and clustering of particles (Fig. 7.5B). Fast sedimenting particles (> 110 S, Fraction III in Fig. 7.2F) appeared in the electron microscope as dimers, trimers and tetramers of particles with different diameters apart from half shells (Fig. 7.5C). $A_{260\ nm}/A_{280\ nm}$ ratios of fast sedimenting particles did not indicate RNA/protein ratios different from virus.

Fractionation of a sucrose gradient after rate zonal centrifugation of assembled nucleoproteins and SDS polyacrylamide gel electrophoresis of the fractions revealed the following RNA contents. Particles sedimenting from 70 to 80 S contained mainly RNA-3, while both RNA-3 and -4 were found as the main RNA molecules in the fraction > 110 S. The fraction containing 80 S particles showed all four RNA molecules although less RNA-3 and -4 than in the original virus preparation. Assembly experiments with separate CCMV-RNA molecules, isolated by rate zonal centrifugation (Fig. 7.6), and assembled with coat protein at low ionic strength, pH 7.4, with Mg^{++} confirmed that low molecular weight RNAs caused heterogeneity of the assembled products. RNA-1+2 formed 80 S particles and RNA-3 and -4 separately or mixed formed 70 to 80 S particles (Fig. 7.7). All three classes of RNA formed nucleoproteins sedimenting faster than 80 S, however RNA-3 and -4 caused most aggregation.

The heterogeneity of the assembled products showed little if any correlation with the age of the virus or the protein preparation. Protein prepared from freshly isolated virus or from virus stored for two months at $4^{\circ}C$ gave identical results (Fig. 7.8). Also fresh protein isolated gel-filtration from virus which had been incubated for two h at $4^{\circ}C$ in 1 M NaCl buffered at pH 7.4 did not prevent heterogeneity and was comparable in encapsidation to protein stored for 2 months at pH 5.0 at $4^{\circ}C$.

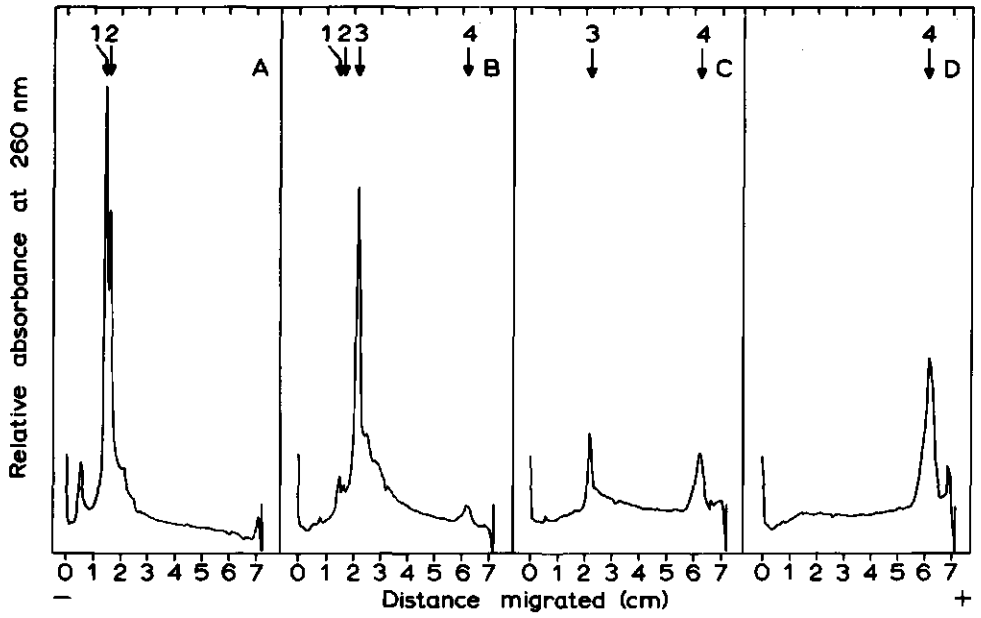


Fig. 7.6. Electrophoresis patterns of isolated and separated CCMV-RNAs in 2.6% (w/v) polyacrylamide gels at 60°C. (A) RNA-1+2, (B) RNA-3, (C) RNA-3+4, and (D) RNA-4. For details of electrophoresis see Fig. 7.3.

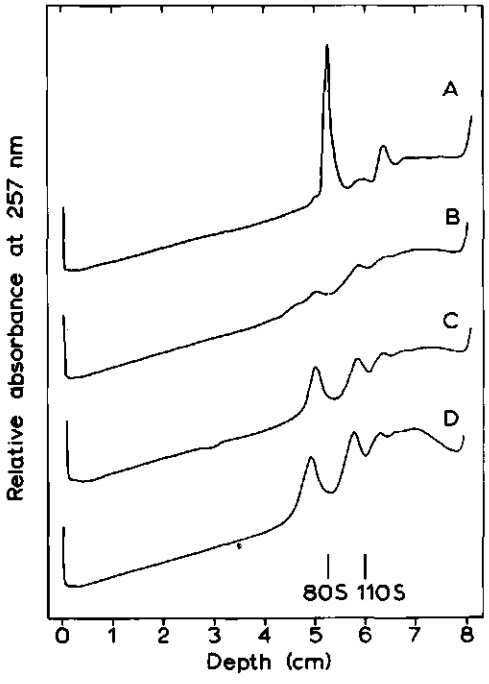


Fig. 7.7. Rate zonal centrifugation analysis of nucleoproteins assembled from isolated and separated CCMV-RNA components and CCMV-protein. One part by weight of RNA component was added to 3 parts of protein, assembled (Method B), and analysed as described in Fig. 7.2 (A) RNA-1+2, (B) RNA-3, (C) RNA-3+4, and (D) RNA-4.

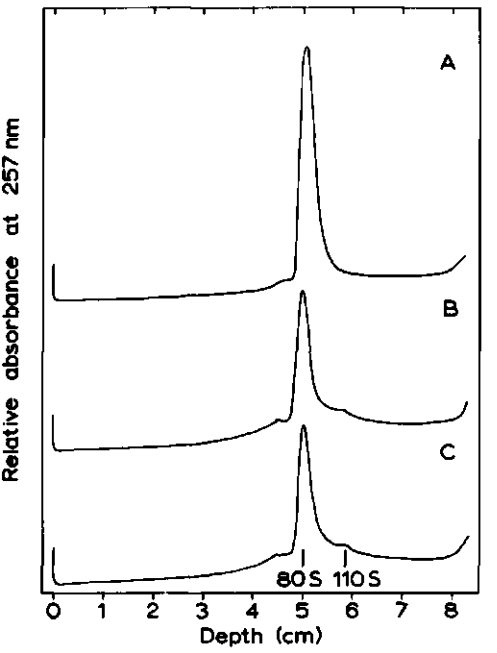


Fig. 7.8. Rate zonal centrifugation analysis of CCMV nucleoprotein particles; the effect of the protein preparation method. (A) CCMV, (B) CCMV assembled (Method B) from RNA and fresh protein obtained by gel-filtration (C) CCMV assembled (Method B) from RNA and protein, stored for 2 weeks at pH 5.0. For details of assembly and centrifugation see Fig. 7.2.

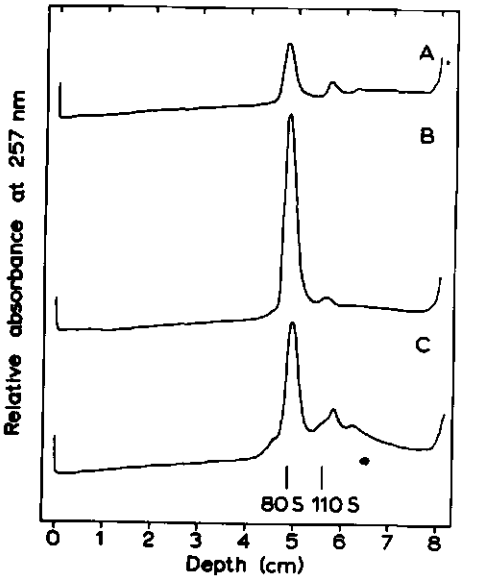


Fig. 7.9. Rate zonal centrifugation analysis of nucleoprotein particles obtained after dissociation and reassociation of bromoviruses. (A) BBMV, (B) BMV, and (C) CCMV (1 mg/ml) were dialysed against 1 M NaCl, pH 7.4 (dissociation buffer) for 24 h at 4°C and subsequently 2 h against low ionic strength, pH 7.4, with Mg^{+} (assembly buffer, method B) and 2 h against virus buffer both at 4°C. Details of centrifugation are described in Fig. 7.2.

Rate zonal centrifugation analysis of nucleoprotein particles obtained after dissociation and reassociation of virus.

In chapter 6 it was shown that CCMV, after dialysis for 24 h at 4°C against 1 M NaCl, pH 7.4, was dissociated into protein dimers and RNA-protein complexes containing 4 to 8 protein subunits per RNA molecule. Reassociation of this dissociated mixture under conditions of low ionic strength, pH 7.4, with Mg^{++}, resulted in nucleoprotein particles sedimenting around 80 S (Fig. 7.9). The RNA remained intact during the dissociation-association procedure (results not shown but comparable to Fig. 7.3). Also with this reassociation procedure particles sedimenting from 70 to 80 S and > 110 S were observed. SDS polyacrylamide gel electrophoresis of 70 to 80 S, 80 S and > 110 S nucleoprotein fractions revealed the same RNA contents as found with assembly from isolated RNA and protein.

Isopycnic centrifugation analysis of nucleoprotein particles assembled from isolated viral RNA and coat protein.

Apart from the RNA content and the sedimentation pattern of the assembled products, their stability in RbCl was tested. When the assembled product of CCMV was centrifuged to equilibrium in 35% (w/w) RbCl at 5°C, two bands were observed by visual inspection of the centrifuge tube, independent of the assembly procedures (Method A or B). The 257 nm absorption pattern of the contents of the tubes are shown in Fig. 7.10B. One band corresponds to the average density of the virus,

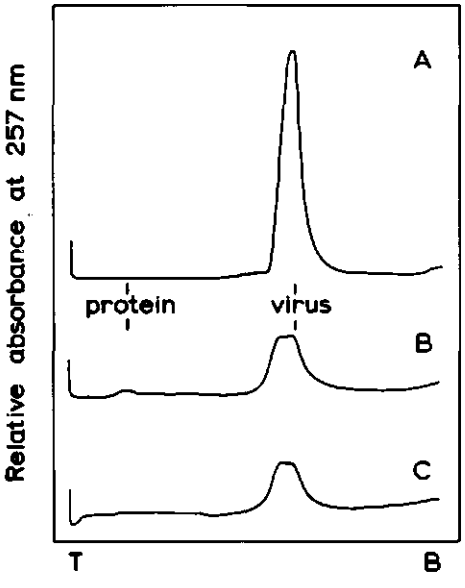


Fig. 7.10. Isopycnic centrifugation analysis of CCMV nucleoprotein particles; the stability of the assembled particles in RbCl. The samples are identical to the ones shown in Fig. 7.8. (A to C) 250 µl (250 µg) of each nucleoprotein preparation was layered onto 6 gram of 35% (w/w) RbCl and centrifuged for 60 h in a MSE SW 60 rotor at 39,000 rev/min and 5°C. The contents of the tubes were monitored at 257 nm. The main bands are indicated as protein and virus.

and the other to the protein indicating that part of the assembled nucleoproteins were unstable in RbCl. With different preparations of each of the bromoviruses variation in the protein band intensity occured. Assembly at low ionic strength, pH 7.5, with Mg^{++} produced some stable nucleoprotein particles, however the amount never exceeded 40% of the nucleoprotein particles found in sucrose gradients, SDS polyacrylamide gel electrophoresis of the nucleoprotein fraction, which is stable in RbCl, revealed less RNA-3 and -4 than in the original virus. At 25°C in RbCl nucleoproteins dissociated into protein and RNA, which would be expected if particles were stabilised mainly by electrostatic interactions.

Isopycnic centrifugation analysis of nucleoprotein particles obtained after dissociation and reassociation of virus.

The nucleoprotein particles obtained after dissociation and reassociation of virus were remarkably stable in RbCl at 5°C with the exception of BBMV. Even upon centrifugation at 25°C in the analytical ultracentrifuge the assembled nucleoprotein particles appeared to be stable in RbCl indicating another type of particle stabilization (Fig. 7.11). BBMV (Fig. 7.11A upper diagram) showed a loss of total

Fig. 7.11. Schlieren patterns of the isopycnic centrifugation of bromovirus nucleoproteins in RbCl after 40 h at 40,000 rev/min and 25°C; the effect of dissociation and association on nucleoprotein distribution. (A) BBMV, (B) BMV, and (C) CCMV; upper patterns: dissociated and reassociated nucleoprotein particles, lower patterns: untreated nucleoprotein particles. Centrifugal force to the left; concentration: 0.25 mg/ml, bar angle: 70°. The rate zonal centrifugation analysis of the same preparations of nucleoproteins obtained after dissociation and reassociation of virus is shown in Fig. 7.9.

material, caused by dissociation of nucleoprotein into RNA and protein. The latter was visible as a negative peak at the meniscus. Both BMV and CCMV (upper diagrams in Fig. 7.11B and C) showed less medium dense nucleoproteins containing RNA-3+4.

Apparently nucleoprotein particles containing RNA-3 or several copies of RNA-4 were not stable in RbCl but dissociated into protein, visible at the meniscus and RNA, which had sedimented at the bottom of the cell. Schlieren patterns were interpreted according to Johnson (1973). The original virus preparations are shown in the lower diagrams of Fig. 7.11.

In cases were the > 110 S CCMV-nucleoproteins were stable it was shown that the density was lower than the original virus. The > 110 S particles may be comparable with the IC_1 and IC_2 particles found by Herzog et al. (1976) after dissociation of BMV in 0.5 M $CaCl_2$.

DISCUSSION

When bromovirus coat proteins and nucleic acids were mixed and incubated or dialysed at low ionic strength at pH 7.4 or pH 6.0 (initially mixed at pH 7.5) mainly nucleoproteins sedimenting around 80 S were formed. This particle has also been found by Bancroft and Hiebert (1967) and Adolph and Butler (1977). Also variable amounts of nucleoproteins sedimenting between 70 and 80 S and > 110 S were observed. The proportion of these aberrant nucleoproteins was not affected by the age of virus, the age of protein or the protein preparation method.

SDS polyacrylamide gel electrophoresis of 70 to 80 S particles revealed the presence of RNA-3, while > 110 S particles contained mainly RNA-3+4. Assembly with separate RNA molecules also demonstrated that RNA-3 and -4 caused heterogeneity of the assembled products. CCMV-RNA degraded by a mixture of pancreatic RNase and RNase T_1 into fragments with an average molecular weight of 0.55×10^6, and assembled at low ionic strength, pH 7.4, with Mg^{++}, also formed 70 to 80 S nucleoprotein particles.

This suggests that the slow sedimenting nucleoprotein (70 to 80 S) are nucleoproteins containing RNA-3 or three and four molecules of RNA-4. The sedimentation coefficients would be 71 S, 70 S and 76 S, for such particles, assuming the same frictional coefficient as for the 78 S virus particles or 81 S, 79 S and 85 S using the frictional coefficient of the 88 S particles.

The nucleoprotein sedimenting > 110 S must be aggregates of improperly assembled nucleoproteins containing RNA-1 or -2 but mainly RNA-3 or several copies of RNA-4.

Sedimentation of the assembled products to equilibrium in RbCl showed that only 40% of the material sedimenting in a sucrose gradient was stable. Original virus is completely stable under these conditions. The stable assembled nucleoproteins contained mainly RNA-1 and -2, indicating that the nucleoproteins containing RNA-3 and -4 and sedimenting from 70 to 80 S and > 110 S were unstable

in RbCl.

Assembly at low ionic strength, pH 7.4, with Mg^{++}, first described by Bancroft and Hiebert (1967), appeared to be the best method to form 80 S nucleoprotein particles containing undegraded RNA molecules. The initial mixing at pH 7.5 in the absence of Mg^{++} as performed by Adolph and Butler (1977) may be sufficient to form 80 S particles, but the absence of Mg^{++} is harmful to the RNA. The dialysis at pH 6.0 would only stabilise the nucleoproteins but not leave the RNA intact. Although monovalent cations can compete with Mg^{++} upon stabilising the secondary structure of RNA (Boedtker, 1962), degradation of RNA has been observed. Chauvin et al. (1978) demonstrated that BMV at pH 5.5 in 0.2 M NaCl backtitrated from pH 7.5 had a larger overall radius in the presence of EDTA than in the presence of Mg^{++} ($MgCl_2$, 0.01 M), indicating a small specific effect of Mg^{++} even in the presence of 0.2 M KCl. Mg^{++} prevents degradation of RNA either by stabilising the secondary structure of RNA or by suppressing the radial expansion of protein and RNA (Durham and Pfeiffer, 1977), resulting in disruption of RNA-protein bonds.

In contrast to the instability of assembled particles in RbCl, nucleoproteins obtained after dissociation and reassociation of virus were almost for 90% stable in RbCl, with the exception of BBMV. The reassociated particles sedimented also around 80 S and contained variable amounts of 70 to 80 S and > 110 S particles, although less than with assembly from purified RNA and protein. Also in this case the unstable RbCl fraction contained RNA-3 and -4. Still particles containing RNA-3 and -4 were found, demonstrating that correct medium dense nucleoproteins can be formed. Probably other RNA-4 to RNA-3 ratios are needed during assembly to obtain sufficient particles containing RNA-3+4, since higher RNA-4 to RNA-3 ratios have been found during CCMV multiplication in vivo (Dawson, 1978).

The protein-nucleic acid complex obtained after dissociation of the virus at pH 7.4 in 1 M NaCl seems to have an effect on proper assembly. Either some secondary structure of the RNA had been preserved or the protein subunits, still bound to the RNA, act as an initiation complex for correct addition of more protein subunits.

At present there is no explanation for the exceptional aggregation of BBMV, but BBMV protein also behaved differently in the formation of empty protein shells, which it failed to form.

8. GENERAL DISCUSSION

In preliminary assembly experiments to obtain information about the recognition of CCMV-RNA by its coat protein, two problems arose. At first it appeared to be impossible to obtain RNA, which had not been degraded to some extent. Secondly the protein, due to RNA degradation in the original virus preparation could not be obtained free of RNA and therefore any search for specific protein-RNA interaction seemed to be meaningless.

Therefore the observed RNA degradation has been studied in more detail as described in chapter 3. The RNA appears to be degraded in situ by a reaction involving oxygen, radicals and traces of metals. The degradation could efficiently be prevented by complexing the metal ions, which probably catalyse the auto-oxidation of thiols and other reducing agents, with chelating agents like EDTA. In situ CCMV-RNA was more readily degraded than isolated RNA in solution. This could easily be explained by assuming a stronger chelating capacity of the isolated RNA.

In recent experiments I have been able to simulate the in situ CCMV-RNA degradation. When virus, purified and kept with EDTA, was incubated in 0.1 M sodium acetate pH 5.0, containing 1 mM EDTA, 2 mM $FeSO_4$ and 10 mM ascorbic acid at 37°C for 1 h RNA degradation in situ occurred. 2-Mercaptoethanol (2-ME) had an effect similar to ascorbic acid. The molar ratio of $FeSO_4$ to EDTA had to be 2 to 1. Higher and lower ratios simulated less RNA degradation. However the concentration of both compounds could be reduced tenfold without a decrease in RNA degradation. Also isolated RNA could be effectively degraded by this incubation mixture. Ascorbic acid has been known as a virus inactivating compound for Influenza A virus (Klein, 1945), tobacco necrosis virus (TNV) (Bawden and Pirie, 1957) and TMV (Bawden and Pirie, 1959). In the presence of Fe^{++} and oxygen, ascorbic acid can bring about hydroxylation of compounds with aromatic character. This reaction is considerably enhanced by certain concentrations of EDTA (Udenfriend et al.,1954). In both reactions: inactivation and hydroxylation, the role of radicals has not been tested, although the need for

oxygen has been demonstrated. The identification of the inactivating compound most likely the hydroxyl radical ($OH^{\cdot}$)(Scholes et al., 1960), will be subject of further studies. Similar radical mediated inactivation might explain the observed inactivation of bacteriophage T_5 and myxoviruses by Bachrach et al. (1971) and Bachrach (1971). The authors examined the inactivating effect of aldehydes obtained by oxidation of polyamines. As aldehydes are easily oxidised by a single electron transfer, the generation of a radical is most likely. Also the inactivation of pseudorabies by dithiothreitol (DTT)(Grainer et al., 1971), the thermal inactivation of rabies and other rhabdoviruses (Michalsky et al., 1976) might be explained by a radical mediated inactivation.

When diethyldithiocarbamate (DIECA) ia added to the leaf homogenate of cucumber infected with prunus necrotic ringspot virus, this virus does not inactivate. On the other hand not DIECA but 2-ME stabilises another labile plant virus, tulare apple mosaic virus (Barnett and Fulton, 1971). This different response to stabilising agents shows that both DIECA and 2-ME might show other effects than those demonstrated in this thesis. Nevertheless the inactivation of these labile viruses should be reinvestigated in view of the radical mediated auto-oxidation of 2-ME and DIECA.

During manipulations with viruses in the presence of reducing agents such as DTT, 2-ME or ascorbic acid, addition of 0.1 to 1.0 mM chelating agent, is suggested to prevent RNA degradation. In cases where the virus particle is stabilised by divalent cations like Mg^{2+} and Ca^{2+} (southern bean mosaic virus, Hsu et al., 1976) an appropiate ratio of divalent cations to chelating agent, based on the equilibrium constant, should be used to keep the free divalent ion concentration sufficiently high for stabilization. The chelating agent will still complex other metal ions, involved in the catalysis of auto-oxidation of thiols, because of the large binding constants of these metals.

While studying the in situ RNA degradation several protein preparation methods have been investigated (chapter 4). With $CaCl_2$ RNA free protein could be obtained, even from virus with in situ degraded RNA. These preparations of dissociated coat protein subunits were used to study the association into pseudo top components (PT) i.e. empty protein shells. The partition of protein among PT and dissociated protein depended on the pH and differed in the association and dissociation reaction. The pH at which 50% of the protein was present as PT was about 5.5 in the association reaction and about 6.0 in the dissociation reaction (chapter 4). This hysteresis effect has also been observed in acid-base titration studies of both protein and virus (Jacrot, 1975) and we have recently

confirmed this observation (Verduin and Penners, in manuscript). The effect can be explained by assuming two conformations of the coat protein. Around pH 5.0 the coat protein subunits occur in a compact form, but around pH 7.5 in a loose conformation (chapter 6). A large change in the secondary structure of the coat protein, if the pH was increased from 5.0 to 7.5, could not be demonstrated however by means of circular dichroism measurements (chapter 5). Therefore the conformational change is assumed to be some rearrangement of a random coil structure.

Adolph and Butler (1975, 1976, 1977) have tried to obtain specific assembly of CCMV by making use of the hysteresis phenomenon, but they failed. No conditions have been found where protein in the compact conformation remains unaggregated. Compact protein forms PT, which precipitates after addition of RNA.

On the analogy of the assembly of TMV, where a disk-like aggregate of 34 subunits plays a key-role in the specific initiation of assembly of virus particles, Adolph and Butler (1974) have looked for an aggregate of the coat protein of CCMV by varying the pH, ionic strength, temperature and concentration of protein, which might play a similar role in the assembly of CCMV particles. Such aggregate of size intermediate between the dissociated protein and the capsid has however not been found (Adolph and Butler, 1974). Neither has it been possible to detect proteins, different from coat protein and occuring in only one or a few copies per virus particle, which might achieve the specific assembly of CCMV-RNA and protein into nucleoprotein particles (Rice, 1974).

Therefore, as described in chapter 6, we have undertaken a study of the dissociation of CCMV particles under controlled conditions of pH and ionic strength to examine whether an intermediate aggregate of protein or RNA and protein could be detected upon dissociation, which might be important also in the reversed process i.e. assembly. Such dissociation studies had become feasible as we had found conditions, which prevented the degradation of RNA upon incubation during longer periods.

Taking the line that the protein occurs in the compact formation if aggregated into a protein shell or a virus nucleoprotein particle, we assumed that it might be possible to detect intermediate stages in the dissociation reaction, in which the protein had still maintained the compact conformation.

Four types of intermediate were detected upon dissociation: unfolded virus and dissociation intermediates, both containing protein-RNA ratios almost equal to that in complete virus; a protein aggregate sedimenting around 8 S and a protein-RNA complex consisting of a RNA strand with 4 to 8 protein subunits

per RNA (chapter 6). The first two intermediates have not been studied in more detail and tested in assembly experiments. The protein aggregate was shortlived and we have not yet been able to determine whether the protein occurs in the compact or loose conformation in the aggregate, neither have we been able to use this aggregate in assembly studies.

The amount of protein in the protein-RNA complexes was too small for determining also the conformation, but assembly of CCMV from a mixture of protein and this protein-RNA complex resulted in nucleoprotein particles, which were more stable in RbCl or CsCl than nucleoprotein from isolated protein and RNA (chapter 7). Presumably the protein subunits, bound to the RNA, retain the compact conformation and regulate the correct addition of protein subunits from the solution. Another explanation would be that the bound protein subunits preserve a defined secondary structure in the RNA molecule. A possible candidate for this defined structure might be the tRNA-like structure of the 3'-terminus of the RNA molecules (Bastin et al., 1976). Preservation of a certain poly (A) structure at the 5'-terminus is unlikely as CCMV-RNA contains no such poly (A) stretches (Semancik, 1974). A further characterization and isolation of this RNA-protein complex with be needed to test whether this complex competes with heterologous RNA molecules of the same size in the assembly process.

Up till now the main criterium for proper assembly of CCMV particles has been encapsidation of the RNA as checked by infectivity tests and sucrose gradient analyses (Bancroft and Hiebert, 1967; Adolph and Butler, 1977). The formation of particles sedimenting around 80 S, as analysed by sucrose gradients, only indicates that an amount of RNA equivalent to 1.1×10^6 g/mol is encapsulated. However, the sedimentation coefficient does not give information about the integrity of the RNA. It has been shown in chapter 3, that even when all RNA molecules inside the virus particles are degraded into small fragments, there is no change in the sedimentation coefficient.

The infectivity of free RNA in solution almost equals that of complete virus particles. Therefore, enhancement of infectivity cannot be used to demonstrate encapsidation but only to measure RNA integrity. Testing the resistance of assembled particles against an exonuclease, a mixture of an exo- and an endonuclease would be more efficient, tells us something about the protection of the RNA by the coat protein but not whether the RNA is correctly located in the protein shell. The only way to find out whether RNA-1, RNA-2 and RNA-3+4 are properly encapsidated in heavy, light and medium dense nucleoprotein particles, respectively, like the original virus preparation, is to use a combination of isopycnic centrifugation analysis and RNA gel electrophoresis

to analyse the RNA content of the assembled nucleoprotein particles as described in chapter 7.

Herzog and Hirth (1978) have also analysed the RNA content of assembled nucleoprotein particles after fractionation of the assembly products on sucrose gradients. These authors found mainly RNA-3 and RNA-4 in the 70 S fraction, which agrees with our finding for CCMV. However they found all four RNAs in the fractions sedimenting around 80 S and 110 S, while the > 110 S fraction of CCMV contained mainly RNA-3 and -4. Interpreting their results one should also bear in mind that the assembly mixture was treated with 6% PEG (MW = 20,000) in the presence of 0.1 M NaCl to concentrate the assembly products. In control experiments has to be tested whether the addition of PEG and subsequent concentration of the assembled products affected encapsidation. The authors have demonstrated undoubtedly, with their methods, that small RNA molecules are readily encapsidated. Preferential encapsidation of small RNA molecules has also been observed in assembly experiments with CCMV protein (Adolph and Butler, 1977) and AMV protein (Driedonks, 1978). In our experiments we have not looked for any preferential encapsidation of RNA-3 or -4, but we were more concerned about the stability of the assembled nucleoproteins. All four RNA molecules are efficiently encapsidated, but when the stability of these nucleoproteins as tested in RbCl it appears that almost 50% of the particles containing RNA-3+4, RNA 3 or several copies of RNA-4,was unstable and dissociated (chapter 7). Also in pH and ionic strength dependent dissociation studies it has been demonstrated that RNA-4 was released from the nucleoprotein particles before any significant amounts of RNA-1, -2 and -3 were released (chapter 6).

In my opinion a high affinity of the coat protein for its own messenger, RNA-4, would be very inefficient. This implicates that after synthesis of small amounts of coat protein, the coat protein blocks its further synthesis by binding of subunits to the messenger RNA coding for it. One would expect a higher affinity of the coat protein for RNA-1, RNA-2 and RNA-3, which are presumed to code for non-structural viral proteins synthesized in early infection. Inhibition of the synthesis of non-structural coat proteins by coat protein has been observed for bacteriophage MS_2 (Sugiyama and Nakada, 1968). The high affinity of the coat protein for the four RNA molecules can be demonstrated by means of kinetic and competition experiments with other RNAs. To eliminate the effect of size of the RNA molecules in the assembly process it will be necessary to perform the experiments with heterologous RNA molecules of equal size.

The question, how RNA-3 and -4 are encapsidated in one nucleoprotein particle,

remains unanswered. In vitro assembly of equimolar amounts of RNA-3 and RNA-4 results in nucleoprotein particles of which less than 50% are stable in RbCl and which contain both RNA-3 and RNA-4. In vivo RNA-3 and -4 are synthesized in larger amounts than RNA-1 and -2 (Dawson, 1978). So higher concentrations of RNA-3 and -4 or a RNA-3 to -4 ratios different from that found in purified virus preparation may be needed to obtain correct in vitro assembly. The only reason probably why RNA-3 has to be encapsidated together with RNA-4 is to obtain a nucleoprotein particle of maximal stability.

In an attempt to explain why no initiation complex can be formed from dissociated protein and isolated RNA, although evidence for such a complex has been obtained from dissociation studies, I propose the following hypothesis for a structural model of the dimer of the CCMV coat protein subunit.

The coat protein dimer at pH 7.5 consists of two polypeptide chains folded into an ellipsoid so that the ratio of polar and apolar amino acid residues at the surface of the ellipsoid allows solubilization of the dimer in 0.2 M NaCl. The conformational change accompanying the transistion from the loose to the compact conformation is visualized by the formation of a carboxyl cage in the hydrophobic interior of the coat protein subunit. This carboxyl cages contains at least two protonated acidic amino acid residues and one lysine residue while close to this cage one lysine and one or a few basic amino acid residues must be located.

The different elements of the hypothetical model are based on the following arguments.

The size of the coat protein monomer is too small for the occurence of one or a few structural domains linked by a flexible hinge (Wetlaufer, 1973). Such structure has been succesfully applied to interpret the structural data of tomato bushy stunt virus (Harrison et al., 1974; Rossman and Liljas, 1974; Winkler et al., 1977) and also for antibody molecules (Huber, 1976). Therefore the protein monomer has been considered as one structural domain.

The folding problem of a globular protein into a structural domain, is how to gain enough energy from hydrophobicity, hydrogen bonds and other ionic interactions to overcome the loss of configurational entropy and the steric strain that occurs in the folded state (Chotia, 1975). In a comparison of four well-studied proteins Klotz (1970) has shown that, although almost all ionic or polar groups are exposed to solvent, apolar residues may be buried in the protein interior or exposed to the solvent. In fact, in all four proteins at least 45% of the apolar residues were partially or fully exposed to the solvent. To the extent that these proteins are respresentative of other proteins, many hydrophobic

amino acid residues may occur at the surface of a protein subunit. It is assumed that the monomer of the coat protein of CCMV is not soluble in aqueous solutions because of the large amount of apolar residues at the surface of the protein. However in the coat protein dimer, where many hydrophobic amino acid residues are presumed to be involved in intersubunit linkage, the ratio of exposed polar and apolar amino acid residues would be high enough to allow solubilization of this aggregate.

The carboxyl cage is assumed on the basis of abnormal acid-base titrations of both protein and nucleoprotein (Jacrot, 1975) analogous to TMV (Butler and Durham, 1977; Stubbs et al., 1977). In the case of TMV this abnormal titration behaviour has been explained by assuming a carboxyl cage in which acidic and basic amino acid residues have been located in a hydrophobic environment in the interior of the protein. This hydrophobic environment causes the delayed deprotonation of carboxyl groups of certain amino acids. According to Chothia (1974), one or a few polar amino acid residues may be buried in the interior of the protein without loosing free energy, if these residues form hydrogen bonds. Therefore it is not unlikely that two carboxyl groups of CCMV protein are hydrogen bonded to other acidic or basic amino acid residues in a hydrophobic environment.

Location of a lysine residue in the carboxyl cage was based on the properties of a mutant of CCMV in which lysine had been replaced by arginine (Bancroft et al., 1973). This mutant did not dissociate at pH 7.0 and one of the abnormally titrating carboxyl groups showed a pK of 4.6. Most likely the arginine replaced the lysine with its positive charge but also donated a proton thereby making one carboxyl group in the cage superfluous.

A second lysine must be present close to the cage. This lysine residue can be labelled with pyridoxal phosphate at pH 5.0 and this label becomes more mobile when the labelled virus was incubated at pH 7.0, where the abnormal carboxyl group titrates. The mobility of the label at pH 7.0 appeared to be strongly affected by the presence of both RNA and Mg^{2+} (Krüse, Verduin and Visser, in manuscript).

With the hypothetical model of the coat protein dimer PT formation at pH 5.0 and nucleoprotein formation at pH 7.5 can be explained as follows:

Decreasing the pH of a coat protein dimer solution at pH 7.5 causes protonation of the two acidic amino acid residues, i.e. the carboxyl cage forms and the lower ratio of polar to apolar amino acid residues at the surface of the protein dimer forces the dimer into PT, stabilised by hydrophobic interactions. The

formation of nucleocapsids at pH 7.5 in the presence of RNA is caused by the neutralisation of some basic amino acid residues of the protein by the RNA resulting in ionic linkages between RNA and protein, while the change in the ratio of polar to apolar residues at the surface of the protein dimer forces the protein into a spherical nucleoprotein particle stabilised by weak hydrophobic interactions.

Also the effect of divalent ions can easily be explained with the hypothetical model. First of all Mg^{2+} and Ca^{2+} should be able to replace the proteins in the carboxyl cage, which in fact was demonstrated by Pfeiffer and Durham (1977). Secondly the divalent cations may stabilise the secondary structure of the RNA (Boedtker, 1962; Gordon, 1965). From the CD measurements (chapter 5) it is concluded that in situ RNA contains a large proportion of base pairing and base stacking. At low ionic strength base stacking is favoured and accompanied by expansion of the RNA strand while at high ionic strength more base pairing occurs (Powell and Richards, 1972).

The change of secondary and tertiary structure of CCMV-RNA by different mono and divalent cations has clearly been demonstrated by Dickerson and Trim (1978). Also polyamines, occuring in plants, are known to influence the structure of RNA, but the bromoviruses appear to contain no detectable amount of these polycations (Nickersona and Lane, 1977). Changes in the secondary and tertiary structure of CCMV-RNA, which is bound to the protein subunits by strong ionic linkage, strongly affects the protein and its carboxyl cage. Radical extension of the protein subunits, which has been observed during the swelling process (Chauvin et al., 1978) may be caused mainly by the extension of the RNA to which the protein is bound.

On the basis of this model the following experiments are suggested to test its validity. First of all we have to focus our attention on the hydrophobicity of the coat protein.

By means of in vivo studies (Kim, 1977) more information has to be obtained about the sites in the cell where synthesis of RNA and protein and subsequent assembly takes place. These studies will contribute to the understanding of the role of membranes in plant virus assembly. The involvement of membranes for assembly of filamentous bacterophages has been demonstrated (Ino-viridae; Marvin and Wachtel, 1975). In a process analogous to that observed with the Ino-viridae the hydrophobic nature of membranes might be able to solubilise monomers of CCMV, thereby exposing parts of the polypeptide chain which recognize the RNA. Another approach would be the determination of the hydrophobicity of the coat protein by testing the affinity of the coat protein for alkyl-substituted agarose in

hydrophobic chromatography as described for the capsid protein of Moloney-Murine Leukemia Virus (Swanson et al., 1978).

Membranes may also be involved in controlling the concentrations of monovalent and divalent ions as suggested by Brady et al. (1977) and Pfeiffer and Durham (1977). Several examples can be shown in which small variations in the concentrations of ions regulate dissociation or association of protein-nucleic acid complexes (Brady et al., 1977; Powell, 1971; Seto and Thomasz, 1976; Weber et al., 1977). Binding studies of mono-and divalent ions to CCMV may elucidate the role of these ions in both dissociation i.e. uncoating and assembly and their influence on the assumed ionic protein-RNA linkages.

The involvement of certain amino acids residues in the assembly process can at present be studied by techniques such as nuclear magnetic resonance (De Wit, 1978), electron spin resonance, fluorescence, and laser Raman spectroscopy (Hartman et al., 1973, 1978; Shie et al., 1978), which allow us to assign individual amino acid residues to the observed molecular interactions in solution.

Also by the use of mutants, where one or two amino acid residues have been replaced (Lane, 1974), the role of certain amino acid residues in protein-protein and protein-nucleic acid interaction suggested in the model may be demonstrated.

SUMMARY

This thesis decribes the conditions for isolation of cowpea chlorotic mottle virus (CCMV), its ribonucleic acid (RNA) and the coat protein, the characterization of the virus and its constituents (chapter 3, 4 and 5) and the dissociation and assembly behaviour of the virus (chapter 6 and 7).

The aim of the investigation and a literature review pertaining to RNA-protein interactions, which are met with the tobamoviruses, the Leviviridae and the bromoviruses are given in chapter 1 and 2.

CCMV, isolated and purified in the prescence of reducing agents such as ascorbic acid and mercaptoethanol, contained variable amounts of degraded RNA. At first RNA-2 was cleaved into two fragments but subsequently all RNA molecules were cleaved at random sites. The buoyant density in RbCl and the sedimentation coefficient of the virus remained unchanged i.e. the degraded RNA was still bound to the protein coat and did not change the stability of the nucleoprotein particles.

The degradation of the RNA was stimulated when virus was incubated at 37°C in the presence of reducing agents such as mercaptoethanol. Besides reducing agents also oxygen and traces of metals appeared to play a role in the degradation process. Addition of chelating agents, such as 1 mM EDTA, to the homogenization buffer and the buffers in which the virus was kept, prevented in situ RNA degradation. Problably the degradation is caused by radicals, which are formed during the auto-oxidation of reducing agents by oxygen, catalysed by traces of metals (chapter 3).

In chapter 4 a describtion is given of three coat protein isolation methods and the influence of the isolation method on the formation of pseudo top component (PT) i.e. an empty protein shell without RNA. By means of $CaCl_2$ RNA free coat protein could be isolated from virus, even when the virus particles contained exstensively degraded RNA. The formation of PT and its dissociation were pH dependent and both processes showed a remarkable hysteris effect. This effect can be explained by assuming two stable conformations of the coat protein.

In chapter 5 the results of partial specific volume, ciruclar dichroism (CD) and sedimentation equilibrium measurements of CCMV are given. The apparent partial specific volume of the dissociated protein in 0.5 M $CaCl_2$ pH 7.5, mainly the dimer of the coat protein subunit, changed from 0.737 cm^3/g to 0.728 cm^3/g in 0.2 M NaCl, 0.01 M $CaCl_2$ pH 5.0, mainly PT. Calculation of the partial specific volume of CCMV from the experimentally determined volumes of RNA, 0.476 cm^3/g and coat protein, 0.745 cm^3/g in 0.2 M NaCl, 1 mM EDTA pH 5.0 resulted in a value of 0.680 cm^3/g, which is lower than the experimentally determined partial specific volume of CCMV, 0.719 cm^3/g. The difference is caused by RNA-protein interaction.

The CD measurements of protein dimers and PT showed little difference between the secondary structure of both protein subunit aggregates. The α-helix content was in both cases smaller than 1%. The structure of the RNA, both free in solution and inside the virus particle showed a large amount of base pairing and base stacking. Small changes in the secondary structure occured, when the virus was swollen and dissociated.

Chapter 6 describes the pH and ionic strength dependent dissociation of CCMV. Upon increasing the pH from 5.0 to 7.5 at 1 M NaCl, CCMV formed RNA-protein complexes, which sedimented slower than the intact virus particles but still retained an RNA-protein ratio identical to virus. When CCMV was incubated at pH 7.5 with increasing concentrations of NaCl, at first unfolding of the RNA occured, while all the protein subunits were still bound to the RNA, followed by a gradual release of protein subunits. In 1 M NaCl the RNA retained 4 to 8 protein subunits per RNA molecule. This RNA-protein complex is probably involved in the recognition of the protein by the viral RNA.

In chapter 7 is described how this RNA-protein complex has been used for assembly of virus particles, 90% of which is stable in RbCl. These particles obtained after dissociation and reassociation of CCMV were characterised with respect to RNA content and compared with virus particles obtained after assembly of isolated RNA and coat protein.

After centrifugation in a sucrose gradient both reassociated and assembled virus showed a band with a sedimentation coefficient of about 80 S at the main product. These particles contained RNA-1 and -2, comparable to the original virus preparation but less RNA-3 and -4. Two other classes of products were observed. On one hand a fraction sedimenting between 70 and 80 S, which contained mainly particles with RNA-3 and on the other hand a fraction with a

sedimentation coefficient > 110 S, in which some particles with RNA-1 and -2 occured, but mainly particles with RNA-3 and -4. Only 40% of the nucleoprotein particles assembled from isolated RNA and protein appeared to be stable in RbCl.

Probably RNA-1 and RNA-2 can form stable virus particles by means of the RNA-protein complex, while RNA-3 also has to make a link with RNA-4, before a stable nucleoprotein particle sedimenting at 80 S is formed. The assembly products of CCMV are compared in this chapter with those of broad bean mottle virus and brome mosaic virus, two other bromoviruses.

Possible assembly mechanisms, a model for the coat protein dimer and future assembly research are discussed in chapter 8.

SAMENVATTING

Dit proefschrift beschrijft de omstandigheden voor de isolatie van cowpea chlorotic mottle virus (CCMV), het ribonucleïnezuur (RNA) en het manteleiwit, de karakterisering van het virus en zijn bestanddelen (hoofdstuk 3,4 en 5) en het dissociatie en assemblage gedrag van het virus (hoofdstuk 6 en 7).

De probleemstelling van het onderzoek en een literatuuroverzicht met betrekking tot RNA-eiwit interacties bij de tobamovirussen, de Leviviridae en de bromovirussen zijn weergegeven in hoofdstuk 1 en 2.

CCMV, geïsoleerd en gezuiverd in aanwezigheid van reducerende stoffen zoals ascorbinezuur en mercaptoethanol, bevatte variable hoeveelheden afgebroken RNA. Allereerst ontstond een breuk in RNA-2, doch vervolgens traden op willekeurige plaatsen breuken op in alle RNA moleculen. De dichtheid in RbCl en de sedimentatie coefficiënt van het virus bleven onveranderd m.a.w. het afgebroken RNA bevond zich nog in de eiwitschil en veranderde de stabiliteit van de deeltjes niet.

De afbraak van het RNA werd bevorderd door het gezuiverde virus bij 37°C met reducerende stoffen, zoals mercaptoethanol te incuberen. Naast reducerende stoffen bleken ook zuurstof en spoortjes metalen een rol te spelen in het afbraakproces. Het toevoegen van chelerende stoffen zoals 1 mM EDTA aan de homogenisatie buffer en de buffers waarin het virus werd bewaard, voorkwam in situ RNA afbraak. Waarschijnlijk vindt de afbraak plaats door radicalen, die worden gevormd bij de auto-oxidatie van reducerende stoffen onder invloed van zuurstof, gekatalyseerd door spoortjes metalen (hoofdstuk 3).

In hoofdstuk 4 worden een drietal eiwit isolatie methoden beschreven en het effect van de isolatiemethode op de vorming van pseudo top komponent (PT) i.e. een lege eiwitschil zonder RNA. Met behulp van $CaCl_2$ kon uit virus, zelfs als dit veel afgebroken RNA bevatte, eiwit vrij van RNA geïsoleerd worden. De vorming van PT en de dissociatie ervan zijn afhankelijk van de pH en de beide processen vertonen een opvallend hysterese effect. Dit effect kan verklaard worden door aan te nemen dat het eiwit in twee stabiele eiwitconformaties kan voorkomen.

In hoofdstuk 5 worden de resultaten gegeven van partieel specifiek volume, circular dichroism (CD) en sedimentatie-evenwicht bepalingen. Het schijnbare partieel specifiek volume van het gedissocieerde eiwit in 0,5 M $NaCl_2$ pH 7,5, voornamelijk de dimeer van de struktuureenheid van het manteleiwit, verandert van 0,737 cm^3/g naar 0,728 cm^3/g in 0,2 M NaCl, 0,01 M $CaCl_2$ pH 5,0, waarbij het eiwit hoofdzakelijk voorkomt als PT. Berekening van het schijnbare partieel specifiek volume van CCMV uit de experimenteel bepaalde volumina van RNA, 0,476 cm^3/g en eiwit, 0,745 cm^3/g in 0,2 M NaCl, 1 mM EDTA pH 5,0 leverde een waarde van 0,680 cm^3/g, welke lager is dan het experimenteel bepaalde schijnbare partieel specifiek volume van CCMV, 0,719 cm^3/g. Het verschil moet worden toegeschreven aan de interactie van het RNA met het eiwit.

De CD metingen aan eiwit dimeren en PT toonden weinig verschil tussen de secundaire structuur van beide eiwit aggregaten. Het α-helix gehalte is in beide gevallen kleiner dan 1%. De structuur van het RNA, zowel vrij in oplossing als in het virusdeeltje vertoonde een grote mate van basenparing en basenstapeling, waarin geringe veranderingen optreden bij het zwellen en dissociëren van het virus.

Hoofdstuk 6 beschrijft de dissociatie van CCMV in afhankelijkheid van veranderingen in pH en ionensterkte. Bij een toename in de pH tussen 5,0 en 7,5 bij een NaCl concentratie van 1 M vormde CCMV RNA-eiwit complexen die langzamer sedimenteerden dan het virus maar toch eenzelfde RNA-eiwit verhouding bezaten als het virus. Wanneer CCMV werd geïncubeerd bij pH 7,5 met toenemende concentraties aan NaCl, trad allereerst een ontvouwing op van het RNA, met nog vrijwel al het eiwit gebonden, gevolgd door een geleidelijke dissociatie van het eiwit. In 1 M NaCl werden nog 4 tot 8 eiwitmoleculen per RNA molecuul aangetoond. Dit RNA-eiwit complex speelt waarschijnlijk een rol bij de herkenning van het eiwit door het virus RNA.

In hoofdstuk 7 wordt beschreven hoe dit RNA-eiwit complex kon worden gebruikt voor assemblage van virusdeeltjes met een grote stabiliteit in RbCl. Deze deeltjes verkregen na dissociatie en reassociatie van CCMV, werden gekarakteriseerd ten aanzien van hun RNA inhoud en vergeleken met virusdeeltjes verkregen door assemblage van geïsoleerd RNA en manteleiwit.

Zowel gereassocieerd als geassembleerd virus vertoonden na centrifugatie in een suikergradient als voornaamste produkt een band met een sedimentatie coefficiënt van ongeveer 80 S. Deze deeltjes bevatten RNA-1 en -2, vergelijkbaar met het originele viruspreparaat en in mindere mate RNA-3 en -4. Twee andere klassen van produkten werden waargenomen. Enerzijds een fraktie sedi-

menterend van 70 tot 80S, welke voornamelijk deeltjes met RNA-3 bevatte en anderzijds een fraktie met een sedimentatie coefficiënt groter dan 110 S, waarin deeltjes voorkwamen die RNA-1 en -2 bevatten, doch vooral veel RNA-3 en -4. Slechts 40% van de geassembleerde virusdeeltjes bleken stabiel in RbCl.

RNA-1 en RNA-2 kunnen uitgaande van het RNA-eiwit complex stabiele virusdeeltjes vormen, terwijl RNA-3 op nog onbekende wijze RNA-4 in een virusdeeltje moet vangen, alvorens een stabiel deeltje met een sedimentatie coefficiënt van ongeveer 80 S ontstaat. De assemblage produkten van CCMV worden in dit hoofdstuk vergeleken met die van broad bean mottle virus en brome mosaic virus, twee andere bromovirussen. Mogelijke assemblage mechanismen, een model voor het manteleiwit dimeer en toekomstig assemblage onderzoek worden besproken in hoofdstuk 8.

Adams, jr., E.T. (1967). Analysis of self-associating systems by sedimentation equilibrium experiments. Fractions, 1967, No. 3, 1-18.

Adler, A.J., Fasman, G.D. and Tal, M. (1970). Circular dichroism of Escherichia coli ribosomes: Effect of heating and metal ions. Biochimica et biophysica acta 213, 424-436.

Adolph, K.W. (1975a). The conformation of the RNA in cowpea chlorotic mottle virus: dye-binding studies. European Journal of Biochemistry 53, 449-455.

Adolph, K.W. (1975b). Conformational features of cowpea chlorotic mottle virus RNA and the stability of the virus. Journal of General Virology 28, 137-145.

Adolph, K.W. (1975c). Structural transitions of cowpea chlorotic mottle virus. Journal of General Virology 28, 147-154.

Adolph, K.W. and Butler, P.J.G. (1974). Studies on the assembly of a spherical plant virus. I. States of aggregation of the isolated protein. Journal of Molecular Biology 88, 327-341.

Adolph, K.W. and Butler, P.J.G. (1975). Reassembly of a spherical virus in mild conditions. Nature, London 255, 737-738.

Adolph, K.W. and Butler, P.J.G. (1976). Assembly of a spherical plant virus. Philosophical Transactions of the Royal Society, London. B. 276, 113-122.

Adolph, K.W. and Butler, P.J.G. (1977). Studies on the assembly of a spherical plant virus. III. Reassembly of infectious virus under mild conditions. Journal of Molecular Biology 109, 345-357.

Atabekova, T.I., Taliansky, M.E. and Atabekov, J.G. (1975). Specificity of protein-RNA and protein-protein interaction upon assembly of TMV in vivo and in vitro. Virology 67, 1-13.

Bachrach, U. and Don, S. (1971). Inactivation of myxoviruses by oxidized polyamines. Journal of General Virology 11, 1-9.

Bachrach, U., Don, S. and Wiener, H. (1971). Antivirus action of acrolein, glutaraldehyde and oxidized spermine. Journal of General Virology 13, 415-422.

Bancroft, J.B. (1970a). Brome mosaic virus. CMI/AAB Descriptions of plant viruses. No. 3.

Bancroft, J.B. (1970b). The self-assembly of spherical plant viruses. Advances in Virus Research 16, 99-134.

Bancroft, J.B. (1971a). Cowpea chlorotic mottle virus. CMI/AAB Descriptions of plant viruses. No. 49.

Bancroft, J.B. (1971b). The significance of the multicomponent nature of cowpea chlorotic mottle virus. Virology 45, 830-834.

Bancroft, J.B. and Flack, I.H. (1972). The behaviour of cowpea chlorotic mottle virus in CsCl. Journal of General Virology 15, 247-251.

Bancroft, J.B. and Hiebert, E. (1967). Formation of an infectious nucleoprotein from protein and nucleic acid isolated from a small spherical virus. Virology 32, 354-356.

Bancroft, J.B., Bracker, C.E. and Wagner, G.W. (1969a). Structures derived from cowpea chlorotic mottle and brome mosaic virus protein. Virology 38, 324-335.

Bancroft, J.B., Hiebert, E. and Bracker, C.E. (1969b). The effects of various polyanions on shell formation of some spherical viruses. Virology 39, 924-930.

Bancroft, J.B., Hills, G.J. and Markham, R. (1967). A study of the self-assembly process in a small spherical virus. Formation of organized structures from protein subunits in vitro. Virology 31, 354-379.

Bancroft, J.B., McDonald, J.G. and Rees, M.W. (1976). A mutant of cowpea chlorotic mottle virus with a perturbed assembly mechanism. Virology 75, 293-305.

Bancroft, J.B., Wagner, G.W. and Bracker, C.E. (1968a). The self-assembly of a nucleic-acid free pseudo-top component for a small spherical virus. Virology 36, 146-149.

Bancroft, J.B., Hiebert, E., Rees, M.W. and Markham, E. (1968b). Properties of cowpea chlorotic mottle virus, its protein and nucleic acid. Virology 34, 224-239.

Bancroft, J.B., McLean, G.D., Rees, M.W. and Short, M.N. (1971). The effect of an arginyl to a cysteinyl replacement on the uncoating behaviour of a spherical plant virus. Virology 45, 707-715.

Bancroft, J.B., Rees, M.W., Johnson, M.W. and Dawson, J.R.O. (1973). A salt-stable mutant of cowpea chlorotic mottle virus. Journal of General Virology 21, 507-513.

Bancroft, J.B., Rees, M.W., Dawson, J.R.O., McLean, G.D. and Short, M.N. (1972). Some properties of a temperature-sensitive mutant of cowpea chlorotic mottle virus. Journal of General Virology 16, 69-81.

Barnett, O.W. and Fulton, R.W. (1971). Differential response of prunus necrotic ringspot and tulare apple mosaic virus to stabilizing agents. Virology 46, 613-619.

Bastin, M., Dasgupta, R., Hall, T.C. and Kaesberg, P. (1976). Similarity in structure and function of the 3'-terminal region of the four brome mosaic virus RNAs. Journal of Molecular Biology 103, 737-745.

Bawden, F.C. and Pirie, N.W. (1957). A virus-inactivating system from tobacco leaves. Journal of General Microbiology 16, 696-710.

Bawden, F.C. and Pirie, N.W. (1959). The infectivity and inactivation of nucleic acid preparations from tobacco mosaic virus. Journal of General Microbiology 21, 438-456.

Bernardi, A. and Spahr, P.F. (1972). Nucleotide sequence at the binding site for coat protein on RNA of bacteriophage R17. Proceedings of the National Academy of Sciences of the United States of America 69, 3033-3037.

Bobst, A.M., Pan, Y.E. and Philips, D.J. (1974). Comparative optical property studies on polycistronic R17 phage ribonucleic acid and rabbit globin messenger ribonucleic acid. Biochemistry 13, 2129-2133.

Bockstahler, L.E. and Kaesberg, P. (1962). The molecular weight and other biophysical properties of bromegrass mosaic virus. Biophysical Journal 2, 1-9.

Boedtker, H. (1962). Configurational properties of tobacco mosaic virus ribonucleic acid. Journal of Molecular Biology 2, 171-188.

Bouck, G.B. and Brown, D.L. (1976). Self-assembly in development. Annual Review of Plant Physiology 27, 71-94.

Brady, J.N., Winston, V.D. and Consigli, R.A. (1977). Dissociation of polyoma virus by the chelation of calcium ions found associated with purified virions. Journal of Virology 23, 717-724.

Brakke, M.K. (1971). Degradation of brome mosaic and tobacco mosaic virus in bentonite. Virology 46, 575-585.

Brandts, J.F., Oliveira, R.J. and Westort, C. (1970). Thermodynamics of protein denaturation. Effect of pressure on the denaturation of ribonuclease. Biochemistry 9, 1038-1047.

Budzynski, A.Z. (1970). Circular dichroism of two TMV mutants with identical single amino acid replacement in different position in polypeptide chain. Biochemical and Biophysical Research Communications 41, 841-845.

Bull, H.B. and Breese, K. (1968). Temperature coefficients of protein partial volumes. The Journal of Physical Chemistry 72, 1817-1819.

Bull, H.B. and Breese, K. (1973). Temperature dependence of partial volumes of proteins. Biopolymers 12, 2351-2358.

Bush, C.A. and Scheraga, H.A. (1967). Optical rotatory dispersion and RNA base pairing in ribosomes and in tobacco mosaic virus. Biochemistry 6, 3036-3042.

Butler, P.J.G. and Durham, A.C.H. (1977). Tobacco mosaic virus protein aggregation and virus assembly. Advances in Protein Chemistry 31, 187-251.

Butler, P.J.G. and Klug, A. (1971). Assembly of the particle of tobacco mosaic virus from RNA and disks of protein. Nature New Biology 229, 47-50.

Butler, P.J.G., Finch, J.F. and Zimmern, P. (1977). Configuration of tobacco mosaic virus RNA during virus assembly. Nature, London 265, 217-219.

Casassa, E.F. and Eisenberg, H. (1964). Thermodynamic analysis of multicomponent solutions. Advances of Protein Chemistry 19, 287-395.

Casjens, S. and King, J. (1975). Virus assembly. Annual Review of Biochemistry 44, 555-611.

Caspar, D.L.D. (1963). Assembly and stability of the tobacco mosaic virus particle. Advances in Protein Chemistry 18, 37-121.

Caspar, D.L.D. (1966a). Design and assembly or organized biological structures. In: "Molecular Architecture in Cell Physiology", Symposium of the Society of General Physiologists, p. 191-207. Ed. by T. Hayashi and A.G. Szent-Györgyi. New York: Prentice-Hall.

Caspar, D.L.D. (1966b). Design principles in organized biological structures. In Ciba Foundation Symposium on Principles of Biomolecular Organization, 7-39.

Caspar, D.L.D. and Klug, A. (1962). Physical principles in the construction of regular viruses. Cold Spring Harbor Symposia on Quantitative Biology 27, 1-24.

Cecil, R. and McPhee, J.R. (1959). The sulfur chemistry of proteins. Advances in Protein Chemistry 14, 255-389.

Champness, J.N., Bloomer, A.C., Bricogne, C., Butler, P.J.G. and Klug, A. (1976). The structure of the protein disk of tobacco mosaic virus to 5 Å resolution. Nature, London 259, 20-24.

Chauvin, C., Pfeiffer, P., Witz, J. and Jacrot, B. (1978). The structural polymorphism of bromegrass mosaic virus: a neutron small angle scattering investigation. Virology 88, 138-148.

Chen, Y.H., Yang, J.T. and Martinez, H.M. (1972). Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. Biochemistry 11, 4120-4131.

Chothia, C. (1974). Hydrophobic bonding and accessible surface area in proteins. Nature, London 248, 338-339.

Chothia, C. (1975). Structural invariants in protein folding. Nature, London 254, 304-308.

Chothia, C. and Janin, J. (1975). Principles of protein-protein recognition. Nature, London 256. 705-708.

Chroboczek, J., Peitrzak, M. and Zagórski, W. (1973). Specificity of formation of complexes between coat protein and bacteriophage f_2 RNA. Journal of Virology 12, 230-240.

Chroboczek, J. and Zagórski, W. (1975). Hexamer of bacteriophage f_2 coat protein as a repressor of bacteriophage RNA polymerase synthesis. Journal of Virology 16, 228-236.

Cox, R.A. and Hirst, W. (1976). A study of the influence of magnesium ions on the conformation of ribosomal ribonucleic acid and on the stability of the larger subribosomal particles of rabbit reticulosystes. Biochemical Journal 160, 505-519.

Crane, H.R. (1950). Principles and problems of biological growth. The Scientific Monthly 70, 376-389.

Crick, F.H.C. and Watson, J.D. (1956). Structure of small viruses. Nature, London 177, 473-475.

Crowther, R.A. and Klug, A. (1975). Structural analysis of macromolecular assemblies by image reconstruction from electron micrographs. Annual Review of Biochemistry 44, 161-182.

Crowther, R.A., Amos, L.A. and Finch, J.T. (1975). Three-dimensional image reconstruction of bacteriophages R_{17} and f_2. Journal of Molecular Biology 98, 631-635.

Dawson, W.O. (1978). Time-course of cowpea chlorotic mottle virus RNA replication. Intervirology 9, 119-128.

Dawson, W.O. and Kuhn, C.W. (1974). Kinetics of multiplication, inactivation, and particle-breakdown of cowpea chlorotic mottle virus in cowpea. Phytopathology 64, 951-957.

De Wit, J.L. (1978). Nuclear magnetic resonance of tobacco mosaic virus. PhD Thesis, Agricultural University, Wageningen, The Netherlands.

Dodds, J.A. and Hamilton, R.I. (1974). Masking of the RNA genome of tobacco mosaic virus by the protein of barley stripe mosaic virus in doubly infected barley. Virology 59, 418-427.

Dickerson, P.E. and Trim, A.R. (1978). Conformational states of cowpea chlorotic mottle virus ribonucleic acid components. Nucleic Acid Research 5, 987-998.

Driedonks, R.A. (1978). A study on the assembly of alfalfa mosaic virus. PhD Thesis, University of Leiden, Leiden, The Netherlands.

Dunker, A.K. (1974). Structure of isometric viruses containing non identical polypeptide chains. Journal of Virology 14, 878-885.

Durham, A.C.H. and Abou Haidar, M. (1977). Cation binding by tobacco rattle virus. Virology 77, 520-523.

Eisenberg, D. and Kauzmann, W. (1969). The structure and properties of water. Oxford: Oxford University Press.

Eiserling, F.A. and Dickson, R.C. (1972).Assembly of viruses. Annual Review of Biochemistry 41, 467-502.

Favre Bonvin, G., Breysse, C., Wallach, J., Ville, A. and Hanss, M. (1974). Interactions entre les cations alcalins et les rRNA de foie de rat. Biochimie 56, 1197-1201.

Fenner, F. (1976). Classification and nomenclature of viruses. Intervirology 7, 1-115.

Finch, J.T. and Bancroft, J.B. (1968). Structure of the reaggregated protein shells of two spherical viruses. Nature, London 220, 815-816.

Fishbach, F.A. and Anderegg, J.W. (1976). Comparisons of the low resolution structure of several small RNA-containing viruses. Biochimica et biophysica acta 432, 404-408.

Fowlks, E. and Young, R.J. (1970). Detection of heterogeneity in plant viral RNA by polyacrylamide gel electrophoresis. Virology 42, 548-550.

Fraenkel-Conrat, H. (1957). Degradation of tobacco mosaic virus with acetic acid. Virology 4, 1-4.

Fraenkel-Conrat, H. (1970). Reconstitution of viruses. Annual Review of Microbiology 24, 463-478.

Fraenkel-Conrat, H. and Williams, R.C. (1955). Reconstitution of active tobacco mosaic virus from the inactive protein an nucleic acid components. Proceedings of the National Academy of Sciences of the United States of America 41, 690-698.

Freese, E. and Freese, E.B. (1966). Mutagenic and inactivating DNA alterations. Radiation Research Supplement 6, 97-140.

Fulton, R.W. (1975). Inheritance and recombination of strain specific characters in tobacco streak virus. Virology 50, 810-820.

Gainer, J.H., Long, jr., J., Hill, P. and Capps, W.I. (1971). Inactivation of the Pseudorabies virus by dithiothreitol. Virology 45, 91-100.

Geelen, J.L.M.C., Van Kammen, A. and Verduin, B.J.M. (1972). Structure of the capsid of cowpea mosaic virus. The chemical subunit, molecular weight and number of subunits per particle. Virology 44, 205-213.

Gibbs, A.J. (1972). Broad bean mottle virus. CMI/AAB Descriptions of plant viruses. No. 101.

Goodman, R.M. and Ross, A.F. (1974). Enhancement by potato virus Y of potato virus X synthesis in doubly infected tobacco depends on the timing of invasion by the viruses. Virology 58, 263-271.

Gordon, J.A. (1965). Stabilizing effectiveness of alkali metals toward ribonucleic acid from the bacterial virus R_{17}. Biopolymers 3, 5-14.

Gratzer, W.B. and Richards, E.G. (1971). Evaluation of RNA conformation from circular dichroism and optical rotatory dispersion data. Biopolymers 10, 2607-2614.

Harrison, B.D., Finch, J.T., Gibbs, A.J., Hollings, M., Shepherd, R.J., Valenta, V. and Wetter, C. (1971). Sixteen groups of plant viruses. Virology 45, 356-363.

Harrison, S.C. (1978). Structure of simple viruses: specificity and flexibility in protein assemblies. Trends in Biochemical Sciences 3, 3-7.

Harrison, S.C. and Jack, A. (1975). Structure of tomato bushy stunt virus. III. Three-dimensional X-ray diffraction analysis at 16 Å resolution. Journal of Molecular Biology 97, 173-191.

Harrison, S.C., Jack, A., Goodenough, D. and Sefton, B.M. (1974). Structural studies of spherical viruses. Journal of supramolecular Structure 2, 486-495.

Hartman, K.A., McDonald-Ordzie, P.E., Kaper, J.M., Prescott, B. and Thomas, jr., G.J. (1978). Studies of virus structure by laser-Raman spectroscopy. Turnip yellow mosaic virus and capsids. Biochemistry 17, 2118-2124.

Heijtink, R.A., Houwing, C.J. and Jaspars, E.M.J. (1977). Molecular weights of particles and RNAs of alfalfa mosaic virus. Number of subunits in protein capsids. Biochemistry 16, 4684-4693.

Henkens, R.W. and Middlebrook, J.L. (1973). Optical and hydrodynamic studies of the structure of bacteriophage f_2. Biochemistry 12, 2910-2915.

Herrmann, R., Schubert, D. and Rudolph, U. (1968). Self-assembly of protein subunits from bacteriophage f_r. Biochemical Biophysical Research Communications 30, 576-581.

Hershko, H. and Fry, M. (1975). Post-translational cleavage of polypeptide chains: role in assembly. Annual Review of Biochemistry 44, 775-797.

Herzog, M. and Hirth, L. (1978). In vitro encapsidation of the four RNA species of brome mosaic virus. Virology 86, 48-56.

Herzog, M., Pfeiffer, P. and Hirth, L. (1976). Pathways of degradation of brome mosaic virus particles as influenced by their RNA content. Virology 69, 394-407.

Hiebert, E. (1969). A study of the self-assembly process in vitro of three spherical plant viruses. PhD Thesis, Purdue University, Lafayette, USA.

Hiebert, E. and Bancroft, J.B. (1969). Factors effecting the assembly of some spherical viruses. Virology 39, 296-311.

Hiebert, E., Bancroft, J.B. and Bracker, C.E. (1968). The assembly in vitro of some small spherical viruses, hybrid viruses and other nucleoproteins. Virology 34, 492-508.

Hindley, J. (1973). Structure and strategy in phage RNA. Progress in Biophysics and Molecular Biology 26, 271-321.

Hohn, T. (1967). Selfassembly of defective particles of the bacteriophage f_r. European Journal of Biochemistry 2, 152-155.

Hohn, T. (1969a). Role of RNA in the assembly process of phage f_r. Journal of Molecular Biology 43, 191-200.

Hohn, T. (1969b). Studies on a possible precursor in the self-assembly of the bacteriophage f_r. European Journal of Biochemistry 8, 552-556.

Hohn, T. and Hohn, B. (1970). Structure and assembly of simple RNA bacteriophages. Advances in Virus Research 16, 43-98.

Holmes, K.C., Stubbs, G.J., Mandelkow, E. and Gallwitz, U. (1975). Structure of tobacco mosaic virus at 6.7 Å resolution. Nature, London 254, 192-196.

Horne, R.W. and Wildy, P. (1961). Symmetry in virus architecture. Virology 15, 348-373.

Howlett, G.J. and Nichol, L.W. (1972). An examination of the overspeeding technique in relation to sedimentation equilibrium. Journal of Physical Chemistry 76, 2740-2743.

Hsu, C.H., Sehgal, O.P. and Pickett, E.E. (1976). Stabilizing effect of divalent metal ions on virions of southern bean mosaic virus. Virology, 69, 587-595.

Huber, R. (1976). Antibody structure. Trends in Biochemical Sciences 1, 174-178.

Hull, R. (1972). The multicomponent nature of broad bean mottle virus and its nucleic acid. Journal of General Virology 17, 111-117.

Hung, P.P. and Overby, L.R. (1969). The reconstitution of infective bacteriophage Qβ. Biochemistry 8, 820-828.

Hunter, M.J. (1966). A method for the determination of protein partial specific volumes. The Journal of Physical Chemistry 70, 3285-3292.

Huxley, P.A. and Summerfield, R.J. (1976). Photomorphogenetic effects of lamp type on growth of some species of tropical grain legumes in controlled environment growth cabinets. Plant Science Letters 6, 25-33.

Hvidt, A. (1975). A discussion of pressure-volume effects in aqueous protein solutions. Journal of Theoretical Biology 50, 245-252.

Ifft, J.B., Voet, D.H. and Vinograd, J. (1961). The determination of density distributions and density gradients in binary solutions at equilibrium in the ultracentrifuge. Journal of Physical Chemistry 65, 1138-1145.

Incardona, N.L. (1968). Alkaline degradation of bromegrass mosaic virus. Biophysical Journal 8, A-69.

Incardona, N.L. and Kaesberg, P. (1964). A pH-induced structural change in bromegrass mosaic virus. Biophysical Journal 4, 11-21.

Incardona, N.L., McKee, S. and Flanegan, J.B. (1973). Noncovalent interactions in viruses: Characterization of their role in the pH and thermally induced conformational changes in brome mosaic virus. Virology 53, 204-214.

Isenberg, H., Cotter, R.I. and Gratzer, W.B. (1971). Secondary structure and interaction of RNA and protein in a bacteriophage. Biochimica et biophysica acta 232, 184-191.

Jack, A., Harrison, S.C. and Crowther, R.A. (1975). Structure of tomato bushy stunt virus. II. Comparison of results obtained by electron microscopy and X-ray diffraction. Journal of Molecular Biology 97, 163-172.

Jacrot, B. (1975). Studies on the assembly of a spherical plant virus. IV. The mechanism of protein aggregation and virus swelling. Journal of Molecular Biology 95, 433-446.

Jacrot, B., Chauvin, C. and Witz, J. (1977). Comparative neutron small-angle scattering study of small spherical RNA viruses. Nature, London 266, 417-421.

Jacrot, B., Pfeiffer, P. and Witz, J. (1976). The structure of a spherical plant virus (bromegrass mosaic virus) established by neutron diffraction. Philosophical Transactions of the Royal Society, London B 276, 109-112.

Jaenicke, R. (1971). Volume changes in the isoelectric heat aggregation of serum albumin. European Journal of Biochemistry 21, 110-115.

Jirgensons, B. (1973). Optical activity of proteins and other macromolecules. Berlin: Springer Verlag.

Johnson, M.W. (1973). Interpretation of Schlieren density gradient photographs. Journal of General Virology 18, 207-209.

Johnson, M.W., Wagner, G.W. and Bancroft, J.B. (1973). A titrimetic and electrophoresis study of cowpea chlorotic mottle virus and its protein. Journal of General Virology 19, 263-273.

Kaper, J.M. (1960). Preparation and characterization of artificial top component from turnip yellow mosaic virus. Journal of Molecular Biology 2, 425-433.

Kaper, J.M. (1972). Experimental analysis of the stabilizing interactions of simple RNA viruses: a systematic approach. In: RNA viruses: replication and structure. FEBS symposia Vol. 27, 19-41. Ed. by E.M.J. Jaspars and A. van Kammen. Amsterdam North-Holland/American Elsevier.

Karpel, R.L., Miller, N.S., Lesk, A.M. and Fresco, J.R. (1975). Stabilization of the native tertiary structure of yeast $tRNA^{Leu}_{3}$ by cationic metal complexes. Journal of Molecular Biology 97, 519-532.

Katz, S. and Miller, J.E. (1971). Structure-volume relationships for proteins. Comparative dilatometric study of acid-base reactions of lysozyme and ovalbumin in water and denaturing media. Biochemistry 10, 3569-3574.

Katz, S. and Miller, J.E. (1972). Medium effects of urea and guanidine hydrochloride on the volume changes produced by protonation of carboxylate groups. Journal of Physical Chemistry 76, 2778-2780.

Kauzmann, W. (1959). Some factors in the interpretation of protein denaturation. Advances in Protein Chemistry XIV, 1-63.

Kay, C.M., Colter, J.S. and Oikawa, K. (1970). Circular dichroism studies on mengo virus variants and their constituent ribonucleates. Canadian Journal of Biochemistry 48, 940-943.

Kellenberger, E. (1972). Assembly in biological systems. In Ciba Foundation Symposium on Polymerization in Biological Systems, 189-202.

Kim, K.S. (1977). An ultrastructural study of inclusions and disease development in plant cells infected by cowpea chlorotic mottle virus. Journal of General Virology 35, 535-543.

Kirby, K.S. (1965). Isolation and characterization of ribosomal ribonucleic acid. Biochemical Journal 96, 266-269.

Klein, M. (1945). The mechanism of the virucidal action of ascorbic acid. Science, New York 101, 587-589.

Klotz, I.M. (1970). Comparison of molecular structures of proteins: helix content; distributions of apolar residues. Archives of Biochemistry and Biophysics 138, 704-706.

Klug, A. (1967). The design of self-assembling systems of equal units. In Formation and fate of cell organelles. Ed. by K.B. Warren. Symposia of the International Society for Cell Biology 6, 1-18.

Klug, A. and Caspar, D.L.D. (1960). The structure of small viruses. Advances in Virus Research 7, 225-325.

Knolle, P. and Hohn, T. (1975). Morphogenesis of RNA phages. In RNA phages, 147-201. Ed. by N.D. Zinder. Cold Spring Harbor, Cold Spring Harbor Laboratory.

Krahn, P.M., O'Callaghan, R.J. and Paranchych. (1972). Stages in phage R_{17} infection. VI. Infection of A protein and RNA into the host cell. Virology 47, 628-637.

Kratky, O., Leopold, H_6 and Stabinger, H_3 (1969). Dichtemessungen an Flüssigkeiten und Gasen auf 10^{-6} g/cm^3 bei 0,6 cm^3 Präparatvolumen. Zeitschrift für angewandte Physik 27, 273-277.

Krüse, J., Verduin, B.J.M. and Visser, A.J.W.G. Intrinsic fluorescence of cowpea chlorotic mottle virus protein. In manuscript.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, London 227, 680-685.

Lane, L.C. (1974). The bromoviruses. Advances in Virus Research 19, 151-220.

Lane, L.C. and Kaesberg, P. (1971). Multiple genetic components in bromegrass mosaic virus. Nature New Biology 232, 40-43.

Latt, S.A. and Sober, H.A. (1967). Protein-nucleic acid interactions. III. Cation effect on binding strength and specificity. Biochemistry 6, 3307-3314.

Lauffer, M.A. (1975). Entropy-Driven Processes in Biology. Berlin, Springer Verlag.

Leberman, R. (1968). The disaggregation and assembly of simple viruses. In The molecular biology of viruses. Symposium of the Society of General Microbiology 18, 183-205.

Lebeurier, G., Nicolaieff, A. and Richards, K.E. (1977). Inside-out model for self-assembly of tobacco mosaic virus. Proceedings of the National Academy Science of the United States of America 74, 149-153.

Lecanidon, R. and Richards, E.G. (1975). The thermodynamics and kinetics of conformational changes in 5-S RNA from Escherichia coli. European Journal of Biochemistry 57, 127-133.

Lee, J.C. and Timasheff, S.N. (1974). Partial specific volumes and interactions with solvent components of proteins inguanidine hydrochloride. Biochemistry 13, 257-265.

Lewin, S. (1974). Displacement of water and its control of biochemical reactions. London, Academic Press.

Lim, V.I. (1978). Polypeptide chain folding through a highly helical intermediate as a general principle of globular protein structure formation. FEBS Letters 89, 10-14.

Loening, U.E. (1967). The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel-electrophoresis. Biochemical Journal 102, 251-257.

Loening, U.E. (1969). The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel-electrophoresis. Biochemical Journal 113, 131-138.

Lot, H., Jonard, G. and Richards, K. (1977). Cucumber mosaic virus RNA 5. Partial characterization and evidence for no large sequence homologies with genomic RNA 5. Fingerprint of CMV-S CARNA 5. FEBS Letters 80, 395-400.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

Maestre, M.F. and Tinoco, jr., I. (1967). Optical rotatory dispersion of viruses. Journal of Molecular Biology 23, 323-335.

Markham, R. and Smith, K.M. (1949). Studies on the virus of turnip yellow mosaic. Parasitology 39, 330-342.

Marvin, D.A. and Wachtel, E.J. (1975). Structure and assembly of filamentous bacterial viruses. Nature, London 253, 19-23.

Matthews, K.S. and Cole, R.D. (1972a). Shell formation by capsid protein f_2 bacteriophage. Journal of Molecular Biology 65, 1-15.

Matthews, K.S. and Cole, R.D. (1972b). Effects of chemical modification of f_2 viral coat protein on its shell forming activity. Journal of Molecular Biology 65, 17-23.

McCammon, J.A., Deutch, J.M. and Felderhof, B.U. (1975). Frictional properties of multisubunit structures. Biopolymers 14, 2613-2623.

McCord, J.M. and Fridovich, J. (1969). The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interactions of sulfite, dimethyl sulfoxide and oxygen. Journal of Biological Chemistry 244, 6056-6063.

McMullen, D.W., Jaskunas, S.R. and Tinoco, jr., I. (1967). Application of matrix rank analysis to the optical rotatory dispersion of TMV RNA. Biopolymers 5, 589-613.

Michalski, F., Parks, N.F., Sokol, F. and Clark, H.F. (1976). Thermal inactivation of rabies and other rhabdoviruses: stabilization by the chelating agent ethylenediaminetetraacetic acid at physiological temperatures. Infection and Immunity 14, 135-143.

Murata, A. and Kitagawa, K. (1973a). Mechanism of inactivation of bacteriophage J_1 by ascorbic acid. Agricultural and Biological Chemistry 37, 1145-1151.

Murata, A. and Kitagawa, K. (1973b). Mechanism of inactivation of bacteriophage J_1 by dithiothreitol, cysteine, mercaptoethanol, or thioglycollate. Agricultural and Biological Chemistry 37, 2159-2165.

Murata, A., Kitagawa, K. and Saruno, R. (1971). Inactivation of bacteriophages by ascorbic acid. Agricultural and Biological Chemistry 35, 294-296.

Murata, A., Kitagawa, K., Inmaru, H. and Saruno, R. (1972a). Inactivation of bacteriophage by thiol reducing agents. Agricultural and Biological Chemistry 36, 1065-1067.

Murata, A., Kitagawa, K., Inmaru, H. and Saruno, R. (1972b). Inactivation of single-stranded DNA and RNA phage by ascorbic acids and thiol reducing agents. Agricultural and Biological Chemistry 36, 2597-2599.

Murata, A., Kitagawa, K., Otokonari, K. and Saruno, R. (1973). Mechanism of inactivation of bacteriophage J_1 by glutathione. Agricultural and Biological Chemistry 37, 1707-1712.

Neal, W.M. and Florini, J.R. (1972). Effect of sucrose gradient composition on resolution of RNA-species. Analytical Biochemistry 45, 271-276.

Nickerson, K.W. and Lane, L. (1977). Polyamine content of several RNA plant viruses. Virology 81, 455-459.

Noguchi, H., Arya, S.K. and Yang, J.T. (1971). Volume changes accompanying the complex formation of polyadenylic and polyuridylic acids. Biopolymers 10, 2491-2498.

O'Callaghan, R., Bradley, R. and Paranchych, W. (1973). Controlled alterations in the physical and biological properties of R_{17} bacteriophage induced by guanidine hydrochloride. Virology 54, 476-494.

Ohno, T., Takahashi, M. and Okada, Y. (1977). Assembly of tobacco mosaic virus in vitro: elongation of partially reconstituted RNA. Proceedings of the National Academy of Sciences of the United States of America 74, 552-555.

Ohno, T., Sumita, M. and Okada, Y. (1977). Location of the initiation site on tobacco mosaic virus RNA insolved in assembly of the virus in vitro. Virology 78, 407-414.

Oriel, P.J. and Koenig, J.A. (1968). The optical rotatory dispersion of MS_2 bacteriophage. Archives of Biochemistry and Biophysics 127, 274-282.

Oriel, P.J., Lindsey, P. and Schueneman, C. (1971). Conformational changes in the reassembly of MS_2 protein subunits. Biopolymers 10, 1661-1672.

Otsuki, Y., Takebe, I., Ohno, T., Fukuda, M. and Okada, Y. (1977). Reconstitution of tobacco mosaic virus rods occurs bidirectionally from an internal initiation region: Demonstration by electron microscopic serology. Proceedings of the National Academy of Sciences of the United States of America 74, 1913-1917.

Page, M.I. and Jencks, W.P. (1971). Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. Proceedings of the National Academy of Science of the United States of America 68, 1678-1683.

Paul, H.L. (1961). Physikalische und Chemische Untersuchungen am broad bean mottle-Virus. Zeitschrift für Naturforschung 16b, 786-791.

Paul, H.L. and Buchta, U. (1971).Molecular weights of plant virus protein subunits determined by the meniscus depletion method using solvents containing urea. Journal of General Virology 11, 11-16.

Pearce, T.C., Rowe, A.J. and Turnock, G. (1975). Determination of the molecular weights of RNAs by low-speed sedimentation equilibrium: 16 S ribosomal RNA as a model compound. Journal of Molecular Biology 97, 193-205.

Perry, R.P., La Torre, J., Kelly, D.E. and Greenberg, J.R. (1972). On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. Biochimica et biophysia acta 262, 220-226.

Pfeiffer, P. (1978). The structural polymorphism of bromegrass mosaic virus: I. Some effects of cation binding on bromegrass mosaic virus structure. Virology in press.

Pfeiffer, P. and Durham, A.C.H. (1977). The cation binding associated with structural transitions in bromegrass mosaic virus. Virology 81, 419-432.

Pfeiffer, P. and Hirth, L. (1974a). Formation of artificial top component from brome mosaic virus at high salt concentrations. Virology 58, 362-368.

Pfeiffer, P. and Hirth, L. (1974b). Aggregation states of brome mosaic virus protein. Virology 61, 160-167.

Pfeiffer, P. and Hirth, L. (1975). The effect of conformational changes in brome mosaic virus upon its sensitivity to trypsin, chymotrypsin and ribonuclease. FEBS Letters 56, 144-148.

Phillips, D.J. and Bobst, A.M. (1972). Circular dichroism melting studies on R_{17} phage RNA. Biochemical and Biophysical Research Communications 47, 150-156.

Piazzolla, P., Guantieri, V., Vovlas, C. and Tamburro, M. (1977). Circular dichroism studies of chicory yellow mottle virus. Journal of General Virology 37, 359-372.

Powell, C.A. (1975). The effects of cations on the alkaline dissociation of tobacco mosaic virus. Virology 64, 75-85.

Powell, J.T. and Richards, E.G. (1972). Specific effects of Li^+ on stacking equilibria in polynucleotides. Biochimica et biophysica acta 281, 145-151.

Privalov, P.L., Filimonov, V.V., Venkstern, T.V. and Bayer, A.A. (1975). A calorimetric investigation of $tRNA_1^{Val}$ melting. Journal of Molecular Biology 97, 279-288.

Rayment, I., Argos, P. and Johnson, J.E. (1977). Crystalline cowpea chlorotic mottle virus. Journal of Ultrastructural Research 61, 240-242.

Record, M.T., Lohman, T.M. and Dehaseth, P. (1976). Ion effects on ligand-nucleic acid interactions. Journal of Molecular Biology 107, 145-158.

Reijnders, L., Aalbers, A.M.J., Van Kammen, A. and Thuring, R.W.J. (1974). Molecular weights of plant viral RNAs determined by gel electrophoresis under denaturing conditions. Virology 60, 515-521.

Rice, R.H. (1974). Minor protein components in cowpea chlorotic mottle virus and satellite of tobacco necrosis virus. Virology 61, 249-255.

Robertz, J.W. and Steitz, J.A. (1967). The reconstitution of infective bacteriophage R_{17}. Proceedings of the National Academy of Sciences of the United States of America 58, 1416-1421.

Rochow, W.F. (1969). Chenopodium hybridum as a local lesion assay host for brome mosaic virus. Phytopathology 49, 126-130.

Rossmann, M.G. and Liljas, A. (1974). Recognition of structural domains in globular proteins. Journal of Molecular Biology 85, 177-181.

Rossmann, M.G., Smiley, I.E. and Wagner, M.A. (1973). Crystalline cowpea chlorotic mottle virus. Journal of Molecular Biology 74, 255-256.

Rueckert, R.R. (1976). On the structure and morphogenesis of picornaviruses. In "Comprehensive Virology". Ed. by H. Fraenkel-Conrat and R. Wagner, Vol. 6, 131-213. New York: Academic Press.

Saha, B.K. (1974). Cleavage of 23 S RNA in 50 S ribosomes into a 5'-terminal and a 3'-terminal fragment by pancreatic ribonuclease. Biochimica et Biophysica acta 353, 292-300.

Samejima, T., Hashizume, H., Imahori, K., Fujii, I. and Miura, K. (1968). Optical rotatory dispersion and circular dichroism of rice dwarf virus ribonucleic acid. Journal of Molecular Biology 34, 39-48.

Samuelson, G. and Kaesberg, P. (1970). An artificial top component of R_{17} bacteriophage. Journal of Molecular Biology 47, 87-91.

Sander, C. and Ts'O, P.O.P. (1971). Interaction of nucleic acids. VIII. Binding of magnesium ions by nucleic acids. Journal of Molecular Biology 55, 1-21.

Schachman, H.K. and Edelstein, J. (1966). Ultracentrifuge studies with absorption optics. IV. Molecular weight determinations at the microgram level. Biochemistry 5, 2681-2705.

Scholes, G., Ward, J.F. and Weiss, J. (1960). Mechanism of the radiation-induced degradation of nucleic acids. Journal of Molecular Biology 2, 379-391.

Schramm, G., Schumacher, G. and Zillig, W. (1955). Über die Struktur des Tabakmosaikvirus. III. Der Zerfall in alkalischer Lösung. Zeitschrift für Naturforschung 10b, 481-492.

Schubert, D. (1969). Conformation at changes during reversible depolymerization of the protein coat from bacteriophage fr. Biochemica et Biophysica acta 188, 147-154.

Schubert, D. and Krafczyk, B. (1969). Circular dichroism studies on the reversible depolymerization of tobacco mosaic virus protein. Biochimica et Biophysica acta 188, 155-157.

Semancik, J.S. (1966). Studies on electrophoretic heterogeneity in isometric plant viruses. Virology 30, 698-704.

Semancik, J.S. (1974). Detection of polyadenylic acid sequences in plant pathogenic RNAs. Virology 62, 288-291.

Seto, H., and Tomasz, A. (1976). Calcium-requiring step in the uptake of deoxyribonucleic acid molecules through the surface of competent pneumococci. Journal of Bacteriology 126, 1113-1118.

Shaw, J.G., (1977). Hydrogen-ion titration of potato virus X protein. Journal of General Virology 36, 357-360.

Shie, M., Dobrov, E.N., and Tikchonenko, T.I. (1978). A comparative study of the structure of tobacco mosaic virus and cucumber virus 4 by laser raman spectroscopy. Biochemical and Biophysical Research Communications 81, 907-914.

Siegel, A. (1971). Pseudovirious of Tobacco Mosaic Virus. Virology 46, 50-59.

Siegel, A. and Huber, R. (1970). Complementary regions to tobacco mosaic virus RNA in host DNA. Biochemical and Biophysical Research Communications 40, 350-355.

Simmons, N.S. and Blout, E.R. (1960). The structure of tobacco mosaic virus and its components: Ultraviolet optical rotatory dispension. Biophysical Journal 1, 55-62.

Spahr, P.F., Farber, M. and Gesteland, R.F. (1969). Binding site on R_{17} RNA for coat protein. Nature, London 222, 455-458.

Srinivasan, S. and Lauffer, M.A. (1970). Reconstruction of tobacco mosaic virus: Calorimetric and related studies. Biochemistry 9, 2173-2180.

Stauffer, H., Srinivasan, S. and Lauffer, M.A. (1970). Calorimetric studies on polymerization-depolymerization of tobacco mosaic protein. Biochemistry 9, 193-200.

Steitz, J.A. (1968a). Identification of the A-protein as a structural component of bacteriophage R_{17}. Journal of Molecular Biology 33, 923-936.

Steitz, J.A. (1968b). Isolation of the A-protein from bacteriophage R_{17}. Journal of Molecular Biology 33, 937-946.

Steitz, J.A. (1968c). A slowly sedimenting, infective form of bacteriophage R_{17}. Journal of Molecular Biology 33, 947-951.

Stevens, C.L. and Lauffer, M.A. (1965). Polymerization-depolymerization of tobacco mosaic virus protein. IV. The role of water. Biochemistry 4, 31-36.

Stubbs, G., Warren, S. and Holmes, K. (1977). Structure of RNA and RNA binding site in tobacco mosaic virus from 4-Å map calculated from X-ray fibre diagrams. Nature, London 267, 216-221.

Suck, D., Rayment, J., Johnson, J.E. and Rossmann, M.G. (1978). The structure of southern bean mosaic virus at 50Å resolution. Virology 85, 187-197.

Sugiyama, T. and Nakada, D. (1967). Control of translation of MS_2 RNA cistrons by MS_2 coat protein. Proceedings of the National Academy of Sciences of the United States of America 57, 1744-1750.

Sugiyama, T. and Nakada, D. (1968). Translational control of bacteriophage analysis of proteins synthesized in vitro. Journal of Molecular Biology 31, 431-440.

Sugiyama, T., Hebert, R.R., and Hartman, K.A. (1967). Ribonucleoprotein complexes formed between bacteriophage MS_2 RNA and MS_2 protein in vitro. Journal of Molecular Biology 25, 455-463.

Sun, S.F., Chang, T.S., and Del Rosario, N.O. (1974). Bovine serum albumin in lithium chloride solutions. Changes in conformation. International Journal of Peptine and Protein Research 6, 87-94.

Suzuki, K. and Taniguchi, Y. (1972). Effect of pressure on biopolymers and model systems. Symposia of the Society for experimental biology 26, 103-124.

Swanson, S.K., Sulkowski, E. and Manly, K.F. (1978). Hydrophobic binding site(s) on moloney-murine leukemia virus P30. Virology 85, 211-221.

Taliansky, M.E., Atabekova, T.I. and Atabekov, J.G. (1977). The formation of phenotypically mixed particles upon mixed assembly of some tobacco mosaic virus strains. Virology 76, 701-708.

Tanford, C. (1973). The hydrophobic effect: formation of micelles and biological membranes. New York: John Wiley and Sons.

Tinoco, I., jr., Borer, P.N., Dengler, B., Levine, M., Uhlenbeck, O.C., Crothers, D.M. and Gralla, J. (1973). Improved estimation of secondary structure in ribonucleic acids. Nature New Biology 246, 40-41.

Udenfriend, S., Clark, C.T., Axelrod, J. and Brodie, B.B. (1954). Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. Journal of Biological Chemistry 208, 731-739.

Van Hemmen, J.J. and Meuling, W.J.A. (1975). Inactivation of biologically active DNA by γ-ray-induced superoxide radicals and their dismutation products singlet molecular oxygen and hydrogen peroxide. Biochimica et Biophysica acta 402, 133-141.

Van Holde, K.E., Rossetti, G.P. and Dyson, R.D. (1969). Sedimentation equilibrium of low-molecular weight associating solutes. Annals of the New York Academy of Sciences, 279-293.

Van Kammen, A. (1967). Purification and properties of the components of cowpea mosaic virus. Virology 31, 633-642.

Van Vloten-Doting, L., Dingjan-Versteegh, A. and Jaspars, E.M.J. (1970). Three nucleoprotein components of alfalfa mosaic virus necessary for infectivity. Virology 40, 419-430.

Verduin, B.J.M. (1974). The preparation of CCMV-protein in connection with its association into a spherical particle. FEBS letters 45, 50-54.

Verduin, B.J.M. (1978a). Riversible change in the nucleoprotein composition of bromoviruses after multiplication in Chenopodium hybridum L. Journal of General Virology 38, 571-575.

Verduin, B.J.M. (1978b) Degradation of cowpea chlorotic mottle virus RNA in situ. Journal of General Virology 39, 131-147.

Verduin, B.J.M. and Bancroft, J.B. (1969). The infectivity of tobacco mosaic virus RNA in coat proteins from spherical viruses. Virology 37, 501-506.

Verduin, B.J.M. and Penners, G. Acid-base titrations of the bromoviruses and their isolated coat proteins. In manuscript.

Verduin, B.J.M., Van Dongen, W.H.A.M. and Krüse, K.M.(1978). The effect of age of infection on the properties of cowpea chlorotic mottle virus and comparison with quinone modified virus (Abstract). Acta Botanica Neerlandica 27, 150-151.

Verma, I.M., Kay, C.M. and Littauer, U.Z. (1971). Circular dischroism of mitochondrial ribosomal RNA from Trichoderma viride. FEBS Letters 12, 317-321.

Vogel, H.J. (Ed). (1977). Nucleic acid-protein recognition. London: Academic Press.

Von Hippel, P.H., and Schleich, T. (1969). The effects of neutral salts or macromolecules. In "Structure and stability of Biological Macromolecules". Ed. by S.N. Timasheff and G.D. Fasman. 417-574. New York: Dekker.

Wagner, G.W. and Bancroft, J.B. (1968). The self-assembly of spherical viruses with mixed coat proteins. Virology 34, 748-756.

Wagner, G.W. and Bancroft, J.B. (1971). The assembly of RNA-free mixed-coat capsids of small spherical viruses. Virology 45, 321-323.

Weber, L.A., Hickey, E.D., Nuss, D.L. and Baglioni, C. (1977). 5'-terminal 7-methyl-guanosine and mRNA function: Influence of potassium concentration on translation in vitro. Proceedings of the National Academy of Sciences of the United States of America 74, 3254-3258.

Wetlaufer, D.B. (1973). Nucleation, rapid folding, and globular intrachain regions in proteins. Proceedings of the National Academy of Sciences of the United States of America 70, 697-701.

Wickner, W. (1976). Asymmetric orientation of phage M_{13} coat protein in Escherichia coli cytoplasmic membranes and in synthetic lipid vesicles. Proceedings of the National Academy of Sciences of the United States of America 73, 1159-1163.

Wilson, T.M.A., Perham, R.N., Finch, J.T. and Butler, P.J.G. (1976). Polarity of the RNA in the tobacco mosaic virus particle and the direction of protein stripping in sodium dodeyl sulphate. FEBS Letters 64, 285-289.

Winkler, F.K., Schutt, C.E., Harrison, S.C. and Bricogne, G. (1977). Tomato bushy stunt virus at 5.5-Å resolution. Nature, London 265, 509-513.

Woods, T.L. and Agrios, G.N. (1974). Inhibitory effects of a polyphenol-polyphenol-oxidase system on the infectivity of cowpea chlorotic mottle virus ribonucleic acid. Phytopathology 64, 35-37.

Wyatt, S.D. and Kuhn, C.W. (1975). The isolation of highly infectious RNA from cowpea chlorotic mottle virus with low specific infectivity (Abstract). Proceedings of the American Phytopathological Society 2, 42-43.

Yagi, K. (1972). Degradation of nucleic acid constituents by free radical systems. Agricultural and Biological Chemistry 36, 1803-1807.

Yamazaki, H. and Kaesberg, P. (1963). Degradation of bromegrass mosaic virus with calcium chloride and the isolation of its protein and nucleic acid. Journal of Molecular Biology 7, 760-762.

Yamazaki, H., Bancroft, J. and Kaesberg, P. (1961). Biophysical studies of broad bean mottle virus. Proceedings of the National Academy of Sciences of the United States of America 47, 979-983.

Yphantis, D.A. and Roark, D.E. (1972). Equilibrium centrifugation of nonideal systems. Molecular weight moments for removing the effects of nonideality. Biochemistry 11, 2925-2934.

Zagórska, L., Chroboczek, J. and Zagórski, W. (1975). Template activity of complexes formed between bacteriophage f_2 RNA and coat protein. Journal of Virology 15, 509-514.

Zagórski, W., Filipowicz, W., Wodnar, A., Leonowicz, A., Zagórska, L. and Szafránski, O. (1972), The effect of magnesium-ion concentration on the translation of phage f_2 RNA in a cell-free system of Escherichia coli. European Journal of Biochemistry 25, 315-322.

Zelazo, P.O. and Haschemeyer, R.H. (1970). Spherical protein shell formation from an 11 S subunit of bacteriophage f_2. Science, New York 168, 1461-1462.

Zimmern, D. and Wilson, T.M.A. (1976). Location of the origin for viral reassembly on tobacco mosaic virus RNA and its relation to stable fragment. FEBS Letters 71, 294-298.

Zulauf, M. (1977). Swelling of brome mosaic virus as studied by intensity fluctuation spectroscopy. Journal of Molecular Biology 114, 259-266.

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CURRICULUM VITAE

Benedictus Jacobus Maria Verduin is geboren op 3 september 1946 te Heemskerk. In 1964 behaalde hij het HBS-B diploma aan het Pius-X College te Beverwijk. In datzelfde jaar begon hij zijn studie aan de Landbouwhogeschool te Wageningen.

Van april tot en met november 1968 verbleef hij in de Verenigde Staten, Purdue University, Lafayette en werkte met Dr. J.B. Bancroft aan de assemblage van TMV-RNA met de manteleiwitten van de bromovirussen. In januari 1971 behaalde hij het doctoraal examen in de studierichting Plantenziektenkunde (cum laude), met als hoofdvak de Virologie en als bijvakken de Biochemie (verzwaard) en de Kolloïdchemie.

Vanaf februari 1971 was hij elf maanden wetenschappelijk medewerker bij de Vakgroep Biochemie van de Landbouwhogeschool. Van 1 januari 1972 tot en met 31 december 1974 maakte hij deel uit van de Werkgemeenschap Eiwitten van de Stichting Scheikundig Onderzoek in Nederland in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek. Vanaf 1975 is hij als wetenschappelijk medewerker verbonden aan de vakgroep Virologie van de Landbouwhogeschool te Wageningen.

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