578.865.1:578.233.44:578.24:576.32

MEDEDELINGEN LANDBOUWHOGESCHOOL
WAGENINGEN • NEDERLAND • 79-15 (1979)

PROTEINS SYNTHESIZED IN TOBACCO MOSAIC VIRUS INFECTED PROTOPLASTS

(with a summary in Dutch)

ROSELLA HUBER

Department of Molecular Biology, Agricultural University, Wageningen, The Netherlands

(received 6-VII-79)

H. VEENMAN & ZONEN B.V. - WAGENINGEN - 1979

Mededelingen Landbouwhogeschool
Wageningen 79-15 (1979)
(Communications Agricultural University)
is also published as a thesis

CONTENTS

SCOPE OF THE INVESTIGATION 1

1. LITERATURE REVIEW. POLYPEPTIDES SYNTHESIZED UNDER THE DIRECTION OF THE TMV GENOME IN VITRO AND IN VIVO 3
   - 1.1. Introduction 3
   - 1.2. Structure and properties of TMV 4
   - 1.3. Translation of TMV RNA in cell-free protein synthesizing systems and inside oocytes 5
   - 1.4. Genetic map of TMV 7
   - 1.5. Synthesis of polypeptides as a result of TMV multiplication in tobacco leaves and tobacco protoplasts 9

2. MATERIALS AND METHODS 12
   - 2.1. Cultivation of the tobacco plants 12
   - 2.2. Isolation of tobacco protoplasts 14
   - 2.3. Viruses and virus purification 15
   - 2.4. Infection of tobacco protoplasts with TMV 16
   - 2.5. Incubation of tobacco protoplasts 16
   - 2.6. Isolation of cowpea protoplasts 17
   - 2.7. Infection of cowpea protoplasts with TMV or C-TMV 17
   - 2.8. Incubation of cowpea protoplasts 18
   - 2.9. Infectivity assay of tobacco and cowpea protoplast extracts 18
   - 2.10. Determination of the frequency of infection of tobacco and cowpea protoplasts 18
   - 2.11. Determination of uptake of [^{3}H]-labeled amino acids in tobacco protoplasts and incorporation of [^{3}H]-labeled amino acids into tobacco protoplast protein. . . 19
   - 2.12. Determination of the specific activity of protein of subcellular fractions of tobacco protoplasts 19
   - 2.13. Determination of the specific activity of TMV coat protein 20
   - 2.14. Electron microscopy 20
   - 2.15. Analysis of protein from TMV and mock-infected tobacco and cowpea protoplasts by SDS-polyacrylamide slabgel electrophoresis 21
   - 2.16. Translation of TMV RNA in cell-free protein synthesizing systems 23
   - 2.17. Isolation of RNA 24
   - 2.18. Chemicals 24

3. TMV MULTIPLICATION IN TOBACCO PROTOPLASTS 26
   - 3.1. Introduction 26
   - 3.2. Results and discussion 27

4. INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEIN OF TOBACCO PROTOPLASTS 31
   - 4.1. Introduction 31
   - 4.2. Uptake of [^{3}H]-leucine by tobacco protoplasts and incorporation into protoplast protein 31
   - 4.3. Factors influencing the incorporation of [^{3}H]-leucine into protoplast protein . . 33
   - 4.4. Incorporation of [^{3}H]-leucine into protein of subcellular fractions 36
   - 4.5. Specific activity of TMV coat protein 37
   - 4.6. Discussion 39

5. TMV MULTIPLICATION IN COWPEA PROTOPLASTS 41
5.1. Introduction 41
5.2. Time course of TMV and C-TMV synthesis in cowpea protoplasts 42
5.3. Frequency of infection of cowpea protoplasts with TMV and C-TMV 43
5.4. Electron microcopy of TMV and of C-TMV infected cowpea protoplasts . . . 46
5.5. Factors influencing the infection of cowpea protoplasts with TMV 46
5.6. Discussion 48

6. POLYPEPTIDES SYNTHESIZED AS A RESULT OF TMV MULTIPLICATION IN TOBACCO PROTOPLASTS 50
6.1. Introduction 50
6.2. Electrophoretic analysis of protein from TMV infected and mock-infected tobacco protoplasts 50
6.3. Polypeptides synthesized as a result of TMV multiplication in tobacco protoplasts 52
6.4. Discussion 57

7. POLYPEPTIDES SYNTHESIZED AS RESULT OF TMV MULTIPLICATION IN COWPEA PROTOPLASTS 60
7.1. Introduction 60
7.2. Electrophoretic analysis of protein from TMV infected and mock-infected cowpea protoplasts 60
7.3 Polypeptides synthesized as a result of TMV multiplication in cowpea protoplasts 61
7.4. Discussion 68

8. POLYPEPTIDES IN RNA-DEPENDENT RNA POLYMERASE PREPARATIONS FROM C-TMV INFECTED COWPEA LEAVES 72
8.1 Introduction 72
8.2. Isolation of RNA-dependent RNA polymerase preparations from cowpea leaves 73
8.3. Comparison of polypeptides in RNA-dependent RNA polymerase preparations from healthy and C-TMV infected cowpea leaves. 78
8.4. Discussion 82

CONCLUSION 85

SUMMARY 87

SAMENVATTING 91

REFERENCES 95

ABBREVIATIONS 103

SCOPE OF THE INVESTIGATION

Much research has been carried out on the tobacco mosaic virus (TMV). TMV is an extremely stable virus and multiplies with great ease in infected tobacco plants. For that reason it is relatively simple to prepare large amounts of purified TMV and the properties of the virus particles have been extensively studied. TMV is a rod-shaped plant virus, 300 nm long and about 18nm in diameter, built up of a monopartite single-stranded RNA of molecular weight 2.1×10^6 and encapsidated by approximately 2,100 similar protein molecules of molecular weight 17,500.

Although much is known of the structure of the TMV particles and their physical and chemical properties, little is known about the mechanism of the virus multiplication process in the infected host cell. Studies of plant virus multiplication have been lagging behind similar studies with bacteriophages and animal viruses because of the absence of cultures of isolated cells which could be infected synchronously.

In 1969 TAKEBE and co-workers developed an enzymatic method for the preparation of large numbers of protoplasts from tobacco leaf tissue and they have demonstrated that these isolated protoplasts can be infected with TMV. The virus multiplication in the cells is approximately synchronous. Thus in essence a cellular system is available for studies of the multiplication of plant viruses.

It has been shown that plant protoplasts can be infected with many plant viruses. However, only a few studies have been made of the virus multiplication process in the protoplasts, of the identification of proteins coded by the virus and of their possible functions in the virus multiplication process. Although not much work has been done in this field it has revealed that there are in fact a few proteins which are synthesized as a result of TMV multiplication in the infected protoplasts.

In recent years efforts have been made to identify the proteins encoded in the TMV RNA by using TMV RNA as a messenger in cell-free protein synthesizing systems. These *in vitro* studies have yielded much information, but at the same time they have raised various questions about the mechanism of virus RNA replication and viral protein synthesis. This situation necessitates *in vivo* studies of TMV multiplication.

This thesis deals with research on the multiplication of TMV in leaf cell protoplasts. An attempt has been made to answer the following questions: 1. Which proteins are synthesized in TMV infected protoplasts as a result of TMV multiplication? 2. Which of the synthesized proteins are made under the direction of the TMV genome and, if any, which of the proteins are host specific? 3. In which functions are these proteins involved?

In chapter 1 the data from the literature relating to the proteins synthesized under the direction of the TMV genome *in vitro* and *in vivo* are reviewed.

Materials and methods are described in chapter 2.

Chapter 3 deals with the influence of the culture conditions of the tobacco plants on the viability and susceptibility to infection of the protoplasts.

Chapter 4 is concerned with the detection capacity for proteins which are synthesized after TMV infection of tobacco protoplasts.

In chapter 5 the influence of various conditions on the TMV infection in cowpea protoplasts is reported.

Chapter 6 describes proteins synthesized as a result of TMV multiplication in tobacco protoplasts.

Subsequently, cowpea protoplasts were used for the same purpose. The proteins detected in this protoplast system are described in chapter 7. In order to determine which of the proteins are TMV coded and which ones are host specific, a comparison was made between the proteins synthesized as a result of TMV multiplication in TMV infected cowpea protoplasts and those proteins synthesized in TMV infected tobacco protoplasts. The TMV coded proteins, synthesized in the different hosts are supposed to be similar, while the host specific proteins synthesized as a result of TMV multiplication differ from host to host.

Chapter 8 deals with the question in which process of the TMV replication the proteins might be involved.

1. LITERATURE REVIEW. POLYPEPTIDES SYNTHESIZED UNDER THE DIRECTION OF THE TMV GENOME IN VITRO AND IN VIVO

1.1. INTRODUCTION

A detailed investigation into the virus infection of plant cells was much impeded, by the fact that this work could only be done with intact leaves or fragments of leaf tissue. After inoculation of the leaves the virus infection does not run synchronously. If the TMV infection and multiplication is synchronous, it is possible to detect proteins synthesized in small amounts as a result of this process and to follow the course of their synthesis after infection. Thus for example the synthesis of MS_2 bateriophage coded proteins has been investigated in *Escherichia coli* spheroplasts, in which the MS_2 phage infection and multiplication runs synchonously (VIÑUELA *et al.*, 1967; KOZAK and NATHANS, 1972).

Various efforts have been made to develop a plant system in which the virus infection and multiplication have a synchronous course. It has been attempted to separate cells from tobacco leaves (ZAITLIN, 1959) or culture tobacco callus tissue and then to infect these cells with TMV (MOTOYOSHI and OSHIMA, 1968; MURAKISHI *et al.*, 1970, 1971). DAWSON and SCHLEGEL (1976) tried to synchronize the course of TMV multiplication in tobacco plants by infecting leaves systematically at non-permissive temperature. After infection the plants were cultivated at 25° C. COCKING and co-workers (COCKING, 1966; COCKING and PONJAR, 1968) isolated protoplasts from tomato fruit tissue for the same purpose.

All these systems did not meet the demands for a detailed study. TAKEBE succeeded in isolating cells from tobacco leaves, digesting the cell wall and infecting the protoplasts with TMV (TAKEBE and OTSUKI, 1969; OTSUKI and TAKEBE, 1969). In this way a suspension of leaf cell protoplasts may be obtained of which a great number is present and of which a high percentage can be infected. The exponential virus multiplication has a synchronous course. This system provides the possibility to study the proteins synthesized as a result of TMV multiplication *in vivo*.

However, the host protein synthesis is not suppressed by the TMV replication nor can it be inhibited effectively. Furthermore the host reacts to the virus multiplication by quantitative changes of the host protein constitution (VAN LOON, 1975, 1976; GIANINAZZI *et al.*, 1977) and may participate in this process. The involvement of the host in the process of the TMV multiplication is indicated by the fact that actinomycin D inhibits the TMV multiplication, when added at an early stage of infection (DAWSON and SCHLEGEL, 1976) Therefore the proteins synthesized upon TMV infection *in vivo*, may be host or TMV coded proteins.

An answer to the question which proteins are synthesized under the direction of the TMV genome has been yielded by studying which products are synthesized under the direction of the TMV RNA in cell-free protein synthesizing systems. A

protein synthesized *in vivo* as a result of the TMV multiplication may be identified to be either virus coded or host coded by determining whether the protein synthesized *in vivo* is similar to a product synthesized *in vitro* under the direction of the TMV RNA.

On the other hand research on proteins synthesized in cell-free protein synthesizing systems leaves open the question whether the products synthesized *in vitro* are of functional importance in the process of the virus infection and multiplication. The investigation on virus proteins formed *in vivo* and *in vitro* therefore support each other.

1.2. Structure and properties of TMV

The rod-shaped TMV particle, about 300 nm long, with a diameter of 18 nm, is composed of about 95 % protein and 5.1 % RNA. The intact virus consists of a helical rod of 2,130 similar coat protein monomers, having 16 1/3 coat protein subunits per turn, with the single-stranded RNA intercalated between successive turns at a radius of 4.0 nm. This structure is extremely compact, with the protein subunits in tight contact, affording maximum protection to the RNA. The native and reconstituted TMV particles have been found to be ribonuclease-resistant (Fraenkel-Conrat, 1956). The M.W. of the TMV particle is 39.4×10^6. The structure and properties of TMV have been reviewed in detail by several authors (Kozloff, 1960; Reddi, 1972; Zaitlin and Israel, 1975; Markham, 1977).

TMV multiplies with great ease in leaves of tobacco plants of various cultivarieties. During 5 days following inoculation about 1 mg virus per g tobacco leaf can be synthesized (Reddi, 1972). It is a monopartite genome virus. Intact TMV RNA of M.W. 2.1×10^6 is infectious (Gierer and Schram, 1956; Fraenkel-Conrat, 1956; Gierer, 1958).

The 5′-end of TMV RNA has a cap structure. The cap has the formula $m^7G^{5'}ppp^{5'}Gp$ (Zimmern, 1975; Keith and Fraenkel-Conrat, 1975). The 3′-end of TMV RNA can be enzymatically aminoacylated with histidine (Öberg and Philipson, 1972; Salomon *et al.*, 1976). Removal of 5 to 10 nucleotide residues from the 3′-terminus results in loss of infectivity. This may indicate that the site of the 3′-end of TMV RNA is involved in the TMV infection and/or multiplication (Salomon *et al.*, 1976).

The TMV coat protein of M.W. 17,533 is composed of 158 amino acids. The total amino sequence is known (Anderer *et al.*, 1960, 1965; Zaitlin and Israel, 1975).

Already in 1962 it has been shown by analysis of amino acid exchanges in the coat protein of many mutants induced by HNO_2, that the viral RNA is of the (+) type and contains the sequence equal to the coding sequence of the TMV coat protein (Wittmann, 1959; Wittmann-Liebold and Wittmann, 1965; Wittmann and Wittmann-Liebold, 1966). Nucleotide sequence analysis of TMV RNA (Richards *et al.*, 1974; Guilley *et al.*, 1975a, 1975b) has confirmed the results of 1962.

1.3. TRANSLATION OF TMV RNA IN CELL-FREE PROTEIN SYNTHESIZING SYSTEMS AND INSIDE OOCYTES

The TMV RNA will code for more proteins than only coat protein, because the size of TMV RNA suggests a coding capacity for a protein of approximately 230,000 daltons, estimated on the 6,394 bases of TMV RNA (CASPAR, 1963; ZAITLIN and ISRAEL, 1975) and an average M.W. of the amino acids of 110. When it was first examined which products are synthesized under the direction of TMV RNA in cell-free protein synthesizing systems, the expectation was, that coat protein as one of the main translation products would be found. However, translation from TMV RNA in various cell-free protein synthesizing systems did not yield a detectable amount of a product similar to coat protein. At the most a small fraction of the products synthesized *in vitro* contained amino acid sequences in common with coat protein (SCHWARTZ, 1967; ROBERTS *et al.*, 1973; EFRON and MARCUS, 1973; DAVIES and KAESBERG, 1974; FRAENKEL-CONRAT *et al.*, 1977: DAVIES, 1979).

The solution to the dilemma that coat protein is not synthesized *in vitro* under the direction of TMV RNA extracted from virus particles and, in contrast to this, is synthesized in large amounts in TMV infected leaves, has been obtained from the study of the synthesis of TMV RNA in inoculated tobacco leaves.

Besides the viral RI, RF and the RNA of M.W. 2×10^6, a definite sized low molecular weight component (termed LMC) of estimated M.W. 350,000 has been found (JACKSON *et al.*, 1972). The LMC is not actinomycin D sensitive, not ribonuclease resistant and anneals with denatured double-stranded TMV RNA. Therefore this RNA component is a fragment of total TMV RNA. The LMC cannot be encapsidated *in vivo* (SIEGEL *et al.*, 1973).

It has been possible to purify the LMC in sufficient quantity for *in vitro* translation studies. When LMC is added to the wheat germ cell-free protein synthesizing system or injected into Xenopus oocytes, a product is synthesized that comigrates with coat protein purified from TMV particles, while the polypeptide patterns of translation products of RNA extracted from virus particles lack a polypeptide with M.W. 17,500 (KNOWLAND *et al.*, 1975; HUNTER *et al.*, 1976; SIEGEL *et al.*, 1976). The product formed *in vitro* under the direction of LMC is coat protein as has been demonstrated by immunoprecipitation (SIEGEL *et al.*, 1976), fingerprinting of tryptic digests and reconstitution experiments (KNOWLAND *et al.*, 1976; HUNTER *et al.*, 1976).

The LMC will be the genuine mRNA for coat protein *in vivo*, for it contains a cap at the 5′-terminus as has been demonstrated by GUILLEY *et al.* (1979). It has been reported that RNA, which resembles LMC, is present in polyribosomes (BABOS, 1971; BEACHY and ZAITLIN, 1975; SKOTNICKI *et al.*, 1976). Moreover, other strains than the common strain of TMV have a small messenger RNA of similar size, which can be translated into coat protein *in vitro*. This has been demonstrated for the cowpea strain of TMV, termed C_c-TMV or B-TMV, by an American and an Australian group of workers, respectively (BRUENING *et al.*, 1976; BEACHY *et al.*, 1977; WHITFELD *et al.*, 1976), termed C-TMV in this thesis,

and a strain of TMV originally isolated from wheat in Kansas, termed K-TMV (BEACHY *et al*, 1977).
The subgenomic mRNA for the coat protein of C-TMV is encapsidated into rods of 40 nm. The encapsidation has facilitated the separation of the small RNA from total virion RNA. For this reason it has been preferred to use C-TMV to characterize the subgenomic mRNA for coat protein.

TMV coat protein is not synthesized under the direction of virion TMV RNA in cell-free protein synthesizing systems. However, two other products are reproducibly formed. When virion TMV RNA is translated inside Xenopus oocytes or in rabbit reticulocyte lysates a polypeptide of M.W. approximately 140,000 to 100,000 (designated p135) is the main product, detected after SDS-PAGE. Both systems may produce under proper conditions a small amount of a second large polypeptide of M.W. about 165,000 (designated p165) (KNOWLAND, 1974; HUNTER *et al.*, 1976).
In later studies, when the wheat germ cell-free system was optimalized for translation into large polypeptides, TMV RNA could be translated also into a product p165 in addition tot p135 and smaller products. However, the appearance of the largest product is variable and trace amounts are formed only (KNOWLAND *et al.*, 1975; ZAITLIN *et al.*, 1976; DAVIES *et al.*, 1977). Since the sum of the M.W. of p165 and p135 exceeds the coding capacity of TMV RNA p165 and p135 are translated from an overlapping nucleotide sequence.
It is possible that the smaller polypeptide arises by proteolytic cleavage of the larger polypeptide. This is inconsistent with their stability during prolonged chases with unlabeled amino acids *in vitro* (PELHAM, 1978). It has been shown by tryptic peptide fingerprinting of the p165 and p135 that the polypeptides give similar patterns except for the presence of two extra polypeptides in the pattern of the largest product. When amber suppressor tRNA, purified from appropriate strains of yeast, is added to the rabbit reticulocyte lysate, the amount of p165, synthesized under the direction of TMV RNA, increases manifold. Therefore the p165 is most probably a readthrough product of p135 (PELHAM, 1978).

When small rods of C-TMV are purified from C-TMV preparations, another class of rods, less than full-length viral rods, have been detected. RNA of different sizes are extracted from these rods. One class of RNA has a M.W. 0.65×10^6, designated I_2-RNA. RNA of similar M.W. as I_2 C-TMV RNA can be extracted from less than full-length TMV and K-TMV rods. When I_2-RNA of C-TMV, TMV or K-TMV is added to wheat germ cell-free protein synthesizing extracts, the RNA is translated into a predominant product of M.W. 30,000, or two products of M.W. 30,000 and 29,000, which have similar tryptic digest maps. Thus, these two products have overlapping amino acid sequences (BEACHY and ZAITLIN, 1977). Furthermore, the translation products from I_2-RNA and coat protein do not share common amino acid sequences, as has been shown by comparison of the fingerprints after tryptic digestion.

The products synthesized under the direction of I_2-RNA from the three strains have similar M.W. It has been shown, however, that the translation products from I_2-RNA of C-TMV and TMV have no common tryptic peptides. The

products formed under the direction of RNA, extracted from full-length rods of the three strains, also have similar M.W. (BEACHY and ZAITLIN, 1977).

The mRNAs of TMV and the products synthesized under their direction in a cellfree synthesizing system are shown in figure 1.1.

In summary the TMV genome contains the coding sequence of five products. Two polypeptides of M.W. approximately 165,000 and 135,000 are formed from large RNA (L-RNA) in cell-free systems, the larger is a readthrough product of the smaller. Two polypeptides of M.W. approximately 30,000 and 29,000 are synthesized under the direction of I_2-RNA *in vitro*; these two polypeptides have nearly similar peptide patterns after tryptic digestion. And coat protein is synthesized under the direction of LMC (S-RNA).

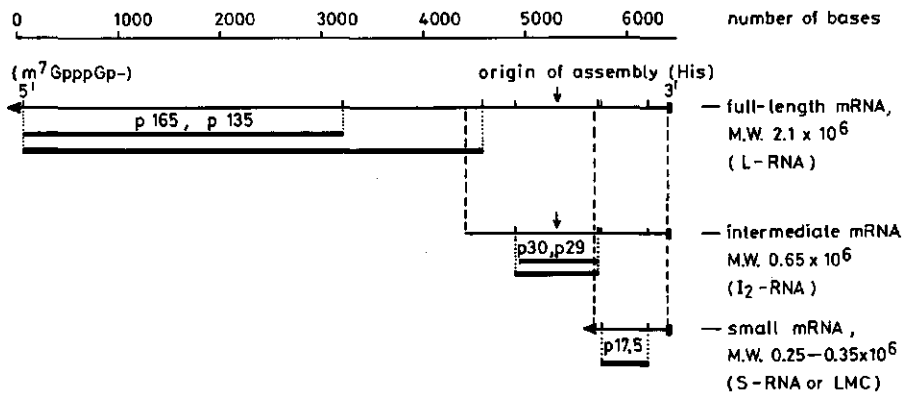


FIG. 1.1. Genetic map of TMV.
The positions of the nucleotide sequences coding for p165, p135, p30, p29 and p17,5 (coat protein) are indicated on full-length, intermediate and small TMV mRNA respectively, and nucleotide sequences corresponding with the mRNAs and with the cistrons are marked. The number of nucleotides per cistron are calculated from the estimated M.W. of the polypeptide, multiplied by 3/110 (assuming a mean M.W. of amino acids of 110). The M.W. of p135 is estimated 116,500 instead of 135,000 based on the revised M.W. of β-galactosidase, which is 116,200 daltons, calculated from its amino acid composition (FOWLER and ZABIN, 1977). The position of the cistrons, with the exception of the coat protein cistron, and of the site from where the assembly of TMV RNAs and coat protein starts are as yet not known with precision. The results of GUILLEY *et al.* (1979) have provided the possibility that the termination site of the cistron of p30 is only separated two nucleotides from the cistron of coat protein. These authors have further shown that the S-RNA contains a cap and consists of about 720 nucleic acids. The five polypeptides shown in the figure are translated in cell-free protein synthesizing systems under the direction of the TMV (subgenomic) mRNAs. It has not been established with certainty if p165, p30 and p29 are also synthesized *in vivo*.

1.4. GENETIC MAP OF TMV

Figure 1.1. gives the three known mRNAs of TMV and the five known polypeptides synthesized under the direction of the respective mRNAs in cell-free protein synthesizing systems; and the figure shows the location of the coding sequence or the sequences equal to the coding sequence on the different RNAs.

The S- and also I_2-RNA have sequence relationship to L-RNA; moreover, S- and I_2-RNA have sequences in common. This has been demonstrated by hybridization competition experiments (BRUENING *et al.*, 1976; BEACHY and ZAITLIN, 1977). Furthermore the S- and I_2-RNA both have relationship to the traject at the 3′-end of L-RNA and the S-RNA is a subset RNA, which has sequences in common with the traject at the 3′-end of I_2-RNA. It has been shown that S- and I_2-RNA can be enzymatically aminoacylated to the same extent as L-RNA. SALOMON *et al.* (1976) demonstrated that aminoacylation of TMV RNA is possible only at the 3′-terminus.

Definite proof that the sequence equal to coat protein mRNA of TMV is near the 3′-terminus of the full-length RNA has been given by HUNTER *et al.* (1976). This RNA of about 750 nucleotides has oligonucleotides in common with an alkali-stable fragment of TMV RNA, which contains 1,000 nucleotides from the 3′-terminal of TMV RNA (ÖBERG and PHILIPSON, 1972; PELHAM and WILSON, 1976) and codes for several residues of coat protein. Moreover, the purified RNA coding for coat protein probably contains a short sequence similar to part of the last 20 nucleotides from the 3′-end of intact virion RNA (GUILLEY *et al.*, 1975). In 1979 these authors have determined the sequence of 1,000 nucleotides at the 3′-end of TMV RNA, demonstrating the coat protein cistron to be situated 204 nucleotides from the 3′-terminus.

It has been thought for several years that the coat protein gene (KADO and KNIGHT, 1968) and also RNA fragments of known nucleotide sequence (GUILLEY *et al.*, 1974) and the nucleotide sequence at the origin of assembly (BUTLER and KLUG, 1971; GUILLEY *et al.*, 1971) were located at the opposite site of TMV RNA, namely near the 5′-end. These locations have been determined after partial removal of the coat protein by SDS (MAY and KNIGHT, 1956) or disassembly in akali (ONDA *et al.*, 1970). The sequential loss of protein initiates predominantly from one end of the TMV particles. The exposed RNA-terminus has been identified by phosphodiesterases. The results with these enzymes have been misleading, owing to the fact that TMV RNA contains a cap structure at the 5′-end as has been shown later (ZIMMERN, 1975; KEITH and FRAENKEL-CONRAT, 1975). Therefore polar stripping of coat protein by SDS or alkaline treatment proceeds from the 5′-end in the direction of the 3′-end of TMV RNA, contrary to previous conclusions (PELHAM and WILSON, 1976; WILSON *et al.*, 1976; OHNO and OKADA, 1977).

In figure 1.1. the sequence coding for p135 and p165 is located between the 5′-terminus of L-RNA and the traject of S-RNA. The reason of this location is that a C-TMV RNA fragment of full-length RNA of M.W. 1.4×10^6, which contains the nucleotide sequence between the 5′-terminus of L-RNA and the traject of S-RNA, has the capacity to code for a product *in vitro* with electrophoretic mobility equal to the electrophoretic mobility of the polypeptide of M.W. 135,000 synthesized in C-TMV infected leaves (BEACHY *et al.*, 1976). TMV RNA with small deletions at the 5′-terminus cannot be translated into a product of similar size (HUNTER *et al.*, 1976). Moreover, partially stripped virus of TMV, of which the 5′-end of the RNA is exposed, can bind wheat ribosomes (HASHIMOTO and

OKAMOTO, 1975) and nucleic acid sequence studies have demonstrated that the first initiation codon is situated 68 nucleotides from the 5'-terminus of TMV RNA (RICHARDS *et al.*, 1978; JONARD *et al.*, 1978). Therefore it seems likely that the sequence coding for the large polypeptides starts at the initiation codon 68 nucleotides from the 5'-end of full-length RNA.

To date it is unknown how subgenomic mRNAs are generated from full-length RNA. It has been suggested that S-RNA is copied from L-RNA (WHITFELD and HIGGINS, 1976). When L-RNA of C-TMV, highly purified by repeated sucrose gradient centrifugation, is inoculated on bean leaves, S- and L-rods are formed. Furthermore the results do not indicate why full-length RNA does not function as messenger for coat protein and p30, and why coat protein is not synthesized under the direction of I_2-RNA.
Which of the five *in vitro* translation products are similar to polypeptides synthesized *in vivo* is dealt with below.

1.5. SYNTHESIS OF POLYPEPTIDES AS A RESULT OF TMV MULTIPLICATION IN TOBACCO LEAVES AND TOBACCO PROTOPLASTS

Five polypeptides have been detected in a first investigation on polypeptides, synthesized upon TMV multiplication in tobacco leaves some days after inoculation. The polypeptides have been distinguished after SDS PAGE as variations in ratio plots of [^{3}H]- and [^{14}C]- leucine incorporated into polypeptides of TMV or mock-inoculated leaves. The molecular weights of the polypeptides have been estimated 245,000, 195,000, 155,000, 37,000 and 17,500. The polypeptide of M.W. 17,500 comigrates with coat protein and is apparently coat protein.

TABLE 1.1. Polypeptides synthesized as a result of TMV multiplication in TMV infected tobacco leaves and protoplasts.

M.W. × 10^{-3}					References
TMV specific polypeptides in leaves					
245	195	155	37	17,5	ZAITLIN and HARIHARASUBRAMANIAN (1970, 1972)
–	–	–	–	17,5	SINGER (1971, 1972); SINGER and CONDIT (1974)
–	150	130	–	n	SCALLA *et al.* (1976)
–	n	130	–	n	SCALLA *et al.* (1978)
TMV specific polypeptides in protoplasts					
–	–	140	–	17,5	SAKAI and TAKEBE (1972)
–	180	140	–	17,5	SAKAI and TAKEBE (1974)
–	165	135	–	17,5	PATERSON and KNIGHT (1975)
–	160	135	–	17,5	SIEGEL *et al.* (1978)

The M.W. of the TMV specific polypeptides have been estimated by means of SDS-PAGE, described by LAEMMLI (1970) of MAIZEL (1971). The polypeptide of M.W. 17,500 is coat protein.
–: the polypeptide has not been observed.
n: the polypeptide has not been determined.

It is synthesized in large amounts (ZAITLIN and HARIHARASUBRAMANIAN, 1970, 1972). It is always possible to find back coat protein (SINGER, 1971, 1972; SINGER and CONDIT, 1974). The polypeptides of M.W. 195,000 and 155,000 have been found back after improving the detectability. In that case polypeptide patterns in SDS-polyacrylamide gels have been visualized by staining of the gels or autoradiography (SCALLA *et al.*, 1976, 1978), so that individual polypeptides of TMV and mock-inoculated leaves were compared by direct observation. The polypeptides of M.W. 245,000 and 37,000, designated p245 and p37 respectively, have not been detected again, see table 1.1. A reason for this is, that the large amount of different host polypeptides formed independently from the TMV infection, obstructs the detection of the TMV specific polypeptides.

The predominating host protein synthesis might be the cause that in TMV infected tobacco protoplasts only the three polypeptides have been detected that have repeatedly been demonstrated in tobacco leaves. It has been tried to inhibit the host protein synthesis by U.V. irradiation of the protoplasts, but effective inhibition is accompanied by a strong decrease of the virus multiplication.
Table 1.1. shows that the estimated M.W. of corresponding polypeptides are rather verging. This is caused by the method of detection of the virus specific polypeptides, used by ZAITLIN and HARIHARASUBRAMANIAN (1970, 1972) and SAKAI and TAKABE (1972, 1974).
For the purpose of this thesis the two polypeptides of M.W. 195,000 to 160,000 and of M.W. 155,000 to 130,000 synthesized in tobacco leaves and protoplasts after TMV infection will be referred to as p165 and p135 respectively, the latter having a M.W. nearly equal to *Escherichia coli* β-galactosidase of M.W. 130,000, estimated by SDS-PAGE (WEBER and OSBORN, 1969).

It has been demonstrated by means of coelectrophoresis and immunoprecipitation that the polypeptide of M.W. 17,5000 synthesized in TMV infected protoplasts is coat protein. Moreover, the polypeptide of M.W. 17,500, termed p17,5, does not contain methionine and histidine, amino acids, which are lacking in coat protein.

By means of protoplasts which are infected with TMV it has been possible to investigate the differential synthesis of p165, p135, p17,5 and of TMV RNA and virus particles.
This made it possible to obtain an indication, that TMV specific proteins are involved in the TMV multiplication. The synthesis of p135 and TMV RNA preceeds the production of virus particles by 4–5 hours and the time course of synthesis of p135 and TMV RNA is similar (AOKI and TAKEBE, 1975). The rate of synthesis of p165 and p135 increases the first 24 hours post infection and declines thereafter, while the rate of synthesis of coat protein and virus particles continues for hours. The rate of synthesis of p165 lags behind the rate of synthesis of p135 (PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978). Since the time course of the synthesis of p135 and of TMV RNA nearly coincides, it is surmised that p135 is involved in the RNA replication.
The host protein synthesis in protoplasts cannot be effectively inhibited; therefore it cannot be decided whether p165 and p135 are either virus coded or host

coded polypeptides, although their synthesis is correlated to the TMV replication.
The answer to this, with respect to p135, has been found by comparing p135 synthesized in TMV infected tobacco leaves to p135 synthesized in a cell-free protein synthesizing system under the direction of TMV RNA. The similarity of their electrophoretic mobility and cyanogen splicing products is very convincing. Therefore it has been concluded that p135 synthesized in TMV infected tobacco protoplasts and leaves is a TMV coded polypeptide (SCALLA *et al.*, 1978).

Such a comparison has not been made between the p165 formed *in vivo* and synthesized *in vitro* under the direction of TMV RNA. Therefore it has not been ascertained whether p165 synthesized *in vivo* is a virus coded protein and whether the readthrough product synthesized *in vitro* is of functional importance. Nor has it been ascertained whether p30 synthesized under the direction of the TMV RNA in a cell-free protein synthesizing system is the same as the polypeptide of M.W. 37,000, presumably present in TMV infected tobacco leaves. It is certain of coat protein and of p135 only that these are TMV coded proteins. It has not yet been determined how the remaining coding capacity of the TMV RNA corresponding to one or more polypeptides, not exceeding 100,000 daltons, is utilized.

2. MATERIALS AND METHODS

2.1. CULTIVATION OF THE TOBACCO PLANTS

The tobacco cultivarieties that were used for isolation of leaf protoplasts were *Nicotiana tabacum* L. cv. Samsun, Samsun NN and Xanthi nc. The plants were cultivated in a phytotron under the following conditions.

Sowing. Tobacco seed was used which was stored dry in a ventilated room and which had been harvested at most 18 months before. The seed was thinly spread in earthenware flowerpots of 12 cm diameter and 12 cm height. The soil was then pressed down rather firmly and carefully watered. The pots were put into the phytotron, covered with a sheet of paper to prevent drying out. As soon as the seed had visibly germinated after ± 5 days the sheet was removed (figure 2.1.).

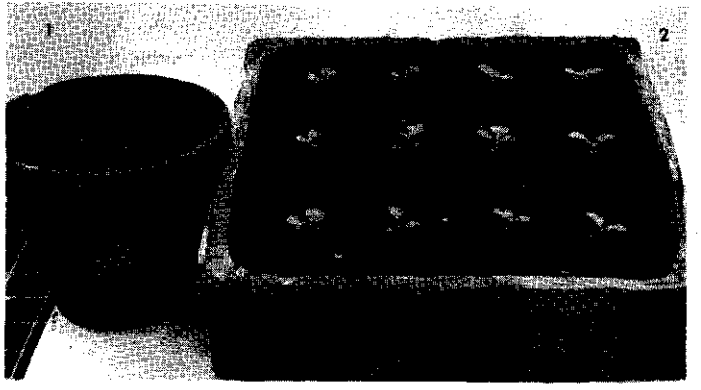
Pricking out. Two weeks after the date of sowing 16 of the largest plants having 5 leaves were pricked out in a square earthenware container of 30 by 30 cm and 10 cm height. The soil was pressed rather firmly and watered (figure 2.2.).

Potting. After another two weeks 6 to 8 plants, with a root system that was extensively branched, were selected from these. They were potted off in flower pots of 12 cm diameter and 12 cm height, taking care not to damage the root system. The soil was watered and the flower pots were put into damp peat litter (figure 2.3.). Finally, after another two weeks those tobacco plants were potted off again which at the outside of the clod showed a well-developed branching of the roots. Before potting the large flowerpots of 20 cm diameter and 20 cm height were thoroughly wetted, in this way not too much water was drawn from the soil after potting off. The soil of the plants to be potted off was watered, so the contents could be taken out as a whole. The soil in the large pots was pressed down gently and watered after potting off. The large pots were put into containers filled with damp peat litter (figure 2.4.).

Watering the soil was watered with tap water of ± 25 °C once a day. Up till six weeks after sowing the soil was kept moderately damp, the plants in the large pots were watered more plentifully along the rim of the pot, so the soil did not silt up. The peat was kept damp in such a way that there was no water at the bottom of the container.

Peat and kind of soil. The peat was kept loose and renewed every two months. No fertilizer was added. The soil was used only once, and was brought onto room temperature before using. The soil used for sowing and for the next two stages consisted of three quarters Triomf no 17S pot soil and one quarter sharp sand. The tobacco plants were then put into large pots containing Triomf no 17S (TRIO B.V., Westerhaar, the Netherlands) and home-made pot soil (1:1).

Samples of Triomf no 17S were analysed by the Proefstation voor Groente en Fruitteelt onder glas (Research Station for hothouse horticulture), Zuidweg 38, Naaldwijk, The Netherlands. It appeared that the soil had a pH of ± 5.5, a normal content of organic ingredients, a moderate amount of calcium carbonic



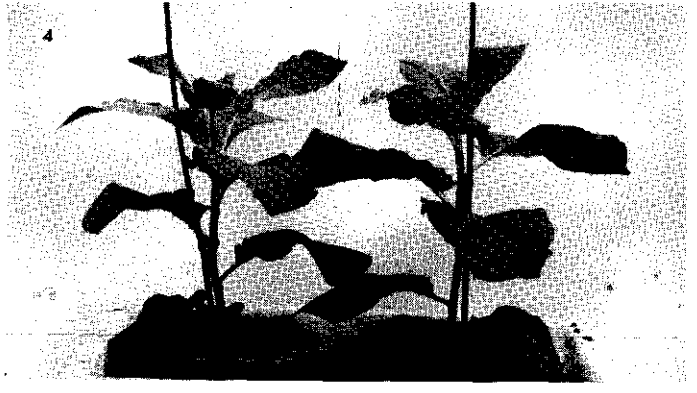


FIG. 2.1. to 2.5. Cultivation of tobacco plants, *Nicotiana tabacum* L. cv. Xanthi nc for isolation of protoplasts. The leaf, indicated by an arrow (figure 2.5.) could be used for protoplast isolation.

acid. The total content of salt and chlorine was low. The soil was rich in nitrogen and potassium and contained a fair amount of phosphor and magnesium.

Temperature, humidity and ventilation. The temperature in the phytotron was kept at 25° C. The same temperature was kept on during the day and the night. The relative humidity was about 80 %. The air was ventilated.

Lighting. The tobacco plants were cultivated under super high pressure mercury lamps of type HPLN of 400 Watt (Philips Nederland n.v., Eindhoven, The Netherlands). The light intensity at the top of the plant amounted to 22,000 erg/cm²/sec, measured with a micro-ampère meter MX309A (Société des Produits Industriels ITT, Annecy, France). The six weeks old Samsun NN and Xanthi nc tobacco plants were placed under high pressure sodium vapour lamps, type SON, of 400 Watt (Philips Nederland n.v., Eindhoven, The Netherlands) at a light intensity of 15,000 erg/cm²/sec, and after a week at a light intensity of 22,000 erg/cm²/sec, measured at the top of the plant. Glow lamps, in addition to HPLN and SON lamps, did not appear to be necessary. The pots were placed on adjustable platforms, so the light intensity at the top of the plant could be kept constant during growth.
The daylight period was 14 hours.
Five mm thick perspex sheets of founded acrylate (Vink, Didam, The Netherlands) were placed under the the lamps, absorbing light of wavelength below 390 nm.

2.2. Isolation of tobacco protoplasts

Selection of the tobacco leaf. From one tobacco plant of approximately 60 days, one or two leaves could be picked for isolation of protoplasts. Sometimes it was possible to harvest another one or two leaves of the same tobacco plant. In many cases that leaf was selected that grew one or two internodia higher on the main stem than the largest leaf that was fully stretched. The leaf selected was nearly fully stretched (figure 2.5.). The stem of such a leaf was more supple than of the fully stretched leaves. The light intensity on the surface of the leaf to be harvested was 15,000 to 21,000 erg/cm²/sec. The leaves were harvested from those tobacco plants which had a firm stem, were expected to grow in length still quite a bit, did not show any flower buds and had a well-developed root system.

Isolation of tobacco protoplasts. The tobacco protoplasts were isolated by means of the two step method as described by Takeby *et al.* (1968) and by Takeby and Otsuki (1969) or of the method as described by Meyer with some modifications (Meyer 1974; Meyer and Abel, 1975). Using the latter method the lower leaf epidermis was peeled off with a forceps and pieces of one leaf were placed in a 100 ml Erlenmeyer flask containing 20 ml enzyme solution (0.7 M mannitol, 0.04 % pectinacidtranseliminase (PATE), pH 6.5). All glassware was sterilized. The leaf tissue was infiltrated *in vacuo* for 5 min. with this medium and then the flasks were placed in a shaker water bath at a frequency of 120 excursions per min. for 10 min. at 25 °C. The maceration medium, which contained some broken cells,

was decanted and replaced by 20 ml of a sterilized salt enzyme mixture (2.5 % KCl, 1 % cellulase and 0.05 % pectinol fest, adjusted to pH 5.6 with KOH; before adjusting the pH the solution was centrifuged at 10,000 rpm in a Sorvall SS34 rotor for 15 min. to remove insoluble material, thereafter the solution was adjusted to pH 5.6 and sterilized by filtration through millipore filters, pore size 0.45 μm). Then the incubation was continued for 1½ to 2 hours at room temperature. The mixture containing released protoplasts was filtered through nylon gauze and the protoplasts were collected by centrifugation at 100 x*g* for 2 min. in a clinical centrifuge. The protoplasts were washed at least three times by resuspending them in sterilized 0.7 M mannitol solution and sedimenting at 100 x*g* for 1 min. The concentration of protoplasts in the suspension and the number of viable protoplasts were determined by counting in a Fuchs Rosenthal haemocytometer (Erma Tokyo) under the light microscope.

2.3. Viruses and virus purification

TMV. The common strain of TMV used was the U_1-strain present in Wageningen. TMV was prepared from Samsun tobacco leaves by a combination of methods as described by Knight (1962) and Hebert (1963). Frozen leaf tissue was homogenized with a mortar and pestle on ice. Three percent (w/w) solid K_2HPO_4 and 100 ml of 0.1 M NaH_2PO_4 buffer (pH 7) were added to 100 g of leaf material during homogenization for maintaining the pH near neutrality. The sap was separated from the solid debris by squeezing through two layers of cheese cloth and centrifuging at 10,000 x*g* for 15 min., at 2°C. The virus was precipitated by polyethylene glycol 6000 and NaCl, which were added to a final concentration of 4 % (w/v) and 0.2 M respectively, under continuous stirring. The solution was stirred 1 hour at room temperature. All succeeding steps were carried out at 0° to 4°C. The precipitate was collected by centrifugation at 10,000 x*g*, for 15 min. The pellet was resuspended in 25 ml of 0.1 M phosphate buffer (pH 7) and the solution was centrifuged at 10,000 x*g* for 15 min. The supernatant was saved and centrifuged at 105,000 x*g* for 1 hour. The pellet was covered with 3 ml of 0.01 M phosphate buffer (pH 7) and allowed to resuspend overnight. TMV was further purified by repeating two cycles of differential centrifugation. TMV was kept in 0.01 M phosphate buffer pH 7. The concentration of the purified TMV suspension was determined spectrophotometrically, based on an extinction coefficient $E_{260}^{1\,cm}$ 0.01 % = 2.8.

U.V. inactivated TMV. TMV was inactivated by means of ultraviolet irradiation (Dijkstra, 1964). A TMV solution containing 0.5 mg virus per ml and 0.066 M NaH_2PO_4 (pH 7) was irradiated by a U.V. lamp (Philips TUV, 15 W). A volume of 1.5 ml of the TMV solution was rocked in a petri dish of 9 cm diameter for 32 min. at a distance of 20 cm from the U.V. tube. The inactivation of TMV was controlled by local lesion assay.

Cowpea strain of TMV. The cowpea strain of TMV, referred to as C-TMV in this thesis, was kindly provided by professor R. M. Lister, Purdue University, West

Lafayette, Indiana, U.S.A. We used the 'bean form' of the virus mixture originally isolated in 1950. The 'bean form' of C-TMV does not produce systemic infection in tobacco.

C-TMV was prepared from Samsun tobacco or cowpea leaves (*Vigna unguiculata* (L) Walp. var. 'Blackeye Early Ramshorn') by a combination of the methods described by WHITFELD and HIGGINS (1976) and by BRUENING *et al.* (1976). Leaves were frozen on dry ice and pulverized with mortar and pestle. To the leafpulp 0.02 g $NaHCO_3$ per g of leaf was added. The pulp was further homogenized on ice, while buffer H, containing 0.04 M sodium EDTA, 0.036 M NaOH and 1 µl/ml of β-mercaptoethanol (pH 7.3), was added. The leaf debris was separated from the sap by filtration through two layers of miracloth and centrifugation at 3,000 x*g* for 10 min., at 4°C. The supernatant was saved and the pellet and leaf debris were again homogenized in 0.35 to 0.5 ml of buffer a. per g of original leaf material. This homogenate was filtrated and centrifuged at 3,000x*g* for 10 min., at 4°C. After combination of the recovered supernatant and the first supernatant they were centrifuged at 10,000 x*g* for 30 min., at 4°C. Solid NaCl and polyethylene glycol were added to the supernatant under continuous stirring to give final concentrations of 0.2 M and 4% (w/v) respectively. The suspension was stirred for 1 hour at room temperature or for 4 hours at 4° C. All succeeding steps were carried out at 0° to 4° C. The precipitate was collected by centrifuging at 10,000 x*g* for 30 min. The pellet was covered with 5 to 10 ml of buffer H per 70 g of leaf material and allowed to resuspend overnight. The suspension was then centrifuged at 10,000 x*g* for 10 min. The supernatant was saved and the pellet was again resuspended in 5 ml buffer a. per 70 g of leaf tissue and the suspension was centrifuged as before. The supernatant was combined with the supernatant which was saved. The virus was further purified by sedimenting through a cushion of 30% (w/v) sucrose in buffer H, in a Beckman Type 30 rotor at 27,000 rpm for 5 hours. The pellet was dissolved in 0.066 M phosphate buffer (pH 7) and the virus solution was finally clarified by centrifugation at 14,000 rpm for 15 min. in a Sorvall SS34 rotor. The concentration of C-TMV solution was calculated assuming an extinction coefficient $E_{260}^{1cm}\,0.1\,\% = 2.8$, by analogy with the common strain of TMV.

2.4. INFECTION OF TOBACCO PROTOPLASTS WITH TMV

The tobacco protoplasts were infected with TMV or mock-infected with U.V. inactivated TMV by the procedure as described by TAKEBE and OTSUKI (1969).

2.5. INCUBATION OF TOBACCO PROTOPLASTS

The tobacco protoplasts were suspended in incubation medium as used by TAKEBE *et al.* (1968) with a slight modification. It had the following composition: 0.7 M mannitol, 0.2 mM KH_2PO_4, 1 mM KNO_3, 0.1 mM $MgSO_4$, 0.1

mM $CaCl_2$, 1 μM KI, 0.01 μM $CuSO_4$, 1 μg/ml 2,4-dichlorophenoxyacetic acid, adjusted to pH 5.6 with KOH. Before incubation of the protoplasts 5μg/ml aureomycin was added to protect the protoplast suspension from contamination by micro-organisms. The protoplasts were suspended at a concentration of 2 to 5 $\times$ 10^5 viable protoplasts/ml in 5 to 10 ml portions, brought in 50 or 100 ml Erlenmeyer flasks respectively, at 25°C under continuous lighting at 9,300 erg/cm^2/sec provided by TL 33 tubes (Philips Nederland n.v., Eindhoven, The Netherlands).
Once the influence of incubation medium on amino acid incorporation was tested, the protoplasts were incubated in saline medium used by MEYER (1974) which was composed of: 1.84 mM KH_2PO_4, 89.01 mM KNO_3, 6.09 mM $MgSO_4.7H_2O$, 81.37 mM $CaCl_2.2H_2O$, 146.07 mM sucrose, 160.94mM KCl, 4.52 $\times$ 10^{-3} mM 2,4-dichlorophenoxy acetic acid (pH 5.6).

2.6. ISOLATION OF COWPEA PROTOPLASTS

Protoplasts from the primary leaves of cowpeas (*Vigna unguiculata* (L) Walp. var. 'Blackeye Early Ramshorn') were isolated as described by HIBI *et al.*, (1975) and ROTTIER *et al.*, (1979). After isolation the cowpea protoplasts were washed three times by resuspending the pellets of protoplasts in 0.6 M mannitol solution and sedimenting at 600 rpm for 2 min. at room temperature.

2.7. INFECTION OF COWPEA PROTOPLASTS WITH TMV OR C-TMV

A known number of cowpea protoplasts, of which at least 75% were living, were collected by centrifugation at 600 rpm for 2 min. from the 0.6 M mannitol solution. The pellets of protoplasts were resuspended in sterilized 0.1 M potassiumphosphate buffer (pH 5.4), containing 0.6. M mannitol and 0.75 μg poly-D-lysine/ml, to a concentration of 4 $\times$ 10^5 living protoplasts/ml. The protoplasts were preincubated for 7.5 min. at 0°C. Concurrently TMV, C-TMV or U.V.-inactivated TMV in a concentration of 2 μg/ml was preincubated in 0.1 M potassiumphosphate buffer (pH 5.4), containing 0.6 M mannitol and 2μg/ml poly-D-lysine during 7.5 min. at room temperature.
After preincubation the protoplasts were sedimented by centrifugation at 600 rpm for 2 min., and the pellets of protoplasts were immediately resuspended in 0.1 M potassiumphosphate buffer (pH 5.4), containing 0.6 M mannitol at 0° C and mixed with an equal volume of preincubation medium of virus and poly-D-lysine. The final concentration of cowpea protoplasts, virus and poly-D-lysine were 2 $\times$ 10^5/ml, 1 μg/ml and 1 μg/ml respectively, in the medium during inoculation. The suspension was kept on ice during 7.5 min. Thereafter the protoplasts were washed four times with sterilized 0.6 M mannitol solution containing 10 mM $CaCl_2$ to remove poly-D-lysine and not-adsorbed virus.

2.8. Incubation of cowpea protoplasts

The cowpea protoplasts were incubated in a medium similar to the medium used for incubation of tobacco protoplasts except that the medium contained 0.6 M mannitol instead of 0.7 M mannitol and under the same conditions as described under section 2.5.

2.9. Infectivity assay of tobacco and cowpea protoplast extracts

When protoplasts were incubated for appropriate periods after infection a known number of viable protoplasts were collected by centrifugation and stored at –20°C till they were used for infectivity assay on leaves of *Nicotiana glutinosa* L. Protoplast extracts were prepared from frozen pellets, which were resuspended in 0.1 M phosphate buffer (pH 7.0). When the protoplasts were incubated for 36 hours after inoculation for example, tobacco protoplasts were resuspended at a concentration of 0.2×10^5 viable protoplasts/ml buffer and cowpea protoplasts were resuspended at a concentration of 2×10^5 viable protoplasts/ml buffer. The cells were homogenized with an all-glass microsize Potter-Elvehjem tissue grinder, at 0°C. The homogenate was centrifuged at 17,000 x*g* for 10 min.
When six half leaves were inoculated with a protoplast extract of this concentration and the opposite half leaves with a control solution of 0.2 µg purified TMV/ml 0.1 M phosphate buffer (pH 7), the number of local lesions produced by the protoplast extract was in the order of magnitude of the number of lesions produced by the control solution. The calculation of the number of TMV particles per infected protoplast was made by assuming a M.W. of 40×10^6 daltons for TMV and assuming that the TMV particles in the crude protoplast extract were as infectious as those in the control solution. The relative infectivity of a protoplast extract is:

$$\frac{\text{the number of local lesions of an extract from } 1 \times 10^5 \text{ protoplasts/ml buffer} \times 100.}{\text{the number of local lesions of 0.2 µg TMV/ml buffer}}$$

2.10 Determination of the frequency of infection of tobacco and cowpea protoplasts

Antiserum to TMV and C-TMV was prepared and partially purified. Thereafter antibodies were conjugated with fluorescein isothiocynate (FITC) by a combination of methods described by Spendlove (1967) and Otsuki and Takebe (1969). Samples of protoplasts were stained with FITC-conjugated antibodies as described by Otsuki and Takeby (1969), except that 96 % alcohol was used for fixation instead of aceton. The protoplasts were examined under a Wild fluorescence microscope.

2.11. Determination of uptake of [^{3}H]-labeled amino acids in tobacco protoplasts and incorporation of [^{3}H]-labeled amino acids into tobacco protoplast protein

[^{3}H]-leucine (specific activity 40–60 Ci/mmol) or/and [^{3}H]-valine (specific activity 15–30 Ci/mmol) were added to the incubation medium containing 2.5 to 5 $\times$ 10^5 tobacco protoplasts/ml. When different samples of protoplasts had to be compared all samples had the same concentration of living protoplasts. After the labeling period portions of 2.5 to 5 $\times$ 10^5 protoplasts were collected by centrifugation at 100 x*g* for 2 min. and washed once. The pellets were stored at –20 °C for analysis of radioactivity. Pelleted protoplasts were thawed quickly and resuspended in 0.5 ml ice-cold 0.01 M phosphate buffer (pH 7.1) containing 0.5 mM phenylmethylsulfonylfluoride (PMSF), an inhibitor of proteolytic enzymes. Samples of this suspension were homogenized by hand in an all-glass microsize Potter-Elvehjem tissue grinder at 0 °C.

To determine the uptake of radioactivity 0.05 ml of the suspension was analysed in Bruno Christian scintillation fluid, Aquasol or Hydroluma by a Packard Tricarb scintillation spectrometer.

To determine the incorporation of [^{3}H]-labeled amino acids into protein of the protoplasts, trichloroacetic acid was added to a final concentration of 5 % to the suspension. The mixture was heated for 3 min. at 90 ° C. After standing at 0 ° to 4 ° C during some hours the precipitate was washed subsequently with 96 % hot ethanol, a mixture of 96 % ethanol, chloroform and ether (2:1:1 v/v) and with ether. After drying the precipate was dissolved in 62.5 mM Tris-HCL buffer (pH 6.8) containing 2 % SDS, 2mM DTE and 8 M ureum and radioactivity was analysed as described before.

2.12 Determination of the specific activity of protein of subcellar fractions of tobacco protoplasts

After incubation of the tobacco protoplasts with radioactive amino acids, portions of 5 to 10 million living protoplasts were sedimented by centrifugation at 100 x*g* for 2 min. Freshly collected protoplasts were resuspended in cold 50 mM Tris-HCL buffer (pH 8.2) containing 10 % (w/w) sucrose, 50 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 10 mM DTE and 0.5 mM PMSF (buffer A) and were homogenized by hand in an all-glass microsize Potter-Elvehjem tissue grinder. By this procedure the protoplasts were disrupted and the chloroplasts were still intact as examined by light microscopy. The homogenate was centrifuged at 2,500 x*g* for 10 min. at 4 ° C. The supernatant was removed and again centrifuged at 20,000 x*g* for 30 min. at 4 ° C. Thus a supernatant containing soluble proteins and a 20,000 x*g* pellet containing membranes and mitochondria were obtained.

The 2,500 x*g* pellet which contained the chloroplasts and nuclei was resuspended with the aid of the homogenizer in the above mentioned buffer A and

layered onto a discontinuous sucrose gradient of 3 × 10 ml of 20, 45 and 60 % (w/w) sucrose in buffer A without EDTA. The gradients were centrifuged in the SW27.1 rotor at 22,000 rpm for 3 hours at 4 °C in a Beckman Model L5–65 ultra centrifuge. The rotor came to a standstill without the use of the brake. In this way the 2,500 x*g* pellet was fractionated into a chloroplast fraction, a fraction of broken cells and membranes, and a nuclear fraction, situated between the 20 and 45 % sucrose layer, 45 and 60 % sucrose layer and at the bottom of the centrifuge tube respectively. The radioactivity incorporated into the proteins of the different fractions was determined by the same procedure as used for pelleted protoplasts described under section 2.11. The amount of protein was determined by the method as described by LOWRY (1951).

2.13. DETERMINATION OF THE SPECIFIC ACTIVITY OF TMV COAT PROTEIN

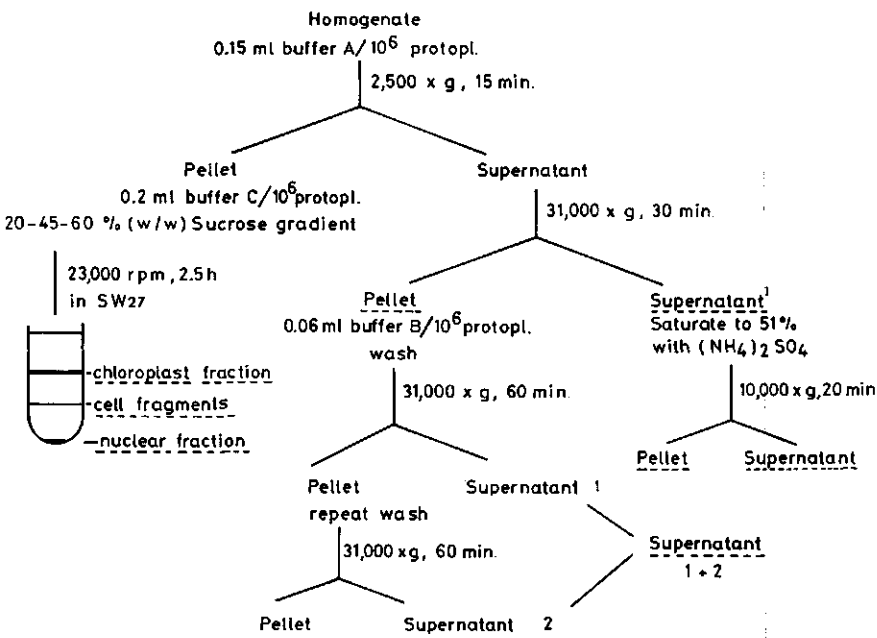
After infection with TMV the tobacco protoplasts were incubated at a concentration of 0.5 × 10^6 protoplasts/ml. [^{3}H]-amino acid was added to the incubation medium 3 hours after inoculation. After the labeling period the protoplasts were collected, washed once with 0.7. M mannitol solution containing 1 mM leucine and stored at -20 ° C. Frozen pellets of 8 to 10 × 10^6 protoplasts were thawed, homogenized with an all-glass microsize Potter-Elvehjem tissue grinder in 0.7 ml of 80 mM potassiumphosphate buffer (pH 7.8), 50 m KCl, 10mM $MgCl_2$, 10 % (w/v) sucrose, 1 mM leucine and centrifuged at 27,000 x*g* for 15 min. at 4 °C. To purify the TMV the 27,000 x*g* supernatant was pipetted onto a layer of 1.8 ml of 20 % (w/v) sucrose solution on top of 9.1 ml CsCl solution with a density of 1.28 g/cm^3 at 25 °C in 10 mM phosphate buffer (pH 7.0). The samples were centrifuged in a SW41 rotor at 30,000 rpm for 20 hours at 10 ° C in a Beckman Ultra centrifuge. The gradients were fractionated by collecting 12 to 15 fractions of 0.7 ml. The density of the fractions was calculated from the refraction index after determination with a refractometer. The absorbancy at 260 nm was determined with a Zeiss spectrophotometer. Aliquots of 10 µl of each fraction were analysed for radioactivity by counting in 10 ml Aquasol by a Packard Tri-carb scintillation spectrometer.
The specific activity of TMV coat protein was calculated as follows: the fractions in the peak area at density of 1.30 g/cm^3 were pooled and dialysed against 10 mM phosphate buffer (pH 7.0.). Then the amount of TMV was calculated from the extinction at 260 nm assuming an extinction coefficient $E^{1\,cm}_{260}$ 0.1 % = 2.8. Aliquots of the dialysed solution were analysed for radioactivity..

2.14 ELECTRON MICROSCOPY

Samples of cowpea protoplasts infected with TMV, C-TMV or mock-infected protoplasts were prepared and stained for electron microscopy as described by HIBI and YORA (1972), except that Spurr medium was used for embedding instead of Epon 812. The ultrathin sections of pellets of cowpea protoplasts were examined under the Hitachi HU-12 electron microscope.

2.15 Analysis of protein from TMV and mock-infected tobacco and cowpea protoplasts by SDS-polyacrylamide slabgel electrophoresis

TMV and mock-infected tobacco protoplasts and TMV and mock-infected cowpea protoplasts were incubated in a concentration of about 3×10^5 protoplasts/ml in the presence of 15 to 20 µl/ml [^{35}S]-methionine (specific activity about 240 Ci/mmol) during 16–19 hours. The labeling period started 8 hours after the onset of incubation of the tobacco protoplasts. The labeling period of protein from cowpea protoplasts started at least 8 hours later than that of tobacco protoplasts. Samples of 2.5 to 5×10^6 protoplasts were collected by centrifugation at 100 x*g* for 2 min. at room temperature and were immediately thereafter resuspended and homogenized with the aid of a small Thomas homo-



Buffer A contained: 0.05 M Tris-HCl (pH 7.4), 0.01 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer B contained: 0.05 M Tris-HCl (pH 8.2), 25 % v/v glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer C contained: 0.05 M Tris-HCl (pH 8.2), 10 % w/w sucrose, 0.001 M $MgCl_2$ 0.05 M KCl, 0.01 M DTE and 0.5 mM PMSF.

Fig. 2.6. Scheme of the subcellular fractionation of the protoplast homogenates.

[1]) The 31,000 x*g* supernatant was centrifuged at 105,000 x*g* for 2 hours; the 105,000 x*g* supernatant was saturated to 51 % with $(NH_4)_2 SO_4$.

genizer or by hand in an all-glass microsize Potter-Elvehjem tissue grinder in about 0.15 ml/10^6 protoplasts of 50 mM Tris.HCl buffer (pH 7.4) containing 10 mM KCl, 1 mM EDTA, 10 mM DTE and 0.5 mM PMSF (buffer A), at 0°C. The homogenate was examined under the light microscope to check that nearly all protoplasts were disrupted and the chloroplasts were still intact.
The homogenate of protoplasts was fractionated as shown in figure 2.6. All operations were at 0° to 4°C. The suspension was centrifuged at 2,500 x*g* for 15 min. The supernatant was adjusted to 25 % (v/v) glycerol and centrifuged at 31,000 x*g* for 30 min. The 31,000 x*g* supernatant was stored at -70° C. In some cases the proteins of the 31,000 x*g* supernatant were divided further into two fractions. The supernatant was centrifuged at 105,000 x*g* for 2 hours and subsequently the 105,000 x*g* supernatant was saturated to 51 % with $(NH_4)_2SO_4$ and incubated under continuous stirring during 4 hours. The precipitate was collected by centrifuging at 10,000 x*g* for 20 min. The precipitate and supernatant were stored at -70° C.
The 31,000 x*g* pellet of cowpea protoplast homogenates was resuspended in 0.06 ml/10^6 protoplasts of 50 mM Tris-HCL buffer (pH 8.2) containing 25 % (v/v) glycerol, 50 mM KCl, 1 mM EDTA, 10 mM DTE and 0.5 mM PMSF (buffer B) and incubated under continuous stirring for 60 min. The suspension was clarified by centrifuging at 31,000 x*g* for 60 min. The supernatant (1) was saved and the pellet was again resuspended in 0.04ml/10^6 protoplasts of buffer B and incubated under continuous stirring for 30 min. Thereafter the suspension was centrifuged at 31,000 x*g* for 60 min. The supernatant (2) was combined with supernatant (1) and stored at –70°C. The 2,500 x*g* pellets of tobacco protoplast homogenates were resuspended with the aid of a homogenizer in about 0.2 ml/10^6 protoplasts of 50 mM Tris-HCL buffer (pH 8.2) containing 10 % (w/w) sucrose, 50 mM HCl, 1 mM $MgCl_2$, 10 mM DTE, 0.5 mM PMSF (buffer C) and layered onto a discontinuous sucrose gradient of 3 × 10 ml of 20, 45 and 60 % (w/w) sucrose in buffer C. The gradients were centrifuged as described under section 2.12.
The nuclear fraction at the bottom of the tube was stored at -70° C. The fraction of chloroplasts and of cell fragments situated between the 20–45 % sucrose layer and 45–60 % sucrose layer respectively, were diluted twice with buffer C and the suspensions were clarified by centrifuging at 15,000 x*g* for 20 min. The pellets were saved and stored under nitrogen at -70° C.

Proteins of subcellular fractions of protoplasts infected with TMV, U.V. inactivated TMV or C-TMV, were analysed by electrophoresis on polyacrylamide slabgels according to the method as described by LAEMMLI (1970) or on gradient polyacrylamide slabgels according to a combination of the procedures as described by LAEMMLI (1970) and MARSDEN *et al.*, (1976). Samples of supernatant and of pellets, resuspended in 50 mM Tris-HCl buffer (pH 6.8) containing 12 % (v/v) glycerol, were adjusted to 5 % β-ME and 2 % SDS. Protein samples of about 50 µl were analysed, containing at least 300,000 cpm and less than 30 µg protein. An equal number of cpm incorporated into protein of corresponding subcellular fractions of TMV and of mock-infected protoplasts were brought on gel. Just

before loading, the samples were incubated in a boiling waterbath for 3 min. Thereafter any material not dissolved was pelleted by centrifuging at 10,000 xg for 15 min. at room temperature in a Janetzki TH12 centrifuge. A 2.5 µl of 0.001 % bromophenol blue solution was added to the samples, before they were brought on gel.

Separating gels of about 10 and 12 cm long were used of different acrylamide concentrations ranging from 7.5. to 17.5 % or linear gradient gels of 7 to 15 % or 7 to 18 % acrylamide were prepared. A spacer gel of 1 cm long of 5 % acrylamide was poured onto the separating gel. The gels were run in a Pharmacia Gelelectrophoresis Apparatus GE4. The upper and lower buffer were the same, containing 25 mM Tris, 0.192 M glycine and 0.1 % SDS. Electrophoresis was performed at 15° C and at constant voltage, during the first hour at 50 to 70 V. and thereafter, when the bromophenol blue band had passed the spacer gel at about 120 to 190 V. for 5 to 8 hours.

After electrophoresis the polypeptides were stained with coomassie brilliant blue R in a solution, containing 0.2 % (w/v) coomassie brilliant blue R in methanol, water, acetic acid (50:50:7 $^{v}/_{v}$) for at least 4 hours at room temperature. Thereafter the gels were washed several times with a mixture of methanol, water, acetic acid (50:880:7 v/v) at 50°C. Then they were processed for autoradiography. The dried gels were exposed to Sakura X-Ray film (Konishiroku photo Ind. Co. Ltd., Japan) for 3 to 30 days. The films were processed using Kodak DX-80 developer and Kodak FX-40 X-Ray liquid fixer.

The polypeptides synthesized as a result of TMV multiplication in tobacco and cowpea protoplasts, were distinguished in autoradiograms by comparison of polypeptide patterns of protein from corresponding subcellular fractions of protoplasts, infected with TMV or with U.V. inactivated TMV. Polypeptides from TMV infected protoplasts were synthesized as a result of TMV multiplication, when polypeptides with a similar electrophoretic mobility from protein of mock-infected protoplasts were either absent or present to a far less extent.

The M.W. of a polypeptide synthesized as a result of TMV multiplication in protoplasts was established by comparison of its electrophoretic mobility with those of the following marker proteins: DNA-RNA polymerase of *Escherichia coli* (M.W. 165,000, 155,000, 95,000 and 39,000), β-galactosidase (M.W. 116,200), phosphorylase A (M.W. 92,500), transferrin (M.W. 80,000), bovine serum albumin (M.W. 68,000), catalase (M.W. 57,500), γ-globulin (M.W. 54,000 and 23,500), ovalbumin (M.W. 46,000), lactate dehydrogenase (M.W. 35,000), TMV coat protein (M.W. 17,500), Lysozyme (M.W. 17,200), RNAse A (M.W. 12,600) and cytochrome C (M.W. 12,500). The markers were visualized by staining.

2.16 Translation of TMV RNA in cell-free protein synthesizing systems

Products were translated from TMV RNA in the rabbit reticulocyte lysate and in a wheat germ cell-free protein synthesizing extract as described by Pelham and

JACKSON (1976) and DAVIES *et al.* (1977) respectively. The translation products were labeled with [^{35}S]-methionine.

2.17. ISOLATION OF VIRUS RNA

RNA was isolated from purified TMV and C-TMV suspended at a concentration of about 20 mg/ml in 0.1 M phosphate buffer by the method as described by HUNTER *et al.* (1976). One volume of water saturated, redistilled phenol and one volume of buffer, containing 50 mM Tris, 10 mM EDTA, 2% SDS (pH 8.8) were added and the mixture was heated at 65° C for 1 min. Thereafter RNA was extracted by shaking at room temperature. After phase separation the aqueous phase was extracted three times with an equal volume water saturated phenol. RNA was precipitated from the final aqueous phase, which was made 0.24 M ammonium acetate, with two volumes of cold alcohol. RNA was subsequently precipitated once or twice more, dried under N_2 and dissolved in water. The concentration of RNA was calculated from the extinction at 260 nm, assuming an extinction coefficient $E\ {}^{1\,cm}_{260}\,0.1\,\% = 21$.

2.18. CHEMICALS

acrylamide, Serva Feinbiochemica, Heidelberg.
actinomycin D, donated by Merck, Sharp and Dohme, U.S.A.
aquasol, New England Nuclear, Boston Mass.
aureomycin (chlorotetracycline HCL), Nutritional Biochemicals Corporation, Cleveland Ohio.
6-benzyladenine, Sigma chemical Co., St. Louis Miss.
bovine serum albumin, Schwarz/Mann, Orangeburg N.Y.
catalase, Boehringer, Mannheim GmbH.
cellulase Onozuka R-10, Yakult Biochemicals, Japan.
coomassie brilliant blue G-250, Serva Feinbiochemica, Heidelberg.
coomassie brilliant blue R, Sigma Chemicals Co., St. Louis Miss.
cytochrome C, Calbiochem, Luzern, Switserland.
DEAE-BioGelA, BioRad Laboratories, Richmond Calif.
DTE, Sigma Chemicals Co., St Louis Miss.
β-galactosidase (from *Escherichia coli*, grade IV), Sigma Chemicals Co., St. Louis Miss.
γ-globuline (human), Sigma chemicals Co., St. Louis Miss.
hydroluma, Lumac, the Netherlands.
instafluor and instagel, Packard Instrument Co., Belgium.
lactate dehydrogenase (from rabbit muscle), Boehringer, Mannheim GmbH.
lysozyme, Sigma Chemicals Co., St. Louis Miss.
L-[4,5,-^{3}H]-leucine (specific activity 40 to 60 Ci/mmol), The Radiochemical Centre, Amersham England.

macerozyme R10, Yakult Biochemicals, Japan.
[^{35}S]-methionine (specific activity $\pm$ 240 Ci/mmol) The Radiochemical Centre, Amersham, England.
methylene bisacrylamide, Serva Feinbiochemica, Heidelberg.
ovalbumin, Sigma Chemicals Co., St. Louis Miss.
PATE, Hoechst A.G., Frankfurt FRG Germany.
pectinol fest, Röhm GmbH, Darmstadt FRG Germany.
phosphorylase A (from rabbit muscle), Boehringer, Mannheim GmbH.
poly-D-lysine, Sigma Chemicals Co., St. Louis Miss.
polyethylene glycol (Carbowax 6000), Heybroek, Amsterdam.
poly-L-ornithine (M.W. 130,000) Pilot Chemicals Inc., U.S.A.
rimocidin, Charles Pfizer and Co. Groton Connecticut.
ribonucleaside triphosphates (unlabeled), Sigma Chemicals Co., St. Louis Miss.
RNAse A (from bovine pancreas), Sigma Chemicals Co., St. Louis Miss.
RNA polymerase of *Escherichia coli*, Boehringer, Mannheim GmbH.
Soluene-350, Packard Instrument Company, Belgium.
TEMED, Koch-Light Labs Ltd.
transferrin, Sigma Chemicals Co., St. Louis Miss.
ureum, Schwarz/Mann, Orangeburg N.Y.
[5-^{3}H]-UTP (ammonium salt, specific activity 11 to 24 Ci/mmol), The Radiochemical Centre, Amersham England.
L-[2,3-^{3}H]-valine (specific activity 15 to 30 Ci/mmol), The Radiochemical Centre, Amersham England.

All other chemicals were obtained from E. Merck, Darmstadt Germany and BDH Biochemical Ltd., Poole England.

3. TMV MULTIPLICATION IN TOBACCO PROTOPLASTS

3.1. INTRODUCTION

Protoplasts from higher plant material can be isolated on a large scale since commercial enzyme preparations are available. GREGORY and COCKING (1963, 1965) isolated by means of enzymes protoplasts from tomato fruit tissue, which could be infected with TMV. Thereupon electron microscopic studies of the initial stages of TMV infection became possible (COCKING, 1966; COCKING and POJNAR, 1968).

TAKEBE and co-workers developed the enzymatic method by which protoplasts can be isolated from tobacco leaves. This method made it possible to investigate in detail the TMV multiplication (TAKEBE *et al.*, 1968; TAKEBE and OTSUKI, 1969, 1969a; SAKAI and TAKEBE, 1972, 1974; AOKI and TAKEBE, 1975). The protoplasts were prepared from tobacco leaves by first isolating the plasmolyzed cells with the aid of a polygalacturonase preparation and then removing the cell wall with cellulase. About 10^7 protoplasts could thus be obtained from 1 g tobacco leaf tissue, of which 90% could be infected with TMV. The virus replication started simultaneously in all the infected protoplasts 6 hours after infection and the vigorous virus replication lasted for more than 48 hours. The final yield of TMV particles per protoplast was over 10^6 (TAKEBE, 1975, 1975a). To date various procedures for the isolation of protoplasts of higher plants are available, which are dealt with in a review article by SARKAR (1977).

The tobacco protoplasts can now likewise be infected with other plant viruses, e.g. CMV (OTSUKI and TAKEBE, 1973), CCMV (MOTOYOSHI *et al.*, 1973, 1975; BANCROFT *et al.*, 1975), BMV variant V5 (MOTOYOSHI *et al.*, 1974), PEMV (MOTOYOSHI and HULL, 1974), PVX (OTSUKI *et al.*, 1974), AMV (MOTOYOSHI *et al.*, 1975), TRN (KUBO *et al.*, 1975; MAYO and ROBERTS, 1978), CGMV (SUGIMURA and USHIYAMA, 1975), CPMV (HUBER *et al.*, 1977). In most cases it is possible to infect the tobacco protoplasts for more than 60% with the viruses mentioned above. However, up till now detailed investigation of the virus replication has yielded only few data on proteins, synthesized as a result of the virus multiplication, and on the course of the virus RNA replication upon infection of the protoplasts (BANCROFT *et al.*, 1975; HARRISON *et al.*, 1976; SAKAI *et al.*, 1979; TAKANAMI *et al.*, 1977; FRITSH *et al.*, 1978; SAKAI *et al.*, 1979). Investigations are seriously hampered by the fact that various factors in the cultivation of the tobacco plants may have a disadvantegeous influence on the quality of the protoplasts (WATTS *et al.*, 1974; KASSANIS and WHITE, 1974; MOTOYOSHI *et al.*, 1974; ZAITLIN and BEACHY, 1974; KUBO *et al.*, 1975a). It is essential for the investigation of virus specific proteins that a large amount of protoplasts should be present, of which a large percentage can be infected and which are viable for at least 40 hours.

For the present study it was necessary to standardize the cultivation of the

tobacco plants in such a way that protoplasts with the above qualities can be continuously isolated.

3.2. Results and discussion

Initially protoplasts were isolated from leaves of tobacco plants cv. Samsun, Samsun NN and Xanthi nc, cultivated in a greenhouse. It appeared that the protoplast preparations differed every time with respect to yield, infectibility and/or viability.

The protoplasts were isolated from tobacco leaves, infected with TMV and then incubated in medium, according to the method described by Takebe (Takebe and Otsuki, 1969) described under Materials and Methods.

At first it was tried to improve the quality of the protoplasts by modifying the isolation or the infection procedure, but the quality remained precarious. Experience showed that variations in the cultivation of the tobacco plants as a result of, among other things, weather conditions strongly influenced the quality. Standardization appeared feasible by cultivating the tobacco plants in a phytotron. In chapter 2 a very detailed description of the cultivation of tobacco plants cv. Samsun, Samsun NN and Xanthi nc in the phytotron has been given. In this way of cultivation it was achieved that the quality of the tobacco protoplasts mostly was as follows:

1. the yield of protoplasts, determined before or after infection with TMV, is more than 2×10^6 viable protoplasts per tobacco leaf,
2. the percentage of protoplasts which are viable 24 hours after infection is at least 60%,
3. the percentage of protoplasts which are infected with TMV is over 50%, determined 24 hours after inoculation,
4. The amount of infectious TMV, synthesized in the infected protoplasts after $\pm$ 32 hours of incubation, corresponds with 0.25×10^6 particles/infected protoplast.

The time course of TMV synthesis during the first 35 hours after inoculation of the tobacco protoplasts is shown in fig. 3.1. In the first 6 hours after the onset of incubation of the infected protoplasts the amount of TMV hardly increases, after approximately 9 hours the virus multiplication runs exponential.

The intensity and quality of the light influenced the yield of viable protoplasts and the infectibility. Because U.V. light probably has a harmful influence on the quality of the protoplasts, perspex sheets were placed under the lamp-house of the phytotron. These sheets absorb light of wavelength lower than 390 nm. When perspex sheets were present, the light intensity could be heightened from 18,000 erg/cm^2/sec to 31,000 erg/cm^2/sec.

Table 3.1 gives the effect of perspex sheets under the lamps on the quality of the protoplasts. It appeared that the yield of vaible protoplasts is considerably enlarged by the presence of perspex sheets, that the number of protoplasts lost as a result of the infection procedure is less, and that the percentage of infected protoplasts does not become too low.

TABLE 3.1. Effect of perspex sheets placed under the lamp-house of the phytotron on the quality of the tobacco protoplasts.

perspex sheet	after the isolation procedure		after the infection procedure		after 36 hours of incubation	
	% viable protoplasts	yield of protoplasts per leaf $\times 10^{-6}$	% viable protoplasts	yield of protoplasts per leaf $\times 10^{-6}$	% viable protoplasts	% infected protoplasts
present	69	7	63	7	60	53
absent	71	2.4	74	0.7	70	72

Samsun tobacco plants were cultivated in the phytotron under conditions as described under Materials and Methods, except the change dealt with in the table.

The spectral quality of the light of the different lamps under which the tobacco plants were cultivated influenced the quality of the protoplasts. Samsun NN tobacco plants yielded best protoplast preparations if they were cultivated under SON lamps during their later growing period, whereas Samsun tobacco plants could be cultivated better under HPLN lamps. Cultivation under TL tubes (Philips fluorescent tubes) never yielded a satisfactory result.

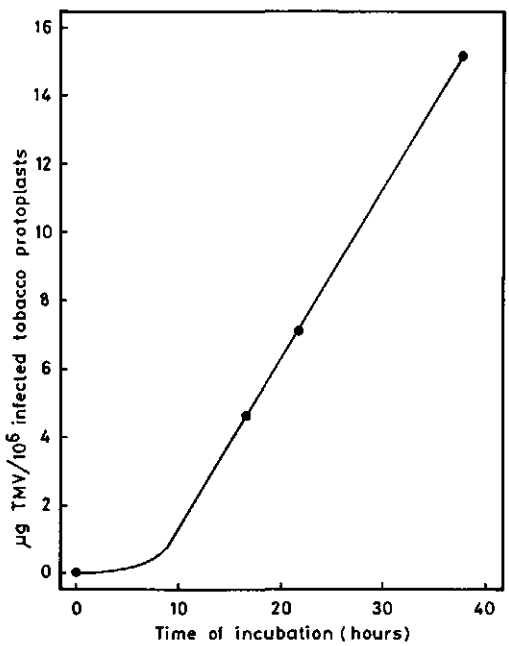


FIG. 3.1. Time course of TMV synthesis in tobacco protoplasts.
The protoplasts were isolated, infected and incubated as described by TAKEBE and OTSUKI (1969). After different periods of incubation samples of protoplasts were harvested. Protoplast extracts were prepared and assayed as described in chapter 2.

The manner of watering the tobacco plants was of strong influence on the quality of the protoplasts. It became clear that the plants should be watered regularly every day, that the water should be at room temperature and that the soil shoul be watered as described under Materials and Methods.

The age of the tobacco plants and of the leaves affected the quality of the protoplasts. From one or two nearly full-grown leaves of a tobacco plant, 58 to 64 days old, protoplasts meeting all criteria could be isolated. The yield of the protoplasts per leaf was too low if a leaf of a plant younger than 53 days, was used. Protoplasts that were difficult to infect were obtained from a nearly full-grown leaf of a plant over 66 days old. Likewise, protoplasts that were difficult to infect were obtained if a leaf that was too old was harvested from a plant of correct age, whereas too few protoplasts resisted the infection procedure with TMV if a leaf was chosen that was too young and which could have expanded further.

TABLE 3.2. Comparison between two methods for isolation of tobacco protoplasts.

	after the isolation procedure	39 hours post inoculation		
Isolation	yield of viable protoplasts per leaf $\times 10^{-6}$	% viable protoplasts	% infected protoplasts	amount of virus particles per infected protoplast $\times 10^{-6}$
Method used by MEYER (1974)	12	86	64	0.34
Method used by TAKEBE (1969)	4	87	55	0.28

The amount of protoplasts isolated per leaf could be increased by using the method for isolation of tobacco protoplasts as described by MEYER (1974), with some modifications (see Materials and Methods), instead of the method as described by TAKEBE. The two methods differ mainly as regard to the use of enzymes and osmoticum. In MEYER's method the pectinase preparations PATE and pectinol fest instead of macerozyme were used and 2.5% KCl and 1% $MgSO_4$ solution instead of 0.7 M mannitol solution were used. Furthermore, in MEYER's method the tobacco protoplasts are isolated in one step instead of in two. As is given in table 3.2., the yield of protoplasts per tobacco leaf became three times higher if the protoplasts were isolated by MEYER's method instead of by the method used by TAKEBE. 12×10^6 vaible protoplasts per tobacco leaf were isolated. This method of isolation hardly influenced the quality of the protoplast preparations in other respects; approximately 60% of the tobacco protoplasts could be infected with TMV and at least 0.30×10^6 virus particles per infected protoplast ($\pm$ 20µg/10^6 infected protoplasts) were synthesized. For this reason, and since the isolation procedure as used by MEYER was less laborious

than TAKEBE's method, MEYER's method was used for the study of protein synthesis in TMV infected tobacco protoplasts.

In comparison with the results reported by TAKEBE the yield of tobacco protoplasts under our cultivating conditions was approximately equal, the percentage of protoplasts infected with TMV was approximately 25 % less and the amount of infectious TMV synthesized per infected protoplast was one third.

4. INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEIN OF TOBACCO PROTOPLASTS

4.1. INTRODUCTION

For the investigation of protein synthesis induced by TMV infection, tobacco protoplasts have been used in several studies. It appeared that the incorporation of radioactive amino acids into the newly synthesized proteins is of crucial importance for the detection of TMV induced proteins, because they have to be distinguished among many radioactive labeled host proteins.

It should be noted, however, that factors influencing the incorporation of amino acids into protoplast proteins have hardly been studied. SAKAI *et al.*, (1970) have published data on uptake of [^{14}C]-amino acids by tobacco protoplasts and incorporation into protein. The influence of various factors on the uptake of [^{14}C]-amino acids by isolated tobacco leaf cells and the incorporation into cell protein has been reported by JENSEN *et al.* (1971) and by FRANCKI *et al.* (1971).

Within the scope of our work we were interested in the possibility to detect TMV specific proteins. This can achieved by determining the specific activity of TMV coat protein, synthesized after infection of tobacco protoplasts with TMV. Before investigating the specific activity of TMV coat protein it was necessary to examine in greater detail some factors influencing the amino acid incorporation.

4.2. UPTAKE OF [^{3}H]-LEUCINE BY TOBACCO PROTOPLASTS AND INCORPORATION INTO PROTOPLAST PROTEIN

The synthesis of proteins as a result of virus multiplication in infected tobacco protoplasts has been studied by labeling proteins with radioactive amino acids during shorter or longer periods, starting at various times post inoculation of the protoplasts.

Therefore the course of the incorporation of [^{3}H]-leucine into protoplast protein was followed during 38 hours post incubation. Tobacco protoplasts were isolated and incubated by the method of TAKEBE *et al.*, (1969) and [^{3}H]-leucine was added to the incubation medium after about 2 hours of incubation. The uptake of [^{3}H]-leucine and the incorporation into protoplast protein were determined as described under Materials and Methods. In fig. 4.1. the time course of the incorporation of [^{3}H]-leucine into protoplast protein is shown. 1 μCi [^{3}H]-leucine (specific activity 45 Ci/mmol) was added per ml incubation medium. In this experiment the incorporation of [^{3}H]-leucine increased for 24 hours after addition, thereafter it levelled off. The time course of the uptake of [^{3}H]-leucine in the protoplasts is also given in fig. 4.1. The uptake of [^{3}H]-leucine increased for 24 hours after addition. Thereafter the amount of amino acids taken up by the

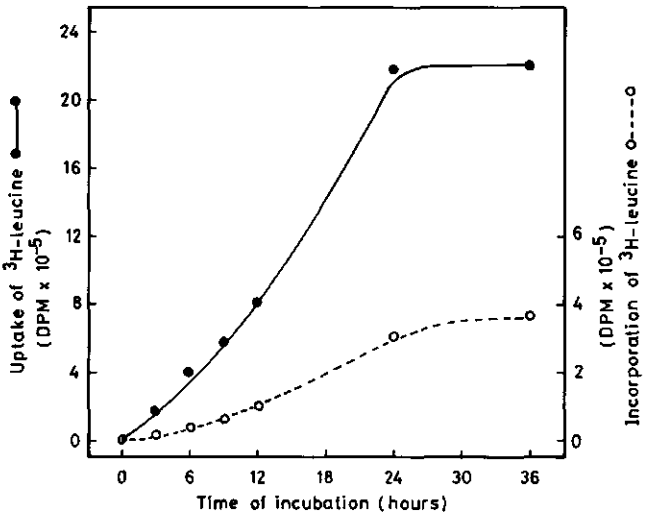


FIG. 4.1. Time course of uptake of [^{3}H]-leucine in tobacco protoplasts and incorporation into protein. Protoplasts were incubated in standard medium at a concentration of 5×10^5 protoplasts/ml. At time zero 1 μCi [^{3}H]-leucine/ml (specific activity 45 Ci/mmol) was added. Samples of 1 ml portions were analysed for uptake and incorporation of [^{3}H]-leucine.
(–●–●–) uptake of [^{3}H]-leucine in dpm/10^5 protoplasts; (–○– –○ –) incorporation of [^{3}H]-leucine in dpm/10^5 protoplasts.

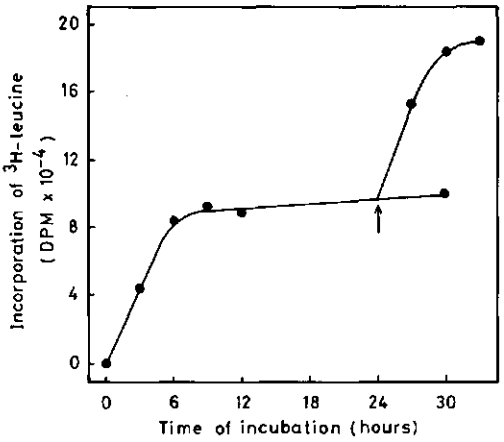


FIG. 4.2. Time course of incorporation of [^{3}H]-leucine into protein of tobacco protoplasts. Protoplasts were incubated in standard medium at a concentration of 6×10^5 protoplasts/ml. At time zero 0.5 μCi [^{3}H]-leucine/ml (specific activity 45 Ci/mmol) was added. After 24 hours of incubation 0.5 μCi [^{3}H]-leucine/ml was added to protoplast samples for a second time (indicated by an arrow). Samples of 1 ml were analysed for incorporation of [^{3}H]-leucine into protein. The incorporation of [^{3}H]-leucine is given as dpm/6 × 10^5 protoplasts.

protoplasts did not increase any longer. After 24 hours 80 % of the amount of [^{3}H]-leucine added to the incubation medium had entered the protoplasts, but only 14 % of this was incorporated into protein. In other experiments the percentage of [^{3}H]-leucine that was taken up by the protoplasts in 24 hours, was always over 50 %; the amount of [^{3}H]-leucine incorporated into protein, however, varied in the different experiments from 15 to 50 % of the uptake after 24 hours. This variation in the incorporation was nearly absent later on, when the cultivation of the tobacco plants was standardized.

The incorporation of [^{3}H]-leucine into protein levelled off after 24 hours of incubation of the protoplasts, in spite of the fact that 50 % or more of the amount of [^{3}H]-leucine that had entered the protoplasts, still was not incorporated. This was not due to a decreased ability of protein synthesis of the protoplasts, for the addition of an equal amount of [^{3}H]-leucine at the plateau phase resulted in a doubled incorporation, as the results of another experiment show (fig. 4.2). Probably the [^{3}H]-leucine which had entered the protoplasts was only available for incorporation into protein during a definite period after it had been taken up in the protoplasts. Therefore it did not seem useful to incubate the protoplasts for longer than 22 hours in the presence of [^{3}H]-leucine in order to label the protoplast proteins. The same amount of [^{3}H]-leucine was incorporated into protoplast proteins when the protoplasts were incubated during a certain labeling period, starting after different periods post incubation.

4.3. Factors influencing the incorporation of [^{3}H]-leucine into protoplast protein

Several factors, which might influence the incorporation of [^{3}H]-leucine into protein were explored: method of protoplast isolation (a), composition of the incubation medium (b), poly-L-ornithine (c), light intensity (d), protoplast concentration (e), antibiotics (f).

a. It appeared that differences in the method of protoplast isolation did not influence the incorporation of [^{3}H]-leucine into protoplast protein.

b. The composition of the incubation medium affected the cultivation of callus tissue (Murashige and Skoog, 1962) and the regeneration of cell wall and the cell division of protoplasts, isolated from tobacco callus tissue (Uchimiya and Murashige, 1976). Because the medium used by Takebe for incubating TMV infected tobacco leaf protoplasts is a modified medium of Murashige and Skoog (1962) and is very poor, the composition of this medium might influence the incorporation of [^{3}H]-leucine into protoplast protein. For this reason the incorporation of [^{3}H]-leucine into protein of protoplasts incubated in the medium as described by Takebe without 6-benzyladenine (see Materials and Methods) was compared with the incorporation into protein of protoplasts incubated in media as mentioned hereafter. The protoplasts were incubated in the medium to be tested at least 4 hours before and 5 hours during the labeling period.

The following media were compared:
1. TAKEBE's medium without 6-benzyladenine and enriched with 1 % sucrose or 10 mg/ml KCl. The incorporation of [^{3}H]-leucine was not enhanced further, in spite of the presence of a carbon source or a higher potassium concentration.
2. The medium developed by MEYER, in which the tobacco protoplasts de-differentiate, while preserving both their ability to regenerate a cell wall and to divide (MEYER, 1974). This medium, compared with the medium of TAKEBE, contains more of various essential salts, contains sucrose and has salts as osmoticum instead of mannitol. However, the incorporation of [^{3}H]-leucine was not changed.
3. The medium as described by TAKEBE, containing 1 to 5 µg/ml of the cytokinin 6-benzyladenine. In this case the incorportation of [^{3}H]-leucine was enhanced by 25 % in the presence of 3 to 5 µg/ml 6-benzyladenine. However, the virus multiplication was slightly inhibited by these concentrations of 6-benzyladenine. Therefore we decided on using the medium of TAKEBE without 6-benzyladenine, since its presence did not improve the viability of the protoplasts.
4. Media as described by TAKEBE, but with different pH's, ranging from 5 to 8 (adjusted with KOH). This change did not affect the incorporation of [^{3}H]-leucine into protoplast proteins.
5. Media consisting of mannitol only. We tried media of various concentrations: 0.5 M, 0.7 M and 0.9 M mannitol. Incubation of the protoplasts in media containing only mannitol and of different osmolarity did not influence the incorporation of [^{3}H]-leucine.
From these results we concluded that a poor medium such as TAKEBE's medium, used by us, is not harmful to the incorporation of [^{3}H]-leucine into protoplast proteins.

c. Poly-L-ornithine is necessary to infect protoplasts with TMV, although it is harmful to the protoplasts. Protoplasts were mock-infected (see Materials and Methods) without of with 0.5 to 2 µg poly-L-ornithine per ml infection medium. [^{3}H]-leucine was added to the incubation medium at least 5 hours after poly-L-ornithine treatment. It appeared that the incorporation of [^{3}H]-leucine into protein of the poly-L-ornithine treated protoplasts was 25 to 40 % less than the incorporation into protein of untreated protoplasts. Thus poly-L-ornithine was disadvantageous to incorporation of animo acids. However, polycations less harmfull to protoplasts. could not be used to infect the protoplasts withTMV.

d. Samples of protoplasts were incubated at different light intensities. After 15 hours [^{3}H]-leucine was added and 6 hours later the protoplasts were harvested. The light intensity two times higher than 9,300 erg/cm^2/sec (9,300 erg/cm^2/sec was used in all other experiments) did not affect the amount of [^{3}H]-leucine incorporated into protein. When samples of protoplasts were incubated either in the light or in the dark it appeared that the incorporation into protein of protoplasts incubated in the dark, was not affected at all, or was 10 to 50 % higher than that of protoplasts incubated at 9,300 erg/cm^2/sec. We preferred to incubate the protoplasts in the light, for incubation in the dark appeared sometimes to decrease the viability of the protoplasts.

TABLE 4.1. Effect of the concentration of protoplasts on the incorporation of [^{3}H]-leucine into protein.

number of protoplasts/ml x 10^{-5}	amount of ^{3}H-leucine incorporated into protein of 10^5 protoplasts dpm $\times 10^{-6}$	percentage of added leucine, incorporated into protoplast protein
1	3.1	8
2	2.55	11.5
4	1.4	12.7

Samples of protoplasts were incubated in the presence of 20 μCi/ml [^{3}H]-leucine (specific activity 45 Ci/mmol) during 7 hours.

e. The effect of concentration of the protoplasts during the labeling period with [^{3}H]-leucine is shown in table 4.1. The specific activity of protein of the protoplasts incubated at a concentration of 1×10^5 protoplasts/ml was at least two times higher than the specific activity of protein of protoplasts incubated at a concentration of 4×10^5 protoplasts/ml. Only 8 to 13 % of the added leucine was incorporated into protein of protoplasts, incubated during 7 hours in the presence of [^{3}H]-leucine at a concentration of 1 and of 4×10^5 protoplasts/ml respectivily. The amount of [^{3}H]-leucine added to the incubation medium was 20 μCi (specific activity 45 Ci/mmol); thus less than 0.44×10^3 μmol leucine per ml was present in the medium. Therefore this result can be explained by the fact that a limiting amount of leucine was present in the medium and the available amount had to be distributed over the protoplasts present.

f. It was found that addition to the incubation medium of the antibiotic(s) aureomycine (5 μg/ml) or aureomycine 5 μg/ml in combination with rimocidin (10 μg/ml), was very effective in inhibiting growth of micro-organism. However, aureomycine, which acts on prokaryotic and eukaryotic systems (VAZQUES, 1974) reduced at this concentration the incorporation of [^{3}H]-leucine into protoplast protein by 25 %. When 10 μg/ml rimocidin was added in addition to aureomycine no further inhibition occurred.
The reduced incorporation of [^{3}H]-leucine was probably not due to inhibition of growth of micro-organism, because in the presence of 1 μg/ml cycloheximide – when there is only prokaryotic protein synthesis – the incorporation of [^{3}H]-leucine into protoplast protein was only 5 % of the incorporation in the absence of any inhibitor. Furthermore the incorporation of [^{3}H]-leucine was not affected by 100 μg/ml chloramphenicol, an inhibitor of prokaryotic protein synthesis.
We decided to use 5 μg/ml aureomycine during incubation of the protoplasts, which antibiotic at this concentration certainly did not inhibit TMV multiplication.

4.4. INCORPORATION OF [^{3}H]-LEUCINE INTO PROTEIN OF SUBCELLULAR FRACTIONS

One million protoplasts contained approximately 690 µg protein. Distribution of the amount of protein and of the radioactive amino acids incorporated over different subcellular fractions is presented in table 4.2. The preparation of the subcellular fractions and the determination of the amount of protein is described under Materials and Methods.

TABLE 4.2. The relative amount of protein and of [^{3}H]-amino acid incorporated into protein in the subcellular fractions of tobacco protoplasts.

subcellular fractions	amount of protein	amount of ^{3}H-amino acid incorporated into protein
	%	%
nuclear	1	1
broken cells and membranes[1)]	3.4	3.5
chloroplast	49	18
20,000 xg pellet	3	9
20,000 xg supernatant	43	68
Sum	± 100	± 100

The procedure for subcellular fractionation and determination of the amount of protein and [^{3}H]-amino acid incorporated is described in chapter 2.

[1)] The fraction of broken cells and membranes was harvested from the layer between the 45 and 60 % sucrose layer after centrifugation of the 2,500 xg pellet in a discontinuous sucrose gradient.

Nearly half of the total amount of protoplast protein was present in the chloroplast fraction (this protein was extracted from the green material layered between the 20 and 45 % sucrose layer after gradient centrifugation of the 2,500 xg pellet), whereas the relative amount of [^{3}H]-leucine incorporated into protein of this fraction was less than 20 %.

The specific activity of the soluble proteins of the 20,000 xg supernatant and of the membrane proteins of the 20,000 xg pellet was higher than the specific activity of proteins in the other fractions.

The incorporation of [^{3}H]-leucine into protein of corresponding subcellular fractions of TMV and mock-infected protoplasts was compared. The protoplasts were labeled in the period from 4 to 24 hours after inoculation. More than 70 % of the protoplasts were infected. Differences between incorporation of [^{3}H]-leucine into protein of the subcellular fractions from TMV and from mock-infected protoplasts were not observed.

4.5. Specific activity of TMV coat protein

The specific activity of TMV coat protein was calculated after the virus had been isolated from infected protoplasts by CsCl gradient centrifugation. For this purpose the 27,000 xg supernatant of protoplast lysates was pipetted onto a sucrose layer on top of the CsCl solution. The experiments are described under Materials and Methods and the results are shown in fig 3.4. and 4.4. This method enabled us to discriminate between TMV coat protein and the soluble protoplast proteins in the 27,000 xg supernatant in one single centrifugation step.

The amount of TMV in the peak area at density 1.30 g/cm^3 was calculated on account of the optical density at 260 nm. This was possible because control experiments (fig. 4.3.) showed that 70 % of the amount of purified TMV layered on top of the sucrose cushion was detected in the peak area. In these control experiments the amount of purified TMV before and after gradient centrifugation was calculated on account of the optical density. The 27,000 xg supernatant of TMV infected protoplasts was layered on top of the sucrose cushion, instead of purified TMV in other control experiments. It appeared that again 70 % of the amount of TMV loaded on top of the gradient was present in the peak area. In this type of experiment the amount of TMV in the 27,000 xg supernatant was determined by infectivity assay and in the peak area this was done on

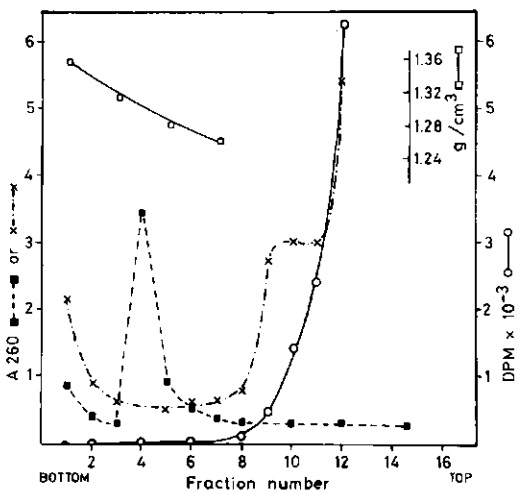


FIG. 4.3. Sedimentation profiles of purified TMV and of the 27,000 xg supernatant of mock-inoculated tobacco protoplasts after CsCl centrifugation.
The procedures are described in chapter 2. An amount of 135 µg purified, non-radioactive TMV was layered on top of the sucrose cushion. The sedimentation profile was determined by absorbancy at 260 nm (– ■— ■–). TMV sedimented to a density of 1.30 g/cm^3 in the CsCl gradient. Density (–□–□–). The 27,000 xg supernatant was prepared from 8 × 10^6 mock-inoculated protoplasts, which were incubated with 1 µCi [^{3}H]-leucine (specific activity 45 Ci/mmol) for 41 hours. It contained 10.5 × 10^6 dpm. The sedimentation profile was determined by absorbancy at 260 nm (– × – · – × –) and by radioactivity (O–O).

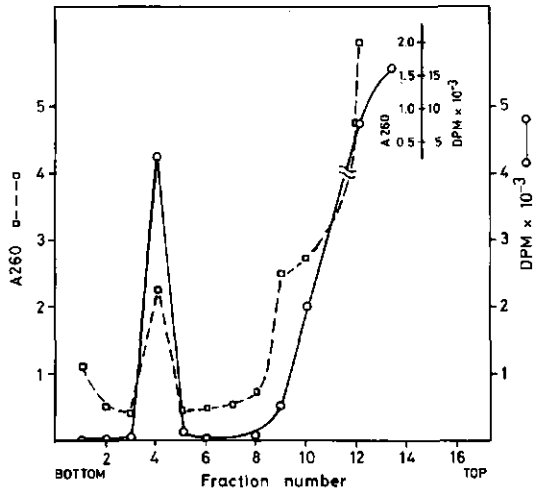


FIG. 4.4. Sedimentation profile of the 27,000 xg supernatant of TMV infected tobacco protoplasts after CsCl centrifugation.
The 27,000 xg supernatant was prepared of 10^7 TMV infected protoplasts, which were incubated with 1 μCi [^{3}H]-leucine/ml (specific activity 45 Ci/mmol) for 41 hours; 73 % of the protoplasts were infected with TMV. The 27,000 xg supernatant contained 7.7×10^6 dpm. The sedimentation profiles were determined by absorbancy at 260 nm (□ - - - □) and radioactivity (O– O).

account of the optical density at 260 nm. The amount of TMV could not be calculated by infectivity assay, for the infectivity of TMV was reduced after CsCl gradient centrifugation.
Moreover it is shown (fig. 4.3.) that the absorbance at 260 nm of material of mock-infected protoplasts in the position of the peak area is very low. Any labeled material of mock-infected protoplasts was absent at the position of the peak area. Furthermore contamination of the peak area by radioactive labeled protein other than TMV coat protein was checked by polyacrylamide gel electrophoresis of the peak fractions. The 27,000 xg supernatant of TMV infected labeled protoplasts was loaded onto the sucrose cushion and centrifuged (fig. 4.4). Thereafter the peak fractions were dialysed and incubated with 2 % SDS and 2 % β-ME for 3 hours at 37° C. After electrophoresis 90 % of the [^{3}H]-amino acid labeled material loaded on top of a 10 % gel was present at the position of TMV coat protein.
Thus our method anabled us to determine correctly both the amount of TMV coat protein and the amount of [^{3}H]-amino acid incorporated into coat protein.

When TMV infected protoplasts were incubated during 42 hours after infection and labeled with 1 μCi [^{3}H]-leucine per ml during the last 33 hours, the amount of coat protein in the 27,000 xg supernatant was 2.5 % of the total amount of protein in the supernatant and the amount of radioactive amino acids incorporated into coat protein was 10 % of the amount of [^{3}H]-leucine incorporated into protein of the 27,000 xg supernatant. In these experiments 50 to 70 % of the protoplasts were infected with TMV and 74 % of the protoplasts were

alive after the incubation period. Table 4.3. shows the specific activity of TMV coat protein and gives the specific activity of the proteins in the 27,000 x*g* supernatant for reasons of comparison. The specific activity of TMV coat protein was about four times the specific activity of the amount of protein of the 27,000 x*g* supernatant.

TABLE 4.3. Specific activity of TMV coat protein and of the total amount of protein from the 27,000 x*g* supernatant of infected tobacco protoplasts.

experiment	labeling period hours	concentration of [^{3}H]-leucine in the incubation medium µCi/ml	specific activity dpm/µg protein	
			TMV coat protein	27,000 x*g* supernatant proteins
1	38	1	7,400	1,900
2	17	0.55	8,700	2,100

The procedure for the determination of the specific activity of TMV coat protein and of the supernatant proteins synthesized in the infected protoplasts is described in chapter 2.

4.6. DISCUSSION

In this study it has been demonstrated that the specific activity of TMV coat protein is four times higher than the specific activity of the proteins from the 27,000 x*g* supernatant of the infected tobacco protoplasts. TMV is separated from the 27,000 x*g* supernatant material by CsCl centrifugation. This appeared to be the most simple and accurate method for this type of investigation.

Ten percent of the [^{3}H]-leucine, incorporated into protein of the 27,000 x*g* supernatant of TMV infected protoplasts, is incorporated into coat protein after a labeling period of 33 hours and an incubation period of 42 hours post infection. PATERSON and KNIGHT (1975) mentioned a nearly equal percentage. In this case the TMV infected tobacco protoplasts were incubated for 12 hours in the presence of [^{14}C]-leucine up till 72 hours post infection. It has been found by SIEGEL *et al.* (1978) that 50 to 70% of the [^{3}H]-leucine, incorporated into protoplast protein, is incorporated into TMV coat protein. The TMV infected tobacco protoplasts were incubated with radioactive amino acids during 2 hours. This considerable difference with our results may hardly be attributed to differing conditions in protoplast isolation, or to different conditions during incubation. It appeared in fact that when our conditions are modified the incorporations of radioactive amino acids is hardly influenced by these changes.

The amount of TMV coat protein, synthesized in 42 hours following in-

oculation of the tobacco protoplasts, is 2.5% of the amount of supernatant proteins. Considerable smaller amounts of the two other TMV induced proteins are synthesized; the maximal rate of synthesis is just as large, or even larger than the rate of synthesis of coat protein during a short period after inoculation (TAKEBE *et al.*, 1975; PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978). Therefore it may be assumed that not only TMV coat protein, but other TMV induced proteins as well have a specific activity of at least four times the activity of the supernatant proteins, if the protoplasts are incubated with radioactive amino acid precisely during the period that the TMV specific proteins are synthesized with great velocity.

Thus it seems probable that proteins synthesized as a result of TMV multiplication, being present in a small amount compared to the host proteins, may be detected after separation by means of SDS-polyacrylamide gel electrophoresis. Even when a virus protein coincides with several host proteins it may be possible that the virus protein is distinguished as a result of its higher specific radioactivity.

5. TMV MULTIPLICATION IN COWPEA PROTOPLASTS

5.1. INTRODUCTION

HIBI *et al.* (1975) have developed a procedure for isolation of mesophyll protoplasts from primary leaves of cowpeas (*Vigna unguiculata* (L) Walp. var. 'Blackeye Early Ramshorn'). The cowpea protoplasts can be infected with CPMV and are of such a quality that the CPMV replication in these protoplasts can be investigated (ROTTIER *et al.*, 1979). It is also possible to infect the cowpea protoplasts for example with CMV (KOIKE *et al.*, 1975), AMV (ALBAS and BOL, 1977) and clover yellow mosaic virus (RAO and HIRUKI, 1978). It has been described by KOIKE *et al.* (1976), that cowpea protoplasts can be infected with TMV.

For various reasons it seemed interesting to include cowpea protoplasts in our work. As has been described in chapter 3 the cultivation of tobacco plants for preparation of protoplasts demands great care and leaves for the isolation of protoplasts can be taken only from 60 days old tobacco plants. In contrast to this cowpea plants can be cultivated in conditions easier to control and the primary leaves are already in optimal condition for protoplast isolation after 10 days.

Furthermore, availability of cowpea protoplasts that can be infected with TMV opens the possibility for investigation of virus specific proteins in another host than tobacco.

Cowpeas are not natural hosts for the common strain of TMV. Leaves inoculated with TMV do not show any symptoms and the virus does not multiply within the plants.

Since tobacco is not a natural host to cowpea mosaic virus (CPMV), the course of CPMV multiplication in tobacco protoplasts was studied and compared with the course of the TMV multiplication in these protoplasts. It appeared that the infection of tobacco protoplasts with CPMV is characterized by a much longer latent period than is found at infection of tobacco protoplasts with TMV (HUBER *et al.*, 1977).

It seemed of interest to trace whether TMV multiplication in cowpea protoplasts is influenced by the fact that cowpea is not a natural host to TMV. The so-called cowpea strain of TMV (C-TMV) produces symptoms on the cowpea leaves after infection and multiplies within the plants. The infection of cowpea protoplasts with C-TMV was therefore included in our work for comparison.

When the procedure of KOIKE *et al.* (1976) was followed for the infection of cowpea protoplasts with TMV, a small percentage of the protoplasts was infected with TMV and a small percentage of these survived the infection procedure. Therefore it was necessary to improve the conditions of the infection of cowpea protoplasts with TMV.

5.2. Time course of TMV and C-TMV synthesis in cowpea protoplasts

Cowpea protoplasts were incubated for 0, 8, 16, 24, 32 and 40 hours post inoculation and extracts of samples of protoplasts were assayed for infectivity. A growth curve of TMV is shown in figure 5.1. The rapid synthesis of TMV started not earlier than 12 hours after the onset of incubation. The rate of synthesis of TMV remained unchanged for 40 hours. Within 40 hours 60,000 infectious virus particles per protoplast were synthesized (4 μg TMV/10^6 infected protoplasts).

The course of the TMV multiplication in cowpea protoplasts was compared in the same experiment with the course of multiplication of C-TMV. Figure 5.1. shows that the time courses of TMV and C-TMV multiplication in cowpea protoplasts run parallel.

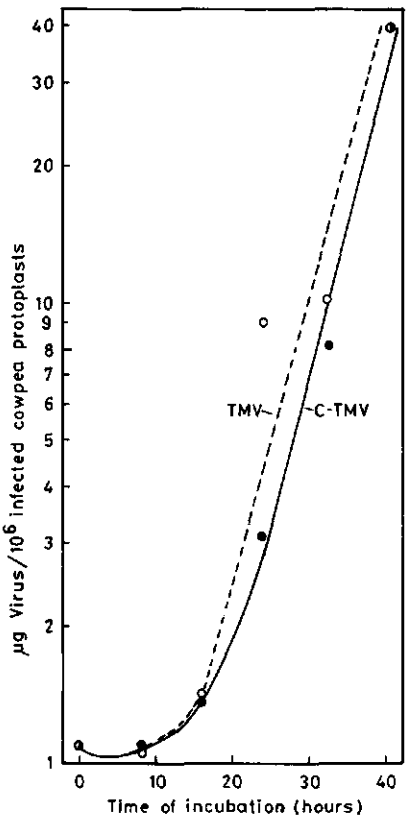


Fig. 5.1. The time course of TMV and C-TMV synthesis in cowpea protoplasts.
The amount of virus in the infected protoplasts was calculated from the infectivity of the extracts, determined by means of local lesion assay of protoplast extracts, and the final percentage of protoplasts which are infected.

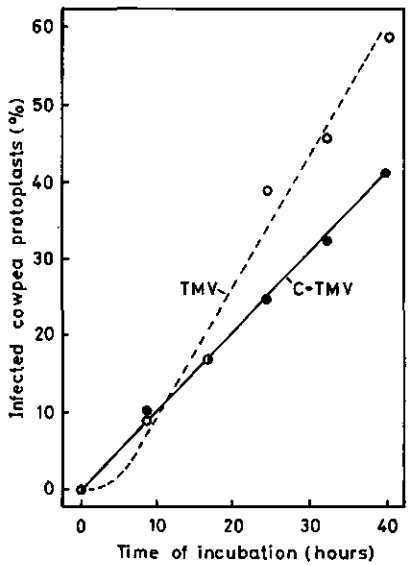


FIG. 5.2. The time course of the percentage of cowpea protoplasts, which are infected with TMV and C-TMV.
Samples of protoplasts were fixed at various times after inoculation and were stained with fluorescent antibodies prepared against TMV and against C-TMV.
The percentage of protoplasts which were infected, was determined under the fluorescence microscope.

5.3. FREQUENCY OF INFECTION OF COWPEA PROTOPLASTS WITH TMV AND C-TMV

At the same time that samples of protoplasts were taken for local lesion assay, protoplasts were fixed for staining by fluorescent antibodies. The increase of the percentage of cowpea protoplasts stainable with fluorescent antibodies prepared against TMV is shown in figure 5.2. As soon as 8 hours after the onset of incubation characteristic fluorescent dots became visible in some protoplasts. The density of the fluorescent dots increased in the infected protoplasts during prolonged incubation. Thirty-two hours post inoculation the majority of the infected protoplasts were stainable with fluorescent antibodies (figure 5.3.), The number of stainable protoplasts increased linear up to at least 40 hours after the onset of incubation. For comparison the increase of the percentage of C-TMV infected protoplast has been traced (in the same experiment), detectable after staining by fluorescent antibodies prepared against C-TMV. The course of the increase of the percentage of protoplasts infected with TMV or C-TMV was similar. In this experiment 42 hours following inoculation with TMV or C-TMV, 59 and 41 % of the protoplasts were infected respectively. In various other experiments, in which cowpea protoplasts were infected on the one hand with

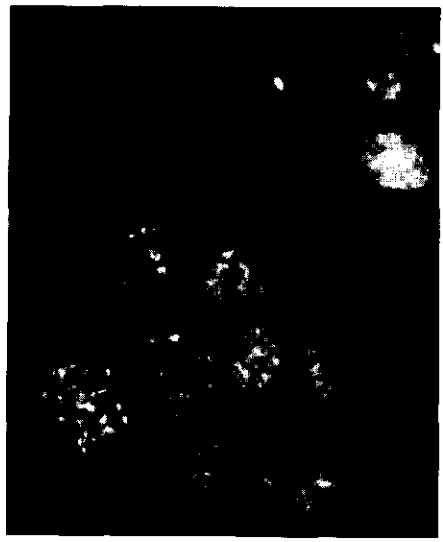


FIG. 5.3. Fluorescence micrograph of cowpea protoplasts infected with TMV.
A sample of protoplasts was fixed at 43 hours post inoculation and stained with fluorescent antibodies prepared against TMV. Several masses of TMV conjugated with fluorescent antibodies were visible, situated between the chloroplasts (± 960 x).

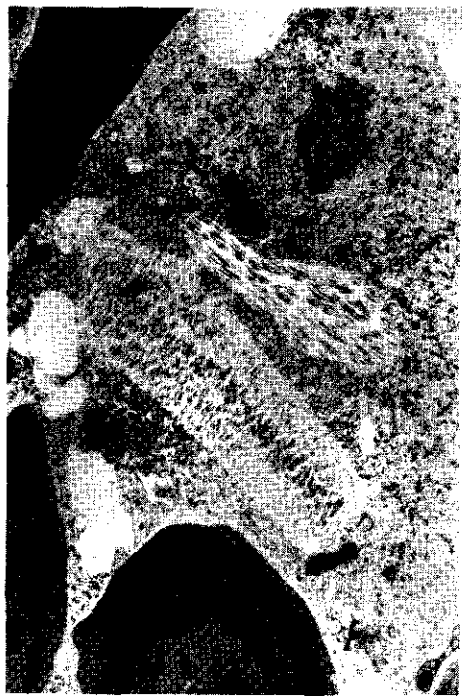


FIG. 5.4. Electron micrograph of cowpea protoplasts infected with TMV.
Samples of protoplasts were fixed at 24 hours post inoculation. Large aggregates of TMV, indicated by arrows, are seen in the cytoplasm (20,000 x).

TMV and on the other with C.TMV, the percentage of TMV and C-TMV infected protoplasts varied but a little.
The TMV infected protoplasts could not be stained by fluorescent C-TMV antibodies and *vice versa.*

5.4. Electron microscopy of TMV and C-TMV infected cowpea protoplasts

Samples of cowpea protoplasts were collected after 24 hours of incubation and prepared for examination under the electron microscope. At many sites in the cytoplasm of TMV infected protoplasts small aggregates of virus were observed. Large aggregates of virus were also frequently present (figure 5.4.). In some ultrathin sections of the infected protoplasts the virus aggregates seemed to be surrounded by endoplasmatic reticulum. When sections of TMV and C-TMV infected cowpea protoplasts were compared no differences were observed. Nor were evident cytopathic effects detected due to TMV multiplication, when ultrathin sections of control protoplasts and infected protoplasts were compared.

5.5. Factors influencing the infection of cowpea protoplasts with TMV

Effect of polycation. The presence of polycation in the inoculation medium was necessary for the infection of cowpea protoplasts with TMV. Table 5.1. shows that the frequency of infection doubles, when poly-L-ornithine is replaced by poly-D-lysine, whereas the amount of TMV synthesized in the infected protoplasts does not change. Pre-incubation of TMV with poly-D-lysine was necessary, but pre-incubation of the protoplasts with polycation was not. Polycations are harmful to the protoplasts. Poly-D-lysine concentrations higher than 1 µg/ml caused a strong decrease of the number of protoplasts, surviving the infection procedure.

Effect of buffer. Table 5.2. shows the result of infection of the cowpea protoplasts with TMV in the presence of potassium phosphate buffer (0.1 M, pH 5.4) at 0° C, instead of potassium citrate buffer (0.01 M, pH 5.2) at 25° C. The amount of TMV synthesized in the infected protoplasts was at least three times higher when potassium phosphate buffer was used, whereas the frequency of infection did not change.

Doubling or halving of the potassium phosphate concentration was of no influence on the frequency or the amount of TMV synthesized. The amount of virus synthesized in the infected protoplasts increased by approximately 30%, when infection in the presence of potassium phosphate buffer took place at 0° C instead of at room temperature.

Effect of pH. When the protoplasts were infected at pH of 5.4 both the percentage of protoplasts infected with TMV and the amount of TMV synthe-

Table 5.1. to 5.6. Effect of factors influencing the infection of cowpea protoplasts with TMV.

Table	Factors of the inoculation medium	% viable protoplasts	% infected protoplasts	µg TMV/10^6 infected protoplasts
1.	POLYCATION[1)]	20 hours post inoculation		
	poly-L-ornithine 0.75 µg/ml during pre-inoculation 1 µg/ml during inoculation	70	24	0.19
	poly-D-lysine 0.75 µg/ml during pre-inoculation 1 µg/ml during inoculation	62	69	0.2
2.	BUFFER	hours post inoculation		
		40	24	24
	potassium phosphate 0.1 M; pH 5.4, at 0° C	53	59	1
	potassium citrate 0.01 M; pH 5.2, at 25° C	58	57	0.29
3.	CONCENTRATION OF TMV	24 hours post inoculation		
	µg/ml			
	1	68	59	1
	2	72	28	0.7
	4	70	38	1
	6	68	39	1
4.	CONCENTRATION OF PROTOPLASTS	24 hours post inoculation		
	10^{-5}/ml			
	2	68	59	1
	4	59	40	1.4
	6	63	30	1.1
	8	74	36	0.95
	factors of the incubation medium			
5.	6-BENZYLAMINOPURINE	41 hours post inoculation		
	µg/ml			
	0	59	54	4.6
	1	56	68	1.7
	3	48	70	0.5
6.	ANTIBIOTICS	41 hours post inoculation		
	–	48	50	4.4
	aureomycine 5 µg/ml	50	54	4.6
	aureomycine 5 µg/ml + rimocidin 10 µg/ml	50	79	2.9
	gentamicin 2.5 µg/ml	45	62	0.06

The inoculation of cowpea protoplasts with TMV, the incubation of the protoplasts, the determination of the percentage of protoplasts which are viable, of the frequency of infection and of the amount of TMV synthesized in the infected protoplasts are described in chapter 2.

[1)]The pH of the inoculation medium was 5.9 instead of 5.4.

sized in the infected protoplasts were highest, tested after 24 hours of incubation. The protoplasts were severely damaged at pH's lower than 5.4. At a pH higher than 5.4 the percentage of infected protoplasts decreased, the amount of virus synthesized in the infected protoplasts did not change, the number of surviving protoplasts increased however.

Effect of concentration of mannitol. The cowpea protoplasts were incubated both during the pre-inoculation and during the inoculation period at a mannitol concentration of 0.6 M. When the mannitol concentration was raised to 0.7 M or 0.8 M during the inoculation period the result was that the amount of TMV, synthesized in the infected protoplasts after 24 hours, decreased by 50 %, although the percentage of protoplasts infected after 24 hours did not diminish.

Effect of concentration of TMV. Table 5.3. shows that at an increasing TMV concentration during inoculation the frequency of infection, tested 24 hours after the onset of incubation, decreases. The amount of virus in the infected protoplasts, synthesized during the 24 hours of incubation, remained unchanged. After 40 hours of incubation the frequency of infection of the various protoplast samples was the same. This points to the fact that at an increasing TMV concentration in the infection medium less TMV particles enter a protoplast or start multiplying after entering.

Effect of concentration of protoplasts. Table 5.4. shows that at an increase of the protoplast concentration during pre-inoculation and inoculation period the percentage of infected protoplasts after 24 hours, decreases. The amount of TMV, synthesized during 24 hours of incubation in the infected protoplasts, remained unchanged. The frequency of infection tested after 40 hours of incubation is approximately the same for the various samples of protoplasts. Presumably the concentration of protoplasts during the infection procedure influences the TMV infection in the same manner as the concentration of TMV during the infection procedure.

Effect of 6-benzylaminopurine and of antibiotics in the medium during TMV multiplication. After inoculation of the cowpea protoplasts with TMV the protoplasts were incubated in minimal medium as described by TAKEBE *et al.* (1968) for tobacco protoplasts, except that a mannitol concentration of 0.6 M was used instead of 0.7 or 0.8 M. The TMV multiplication was inhibited by 50 to 85 % by 6-benzylaminopurine in the medium in a concentration of 1 µg/ml or higher, as is shown in Table 5.5. The survival of the protoplasts during the incubation was not raised by 6-benzylaminopurine. Therefore this compound was not added to the incubation medium.

It became apparent that the antibiotic added to the medium should be selected most carefully. As is shown in Table 5.6. the TMV multiplication was not suppressed by aureomycine (5 µg/ml). Inhibition of the TMV multiplication occured when aureomycine (5 µg/ml) was added in combination with rimocidin (10 µg/ml). When gentamycin (2.5 µg/ml) was present in the medium the TMV multiplication was also inhibited.

5.6. Discussion

The conditions for infection of cowpea protoplasts with TMV were optimalized in the study described here. Fifty to 70 % of the cowpea protoplasts can be infected with TMV and 4 μg/10^6 infected protoplasts is synthesized after 40 hours of incubation. Best results were obtained if both the cowpea protoplasts and the TMV are incubated with poly-D-lysine prior to infection.
The concentration of protoplasts and of TMV is low during infection, i.e. 2×10^5 protoplasts/ml and 1 μg TMV/ml, whereas the concentration of poly-D-lysine (1 μg/ml) is as high as possible. At the infection procedure of cowpea protoplasts (*Vigna sinensis)* with TMV, as described by Koike *et al.* (1976), a less aggressive treatment of the protoplasts with polycation was applied. They used 0.5 μg/ml poly-L-ornithine during infection and the protoplasts were not pre-incubated with polycation prior to infection. Cowpea protoplasts can be infected with CPMV without polycation, however.

The growth curve of TMV in cowpea protoplasts shows that the amount of TMV starts to increase only 12 hours after incubation with great velocity and that the TMV synthesis is linear for longer than 36 hours. However, some of the TMV infected protoplasts are stained as early as 8 hours after incubation with fluorescent TMV antibodies, visible as tiny fluorescent dots. After that time both the amount of fluorescence in the infected protoplasts increases and the percentage of infected protoplasts. Therefore it is probable that the TMV multiplication starts already earlier than 8 hours after incubation and that the slow start is a dose effect. Very few virus particles may have entered into most of the protoplasts or/and proceeded to virus multiplication after infection.

Whereas it is not possible to infect cowpea plants with TMV, in leaf cell protoplasts the infection and multiplication of TMV take place unimpared. It appeared that the course of the TMV and C-TMV synthesis in the infected protoplasts is the same and that changes in the conditions of infection have a similar effect on the C-TMV as on the TMV infection.
It is not clear which factors prevent the infection of cowpea leaves with TMV. Possibly at inoculation of leaves a number of cells are in fact infected, but no spread of virus to neighbouring cells occurs. In some experiments we isolated protoplasts from cowpea leaves that were inoculated with TMV, TMV RNA and TMV preincubated with poly-D-lysine. In the protoplast preparations it appeared not possible to detect cells stained by fluorescent antibodies. If there is any infection at all of cells after inoculation of the leaves this will be in a very small number of cells and the infection does not seem to spread to the other cells in the leaf. These preliminary results indicate that leaf tissue and isolated leaf cells respond differently to virus infection.

The percentage of cowpea protoplasts infected with TMV is approximately equal to the percentage of tobacco protoplasts infected with TMV. Nor does the course of the TMV multiplication in cowpea protoplasts differ very much from the course of the TMV multiplication in tobacco protoplasts. In cowpea protoplasts TMV starts to be synthesized rapidly 6 to 8 hours later than in tobacco

protoplasts and the rapid synthesis continues for a longer period. The amount of TMV synthesized in cowpea protoplasts after 40 hours of incubation is one fifth of the amount of TMV synthesized in tobacco protoplasts. A possible explanation for the smaller amount of TMV produced per infected protoplast is that cowpea protoplasts are smaller than tobacco protoplasts.

Ultrathin sections of TMV infected cowpea protoplasts examined under the electron microscope did not show any other cytopathic effect than virus aggregations, comparable to what has been mentioned of electron microscopic examination of TMV infected tobacco protoplasts (HIBI and YORA, 1972; OTSUKI *et al.*, 1973).

It seems probable therefore that cowpea protoplasts are very suitable for comparison of the virus specific proteins, synthesized upon TMV infection therein, with the protiens synthesized as a result of the TMV infection in tobacco protoplasts.

6. POLYPEPTIDES SYNTHESIZED AS A RESULT OF TMV MULTIPLICATION IN TOBACCO PROTOPLASTS

6.1. INTRODUCTION

The search for proteins synthesized as a result of TMV infection in tobacco leaves and protoplasts has not led to unequivocal results. At first it was reported that the synthesis of five polypeptides of molecular weights 245,000, 195,000, 155,000, 37,000 and 17,500 (coat protein), not found in uninfected leaves, occurred in TMV infected tobacco leaves (ZAITLIN and HARIHARASUBRAMANIAN, 1970, 1972); see table 1.1. Subsequent studies were unable to reproduce these results (SINGER, 1971, 1972; SINGER and CONDIT, 1974) but SCALLA *et al.* (1976, 1978) reported the occurrence of three polypeptides of M.W. 150,000 and 130,000 similar to the polypeptides of M.W. 195,000 and 155,000 respectivily and coat protein. These three polypeptides were the only ones which were also detected in TMV infected tobacco protoplasts (SAKAI and TAKEBE, 1974; PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978).

The search for proteins synthesized as a result of TMV infection is hampered by the large number of host proteins synthesized at the same time. Host protein synthesis is not suppressed by TMV infection and inhibition by means of U.V. irradiation resulted in a considerable impairing of the virus multiplication (PATERSON and KNIGHT, 1975).

In this chapter it is shown that it is possible to demonstrate ten polypeptides in TMV infected tobacco protoplasts, which are not or to a far less extent synthesized in mock-infected protoplasts. These results were obtained by improving the detection capacity by means of subcellular fractionation of the homogenates of TMV infected and healthy protoplasts before analysis of the proteins by SDS-polyacrylamide slabgel electrophoresis.

6.2. ELECTROPHORETIC ANALYSIS OF PROTEIN FROM TMV INFECTED AND MOCK-INFECTED TOBACCO PROTOPLASTS

The proteins of TMV infected and mock-infected tobacco protoplasts were labeled with [^{35}S]-methionine. The growth curve of TMV in tobacco protoplasts (figure 3.1.) is characterized by a period of about 6 hours after the onset of incubation, during which there is hardly any increase of virus, followed by an exponential multiplication of TMV for about 22 hours. The proteins of the protoplasts were labeled by adding [^{35}S]-methionine to the incubation medium 8 hours after the onset of incubation, at the beginning of the exponential virus growth, and the labeling was continued for 16 hours.

The host protein synthesis is not inhibited by the TMV infection, therefore a large variety of host proteins are also labeled. When the proteins from TMV

infected protoplasts were analysed by SDS-PAGE, polypeptide patterns contained a large number of bands, which were very close to each other and even overlapped. This hampered the detection of polypeptides newly synthesized upon virus infection, in particular in the M.W. range of 100,000 to 35,000. It appeared possible to achieve an improvement of the detection capacity by subcellular fractionation of the protoplast homogenates before electrophoretic analysis.

After homogenization of the protoplasts the homogenate was divided into a 2,500 x*g* pellet, a 31,000 x*g* pellet and a 31,000 x*g* supernatant (see chapter 2.). Bands of polypeptides from the 31,000 x*g* supernatant appeared clearly distinguishable in the autoradiograms, although many bands were present. The resolution could be improved by means of $(NH_4)_2SO_4$ precipitation of protein from the 31,000 x*g* supernatant. The protein fraction which precipitated and the fraction which remained in solution appeared to yield polypeptide patterns of which the bands were less tightly packed. Protein of the 31,000 x*g* pellet showed a good polypeptide pattern, although a grey background was present in the autoradiogram.

In autoradiograms of protein of the 2,500 x*g* pellet a predominant grey background was present. Therefore this pellet was resuspended and thereupon fractionated by means of centrifugation in a discontinuous sucrose gradient. This consisted of a 20%, 45% and 60% (w/v) sucrose layer. Three fractions were obtained after centrifugation: a nuclear fraction at the bottom of the centrifuge tube, a mixture of membrane fragments and remains of chloroplasts (rest fraction), present between the 60 and 45% sucrose layer, and a chloroplasts fraction, present between the 45 and 20% sucrose layer. The polypeptide patterns of these fractions were clear, although a grey background remained. This background was more pronounced, when during preparation the samples were not held severely reduced by 2% β-ME or 5 mM DTE in the medium and when they were not stored under nitrogen.

In the foregoing chapter it appeared that the specific radioactivity of protein from the chloroplast fraction was less than that of protein of any other fractions. Polypeptides synthesized upon TMV infection in inoculated tobacco leaves have been most clearly distinguished in electropherograms of protein from the 1,000 x*g* pellet, containing chloroplasts and nuclei (ZAITLIN and HARIHARASUBRAMANIAN, 1972). Therefore [^{35}S]-methionine was added to the incubation medium in such a quantity that autoradiograms with a clear polypeptide pattern of the chloroplast fraction were obtained.

When tobacco protoplasts were incubated with 20 μCi [^{35}S]-methionine (specific activity 240 Ci/mmol) for 16 hours under conditions as described in chapter 2., the incorporation of [^{35}S]-methionine into protein was such that 30 μg protein from the chloroplast fraction could be brought on gel with 300,000 cpm.

6.3. Polypeptides synthesized as a result of TMV multiplication in tobacco protoplasts

In polypeptide patterns of protein from the subfractions of the 2,500 x*g* pellet of TMV infected protoplasts four polypeptides were distinguished that were not or to a far less extent present in polypeptide patterns of protein from corresponding fractions of mock-infected protoplasts. The molecular weights of these polypeptides were approximately 170,000, 116,5000, 72,000 and 30,000, in the following denoted by p170, p116,5, p72, p30, respectively. In fig. 6.1. electrophoretic patterns of protein of the chloroplast fraction of TMV infected and mock-infected protoplasts are given. Polypeptides smaller than approximately 20,000 daltons had migrated from this gel, with the result that among others coat protein is not present in this gel.

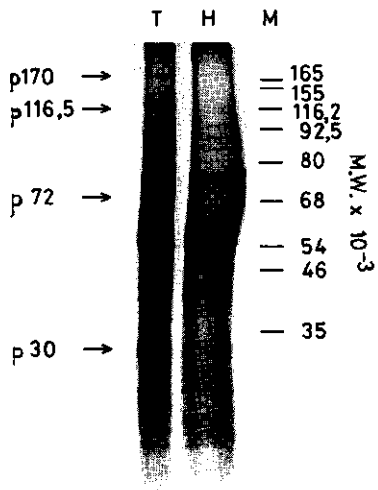


FIG. 6.1. Comparison of the electrophoretic patterns of protein from the chloroplast fraction of TMV and mock-infected tobacco protoplasts.
TMV infected and mock-infected protoplasts were incubated in standard medium (2.8×10^5 protoplasts/ml). Eight hours after the onset of the incubation [^{35}S]-methionine (20 μCi [^{35}S]-methionine/ml) was added. After 16 hours the protoplasts were collected by centrifugation at 100 x*g* for 2 min. The protoplasts were homogenized and the homogenate was centrifuged at 2,500 x*g* for 15 min. The 2,500 x*g* pellet was resuspended and loaded onto a discontinuous sucrose gradient as described in chapter 2. The chloroplast fractions were prepared for SDS-PAGE on 12.5 % gel. The samples of the chloroplast fraction of TMV and mock-infected protoplasts, applied to the gel, contained equal amounts of cpm. Electrophoresis was carried out during 9 hours at constant voltage, 1 hour at 50 V and 8 hours at 100 V. Polypeptides smaller than approximately 20,000 daltons had migrated from the gel, therefore coat protein is not present in this gel. The slabgel was stained, dried and autoradiographed.
The positions of p170, p116,5, p72 and p30, synthesized as a result of TMV multiplication, are marked by arrows.
T: TMV infected protoplasts.
H: mock-infected protoplasts.
M: marker proteins.

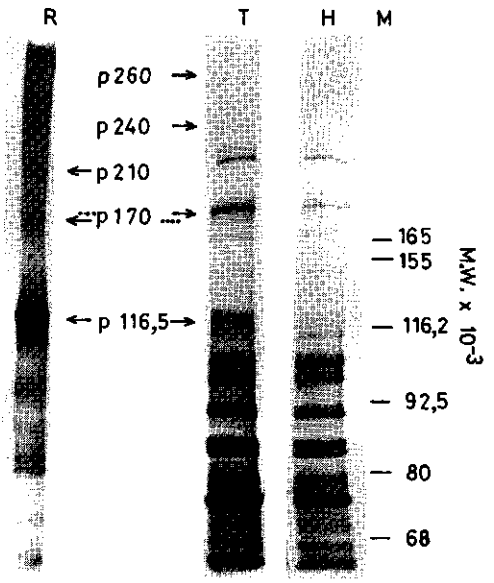


FIG. 6.2. Autoradiogram of protein of the 31,000 x*g* supernatant from TMV infected and mock-infected tobacco protoplasts and of the products synthesized in the reticulocyte lysate with TMV RNA as messenger.

TMV infected and mock-infected tobacco protoplasts were incubated in standard medium (2.8×10^5 protoplasts/ml). Eight hours after the onset of incubation [^{35}S]-methionine (20 μCi [^{35}S]-methionine/ml) was added. After 16 hours the protoplasts were harvested. Samples of the 31,000 x*g* supernatant fraction from TMV and mock-infected protoplasts, containing equal amounts of cpm, were prepared for SDS-PAGE. Samples of [^{35}S]-methionine labeled products translated from TMV mRNA in the reticulocyte lysate were prepared in the same way for SDS-PAGE. The samples were analysed on a 7.5 % slabgel at constant voltage, during 1 hour at 50 V and 6 hours at 160 V. The gels were stained, dried and autoradiographed.

The positions of the polypeptides synthesized as a result of TMV multiplication in the protoplasts, p260, p240, p170 and p116,5, and of the *in vitro* translation products, p210, p170 and p116,5, are indicated by arrows. The dotted lines (.....) above and below the arrows of p170 mark te position of p170 synthesized in vivo and in vitro respectively.

R: products synthesized in the reticulocyte lysate.
T: TMV infected protoplasts.
H: mock-infected protoplasts.
M: marker proteins.

A polypeptide of electrophoretic mobility similar to p170 was present in polypeptide patterns of nuclear, rest and chloroplast fractions from mock-infected protoplasts. The band in the polypeptide pattern of mock-infected protoplasts was weaker than the corresponding band in the pattern from TMV infected protoplasts.

P116,5, p72, p30 could only be distinguished in electrophoretic patterns of protein of TMV infected protoplasts. The darkest band in polypeptide patterns of the pellet fractions of TMV infected protoplasts was the band of p116,5.

The p116,5, p72 and p30 were also observed in polypeptide patterns of protein of the 31,000 x*g* pellet of TMV infected protoplasts (not shown). The p170, how-

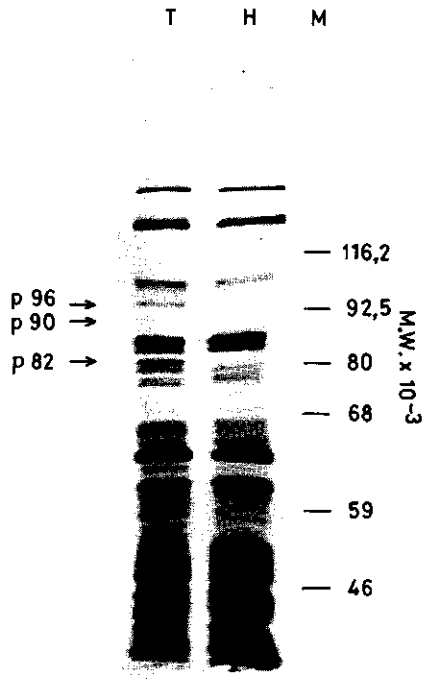


FIG. 6.3. Comparison of the electrophoretic patterns of protein, precipitated by $(NH_4)_2SO_4$, of the 105,000 xg supernatant from TMV infected and mock-infected tobacco protoplasts.
TMV infected and mock-infected protoplasts were incubated in standard medium (3×10^5 protoplasts/ml). Eight hours after the onset of incubation [^{35}S]-methionine (20 μCi [^{35}S]-methionine/ml) was added. After 16 hours the protoplasts were collected. The protoplasts were homogenized and the homogenate was fractionated. The 105,000 xg supernatant was saturated to 51 % with $(NH_4)_2SO_4$. The precipitate was pelleted and the pellet was prepared for SDS-PAGE. Samples of TMV and mock-infected protoplasts containing equal amounts of cpm were analysed on a 7.5 % slabgel at constant voltage during 6 hours, 1.5 hours at 50 V and 4.5 hours at 180 V. Thereafter the gels were stained, dried and autoradiographed.
The positions of p96, p90 and p82, synthesized as a result of TMV multiplication in the protoplasts, are marked by arrows.
T: TMV infected protoplasts.
H: mock-infected protoplasts.
M: marker proteins.

ever, was not distinguished in the electrophoretic pattern of protein of the 31,000 xg pellet, since a polypeptide of the corresponding fraction from mock-infected protoplasts comigrated with the p170 of TMV infected protoplasts and the band was as strong in the pattern of protein of TMV infected protoplasts as in the pattern of mock-infected protoplasts.

In polypeptide patterns of protein of the 31,000 xg supernatant of TMV infected protoplasts the p170, 116,5, p72, p17,5 and the polypeptides of molecular weights approximately 260,000, 240,000, 96,000, 90,000 and 82,000, thereupon denoted by p260, p240, p96, p90 and p82 respectively, were detected.

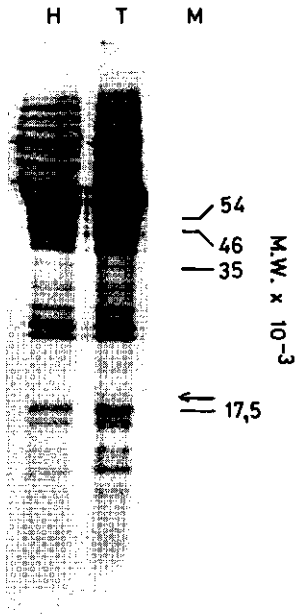


FIG. 6.4. [^{35}S]-methionine labeled TMV coat protein in the electrophoretic pattern of protein from the 31,000 x*g* supernatant fraction of TMV infected tobacco protoplasts.
TMV infected and mock-infected protoplasts were incubated in standard medium (3×10^5 protoplasts/ml). Seven hours after the onset of incubation [^{35}S]-methionine was added (20 μCi [^{35}S)-methionine/ml) After 16 hours the protoplasts were collected and homogenized. The samples of the 31,000 x*g* supernatant were prepared for SDS-PAGE. Samples of TMV infected and mock-infected protoplasts, containing an equal amount of cpm, were analysed on a 12.5 % slabgel at constant voltage during 6 hours, 1.5 hours at 50 V, 1 hour at 150 V and 3.5. hours at 220 V. The gel was stained, dried and autoradiographed. The position of the [^{35}S]-methionine labeled TMV coat protein synthesized in the infected protoplasts is marked by an arrow.
H: mock-infected protoplasts.
T: TMV infected protoplasts.
M: marker proteins.

Polypeptides with similar mobility were not or to a far less extent present in electrophoretic patterns of protein of mock-infected protoplasts.

In fig. 6.2., in the tract of protein of the 31,000 x*g* supernatant of TMV infected protoplasts,the p260, p240, p170 and p116,5 were observed. Polypeptides smaller than approximately 68,000 daltons had migrated from the 7.5 % gel. The p72 band had probably become invisible by the presence of the other bands of the gel. A polypeptide from mock-infected protoplasts comigrated with the p170 from TMV infected protoplasts. The band in the polypeptide pattern of mock-infected protoplasts was weaker than the corresponding band from TMV infected protoplasts. The bands of p260 and p240 were very weak. The pl16,5 was much clearer in other electrophoretic patterns of protein from the 31,000 x*g* supernatant.

Fig. 6.3. shows polypeptide patterns of protein from the 31,000 x*g* supernatant that remained in solution after the supernatant had been saturated to 51 % with

$(NH_4)_2SO_4$ at 0° C. In the tract of protein from TMV infected protoplasts the p96, p90 and p82 were distinguished. Polypeptides from mock-infected protoplasts comigrated with these three polypeptides from TMV infected protoplasts. The bands of these three polypeptides of mock-infected protoplasts were much weaker than the corresponding bands of TMV infected protoplasts. The p96 and p90 could not be distinguished in electropherograms of proteins of the 31,000 x*g* supernatant, when this supernatant had not been further fractionated. The p82 is not seen in the electrophoretic pattern, given in fig. 6.2. However, in other electrophoretic patterns of protein from the 31,000 x*g* supernatant, not further fractionated, p82 could be detected indeed. The bands of p96 and p90 belonged to the less strong bands of the pattern. After fractionation of the 31,000 x*g* supernatant proteins the p260, p240 and p170 were further left out of consideration. The p72 and p116,5 were observed in electrophoretic patterns of the protein fraction precipitating with $(NH_4)_2SO_4$.

After staining the gels with coomassie brilliant blue R the stained polypeptide patterns of protein from TMV and mock-infected protoplasts were nearly similar. However, one polypeptide, the p17,5, in patterns of protein from TMV infected protoplasts did not comigate with a polypeptide from mock-infected protoplasts. The p17,5 comigrated in fact with coat protein, serving as marker, and was for this reason apparently coat protein. A [^{35}S]-methionine labeled polypeptide with a migration velocity somewhat less than the stained coat protein from the same gel tract, was detected in autoradiograms with polypeptide patterns of protein from the 31,000 x*g* supernatant of TMV infected protoplasts. A polypeptide labeled with [^{35}S]-methionine from mock-infected protoplasts did not comigrate with the p17,5 (fig. 6.4.). The identification to TMV coat protein of this [^{35}S]-methionine labeled polypeptide will be described in the next chapter.

In order to identify p170 and p116,5 their electrophoretic mobility was compared with the mobility of the largest products *in vitro* synthesized under the direction of the TMV RNA in the rabbit reticulocyte lysate (see Materials and Methods). In the rabbit reticulocyte lysate two polypeptides of M.W. of approximately 165,000 and 140,000 (KNOWLAND *et al.*, 1974) of 160,000 and 110,000 (PELHAM, 1968) respectively, were reproducibly synthesized under the direction of TMV RNA.

Fig. 6.2. shows in addition to electrophoretic patterns of protein from the 31,000 x*g* supernatant of TMV and mock-infected protoplasts, the polypeptide pattern of products *in vitro* synthesized from TMV RNA as messenger. The products formed *in vitro* had a M.W. of approximately 210,000, 170,000 and 116,500 (in the following denoted p210, p170 and p116,5). Comparing the polypeptide patterns of protein synthesized *in vivo* and *in vitro* it appeared that the electrophoretic mobility of the p170 synthesized *in vivo* is not quite the same as the mobility of the p170 synthesized *in vitro*. The electrophoretic mobility of p116,5 from TMV infected protoplast was similar to the electrophoretic mobility of the *in vitro* translation product p116,5. None of the polypeptides synthesized *in vivo* in consequence of TMV multiplication comigrated with the p210 formed *in vitro*.

TABLE 6.1. Polypeptides synthesized as a result of TMV multiplication in tobacco protoplasts and the subcellular fractions in which the polypeptides are localized.

M.W × 10^{-3} of polypeptides, synthesized as a result of TMV multiplication	polypeptides present (+) or absent (–) in		
	31,000 xg supernatant	31,000 xg pellet	chloroplast, rest and nuclear fractions from the 2,500 xg pellet
260	+	–	–
240	+	–	–
170	+ h	–	+ h
116,5	+	+	+
96	+ h	–	–
90	+ h	–	–
82	+ h	–	–
72	+	+	+
30	–	+	+
17,5 (coat protein)	+	n	n

n: not determined.

h: a polypeptide of similar M.W. was present in the corresponding fraction of mock-infected tobacco protoplasts. The band of the polypeptide from mock-infected protoplasts was weaker than the band of the polypeptide from TMV infected protoplasts.

6.4. DISCUSSION

In this chapter we describe that ten polypeptides are observed in TMV infected tobacco protoplasts, which are not or to a far less extent present in mock-infected protoplasts (see table 6.1.). The molecular weights of the polypeptides were estimated 260,000, 240,000, 170,000, 116,500, 96,000, 90,000, 82,000, 72,000, 30,000 and 17,500 (coat protein). Such a large number could be observed because the protoplast homogenates were fractionated into six subcellular fractions. This improved the detection capacity.

It was shown that the p116,5 mentioned above and the p116,5 synthesized *in vitro* from TMV RNA as messenger comigrated. Therefore the p116,5 and p170 synthesized in TMV infected protoplasts will correspond with polypeptides reported by others: p135 and p165 respectively, from TMV inoculated leaves and TMV infected protoplasts (ZAITLIN and HARIHARASUBRAMANIAN, 1972; SAKAI and TAKEBE, 1974; PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978). Possibly the p260 or p240 and p30 observed here correspond with the polypeptides found by ZAITLIN and HARIHARASUBRAMANIAN (1970, 1972) in TMV inoculated tobacco leaves, the p245 and p37 respectively.

Furthermore the subcellular fractionation of the protoplast homogenates holds the possibility to determine whether proteins synthesized upon TMV multiplication are present in supernatant and in pellet fractions. The membrane fraction will not be contaminated by supernatant proteins, aggregated to ma-

terial of the membranes, as a result of the choice of buffer, without Mg^{2+} and with EDTA. On the other hand proteins, weakly bound to membranes, are possibly solubilized. Membrane-bound proteins will not be present in the 31,000 x*g* supernatant, for electrophoretic patterns of protein of the 31,000 x*g* supernatant did not change after centrifugation at 105,000 x*g*. Table 6.1. gives a survey the subcellular fractions, in which the polypeptides synthesized as a result of TMV multiplication were observed. It appears that p260, p240, p96, p90, and p82 are detected exclusively in polypeptide patterns of 31,000 x*g* supernatant proteins, that the p170, p116,5 and p72 are distinguished in polypeptide patterns of protein from all fractions and that the p30 is observed exclusively in patterns of the pellet fractions. Coat protein is present in all fractions since TMV particles are present in very large amounts in the protoplast homogenate. It has been assumed that specific polypeptides from different subcellular fractions having the same electrophoretic mobility, are similar polypeptides. However, the migration velocity of p72 from supernatant and pellet fractions were not compared in one slabgel. It has been demonstrated by SCALLA *et al.* (1978) that pl16,5, occurring in supernatant and in pellet fractions of TMV inoculated tobacco leaves, is the same polypeptide.

The sum of the molecular weights of all polypeptides observed exceeds by far the coding capacity of the TMV RNA; therefore only a few of the polypeptides distinguished may be coded by the TMV genome. The largest *in vitro* product, being translated from the TMV RNA in the reticulocyte lysate, has a M.W. of approximately 210,000. The sum of the 210,000 daltons of this product and of the 17,500 daltons of coat protein approximates the coding capacity of TMV RNA, estimated on the 6,390 bases of TMV RNA (CASPAR, 1963; ZAITLIN and ISRAEL, 1975) and a mean M.W. of 110 for amino acids.

The M.W. of p260 and p240 exceed the coding capacity of TMV RNA. It is therefore likely that these polypeptides are host polypeptides. The p96, p90 and p82 from TMV infected protoplasts comigrated with polypeptides from mock-infected protoplasts that were present to a smaller extent. The p170 also co-migrated with a polypeptide from mock-infected protoplasts that was present to a smaller extent. In this way an indication is obtained that the p96, p90 and p82 (all three polypeptides were exclusively observed in the polypeptide patterns of protein of the 31,000 x*g* supertanant) and the p170 are host polypeptides.

Others conjectured that the p170 synthesized *in vivo* and *in vitro* might have a similar electrophoretic mobility and therefore might be a TMV coded polypeptide (HUNTER *et al.*, 1976; SIEGEL *et al.*, 1978). It was demonstrated that p170, observed in this study, practically comigrates with the *in vitro* product synthesized under the direction of TMV RNA in the reticulocyte lysate. Therefore this did not indicate whether or not the p170, formed *in vivo*, is synthesized under the direction of the TMV RNA. Moreover the p170 formed *in vitro* is a readthrough product, of which under certain conditions only trace amounts are synthesized (BRUENING *et al.*, 1976; PELHAM, 1978). It is totally uncertain whether read-through products may be synthesized in tobacco plants.

The other polypeptides, viz. the p116,5, p72, p30 and p17,5 did not comigrate

with a polypeptide from mock-infected protoplasts. This suggests that not only the p116,5 and coat protein are synthesized under the direction of the TMV genome, as has been proved by WITTMANN and WITTMANN-LIEBOLD (1966), GUILLEY *et al.* (1975) and SCALLA *et al.* (1978), but also that the p72 and p30 are virus coded polypeptides. It has been demonstrated (BRUENING *et al.*, 1976; HIGGINS *et al.*, 1976; BEACHY and ZAITLIN, 1977) that a product of 30,000 daltons is synthesized under the direction of a subgenomic TMV mRNA in the wheat germ cell-free protein synthesizing system. It is conceivable, that p30 synthesized in TMV infected protoplasts is similar to the *in vitro* product. For this reason p30 might be a TMV coded product.

It is still unknown what the function is of the polypeptides synthesized upon TMV multiplication. The p116,5 is supposed to be the replicase or part of this (ZAITLIN *et al.*, 1973; TAKEBE *et al.*, 1975; HUNTER et al., 1976). Since p30 is exclusively present in the membrane fractions it may be involved in the synthesis of TMV RNA in addition to the replicase. RI associated with polyribosomes, RF and replicase activity have been found in the membrane fractions (BEACHY and ZAITLIN, 1975; SKOTNICKI, 1976; RALPH and WOIJCIK, 1969; JACKSON *et al.*, 1971; NILSSON-TILLGREN *et al.*, 1974; BRADLEY and ZAITLIN, 1971; ZAITLIN *et al.*, 1973; SELA and HAUSCHNER, 1975; WHITE and MURAKISHI, 1975). A virus specific polypeptide, not the RNA polymerase, possibly regulates the replication of the semliki forest RNAs (SAWICKI *et al.*, 1978) for example.

In summary the results suggest that p170 may not be coded by the host genome and p72 may be synthesized under the direction of the TMV RNA. In the study dealt with in the next chapter p170 and p72 were further identified.

7. POLYPEPTIDES SYNTHESIZED AS A RESULT OF TMV MULTIPLICATION IN COWPEA PROTOPLASTS

7.1. INTRODUCTION

In TMV infected tobacco protoplasts a large number of polypeptides, synthesized as a result of TMV multiplication, were observed (previous chapter). It has been conclusively demonstrated that two of the polypeptides, coat protein (p17,5) and p116,5 are coded by the TMV genome (WITTMANN and WITTMAN-LIEBOLD, 1966; SCALLA *et al.*, 1978). All other polypeptides may have been synthesized under the direction of either the TMV genome or the host genome.

In an effort to identify TMV coded polypeptides, the infection of cowpea protoplasts with TMV was studied (chapter 5). Cowpea and tobacco belong to different families, viz. *Papilionaceae* and *Solanaceae* respectively. As a result of the genetic difference it is very likely that the host polypeptides synthesized as a result of TMV infection in cowpea or tobacco protoplasts have different electrophoretic mobilities. The TMV coded polypeptides synthesized after infection will be similar, however. A comparison of the polypeptides synthesized in cowpea and tobacco protoplasts infected with TMV may therefore help to discriminate between TMV coded and host specific proteins.

In the study described hereafter the proteins of TMV infected and mock-infected cowpea protoplasts were electrophoretically analysed by the same method as the proteins of TMV and mock-infected tobacco protoplasts were analysed. Thereupon the electrophoretic mobility of polypeptides synthesized as a result of TMV multiplication in cowpea and tobacco protoplasts were compared.

7.2. ELECTROPHORETIC ANALYSIS OF PROTEIN FROM TMV INFECTED AND MOCK-INFECTED COWPEA PROTOPLASTS

Protein of cowpea protoplasts was labeled with [^{35}S]-methionine during a period that the increase of TMV was comparable with the increase of TMV in tobacco protoplasts. In tobacco protoplasts the amount of TMV began to increase rapidly 8 hours after the incubation and in cowpea protoplasts the amount of TMV began to increase 16 hours after the onset of the incubation period (fig. 7.1.); therefore the labeling period of cowpea protoplasts started at least 8 hours later than that of tobacco protoplasts.

The incorporation of radioactive amino acids into protein of cowpea protoplasts was about 1.25 times as much as in protein of tobacco protoplasts, when labeling was done for the same periods and under similar conditions; see Materials and Methods.

A similar method was used for the subcellular fractionation of homogenates of

cowpea protoplasts as for homogenates of tobacco protoplasts. The electrophoretic patterns of protein from the 31,000 x*g* pellet fraction of cowpea protoplasts were less clear than those of protein from corresponding fractions of tobacco protoplasts. Since this polypeptide pattern may be disturbed by lipids for instance, the 31,000 x*g* pellet was extracted with Tris buffer, containing EDTA and lacking Mg^{2+}, see chapter 2. Proteins weakly bound to membranes were solubilized. Their polypeptide patterns were very clear.

The electrophoretic patterns of protein from the 2,500 x*g* pellet of cowpea protoplasts were not clear at all. Further fractionation of the 2,500 x*g* pellet by means of a discontinuous sucrose gradient, a method which yielded an improvement of the polypeptide patterns of protein from the corresponding fraction of tobacco protoplasts, did not result in improvement of the polypeptide pattern of this fraction of cowpea protoplasts. Therefore the 2,500 x*g* pellet fraction of cowpea protoplasts was not further investigated.

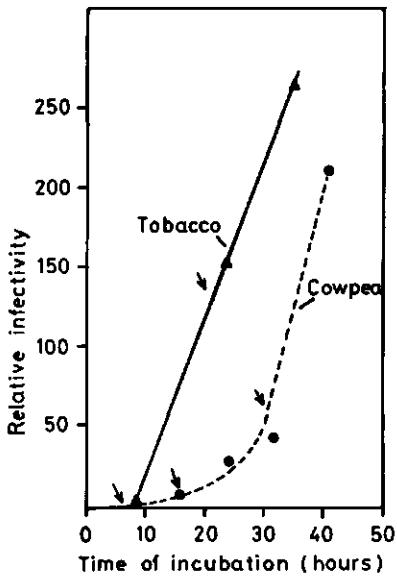


FIG. 7.1. Comparison of the time course of TMV synthesis in TMV infected cowpea and tobacco protoplasts.
Cowpea protoplasts and tobacco protoplasts were infected with TMV. Samples of protoplasts were harvested at various times after the onset of incubation. The infectivity of the protoplast extracts was tested by local lesion assay as described in chapter 2. The arrows indicate the periods during which the tobacco protoplasts and cowpea protoplasts were incubated in the presence of [^{35}S]-methionine.

7.3. POLYPEPTIDES SYNTHESIZED AS A RESULT OF TMV MULTIPLICATION IN COWPEA PROTOPLASTS

Eight polypeptides were detected in polypeptide patterns of protein from the 31,000 x*g* supernatant of TMV infected cowpea protoplasts which were absent

or present to a far less extent in polypeptide patterns of corresponding fractions of mock-infected protoplasts. The molecular weights of the polypeptides observed were estimated 150,000, 116,500, 86,000, 72,000, 17,500, 16,000, 14,000 and 10,000, in the following designated p150, pl16,5, p86, p72, p17,5, p16, p14 and p10 respectively. Fig. 7.2. gives polypeptide patterns of protein from the 31,000 xg supernatant of TMV and mock-infected cowpea protoplasts and shows the p150, p116,5 and p86. Polypeptides, smaller than 68,000 daltons, were migrated from the 7.5% gel, therefore polypeptides with highest M.W. could be clearly

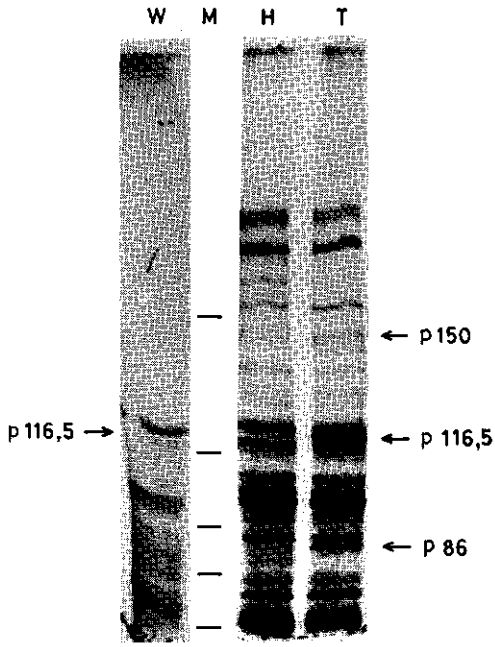


FIG. 7.2. Comparison of the electrophoretic patterns of [^{35}S]-methionine labeled protein from the 31,000 xg supernatant fraction of TMV infected and mock-infected cowpea protoplasts and of the products of translation of TMV RNA in a wheat germ extract.
TMV infected an mock-infected protoplasts were incubated in standard medium (2.5×10^5 protoplasts/ml). Sixteen hours after the onset of incubation [^{35}S]-methionine (20 μCi [^{35}S]-methionine/ml) was added. After 8 hours the protoplasts were harvested. Subcellular fractions were prepared. Samples of the 31,000 xg supernatant fraction of TMV and mock-infected cells containing an equal amount of cpm were prepared for SDS-PAGE.[^{35}S]-methionine labeled translation products in wheat germ extract with TMV RNA as messenger were prepared in the same way for SDS-PAGE. The samples were analysed on 7.5% slabgels at constant voltage, during 1 hour at 50 V and 4 hours at 190 V. The slabgels were stained, dried and autoradiographed. The positions of the polypeptides synthesized as a result of TMV multiplication or synthesized *in vitro* are indicated.

T: TMV infected protoplasts.
H: mock-infected protoplasts.
M: markers, the polypeptide of M.W. 170,000 of healthy tobacco protoplasts, β galactosidase (M.W. 116,200), phosphorylase A (M.W. 92,500), transferrin (M.W. 80,000) and bovine serum albumin (M.W. 68,000).
W: *in vitro* products, translated in the wheat germ extract.

distinguished, although in consequence thereof the band of p72 probably coincided with other bands at the bottom of the gel.
Polypeptides from mock-infected protoplasts comigrated with the p150 and p86 from TMV infected protoplasts. The corresponding bands of the polypeptides from mock-infected protoplasts were weaker than the bands from the TMV infected protoplasts. The band of p150 is much weaker than the band of p116,5. No polypeptide from mock-infected protoplasts comigrated with the p116,5. Fig. 7.3. shows the electrophoretic pattern of protein from the 31,000 x*g* supernatant of TMV and mock-infected cowpea protoplasts, analysed on 17.5% slabgels. The p17,5, p16, p14 and p10 were observed in the polypeptide patterns of protein from TMV infected protoplasts. A polypeptide from mock-infected protoplasts comigrated with p10; the band of the polypeptide from mock-infected protoplasts was much weaker than that of the p10 from TMV infected protoplasts. No polypeptides from mock-infected protoplasts comigrated with

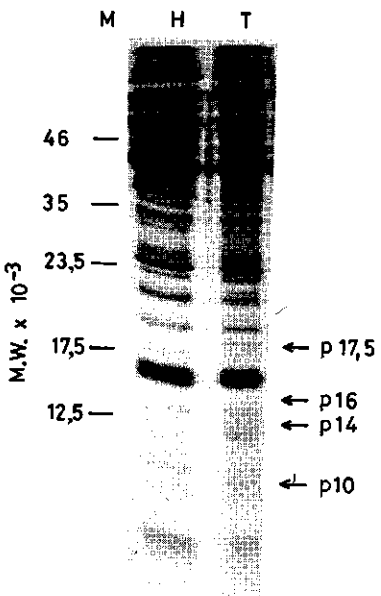


FIG. 7.3. Comparison of the electrophoretic patterns of [^{35}S]-methionine labeled protein from the 31,000 x*g* supernatant of TMV infected and mock-infected cowpea protoplasts.
TMV infected and mock-infected protoplasts were incubated in standard medium (3.5×10^5 protoplasts/ml). Nineteen hours after the onset of incubation [^{35}S]-methionine (15 μCi [^{35}S]-methionine/ml) was added. After 12 hours the protoplasts were harvested. Subcellular fractions were prepared. Samples of the 31,000 x*g* supernatant fraction of TMV and mock-infected cells containing an equal amount of cpm were prepared for SDS-PAGE. The samples were analysed on a 17.5% slabgel at constant voltage during 1.5 hours at 50 V and 6 hours at 140 V. The gels were dried and autoradiographed. The positions of the polypeptides synthesized as a result of TMV multiplication are indicated.
T: TMV infected protoplasts.
H: mock-infected protoplasts.
M: markers.

the p17,5, p16 and p14. The bands of p16 and p14, however, were weak. In most autoradiograms the electrophoretic mobility of p17,5 was slightly smaller than the polypeptide of 17,500 daltons from the same geltract, visible after staining with coomassie brilliant blue R. The stained polypeptide of 17,500 daltons comigrated with coat protein that was used as marker and isolated from TMV particles, purified from TMV inoculated tobacco leaves. The stained polypeptide apparently was coat protein.

Fig. 7.4. shows the polypeptide patterns of protein from protoplasts infected with the cowpea strain of TMV (C-TMV), next to patterns of mock-infected and TMV infected cowpea protoplasts. In the autoradiogram, in the geltract of protein from C-TMV infected protoplasts, a polypeptide of M.W. approximately 18,500 (p18,5) was present. The p18,5 migrated somewhat slower than C-TMV coat protein, visible after staining.

With the aid of antiserum prepared against C-TMV or TMV (see Materials and

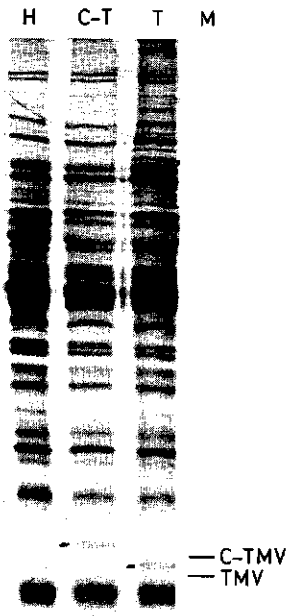


FIG. 7.4. [^{35}S]-methionine labeled TMV and C-TMV coat protein in electrophoretic patterns of protein from the 31,000 x*g* supernatant fraction of cowpea protoplasts infected with TMV and C-TMV respectively.

Protoplasts infected with TMV and C-TMV and mock-infected protoplasts were incubated and prepared for SDS-PAGE as described in the legend of fig. 7.3. Samples were analysed on a 7.5 % slabgel at constant voltage during 1.5 hours at 50 V and 6 hours at 140 V. The gels were stained, dried and autoradiographed. The positions of [^{35}S]-methionine labeled C-TMV and TMV coat protein synthesized in the infected protoplasts are marked (–).

H: mock-infected protoplasts.
C-T: C-TMV infected protoplasts.
T: TMV infected protoplasts.
M: C-TMV and TMV coat protein from purified virus particles.

Methods) it was investigated, whether p18,5 and p17,5 respectively, were coat proteins. C-TMV antiserum was added to the 31,000 x*g* supernatant of C-TMV infected protoplasts and TMV antiserum was added to the 31,000 x*g* supernatant of TMV infected protoplasts and of mock-infected protoplasts to which TMV, purified from infected leaves, was added. The immunoprecipitate from the 31,000 x*g* supernatants was pelleted by centrifugation and the protein from the pellets was analysed by SDS-gelelectrophoresis. The p18,5 and p17,5 were detected in the autoradiograms of the protein from C-TMV and TMV infected protoplasts respectively, precipitated by means of virus antibodies. No polypeptide of the size of these polypeptides was found in the autoradiogram of the protein precipitate of mock-infected protoplasts. The p18,5 and p17,5 apparently are coat protein of C-TMV and TMV respectively, in which [^{35}S]-methionine was incorporated.

The p116,5 and p72 and p17,5 were also observed in polypeptide patterns of protein from the 31,000 x*g* pellet of TMV infected protoplasts. Polypeptides from the 31,000 x*g* pellet of mock-infected protoplasts did not comigrate with these polypeptides from TMV infected protoplasts. The electrophoretic patterns

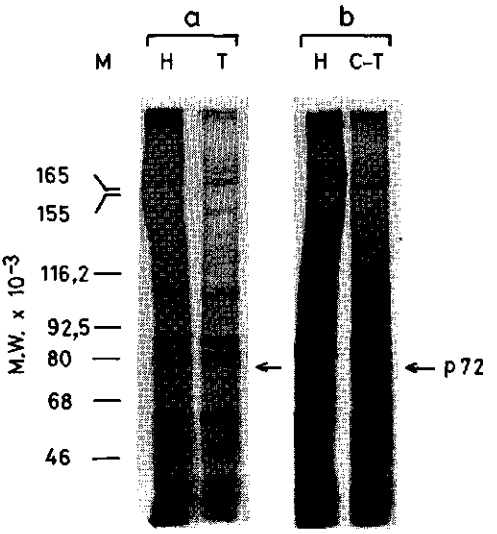


FIG. 7.5. Comparison of the electrophoretic patterns of [^{35}S]-methionine labeled protein from the extracts of the 31,000 x*g* pellet fraction of TMV, C-TMV and mock-infected cowpea protoplasts. Protoplasts infected with TMV and C-TMV and mock-infected protoplasts were incubated in standard medium (3.5 × 10^5 protoplasts/ml). Seven and a half hours after the onset of incubation [^{35}S]-methionine (15 μCi [^{35}S]-methionine/ml) was added. After 11 hours the protoplasts were harvested. Samples of the extracts of the 31,000 x*g* pellet fraction of TMV, C-TMV and mock-infected protoplasts containing an equal amount of cpm were prepared for SDS-PAGE. The samples were analysed on a 7.5 % slabgel at constant voltage during 1 hour at 50 V and 10 hours at 120 V. The slabgels were stained and autoradiographed. The position of p72 synthesized as a result of TMV and C-TMV multiplication is indicated by arrows.

a: comparison of polypeptide patterns from mock-infected (H) and TMV infected (T) protoplasts.
b: comparison of polypeptide patterns from mock-infected (H) and C-TMV infected protoplasts.
M: markers.

of protein from the pellet fraction showed a predominant, grey background, therefore it was tried to extract protein from the membranes. The proteins that were solubilized showed a clear electrophoretic pattern (fig. 7.5.). Fig. 5a shows polypeptide patterns of protein extracted from the 31,000 x*g* pellet of TMV and mock-infected protoplasts. The p72 was detected in the polypeptide patterns of

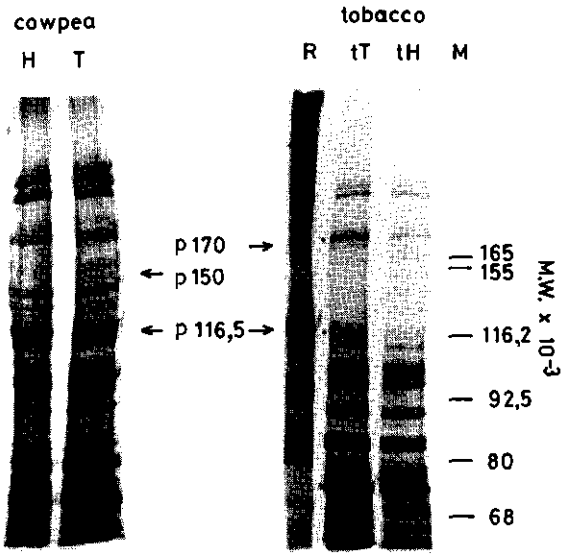


FIG. 7.6. Autoradiogram of the products synthesized under the direction of TMV RNA in the reticulocyte lysate and of protein of the 31,000 x*g* supernatant from TMV infected and mock-infected cowpea protoplasts and from TMV infected and mock-infected tobacco protoplasts.
TMV infected and mock-infected cowpea protoplasts were incubated in standard medium (3×10^5 protoplast/ml). Seventeen hours after the onset of incubation [^{35}S]-methionine (20 μCi [^{35}S]-methionine/ml) was added. After 18 hours the cowpea protoplasts were harvested. TMV infected and mock-infected tobacco protoplasts were incubated in standard medium at a protoplast concentration of 2.8×10^5 protoplasts/ml. Eight hours after the onset of incubation [^{35}S]-methionine (20 μCi [^{35}S]-methionine/ml) was added. After 16 hours the tobacco protoplasts were harvested. Samples of the 31,000 x*g* supernatant of TMV and mock-infected protoplasts,containing about the same amount of cpm were prepared for SDS-PAGE. Samples of [^{35}S]-methionine labeled products synthesized under the direction of TMV RNA in the reticulocyte lysate were prepared in the same way for SDS-PAGE. Samples were analysed on a 7.5 % slabgel at constant voltage, during 1 hour at 50 V and 6 hours at 160 V. The gels were stained, dried and autoradiographed.
The positions of the products synthesized *in vitro* under the direction of TMV RNA of M.W. 170,000 and 116,500, and the position of the polypeptides synthesized as a result of TMV multiplication in TMV infected cowpea protoplasts of M.W. 150,000 and 116,500, are indicated with arrows. The positions of the polypeptides synthesized as a result of TMV multiplication in TMV infected tobacco protoplasts of M.W. 170,000 and 116,500 are marked by a dot.
H: mock-infected cowpea protoplasts.
T: TMV infected cowpea protoplasts.
R: products synthesized in the reticulocyte lysate.
tT: TMV infected tobacco protoplasts.
tH: mock-infected tobacco protoplasts.
M: markers.

the TMV infected protoplasts and did not comigrate with a polypeptide from mock-infected protoplasts. Fig. 5b shows polypeptide patterns of protein, extracted from the 31,000 x*g* pellet of C-TMV and mock-infected protoplasts. The p72 was also observed in the polypeptide pattern of C-TMV infected protoplasts, whereas a polypeptide with a similar electrophoretic mobility was absent in mock-infected protoplasts.

The p72 in TMV and C-TMV infected cowpea protoplasts was easier to detect in electrophoretic patterns of protein, labeled with [^{35}S]-methionine, during the period of $7\frac{1}{2}$ to 18 hours after the onset of incubation, than in the period of 16 to 31 hours. In the period of $7\frac{1}{2}$ to 18 hours after the onset of incubation the amount of virus that was synthesized was still small, as is demonstrated in fig. 7.1. The p116,5 was not detected in electrophoretic patterns of protein from the 31,000 x*g* pellet that was solubilized.

The p150 and p116,5 were further charaterized by comparison of their electrophoretic mobility with the largest products translated *in vitro* under the direction of TMV RNA in the rabbit reticulocyte lysate. Fig. 7.6., geltract R. shows the electrophoretic pattern of the products, translated under the direction of TMV RNA. A polypeptide of M.W. approximately 170,000 (p170) and of 116,500 (p116,5) were synthesized in the reticulocyte lysate. The p170 and p116,5, synthesized *in vitro* from TMV RNA as messenger, are the same products as mentioned by KNOWLAND (1974), PELHAM (1978) and DAVIES *et al.* (1977). Apart from these two polypeptides another polypeptide of M.W. approximately 210,000 (p210) was observed in the polypeptide pattern of the *in vitro* products.

Fig. 7.6. shows, in addition to the polypeptide pattern of the *in vitro* products, the polypeptide patterns of protein from the 31,000 x*g* supernatant of TMV and mock-infected cowpea protoplasts (geltract T and H). It appeared that the electrophoretic mobility of the p150, detected in the polypeptide pattern of TMV infected cowpea protoplasts, did not correspond with the electrophoretic mobility of p170 synthesized *in vitro*. The p116,5, found in TMV infected protoplasts, and the p116,5, synthesized *in vitro*, had a similar mobility. The p116,5 synthesized *in vivo* stands out better in fig. 7.2. which shows the polypeptide pattern of protein from the 31,000 x*g* supernatant fraction of TMV and mock-infected cowpea protoplasts. Fig. 7.2. geltract W, shows in addition the electrophoretic pattern of the products synthesized *in vitro* under the direction of the TMV RNA. The electrophoretic mobility of the p116,5 synthesized *in vitro* and *in vivo* corresponded also in this autoradiogram.

In fig. 7.6. the electrophoretic patterns of the 31,000 x*g* supernatant from TMV and mock-infected cowpea protoplasts (geltract T and H) are compared with the electrophoretic patterns of corresponding fractions from TMV and mock-infected tobacco protoplasts (geltract tT and tH). It appeared that the p150 of TMV infected cowpea protoplasts did not comigrate with p170 of TMV infected tobacco protoplasts, but the p116,5 of TMV infected cowpea and tobacco protoplasts in fact had similar mobilities.

TABLE 7.1. Polypeptides synthesized as a result of TMV multiplication in cowpea protoplasts and the cell-fractions in which these polypeptides are localized.

M.W. × 10^{-3} of polypeptides synthesized as a result of TMV multiplication	Polypeptides present (+) or absent (–) in the 31,000 xg	
	supernatant	pellet
150	+ h	–
116,5	+	+
86	+ h	–
72	+	+
17,5 (coat protein)	+	+
16	+	n
14	+	n
10	+ h	n

h: A polypeptide of similar M.W. was present in the corresponding fraction of mock-infected cowpea protoplasts. The band of the polypeptides from mock-infected protoplasts was weaker than the band of the polypeptide from TMV infected protoplasts.

n: not determined.

7.4. DISCUSSION

It appeared from the investigation, as described in this chapter, that eight polypeptides are synthesized as a result of TMV multiplication in TMV infected cowpea protoplasts. These polypeptides were absent, or they were present to a far less extent in mock-infected cowpea protoplasts. The molecular weights of these polypeptides, electrophoretically estimated, were 150,000, 116,500, 86,000, 72,000, 17,500 (coat protein), 16,000, 14,000 and 10,000.

The p116,5 synthesized in TMV infected cowpea protoplasts comigrates with the *in vitro* translation product, synthesized from TMV RNA as messenger, therefore it is similar to the p135 found by others in TMV infected tobacco protoplasts and TMV inoculated leaves (ZAITLIN and HARIHARASUBRAMANIAN, 1972; SAKAI and TAKEBE, 1975; PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978).

Table 7.1. shows in which subcellular fractions the polypeptides synthesized in consequence of TMV multiplication were observed. The p150, p86, p17 and p10 are exclusively distinguished in electrophoretic patterns of the proteins from the 31,000 xg supernatant and the p116,5, p72 and p17,5 are detected in polypeptide patterns of protein from supernatant as well as from 31,000 xg pellet.

An obvious question is now which polypeptides, synthesized in consequence of the TMV multiplication, are synthesized under the direction of the host genome and which under the direction of the TMV genome. This question concerns both the polypeptides detected in TMV infected cowpea and tobacco protoplasts. The host polypeptides, synthesized as a result of TMV multiplication in cowpea or tobacco protoplasts, are polypeptides differing from host to

host. It is likely that the electrophoretic mobility of these polypeptides is not similar. On the other hand, similar TMV coded proteins, performing an essential function in the TMV multiplication process, will be synthesized in both hosts. These have a similar electrophoretic mobility. This was used for distinguishing between host and TMV coded polypeptides.
Table 7.2. gives the polypeptides, synthesized as a result of TMV multiplication in cowpea and tobacco protoplasts. It appears that p150 and p86 from TMV infected cowpea protoplasts do not correspond with polypeptides from TMV infected tobacco protoplasts. It also appears that p170, p96, p90 and p82 from TMV infected tobacco protoplasts are not present in TMV infected cowpea protoplasts. For this reason it may be assumed that these polypeptides from cowpea and tobacco protoplasts are host polypeptides. Moreover, polypeptides from mock-infected cowpea and tobacco protoplasts comigrated with the polypeptides just mentioned from TMV infected cowpea or tobacco protoplasts respectively. This is in support of their being coded by the host genome.

It was demonstrated that p170, synthesized in TMV infected tobacco protoplasts, does not comigrate with p150 from TMV infected cowpea protoplasts. However, it appeared that the electrophoretic mobility of p170 from TMV infected tobacco protoplasts is almost similar to p170, synthesized *in vitro* under the direction of the TMV RNA in the reticulocyte lysate. There is a possibility that the correspondence in electrophoretic mobility of p170 from TMV infected tobacco protoplasts and of p170, translated from the TMV RNA in the reticulocyte lysate, is a coincidence.
Moreover, p170, synthesized *in vitro*, is a readthrough product, caused by suppression of the UAG stop codon of p116,5. It is only synthesized *in vitro* under special conditions (PELHAM, 1978; DAVIES *et al.*, 1977). A readthrough product is also synthesized under the direction of the Qβ RNA (WEBER and KONIGSBERG, 1975) and the RNA of leukemia and sarcoma viruses (JANJOOM *et al.*, 1977; OPPERMAN *et al.*, 1977; PHILIPSON *et al.*, 1978) in the reticulocyte lysate. These readthrough products are also synthesized *in vivo*. However, no function of these readthrough products has been discovered yet. Since p170 is not synthesized in TMV infected cowpea protoplasts, it is probable that p170, whenever it is synthesized under the direction of the TMV genome in TMV infected tobacco protoplasts, has no function at the TMV multiplication.

Nor was p30, synthesized in TMV infected tobacco protoplasts, observed in TMV infected cowpea protoplasts, although p30 might be a virus coded polypeptide (previous chapter). The p30 is distinguished in electropherograms of protein from membrane fractions of TMV infected tobacco protoplasts and is strongly membrane bound. A possible explanation for the fact that p30 is not detected in electrophoretic patterns of TMV infected cowpea protoplasts, is that polypeptide patterns of protein from membrane fractions of cowpea protoplasts are obliterated by a grey background, which is increasing toward the bottom part of the gel.

Furthermore it appears from table 7.2. that the polypeptides, synthesized in consequence of TMV multiplication, p116,5, p72 and p17,5, are found both in

TABLE 7.2. Polypeptides synthesized as a result of TMV multiplication in cowpea and tobacco protoplasts and polypeptides translated under the direction of TMV RNA in a cell-free protein synthesizing system.

M.W. × 10^{-3}		
polypeptides of TMV infected		polypeptides synthesized
cowpea protoplasts	tobacco protoplasts	*in vitro*[1]
–	260	
–	240	
–	–	
–	170 h	165
150 h	–	
116,5	116,5	116,5
–	96 h	
–	90 h	
86 h	–	
–	82 h	
72	72	
–	30	29-30
17,5(m)	17,5(m)	17,5(m)
16	n	
14	n	
10 h	n	

1. The polypeptides of M.W. 30,000, 29,000 and 17,500 are products translated under the direction of subgenomic TMV mRNAs in the wheat germ cell-free protein synthesizing system (SIEGEL *et al.*, 1976; HUNTER *et al.*, 1976; BEACHY and ZAITLIN, 1977).

h: A polypeptide of similar M.W. was present in mock-infected protoplasts. The band of the polypeptide from mock-infected protoplasts was weaker than the band of the polypeptide of TMV infected protoplasts.

m: TMV coat protein.

n: not determined.

cowpea and in tobacco protoplasts. This points to the fact that these polypeptides are being coded by the TMV genome. It has been proved (chapter 2.) that p116,5 from TMV infected tobacco protoplasts, like coat protein, is coded by the TMV RNA.

Besides p116,5 and coat protein, p72 is the only other polypeptide that appears to be coded by the TMV genome, based on this comparison. It is striking that only p72, in addition to p116,5 and coat protein from TMV infected cowpea protoplasts, is present in electrophoretic patterns of protein from supernatant and pellet. All other polypeptides, synthesized as a result of TMV multiplication and supposed to be coded by the cowpea genome, were exclusively present in polypeptide patterns of protein from the supernatant (table 7.1.). The p72 of TMV infected cowpea and tobacco protoplasts were present in corresponding fractions. In case of p72 being a gen product of TMV RNA, the question is forced upon us how it is translated from the TMV RNA. There are three possibilities:

1. it is a primary translation product, 2. it is translated out of phase, such as proteins of bacteriophage φX174 (SANGER *et al.*, 1978; SHAW *et al.*, 1978) or, 3. p72 originates from a primary gen product by processing.

Our results indicate that [^{35}S]-methionine is incorporated into part of the virus coat protein synthesized in TMV infected cowpea and tobacco protoplasts. This is rather surprising since the coat protein of the isolate of the common strain of TMV used in this study does not contain methionine as has been demonstrated by MOSCH *et al.* (1973).

Also part of the coat proteins synthesized in C-TMV infected protoplasts is labeled by [^{35}S]-methionine, whereas coat protein from purified C-TMV particles does not contain methionine (REES and SHORT, 1975). The labeled coat proteins observed in TMV and C-TMV infected protoplasts migrate somewhat slower than coat protein from purified TMV and C-TMV particles respectively, and therefore they appear to be slightly larger than the coat proteins of the viruses. WHITFELD and HIGGINS (1976) have shown that C-TMV coat protein synthesized *in vitro* under the direction of the messenger RNA for coat protein, is also larger than the largest of the two coat proteins of purified virus particles and can be labeled with [^{35}S]-methionine. They supposed that the messenger RNA for coat protein directs the *in vitro* synthesis of coat protein chains containing extra amino acids at the NH_2-terminus. *In vivo* the extra amino acids are split off, resulting in coat protein lacking methionine.

Other explanations may be that our TMV strain has been contaminated with a variant containing methionine in its coat or that the [^{35}S]-methionine is sticking to the coat protein synthesized in the protoplasts. These possibilities cannot fully be excluded, but appear less plausible.

8. POLYPEPTIDES IN RNA-DEPENDENT RNA POLYMERASE PREPARATIONS FROM C-TMV INFECTED COWPEA LEAVES

8.1. INTRODUCTION

In the previous two chapters it has been described that both in tobacco protoplasts and in cowpea protoplasts a large number of polypeptides are synthesized in consequence of TMV multiplication. Of all these polypeptides p17,5, 116,5p and probably p72, are TMV coded polypeptides. Only of p17,5 a function is known, i.e. it is coat protein; p116,5, supposedly, is involved in the TMV RNA replication (ZAITLIN *et al.*, 1973; TAKEBE *et al.*, 1975; HUNTER *et al.*, 1976).

In cells of leaves, infected with TMV, a synthesis of new virus particles takes place on a massive scale, which process is accompanied with the production of a large amount of TMV coat protein and with a rapid synthesis of TMV RNA. The supposition that in the synthesis of TMV RNA one or more virus coded proteins are involved, seems obvious. A further argument for this supposition is that RNA replicates of bacteriophages and animal viruses, for example Qβ (KAMEN, 1975) and poliovirus (FLANEGAN and BALTIMORE, 1979) contain a virus coded protein.

RNA-dependent RNA polymerase activity is present in TMV infected tobacco leaves and tobacco callus cultures (DUDA *et al.*, 1973; ZAITLIN *et al.*, 1973; SELA and HAUSCHER, 1975; WHITE and MURAKISHI, 1977). The RNA polymerase activity strongly increases in the first days post inoculation of the tobacco leaves with TMV (ZAITLIN *et al.*, 1973). It has been shown that preparations with such activity can be purified to a considerable degree.

A low RNA-dependent RNA polymerase activity has been observed in healthy leaves (ASTIER-MANIFACIER and CORNUET, 1971; ZAITLIN *et al.*, 1973; FRAENKEL-CONRAT, 1976; BOL *et al.*, 1976; LE ROY *et al.*, 1977; STUSSI-GARAUD *et al.*, 1977; IKEGAMI and FRAENKEL-CONRAT, 1978, 1978a; ROMAINE and ZAITLIN, 1978; WHITE and DAWSON, 1978; DUDA, 1979). Up till now no clear-cut difference has been demonstrated between RNA polymerase preparations from healthy and from infected leaves (IKEGAMI and FRAENKEL-CONRAT, 1978; DUDA, 1979). Therefore it is supposed that the synthesis or activity of RNA-dependent RNA polymerase is stimulated after inoculation of the leaves with virus. This should (partly) account for the large rapidity of the TMV RNA synthesis. This has not been proved conclusively, however.

The investigation of proteins in RNA-dependent RNA polymerase preparations, isolated from TMV infected leaves, was of interest for the following reasons. In the case that the increase of the RNA-dependent RNA polymerase activity in infected leaves is based on the increase of protein(s) already present in healthy leaves, one or more proteins will be found in this fraction, which have increased in consequence of the TMV multiplication. Moreover it may be expec-

ted that in the RNA-dependent RNA polymerase preparations from TMV infected leaves one or more virus coded proteins are present, causing the TMV RNA to be multiplied extremely effectively.
Therefore it was examined whether in the leaf fraction with RNA-dependent RNA polymerase activity polypeptides occurred which are increased or newly synthesized as a result of the C-TMV multiplication and whether one or more of the polypeptides observed correspond to polypeptides described in previous chapters.

Since in our laboratory there was already considerable experience with the purification of RNA-dependent RNA polymerase from CPMV infected cowpea leaves (ZABEL *et al.*, 1974, 1976, 1979) this work was done with cowpea leaves. Cowpea plants are no hosts of the common strain of TMV. For this reason cowpea leaves were inoculated with the cowpea strain of TMV (C-TMV).
C-TMV instead of TMV could be used for this study, since in a cell free protein synthesizing system, under the direction of the C-TMV and TMV RNA, products are synthesized with a similar electrophoretic mobility, i.e. the products of M.W. 30,000 and of M.W. 116,500 (BEACHY and ZAITLIN, 1977). P72, synthesized in TMV and C-TMV infected cowpea protoplasts, likewise has a similar mobility (fig. 7.5.). The M.W. of coat protein of the two strains differ only 1,000 daltons (REES and SHORT, 1975).

The procedure, developed for purification of RNA-dependent RNA polymerase from CPMV infected leaves, termed CPMV replicase, was applied for purification of RNA-dependent RNA polymerase preparations from C-TMV and mock-inoculated cowpea leaves.
At the same time this offered the possibility to compare RNA-dependent RNA polymerase preparations from C-TMV infected cowpea leaves with corresponding preparations from CPMV infected cowpea leaves.

8.2. ISOLATION OF RNA-DEPENDENT RNA POLYMERASE PREPARATIONS FROM COWPEA LEAVES

Cowpea plants (*Vigna unguiculata* (L) Walp. var. 'Blackeye Early Ramshorn') were cultivated in a growth chamber under conditions described by ZABEL *et al.* (1979). About one week after sowing primary leaves were inoculated with 1 mg C-TMV/ml in 0.06 M phosphate buffer pH 7.0. RNA-dependent RNA polymerase preparations were isolated from primary cowpea leaves 3 or 4 days after inoculation with C-TMV, according to the procedure for the purification of CPMV replicase, described by ZABEL *et al.* (1974, 1976, 1978, 1979). A scheme of the purification procedure is given in fig. 8.1. Exactly the same method was used for isolation of RNA-dependent RNA polymerase preparations from cowpea leaves, inoculated with CPMV (yellow strain) in a homogenate of infected leaves, or from cowpea leaves mock-inoculated with sterilized 0.06 M phosphate buffer pH 7.0.
After fractionation of the leaf homogenate the 31,000 x*g* pellet was extracted

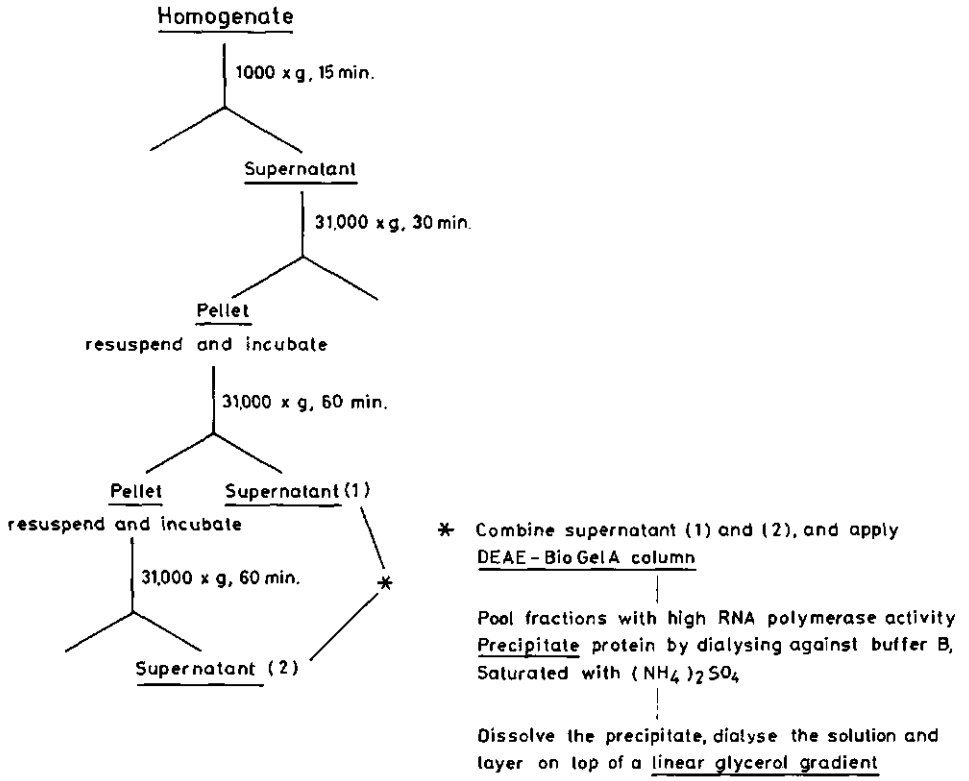


FIG. 8.1. Scheme for the isolation of RNA-dependent RNA polymerase from cowpea leaves.
Portions of 12 g freshly harvested cowpea leaves from which the midribs were removed, were homogenized in buffer A (2 ml/g fresh weight) in a prechilled mortar. All operations were carried out at 2° to 4° C. The homogenate was squeezed through 2 layers of Miracloth and centrifuged at 1,000 xg for 15 min. The 1,000 xg supernatant was adjusted to 25 % (v/v) glycerol and centrifuged at 31,000 xg for 30 min. The 31,000 xg pellet was resuspended in buffer B (1 ml/g fresh weight) and incubated under continuous stirring for 60 min. The suspension was centrifuged at 31,000 xg for 60 min. The supernatant (1) was saved, the pellet was again resuspended in buffer B (0.25 ml/g fresh weight) and incubated under continuous stirring for 30 min. The suspension was centrifuged at 31,000 xg for 60 min. The supernatant (2) was saved. Supernatant (1) and (2) from 70 to 100 g of leaf tissue were combined and applied to a DEAE-BioGel A column (1.6 × 15 cm) equilibrated with buffer B. The column was washed with buffer B and the material bound to the column was eluted with a 50 to 400 mM KCl non-linear increasing gradient in buffer B. About 10 fractions containing the bulk of the RNA polymerase activity were pooled. The protein was precipitated by dialysing the solution against buffer B, saturated with $(NH_4)_2SO_4$ at 2° C. The collected precipitate was dissolved in about 1 ml buffer C and the solution was dialysed against buffer C, layered on top of an 8 to 25 % (v/v) glycerol gradient in buffer C and centrifuged in tubes of 12 ml in a Beckmann SW41 rotor at 36,000 rpm for 16.5 hours (ZABEL, 1978; ZABEL *et al.*, 1979).

Buffer A: 50 mM Tris-hydrochloride (pH 7.4), 10 mM KCl, 1 mM EDTA, 10 mM DTE, 0.5 mM PMSF.

Buffer B: 50 mM Tris-hydrochloride (pH 8.2), 50 mM KCl, 1 mM EDTA, 25 % (v/v) glycerol, 10 mM DTE, 0.5 mM PMSF.

Buffer C: 50 mM Tris-hydrochloride (pH 8.2), 250 mM KCl, 1 mM EDTA, 10 mM DTE, 0.5 mM PMSF.

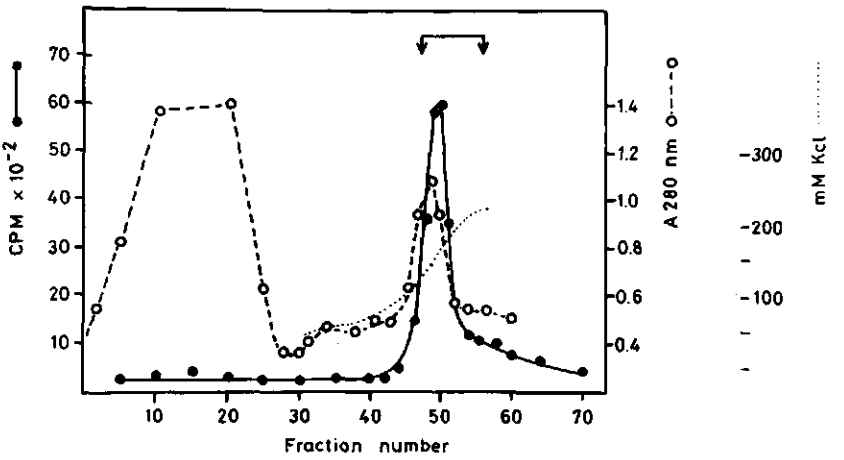


FIG. 8.2. DEAE-BioGel A column chromatography of a polymerase preparation extracted form the 31,000 xg pellet of C-TMV infected cowpea leaves.
A membrane extract containing approximately 45 mg protein in 75 ml buffer B was applied to a DEAE-BioGel A column (1.6 × 15 cm) equilibrated with buffer B. Fractions of about 5 ml were collected. All operations were carried out at 4° C. The unbound material was washed from the column by buffer B. This was followed by elution of the bound material with 80 ml of a non-linear 50 to 400 mM KCl gradient in buffer B. After washing, fractions of about 2.4 ml were collected at a flow rate of 20 ml/hour. Fifty µl samples of the fractions were assayed for RNA polymerase activity in a reaction mixture of 120 µl for 30 min. at 30°. The assay mixture was as described in the legend of table 8.1. Two ml each of the fractions 47 to 56 (⌐¬) were pooled for further purification.
RNA-dependent polymerase activity (–●–●–). $A_{280\ nm}$ (–O– – O–) of samples, diluted 5x with double distilled water, was measured in a spectrophotometer. The KCl molarity (.....) of the samples was measured in a conductivity meter.
Buffer B:50 mM Tris-hydrochloride (pH 8.2), 50 mM KCl, 1 mM EDTA, 25% (v/v) glycerol, 10 mM DTE, 0.5 mM PMSF.

with buffer without Mg^{2+} (50 mM Tris-hydrochloride (pH 8.2), 50 mM KCl, 1 mM EDTA, 25% (v/v) glycerol, 10 mM DTE and 0.5 mM PMSF). The purification proceeded by means of DEAE-BioGel A column chromatography. The column was eluted after loading and washing, with a non-linear increasing KCl gradient in the above mentioned buffer.
As shown in fig 8.2, the RNA polymerase from C-TMV inoculated leaves was eluted from the column at a concentration of 120 to 150 mM KCl. Similar O.D. patterns were obtained from polymerase preparations extracted from the 31,000 xg pellet of CPMV and mock-inoculated leaves after DEAE-BioGel column chromatography, and the RNA-dependent RNA polymerase activity was found in corresponding column fractions. The protein from the fractions as indicated in fig. 8.2. with high specific polymerase activity was precipitated by means of dialysis against the above mentioned buffer saturated with $(NH_4)_2SO_4$ at 2° C. The protein that was precipitated, was dissolved in a small volume of buffer (50 mM Tris-hydrochloride (pH 8.2), 250 mM KCl, 1 mM EDTA, 10 mM DTE and 0.5 mM PMSF), was then loaded on top of a 8 to 25% glycerol gradient and

centrifuged in a polyallomer tube in a SW41 rotor at 36,000 rpm, for 16.5 hours at 2° C. The profile of the RNA-dependent RNA polymerase activity, after glycerol centrifugation, is given in fig. 8.3. The highest RNA polymerase activity was found in all three cases in exactly the same position in the glycerol gradient, while the specific activity varied considerably. This position corresponded with a M.W. approximately 150,000. For estimation of the M.W. katalase (M.W. 247,500), γ-globuline (M.W. 153,000) and transferrin (M.W. 80,000) were used, sedimenting at the same time in glycerol gradients.

Starting with 100 g inoculated leaves, without midribs, 85 mg protein was extracted from the 31,000 xg pellet and only 9 mg protein was collected from the DEAE-BioGel A column fractions with highest specific activity. From this ± 6 mg protein was brought on the glycerol gradient, of which 1.7 to 0.7 mg was recovered from the four glycerol gradient fractions with highest polymerase activity.

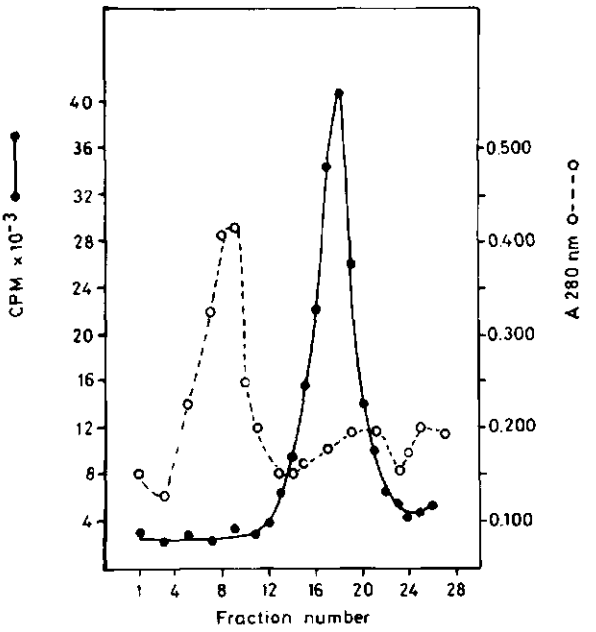


FIG. 8.3. Glycerol gradient centrifugation of RNA-dependent RNA polymerase preparation of C-TMV infected cowpea leaves after purification by DEAE-BioGel A column chromatography.
A solution of approximately 10 mg protein in 0.75 ml buffer C was layered on top of an 11.2 ml 8 to 25% glycerol gradient in buffer C. The gradient was centrifuged in a SW41 rotor at 36,000 rpm for 16.5 hours, at 2 C. The rotor came to a standstill without the use of the brake. Fractions of about 0.5 ml were collected from the bottom of the tube. Fifty µl samples of the fractions were assayed for polymerase activity in a reaction mixture of 240 µl for 1 hour at 30°. The assay mixture was as described in the legend of table 8.1.
Polymerase activity (–●–●–). $A_{280\ nm}$ (–O—O–) of samples, diluted five times with double distilled water, measured in a spectrophotometer.
Buffer C:50 mM Tris-hydrochloride (pH 8.2), 250 mM KCl, 1 mM EDTA, 10 mM DTE and 0.5 mM PMSF.

The RNA polymerase activity was tested under nearly similar conditions as has been described by ZABEL *et al.* (1976) for CPMV replicase, see table 8.1. However, the specific activity of [^{3}H]-UTP was five times higher in the assay medium for determining low RNA-dependent RNA polymerase activity of healthy control leaves. The $MgCl_2$ concentration of the assay medium was 5 mM, since it has been found by ZAITLIN *et al.* (1973) that the activity of the RNA polymerase from TMV infected tobacco leaves, solubilized and purified by glycerol gradient, is maximal at 5 mM and decreases at higher $MgCl_2$ concentration. The activity of CPMV replicase is optimal at a Mg^{2+} concentration of 10 mM, therefore the CPMV replicase activity was tested sub-optimally. The assay conditions for the RNA-polymerases, purified from C-TMV and mock-infected leaves, were not optimalized.

TABLE 8.1. Specific activity of RNA-dependent RNA polymerase preparations from mock-, C-TMV and CPMV inoculated cowpea leaves after successive purification by DEAE-BioGel column chromatography and glycerol gradient centrifugation.

RNA-dependent RNA polymerase from cowpea leaves inoculated with	Specific activity (units/mg)[2] after successive purification by means of	
	DEAE-BioGel column chromatography	Glycerol gradient centrifugation
Buffer	29	69
C-TMV	44	393
CPMV	390	–

The assay mixture of 120 µl contained 50 mM Tris-hydrochloride pH 8.2, 10% glycerol, 5 mM $MgCl_2$, 25 mM KCl, 60 mM $(NH_4)_2SO_4$, 1 mM EDTA, 4 mM DTE, 1 mM each of ATP, GTP and CTP, 0.008 mM UTP, 41.5 µCi [^{3}H]-UTP (specific activity 14 to 16 Ci/mmol) per ml, 20.75 µg actinomycin D/ml, 33 to 100 µg C-TMV or TMV RNA/ml and 25 µl enzym preparation per 120 µl assay mixture. The reaction mixture was incubated for 30 min. of 1 hour at 30°C. The incubation was terminated by spotting the samples on Whatman 3MM filter discs, which were immediately immersed in 5% trichloroacetic acid containing 2% $Na_4P_2O_7$ and 2% NaH_2PO_4 at 2°C. This was followed by several washings in this medium and one time in 1N HCl containing 100 mM $Na_4P_2O_7$, two times in 80% ethanol at 2°, and finally two times in ether. The filters were dried and each filter was incubated with 0.75 ml 90% soluene-350 for 1 hour at 50°C and subsequently counted in 5 ml Instafluor. Zero time values were subtracted from the values of the samples. [1]

[1] The specific activity of RNA-dependent RNA polymerase from fractions which had the highest polymerase activity are given.

[2] One unit of RNA polymerase activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of labeled UMP, under the assay conditions described in the legend.

Table 8.1. gives the specific activity of RNA polymerase preparations, purified in a similar way from mock-, C-TMV and CPMV inoculated leaves, tested in fractions of the DEAE-BioGel column and glycerol gradient with highest RNA-dependent RNA polymerase activity. The specific activity of the CPMV replicase was over eight times higher than the specific activity of comparable

preparations from C-TMV inoculated leaves. The specific activity of glycerol gradient purified RNA polymerase from C-TMV inoculated leaves was 5.8 times higher than that of comparable preparations purified from mock-inoculated leaves.
The activity of all three purified RNA polymerases after glycerol gradient centrifugation was completely dependent on added RNA, whereas no specific preference of one of the preparations for CPMV or TMV RNA became apparent. The activity of all three RNA-dependent polymerase preparations was inhibited by pyrophosphate, but not by orthophosphate. On the strength of this and of the way of preparation, being similar to CPMV replicase, it was assumed that the isolated preparations contained RNA-dependent RNA polymerases.

8.3. Comparison of polypeptides in RNA-dependent RNA polymerase preparations from healthy and C-TMV infected cowpea leaves

The RNA-dependent RNA polymerase preparations were analysed both before and after glycerol gradient centrifugation by means of SDS-polyacrylamid slabgel electrophoresis. The proteins were not labeled with radioactive amino acids. They were stained after electrophoresis with coomassie brilliant blue R or/and G-250. This method allows the detection of proteins to a level of 0.25 μg protein per band. Always approximately 30 μg protein was brought on gel.

Fig. 8.4. shows electrophoretic patterns of protein from RNA-dependent polymerase preparations, isolated from C-TMV and mock-inoculated leaves prior to glycerol gradient centrifugation. Six polypeptides were detected in the polypeptide patterns of C-TMV preparations, which were not or to a far less extent present in comparable preparations from mock-inoculated leaves. The molecular weights of these polypeptides were estimated 90,000, 72,000, 46,000, 35,000, 30,000 and 22,000 (in the following designated p90, p72, p46, p35, p30 and p22 respectively). The p90, p35 and p30 comigrated with polypeptides from corresponding RNA-dependent polymerase preparations from mock-inoculated leaves. The bands of the polypeptides from mock-inoculated leaves were much weaker.

Fig. 8.5. shows the result of electrophoresis of RNA-dependent RNA polymerase preparations after glycerol gradient centrifugation. Polypeptide patterns of RNA-dependent RNA polymerase preparations, isolated for C-TMV or mock-inoculated cowpea leaves from glycerol gradient fractions with highest specific activity were compared. In the patterns of C-TMV preparations polypeptides of M.W. approximately 98,000, 90,000, 72,000, and 46,000 were distinguished, which were not or to a far less extent observed in comparable preparations of mock-inoculated leaves. The polypeptides of M.W. 98,000 and 90,000 comigrated with polypeptides from preparations of mock-inoculated leaves, of which the bands, however, were much weaker. It is most plausible that the polypeptides of M.W. 90,000, 72,000 and 46,000 are similar to the p90, p72 and p46, which

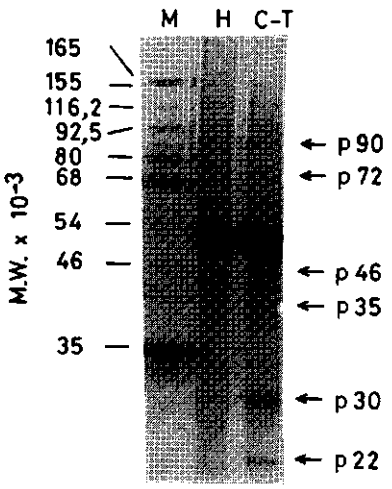


FIG. 8.4. Comparison of electrophoretic patterns of RNA-dependent RNA polymerase preparations from C-TMV and mock-inoculated cowpea leaves before glycerol gradient centrifugation.
DEAE-BioGel column fractions, which had a high RNA-dependent RNA polymerase activity, from C-TMV and mock-inoculated leaves were pooled. The proteins were precipitated by dialyses against buffer A (fig. 8.1.), saturated with $(NH_4)_2SO_4$. The precipitate was collected and the pellet was dissolved in sample buffer. Samples of 30 µg protein were analysed by SDS-PAGE in a 12.5% slabgel at constant voltage during 1.5 hours at 50 V and 6.5 hours at 130 V. After electrophoresis the polypeptides were stained with coomassie brilliant blue R.
The positions of p90, p72, p46, p35, p30 and p22 are indicated.
M: ' markers.
H: RNA-dependent RNA polymerase preparation from mock-inoculated leaves.
C-T: RNA-dependent RNA polymerase preparation from C-TMV infected leaves.

have been observed in polypeptide patterns of preparations prior to glycerol gradient centrifugation (fig. 8.4.).
At comparison of the electrophoretic patterns of protein from different fractions of the glycerol gradient, it appeared that the four polypeptides were most strongly represented in polypeptide patterns of protein from glycerol gradient fractions with high specific RNA-dependent RNA polymerase activity from C-TMV inoculated leaves. Likewise the p98 and p90 from RNA-dependent RNA polymerase preparations of mock-inoculates leaves were most strongly represented in polypeptide patterns of protein from fractions with highest enzyme activity.

Polypeptides of M.W. 35,000, 30,000 and 22,000 were detected in polypeptide patterns from fractions 7–11 of the glycerol gradient, loaded with a RNA-dependent RNA polymerase preparation for C-TMV infected leaves (fig. 8.3.). Polypeptides from corresponding fractions of the glycerol gradient, loaded with an RNA polymerase preparation from mock-inoculated leaves, comigrated with the polypeptides of M.W. 35,000 and 30,000. The bands of the polypeptides from preparations of mock-inoculated leaves were much weaker. Probably the three polypeptides are similar to p35, p30 en p22, which were detected prior to glycerol gradient centrifugation (fig. 8.4.).

Since the low RNA-dependent RNA polymerase activity observed in mock-inoculated leaves has been supposed to increase in consequence of virus infection, it is possible that some of the polypeptides, viz. p98, p90 and p72 and p46, from the RNA-dependent RNA polymerase preparations, isolated from C-TMV infected cowpea leaves, are host proteins, which have increased as a result of the virus infection. In that case it seems possible that similar host proteins are present in RNA-dependent RNA polymerase preparations from cowpea leaves which are infected with a different virus, *e.g.* CPMV, now stimulated by the CPMV infection. It may be expected that these host proteins from RNA-dependent RNA polymerase preparations from C-TMV and CPMV infected cowpea possess a similar electrophoretic mobility.
As has been described in the two previous chapters a polypeptide of the same

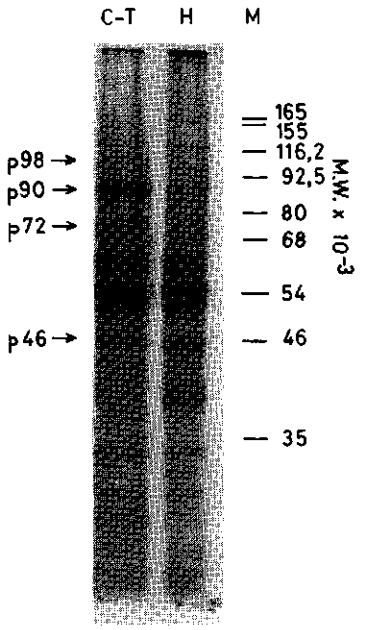


FIG. 8.5. Comparison of electrophoretic patterns of RNA-dependent RNA polymerase preparations from C-TMV and mock-inoculated cowpea leaves after glycerol gradient centrifugation.
RNA-dependent RNA polymerase preparations from C-TMV inoculated leaves and similar preparations from mock-inoculated leaves were purified successively by DEAE-BioGel column chromatography and glycerol gradient centrifugation. Protein from the glycerol gradient fractions which had the highest C-TMV polymerase activity and protein from mock-inoculated leaves from the corresponding gradient fractions was concentrated by precipitation with aceton. The precipitate was dissolved in sample buffer. Samples of approximately 30 µg protein were analysed by SDS-PAGE on a 12.5 % slabgel at constant voltage during 1.5 h at 50 V and 6.5 h at 130 V. After electrophoresis the polypeptides were stained with coomassie brilliant blue R and G-250.
The positions of p98, p90, p72 and p46 are indicated.
C-T: RNA polymerase preparation from C-TMV infected leaves.
H: RNA polymerase preparation from mock-inoculated leaves.
M: markers.

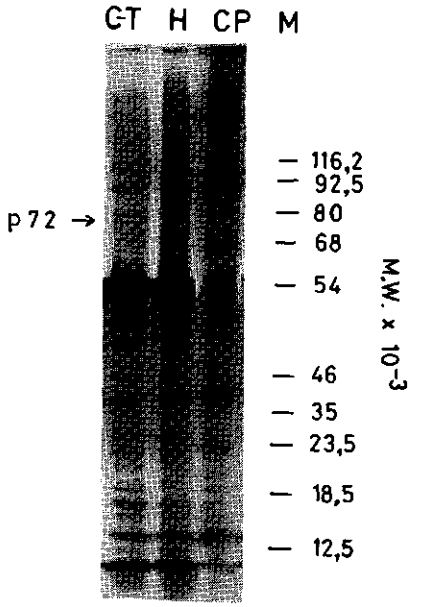


FIG. 8.6. Comparison of electrophoretic patterns of RNA-dependent RNA polymerase preparations from C-TMV and mock-inoculated cowpea leaves and CPMV replicase after DEAE-BioGel column chromatography.
Thirty μg of protein from DEAE-BioGel purified CPMV replicase and similar preparations from mock- and C-TMV inoculated leaves were analysed by SDS-PAGE on 7.5 to 18% gradient gels at constant voltage during 1 hour at 70 V and 7 hours at 120 V. After electrophoresis the polypeptides were stained with coomassie brilliant blue R.
The position of p72 is indicated.
C-T: RNA polymerase preparation from C-TMV infected leaves.
H: RNA polymerase preparation from mock-infected leaves.
CP: CPMV replicase.
M: markers.

M.W. as the p72 from C-TMV RNA-dependent RNA polymerase has been detected both in tobacco protoplasts infected with TMV and in cowpea protoplasts infected with TMV or C-TMV. In the previous chapter it was assumed that the polypeptide observed in infected protoplasts is coded by the virus genome. For this reason it was of importance to trace whether p72 from RNA-dependent RNA polymerase, isolated from C-TMV infected leaves, comigrated with a polypeptide from CPMV replicase, synthesized in consequence of the CPMV infection. Fig. 8.6. shows the polypeptide pattern of RNA-dependent RNA polymerase preparations, which have been isolated in a similar way from C-TMV, mock- or CPMV inoculated cowpea leaves after DEAE-BioGel A column chromatography. It can be seen that p72 from the C-TMV infected leaves did not comigrate with a specific polypeptide from CPMV replicase. Nor was a CPMV specific polypeptide of M.W. 72,000 detected after further purification of the CPMV replicase by means of glycerol gradient centrifugation, while

the specific activity of the CPMV replicase was much higher than RNA-dependent RNA polymerase from C-TMV infected cowpea leaves, isolated in a similar way.

In contrast to the above, however, at comparison of electrophoretic patterns of CPMV replicase and RNA-dependent RNA polymerase from mock-inoculated leaves, purified in a similar way, polypeptides of M.W. 98,000 and 90,000 in fact have been observed in addition to CPMV specific polypeptides. The polypeptides of M.W. 98,000 and 90,000 comigrated with polypeptides from corresponding fractions with RNA-dependent RNA polymerase activity from mock-inoculated cowpea leaves, but the bands in the patterns of mock-inoculated leaves were much weaker. It is very likely that the polypeptides of M.W. approximately 98,000 and 90,000 from CPMV replicase are similar to p98 and p90 from RNA-dependent RNA polymerase preparations from C-TMV inoculated leaves.

8.4. Discussion

From the investigations as described here it is apparent that a low RNA-dependent RNA polymerase activity is present in mock-infected cowpea leaves. White and Dawson (1978) and Ikegami and Fraenkel-Conrat (1978) likewise have found a RNA polymerase activity in mock-inoculated cowpea leaves. Others have demonstrated a comparable activity in mock-inoculated tobacco leaves (Bol *et al.*, 1976; Le Roy *et al.*, 1977; Ikegami and Fraenkel-Conrat, 1978a; Romaine and Zaitlin, 1978; Clerx and Bol, 1978; Duda, 1979).

The specific activity of RNA-dependent RNA polymerase preparations isolated from the 31,000 x*g* pellet fraction of mock-inoculated cowpea leaves and tested after purification by means of glycerol gradient centrifugation, is one sixth of the specific activity of corresponding RNA-dependent RNA polymerase from C-TMV infected cowpea leaves (table 8.1.) and one nineteenth of CPMV replicase prepared in the same way (Zabel, 1978).

Since the RNA-dependent RNA polymerase activity of mock-inoculated cowpea leaves is extremely low with respect to the CPMV replicase activity, this has not been observed under the assay conditions under which CPMV replicase activity was determined by Zabel *et al.* (1974, 1978, 1979). The specific activity of [^{3}H]-UTP used in their study was five times lower than the specific activity of [^{3}H]-UTP used in the present study. For this reason the incorporation of labeled UMP catalyzed by RNA-dependent RNA polymerase from mock-inoculated leaves becomes negligible against the background.

In RNA-dependent RNA polymerase preparations, isolated from C-TMV infected cowpea leaves four polypeptides, stimulated or newly synthesized as a result of C-TMV infection, have been observed after the last purification step, i.e. after glycerol gradient centrifugation. The molecular weights of these polypeptides were estimated 98,000, 90,000, 72,000 and 46,000.

The p98 and p90 comigrate with polypeptides from RNA-dependent RNA

preparations, purified in a similar manner from healthy control leaves. Furthermore, in corresponding CPMV replicase polypeptides of the same M.W. are present likewise comigrating with polypeptides of preparations prepared in an entirely corresponding manner from mock-inoculated leaves. These polypeptides, observed both in RNA-dependent RNA polymerase preparations from C-TMV and CPMV infected leaves, probably are similar. Therefore it is most plausible that p98 and p90 are host polypeptides. In TMV infected cowpea protoplasts no polypeptides synthesized as a result of the TMV multiplication and corresponding with p98 and p90 were found. A possible explanation for this is that the subcellular fractions of cowpea protoplasts are much less purified than the RNA-dependent RNA polymerase preparations from C-TMV or mock-inoculated cowpea leaves.

The p72 and p46 are exclusively present in RNA-dependent RNA polymerase preparations, isolated fromC-TMV infected cowpea leaves. Polypeptides from preparations, purified in a similar manner from healthy control leaves, do not comigrate with these polypeptides, and no polypeptides of the same M.W. are distinguished in corresponding CPMV replicase, synthesized as a result of the CPMV infection. This is an indication that p72 and p46 are synthesized under the direction of the C-TMV genome.

Further indications that p46 is coded by the C-TMV genome are lacking, since a polypeptide of this size has not been found in TMV or C-TMV infected protoplasts. A possible explanation for this may be that the RNA polymerase preparation is much further purified than any subcellular fraction of the protoplasts. Yet another explanation is, that in TMV or C-TMV infected protoplasts there is not enough [^{35}S]-methionine incorporated in the polypeptide of this size, and therefore it is not detected.

The p72 from RNA-dependent RNA polymerase preparations from C-TMV infected leaves corresponds with a polypeptide of M.W. 72,000, synthesized in consequence of TMV or C-TMV multiplication in cowpea protoplasts. Since a polypeptide of the same M.W. is synthesized in TMV infected tobacco protoplasts in consequence of the TMV multiplication, the conclusion seems justified that the polypeptide synthesized in infected protoplasts is a virus coded polypeptide. The polypeptide of M.W. 72,000 from infected protoplasts is present in the 31,000 x*g* pellet fraction and can be extracted from this with Tris buffer without Mg^{2+}. Therefore it is most plausible that p72 from the RNA-dependent RNA polymerase preparation from C-TMV infected cowpea leaves and the polypeptide from C-TMV and TMV infected protoplasts are similar.

In the RNA-dependent RNA polymerase preparations from C-TMV infected cowpea leaves p116,5 has not been observed. This virus-coded polypeptide has been repeatedly detected in TMV infected and C-TMV infected leaves and protoplasts. It has been supposed that p116,5 is involved in the TMV RNA synthesis, since p116,5 is synthesized during an early period after infection (SAKAI and TAKEBE, 1974; PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978) and is present in the 31,000 x*g* pellet of TMV infected tobacco leaves, from which it can be extracted, like the RNA polymerase (SCALLA *et al.*, 1978). Neither Ro-

MAINE and ZAITLIN (1978) were able to find this polypeptide after further purification by means of DEAE-BioGel column chromatography of the RNA polymerase from TMV infected tobacco leaves. Since it is possible to detect p72 in RNA-dependent RNA polymerase from C-TMV infected cowpea leaves and since this polypeptide is synthesized, like p116,5 during an early period after infection, it appears plausible to suppose that p72 is involved in TMV RNA synthesis.

For further characterization of the polypeptides that are observed, i.e. p98, p90, and p72, p46, further purification of the RNA-dependent RNA polymerase preparations undoubtedly offers most perspectives. A promising approach might be to compare polypeptide patterns of RNA-dependent RNA polymerase preparations, isolated from C-TMV infected tobacco and cowpea leaves. If in both preparations polypeptides with similar M.W. are distinguished, these can be identified for instance by polypeptide mapping after proteolytic cleavage.

CONCLUSION

The work presented here demonstrates a larger number of polypeptides, which are synthesized as a result of TMV multiplication in infected protoplasts, than have been observed before. In search for TMV specific polypeptides, the use of protoplasts instead of leaf tissue has not only the advantage that the course of virus multiplication in infected protoplasts is synchronous, but also that subcellular fractions prepared from the protoplast homogenates are less contaminated by material from other subcellular fractions. The fractionation of protoplast homogenates may therefore result in a stronger enrichment of specific proteins in subcellular fractions than can be achieved by fractionation of leaf tissue.

The large number of polypeptides synthesized as a result of TMV multiplication adds to the difficulty in determining which specific polypeptides are formed under the direction of TMV RNA or of the host genome. Comparison of the polypeptides synthesized upon TMV infection in different hosts provides the possibility of recognizing polypeptides encoded in the TMV RNA which have not been observed when TMV RNAs are translated in cell-free protein synthesizing systems. On the other hand, it may not be excluded that a host polypeptide, synthesized as a result of TMV multiplication, has a similar size in both hosts.

With regard to the size of the TMV RNA only a small number of the TMV specific polypeptides observed here may have been coded by the TMV RNA. P116,5 and coat protein, which have been shown to be TMV coded polypeptides (WITTMANN-LIEBOLD and WITTMANN, 1965; RICHARDS *et al.*, 1974; GUILLEY *et al.*, 1975a, 1975b; SCALLA *et al.*, 1978) are detected here too, both in TMV infected tobacco and cowpea protoplasts.

P30 is observed in polypeptide patterns of proteins from the pellet fractions of TMV infected tobacco protoplasts. This polypeptide may be similar to the *in vitro* product of a same size. P30 is therefore possibly a TMV coded polypeptide. ZAITLIN and HARIHARASUBRAMANIAN (1970, 1972) reported a polypeptide of 37,000 daltons, found in polypeptide patterns of protein from pellet fractions of TMV infected tobacco leaves. The size of the polypeptide synthesized in TMV infected tobacco leaves rather deviates from that of p30, which may be due to the molecular weight determination from seperate gels in tubes.

P170 is observed in TMV infected tobacco protoplasts and tobacco leaves (ZAITLIN and HARIHARASUBRAMANIAN, 1970, 1972; SAKAI and TAKEBE, 1974; PATERSON and KNIGHT, 1975; SCALLA *et al.*, 1976; SIEGEL *et al.*, 1978), but p170 is not synthesized in TMV infected cowpea protoplasts. The p170 formed in TMV infected tobacco protoplasts comigrates with a polypeptide from mock-infected tobacco protoplasts. Moreover, p170 has an electrophoretic mobility, which is not exactly similar to the mobility of the translation product synthesized in reticulocyte lysates with TMV RNA as messenger. Therefore it seems reasonable to suppose that p170 synthesized in TMV infected tobacco protoplasts and

leaves is a host polypeptide of which the synthesis is stimulated upon TMV infection. This suggests that p170 is not synthesized *in vivo* as a result of TMV multiplication, whereas a product of this size is formed *in vitro* under the direction of TMV RNA. Although a p170 is not synthesized in TMV infected cowpea protoplasts, it cannot be excluded that p170 is still synthesized in TMV infected tobacco cells. In this case the TMV coded p170 may have no functional significance for the virus multiplication.

A p72 is synthesized in both TMV infected tobacco and TMV infected cowpea protoplast. Therefore an indication is obtained, that p72 is synthesized under the direction of the TMV genome. This is further supported by the fact that a p72 is found in RNA-dependent RNA polymerase preparations of C-TMV infected cowpea leaves, whereas p72 is not observed in mock-inoculated cowpea leaves. Moreover, when C-TMV and CPMV RNA-dependent RNA polymerase preparations are compared, p72 appears to be exclusively present in prepararions from C-TMV infected leaves.

A specific polypeptide of similar size has not been reported before in research on polypeptides synthesized under the direction of TMV RNA *in vitro* and *in vivo*. The size of TMV RNA is large enough to contain the nucleotide sequence of p72 in addition to the sequence of pl16,5, p30 and coat protein (see fig. 1.1.). The region involved in the synthesis of p72 may be situated between the cistron of pl16,5 and the region corresponding to p30. This may be especially the case since the results of GUILLEY *et al.* (1979) have pointed out that the region of p30 may be separated by only two nucleotides from the region of coat protein. Thus it is possible that not only coat protein and p30 are synthesized from subgenomic TMV mRNAs, but that p72 is synthesized likewise. An interesting possibility is that the subset TMV RNAs of 1.1 or 1.4×10^6 daltons mentioned by SIEGEL *at al.* (1976) and by BEACHY and ZAITLIN (1977) may contain the cistron of p72.

Other possibilities are that overlapping sequences are involved in the synthesis of p72 or that a precursor polypeptide is synthesized, which is cleaved to p72. At present there is no evidence to support these suggestions.

Assuming p72 to be encoded by the TMV RNA a virus coded polypeptide possibly plays a part in the TMV RNA synthesis, because p72 is present in RNA-dependent RNA polymerase preparations, which are purified to a far extent.

It is still uncertain how the host is involved in the process of TMV multiplication. Our results show, however, that two polypeptides, p98 and p90, are present in a more pronounced extent in RNA-dependent RNA polymerase preparations isolated from C-TMV and CPMV infected cowpea leaves than in preparations from mock-inoculated leaves. Therefore host proteins may be involved in virus RNA synthesis.

The p170 and p82 synthesized in TMV infected tobacco protoplasts and p86 formed in TMV infected cowpea protoplasts may be host proteins, the synthesis of which is induced upon TMV infection. These polypeptides are among those specific polypeptides, which are distinguishable most clearly. Charaterization of these polypeptides may help to provide an insight into how the host is involved in the process of TMV infection.

SUMMARY

The study described here concerns the proteins, synthesized as a result of tobacco mosaic virus (TMV) multiplication in tobacco protoplasts and in cowpea protoplasts. The identification of proteins involved in the TMV infection, for instance in the virus RNA replication, helps to elucidate the infection process in the plant cell. Not only virus coded proteins, but possibly also host coded proteins may play a part in the TMV multiplication.
Research on proteins encoded by the TMV RNA, carried out in cell-free protein synthesizing systems, has revealed that five polypeptides are synthesized under the direction of TMV (subgenomic) mRNAs (see table 1.2., chapter 1.). Whether the polypeptides, synthesized *in vitro* with TMV RNA as messenger, are of functional significance for the TMV infection may only be determined by means of investigating TMV infected leaves and protoplasts.
The TMV multiplication runs synchronously in all protoplasts that are infected. Therefore, proteins synthesized in small amounts upon infection, may be thus detected.

The search for proteins sythesized in protoplasts as a result of TMV infection has long been hindered by the fact that various factors in the cultivation of the tobacco plants may adversely influence the quality of the protoplasts. The cultivation of the tobacco plants: *Nicotiana Tabacum* cv. L. Samsun, Samsun NN and Xanthi nc, could be standardized however, as described in chapter 2. When the tobacco plants were cultivated in this way, at least 50 % of the tobacco protoplasts could be infected with TMV and 70 % or more of the protoplasts survived the subsequent incubation period of 36 hours. This could be achieved every time the protoplasts were isolated. The intensity and quality of the light, the way of watering, the age of the tobacco plants and of the leaf, from which the protoplasts are isolated, among others, appeared to affect the quality of the protoplasts (chapter 3.).

The proteins, synthesized upon TMV infection, have to be distinguished among a great variety of host proteins. For this reason it is important to determine the incorporation of radioactive amino acids into protein synthesized as a result of TMV multiplication, in comparison with the incorporation into host proteins that are formed independently from the virus infection. Therefore the specific activity of TMV coat protein (cpm/mg protein) and of the proteins of the 27,000 x*g* supernatant fraction, synthesized in infected tobacco protoplasts were compared. It appeared that the specific activity of TMV coat protein was at least four times higher than of the proteins in the 27,000 x*g* supernatant (chapter 4.).

The proteins synthesized as a result of TMV multiplication were studied not only in tobacco protoplasts, but also in protoplasts from the primary leaves of cowpea (*Vigna unguiculata* (L.) Walp. var. 'Blackeye Early Ramshorn'). The method used for the infection of tobacco protoplasts with TMV was not suitable

for the infection of cowpea protoplasts with TMV. Best results were obtained when both protoplasts and virus were incubated in the presence of poly-D-lysine, for 7.5 min. before infection. The protoplasts were pre-incubated in 0.1 M potassium phosphate buffer (pH 5.4) at 0° C, at a concentration of 4×10^5 protoplasts/ml and 0.75 µg poly-D-lysine/ml. TMV was pre-incubated in the same buffer at room temperature at a concentration of 2 µg TMV/ml and 2 µg poly-D-lysine/ml. During infection the cowpea protoplasts were incubated together with TMV and poly-D-lysine in a concentration of 2×10^5 protoplasts/ml, 1 µg TMV/ml and 1 µg poly-D-lysine/ml, for 7.5 min, in the buffer mentioned above at 0° C. In this way 50 to 70 % of the cowpea protoplasts could be infected with TMV.

The course of TMV synthesis in cowpea protoplasts was comparable with that in tobacco protoplasts. The TMV multiplication in cowpea protoplasts was preceeded, however, by a period of 16 hours, during which the increase of TMV is slight, while the TMV multiplication in tobacco protoplasts was preceeded by a lag period of 8 hours. A possible explanation is that a much smaller amount of TMV particles penetrates into cowpea protoplasts during inoculation and/or starts to multiply than is the case in tobacco protoplasts (chapter 5.).

The proteins of TMV infected and mock-infected protoplasts were analysed therupon by means of SDS-polyacrylamide slabgel electrophoresis and the polypeptide patterns were visualized by autoradiography.

Ten polypeptides were distinguished, which are synthesized as a result of TMV multiplication in polypeptide patterns of proteins from infected tobacco protoplasts. The molecular weights were estimated to be 260,000, 240,000, 170,000, 116,500, 96,000, 90,000, 82,000, 72,000, 30,000 and 17,500 (coat protein). Polypeptides of similar molecular weight were absent or were present to much less extent in polypeptide patterns of proteins from mock-infected tobacco protoplasts. Many polypeptides were observed for reason that the detection capacity was improved by means of subcellular fractionation of the protoplast homogenates.

The polypeptides of molecular weight 170,000, 116,500, 72,000 and coat protein were present in the 31,000 x*g* supernatant fraction and the pellet fractions as well. The polypeptide of molecular weight of 30,000 was present exclusively in the pellet fractions. The other polypeptides were observed exclusively in polypeptide patterns of protein of the 31,000 x*g* supernatant fraction (see table 6.1., chapter 6.).

Eight polypeptides were observed, which were synthesized as a result of TMV multiplication in cowpea protoplasts. The molecular weights of the polypeptides were approximately 150,000, 116,500, 86,000, 72,000, 17,500 (coat protein), 16,000, 14,000 and 10,000. Polypeptides of similar molecular weight were absent or present on a far less extent in polypeptide patterns of proteins from mock-infected cowpea protoplasts.

The polypeptides of molecular weight 116,500, 72,000 and coat protein were present in the 31,000 x*g* pellet and 31,000 x*g* supernatant. The other polyeptides were present exclusively in the 31,000 x*g* supernatant (table 7.1., chapter 7.).

It was assumed that the TMV coded polypeptides are similar in different hosts and, on the other hand, that the host polypeptides, synthesized upon TMV infection differ from host to host. When the TMV specific polypeptides, synthesized in infected tobacco protoplasts were compared with the specific polypeptides synthesized in TMV infected cowpea protoplasts, it appeared that only the polypeptides of molecular weight 116,500, 72,000 and coat protein are of similar size in both hosts (table 7.2., chapter 7.). This is an indication that not only the polypeptide of 116,500 daltons and coat protein are TMV coded polypeptides, but that also the polypeptide of 72,000 daltons is encoded in the TMV RNA. It has not been reported that a polypeptide of this size is observed when TMV RNAs are translated in cell-free protein synthesizing systems.
A polypeptide of 170,000 daltons is synthesized *in vitro* under the direction of the TMV RNA. It appeared that the polypeptide synthesized in TMV infected tobacco leaves, has a slightly less electrophoretic mobility than the product of 170,000 daltons synthesized *in vitro* from TMV RNA as messenger. A polypeptide of similar electrophoretic mobility was present to a lesser extent in mock-infected tobacco protoplasts. Furthermore, a polypeptide of 170,000 daltons was not observed in TMV infected cowpea protoplasts. For these reasons it is likely, that the polypeptide of 170,000 daltons, synthesized in TMV infected tobacco protoplasts, is encoded in the genome of tobacco or is encoded in the TMV RNA, but then the polypeptide has no functional significance in the TMV multiplication process.
Further the polypeptide of 30,000 was observed only in TMV infected tobacco protoplasts, whereas a polypeptide of similar molecular weight was shown to be synthesized *in vitro* from a TMV subgenomic mRNA. The polypeptide of 30,000 daltons was detected exclusively in the polypeptide patterns of protein from the pellet fractions of TMV infected tobacco protoplasts. Polypeptide patterns of protein from corresponding fractions of cowpea protoplasts had a predominant, grey background. Due to this the polypeptide of 30,000 daltons may not be distinguished in TMV infected cowpea protoplasts, whereas the polypeptide of 30,000 daltons synthesized in TMV infected tobacco protoplasts can in fact be a polypeptide coded by TMV RNA. The other polypeptides synthesized in infected tobacco protoplasts or cowpea protoplasts as a result of TMV multiplication are presumably synthesized under the genome of tobacco or cowpea respectively.

Finally, it was attempted to examine in what way the polypeptides of 116,500 and 72,000 are involved in the TMV infection process. Both polypeptides were shown to be present in the 31,000 x*g* pellet of TMV infected tobacco and cowpea protoplasts. It was studied whether virus specific polypeptides of similar molecular weight can be observed in RNA-dependent RNA polymerase preparations isolated from the 31,000 x*g* pellet fraction of cowpea leaves infected with the cowpea strain of TMV (C-TMV). The RNA-dependent RNA polymerase preparations were isolated by extraction of the 31,000 x*g* pellet fraction and were further purified by means of subsequent DEAE-BioGel column chromatography and glycerol gradient centrifugation. The purification procedure used was

the same procedure as described for the isolation of RNA-dependent RNA polymerase from cowpea leaves infected with cowpea mosiac virus (CPMV).
Four specific polypeptides of molecular weight of 98,000, 90,000, 72,000 and 46,000 were distinguished in RNA-dependent RNA polymerase preparations from C-TMV infected cowpea leaves, after glycerol gradient purifications. A polypeptide of molecular weight 116,500 was not observed. Polypeptides of molecular weights 72,000 and 46,000 were not found and those of molecular weights 98,000 and 90,000 were distinguished to a less extent in polypeptide patterns of preparations isolated in exactly the same way from mock-inoculated cowpea leaves.
RNA-dependent RNA polymerase activity was also observed in preparations isolated from mock-inoculated cowpea leaves. The specific activity (cpm/mg protein) of the preparation from mock-inoculated leaves was one sixth of the specific activity of the RNA-dependent RNA polymerase preparations from C-TMV infected cowpea leaves. The RNA-dependent RNA polymerase activity in C-TMV infected cowpea leaves might therefore be attributed to the increase of one or several polypeptides, present already before inoculation. Since it was thought that the polypeptide of 72,000 daltons is a TMV coded polypeptide, it was examined which specific polypeptides are present in RNA-dependent RNA polymerase preparations isolated in a similar way from CPMV infected cowpea leaves. It appeared, that in addition to CPMV specific polypeptides, the polypeptides of molecular weight 98,000 and 90,000 were also observed in RNA-dependent RNA polymerase preparations from CPMV infected leaves. The polypeptides of 72,000 and 46,000 daltons were distinguished only in preparations isolated from C-TMV infected cowpea leaves. These results suggest that the polypeptide of 72,000 daltons in involved is the synthesis of TMV RNA (chapter 8.).

SAMENVATTING

Het onderzoek dat hier beschreven is, betreft de eiwitten die gesynthetizeerd zijn tengevolge van tabaksmozaïekvirus (TMV) vermeerdering in geïnfecteerde tabaksprotoplasten en in geïnfecteerde cowpeaprotoplasten. De identificatie van eiwitten, die betrokken zijn bij de TMV infectie, bijvoorbeeld bij de virus RNA replicatie, draagt bij aan het inzicht in het infectieproces in de plantecel. Niet alleen virus gecodeerde, maar mogelijk ook gastheer gecodeerde eiwitten kunnen een rol spelen bij de TMV vermeerdering.
Onderzoek naar TMV gecodeerde eiwitten, uitgevoerd in celvrije eiwit synthetizerende systemen, heeft uitgewezen, dat vijf eiwitten gevormd worden onder invloed van TMV (subgenomische) mRNA's (zie tabel 1.2., hoofdstuk 1.). In hoeverre de eiwitten, *in vitro* gevormd met TMV RNA als messenger, van functionele betekenis zijn voor de TMV infectie kan slechts nagegaan worden door middel van onderzoek in TMV geïnfecteerde bladeren en protoplasten.
De TMV vermeerdering verloopt synchroon in de protoplasten die geïnfecteerd zijn. Daarom zullen eiwitten, die in kleine hoeveelheden gevormd worden na infectie gedetecteerd kunnen worden. Het onderzoek naar eiwitten die gevormd worden ten gevolge van de TMV infectie wordt bemoeilijkt, omdat verschillende factoren van de kweek van de tabaksplanten de kwaliteit van de protoplasten nadelig kunnen beïnvloeden. De kweek van de tabaksplanten: *Nicotiana tabacum* cv. L Samsun, Samsun NN en Xanthi nc, kon echter gestandaardizeerd worden, zoals beschreven in hoofdstuk 2.
Wanneer de tabaksplanten op de beschreven wijze werden gekweekt, konden elke keer dat protoplasten geïsoleerd werden meer dan 50 % van de protoplasten geïnfecteerd worden met TMV en overleefden minstens 70 % van de protoplasten de daaropvolgende incubatieperiode van 36 uur. Onder andere de intensiteit en de kwaliteit van het licht, de wijze van watergeven, de leeftijd van de tabaksplanten en van het blad waaruit de protoplasten geïsoleerd worden, bleken effect te hebben op de kwaliteit van de protoplasten (hoofdstuk 3.).

De eiwitten, die gevormd worden ten gevolge van de TMV vermeerdering moeten onderscheiden worden temidden van een grote variëteit aan gastheer eiwitten. In verband hiermee is het van belang na te gaan hoe de inbouw van radio-actief aminozuur is in eiwit, dat gevormd wordt ten gevolge van de TMV vermeerdering, ten opzichte van de inbouw in gastheer eiwitten, die gevormd worden onafhankelijk van de virusinfectie. De specifieke activiteit van TMV manteleiwit (cpm/mg eiwit) en van de eiwitten uit de 27,000 *xg* supernatant fractie, gevormd in geïnfecteerde tabaksprotoplasten werd daarom vergeleken. Het bleek, dat de specifieke activiteit van TMV manteleiwit minstens vier keer hoger was dan van de eiwitten uit de 27,000 *xg* supernatant (hoofdstuk 4.).
Niet alleen in tabaksprotoplasten, maar ook in protoplasten uit primaire bladeren van cowpea (*Vigna unguiculata* (L.) Walp. var. 'Blackeye Early Ramshorn') werd onderzocht welke eiwitten gevormd worden ten gevolge van de TMV

vermeerdering. De methode, volgens welke tabaksprotoplasten geïnfecteerd worden met TMV, was niet toepasbaar voor de infectie van cowpeaprotoplasten met TMV. Het beste resultaat werd verkregen wanneer zowel de protoplasten als virus gepre-incubeerd werden met poly-D-lysine gedurende 7,5 min. Preincubatie van de protoplasten vond plaats in 0,1 M kaliumfosfaatbuffer (pH 5,4) bij 0° C bij een concentratie van 4×10^5 protoplasten/ml en 0,75 µg poly-D-lysine/ml. TMV werd gepre-incubeerd in dezelfde buffer bij kamertemperatuur in een concentratie van 2 µg TMV/ml en 2 µg poly-D-lysine/ml. Tijdens infectie werden de cowpeaprotoplasten (2×10^5 protoplasten/ml) geïncubeerd met TMV (1 µg/ml) en poly-D-lysine (1 µg/ml) gedurende 7,5 min, in bovengenoemde buffer bij 0 °C. Op deze wijze konden 50 tot 70 % van de cowpeaprotoplasten geïnfecteerd worden met TMV.

Het verloop van de TMV synthese in cowpeaprotoplasten is vergelijkbaar met die in tabaksprotoplasten. De TMV vermeerdering in cowpeaprotoplasten werd echter voorafgegaan door een periode van 16 uur, dat de TMV toename gering is, terwijl de TMV vermeerdering in tabaksprotoplasten voorafgegaan werd door een lag periode van 8 uur. Mogelijk vindt dit zijn oorzaak hierin, dat een veel kleiner aantal TMV deeltjes in cowpeaprotoplasten binnendringt tijdens inoculatie en/of tot de vermeerdering overgaat dan het geval is in tabaksprotoplasten (hoofdstuk 5.).

De eiwitten van TMV geïnfecteerde en schijngeïnfecteerde protoplasten werden vervolgens geanalyseerd door middel van SDS-polyacrylamide slabgel electroforese en de polypeptide patronen werden zichtbaar gemaakt door autoradiografie.

In polypeptide patronen van eiwit uit geïnfecteerde tabaksprotoplasten werden tien polypeptiden onderscheiden, die niet of in veel mindere mate voorkwamen in polypeptide patronen van schijngeïnfecteerde tabaksprotoplasten. De molecuul gewichten werden bepaald op 260000, 240000, 170000, 116500, 96000, 90000, 82000, 72000, 30000 en 17500 (manteleiwit). Zoveel polypeptiden werden onderscheiden, omdat de detectie-capaciteit verhoogd was door subcellulaire fractionering van de protoplasten homogenaten.
De polypeptiden met molecuul gewicht van 170000, 116500, 72000 en manteleiwit kwamen voor in de 31000 x*g* supernatant fractie en in de pellet fracties. De polypeptide met molecull gewicht 30000 werd uitsluitend waargenomen in de pellet fracties. De overige werden uitsluitend waargenomen in de 31000 x*g* supernatant fractie (zie tabel 6.1., hoofdstuk 6.).

In polypeptide patronen van eiwit uit geïnfecteerde cowpeaprotoplasten werden acht polypeptiden onderscheiden, die niet of in veel mindere mate voorkwamen in polypeptide patronen van schijngeïnfecteerde cowpeaprotoplasten. De molecuul gewichten van de polypeptiden, gevormd ten gevolge van de TMV vermeerdering werden bepaald op 150000, 116500, 86000, 72000, 17500 (manteleiwit), 16000, 14000 en 10000. De polypeptiden met molecuul gewicht van 116500, 72000 en manteleiwit kwamen voor in de 31000 x*g* pellet en supernatant. De overige waren uitsluitend in de supernatant aanwezig (tabel 7.1., hoofdstuk 7.). Verondersteld werd, dat de TMV gecodeerde polypeptiden dezelfde zijn

in verschillende gastheren en dat de gastheer gecodeerde polypeptiden, die gevormd worden tengevolge van de TMV vermeerdering verschillen van gastheer tot gastheer. Wanneer de TMV specifieke polypeptiden, die gevormd zijn in geïnfecteerde tabaksprotoplasten, vergeleken werden met de specifieke polypeptiden, die in TMV geïnfecteerde cowpeaprotoplasten gevormd zijn, bleek dat uitsluitend de polypeptiden met molecuul gewicht van 116000, 72000 en manteleiwit dezelfde molecuul gewichten hebben (tabel 7.2., hoofdstuk 7.). Dit is een aanwijzing, dat niet alleen de polypeptide van 116500 daltons en manteleiwit TMV gecodeerde polypeptiden zijn, maar ook de polypeptide van 72000 daltons. Het is niet bekend, dat een polypeptide van deze grootte is waargenomen wanneer TMV RNA's vertaald worden in celvrije eiwit synthetizerende systemen. Een polypeptide van 170000 daltons wordt *in vitro* gevormd onder invloed van het TMV RNA. Een polypeptide, waarvan de electroforetische mobiliteit een weinig geringer bleek te zijn dan het *in vitro* gevormde produkt van 170000 daltons, werd gevormd in TMV geïnfecteerde tabaksprotoplasten. Een polypeptide met eenzelfde elektroforetische mobiliteit was in mindere mate aanwezig in schijngeïnfecteerde tabaksprotoplasten. Bovendien werd geen polypeptide van 170000 daltons waargenomen in TMV geïnfecteerde cowpeaprotoplasten. Het is daarom waarschijnlijk, dat het polypeptide van 170000 daltons, gevormd in TMV geïnfecteerde tabaksprotoplasten, of gevormd is onder invloed van het genoom van tabak, of dat het een TMV gecodeerde polypeptide is, dat geen functionele betekenis heeft bij de virusvermenigvuldiging.

Ook het polypeptide van 30000 daltons werd alleen gevonden in TMV geïnfecteerde tabaksprotoplasten, hoewel een polypeptide van dezelfde grootte gevormd wordt *in vitro* van een TMV subgenomische mRNA. Dit polypeptide werd uitsluitend in de polypeptide patronen van eiwit uit de pellet fracties van TMV geïnfecteerde tabaksprotoplasten waargenomen. Polypeptide patronen van eiwit uit overeenkomstige fracties van cowpeaprotoplasten hadden een sterke grijze achtergrond. Daarom zou de polypeptide van 30000 daltons niet waargenomen kunnen zijn in TMV geïnfecteerde cowpeaprotoplasten, terwijl het polypeptide van 30000 daltons, gevormd in TMV geïnfecteerde tabaksprotoplasten mogelijk wel een TMV gecodeerde polypeptide is.
De overige specifieke polypeptiden, gesynthetizeerd in TMV geïnfecteerde tabaksprotoplasten of cowpeaprotoplasten, zullen waarschijnlijk gevormd zijn onder invloed van het genoom van respectievelijk tabak of cowpea.

Tenslotte werd ingegaan op de vraag welke rol de polypeptiden van 72000 en 116500 zouden kunnen vervullen bij de TMV vermenigvuldiging. Beide polypeptiden kwamen voor in de 31000 x*g* pellet van TMV geïnfecteerde tabaks- en cowpeaprotoplasten. Nagegaan werd of virus specifieke polypeptiden met eenzelfde molecuul gewicht gevonden worden in RNA afhankelijke RNA polymerase preparaten geïsoleerd uit de 31000 x*g* pellet fractie van cowpeabladeren, die geïnfecteerd zijn met de cowpeastam van TMV (C-TMV).
Na extractie van de preparaten uit de 31000 x*g* pellet fractie werden deze verder gezuiverd door middel van achtereenvolgens DEAE-BioGel kolom-chromatografie en glycerol-gradient centrifugatie.

De RNA afhanklijke RNA polymerase preparaten werden geïsoleerd volgens de zuiveringsprocedure beschreven voor RNA afhankelijk RNA polymerase uit bladeren geïnfecteerd met cowpeamozaïekvirus (CPMV). Vier specifieke polypeptiden met molecuul gewicht van 98000, 90000, 72000 en 46000 werden waargenomen in RNA afhankelijke RNA polymerase preparaten uit C-TMV geïnfecteerde cowpeabladeren na glycerol-gradient zuivering. Een polypeptide met molecuul gewicht 116500 werd niet waargenomen. Polypeptiden met molecuul gewicht van 72000 en 46000 werden niet en polypeptiden met molecuul gewicht van 98000 en 90000 werden in mindere mate waargenomen in polypeptide patronen van preparaten op geheel gelijke wijze geïsoleerd uit schijngeïnoculeerde cowpeabladeren.

In preparaten geïsoleerd uit schijn-geïnoculeerde cowpeabladeren werd ook een RNA afhankelijke RNA polymerase activiteit waargenomen. De specifieke activiteit (cpm/mg eiwit) van het preparaat uit schijngeïnfecteerde cowpeabladeren was een zesde van de specifieke activiteit van het preparaat uit C-TMV geïnfecteerde bladeren.

De RNA afhankelijk RNA polymerase activiteit in C-TMV geïnfecteerde cowpeabladeren zou dan ook berusten op de toename van één of meer reeds vóór inoculatie aanwezige eiwitten. Omdat verondersteld was, dat het polypeptide van 72000 daltons een virus gecodeerde polypeptide is, werd nagegaan welke specifieke polypeptiden aanwezig zijn in op gelijke wijze geïsoleerde RNA afhankelijke RNA polymerase preparaten uit CPMV geïnfecteerde cowpeabladeren. Het bleek, dat naast specifieke CPMV polypeptiden, de polypeptiden met molecuul gewicht 98000 en 90000 ook voorkwamen in RNA afhankelijke RNA polymerase preparaten uit CPMV geïnfecteerde cowpeabladeren. De polypeptiden van 72000 en 46000 daltons werden alleen waargenomen in preparaten geïsoleerd uit C-TMV geïnfecteerde bladeren. Deze gegevens maken aannemelijk, dat de polypeptide van 72000 daltons betrokken is bij de synthese van TMV RNA (zie hoofdstuk 8.).

Wie wat vindt heeft slecht gezocht. (Rutger Kopland, 1972)

REFERENCES

ALBLAS, F., and BOL, J.F. (1977). Factors influencing the infection of cowpea mesophyll protoplasts by alfalfa mosaic virus. J. gen. Virol. **36**, 175–185.

ANDERER, F. A., UHRIG, H., WEBER, and SCHRAMM, G. (1960). Primary structure of the protein of TMV. Nature **186**, 922–925.

ANDERER, F. A., WITTMANN-LIEBOLD, B., and WITTMANN, H. G. (1965). Weitere Untersuchungen zur Aminosäuresequenz des Proteins in Tabakmosaikvirus. Z. Naturforsch. **206**, 1203–1213.

AOKI, S., and TAKEBE., (1975). Replication of tobacco mosaic virus RNA in tobacco mesophyll protoplasts inoculated *in vitro*. Virology **65**, 343–354.

ASTIER-MANIFACIER, S., and CORNUET, P. (1971). RNA-dependent RNA polymerase in chinese cabbage. Biochim. Biophys. Acta **232**, 484–493.

BABOS, P. (1971). TMV-RNA associated with ribosomes of tobacco leaves infected with TMV. Virology **43**, 597–606.

BANCROFT, J. B., MOTOYOSHI, F., WATTS, J. W., and DAWSON, J. R. O. (1975). Cowpea chlorotic mottle and brome mosaic virus in tobacco protoplasts. In: Modification of the information content of plant cells. (R. Markham, D. R. Davis, D. A. Hopwood, and R. W. Horne, eds.) North-Holland, Amsterdam, pp. 133–160.

BEACHY, R. N., and ZAITLIN, M. (1975). Replication of tobacco mosaic virus. VI. Replicative intermediate and TMV-RNA-related RNAs associated with polyribosomes. Virology **63**, 84–97.

BEACHY, R. N., ZAITLIN, M., BRUENING, G., and ISRAEL, H. W. (1976). A genetic map for the cowpea strain of TMV. Virology **73**, 498–507.

BEACHY, R. N. and ZAITLIN, M. (1977). Characterization and in vitro translation of the RNAs from less-than-full-length, virus-related, nucleoprotein rods present in tobacco mosaic virus preparations. Virology **81**, 160–169.

BISHOP, D. H. L. (1977). Virion Polymerases. In: Comprehensive virology. (H. Fraenkel-Conrat and R. R. Wagner, eds.) Plenum Publishing Corporation, Vol. **10**, pp. 117–278.

BOL. J. F., CLERX-van HAASTER, C. M., and WEENING, C. J. (1976). Host and virus specific RNA polymerases in alfalfa mosaic virus infected tobacco. Ann. Microbiol. (Inst. Pasteur) **127A**, 183–192.

BRADLEY, D. W., and ZAITLIN, M. (1971). Replication of tobacco mosaic virus. II. The in vitro synthesis of high molecular weight virus-specific RNAs. Virology **45**, 192–199.

BRUENING, G., BEACHY, R. N., SCALLA, R., and ZAITLIN, M. (1976). *In vitro* and *in vivo* translation of the ribonucleic acids of a cowpea strain of tobacco mosaic virus. Virology **71**, 498–517.

BUTLER, P. J. G., and KLUG, A. (1971). Assembly of the particle of tobacco mosaic virus from RNA and disks of protein. Nature New Biology **229**: 47–50.

CASPAR, D. L. D. (1963). Assembly and stability of the tobacco mosaic virus particle. Adv. Protein Chem. **18**, 37–121.

CLERX, C. M. and BOL, J. F. (1978). Properties of solubilized RNA-dependent RNA polymerase from alfalfa mosaic virus-infected and healthy tobacco plants. Virology **91**, 453–463.

COCKING, E. C. (1966). An electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus. Planta (Berl.) **68**, 206–214.

COCKING, E. C., and POJNAR, E. (1968). Appearance of tobacco mosaic virus in thin section using different staining procedures. J. gen. Virol. **2**, 317–318.

COCKING, E. C., and POJNAR, E. (1969). An electron microscopic study of the infection of isolated tomato fruit protoplasts by tobacco mosaic virus. J. gen. Virol. **4**, 305–312.

DAVIES, J. W., and KAESBERG, P. (1974). Translation of virus mRNA: Protein synthesis directed by several virus RNAs in cell-free extract from wheat germ. J. gen. Virol. **25**, 11–20.

DAVIES, J. W., AALBERS, A. M. J., STUIK, E. J., and van KAMMEN, A. (1977). Translation of cowpea mosaic virus RNA in a cell-free extract from wheat germ. FEBS Lett. **77**, 265–269.

DAVIES, J. W. (1979). Translation of plant virus ribonucleic acids in extracts from eukaryotic cells. In:

Nucleic acids in Plants. (T. C. Hall and J. W. Davies, eds.) CRC Press Inco., Fla., Vol. **2**, Section 3.

DAWSON, W. O., and SCHLEGEL, D. E. (1976). The sequence of inhibition of tobacco mosaic virus synthesis by actinomycin D, 2-thiouracil, and cycloheximide in synchronous infection. Phytopathology **66**, 177–181.

DAWSON, W. O., and SCHLEGEL, D. E. (1976). Time-course of tobacco mosaic virus-induced RNA synthesis in synchronously infected tobacco leaves. Phypathology **66**, 437–442.

DIJKSTRA, J. (1964). The early events of tobacco mosaic virus infection in *Nicotiana glutinosa* L. Meded. Landbouwhogeschool, Wageningen **64**, 1–83.

DUDA, C. T., ZAITLIN, M., and SIEGEL, A. (1973). *In vitro* synthesis of double-stranded RNA by an enzyme system isolated from tobacco leaves. Biochem. Biophys. Acta **319**, 62–71.

DUDA, C. T. (1979). Synthesis of double.stranded RNA. II. Partial purification and charaterization of an RNA-dependent RNA polymerase in healthy tobacco leaves. Virology **92**, 180–189.

EFRON, D., and MARCUS, A. (1973). Translation of TMV-RNA in cell-free wheat embryo system. Virology **53**, 343–348.

FLANEGAN, J. B., and BALTIMORE, D. (1979). Poliovirus poly(U) polymerase and RNA replicase have the same viral polypeptide. J. Virol. **29**, 352–360.

FOWLER, A. V., and ZABIN, I. (1977). The amino acid sequence of β-galactosidase of *Escherichia coli*. Proc. Nat. Acad. Sci. USA **74**, 1507–1510.

FRAENKEL-CONRAT, H. (1956). The role of the nucleic acid in the reconstitution of active tobacco mosaic virus. J. Am. Chem. Soc. **78**, 882–883.

FRAENKEL-CONRAT, H. (1976). RNA polymerase from tobacco necrosis virus infected and uninfected tobacco. Purification of the membrane-associated enzyme. Virology **72**, 23–32.

FRAENKEL-CONRAT, H., SALVATO, M., and HIRTH, L. (1977). The translation of large plant viral RNAs. In: Comprehensive Virology. (H. Fraenkel-Conrat and R. R. Wagner, eds). Plenum Press, New York and London, Vol. **11**, pp. 201–235.

FRITSCH, C., MAYO, M. A., and MURANT, A. F. (1978). Translation of the satellite RNA of tomato black ring virus *in vitro* and in tobacco protoplasts. J. gen. Virol. **40**, 587–593.

FUNATSU, G., and FRAENKEL-CONRAT, H. (1964). Location of amino acid exchanges in chemically evoked mutants of tobacco mosaic virus. Biochemistry **3**, 1356–1362.

GIANINAZZI, S., KASSANIS, B., PRATT, H. M., SHEWRY, P. R., and MIFLIN, B. J. (1977). Partial purification and preliminary characterization of soluble leaf proteins, specific to virus-infected tobacco plants. J. gen. Virol. **34**, 345–351.

GIERER, A., and SCHRAMM, G. (1956). Infectivity of Ribonucleic Acid from Tobacco Mosaic Virus. Nature **177**, 702–703.

GIERER, A. (1958). Die Grosse der biologisch activen Einheit der Ribosenucleinsäure des Tabaksmosaikvirus. Z. Naturforsch. **13**, 485–488.

GREGORY, D. W., and COCKING, E. C. (1963). The use of polygalacturonase for the isolation of plant protoplasts and vacuoles. Biochem. J. **88**, 408.

GREGORY, D. W., and COCKING, E. C. (1965). The large-scale isolation of plant protoplasts. J. Cell Biology **24**, 143–146.

GUILLEY, H., STUSSI, C., and HIRTH, L. (1971). Influence de la phosphodiestérose de rate de porc sur la reconstitution *in vitro* du virus de la Mosaïque du Tobac. C.R. Acad. Sci., Ser. D. **272**, 1181–1184.

GUILLEY, H., JONARD, G., RICHARDS, K. E., and HIRTH, L. (1975). Sequence of a specifically encapsidated RNA fragment originating from the tobacco-mosaic-virus coat-protein cistron. Eur. J. Biochem. **54**, 135–144.

GUILLEY, H., JONARD, G., RICHARDS, K. E., and HIRTH, L. (1975). Observations concerning the sequence of two additional specifically encapsidated RNA fragments originated from the tobacco-mosaic-virus coat-protein cistron. Eur. J. Biochem. **54**, 145–153.

GUILLEY, H., JONARD, G., and HIRTH, L. (1975). Sequence of 71 nucleotides at the 3'-end of tobacco mosaic virus RNA. Proc. Nat. Acad. Sci. USA **72**, 864–868.

GUILLEY, H., JONARD, G., KUKLA, B., and RICHARDS, K. E. (1979). Sequence of 1000 nucleotides at the 3'-end of tobacco mosaic virus RNA. Nucleic Acids Research **6**, 1287–1308.

HARRISON, B. D., KUBO, S., ROBINSON, D. J., and HUTCHESON, A. M. (1976). The multiplication cycle of tobacco rattle virus in tobacco mesophyll protoplasts. J. gen. Virol. , 237–248.

HASHIMOTO, J., and OKAMOTO, K. (1975). Partially stripped tobacco mosaic virus-stimulated methionyl-tRNA binding to ribosomes in an in vitro wheat embryo system. Virology **67**, 107–113.

HENNIG, B., and WITTMANN, H. G. (1972). Tobacco mosaic virus. Mutants and strains. In: Principles and techniques in plant virology. (C. I. Kado and H. O. Agrawal, eds.). Van Nostrand Reinhold, New York, pp. 546–672.

HEBERT, T. T. (1963). Precipitation of plant viruses by polyethylene glycol. Phytopathology **53**, 362.

HIBI, T., and YORA, K. (1972). Electron microscopy of tobacco mosaic virus infection in tobacco mesophyll protoplasts. Ann. Phytopathol. Soc. Jap. **38**, 350–356.

HIBI, T., REZELMAN, G., and van KAMMEN, A. (1975). Infection of cowpea mesophyll protoplasts with cowpea mosaic virus. Virology **64**, 308–318.

HIGGINS, T. J. V., GOODWIN, P. B., and WHITFELD, P. R. (1976). Occurrence of short particles in beans infected with the cowpea strain of TMV. II. Evidence that short particles contain the cistron for coat-protein. Virology **71**, 486–497.

HUNTER, T. R., HUNT, T., KNOWLAND, J., and ZIMMERN, D. (1976). Messenger RNA for the coat protein of tobacco mosaic virus. Nature **260**: 759–764.

HUBER, R., REZELMAN, G., HIBI, T., and van KAMMEN, A. (1977). Cowpea mosaic virus infection of protoplasts from Samsun tobacco leaves. J. gen. Virol. **34**, 315–323.

IKEGAMI, M. and FRAENKEL-CONRAT, H. (1978). The RNA-dependent RNA polymerase of cowpea. FEBS Letters **96**, 197–200.

IKEGAMI, M., and FRAENKEL-CONRAT, H. (1978a). RNA-dependent RNA polymerase of tobacco plants. Proc. Natl. Acad. Sci. USA **75**, 2122–2124.

JACKSON, A. O., MITCHELL, D. M., and SIEGEL, A. (1971). Replication of tobacco mosaic virus. I. Isolation and characterization of double-stranded forms of ribonucleic acid. Virology **45**, 182–191.

JACKSON, A. O., ZAITLIN, M., SIEGEL, A., and FRANCKI, R. I. B. (1972). Replication of tobacco mosaic virus. III. Viral RNA metabolism in separated leaf cells. Virology **48**, 655–665.

JAMJOOM, G. A., NASO, R. B., and ARLINGHOUS, R. B. (1977). Further characterization of intracellular precursor polyproteins of rauscher leukemia virus. Virology **7**, 11–34.

JASPERS, E. M. J. (1974). Plant viruses with a multipartite genome. Adv. Virus Res. **19**, 37–149.

JOCKUSCH, H. (1966). Temperatursensitive Mutanten des Tabakmosaik. I. *In vivo*-verhalten. Z. Vererbungsl. **98**, 320–343.

JONARD, G., RICHARDS, K. E., GUILLEY, H., and HIRTH, L. (1977). Sequence from the assembly nucleation region of TMV RNA. Cell **11**, 483–493.

JONARD, G., RICHARDS, K., MOHIER, E., and GERLINGER, P. (1978). Nucleotide sequence at the 5'-extremity of tobacco-mosaic-virus RNA. 2. The coding region. Eur. J. Biochem. **84**, 521–531.

KADO, C. I., and KNIGHT, C. A. (1966). Location of a local lesion gene in tobacco mosaic virus RNA. Proc. Nat. Acad. Sci. USA **55**, 1276–1283.

KADO, C. I., and KNIGHT, C. A.. (1968). The coat protein gene of tobacco mosaic virus. I. Location of the gene by mixed infection. J. Mol. Biol. **36**, 15–23.

KAMEN, R. I. (1975). Structure and function of Q_β RNA replicase. In: RNA phages. (N. D. Zinder, ed.). Cold Spring Harbor Laboratory, pp. 203–234.

KASSANIS, B., and WHITE, R. F. (1974). A simplified method of obtaining tobacco protoplasts for infection with tobacco mosaic virus. J. gen. Virol. **24**, 447–452.

KEITH, J., and FRAENKEL-CONRAT, H. (1975). Tobacco mosaic virus RNA carries 5'-terminal triphosphorylated guanosine blocked by 5'-linked 7-methylguanosine. FEBS Letters **57**, 31–33.

KNIGHT, C. A. (1962). Tobacco mosaic virus. Biochem. Preparations **9**, 132–136.

KNOWLAND, J. (1974). Protein synthesis directed by the RNA from a plant virus in a normal animal cell. Genetics **78**, 383–394.

KNOWLAND, J., HUNTER, T., HUNT, T., and ZIMMERN, D. (1975). Translation of tobacco mosaic virus RNA and translation of the messenger for TMV coat protein. In: *In vitro* transcription and translation of viral genomes. (A. L. Haenni and G. Beaud, eds.) Paris, INSERM **47**, pp. 211–216.

KOIKE, M., HIBI, T., and YORA (1975). Infection of cowpea protoplasts by cucumber mosaic virus. Ann. Phytopathol. Soc. Japan **41**, 243 (Abstract).

KOIKE, M., HIBI, T., and YORA, K. (1976). Infection of cowpea protoplasts by tobacco mosaic virus. Ann. Phytopathol. Soc. Japan. Abstract.

KOZAK, M., and NATHANS, D. (1972). Translation of the genome of a ribonucleic acid bacteriophage. Bacteriol. Rev. **36**, 109–134.

KOZLOFF, L. M. (1960). Biochemistry of viruses. Ann. Rev. Biochemistry **29**, 475–502.

KUBO, S., HARRISON, B. D., ROBINSON, D. J., and MAYO, M. A. (1975). Tobacco rattle virus in tobacco mesophyll protoplasts: infection and virus multiplication. J. gen. Virol. **27**, 293–304.

KUBO, S., HARRISON, B. D., and ROBINSON, D. J. (1975a). Defined conditions for growth of tobacco plants as sources of protoplasts for virus infection. J. gen. Virol. **28**, 255–257.

LAEMMLI, U. K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T_4. Nature **227**, 680–685.

LEROY, C., STRUSSI-GARAUD, C., and HIRTH, L. (1977). RNA-dependent RNA polymerase in uninfected and alfalfa mosaic virus-infected tobacco plants. Virology **82**, 48–62.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. N. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275.

VAN LOON, L. C. (1975). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana Tabacum* var. 'Samsun' and 'Samsun NN'. IV. Similarity of quantitative changes of specific proteins after infection with different viruses and their relationship to acquired resistance. Virology **67**, 566–575.

VAN LOON, L. C. (1976). Specific soluble leaf proteins in virus-infected tobacco plants are not normal constituents. J. gen. Virol. **30**, 375–379.

MAIZEL, J. V. Jr. (1971). Polyacrylamide gel electrophoresis of viral proteins. In: Methods in Virology. (K. Maramorosch and H. Koprowski, eds.). Acad. Press. New York, vol **5**, pp. 179–246.

MARKHAM, R. (1977). Landmarks in plant virology: genesis of concepts. Ann. Rev. Phytopathol. **15**, 17–39.

MARSDEN, H. S., CROMBE, I. K., and SUBAK-SHARPE, J. H. (1976). Control of protein synthesis in herpes virus-infected cells: Analysis of the polypeptides induced by wild type and sixteen temparature-sensitive mutants of HSV strain 17. J. gen. Virol. **31**, 347–372.

MAY, D. S., and KNIGHT, C. A. (1965). Polar stripping of protein subunits from tobacco mosaic virus. Virology **25**, 502–507.

MAYO, M. A., and ROBERTS, I. M. (1978). Inoculation of tobacco protoplasts with tobacco rattle virus: experiments with aggregates formed in inocula. J. gen. Virol. **40**, 19–30.

MEYER, Y. (1974). Isolation and culture of tobacco mesophyll protoplasts using a saline medium. Protoplasma **81**, 363–372.

MEYER, Y., ABEL, W. O. (1975). Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. Planta (Berl.) **125**, 1–13.

MOSCH, W. H. M., HUTTINGA, H., and RAST, A. Th. B. (1973). Some chemical and physical properties of 18 tobacco mosaic isolates from tomato. Neth. J. Pl. Path. **79**, 104–111.

MOTOYOSHI, F., and OSHIMA, N. (1968). Multiplication of tobacco mosaic virus in suspension culture of tobacco callus. Japan. J. Microbiol. **12**, 317–320.

MOTOYOSHI, F., BANCROFT, J. B., WATTS, J. W., and BURGESS, J. (1973). The infection of tobacco protoplasts with cowpea chlorotic mottle virus and its RNA. J. gen. Virol. **20**, 177–193.

MOTOYOSHI, F., BANCROFT, J. B., and WATTS, J. W. (1974). The infection of tobacco protoplasts with a variant of brome mosaic virus. J. gen. Virol. **25**, 31–36.

MOTOYOSHI, F., and HULL, R. (1974). The infection of tobacco protoplasts with pea enation mosaic virus. J. gen. Virol. **24**, 89–99.

MOTOYOSHI, F., WATTS, J. W., and BANCROFT, J. B. (1975). Factors influencing the infection of tobacco protoplasts by cowpea chlorotic mottle virus. J. gen. Virol. **25**, 245–256.

MOTOYOSHI, F., HULL, R., and FLACK, I. H. (1975). Infection of tobacco mesophyll protoplasts by alfalfa mosaic virus. J. gen. Virol. **27**, 263–266.

MURAKISHI, H. H., HARTMANN, J. X., PELCHER, L. E., and BEACHY, R. N. (1970). Improved inoculation of cultured plant cells resulting in high virus titer and crystal formation. Virology **41**, 365–367.

MURAKISHI, H. H., HARTMANN, J. X., BEACHY, R. N., and PELCHER, L. E. (1971). Growth curve and

yield of tobacco mosaic virus in tobacco callus cells. Virology **43**, 62–69.
MURASHIGE, T., and SKOOG, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473–497.
NILSSON-TILLGREN, T., KIELLAND-BRANDT, M. C., and BEKKE, B. (1974). Studies on the biosynthesis of tobacco mosaic virus. VI. On the subcellular localization of double-stranded viral RNA. Mol. Gen. Genet. **128**, 157–169.
ÖBERG, B., and PHILIPSON, L. (1972). Binding of histidine to TMV RNA. Biochem. Biophys. Res. Commun. **48**, 927–932.
OHNO, T., NOZU, Y., and OKADA, Y. (1971). Polar reconstitution of tobacco mosaic virus. Virology **44**, 510–516.
OHNO, T., and OKADA, Y. (1977). Polarity of stripping of tobacco mosaic virus by alkali and sodium dodecyl sulfate. Virology **76**, 429–432.
ONDA, H., TANIGUCHI, T., and MATSUI. (1970). Polar stripping of tobacco mosaic virus by alkali. Virology **42**, 551–553.
OPPERMANN, H., BISHOP, J. M., VARMUS, H. E., and LEVINTOW, L. (1977). A joint product of the genes gag and pol of avian sarcoma virus: A possible precusor of reverse transcriptase. Cell **12**, 993–1005.
OTSUKI, Y., and TAKEBE, I. (1969), Fluorescent antibody staining of tobacco mosaic virus antigen in tobacco mesophyll protoplasts. Virology **38**, 497–499.
OTSUKI, Y., and TAKEBE, I. (1969a). Isolation of intact mesophyll cells and their protoplasts from higher plants. Plant en Cell Physiol. **10**, 917–921.
OTSUKI, Y., TAKEBE, I., HONDA, Y., and MATSUI, C. (1972). Ultrastructure of infection of tobacco mesophyll protoplasts by tobacco mosaic virus. Virology **49**, 188–194.
OTSUKI, Y., and TAKEBE, I. (1973). Infection of tobacco mesophyll protoplasts by cucumber mosaic virus. Virology **52**, 433–438.
OTSUKI, J., TAKEBE, I., HONDA, Y., KAJITA, S., and MATSUI, C. (1974). Infection of tobacco mesophyll protoplasts by potato virus X. J. gen. Virol. **22**, 375–385.
PATERSON, R., and KNIGHT, C. A. (1975). Protein synthesis in tobacco protoplasts infected with tobacco mosaic virus. Virology **64**, 10–22.
PELHAM, H. R. B., and JACKSON, R. J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**, 247–256.
PELHAM, H. R. B. (1978). Leaky UAG termination codon in tobacco mosaic virus RNA. Nature **272**, 469–471.
PERHAM, R. N., and WILSON, T. M. A. (1976). The polarity of stripping of coat protein subunits from the RNA in tobacco mosaic virus under alkaline conditions. FEBS Letters **62**, 11–15.
PHILIPSON, L., ANDERSSON, P., OLSHEVSKY, U., WEINBERG, R., BALTIMORE, D., and GESTELAND, R. (1978). Translation of MuLV and MSV RNA's in nuclease treated reticulocyte extracts: Enhancement of the gag-pol polypeptide with yeast suppressor tRNA. Cell **13**, 189–199.
RALPH, R. K., and WOJCIK, S. J. (1969). Double-stranded tobacco mosaic virus RNA. Virology **37**, 276–282.
RAO, P. V., and HIRUKI, C. (1978). Infection of cowpea mesophyll protoplasts with clover yellow mosaic virus. J. gen. Virol. **38**, 303–311.
REDDI, K. K. (1972). Tobacco mosaic virus with emphasis on the events within the host cell following infection. Adv. Virus. Res. **17**, 51–91.
REES, M. W., and SHORT, M. N. (1975). The amino acid sequence of the cowpea strain of tobacco mosaic virus protein. Biochim. Biophys. Acta **393**, 15–23.
RICHARDS, K. E., GUILLEY, H., JONARD, G., and HIRTH, L. (1974). A specifically encapsidated fragment from the RNA of tobacco mosaic virus: sequence homology with the coat protein cistron. FEBS Letters **43**, 31–32.
RICHARDS, K., GUILLEY, H., JONARD, G., and KEITH, G. (1977). Leader sequence of 71 nucleotides devoid of G in tobacco mosaic virus. Nature **267**, 548–550.
RICHARDS, K., GUILLEY, H., JONARD, G., and HIRTH, L. (1978). Nucleotide sequence at the 5'-extremity of tobacco-mosaic-virus RNA. 1. The noncoding region (Nucleotides 1–68). Eur. J. Biochem. **84**, 513–519.
ROBERTS, B. E., MATHEWS, M. B., and BRUTON, C. J. (1973). Tobacco mosaic virus RNA directs the

synthesis of a coat protein peptide in a cell-free system from wheat. J. Mol. Biol. **88**, 733–741.
ROMAINE, C. P., and ZAITLIN, M. (1978). RNA-dependent RNA polymerase in uninfected and tobacco mosaic virus-infected tobacco leaves: viral-induced stimulation of a host polymerase activity. Virology **86**, 241–253.
ROTTIER, P. J. M., REZELMANN, G., and van KAMMEN, A. (1979). The inhibition of cowpea mosaic virus replication by actinomycin D. Virology **93**, 299–309.
SAKAI, F., and TAKEBE, I. (1970). RNA and protein synthesis in protoplasts isolated from tobacco leaves. Biochim. Biophys. Acta **224**, 531–540.
SAKAI, F., and TAKEBE, I. (1972). A non-coat protein synthesized in tobacco mesophyll protoplasts infected by tobacco mosaic virus. Mol. Gen. Genet. **118**, 93–96.
SAKAI, F., and TAKEBE, I. (1974). Protein synthesis in tobacco mesophyll protoplasts induced by tobacco mosaic virus infection. Virology **62**, 426–433.
SAKAI F., WATTS, J. W., DAWSON, J. R. O., and BANCROFT, J. B. (1977). Synthesis of proteins in tobacco protoplasts infected with cowpea chlorotic mottle virus. J. gen. Virol. **34**, 285–293.
SAKAI, F., DAWSON, J. R. O., and WATTS, J. W. (1979). Synthesis of proteins in tobacco protoplasts infected with brome mosaic virus. J. gen. Virol. **42**, 323–328.
SALOMON, R., SELA, I., SOREQ, H., GIVEON, D., and LITTAUER, U. Z. (1976). Enzymatic acylation of histidine to tobacco mosaic virus RNA. Virology **71**, 74–84.
SANGER, F., COULSON, A. R., FRIEDMANN, T., AIR, G. M., BARRELL, B. G., BROWN, N. L., FIDDES, J. C., HUCHINSON, C. A. III, SLOCOMBE, P. M., and SMITH, M. (1978). The nucleotide sequence of bacteriophage ßX174. J. Mol. Biol. **125**, 225–246.
SARKAR, S. (1977). Use of protoplasts for plant virus studies. In: Methods in Virology. (K. Maramorsch, and H. Koprowski, eds.). Vol. **6**, pp. 435–456.
SAWICKI, D. L., KAÄRIANIN, L., LAMBEK, C., and GOMATOS, P. J. (1978). Mechanism for control of synthesis of semliki forest virus 26 and 42 S RNA. J. Virol. **25**, 19–27.
SCALLA, R., ROMAINE, P., ASSELIN, A., RIGAUD, J., and ZAITLIN, M. (1978). An *in vivo* study of a nonstructural polypeptide synthesized upon TMV infection and its identification with a polypeptide synthesized *in vitro* from TMV RNA. Virology **91**, 182–193.
SCHWARTZ, J. H. (1967). Initiation of protein synthesis under the direction of tobacco mosaic RNA in cell-free extracts of *Escherichia coli*. J. Mol. Biol. **30**, 309–322.
SELA, I., and HAUSCHNER, A. (1975). Isolation and characterization of TMV RNA dependent enzyme from TMV-infected tobacco leaves. Virology **64**, 284–288.
SHAW, D. C., WALKER, J. E., NORTHROP, F. D., BARRELL, B. G., GODSON, G. N., and FIDDES, J. C. (1978). Gene K, a new overlapping gene in bacteriophage G_4. Nature **272**, 510–515.
SHALLA, F., and PETERSON, L. J. (1973). Infection of isolated plant protoplasts with potato virus X. Phytopathol. **63**, 1125–1130.
SIEGEL, A., ZAITLIN, M., and DUDA, C. T. (1973). Replication of tobacco mosaic virus. IV. Further characterization of viral related RNAs Virology **53**, 75–83.
SIEGEL, A., HARI, V., MONTGOMERY, I., and KOLACZ, K. (1976). A messenger RNA for capsid protein isolated from tobacco mosiac virus infected tissue. Virology **73**, 363–371.
SIEGEL, A., HARI, V., and KOLACZ, K. (1978). The effect of tobacco mosaic virus infection on host and virus-specific protein synthesis in protoplasts. Virology **85**, 494–503.
SINGER, B. (1971). Protein synthesis in virus-infected plants. I. The number and nature of TMV-directed proteins detected on polyacrylamide gels. Virology **46**, 247–255.
SINGER, B. (1972). Protein synthesis in virus-infected plants. II. The synthesis and accumulation of TMV and TMV coat protein in subcellular fractions of TMV-infected tobacco. Virology **47**, 397–404.
SINGER, B., and CONDIT, C. (1974). Protein synthesis in virus-infected plants. III. Effect of tobacco mosaic virus mutants on protein synthesis in *Nicotiana Tabacum*. Virology **57**, 42–48.
SKOTNICKI, A., GIBBS, A., and SHAW, D. C. (1976). *In vitro* translation of polyribosome-associated RNAs from tobamovirus-infected plants. Intervirology **7**, 256.
SKOTNICKI, A., GIBBS, A., and SHAW, D. C. (1976). Mixed infection with two tobamoviruses: the formation of particles containing the coat protein messenger RNAs of either virus. Intervirology **7**, 328–337.
SPENDLOVE, R. S. (1967). Microscopic techniques. Preparation of antiserum labeled with fluorescein

isothiocynate by method of optimal proportions. In: Methods of Virology. (K. Maramorosch and H. Koprowski, eds.). Vol. 3, pp. 475–520.

STUSSI-GARAUD, C., LEMIUS, J., and FRAENKEL-CONRAT, H. (1977). RNA polymerase from tobacco necrosis virus-infected and uninfected tobacco. II. Properties of the bound and soluble polymerases and nature of their products. Virology **81**, 224–236.

SUGIMURA, Y., and USHIYAMA, R. (1975). Cucumber green mottle mosaic virus infection and its bearing on cytological alternations in tobacco mesophyll protoplasts. J. gen. Virol. **29**, 93–98.

TAKEBE, I., OTSUKI, Y., and AOKI, S. (1968). Isolation of tobacco mesophyll cells in intact and active state. Plant and Cell Physiol. **9**, 115–124.

TAKEBE, I., and OTSUKI, Y. (1969). Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. Proc. Nat. Acad. Sci. USA **64**, 843–948.

TAKEBE, I., AOKI, S., and SAKAI, F. (1975). Replication and expression of tobacco mosaic virus genome in isolated tobacco leaf protoplasts. In: Modification of the information content of plant cells. (R. Markham, ed.). North-Holland Publishing Co., Amsterdam, pp. 105–125.

TAKEBE, I. (1975). The use of protoplasts in plant virology. Ann. Rev. Phytopathol. **13**, 105–125.

TAKEBE, I. (1975a). Protoplasts from leaf mesophyll and their infection by plant viruses. Rev. Plant Protec. Res. **8**, 136–150.

TAKANAMI, Y., KUBO, S., and IMAIZUMI, S. (1977). Synthesis of single and double stranded cucumber mosaic virus RNAs in tobacco mesophyll protoplasts. Virology **80**, 376–389.

VANDEWALLE, M. J., and SIEGEL, A. (1976). A study of nucleotide sequence homology between strains of tobacco mosaic virus. Virology **73**, 429–430.

VAZQUEZ, D. (1974). Inhibitors of protein synthesis. FEBS Letters **40** Suppl., 63–84.

VIÑUELA, I., ALGRANATI, I. D., and OCHOA, S. (1967). Synthesis of virus-specific proteins in *Escherichia coli* infected with the RNA bacteriophage MS_2. Eur. J. Biochem. **1**, 3–11.

WATTS, J. W., MOTOYOSHI, F., and KING, J. M. (1974). Problems associated with the production of stable protoplasts of cells of tobacco mesophyll. Ann. Bot. **38**, 667–671.

WEBER, K., and KONIGSBERG, W. (1975). In: RNA phages. (N. D. Zinder, ed.). Cold Spring Harbor Laboratory, pp. 51–84.

WEBER, K. and OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. **244**, 4406–4412.

WHITE, J. L., and MURAKISHI, H. H. (1977). In vitro replication of tobacco mosaic virus RNA in tobacco callus cultures: solubilization of membrane-bound replicase and partial purification. J. Virol. **21**, 484–492.

WHITE, J. L., and DAWSON, W. O. (1978). Characterization of RNA-dependent RNA polymerase in uninfected and cowpea chlorotic mottle virus-infected cowpea leaves. Virology **88**, 33–43.

WHITFELD, P. R., and HIGGINS, T. J. V. (1976). Occurrence of short particles in beans infected with the cowpea strain of TMV. I. Purification and characterization of short particles. Virology **71**, 471–485.

WILSON, T. N. A., PELHAM, R. N., FINCH, J. T., and BUTLER, P. J. G. (1976). Polarity of the RNA in the tobacco mosaic virus particle and the direction of protein stripping in sodium dodecyl sylphate. FEBS Letters **64**, 285–289.

WITTMANN, H. G. (1959). Vergleich der Proteine des Normalstammes und einer Nitritmutante des Tabakmosaikvirus. Z. Vererbungsl. **90**, 463–475.

WITTMANN-LIEBOLD, B., and WITTMANN, H. G. (1965). Lokalisierung von Aminosäureaustauschen bei Nitritmutanten des Tabakmosaikvirus. Z. Vererbungslehre **97**, 305–326.

WITTMANN, H. G., and WITTMANN-LIEBOLD, B. (1966). Protein chemical studies of the RNA viruses and their mutants. Cold Spring Harbor Symp. Quart. Biol. **31**, 163–172.

ZABEL, P., WEENER-SWAANS, H., and van KAMMEN, A. (1974). In vitro replication of cowpea mosaic virus RNA. I. Isolation and properties of the membrane-bound replicase. J. Virol. **14**, 1049–1055.

ZABEL, P., JONGEN-NEVEN, I., and van KAMMEN, A. (1976). In vitro replication of cowpea mosaic virus RNA. II. Solubilization of membrane-bound replicase and the partial purification of the solubilized enzyme. J. Virol. **17**, 679–685.

ZABEL, P., JONGEN-NEVEN, I., and van KAMMEN, A. (1979). In vitro replication of cowpea mosaic virus RNA. III. Template recognition by cowpea mosaic virus RNA replicase. J. Virol. **29**, 21–33.

ZABEL, P. (1978). Purification and properties of cowpea mosaic virus RNA replicase. Ph. D. Thesis,

Agricultural University, Wageningen, The Netherlands.
ZAITLIN, M. (1959). Isolation of tobacco leaf cells capable of supporting virus multiplication. Nature **184**, 1002–1003.
ZAITLIN, M., and HARIHARASUBRAMANIAN, V. (1970). Proteins in tobacco mosaic virus-infected plants. Biochem. Biophys. Res. Commun. **39**, 1031–1036.
ZAITLIN, M., and HARIHARASUBRAMANIAN, V. (1972). A gel electrophoretic analysis of proteins from plants infected with tobacco mosaic and potato spindle tuber viruses. Virology, **47**, 296–305.
ZAITLIN, M., DUDA, C. T., and PETTI, M. A. (1973). Replication of tobacco mosaic virus. V. Properties of the bound and solubilized replicase. Virology **53**, 300–311.
ZAITLIN, M., and BEACHY, R. R. (1974). The use of protoplasts and separated cells in plant virus research. Adv. Vir. Res. **10**, 1–35.
ZAITLIN, M., and ISRAEL, H. W. (1975). Tobacco mosaic virus (type strain). C.M.I./A.A.B. Descriptions of plant viruses. No. 151.
ZAITLIN, M., BEACHY, R. N., BRUENING, G., ROMAINE, C. P., and SCALLA, R. (1976). Translation of tobacco mosaic virus RNA. In: Animal Virology. (D. Baltimore, A. S. Huand, and C. F. Fox, eds.). ICN-UCLA Symposia on Molecular and Cellular Biology. Acad. Press, New York, vol. **4**, pp. 567–581.
ZAITLIN, M., BEACHY, R. N., and BRUENING, G. (1977). Lack of molecular hybridization between RNAs of two strains of TMV: a reconsideration of the criteria for strain relationship. Virology **82**, 237–241.
ZIMMERN, D. (1975). The 5′ end group ot tobacco mosaic virus RNA is $m^7G^{5'}$-ppp $^{5'}$Gp. Nucleic Acids Research **3**, 1189–1203.
ZIMMERN, D., and BUTLER, P. J. G. (1977). The isolation of tobacco mosaic virus RNA. Fragments containing the origin for viral assembly. Cell **11**, 455–462.
ZIMMERN, D. (1977). The nucleotide sequence at the origin for assembly on tobacco mosaic virus RNA. Cell **11**, 463–482.

ABBREVIATIONS

A_{280}	optical density at 280 nm
AMV	alfalfa mosaic virus
ATP	adenosine-5′-triphospate
BMV	brome mosaic virus
CCMV	cowpea chlorotic mottle virus
CGMV	cucumber green mottle mosaic virus
Ci	Curie
CMV	cucumber mosaic virus
cpm	counts per minute
CPMV	cowpea mosaic virus
C-TMV	cowpea strain of tobacco mosaic virus
CTP	cytidine-5′-triphosphate
dpm	desintegrations per minute
DTE	dithioerythritol
E_{260}	optical density at 260 nm
EDTA	ethylenediaminetetraacetate
FITC	fluorescein isothiocyanate
g	acceleration of gravity
GTP	guanosine-5′-triphosphate
LMC	low molecular weight component
ME	mercaptoethanol
mmol	millimolar
mRNA	messenger RNA
M.W.	molecular weight(s)
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PEMV	pea enation mosaic virus
PMSF	phenyl methyl sulphonyl fluoride
poly U	polyribouridylic acid
PVX	potatovirus X
RF	replicative form
RI	replicative intermediate
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TMV	tobacco mosaic virus
Tris	tris(hydroxyl)aminomethane
TRV	tobacco rattle virus
tRNA	transfer ribonucleic acid
U.V.	ultraviolet
UTP	uridine-5′-triphosphate
V	volt