A TMV MUTANT WITH NONFUNCTIONAL COAT PROTEIN WHICH CAN REVERT TO A MUTANT WITH FUNCTIONAL PROTEIN

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Jeffrey John Hubert

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Don P. Bourque	April 2, 1974
Harriet R Nelson	April 2, 1974
Karen MacFadden	April 2, 1974
C. K. Eyrane	April 2 1974
R. L. Gilbertson	April 3, 1974

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ABSTRACT

A TMV mutant (PM6) with a nonfunctional coat protein was isolated after treatment of the U1 strain with nitrous acid. The protein was analyzed and comparison to U1 coat protein showed that the protein had an aspartic (or asparagine) to glycine and an alanine to threonine exchange in its primary structure; the alanine to threonine exchange was localized at position 105.

A new mutant (PM6R) which has a functional coat protein was isolated from plants infected with PM6. Analysis of PM6R coat protein showed that it contained the same alanine to threonine exchange at position 105 that was present in PM6 coat protein. In addition, it was observed that the glycine present in PM6 protein had been replaced by aspartic acid (or asparagine), restoring the aspartic acid residue which is normally found in U1 coat protein. Thus, PM6R is thought to have been derived from a low frequency reversion of a codon in the PM6 genome which is responsible for only one of the amino acid exchanges found in PM6 protein.

As a result of the conversion of PM6, with a nonfunctional coat protein, to PM6R with a functional coat protein, it was deduced that an aspartic acid residue found to be localized in tryptic peptide 6 of PM6 coat protein plays an important role in the aggregation and encapsidation properties of TMV coat protein. It is proposed that this aspartic acid residue possesses a carboxyl group that participates in one of two

carboxyl-carboxylate pairs as postulated by others and that controls TMV coat protein aggregation. A probable location for this aspartic acid residue was considered to be position 88 of the TMV coat protein.

Of the two amino acid exchanges found in PM6 coat protein, only the aspartic to glycine exchange is permissible by the presently proposed mechanism of base alteration in TMV RNA by nitrous acid. The second exchange in this protein i.e., alanine to threonine, cannot be rationalized by the action of nitrous acid on TMV RNA. Consequently, it is possible that PM6 may arise from a naturally occurring TMV strain in the U1 population which already possesses an alanine to threonine exchange in its coat protein. Nitrous acid could act on such a strain to produce the second amino acid exchange (i.e., aspartic to glycine) observed in the nonfunctional protein of PM6. The alternatives are that nitrous acid acts by some as yet unknown mechanism to produce the alanine to threonine exchange observed in the PM6 coat protein or that PM6 is present in the U1 population as a naturally occurring variant.

A unique aggregation form is suggested for PM6 coat protein *in vivo*. It is proposed that the protein forms wheel-like aggregates *in vivo* that are constructed of 2½ turn helices utilizing the Butler and Klug model for TMV coat protein aggregation and assembly.

INTRODUCTION AND LITERATURE REVIEW

Structure and Composition of TMV

Tobacco mosaic virus (TMV) was the first virus to be purified to crystalline form and subsequently it has been well characterized physically. The TMV particle (virion) is a very stable, rigid rod averaging 3000 Å in length and 150 Å in diameter with a central cylindrical hole measuring about 36 Å in diameter. The virion is composed of a ribonucleic acid (RNA) molecule (MW ca 2×10^6 daltons) that is covered by a protein coat composed of more than 2200 identical protein subunits (MW 17500 each). In the virus rods the subunits are arranged in a tightly packed, right-handed helical array (Finch, 1972) with 16 1/3 subunits per turn of the helix and each turn of the helix has a pitch of 23 degrees. The TMV RNA molecule is positioned on the inside of the protein cylinder formed by the subunits in a groove that follows the pitch of the helix. There are approximately 50 nucleotides per turn of the helix, or three nucleotides per protein subunit. The protein coat of the virus serves to protect the TMV RNA molecule, which is the infectious component of the virus, from inactivation and subsequent loss of infectivity. Each TMV RNA molecule is composed of approximately 6400 nucleotides and each TMV coat protein subunit is composed of 158 amino acid residues of known sequence. The original literature citations on the structure and composition of TMV are numerous and are available in review form (Lauffer and Stevens, 1968; Siegel and Wildman, 1960; Tsugita and Hirashima, 1972).

TMV Assembly

Polymerization of TMV Coat Protein

In vitro, TMV virions can be disassembled into their component parts, infectious TMV RNA and TMV coat protein subunits, and reassembled again from these components in an aqueous solution of the proper pH, temperature, and ionic strength (Fraenkel-Conrat and Williams, 1955). However, if no TMV RNA is present in the solution, the coat proteins can polymerize to form long, flexuous protein rods of variable lengths, but with a highly ordered quaternary structure that is dependent upon the conditions under which the polymerization occurs (Durham *et al.*, 1971; Durham and Klug, 1971; Durham and Klug, 1972). The manner in which protein rods and TMV virions are assembled are thought to be similar, and consequently both mechanisms have been extensively investigated and some excellent reviews are available on this subject (Klug and Caspar, 1960; Caspar, 1963; Lauffer and Stevens, 1968; Lauffer, 1971). Some of these reviews outline proposed aggregation states of the protein starting with monomers (a single coat protein subunit) and progressing to heptamers and higher aggregates. Some of these aggregates are postulated to play an important role in the assembly of TMV virions, but unfortunately, strong evidence as to exactly what role they play in the assembly process, if any, is severely lacking.

The subunit proteins in the rods described above can be found in two kinds of arrangements (Durham *et al.*, 1971; Durham and Klug, 1972). In low pH environments (pH 5.0 to 6.5), the protein forms rods in which the proteins are in a helical array identical to that found in the

virion i.e., with 16 1/3 coat protein subunits per turn of the helix (Franklin, 1955; Durham et al., 1971). At neutral pH conditions, the proteins are organized into protein rods that have a so-called stacked disk structure (Franklin and Commoner, 1955; Caspar, 1963; Durham et al., 1971; Durham, 1972a; Durham and Klug, 1972). Such rods are composed of protein disks stacked on top of each other. Each disk is currently postulated to contain two closed rings of 17 protein subunits each (Finch et al., 1966; Klug and Durham, 1971), although other work disagrees totally (Markham et al., 1963) or in part (Carpenter, 1970) with these findings. Protein rods have also been observed that have helical and stacked disk regions in the same rod (Carpenter, 1970).

The "Disk" Theory of Virion Assembly

It was first suggested by Caspar (1963) that an interrelationship existed between the stacked disk and helical forms of the protein rods and the assembly of TMV virions. The relationship was further investigated by Klug and his associates and culminated in the "disk" theory of TMV assembly (Butler and Klug, 1971; Butler, 1971). They propose that two rings of 17 subunits stacked on top of each other comprise a "disk" aggregate of 34 subunits that is the protein unit added to TMV RNA during the TMV assembly process. Butler and Klug (1971) use the term "disk" in their work to denote the 34 subunit aggregate as opposed to its use by other workers (Markham et al., 1963; Caspar, 1963; Carpenter, 1970) to denote a closed ring of 16 or 17 subunit proteins. To avoid confusion, the 34 subunit aggregate will hereafter be referred to as a double disk.

Double disks are constructed from coat protein subunits through a pathway of intermediate aggregates of increasing size beginning with a trimer (an aggregate of three subunits) and culminating in a double disk of 34 subunits (see Fig. 1, Durham et al., 1971; Durham and Klug, 1971; Durham, 1972a; Durham and Klug, 1972; Klug and Durham, 1971). The double disk is constructed as a unit, i.e. single disks of 17 subunits are not constructed individually and stacked on top of one another to form double disks but rather double disks are formed as an aggregate. Double disks can stack on top of each other to form protein rods with a stacked disk structure, or they can associate with TMV-RNA to form a virion (Durham et al., 1971; Butler and Klug, 1971).

To initiate virion formation, Butler and Klug (1971) propose that the subunits in the double disk interact with TMV RNA in such a manner that the double disk forms a helix which binds a special sequence of about 50 nucleotides at the 5' end of TMV RNA. Double disks are now added to this complex in a polar manner and each subunit in the most recently added double disk slips down in a sequential manner, at a point of dislocation created at the interface of the helix and the double disk (Harris, 1972), to enclose (encapsidate) the TMV RNA molecule (see Fig. 5, Butler and Klug, 1971; Butler, 1971). The addition of double disks to the growing point of the virion continues until the entire TMV RNA molecule is encapsidated.

The disk theory was recently amended by its originators to include limited growth of virions by the addition of single subunit proteins to those already in place on the virion (Butler, 1972; Butler and Klug, 1973; Butler and Finch, 1973). The amendment was to counter the arguments

of Ohno et al. (1972) and Okada and Ohno (1972) who contend that monomer addition is more important in virion growth than the disk theory proposes. Ohno et al. (1972) contend that a double disk initiates virion assembly, but that single subunits are added thereafter to complete the process. Richards and Williams (1972) claim that single subunits are used throughout the assembly process, but Butler and Klug (1972) interpret the experimental data of Richards and Williams (1972) as favoring their own theory of assembly.

Experimental Evidence for the Disk Theory

The disk theory of TMV assembly is strongly supported by experimental evidence that can be listed as follows: (1) Double disks are the predominant protein aggregate at pH conditions that are optimum for reconstitution of TMV (Klug and Durham, 1971). The addition of double disk units to TMV RNA under reconstitution conditions results in the rapid formation of TMV virions, whereas after the addition of smaller aggregates a protracted period of time is necessary before virion formation can be measured (Butler and Klug, 1971; Butler, 1972). Further, the time lag that occurs when small aggregates are added can be correlated with the time necessary for these aggregates to form double disks. Work in other laboratories seems to varify the importance of the double disk in the TMV assembly process (Thouvenel et al., 1971; Scheele and Schuster, 1971; Favre et al., 1972; Lonchampt et al., 1972; Rodionova et al., 1973); (2) Electron microscopy shows what appear to be double disks being added to the growing TMV virions or stacked disk protein rods (Butler and Klug, 1971; Durham and Finch, 1972). Electron microscope

studies of aggregation states show that double disks and short-stacked disk rods are the predominant aggregation state of TMV coat protein subunits under the narrow range of conditions at which TMV reconstitution occurs (Durham, 1972a); and (3) Short protein rods with a stacked disk structure can be converted to rods with a helical arrangement under the proper pH conditions due to slippage of the disks into helices which anneal to form helical rods (Durham and Finch, 1972). The reversal of this process i.e., the conversion of helical rods to the stacked disk form, does not occur by the same mechanisms, but rather by the dissolution of the helical rods into smaller aggregates which in turn form double disks and stacked-disk rods by the previously mentioned pathway of intermediate aggregation states.

Role of Carboxyl Groups in the Disk Theory

Caspar (1963) has implicated carboxyl groups on amino acid residues in the coat protein as crucial in the TMV assembly process. He showed that four carboxyl groups in each subunit protein are capable of binding two molecules of lead and he also predicted from titration experiments that the carboxyl groups belong to two pairs of aspartic acid residues. Butler and Durham (1972) have predicted the positions of these aspartic acid pairs by surveying the literature on TMV coat protein mutants to see which aspartic acid residues were never exchanged for other amino acids in the coat proteins of these mutants. By the process of elimination, they showed that three aspartic acid residues are always conserved in these proteins and that these residues are located at positions in the TMV coat protein that make it highly probable

that they belong to the two proposed aspartic acid pairs. The site of the fourth residue is unknown, but two alternatives have been proposed (Butler and Durham, 1972).

Klug and his associates have investigated the role carboxyl groups play in the disk theory of TMV assembly and subsequently postulated a switching mechanism (Butler et al., 1972; Butler, 1971) for the conversion of stacked disk protein rods to rods with a helical configuration. It is thought that at neutral pH the four carboxyl groups are charged and the rod is in a loose stacked disk configuration due to the electrostatic repulsion caused by the charged groups; but, when these groups are neutralized by protonation in a low pH environment, the disks in the rods can slip into helices which interlock to form compact protein rods in which the subunit proteins are all in a helical arrangement. This compact arrangement is possible because the repelling charges are no longer present. It is theorized that the presence of TMV RNA also acts to neutralize the charged carboxyl groups on the subunits, thus allowing them to slip out of the disk form and into the helical form and encapsidate a TMV RNA molecule as prescribed by the disk theory of TMV assembly.

TMV RNA thus acts as a biological switch whose presence allows the double disks of protein to encapsidate TMV RNA to form virions and whose absence prevents the double disks from being converted to a configuration (i.e.,helical rods) in which they are unavailable for the use in the TMV assembly process. Consequently, in vivo, helical protein rods are not favored because no means are available to neutralize the charges on the coat protein except the presence of TMV RNA. Therefore, protein rods with a helical configuration are considered to be an in vitro

phenomenon created in the laboratory by manipulating the charge on the carboxyl groups of the coat protein.

TMV Strains

Wild Type TMV Strains

Many biological variants or strains of TMV are known to exist in the wild type population (Hennig and Wittmann, 1972) but the most common form of TMV is the "Vulgare" or U1 strain of the virus (Siegel and Wildman, 1954). The U1 strain and other TMV strains can replicate in tobacco plants and in many other plant species. In the laboratory, TMV is most often grown in the common tobacco plant, Nicotiana tabacum L. var. Turkish Samsun. When such plants become infected with a wild type strain, they typically exhibit systemic symptoms on their leaves consisting of contrasting light and dark green areas known collectively as a mottle or mosaic. It is from these mosaic-like symptoms that the virus derives its name. Tobacco plants (N. tabacum L. var. Samsun NN; N. tabacum L. var. Xanthi-nc; and N. glutinosa L.) possessing the necrotic gene (NN gene) react differently to infection by forming localized virus infections on their leaf surfaces that appear as small, dark, necrotic spots known as "local lesions." In these infections, virus multiplication is considered to be initiated by a single infecting virus particle and all subsequent particles produced in that primary lesion are the progeny of the original infecting particle. Since each local lesion is initiated by a single virus particle, the number of lesions produced on a leaf surface are quantitatively related to the number of infectious virus particles present in the inoculum source. Other tobacco species (N.

tabacum L. var Java and N. sylvestris) are known that react differently to infection by different TMV strains i.e., either by systemic or by local lesion infections depending on the infecting strain (Hennig and Wittmann, 1972; Siegel and Wildman, 1954).

Induced Mutation of TMV by Nitrous Acid

After the primary structure of the TMV coat protein of the U1 strain was determined (Anderer et al., 1960; Tsugita et al., 1960; Funatsu et al., 1964; Anderer et al., 1965; Dayhoff, 1969; Nozu and Okada, 1970; Tsugita and Hirashima, 1972), amino acid analysis of the coat proteins of many other naturally occurring TMV strains yielded information about the biological variation within the TMV population (reviewed in Hennig and Wittmann, 1972). A means was found to induce TMV mutants from TMV strains when it was demonstrated that nitrous acid (HNO_2) was a potent mutagen of TMV (Mundry and Gierer, 1958; Gierer and Mundry, 1958), and that its mechanism of action on the TMV RNA molecule could be understood (Schuster and Schramm, 1958). Nitrous acid was shown to oxidatively deaminate the nucleic acid bases of infectious TMV RNA while leaving the phosphodiester linkages intact. If TMV RNA is treated with HNO_2, deaminations occur that result in the conversion of adenine to hypoxanthine (hypoxanthine pairs like guanine and is later converted to guanine when TMV RNA replication occurs), guanine to xanthine (xanthine has no counterpart in TMV RNA), and cytosine to uracil, although the rate of the latter's conversion is slower than the former two conversions (Schuster and Wilhelm, 1963). Nitrous acid similarly deaminates adenine and cytosine bases in the intact virus particle, but

at a slower rate when compared with free TMV RNA, while leaving the guanine residues unaltered (Schuster and Wilhelm, 1963). Vanderbilt and Tessman (1970) have demonstrated that in the bacteriophage S13, whose genome is single stranded DNA, adenine to guanine and thymine to cytosine conversions are possible, as well as the reversion of guanine and cytosine back to adenine and thymine respectively. Whether similar reversions in HNO_2-mutated TMV RNA bases can take place is not known, but the data accumulated on HNO_2 induced TMV mutants thus far would indicate that they cannot (Hennig and Wittmann, 1972).

Deaminations in the TMV RNA molecule can permanently change the genetic code words for amino acids in the molecule. For example, if cytosine (C) is deaminated and thus converted to uracil (U), it could change one of the code words for serine, UCU, to a code word for phenylalanine, UUU. Such changes may occur in any portion of the TMV RNA molecule and may result in amino acid exchanges in any protein translated from that molecule. These base exchanges may result in the inability of TMV RNA to successfully replicate itself because it is unable to produce the functional proteins necessary for its replicative process. However, if the base exchanges occur in that region of the TMV RNA molecule that codes for the coat protein subunit, it may result in one or more amino acids being exchanged for others in the coat protein subunit.

Nitrous Acid Induced TMV Mutants and the Genetic Code

Many HNO_2 mutants of TMV having changed coat proteins have been isolated and their coat proteins sequenced. In general, these data have been compatible with other data in verifying the non-overlapping nature

of the genetic code as well as the code words for some of the amino acids (Siegel, 1965; Hennig and Wittmann, 1972). Of the 35 HNO_2 mutants whose coat proteins were sequenced, 22 had one amino acid exchange, 12 had two exchanges and one had three exchanges (see Table 20-1 in Kado and Agrawal, 1972). Some of the amino acid replacements in these coat protein mutants could not be accounted for by HNO_2 induced RNA base exchanges and therefore some of these mutants may have been present in the starting material as naturally occurring variants that by chance were not inactivated, or else were more resistant to HNO_2 treatment, and consequently survived the treatment. At least one worker (Bawden, 1959) has argued that HNO_2 acts by inactivating the strains more susceptible to it, thus the more resistant HNO_2 strains are increased in relative number and are selected from the normal population during the screening process. This argument may have some validity, but when all the evidence was reviewed, Siegel (1965) concluded that HNO_2 acts as a powerful mutagenic agent. Experiments of Rappaport and Wildman (1962) further indicate that naturally occurring TMV variants are inactivated by HNO_2 at the same rate as the common strain.

Defective TMV Mutants

Isolation of Defective Mutants. When purified TMV is 99% inactivated by HNO_2 treatment and then inoculated onto a local lesion host plant and the lesions resulting from the TMV survivors are used as individual inoculum sources to infect other host plants, approximately one-third of the other plants fail to become infected. The failure of some of these other plants to become infected has become known as the

"miss" phenomenon (Siegel, 1965). Siegel et al. (1962) investigated the "miss" phenomenon by another method. TMV particles were treated with HNO_2 to .015 to 4.2% survival and inoculated at limit dilution into systemic responding host plants in the seedling stage. At these low dilutions, approximately 50% of the plants remained uninfected, and many of the remaining plants were infected by only one virus particle, as indicated by a single yellow primary infection site. Plants infected in this manner revealed two very slow-growing mutants (Siegel et al., 1962) that were difficult to transfer, except by special techniques (Sarkar, 1963).

Both of these slow-growing mutant strains were shown to have coat proteins that could not encapsidate TMV RNA and were termed "defective strains" PM1 and PM2 (Siegel et al., 1962; Parish and Zaitlin, 1966). Presumably, as a result of their nonfunctional coat proteins, their infectious principle is an unprotected TMV RNA molecule which is very labile during transfer from one host plant to another due to exposure to leaf cell ribonucleases. Coat protein from defective strains will hereafter be referred to as defective or nonfunctional protein.

Defective Mutants with Insoluble Coat Proteins. The first defective mutant, PM1, has an insoluble defective coat protein that is found in the 12,000g (20 min.) pellet of leaf homogenates of PM1 infected plants (Parish and Zaitlin, 1966). PM1 coat protein and the coat proteins of other mutants in this class were purified and were shown to have a number of amino acid replacements in their primary structure, with a general trend showing one less residue of aspartic acid (or asparagine) and one more residue of lysine when compared to the coat

protein of the common strain (Hariharasubramanian et al., 1973). Two of these mutants, PM1 and PM4 (Siegel et al., 1962; Hariharasubramanian et al., 1973), were obtained after HNO_2 treatment of U1, and the third, PM2I, was obtained from a subculture of PM2 (which engenders a soluble, but defective coat protein in its host plant) when PM2 is grown in a host plant at normal or elevated temperatures (Hariharsubramanian et al., 1973; Zaitlin, 1967; Hariharasubramanian and Zaitlin, 1968; Kassanis and Turner, 1972). The nature of the amino acid exchanges in PM1 and PM4 indicates they are not induced by HNO_2, but possibly were mutants derived from a defective strain with a soluble coat protein or were originally present in the inoculum. Consequently, the role HNO_2 plays in the selection of such mutants, if any, is presently uncertain. Although PM2I was obtained from cultures of PM2, amino acid exchanges in the PM2I coat protein are vastly different from those in the PM2 coat protein, suggesting that PM2I may not be a spontaneous mutant of PM2. The number of mutational events necessary to produce such a drastically changed protein make the hypothesis of spontaneous mutation for the origin of PM2I unlikely, although a similar parallel can be found in the seemingly spontaneous occurrence of TMV strains with multiple amino acid exchanges that arise from single lesion isolates of U1 (Bald, 1972 and personal communication of J. G. Bald to M. Zaitlin). Another interesting observation is that the U2 strain, whose coat protein is 26% different in sequence from the U1 coat protein (Hennig and Wittmann, 1972), was originally isolated from a culture of U1 and shown to make up 12.5% of the total extractable virus in this mixed culture (Singer et al., 1951; Siegel and Wildman, 1954). Whether U2 originally arose from U1 in this culture by multiple

spontaneous mutations over an extended period of time is unknown, but there is no doubt that U2 was a contaminant of this particular U1 culture. Consequently, a more restricted theory for the origin of PM2I might be that it is a contaminant of PM2 and that its growth is favored over PM2 growth at high temperatures. Evidence exists that a temperature sensitive strain, Ni 2519, contains about 0.5% wild type contaminates even after repeated attempts to remove the contaminates by serial transfer though local lesion hosts (Jockusch, 1968). Some evidence indicates these defective mutants may exist in the normal TMV population and that high temperatures may act in their selection during the screening process by favoring their growth while inhibiting that of wild type TMV (Hariharasubramanian and Zaitlin, 1968; Kapitsa et al., 1969a; Kapitsa et al., 1969b).

Defective Mutants with Soluble Coat Proteins. PM2, the other defective mutant isolated in the original study of defective mutants (Siegel et al., 1962), proved to have a defective but soluble coat protein that could be purified from the cytoplasmic fraction of leaf cell homogenates prepared from PM2 infected plants. Amino acid analyses of the protein showed threonine to isoleucine and glutamic to aspartic replacements in its primary structure at positions 28 and 95 respectively (Zaitlin and McCaughey, 1965; Wittmann, 1965) when compared to the U1 strain. One of these replacements, glutamic to aspartic, is not compatible with the presumed action of HNO_2 on TMV RNA bases and consequently may not be HNO_2 induced. Since two replacements exist in this protein, it is not known which amino acid exchange is responsible for its defectiveness, but the threonine to isoleucine exchange has been

observed in nondefective mutants previously (see Table I, Siegel, 1965; Table 20-1, Hennig and Wittmann, 1972). A second defective strain, PM5, with a soluble but defective coat protein was isolated by Hariharasubramanian and Siegel (1969); the PM5 coat protein showed an arginine to cysteine exchange at position 112 in its primary sequence. The coat protein subunits from these strains are capable of aggregating in an aberrant manner *in vivo* and *in vitro* to form protein rods similar to, but distince from wild type protein rods in addition to other unique structures (Zaitlin and Ferris, 1964; Siegel *et al.*, 1966).

Defective Mutants with Temperature Sensitive Coat Proteins. After HNO_2 treatment of the U1 strain, Hariharasubramanian *et al.* (1970) isolated another mutant, Ts-38, whose coat protein is capable of encapsidating TMV RNA to form infectious virions only when grown in a host plant at 23°C. When this mutant is grown at 35°C, its coat protein becomes insoluble and nonfunctional and therefore incapable of encapsidating TMV RNA. Both of the amino acid exchanges in this mutant, threonine to alanine and serine to phenylalanine at positions 81 and 143 respectively, can be rationalized by the action of HNO_2 on TMV and RNA. A mutant (Ni 118) having a coat protein with similar properties was isolated from the common strain at high temperature by Jokusch (1968) which has a proline to leucine exchange at position 20. Hariharasubramanian (1970) has also isolated a mutant (HT-4) from U1 at high temperatures whose coat protein is temperature sensitive. A critical amino acid exchange in the coat protein subunits of each of these three mutants presumably causes the polypeptide chain of the subunits to fold abnormally at elevated temperatures resulting in a perturbed teritiary structure and a nonfunctional

coat protein. This mechanism is in contrast to one postulated for the other defective mutants whose soluble or insoluble coat proteins are nonfunctional at all of the temperatures at which they were tested. The relationship of these temperature sensitive mutants to the permanently defective mutants previously discussed is uncertain, but they may occupy a position intermediate between the normally functioning coat protein of the common strain and the permanently nonfunctional coat proteins of the defective mutants.

"Reversion" of Defective Mutants. When certain defective mutants are grown in tobacco plants at elevated summer greenhouse temperatures, some of the plants eventually exhibited systemic symptoms of a U1 type infection and infectious TMV virions could be extracted from these plants. The careful cultural techniques used to propagate and maintain defective mutants in tobacco plants should preclude the possibility that these mutant infected plants had become contaminated with wild type TMV strains of external origin. Consequently, another reason was sought to explain the occurrence of wild type TMV in plants originally infected with only defective mutants. It was proposed that defective mutants might, by means of a reverse mutation, revert back to a mutant (termed a "revertant") whose coat protein is now functional and therefore capable of encapsidating TMV RNA to produce infectious virions. To test this hypothesis, PM5 infected tobacco plants were held at elevated temperatures in the greenhouse during the summer for various periods of time. A TMV isolate was obtained from each PM5 infected plant that exhibited U1 type symptoms during the course of the experiment, but when the coat protein of all such isolates was examined by amino acid analysis, all

isolates proved to have coat proteins identical in amino acid composition to U1 except one, and this exception was inadvertently lost (Zaitlin, 1968, personal communication).

Kapitsa et al. (1969c) have also observed that some tobacco plants infected with a defective TMV mutant, which engenders a nonfunctional insoluble coat protein in its host plant (Kapitsa et al., 1969a), develop U1 type symptoms and that intact TMV particles are in evidence in these plants. These workers have also suggested that these isolates are "revertants" but have not presented any coat protein sequence data to substantiate the assertion.

Goals of this Investigation

Of the preceding defective mutants discussed, only PM2 and PM5 have permanently nonfunctional, but soluble coat proteins that aggregate into protein rods that are similar to those of the wild type protein. These mutant proteins differ from the common coat protein by only one or two amino acid exchanges in their primary structure. Consequently, such proteins are suitable models to determine which amino acid residues of the TMV coat protein are crucial in allowing it to aggregate correctly and encapsidate TMV RNA. This dissertation seeks to further characterize those residues necessary for normal coat protein function by examining a new defective mutant, termed PM6, which engenders a soluble and permanently defective coat protein in its host plant that is capable of aggregating into several well-defined structures.

In an attempt to answer the question as to whether "revertants" exist or do not exist, this dissertation presents evidence from amino

acid analysis that one such "revertant" mutant, termed PM6R, is derived from the defective mutant PM6. It is proposed that PM6 undergoes a reverse mutation affecting a single amino acid residue in its coat protein which results in the formation of a new mutant (PM6R), whose coat protein is functional.

MATERIALS AND METHODS

PM6 Isolation and Propagation

PM6 was obtained upon treatment of the U1 strain with HNO_2 in the following manner (Siegel, 1960): Two parts of a purified U1 suspension (28 mg/ml) were added to 1 part of 4 M $NaNO_2$ and 1 part of 1 M acetate buffer at pH 4.0 and the mixture was allowed to reach for 3 hrs. Aliquots were withdrawn at zero time and at 1, 2, and 3 hrs. during the course of the reaction and then diluted 1:56,000, 1:5600, 1:560, and 1:56 respectively with ice cold 0.066M phosphate buffer (0.0402 M Na_2HPO_4 and 0.0257 M KH_2PO_4, pH 7.0) to stop the reaction. These aliquots were mixed with an abrasive (Celite at 50 mg/ml) and their infectivity was assayed on Xanthi-nc tobacco (N. tabacum var. Xanthi-nc) to determine the level of U1 survival. The aliquot showing the survival level closest to 0.1% was innoculated into a single leaf of each of 100 young Samsun tobacco (N. tabacum var. Samsun) seedlings (at the one or two expanded leaf stage) at a concentration estimated to infect one-half or fewer of the seedlings. Those plants showing symptoms of systemic infection within the first week i.e., 26 plants, were discarded and the remainder i.e., 64 plants, were held for further observation. Two weeks after infection, one of the remaining plants showed a small expanding yellow infection site typical of a defective mutant (Siegel et al., 1962). A portion of this site was cut out and tested serologically by the Ouchterlony double-diffusion test for the presence or absence of excess

TMV coat protein (Hariharasubramanian et al., 1970). In leaf tissue infected with a defective TMV mutant, free coat protein should be detectable. The serological test was strongly positive for excess coat protein, indicating that the isolate was probably a new defective mutant; this isolate was named PM6. The infection was then allowed to spread further and a portion was later removed and ground in a small volume (1 sq.cm. infected leaf tissue per ml) of ice cold TP buffer (0.05 M Tris, 0.05 M Na_2HPO_4, HCl to give pH 8.6, Sarkar, 1963) which had been heated to boiling. Celite was added at 50 mg/ml and the resulting homogenate was brushed onto the leaves of young Samsun tobacco plants to initiate infection. The plants were held at 23°C in a growth room to encourage rapid replication of the mutant.

PM6 was routinely transferred and propagated in this manner. Ten days after inoculation, the plants exhibited primary symptoms as evidenced by small yellow infection sites which expanded slowly, and 30-60 days after inoculation, the plants exhibited secondary symptoms typical of those caused by other defective mutants (Siegel et al., 1962; Hariharasubramanian and Siegel, 1969) in tobacco plants i.e., leaf stunting, distortion, rugosity, and mottling (Fig. 1). The spread of a defective virus infection in its host is slow, due presumably to poor translocation of its infectious RNA in host tissues. In contrast, wild type TMV strains that engender functional virus particles in their host plant are rapidly translocated to other tissues of the plant, which results in a nearly complete systemic infection of the host by these strains. The slow spread of PM6 infections in leaves is either regular, which results in an oak leaf pattern due to the yellowing of infected leaf tissue

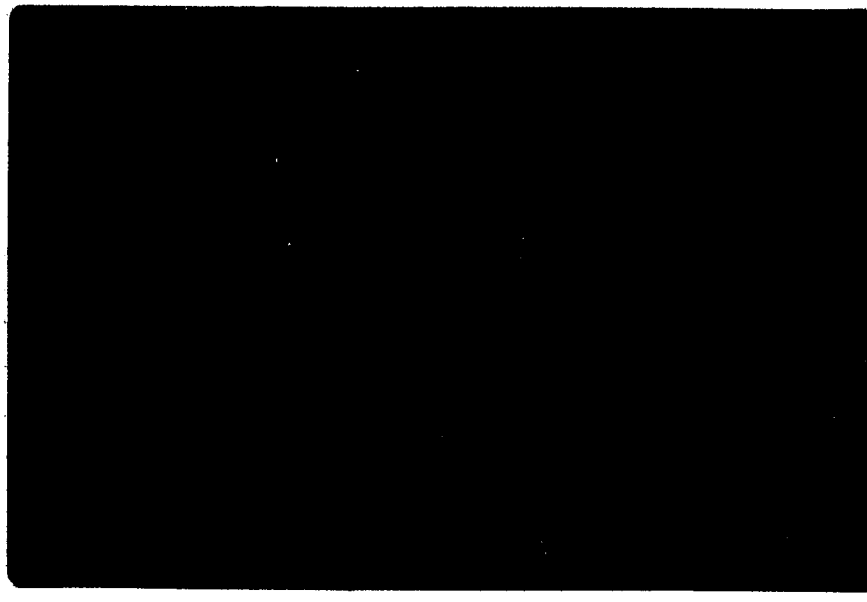

Fig. 1. Symptom diversity in PM6-infected Samsun tobacco plant leaves.

around leaf veins, or irregular, which results in large uninfected areas in the leaves. PM6 infrequently infects the expanding leaves at terminal growing points in tobacco plants in contrast to the rapid and complete infection of such leaves by wild type strains. When PM6 infections do occur in terminal leaves, they are usually of a spotty and localized nature and the majority of the terminal leaves remain uninfected.

Revertant Isolation and Propagation

Revertant mutants (PM6R) of PM6 were isolated in two separate screening experiments. Leaf RNA extracted from PM6 infected tobacco plants after Jackson et al. (1972) was used to infect young tobacco plants at the beginning of each experiment.

In the first screening experiment, one group of 51 PM6 infected plants was placed in a greenhouse where the highest daily summertime temperatures reached were 43-46°C; 15 uninfected tobacco plants were distributed throughout this group of 51 to monitor possible external or cross-contamination of the test plants. A second group of 5 PM6 infected plants was held at a constant 23°C in a growth room. All plants were observed frequently and when systemic symptoms appeared in the terminal leaves of any PM6 infected plant that were similar in appearance to the symptoms caused by a U1 infection (Fig. 2), that plant was removed from the group. A portion of one of the terminal leaves showing systemic symptoms was removed from the plant and ground in 0.066 M phosphate buffer with Celite added (50 mg/ml) and the resulting homogenate was brushed onto the leaves of Samsun tobacco plants to infect them, and the infected plants were placed under greenhouse conditions (26-30°C)

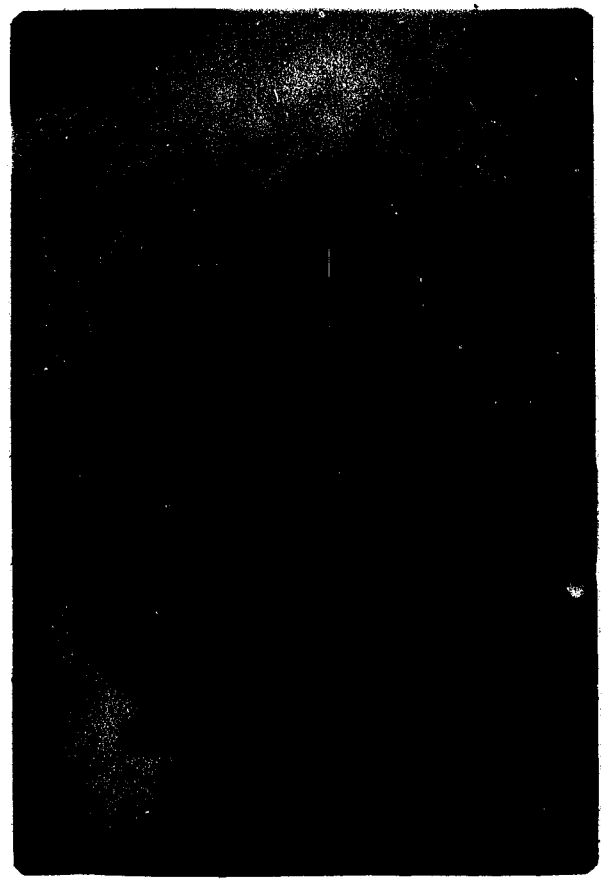

Fig. 2. Samsun tobacco plant showing symptoms of PM6R infection in terminal leaves.

to allow the virus to replicate. Revertant mutants obtained initially were transferred and propagated in this manner, but later when purified virus preparations of the revertant mutants were obtained, the virus was suspended at 1 mg/ml in 0.066 M phosphate buffer with Celite added (50 mg/ml) and then brushed onto Samsun tobacco plant leaves to initiate infection. Each revertant isolate obtained in this manner was given an isolation number of $PM6R_1$, $PM6R_2$, $PM6R_3$, etc.

At a later date, a second screening experiment was conducted that was identical to the first one except that only 45 PM6 infected plants were used in the first group and they were placed in a greenhouse during a period where the highest daily temperature reached was 26-30°C.

PM6 Isolation from Mixed Infections

In earlier studies (Siegel et al., 1962) when defective mutants were isolated, it was found that infectivity could not be recovered from necrotic local lesions formed by these mutants. Consequently when reversion to PM6R occurred in PM6 infected plants, no means were available to rescue PM6 from the mixed infection. This problem was overcome by developing the following technique that will transfer the infectivity in necrotic local lesions induced by PM6 and all other defective TMV mutants that have been isolated. Leaves with mixed PM6 and PM6R infections are ground in ice cold TP buffer and then the homogenate is brushed onto Xanthi-nc tobacco leaves to produce numerous necrotic local lesion infections. As soon as the lesions appeared (about 60 hrs. under our greenhouse conditions), single lesions were selected at random and cut out of the leaf. Each lesion was individually ground between two sterile

glass paddles in 2-3 drops of ice cold TP buffer and the grindate was rubbed immediately onto the leaves of young Samsun tobacco plants with the glass paddles. Plants infected in this manner have either PM6 infections or PM6R infections depending on the origin of the lesion serving as their inoculum source. Since the symptoms produced in Samsun tobacco plants by PM6 or PM6R infections are quite different (Figs. 1 and 3), the PM6 infected plants can easily be separated from the PM6R infected plants.

It is important to use local lesions caused by defective mutants as an inoculum source as soon as the lesions are visible on the leaf surfaces (i.e. one day old lesions) because the transferable infectivity in such lesions falls off rapidly shortly after their appearance (Table 1). The technique developed here for transferring local lesions induced by defective TMV mutants has been used by other workers (Hariharasubramanian et al., 1970) to screen for defective temperature sensitive TMV mutants. U1 was treated with HNO_2 to 0.1% survival and then rubbed onto Xanthi-nc tobacco plants and the resulting lesions were transferred individually to small Samsun tobacco plants as soon as they appeared by the method described above (J. J. Hubert, in Hariharasubramanian et al., 1970). The newly inoculated Samsun tobacco plants were then screened periodically for the appearance of defective mutants as described under the section on PM6 isolation and propagation.

Virus Purification

PM6 Mutant

The infectious principle of defective TMV mutants is a naked TMV RNA molecule (Siegel et al., 1962), consequently, PM6 was obtained as

Fig. 3. Samsun tobacco plant with mixed PM6 and PM6R infection.

Lower leaves show PM6 symptoms and terminal leaves show PM6R symptoms.

Table 1. Infectivity of PM4 induced necrotic local lesions[a] harvested at various times after their appearance.

	Lesion age in days[b]				
	0	1	2	3	4
Number of lesions[c]	3.7	3.6	0.4	1.3	0.3

a. Local lesions of various ages induced by inoculating Xanthi-nc tobacco plants with PM4 on five consecutive days were used as inoculum (as described in the materials and methods section) to test their infectivity on Xanthi-nc tobacco plants in half leaf experiments using a 5X5 greco latin square.

b. Lesion age is considered to begin as soon as necrotic local lesions appear i.e., at about 60 hours under our greenhouse conditions (0 days).

c. Average number of lesions per half leaf from 10 half leaves.

RNA from PM6-infected Samsun tobacco plant leaves as a portion of the total extractable leaf RNA in the following manner: PM6 infected leaves with advanced, high-titer infections were frozen with liquid nitrogen and then ground to a fine powder in a mortar and pestle. Three volumes of phenol, 3 volumes of 0.1 M TNE buffer (0.1 M Tris, 0.1 M NaCl, 0.1 M EDTA and HCl added to pH 7.0), and 0.5 ml of 20% sodium dodecyl sulfate per gram (fresh weight) of leaf tissue were added to the leaf powder. The entire mixture was thawed just enough to liquify the phenol and the resulting emulsion was agitated for a few minutes to facilitate phenol extraction, and then it was centrifuged for 10 min. at 4000 g to break the emulsion. The upper phenol phase was removed and discarded and the aqueous phase was extracted again with 3 volumes of phenol and then centrifuged as before, and the phases separated. Phenol extraction of the aqueous phase was continued until no denatured protein or green color was visible in the phenol phase. The RNA in the aqueous phase was then precipitated by the addition of 1½ volumes of isopropanol and 2 drops of 3 M sodium acetate buffer (3 M NaAc adjusted pH 4.0 with glacial HOAc) and the RNA precipitated was collected by centrifugation and then dissolved in a small volume (5-1 ml) of ice cold TP buffer and stored at -20°C. Aliquots of the frozen RNA preparations were assayed on Xanthi-nc tobacco plant leaves for infectivity and then diluted with TP buffer to obtain the desired infectivity (5-6 infection sites per leaf) prior to use as inoculum. PM6 RNA contained in this partially purified total leaf RNA extract was used to infect Samsun tobacco plants by brushing the RNA in ice cold TP buffer onto their leaf surfaces.

Revertant Mutant and U1 Strain

Virus was purified from tobacco plants infected with either revertant mutants or with the U1 strain by the method of Gooding and Hebert (1967) with some modifications. Leaves from infected tobacco plants that had been frozen and thawed 4-5 times were homogenized in the minimal amount of 0.066 M phosphate buffer necessary to effect homogenization in a Waring blender. The homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 12,000 g for 10 min. and the pellet discarded. To the supernatant polyethyleneglycol (MW 6000) was added to 4% and NaCl to 0.3 M and the mixture was stirred until the virus precipitated (about 3-5 min.). The precipitate of virus particles was collected by centrifuging the solution at 4,000 g for 10 min.; the resulting very soft virus pellets were resuspended in the minimal amount of 0.066 M phosphate buffer necessary to dissolve them. The virus solution was centrifuged at 17,000 g for 15 min. and the resulting pellets of contaminating material discarded. The supernatant containing the virus was subjected to one more cycle of precipitation, and purified as described above and then the virus was pelleted out of solution by centrifuging the suspension at 105,000 g for 1 hr. The resulting gelatinous pellets of virus were clear or slightly amber in color overlying a very small brown plug of contaminating material. The supernatant was discarded and the virus pellets were immediately dissolved in water then centrifuged at 12,000 g for 10 min. to remove the remaining contaminating material as a small black pellet. The concentration of the virus in the water suspension was determined spectrophotometrically by using the extinction coefficient for U1, $E^{1\%}_{260nm} = 3.0$

(1 cm light path), for 1 mg virus per ml. A typical yield was 4.5 mg virus from 1 gm of infected leaves (fresh weight).

Protein Purification

PM6 Coat Protein

All procedures in the purification process of PM6 coat protein were performed in the cold (5-15°C) to avoid denaturation of the protein. PM6 infected tobacco leaves were frozen in liquid nitrogen and then ground to a fine powder with a mortar and pestle. Phosphate buffer (0.066 M) was added (2 ml buffer/gm powdered leaf tissue) to the leaf powder and the resulting slurry was filtered through four layers of cheese cloth. The filtrate was clarified by centrifugation at 12,000 g for 10 min., KCl (0.1M final concentration) was added to the supernatant, and the pH of the solution was adjusted to 4.7 with HCl. The solution was then placed in the cold for 10 min. to allow the coat protein to aggregate, after which time the pH was readjusted to 4.7 with HCl. The solution was clarified by centrifugation at 12,000 g for 10 min. and the resulting supernatant was centrifuged at 105,000 g for 1 hr. to pellet the aggregated coat protein. The resulting clear to amber gelatinous pellet of coat protein was overlayed on a brown plug of contaminating material. To remove the contaminating material, the gelatinous pellet was resuspended immediately in 0.001 M NaOH and then the resulting solution was adjusted to pH 8.0 with HCl to dissociate the coat protein. The protein solution was then centrifuged at 12,000 g for 10 min. to remove contaminating material. The resulting pellet was discarded and the supernatant was then centrifuged at 105,000 g for one hr. and the remaining

contaminating material discarded as a small pellet. The concentration of coat protein in the supernatant solution was estimated spectrophotometrically by using the extinction coefficient for U1 coat protein, $E^{1\%}_{280nm} = 1.27$ (1 cm light path), for 1 mg coat protein per ml. The purified protein was stored at pH 8.0 in the cold or frozen. Yields averaged 0.5 mg coat protein per gm (fresh weight) leaf tissue.

Integral amino acid values obtained for the composition of a newly isolated protein are indicative that the process devised for the purification of the new protein has been successfully used to effect its purification. Consequently, to test the purity of the PM6 coat protein preparation using the above criteria, 1 mg of the protein was hydrolyzed and then analyzed on an amino acid analyzer (see p. 37) to see if integral amino acid values could be obtained for its composition. If the analysis showed the protein was not pure i.e., integral acid values were not obtained for its composition, the protein was recycled (usually only once or twice) through the entire purification procedure, beginning with the addition of KCl, until analysis showed that the values obtained from it were integral. When integral values obtained for the composition PM6 coat protein approached the known integral values for the composition of U1 coat protein to the extent that differences and similarities in the amino acid composition of PM6 coat protein could be detected when compared to the known amino acid composition of U1 coat protein, then the state of purification of the PM6 coat protein was considered to be satisfactory.

Revertant and U1 Coat Protein

The coat protein of revertant mutants and the U1 strain were prepared and purified by the method of Fraenkel-Conrat (1957) with some modifications. The coat protein was stripped from virus particles by adding one volume of a purified virus suspension in water to two volumes of cold glacial acetic acid and stirring the solution until it reached room temperature (about 10 min.). The denatured TMV RNA precipitated and was removed as a pellet by centrifuging the solution at 12,000 g for 10 min. The supernatant of coat protein and acetic acid (HOAc) was dialyzed against repeated changes of distilled water in the cold until the coat protein precipitated in the dialysis sack. The solution was then removed and centrifuged at 4,000 g for 10 min. to collect the protein precipitate as soft white pellets, which were resuspended in a minimal amount of 0.01 M NaOH. The pH of the protein solution was then adjusted to 7.8 with more NaOH. The solution was now centrifuged at 12,000 g for 10 min. to remove the majority of insoluble coat protein (i.e., that coat protein that was irreversibly denatured and consequently remained permanently insoluble) as a pellet and the resulting supernate was centrifuged at 105,000 g for 1 hr. to remove as a pellet any remaining coat protein and virus particles that were not stripped by the HOAc treatment. The concentration and the purity of the coat protein preparations were determined as previously described for PM6 coat protein. If the purity was insufficient, further purification was effected by aggregating the protein in solution by adjusting the pH of the solution to 6.1 and 4.7 for revertant and U1 coat protein respectively with NaOH, and then subjecting the solution to alternate low and high speed

centrifugation as prescribed for the purification of PM6 coat protein. The protein was recycled through this procedure repeatedly until analysis showed integral values for all amino acides.

Tryptic Digestion of Coat Protein

Coat protein (150 mg ca 5 mg/ml) in water adjusted to pH 8.0 with NaOH was digested with 1% trypsin-TPCK (Worthington Biochem. Corp., Freehold, N.J.) at 37°C for 2 hrs.; ammonium hydroxide (NH_4OH) was added during the digestion period to maintain the pH of the reaction mixture at 8.0 (Funatsu, 1964). Tryptic digestion of TMV coat protein results in the cleavage of protein into 12 discrete peptides that are numbered sequentially from 1 through 12 beginning with the N-terminal end of the protein (Funatsu, 1964). The reaction was terminated by adjusting the pH of the mixture to 4.5 with HOAc and then allowing it to stand for 30 min. in an ice bath to precipitate peptide 1 (Funatsu, 1964). The mixture was then centrifuged at 12,000 g for 10 min. and the supernatant with the soluble peptides was lyophilized and stored. The pellet containing peptide 1 (residues 1-41) was dissolved in a minimal amount of dilute NH_4OH, adjusted to pH 7.0 with more concentrated NH_4OH, and further purified by isoelectric precipitation at pH 4.5 (Tsugita et al., 1960) followed by gel filtration chromatography (Stepanov et al., 1961; Zaitlin and McCaughey, 1965).

Subtilisin Digestion of Tryptic Peptides

Tryptic peptide 8 (ca 20 mg) in a minimal amount of dilute NH_4OH at pH 8.0 was digested with 0.5% commercial subtilisin (trade name Nagarse, from Nagase and Co. Ltd., Osaka, Japan; U.S. distributer, Enzyme

Development Corp., New York, N.Y.) at 23°C for 20 min. and then adjusted to pH 9.0 with 0.1 M NH_4OH. This subtilisin digest was put immediately onto an ion exchange column to separate the resulting peptides (Funatsu et al., 1964).

Dilute Acid Hydrolysis of Subtilisin Peptides

Ten mg of a lyophilized subtilisin peptide (residues 104-112) from tryptic peptide 8 (residues 93-112) was dissolved in 8 ml of a mixture of 50% water and 50% ethanol (95%) that was 0.03 M in HCl (final pH 1.52). The mixture was hydrolyzed in vacuo in a sealed glass ampule at 110°C for 12 hrs. (Tsung and Fraenkel-Conrat, 1965). The hydrolysate was removed, lyophilized, and dissolved in a minimal volume of dilute NH_4OH at pH 9.0, and then put onto an ion exchange column to separate the resulting peptides.

Ion Exchange Chromatography of Peptides

Peptides from PM6 and PM6R coat protein were separated by ion exchange chromatography as described by Funatsu (1964) with some modification. Lyophilized peptides were dissolved in a minimal amount of PCA buffer (1% pyridine, 1% collidine 2,4,6-trimethylpyridine, and HOAc to give the desired pH) adjusted to pH 8.8. The addition of a large amount of peptides to a minimal amount of PCA buffer often brought the pH of the buffered solution below 8.8, so 1 M NH_4OH was added to the solution to bring its pH back up to 8.8 if necessary. The solution of subtilisin or dilute acid peptides were applied directly to Dowex 1X2 (200-400 mesh; acetate form) column (0.9 x 150 cm) that had been equilibrated to pH 8.8 with PCA buffer, but solutions of tryptic peptides were first centrifuged

at 12,000 g for 10 min. to remove any peptide(s) or protein that remained insoluble before being applied to the column. A large clear gelatinous pellet was obtained after centrifugation of the soluble tryptic peptides that was primarily peptide 10. This pellet was resuspended in 1 M NH_4OH and peptide 10 was purified from it by repeated isoelectric precipitation at pH 7.0. After application of the peptides to a column that had been equilibrated to pH 8.8 with PCA buffer, the column was developed at room temperature by eluting it in two steps with a decreasing pH gradient formed by mixing PCA buffer with increasing amounts of HOAc (part of this pH gradient was formed in a 9-chambered Autograd gradient maker; Peterson and Sober, 1959). The elution schedule used for each peptide mixture is described in the appropriate figure legend. The eluant was pumped onto the column with a Buchler mini- or peristaltic pump so that the eluant flow rate was 35-40 ml per hr. and 3.3 ml fractions were collected. At the conclusion of each peptide separation on the Dowex column, the column was eluted with 1 liter of 30% HAc followed by 1 liter of 30% HCl as a precautionary measure to make sure that any peptide(s) that might have been strongly bound to the resin were released and then the column was regenerated with PCA buffer at pH 9.5 until the pH of the eluant reached 8.8.

Not all peptides remained soluble after separation by ion exchange chromatography. Tryptic peptide 10 was found to be released from the column at pH 7.2 and fractions containing it (Figs. 4 and 4) had a precipitate in them that was determined to be peptide 10. Tryptic peptides 4 and 8 were released simultaneously from the column at pH 3.0 (Figs. 4 and 5). Peptide 4 was completely soluble, but peptide 8 was

completely insoluble at this pH and was found entirely as a heavy precipitate in the fractions containing peptide 4. The precipitate of peptide 8 was collected by centrifugation from the fractions in which it occurred, dissolved in 1 M NH_4OH, and then further purified by repeated isoelectric precipitation at pH 3.5. Amino acid analysis showed the precipitate to be peptide 7-8.

Folin-Lowry Protein Assay

The Folin-Lowry assay (Lowry et al., 1951) with some modifications was used to estimate the relative quantities of peptides in fractions taken from the eluant of ion exchange columns. Aliquots (0.1 or 0.2 ml) were withdrawn at specified intervals (see Figure legends) from the fractions to be tested and were placed in test tubes to which 1 ml of a reaction mixture (composed of solutions 1 and 2 mixed in a ration of 50:1; solution 1 is 10 gm Na_2CO_3 and 2 gm NaOH in 500 ml water and solution 2 is 1 gm sodium tratrate and 0.5 gm $CuSO_4 \cdot 5H_2O$ in 100 ml water) was added. The reactants were incubated at room temperature for 10 min., and the 0.1 ml of 1 N Folin-Ciocalteau phenol reagent (Fisher Scientific Co.) was added to each tube to develop color. After 30 min. of color development, optical density (O.D.) readings of the colored solutions were made at 750 nm against a blank solution (0.1 ml of pH 8.8 PCA buffer plus all other reactants). The O.D. readings were plotted against their corresponding fraction numbers to produce a profile of the peptides eluted from the ion exchange column. When aliquots were taken from fractions whose HOAc content (above 0.65 N) was high enough to interfere with the assay, the aliquots were first dried down in their respective test tubes

at 110°C in a forced air oven to remove the HOAc and then 1 ml of the reaction mixture was added directly to each tube and the rest of the procedure was carried out as described above.

Paper Chromatography of Peptides

Descending paper chromatography was often used to further purify peptides taken from ion exchange columns prior to their hydrolysis and amino acid analysis. Peptides concentrated in 0.01 M NH_4OH were spotted onto one inch strips of Whatman 3MM paper that had been washed repeatedly with 1 N HCl followed by 1 N HOAc and then rinsed with water to remove any ninhydrin sensitive contaminants. The chromatograms were eluted with n-butanol-HOAc-water; 4:1:5 v/v (Woody and Knight, 1959) or with n-butanol-HOAc-water-pyridine; 30:6:24:20 v/v (Waley and Watson, 1953). The separated peptides were located by cutting paper strips from the edge of chromatograms, dipping these strips in a ninhydrin solution (1.75 gm ninhydrin, 1120 ml 95% ethanol, 525 ml glacial HOAc, and 70 ml dichlorethane) and then heating the strips for 10 min. at 85°C to develop color. The matching strips were used to locate the peptides on the chromatogram and the portions of the chromatogram containing these peptides were cut out and eluted from the paper with either 10% NH_4OH, 0.2 N HOAc, or water (Funatsu and Funatsu, 1967).

Hydrolysis and Amino Acid Analysis of Coat Protein and Its Peptides

Hydrolysis of 0.5-1.0 mg samples of coat protein or coat protein peptides was carried out in 6 N HCl in sealed tubes evacuated under nitrogen that were heated for 24 and 72 hrs. at 110°C (Zaitlin and

McCaughey, 1965). The acid was removed from the hydrolysates under vacuum at 85-90°C and the residue of amino acids was taken up in 2 ml of pH 2.2 sodium citrate buffer (19.6 gm sodium citrate- $2H_2O$, 16.5 ml conc. HCl, 5 ml thiodiglycol, 0.1 ml octanoic acid, and water to a final volume of 1000 ml) and chromatographed on a Beckman 121 automatic amino acid analyzer. Cysteine was determined by chromatographing separately hydrolyzed samples of whole coat protein that had been previously oxidized with performic acid by the method of Hirs (1956). Tryptophan was estimated spectrophotometrically by determining the tyrosine-tryptophan ratio of whole coat protein in 0.1 M NaOH by the method of Beaven and Holiday (1952).

Two sources of error are possible when the amino acid composition of a protein is estimated with an amino acid analyzer: Small errors in the quantitative formulation of the commercially-supplied amino acid standards can cause errors in the estimation of the amounts of some amino acid residues, and instrument error (estimated to be $\pm$ 2%; Beckman Instrument Manual for the operation of the Beckman 121 automatic amino acid analyzer) can result in a calculation error for those residues present in high number in the whole coat protein. The combined weight of both of these errors is usually small (i.e., less than one integral value for the amino acid residue in question), but it is often of sufficient magnitude to prevent an unequivocal determination of the integral value for some amino acid residues in the mutant coat protein when they are compared to the known values in the standard coat protein. Such errors, when applied equally to the amino acid values obtained for a standard and mutant coat protein should result in the same relative

positive or negative percentage error in the amino acid values for both proteins when the proteins differ by only two or three amino acid residues in their primary sequences. Consequently, a method was devised to correct these errors on a percentage basis which provides a means of comparing the amino acid values obtained from mutant coat proteins to those of a standard coat protein on a standardized basis.

A large amount of standard U1 coat protein, whose exact amino acid composition is known (Tsugita et al., 1960), was prepared and a sample from it was hydrolyzed with each sample of PM6 and PM6R coat protein hydrolyzed. The hydrolysate of the U1 coat protein was analyzed on the amino acid analyzer immediately before or after the hydrolysate of the PM6 or PM6R coat protein with which it was hydrolyzed. Any deviation of the amino acid values in the U1 coat protein from their known standard values was noted and used to make a percentage correction of the amino acid values obtained for the mutant coat protein. A hypothetical example of this correction is as follows: If the known value of aspartic acid in the U1 coat protein is 18.00 residues and the calculated value for aspartic acid in the U1 and mutant coat proteins from a 24 hr. hydrolysate is 17.44 and 17.31 respectively, then a correction of the calculated amino acid value of the mutant coat protein can be made on a percentage basis e.g., (17.31) (18.00) / (17.44) = 17.87, which is the adjusted value of aspartic acid in the mutant coat protein. The value of 17.87 residues is now rounded off to 17.9 residues which would be the adjusted value listed in the Tables of values for whole coat proteins. Values for peptides in all Tables are their calculated values rounded off to the nearest tenth of a residue without a correction

applied. The amino acid values in all Tables are from a single analysis made of a typical coat protein or coat protein peptide preparation. Numerous coat protein and coat protein peptide preparations were made, but when these preparations were of sufficient purity, all yielded the same integral amino acid values upon analysis for the coat protein or the coat protein peptide in question. Initially, some difficulty was encountered in obtaining integral amino acid values for serine for the PM6 coat protein, but when amino acid analysis was performed on the protein immediately after its purification from leaf homogenates (i.e., before storage by freezing or prolonged refrigeration), and a percentage correction was applied to the values obtained, the integral value for serine from successive coat protein preparations was found to be the same. Threonine and serine degrade linearly with time upon release from proteins by hydrolysis in 6 N HCl (reviewed by Roach and Gehrke, 1970). Therefore values for these residues were estimated indirectly using threonine and serine values from 24 and 72 hr. hydrolysates to extrapolate back to zero time degradation. Isoleucine is released slowly by acid hydrolysis of TMV coat protein (Tsung and Fraenkel-Conrat, 1965), so values for it in whole coat protein and in peptides 1, 6, 7-8 and 8 were taken from 72 hr. hydrolysates. It was found that residue values for threonine, serine and isoleucine could also be calculated directly from residues values taken from 24 hr. hydrolysates by applying a correction calculated on a percentage basis from the standard U1 protein in the same manner described above for correcting errors in amino acid standards and instrument error. Such corrections are apparently possible because threonine, serine, and isoleucine are degraded or released at the

same rate in PM6, PM6R, and U1 coat protein as evidenced by the same values obtained using either the indirect or direct method for their calculation. The direct method of calculation for those residue values was routinely used as a short cut when analyzing the coat proteins of PM6 and PM6R for purity prior to their enzymatic digestion.

Electron Microscopy of TMV Infected Leaf Cells

Carbon coated grids (400 mesh) were prepared (Hariharasubramanian and Siegel, 1969) and one drop of a negative stain (1% uranyl acetate; Leberman, 1965) was placed on top of each grid and then cut surfaces of PM6 infected or revertant infected tobacco leaves were drawn through the negative stain 2 or 3 times according to the leaf dip method of Hitchborn and Hills (1965). The excess moisture was blotted off the grids and the negatively stained coat protein or virus particles that had been deposited on the grids from the cut leaf surfaces were observed immediately in a Philips EM200 electron microscope.

RESULTS

Characterization of PM6

Confirmation of Defectiveness

Confirmation that PM6 is a defective mutant is directly supported by two lines of evidence. Infectivity of the RNA in a TMV virion is very stable in leaf homogenates due to the protection afforded by its protein coat, but the RNA of defective mutants in leaf homogenates is unprotected by such a coat and is therefore susceptible to rapid inactivation at elevated temperatures (Siegel et al., 1962) due presumably to the increased rate of degradation of the infectious RNA of the defective mutant by leaf ribonucleases. Consequently, the resistance to inactivation of PM6 in leaf homogenates is useful in determining whether the PM6 isolate is a defective mutant. Thus, leaf homogenates were prepared in ice cold TP buffer from PM6 infected, U1 infected, and healthy Samsun tobacco plant leaves. Each homogenate was divided into two equal parts and one part from each homogenate was heated to 60°C for 10 min. and then cooled while the other part was kept at ice bath temperature. The infectivity of these homogenates were compared to one another by half leaf tests on Xanthi-nc tobacco plants. The half leaves were scored positive or negative as to lesion occurrence regardless of the number of lesions produced on a half leaf. The tests showed that infectivity in PM6 leaf homogenates is lost after heat treatment while the infectivity in U1 leaf homogenates remained normal after an identical treatment. Thus, PM6 is

a defective mutant. The defectiveness of this mutant is further indicated by the fact that no virus rods can be extracted from PM6 infected plants and that no virus rods are visible in electron micrographs taken of leaf dips from PM6 infected plants.

Coat Protein Properties

PM6 coat protein can be purified from the soluble cytoplasmic fraction of PM6 infected Samsun tobacco leaf cells where it accumulates presumably because of its inability to encapsidate PM6 RNA. The purified protein was found to be unstable and this instability manifested itself in the continual precipitation of the protein from solution beginning from the time it was purified and continuing until only a very low concentration of the protein was left in solution. Subjecting the purified protein to pH conditions below 4.0, temperatures above 15°C, concentrations above 5 mg/ml, freezing and thawing, and agitation all resulted in a rapid denaturation as indicated by the precipation of the protein from solution. Precipitated protein could often be resolubilized by dissolving it in a 6 M urea solution and then dialyzing away the urea with cold TP buffer. However, only a small amount of the protein could usually be recovered by such a procedure.

The protein was found to have a cation requirement for aggregation at pH 4.7. The chlorides of Mg^{++}, Na^{+}, and K^{+} all worked, but K^{+} was found to be best. The mechanism of the cation effect is unknown, but it does not aid in the assembly of protein rods at pH 4.7 because electron microscopic observation of the protein aggregated at pH 4.7 in the presence of a cation showed no organized structure, but rather small

amorphous aggregates. Absence of the cation during aggregation of the protein at pH 4.7 will result in the eventual precipitation of the protein from solution, but not in permanent denaturation, because if the pH of the solution is raised again to neutrality, the protein will quickly go back into solution.

Coat Protein and Peptide Analysis

Amino acid analysis of the total PM6 coat protein showed it to be high in one residue each of threonine and glycine and low in one each of aspartic (or asparagine) and alanine (Table 2). Tryptic peptides prepared from PM6 protein were also examined by amino acid analysis. Tryptic peptide 1, which was separated from the other peptides by isoelectric precipitation at pH 4.5, showed no amino acid change in composition from wild type peptide 1 (Table 3). Cysteine and tryptophane were not examined in this peptide. The remaining soluble peptides were separated on an ion exchange column (Fig. 4), but not all of the peptides were resolved. Most of the peptide peaks from this separation were identified from their amino acid compositions (Table 4) and peptide 8, which occurs as a precipitate at pH 3.0 under the peptide 4 peak (Fig. 4), was found to be low in one alanine and high in one threonine when compared with U1 coat protein (Table 5), indicating an alanine to threonine exchange. If the alanine to threonine exchange is subtracted from the residue differences found in the whole PM6 coat protein (Table 2), it is evident that an aspartic (or asparagine) to glycine exchange must also exist elsewhere in the PM6 coat protein. Since all tryptic peptides of PM6 protein were not identified, the exact location of the aspartic (or asparagine) to

Table 2. Amino acid composition of PM6 coat protein.

Amino acid residue	Moles amino acid per mole protein		
	24 hour hydrolysis[a]	Integral value	TMV
Lys	2.1	2	2
Arg	11.1	11	11
Asp	16.8	17	18
Thr	16.6	17	16
Ser	16.2	16	16
Glu	16.0	16[b]	16
Pro	8.1	8	8
Gly	7.2	7	6
Ala	13.1	13	14
Cys	0.7	1[c]	1
Val	14.1	14	14
Ile	9.1	9	9
Leu	12.1	12	12
Tyr	4.1	4	4
Phe	8.2	8	8
Try	3.1	3[d]	3

a. Adjusted values obtained by a method described in the results section.

b. Other values calculated on basis of 16 for Glu.

c. Value obtained on separate analyses of performic acid oxidized protein.

d. Value determined spectrophotometrically.

Table 3. Amino acid composition of tryptic peptide 1 from PM6 and $PM6R_1$ coat protein.

Amino acid residue	Moles amino acid per mole peptide for 24-hour hydrolysis			
	PM6	$PM6R_1$	Integral value	TMV
Arg	0.8	0.9	1	1
Asp	4.4	4.4	4	4
Thr	4.1	4.4	4	4
Ser	4.9	5.1	5	5
Glu	6.0	6.0	6[a]	6
Pro	1.8	1.8	2	2
Gly	1.3	1.3	1	1
Ala	4.3	4.3	4	4
Cys	ND[b]	ND[b]	ND[b]	1
Val	1.1	1.1	1	1
Ile	3.2	3.2	3[c]	3
Leu	4.4	4.4	4	4
Tyr	1.0	1.1	1	1
Phe	3.1	3.2	3	3
Try	ND[b]	ND[b]	ND[b]	1

a. Other values calculated on basis of 6 for Glu.

b. Value not determined (ND).

c. Value taken from 72-hour hydrolysis.

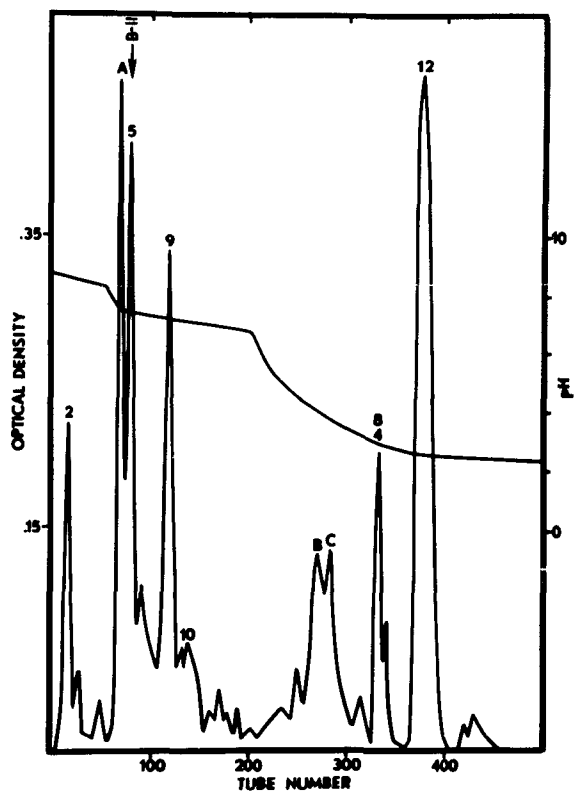

Fig. 4. Chromatography of pH 4.5 soluble tryptic peptides from PM6 protein.

Chromatography performed on a Dowex 1-X2 acetate column (0.9 x 150 cm); 3.3 ml of eluate was collected per tube and 0.1 ml aliquots from every third tube were used for testing with the Folin-Lowry reagent. The color developed was measured at 750 nm in a spectrophotometer as optical density. Peptides are numbered according to their sequence in the TMV protein. Peak $\frac{8}{4}$ contains peptides 8 and 4. The flow rate was 35-40 ml/hour at room temperature. Elution buffers were made of 1% pyridine, 1% collidine (2,4,6-trimethylpyridine), and acetic acid to give different pH's. Elution schedule: Buffer 1(B-1) 250 ml pH 8.8 buffer, Buffer 2(B-II) gradient elution by Autograd gradient maker using 9 chambers with 150 ml in each. Chambers 1-3, pH 7.3 buffer; 4 and 5, 0.02 N acetic acid; 6, 0.2 N acetic acid; 7, 0.35 N acetic acid; 8, 0.50 N acetic acid; and 9, 0.65 N acetic acid. The pH gradient profile is also shown in this figure as a representative example of the pH gradient used in all subsequent peptide separations. The pH profile was established by taking a pH measurement on each fraction that was tested with the Folin-Lowry reagent.

Table 4. Amino acid composition of soluble tryptic peptides of PM6 coat protein.

Amino acid residue	In moles of amino acid per mole peptide after 24-hour hydrolysis					
	2[a]	5	9	10	4	12
Lys	1.1(1)[b]	0.8(1)			1.0(1)	
Arg			2.0(2)	1.0(1)		
Asp			2.0(2)[c]	2.0(2)[c]	2.0(2)[c]	
Thr	1.1(1)		1.0(1)			2.2(2)
Ser				0.7(1)	0.9(1)	6.0(6)[c]
Glu	1.2(1)			0.8(1)		1.3(1)
Pro					1.0(1)	1.1(1)
Gly						2.3(2)
Ala			2.0(2)	1.3(1)		1.2(1)
Val	2.0(2)[c]	1.0(1)[c]	2.1(2)	1.2(1)		1.2(1)
Ile			1.0(1)	2.5(3)		
Leu				2.3(2)		1.1(1)
Tyr		0.8(1)				
Phe					1.9(2)	1.1(1)
Try						ND(1)[d]

a. Peptides listed from left to right in order of their elution from an ion exchange column. Each peptide number indicates the sequential position of that peptide beginning from the N-terminal end of the primary structure in the TMV coat protein (Funatsu, 1964).

b. Values in parentheses are the expected TMV values for that residue taken from Funatsu (1964).

c. Value used as a basis for calculation of other residue values in that peptide.

d. Value not determined (ND).

Table 5. Amino acid composition of tryptic peptide 8 from PM6 and $PM6R_1$ coat protein.

Amino acid residue	Moles amino acid per mole peptide after 24-hour hydrolysis			TMV peptide 8
	PM6	$PM6R_1$	Integral value	
Arg	0.9	0.9	1	1
Asp	3.0	3.0	3[a]	3
Thr	4.6	5.2	5[b]	4
Glu	3.9	3.9	4	4
Pro	1.0	0.7	1	1
Ala	2.0	1.9	2[b]	3
Val	1.0	1.0	1	1
Ile	1.8	1.8	2[c]	2
Leu	1.0	1.0	1	1

a. Other values calculated on basis of 3 for Asp.

b. Indicates change in integral value from TMV peptide 8.

c. Value taken from 72-hour hydrolysis.

glycine exchange is unknown, but it is shown that peptide 6 is not positioned where it might be expected in the peptide profile of PM6 coat protein (Fig. 4) judging from what might be found in U1. If peptide 6 of PM6 had a U1 type composition, it would be positioned between peptides 4 and 12 just as peptide 6 from PM6R is positioned between peptides 4 and 12 in the peptide profile of PM6R coat protein (Fig. 5; peptides 4, 6 and 12 of PM6R coat protein are shown to have a wild type amino acid composition in Table 8). Peptide 6 of PM6 protein is not located between peptides 4 and 12 because it presumably contains an aspartic (or asparagine) to glycine exchange. Such a peptide would have one less ionizable carboxyl group exchange and consequently would be less acidic and would be released earlier from an ion exchange column developed with a decreasing pH gradient. A peptide with such an elution characteristic is located at peak C (Fig. 4) and preliminary analysis of this peptide indicates that it is probably peptide 6. Preliminary amino acid analysis of peaks A and B (Fig. 4) indicate that peak A may contain peptides 3 and 11 and that peak B may contain peptide 3-4 (see Table 8, footnote b for an explanation of the origin and composition of this peptide).

Electron Microscopy

Electron micrographs of leaf dips from PM6 infected leaf tissue show what appears to be the coat protein of PM6 aggregated into rods (Fig. 6) whereas such structures are completely absent in uninfected leaf tissue. These long flexuous rods appear to be made up of units that are loosely joined together, and each unit appears to be a double disk (Fig. 6). Wheel-like structures also appear among the rods in high

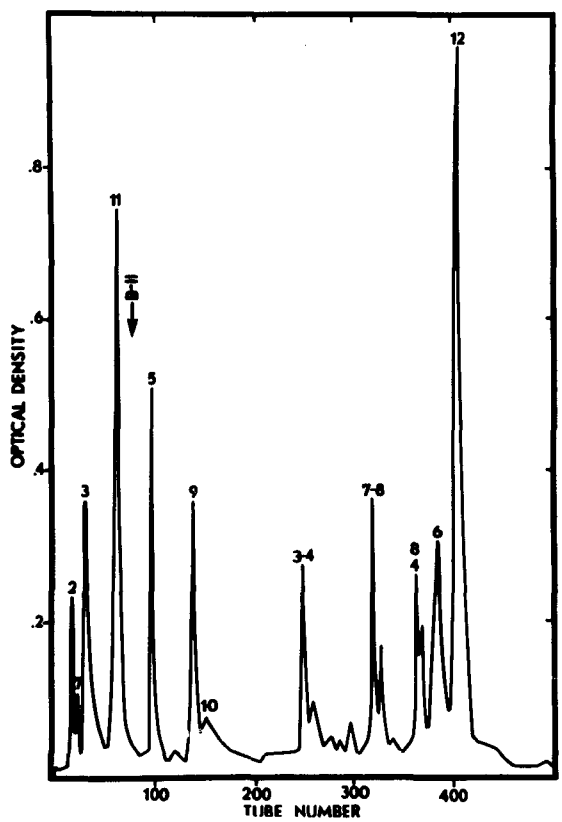

Fig. 5. Chromatography of pH 4.5 soluble tryptic peptides from $PM6R_1$ protein.

Elution and analysis performed exactly as in Fig. 1 except that 135 ml of buffer was used in each chamber of the Autograd gradient maker to make the buffer 2 (B-II) gradient. Peptide numbering is the same as in Fig. 4.

Fig. 6. PM6 protein rod showing region of stacked disks.

The arrow indicates a region in a protein rod where stacked disks are clearly visible. Electron micrograph obtained from a negatively stained leaf dip from a PM6-infected Samsun tobacco plant.

frequency (Fig. 7). They appear to be formed from protein rods of sufficient length to bend back on themselves and join end to end to form an unbroken circle, termed a "wheel" (Fig. 8). Each wheel is made up of a number of units and each unit (Fig. 8), appears to have two electron dense grooves on its outward side and one electron dense groove on its inward side (Fig. 9). A structure for the unit that would be compatible with these observations is a 2½ turn helix. A model of such a helix (Fig. 10) shows its asymmetric nature. The outward side of the helix has three turns of coat protein subunits and the inside of the helix has two. Some of the protein rods also appear to have a few units that are 2½ turn helices (Fig. 11) and some of the wheels also appear to have a few units that are double disks (Fig. 7), although the model of the wheel (Fig. 12) presented here has been constructed entirely of 2½ turn helices. The maximum number of units that can make up a wheel is unknown, but the minimum number of units counted in wheels observed in numerous micrographs was 13 and this is the number used on constructing the wheel model.

Characterization of PM6R

Coat Protein Properties

The coat protein of PM6R isolates, like the coat protein of U1, but unlike the coat protein of PM6, is very stable in pure form. The protein aggregates at pH 6.1, as evidenced by the turbidity of the protein solution at this pH, in contrast to the aggregation of U1 coat protein which occurs at pH 5.0. The aggregated protein can be centrifuged from solution at 105,000 *g* for 30 min. to form clear gelatinous

Fig. 7. PM6 protein in the wheel configuration that appears to be constructed from both stacked disks and 2½ turn helicies.

The arrow indicates a protein wheel whose units of construction appear to be stacked disks on the lower side and 2½ turn helicies on the upper side. Electron micrograph obtained from a negatively stained leaf dip from a PM6-infected Samsun tobacco plant.

Fig. 8. PM6 prtoein wheels that appear to be in intermediate states of formation.

Protein rods in various stages of bending back on themselves and joining end to end to form protein wheels are evident in the field like the one shown in the lower right-hand corner. Electron micrograph obtained from a negatively stained leaf dip from a PM6-infected Samsun tobacco plant.

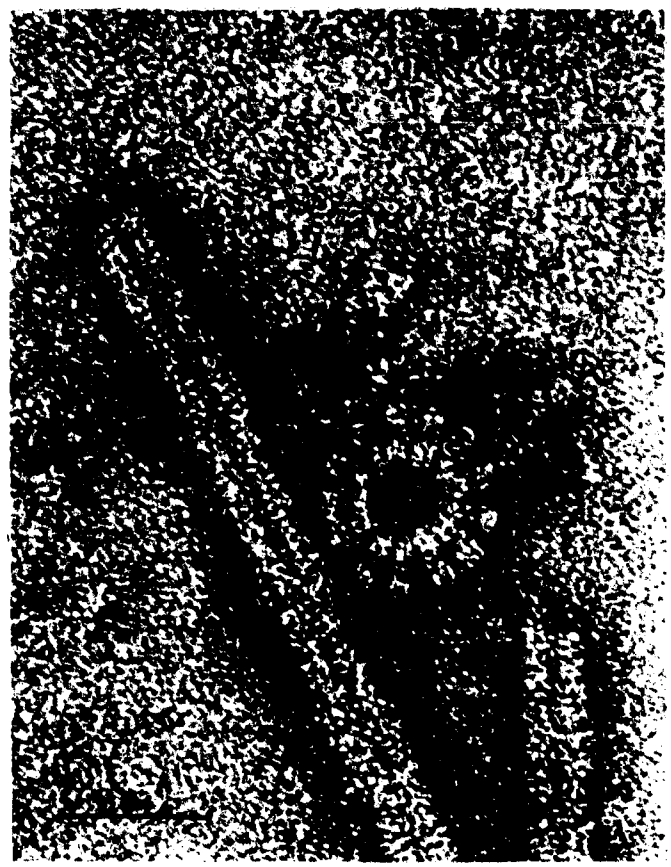

Fig. 9. Two and one-half turn helix in PM6 protein wheel.

Arrow indicates one 2½ turn helix that is clearly visible and which shows two electron dense grooves on its outside and one electron dense groove on its inside. Electron micrograph obtained from a negatively stained leaf dip from a PM6-infected Samsun tobacco plant.

Fig. 10. Model of 2½ turn helix.

The model was constructed from Tygon tubing held together with straight pins and depicts 3 turns of protein subunits on the upper side and 2 turns on the lower side that results in two grooves on the upper and one on the lower side.

Fig. 11. PM6 protein rod showing possible helical region.

Arrow indicates portion of rod that may be composed of helical units, possibly 2½ or 3 turn helicies. Electron micrograph obtained from a negatively stained leaf dip from a PM6-infected Samsun tobacco plant.

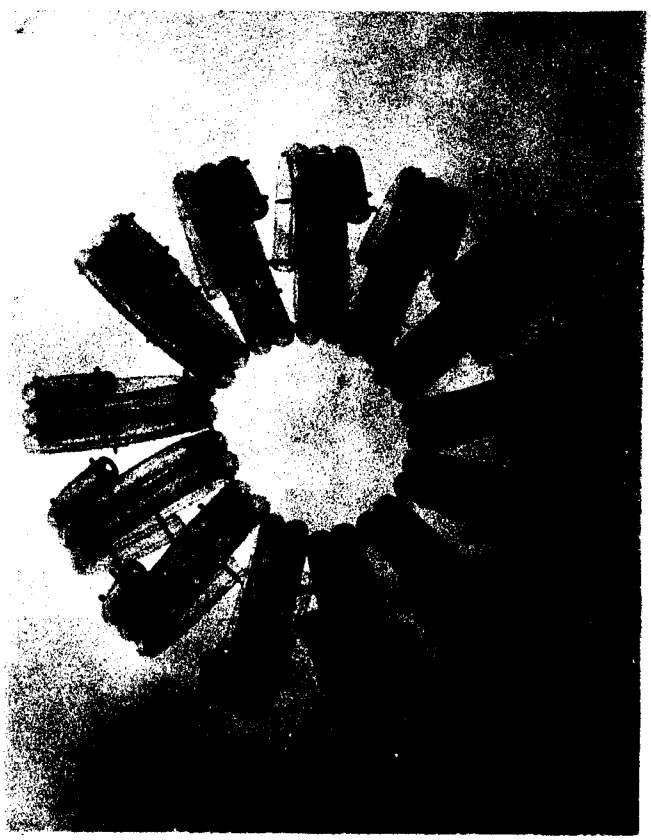

Fig. 12. Model of PM6 protein wheel.

The model was constructed from Tygon tubing held together with straight pins. The model of the proposed wheel configuration shows the unit of construction as a 2½ turn helix. The tubing alignment depicts the subunit proteins in the helicies in two dimensions as they would appear in an electron micrograph of negatively stained PM6 protein wheels.

pellets. Electron microscopic examination of the aggregated protein revealed the aggregates were short protein rods. The protein appears to possess an isoelectric point of about pH 4.7, as evidenced by its precipitation from solution at this pH, in contrast to pH 3.2 for U1 coat protein (Kramer and Wittmann, 1958).

Frequency of PM6R Isolation and Coat Protein Analysis

In the first screening experiment, 35 PM6R isolates were obtained from 51 PM6 infected plants held at 43-46°C over a period of 90 days. Amino acid analysis of 10 of these isolates showed that nine of them were low in one alanine and high in one threonine (Table 6) compared with U1 protein, indicating an alanine to threonine exchange in their primary structure. The coat protein of the tenth isolate was U1-like in composition. Only one of the 15 control plants spread throughout the group of 51 plants became infected with a wild type strain and only one of the five PM6 infected plants held at room temperature in a growth room produced a revertant isolate. The coat proteins of both of these isolates showed an amino acid composition identical to the composition of the first nine PM6R isolates examined. In the second screening experiment, 9 PM6R isolates were obtained from 45 PM6 infected plants held at 26-30°C for 60 days and none of the control plants or the PM6-infected plants held at room temperature in a growth room became infected with a wild type strain or produced any revertant isolates. The coat protein amino acid composition of all nine of these isolates showed that all possessed an alanine to threonine exchange (Table 7). In addition to the revertant isolates obtained in the screening experiments detailed above, five additional

Table 6. Amino acid composition of coat protein from PM6R isolates from screening experiment 1.

Amino acid residue	Moles amino acid per mole protein after 24-hour hydrolysis[a]									Integral value of all isolates
	PM6R$_1$[f]	PM6R$_2$	PM6R$_3$	PM6R$_4$	PM6R$_5$	PM6R$_6$	PM6R$_7$	PM6R$_8$	PM6R$_9$	
Lys	1.9	2.0	2.0	1.9	1.9	2.0	1.9	1.9	2.0	2
Arg	10.9	10.7	11.0	10.8	10.9	10.9	11.0	10.8	10.6	11
Asp	17.9	18.1	18.1	17.9	17.8	18.1	18.1	18.1	17.9	18
Thr	16.8	17.1	17.2	16.8	16.8	17.0	16.8	17.2	17.2	17(+1)[g]
Ser	15.8	16.1	15.5	15.8	15.6	15.8	15.7	15.8	15.5	16
Glu	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16[c]
Pro	7.8	7.7	7.9	7.6	7.5	7.8	8.3	7.8	7.8	8
Gly	6.1	6.1	6.3	6.1	6.0	6.3	6.1	6.0	5.9	6
Ala	12.9	12.9	12.8	12.9	12.9	13.0	13.1	12.9	12.8	13(-1)[g]
Cys	0.9	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	1[d]
Val	13.9	13.9	14.1	13.9	13.8	13.9	14.1	13.9	13.9	14
Ile	8.7	8.9	9.1	9.0	8.9	9.0	9.0	9.1	9.0	9
Leu	12.1	12.1	12.0	12.1	12.0	12.0	11.9	12.1	11.9	12
Tyr	3.9	3.9	4.0	3.9	3.9	4.0	4.0	4.1	4.0	4
Phe	7.9	8.0	8.1	8.1	7.9	8.0	8.0	8.1	8.2	8
Try	3.3	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	3[e]

a. Adjusted values obtained by a method described in the results section.

b. Value not determined (ND).

c. Other values calculated on basis of 16 for Glu.

d. Value obtained on separate analyses of performic acid oxidized protein.

e. Value determined spectrophotometrically.

f. PM6R isolates.

g. Number in parentheses indicates change in integral value from TMV coat protein.

Table 7. Amino acid composition of coat protein from PM6R isolates from screening experiment 2.

Amino acid residue	Moles amino acid per mole protein after 24-hour hydrolysis[a]									Integral value of all isolates
	PM6R$_{10}$[e]	PM6R$_{11}$	PM6R$_{12}$	PM6R$_{13}$	PM6R$_{14}$	PM6R$_{15}$	PM6R$_{16}$	PM6R$_{17}$	PM6R$_{18}$	
Lys	1.9	2.1	2.0	2.0	2.2	2.1	2.1	2.2	2.1	2
Arg	11.2	11.2	11.0	10.9	11.1	11.0	11.3	11.2	11.2	11
Asp	17.9	18.2	17.8	18.0	18.1	17.8	18.0	18.1	18.1	18
Thr	17.2	17.2	16.8	17.1	17.0	16.8	16.9	16.9	17.0	17(+1)[d]
Ser	16.1	15.9	15.9	16.0	15.9	16.0	15.9	16.0	16.1	16[b]
Glu	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16[b]
Pro	8.0	7.9	8.1	7.8	8.0	8.0	7.8	7.8	7.7	8
Gly	6.0	6.2	6.0	6.0	6.1	6.0	6.1	6.0	6.0	6
Ala	13.0	13.3	13.0	12.9	13.2	13.1	12.8	12.9	13.0	13(-1)[d]
Cys	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
Val	14.0	14.1	14.1	14.1	14.1	13.9	14.0	14.0	14.0	14
Ile	9.0	9.1	8.8	9.1	8.9	8.9	8.9	8.9	8.9	9
Leu	12.1	12.2	11.8	12.0	11.9	11.9	12.1	12.1	11.8	12
Tyr	4.0	4.1	4.0	4.0	4.1	4.0	4.1	4.1	4.0	4
Phe	7.8	8.2	8.0	8.1	8.2	8.2	8.1	8.2	8.1	8[c]
Try	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]

a. Adjusted values obtained by a method described in the results section.

b. Other values calculated on the basis of 16 for Glu.

c. Value not determined (ND).

d. Number in parentheses indicates change in integral value from TMV coat protein.

e. PM6R isolates.

revertant isolates were obtained from a group of PM6 infected plants held at room temperature in a growth room for 3-6 months for the routine culture and maintenance of the PM6 strain. The coat proteins fo three of these isolates were examined, and they also showed an alanine to threonine exchange. Although no experiment was conducted specifically to test the effect to temperature on the frequency of reversion, the number of revertant isolates obtained at different temperatures in separate experiments strongly suggests that increased temperatures increase the frequency of PM6 reversion to PM6R.

Peptide Analysis

Tryptic peptides. To localize the exact position of the alanine to threonine exchange in the coat protein of the PM6R mutant, the coat protein of the $PM6R_1$ isolate was sequenced, taking this isolate as a representative example of the coat proteins of all PM6R isolates. Tryptic peptides of the coat protein were prepared, and peptide 1 was separated by isoelectric precipitation from the other peptides. Amino acid analysis of peptide 1 showed it to be identical in composition to peptide 1 of PM6 and U1 (Table 3). The soluble tryptic peptides were separated by ion exchange chromatography, a peptide profile of the eluted peptides was obtained by Lowry assay and the peptides in the resolved peaks (Fig. 5) were identified by their amino acid composition after analysis. Peptide 8, which occurs as a precipitate at pH 3.0 under the peptide 4 peak (Fig. 5), contained an alanine to threonine exchange (Table 5). The remaining peptides all showed analyses identical to their wild type peptides in U1 protein (Table 8). Two peptides, 3-4 and 7-8,

Table 8. Amino acid composition of soluble tryptic peptides of $PM6R_1$ coat protein.

Amino acid residue	Expressed in moles of amino acid per mole peptide after 24-hour hydrolysis											
	2[a]	7	3	11	5	9	10	3-4[b]	7-8[c]	4	6	12
Lys			1.3(1)					2.0(2)		0.9(1)		
Arg	1.2(1)[d]	1.0(1)[e]	1.1(1)	1.0(1)[e]	1.0(1)	1.9(2)[e]	0.9(1)[e]	1.0(1)	1.9(2)[e]		1.0(1)[e]	
Asp		1.0(1)[e]		1.0(1)[e]		2.0(2)[e]	2.0(2)[e]	2.0(2)	4.0(4)[h]	2.0(2)[e]	3.0(3)[e]	
Thr	1.1(1)		1.2(1)	1.0(1)		1.0(1)		1.1(1)	4.9(4)[h]		2.3(2)	2.1(2)[e]
Ser			2.2(2)[e]	1.0(1)			0.7(1)	2.8(3)	3.9(4)	0.8(1)		6.0(6)[e]
Glu	1.1(1)		3.0(3)[e]				0.7(1)	3.0(3)[e]	0.9(1)			1.1(1)
Pro			2.2(2)					2.9(3)		0.9(1)	1.0(1)	1.0(1)
Gly				2.0(2)							1.0(1)	2.3(2)
Ala						2.0(2)	1.3(1)		2.0(3)[i]		2.9(3)	1.1(1)
Val	2.0(2)[e]		3.3(3)		1.0(1)[e]	2.0(2)	0.9(1)	2.9(3)	1.1(1)[f]		1.9(2)	1.1(1)
Ile						1.0(1)	2.5(3)		1.8(2)[f]			
Leu							2.3(2)		1.1(1)		3.6(4)	1.1(1)
Tyr				1.0(1)	1.0(1)					1.6(2)	0.8(1)	
Phe			1.0(1)					2.0(3)			0.8(1)	1.2(1)
Try			ND(1)[g]									ND(1)[g]

a. Peptides listed from left to right in order of their elution from an ion exchange column. Each peptide number indicates the sequential position of that peptide beginning from the N-terminal end of the primary structure in the TMV coat protein (Funatsu, 1964).

b. A peptide (3-4) comprising residues 47-68 that results from incomplete tryptic digestion of the peptide bond between residues 61 and 62 that joins peptides 3 and 4.

c. A peptide (7-8) comprising residues 91-112 that results from incomplete tryptic digestion of the peptide bond between residues 92 and 93 that joins peptides 7 and 8.

d. Values in parentheses are the expected TMV values for that residue taken from Funatsu (1964).

e. Value used as a basis for calculation of other residue values in that peptide.

f. Value taken from 72-hour hydrolysis.

g. Value not determined (ND).

h. Indicates increase of one residue from expected Ul value.

i. Indicates decrease of one residue from expected Ul value.

which result from the incomplete tryptic hydrolysis of the peptide bonds between peptides 3 and 4, and 7 and 8 (see Table 8, footnote b and c for a more detailed explanation of the origin and composition of these peptides), and which are normally present as minor components in the tryptic peptide profile of U1 coat protein (see Fig. 1 in Funatsu, 1964), were present in unusually high concentration. Peptide 7-8 also showed the alanine to threonine exchange (Table 8) found in peptide 8. The peak containing peptide 7-8 (Fig. 5) also contained a considerable precipitate, which was purified as previously described and then analyzed for its amino acid composition. The precipitate analyzed exactly like peptide 7-8 showing that peptide 7-8 is insoluble at high concentrations under these pH conditions.

Subtilisin Peptides. To further localize the alanine to threonine exchange in peptide 8, approximately 40 mg of purified tryptic peptide 8, comprising amino acid residues 93-112, was digested with subtilisin and the resulting peptides separated by ion exchange chromatography. A peptide profile was obtained (Fig. 13) and the peptides in these peaks were identified by amino acid analysis (Table 9). Peak A corresponded to residues 100-103, peak C to residues 104-112, peak D to residues 98-99, and peak E to residues 93-97; peak B was composed of only threonine. Each of the alanine residues in peptide 8, located at positions 100, 105, and 110 (Table 10), are potential candidates for exchange with threonine. Peak A containing residues 100-103 shows no alanine to threonine exchange at position 100, (peak A, Table 9) but peak C containing the remaining two alanine residues, shows that one of the alanine residues in this peptide at either position 105 or 110 is exchanged for

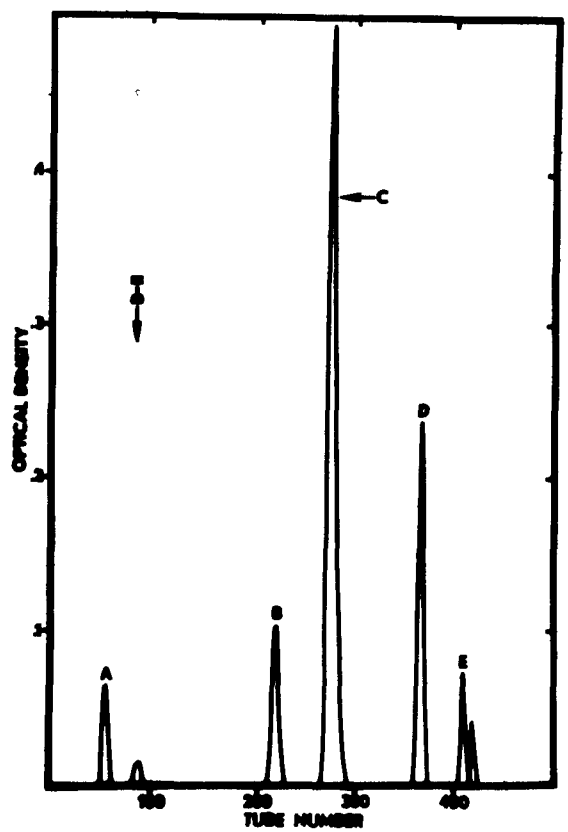

Fig. 13. Chromatography of subtilisin digestion of tryptic peptide 8 from $PM6R_1$ coat protein.

Elution and analysis performed exactly as in Fig. 1 except that 200 ml of buffer were used in each chamber of the Autograd gradient maker to make the buffer 2 (B-II) gradient, and 0.2 ml aliquots were removed from every tube for testing with the Folin-Lowry reagent.

Table 9. Amino acid composition of subtilisin peptides from tryptic peptide 8 of $PM6R_1$ coat protein.

Amino acid residue	Moles amino acid per mole peptide after 24-hour hydrolysis				
	A[a]	B	C	D	E
Arg			0.8(1)		
Asp	1.0(1)[b]		1.0(1)[b]	0.9(1)	
Thr	1.1(1)[c]	0.01[d]	4.1(3)[f]		
Glu			0.9(1)	1.0(1)[b]	2.0(2)[b]
Pro	1.0(1)				
Gly					
Ala	1.0(1)		1.0(2)[g]		
Val					0.7(1)
Leu			0.9(1)		
Tyr					1.6(2)
	100-103[e]	104	104-112	98-99	93-97

a. Peptides listed alphabetically according to their elution peaks from an ion exchange column.

b. Value used as a basis for calculation of other residue values in that peptide.

c. Values in parentheses are expected TMV peptide values for that residue.

d. Value expressed in moles of residue.

e. Residues comprising each peptide obtained upon subtilisin digestion of tryptic peptide 8 of U1 coat protein by Funatsu et al. (1964). The peptide residues are positioned under the subtilisin peptides of tryptic peptide 8 of PM6R coat protein to which their amino acid composition corresponds.

f. Indicates increase of one residue from expected value.

g. Indicates decrease of one residue from expected value.

Table 10. Amino acid sequence of tryptic peptide 8 from U1 coat protein and $PM6R_1$ coat protein.

Residue number :	93	94	95	96	97	↓[b]	98		99		↓[b]	100	101
U1[a]:	Ile-	Ile-	Glu-	Val-	Glu-	-Asp(-NH2)-			Glu(-NH2)-		-Ala-	Asp(-NH2)-	
	102	103	↓[b]	104	105	106	197	108	↓[b]	109	110	111	112
	Pro-	Thr-	-Thr-	Ala-	Glu-	Thr-	Leu-	-Asp-		Ala-	Thr-	Arg	
Residue number:	93	94	95	96	97	↓[b]	98		99		↓[b]	100	101
$PM6R_1$:	Ile-	Ile-	Glu-	Val-	Glu-	-Asp(-NH2)-			Glu(-NH2)-		-Ala-	Asp(-NH2)-	
	102	103	↓[c]	104	↓[b]	105[d]	106	107	108	109	110	111	112
	Pro-	Thr-	-Thr-	-Thr-	Glu-	Thr-	Leu-	Asp-	Ala-	Thr-	Arg		

a. Sequence taken from Fig. 15-3 in Tsugita and Hirashima (1972) and location of subtilisin peptide bond cleavage taken from Funatsu et al. (1964).

b. Arrow indicates subtilisin cleavage of peptide bond.

c. Cleavage of this bond is only partial.

d. Ala exchanged for thr at position 105.

threonine (peak C, Table 9). It is known that subtilisin normally cleaves between the two threonine residues at positions 103 and 104 (Table 10; Funatsu et al., 1964). However, if alanine at position 105 is exchanged for threonine, thus creating three threonine residues in a row, then it is likely that subtilisin would cleave the peptide bond on either side of residue 104, resulting in the liberation of one mole of threonine per mole of peptide and the appearance of a peak of threonine in an ion exchange profile of subtilisin peptides from peptide 8. Although a peak of only threonine is observed (peak B, Fig. 13 and Table 9) which is probably residue 104, analysis of the peptide in peak C (Fig. 13 and Table 9) shows that it contains the normal number of threonine residues plus one additional threonine due to the alanine to threonine exchange in this peptide. These results suggest that the alanine to threonine exchange is at position 105 and that only a small amount of threonine from position 104 is released to create peak B (Fig. 13) due to incomplete subtilisin hydrolysis of the peptide bond between residues 104 and 105 (Table 10) and that the peptide comprising residues 104-112 remains essentially intact.

Dilute Acid Peptides. Normally subtilisin cleaves the peptide bond between residues 108 and 109 in the peptide comprising residues 104-112 taken from U1 coat protein, thus creating two separate peptides (Table 10), each containing one alanine residue at positions 105 and 110. Amino acid analysis of each of these peptides could easily show the exact position of the alanine to threonine exchange in the peptide comprising residues 104-112, but unfortunately, subtilisin does not cleave the peptide bond between residues 108 and 109 in the same peptide taken from

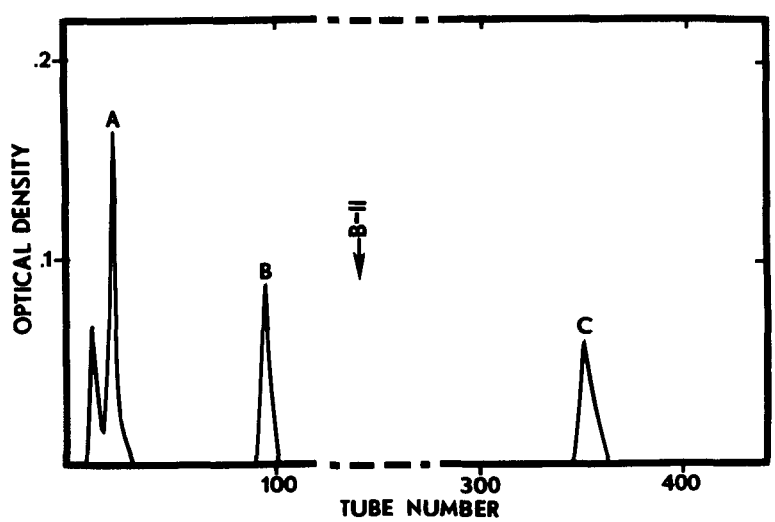

Fig. 14. Chromatography of a dilute acid digestion of a subtilisin peptide from $PM6R_1$ coat protein comprising residues 104 and 112.

Elution and analysis performed exactly as in Fig. 1 except that 135 ml of buffer was used in each chamber of the Autograd gradient maker to make the buffer 2 (B-II) gradient and 0.2 ml aliquots were removed from every tube for testing with the Folin-Lowry reagent.

Table 11. Amino acid composition of dilute acid peptides from a subtilisin peptide of $PM6R_1$ coat protein comprising residue numbers 104-112.

Amino acid residue	Moles amino acid per peptide after 24-hour hydrolysis	
	A[a]	C
Arg	1.0(1)[b]	
Thr	1.1(1)[c]	3.0(2)[b,e]
Glu		1.3(1)
Ala	0.6(1)	
Leu		0.6(1)
	110-112[d]	104-108

a. Peptide peaks alphabetically listed in order of their elution from an ion exchange column.

b. Value used as basis for calculation of other residue values in that peptide.

c. Values in parentheses are expected TMV peptide values for that residue.

d. Residues of U1 coat protein corresponding to the amino acid composition of dilute acid peptides from PM6R coat protein.

e. Indicates increase of one residue from expected value.

$PM6R_1$ coat protein. The failure of subtilisin to cleave between these two residues is probably due to the changed primary structure of this peptide. Consequently, to confirm the alanine to threonine exchange in $PM6R_1$ protein at position 105, the peptide comprising residues 104-112 was cleaved again by dilute acid (0.03 M HCl) which removes the aspartic acid residue at position 109 in the peptide producing two smaller peptides comprising residues 104 to 108 and 110 to 112. These peptides (peaks A and C) along with a degradation product (peak B) were separated by ion exchange chromatography (Fig. 14). Amino acid analysis showed that peak A (Fig. 14), along with the unlabeled peak to the left of A, contained a peptide whose amino acid composition corresponded to residues 110 to 112 (Table 10 and 11) and that peak C (Fig. 14) corresponded to a peptide composed of residues 105 to 108 (Table 10 and 11). These analyses confirmed that the peptide comprising residues 105 to 108 contained the alanine to threonine exchange at position 105 (peak C, Table 10 and 11). The value obtained for alanine in the peptide comprising residues 110 to 112 (peak A, Table 11) was low as expected because alanine is partially released by dilute acid hydrolysis when it is located adjacent to and on the carboxyl side of an aspartic acid residue in a peptide (Tsung and Fraenkel-Conrat, 1965).

DISCUSSION AND CONCLUSIONS

Origin of PM6 and PM6R

PM6 was obtained after the HNO_2 treatment of the common TMV strain, U1. Its coat protein was shown to be nonfunctional and to contain an aspartic (or asparagine) to glycine and an alanine to threonine exchange in its primary structure. HNO_2 alters the TMV genome by converting cytosine (C) to uracil (U) and adenosine (A) to guanine (G) by deaminating C or A in the TMV RNA molecule. This conversion can result in a change in the triplet code words for the amino acid residues. It is not known whether it is aspartic acid or asparagine which is exchanged for glycine in the PM6 coat protein because aspartic acid is indistinguishable from asparagine in the amino acid analysis of the total coat protein. However, as previously noted (p. 50) in the peptide profile of PM6 coat protein (Fig. 4), tryptic peptide 6, which is thought to be contained in peak C, is released earlier than normal from the ion exchange column due presumably to its less acidic nature. Such elution behavior would be consistent with an aspartic to glycine exchange in this peptide but not with an asparagine to glycine exchange. It is improbable that the amino acid exchange in the coat protein is asparagine to glycine because this would necessitate the conversion of one of the code words for asparagine, AAU or AAC, to one of the code words for glycine, GGU, GGC, GGA, or GGG. It is evident that two or three nucleic acid bases would have to be converted in the same code word to effect the asparagine to glycine exchange. Such an amino acid exchange has never been reported

in any coat protein of HNO_2-induced TMV mutants examined (Table 20-1, Hennig and Wittmann, 1972), or in any other TMV strains which have a small number of amino acid exchanges in their coat proteins (Hennig and Wittmann, 1972). However, one TMV mutant, no. 470 (Rombauts and Fraenkel-Conrat, 1968), has been reported with a serine to histidine exchange in its coat protein that can only occur by the conversion of one of the code words for serine, UCU, UCC, AGU, or AGC, to one of the code words for histidine, CAU or CAC. It is evident that two or three nucleotide conversions in one of the serine code words would have to be made to effect the serine to histidine exchange but unfortunately, none of these conversions conform to the known action of HNO_2 on TMV RNA (Fig. 20-5 in Hennig and Wittmann, 1972; Schuster and Schramm, 1958). An aspartic acid to glycine exchange in the PM6 coat protein is much more probable because such an exchange is easily explained by the conversion of either of the code words for aspartic acid, GAU or GAC, to one of the code words for glycine, GGU, GGC, GGA, or GGG. It is obvious that GAU or GAC could be converted to GGU or GGC respectively by the conversion of one nucleotide, or that GAC could be converted to GGU by the conversion of two nucleotides; all of these nucleotide conversions are possible via the deamination of TMV RNA by HNO_2 (Schuster and Schramm, 1958). Furthermore, three other HNO_2-induced TMV mutants (Ni 102, Ni 103, and Ni 116) have been previously reported which have an aspartic acid to glycine exchange in their coat proteins (Wittmann-Liebold and Wittmann, 1965). Consequently, because of the previously discussed migration pattern of PM6 tryptic peptide 6 and because an asparagine to glycine exchange is not likely to have been caused by the action of HNO_2 on TMV RNA, it is

reasonable to conclude that the first amino acid exchange observed in the PM6 coat protein is an aspartic to glycine and not an asparagine to glycine exchange. The origin of the second amino acid exchange in the PM6 coat protein i.e., alanine to threonine, is not easily explained. To exchange alanine for threonine, one of the code words for alanine, GGU, GCC, GCA, or GCG, must be converted to one of the code words for threonine, ACU, ACC, ACA, or ACG, but such a conversion is not possible by the accepted mechanism of HNO_2 action. However, Vanderbilt and Tessman (1970) have reported a case of anomalous conversion of A to G in the single-stranded DNA bacteriophage S13. PM6 is not unique in having an amino acid exchange in its coat protein that would not be predicted from the action of HNO_2 because another defective mutant (PM2) obtained in the same manner as PM6 also has two amino acid exchanges i.e., threonine to isoleucine and glutamic acid to aspartic acid in its coat protein, the second of which cannot be explained by the deaminating action of HNO_2 on the genome of the parent strain (Wittmann, 1965).

It is conceivable that a single nucleotide base in the genome of a TMV variant in the U1 population, which already possessed a naturally occurring alanine to threonine exchange in its functional coat protein, was deaminated by HNO_2 to provide the additional aspartic (or asparagine) to glycine exchange observed in the PM6 coat protein. The existence of two defective mutants (PM2 and PM6) with unexplained amino acid exchanges might mean that HNO_2 acts in some way to select naturally occurring variants from the TMV population that have preexisting amino acid exchanges because the probability is small that two such mutants would be independently isolated. Such a selection mechanism might be explained by some

irregularity in the arrangement of the protein coat in a naturally occurring TMV variant with an alanine to threonine exchange in its subunit coat protein which might allow a selective deamination of some of its RNA bases by HNO_2. Naturally occurring TMV strains are known whose coat protein arrangement in their virions deviates from that of the U1 strain either by a slightly different number of coat protein subunits per turn of helix (reviewed in Caspar, 1963) or by a periodic perturbation in the packing of the subunits near the outside of the helix which leads to a small, but regular deformation of the helix in the axial direction (Caspar and Homes, 1969). It is not known whether PM6 could originate by such a selection mechanism, but preliminary evidence from electron micrographs of PM6R in vivo indicate that PM6R coat protein may have some difficulty encapsidating PM6R-RNA as evidenced by a high frequency of PM6R protein rods among PM6R virions in these micrographs. Whether this observation might mean that there is some irregularity in the arrangement of subunit proteins in the PM6R virions is unknown, but is is a provoking thought. It would be interesting to test the hypothesis that HNO_2 deaminates the RNA in the virions of TMV variants on a more selective basis than it does the RNA in virions of the U1 strain by treating PM6R virions with HNO_2 to a low survival level and then screening the survivors to see if PM6 or any other defective mutant(s) could be generated from PM6R in large numbers. Another alternative for the origin of PM6 is that HNO_2 acts in some way, other than by modifying the genetic code of TMV, to select PM6 as a naturally occurring variant from the U1 population. Such strains might exist in the U1 population and they could be encapsidated by U1 coat protein. A screening of the U1

population for such mutants by taking over 900 local lesions induced by U1 on Xanthi-nc tobacco plants and using the lesions as inoculum sources to infect Samsun tobacco plants by techniques known to preverve the infectivity of local lesions induced by defective mutants (see p. 24) failed to isolate any defective mutants. Although such an experiment is inconclusive, it does indicate that defective mutants may not be plentiful in the U1 population or that they may not exist there at all. This experiment is even more significant when it is realized that PM6 was isolated after screening approximately 100 plants inoculated with HNO_2 treated U1. Another unlikely alternative for the origin of PM6 that should be considered is that HNO_2 did alter the genome of U1 by some unknown mechanism to create the alanine to threonine exchange observed in the PM6 coat protein. Unfortunately, a detailed discussion of this alternative is not possible because a mechanism for such an amino acid exchange is not available.

When tobacco plants heavily infected with PM6 are held at elevated temperatures for short periods of time (about 1 month) in a greenhouse or at room temperature in a growth room for longer periods of time (3-6 months), a new mutant named PM6R appears in them that has a functional coat protein with an alanine to threonine exchange in its primary structure. The alanine to threonine exchange in both PM6 and PM6R coat proteins is in all probability at the same locus. This assertion is supported by the fact that peptide 8 in both proteins contains an alanine to threonine exchange (Table 7). The alanine to threonine exchange found in both of these proteins was used as a genetic marker to identify the

origin of numerous PM6R isolates (Tables 6 and 7) obtained from PM6 infected plants due to careless experimental technique.

The argument that PM6R may be a permanent contaminant of PM6 cultures should also be considered. Jockush (1968) found that a temperature sensitive TMV mutant contained about 0.5% wild type contaminants even after consecutive isolations from single lesions. He suggested that these contaminants may result from large scale mutations and from a selective disadvantage of the temperature sensitive mutant during systemic infection of the plant, but he did not consider the other alternative i.e., that a small amount of the wild type strain may be so closely associated with and replicated with the temperature sensitive strain that the two strains cannot be separated by conventional local lesion techniques. Hariharasubramanian et al. (1973) have noted this alternative in discussing the origin of one of the defective mutants with insoluble coat proteins.

The fact that PM6 can be separated from PM6R by local lesion techniques reported here and that such isolates remain PM6R-free almost indefinitely if the plants with PM6 infections are held at room temperature in a growth room and trimmed back periodically so that they contain only one or two PM6 infected leaves per plant at all times seems to indicate that PM6R is not a permanent contaminant of PM6 cultures, or that such cultural conditions do not favor PM6R growth. This latter argument concerning cultural conditions is not a strong one because in mixed infections of PM6 and PM6R in Samsun tobacco plants under the same cultural conditions described above, PM6R will rapidly become the dominant strain and will eventually totally exclude PM6 infection from the

same plant. If PM6R is a permanent contaminant of PM6 cultures, it could be present in trace amounts in the same host plant with PM6 at replicative sites, but due to the large amount of PM6 present at these same sites, PM6R replication could be surpressed. Viral interference of this type is a well documented phenomenon although the specific mechanism by which it occurs at the cellular level is largely unknown and the supression of one strain by another has never been found to be complete (reviewed by Price, 1964 and by Matthews, 1970, pp. 411-415). Because PM6R, like U1, is highly systemic and very mobile in a host plant, and because PM6 infections are predominately of a localized nature in a host plant, it seems likely that even a trace amount of PM6R in a plant with a heavy PM6 infection would be translocated to some portion of that plant that was uninfected by PM6 where it could then undergo normal replication. Since viral interference has never been shown to be complete and since PM6R is very mobile in its host plant, the possibility that trace amounts of PM6R in PM6 cultures is supressed does not seem feasible. Evidence will be discussed later (p. 82) to show that the conversion of PM6 to PM6R is a rare mutational event, yet PM6R is expressed in plants where this mutational conversion occurs. Consequently, it would seem highly unlikely that in PM6 cultures contaminated by a very minute amount of PM6R, that PM6R would fail to be expressed. Therefore, for the reasons cited above, the argument that PM6R is a permanent contaminant of PM6 cultures does not seem to be a very viable one when all of the phenomena surrounding the conversion of PM6 to PM6R are critically examined.

The results discussed above support the conclusion that PM6 is converted directly to PM6R by a one step reverse mutation in the PM6 genome which restores the aspartic (or asparagine) glycine exchange in the PM6 coat protein by changing glycine back to aspartic (or asparagine) but leaving the alanine to threonine exchange of PM6 unaltered. To describe this entire process the term "reversion" is hereafter employed. A new class of TMV mutants is created by the reversion of PM6 to PM6R and it is proposed that this new mutant class, represented by the prototype PM6R, be termed "revertants" because of their origin. As a result of the proposed reversion phenomenon, it is postulated that most wild type strains appearing in tobacco plants infected with defective strains could be revertant strains and not contaminants. A typical example of this class of TMV mutants is another revertant strain isolated from PM6 plants in this study. This strain had a coat protein whose composition was U1-like, but when the strain was inoculated into host plants, the strain produced symptoms in tobacco that were very different from those produced by U1 or PM6R. Kapitsa et al. (1969a and 1969c) and Zaitlin (1968, personal communication) have also noted the appearance of wild type strains in plants infected with certain defective strains. Kapitsa did not attempt to prove these isolates were really revertants and not contaminants. Zaitlin and Hariharasubramanian (Zaitlin, 1968) looked at the coat protein compositions of many such isolates from plants infected originally with PM5, but they were unable to show that their isolates were truly revertants rather than contaminants. The complete reversion of all of the isolates except one resulted in their having coat proteins that were identical to U1 in composition and thus

there was no means of proving they were truly revertants rather than contaminants. A change in the PM5 coat protein to a composition identical to that of U1 coat protein might be expected because the PM5 coat protein possesses only one amino acid exchange which presumably renders it nonfunctional. Consequently, PM5 is probably not a good candidate with which to investigate the reversion phenomena. However, since PM6R still contains a genetic marker found in PM6, it seems plausible that the isolates obtained by Zaitlin and Hariharasubramanian and by Kapitsa were actually revertants (as in PM6-PM6R) and not contaminants.

It is interesting to speculate that the U1-like symptoms found on plants infected with defective mutants having soluble and insoluble nonfunctional coat proteins (Smith, 1974, personal communication) may be due to revertant mutants. Currently, a mutant from a PM2 infected plant was isolated that produced U1-like symptoms on Samsun tobacco plants. This isolate is considered to be a possible revertant candidate and consequently its coat protein is currently under investigation. It is interesting to note that the coat protein of PM2 contains two amino acid exchanges (Wittmann, 1965) of which only one is permissable by the action of HNO_2 on TMV RNA. Therefore, one might speculate that the critical amino acid residue in the PM2 coat protein may be the residue that could have been substituted by the action of HNO_2 (i.e., threonine to isoleucine) and consequently this residue would be a logical choice for reversion back to the original residue found in the U1 coat protein at this position while leaving the other amino acid exchange (i.e., glutamic to aspartic) that is unexplained by the action of HNO_2

unaltered as a genetic marker in the revertant mutant of PM2 in a situation analogous to the PM6-PM6R reversion.

Plants held at room temperature with small amounts of PM6 infection can be kept PM6R-free by pruning their infected leaf areas to a minimum while plants with heavy PM6 infections held at the same temperatures occasionally become infected with PM6R. Furthermore, plants heavily infected with PM6 that are held at two different summertime greenhouse elevated temperatures for similar periods of time (see results of screening experiments 1 and 2, p. 60) show a much higher incidence of infection with PM6R than PM6 infected plants held at room temperature in a growth room. These data may indicate that the conversion of PM6 to PM6R is a relatively rare phenomenon. Stated more directly, high concentrations of PM6 in host plant leaves may provide more PM6 candidates for conversion to PM6R and thus increase the probability for the chance mutational event to occur which converts PM6 to PM6R. High leaf temperatures in infected leaves might also increase the probability of mutation and also provide a selective advantage favoring PM6R growth after the mutational event has occurred.

It is interesting to note that in all revertant isolates except one examined in this study, PM6 is always converted to PM6R. Reasons for this fact are unknown, but it is possible that only a limited number of mutational alternatives are available to PM6 and that PM6R predominates over the other alternatives because of unknown selection pressures. Another possibility is that the methods used in this study are unable to detect the occurrence of other revertant strains derived from PM6. For example, if PM6 has a conversion rate to PM6R that is higher than or

equal to the conversion rate to other possible revertant strains and a replication rate that is higher than is possible for other possible revertant strains, then PM6R might appear as often or more often than the other revertants and rapidly replicate to such an extent that all other revertant strains would not have a chance to reach a sufficient concentration level to be detected. The reason the second revertant strain found in this study was detected at all might be that PM6R did not appear in the same plant before this alternate revertant strain was well established; such a chance occurrence would be expected to happen occasionally.

Another interesting speculation for the repeated occurrence of PM6R is that there is a mechanism for directing the production of TMV strains by a method other than classical mutation. Such a speculation is not unreasonable in light of the fact that many TMV strains with multiple amino acid exchanges in their coat proteins constantly occur in U1 and defective mutant populations. Bald (1972) and Hariharasubramanian et al. (1973) have noted the occurrence of such strains and A. Siegel (1973, personal communication) has also noted that single lesion isolates taken from pure cultures of U1 eventually produce isolates of the U2 strain. The coat protein of U2 is 26% different in sequence composition from that of U1 (Rentschler, 1967; reviewed in Hennig and Wittmann, 1972). Since it is not likely that such a large number of changes in U2 coat protein composition can take place in a one step mutation and the possibility of reproducing such mutants does not seem possible if the production of these exchanges were a chance mutational phenomenon, then the case for directed mutation would seem to be a strong one. The fact that PM6 almost always reverts to PM6R is also

suggestive of a directed phenomenon, although it is acknowledged that the conversion of PM6 to PM6R probably occurs by a one step mutation.

Localization of an Amino Acid Residue Critical for Coat Protein Aggregation

It has already been noted that the defective (nonfunctional) coat protein of PM6 with an aspartic (or asparagine) to glycine and an alanine to threonine exchange in its primary structure is converted to a functional coat protein by the reversion of the aspartic (or asparagine) exchange. As a result of this deduction it is logical to conclude that the aspartic (or asparagine) to glycine in the coat protein is responsible for the inability of the protein to aggregate correctly and encapsidate PM6-RNA to form normal virions. The change from an aspartic (or asparagine) to a glycine residue would represent a considerable change in the charge of the protein and might be expected to affect the normal functioning of the protein in some manner.

The exact location of the aspartic (or asparagine) to glycine exchange is unknown, but evidence has been presented to show that the exchange is located in tryptic peptide 6 of the PM6 coat protein. Peptide 6 contains one asparagine and two aspartic residues at positions 73, 77, and 88 respectively (Tsung et al., 1964). Since an HNO_2-derived TMV mutant (Nil103) with a functional coat protein has been found (Wittmann-Liebold and Wittmann, 1965) which has an asparagine to serine exchange at position 73, it is unlikely that the asparagine residue at position 73 plays a critical role in the structure and function of the coat protein. The possibility also exists that the serine residue is a satisfactory substitution for the asparagine residue that does not interfere with the

aggregation or encapsidation properties of the protein. This latter alternative is not likely because of the difference of conformation and charge that are inherent in asparagine and serine residues. Furthermore, the difficulty of converting asparagine to glycine by HNO_2 treatment of TMV RNA has been discussed earlier. Consequently, this would mean that one of the aspartic acid residues located either at position 77 or 88 in the protein is exchanged for glycine, implying that the exchanged residue involved is truly an aspartic residue at position 88 and not the asparagine residue located at position 73. Butler and Durham (1972) have proposed two carboxyl-carboxylate pairs as crucial to the normal aggregation of coat protein subunits. Residues at positions 115 and 116 make up one probable pair and residue 145 and one other make up the other probable pair. The fourth carboxyl group is thought to be contributed by either residue 88 or 131, with 88 being the strongest candidate because of conformational data and the fact that residues 87 to 94 are one of the two regions in the poly-peptide chain of coat protein that are completely conserved in all strains (Butler and Klug, 1972). Consequently, an argument can be made that the proposed aspartic to glycine exchange in peptide 6 is located at position 88 and conversely that position 88 is the other member of the second carboxyl-carboxylate pair. A simple histogram (Fig. 15) of the locations of amino acid exchanges in previously isolated coat protein mutants obtained after HNO_2 treatment of U1 also indicate that few amino acid exchanges have ever been found in the peptide 6 region of the coat protein indicating that this region is probably an important one for the normal functioning of the coat protein. It should also be noted that the conserved sequence

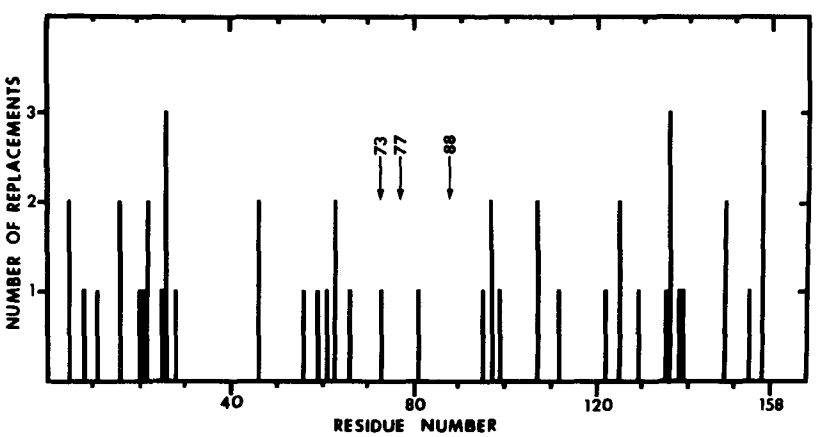

Fig. 15. Histogram of the number of times that each of the 158 amino acid residues in the coat protein of the U1 strain of TMV was replaced.

All amino acid replacements occur in reported nitrous acid induced TMV coat protein mutants compiled in Table 20-1 by Hennig and Wittmann (1972). Arrows indicate the three possible location for the asp (or asn) to gly exchange that occurs in tryptic peptide 6 of PM6 coat protein. Tryptic peptide 6 comprises residue numbers 72-90.

comprising residues 87 to 94 may also constitute part of the RNA binding site (Butler and Durham, 1972) and this may be the real reason for the inability of PM6 coat protein to form virions.

Conversion of PM6 Protein Rods to Protein Wheels *in Vivo*

It has been proposed on the basis of electron micrographic evidence that the coat protein of PM6 aggregates *in vivo* to form protein rods and wheels whose units of construction are double disks and 2½ turn helices respectively. It is interesting to speculate about the manner in which the wheels are formed and of what significance their structure may be to the theory offered by Butler and Klug (1971) for the assembly of protein rods and virions.

Durham and Klug (1971) propose that the growth of stacked disk protein rods takes place *in vivo* and *in vitro* at neutral pH conditions by the stacking of double disks of protein (each double disk is composed of two rings of 17 subunits each). In order for the stacked disk rods to be converted to protein rods with a helical conformation, the two carboxyl-carboxylate pairs on each protein subunit must be neutralized by either lowering the pH *in vitro* or by the addition of TMV RNA *in vivo*. When the pairs of carboxyl groups are neutralized, the disks can dislocate, forming two-turn helices which can interlock forming protein rods *in vitro* in which the protein subunits are in a helical conformation, or virions *in vivo* in which the coat protein subunits are helically arranged (for details see introduction section on TMV assembly, p. 2).

It is possible to speculate from the structure of the wheels about the mechanism by which they are formed. Rods of PM6 coat protein

appear to be composed of double disks that are weakly bonded end to end resulting in rods that are very flexible and loosely held together. Such rods, if of sufficient length, appear capable of bending back on themselves and annealing end to end to form wheels. It is proposed that because of the absence of a missing carboxyl group in the PM6 protein, double disks formed from this protein show a greater tendency to dislocate into two turn helices in vivo than is normally predicted for U1 protein (Butler and Durham, 1972). If the double disks in the protein wheels dislocate into two turn helices with two open ends, then it is possible that single coat protein subunits can be added to one of these ends until steric hindrance prevents further subunit addition as prescribed by the amended theory of TMV assembly (Ohno et al., 1972; Okada and Ohno, 1972). Such helices then would be approximately two and one-half turns and would be permanently locked into the wheel configuration under physiological conditions. In the many wheels that have been observed, the units composing them seem to be composed of both 2½ turn helices and double disks (Fig. 7), but with the 2½ turn helices largely predominate. The rods also seem to have 2 turn helices in regions where they are bent allowing for the addition of single subunits, as evidenced by what appear to be 2½ turn helices in these regions (Fig. 11), but such observations are uncertain because these regions are difficult to resolve.

If units making up the wheels represent mostly two turn helices that have been lengthened by the addition of extra subunits, then it is possible that the 2½ turn helices observed in the wheel configuration represent the lock washer or two turn helix postulated to exist by Butler and Klug (1971) and to play a major role in their theory on TMV assembly.

It is important to realize however, that these speculations presently have no experimental evidence to support them, other than the observation that they fit unusually well into the experimental disk model of TMV assembly promulgated by Butler and Klug. The observation that PM6 protein aggregates into rod and wheel conformations instead of remaining in the double disk form where it would be available for use in virion assembly as proposed by Butler and Klug (1971), may also be one of the reasons why PM6 is a defective strain i.e., the protein is not available for TMV assembly because it is irreversibly bound under physiological conditions in several aberrant aggregation forms.

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