VIRAL PROTEIN SYNTHESIS IN COWPEA MOSAIC VIRUS INFECTED PROTOPLASTS

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(with a summary in Dutch)

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 21 maart 1980
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

H. VEENMAN EN ZONEN B.V. - WAGENINGEN - 1980

STELLINGEN

1.

De theorie, dat meer-komponentenvirussen tijdens de evolutie zijn ontstaan door coïnfektie van twee of meer, oorspronkelijk onafhankelijk replicerende virussen, wordt ontkracht door de recentelijk aangetoonde overeenkomsten in primaire struktuur tussen de verschillende genoomdelen van o.a. alfalfa mozaïekvirus en cowpea mozaïekvirus.

L. Reijnders, Adv. Virus Res. **23**, 79–102, 1978.
E. C. Koper-Zwarthoff, F. Th. Brederode, P. Walstra en J. Bol, Nucleic Acids Res. **7**, 1887–1900, 1979.
J. W. Davies, J. Stanley en A. van Kammen, Nucleic Acids Res. **7**, 493–500, 1979.

2.

De experimenten van Gaard en De Zoeten over de initiatie van de infektie door tabaksratelvirus rechtvaardigen niet de konklusie, dat extracellulaire dissociatie van virus een integraal onderdeel van de infektiecyclus uitmaakt.

G. Gaard en G. A. de Zoeten, Virology **96**, 21–31, 1979.

3.

Het is niet bewezen, dat het vrijmaken van infektieuze virusdeeltjes uit kernpolyeders *in vitro* met darmsap een verbetering is ten opzichte van de methode met alkali.

L. E. Volkman en M. D. Summers, J. Invertebr. Pathol. **30**, 102–103, 1977.
P. V. Vail, C. L. Romine en J. L. Vaughn, J. Invertebr. Pathol. **33**, 328–330, 1979.

4.

De aanwezigheid van een eiwit, covalent gebonden aan het 5′-uiteinde sommige virale genomen, maakt een nadere definiëring van het begrip struktureel eiwit bij virussen wenselijk.

5.

Overeenkomst in klinische symptomen tussen Adema disease (lethaalgen A46) bij runderen en acrodermatitis enteropathica bij de mens impliceert nog geen overeenkomstige moleculaire en/of cellulaire achtergrond.

K. Weismann en T. Flagstad, Acta Dermatovener (Stockholm) **56**, 151–154, 1976.

6.

Ambros, Pettersson en Baltimore leveren niet het experimentele bewijs voor hun konklusie, dat tijdens poliovirusinfektie afsplitsing van VPg een stap is in het maturatieproces van poliovirus boodschapper-RNA.

V. AMBROS, R. F. PETTERSSON en D. BALTIMORE, Cell **15**, 1439–1446, 1978.
R. F. PETTERSSON, J. B. FLANEGAN, J. K. ROSE en D. BALTIMORE, Nature **268**, 270–272, 1977.

7.

Oppositie tegen de verplichte, niet op het proefschrift betrekking hebbende stellingen bij academische promoties getuigt van een geringschatting van het proefschrift en is derhalve beledigend voor de promovendus.

8.

Wie het traditionele huwelijk nog als hoeksteen van de samenleving wil beschouwen zal toch moeten erkennen, dat het bouwwerk in toenemende mate verkrottingsverschijnselen vertoont. Renovatie in de oude staat is niet wenselijk.

PETER ROTTIER
Wageningen, 21 maart 1980

VOORWOORD

Dit proefschrift heb ik kunnen maken mede dank zij de vele mensen, die er op één of andere manier aan hebben meegewerkt. Hen allen wil ik daarvoor oprecht bedanken, waarbij ik enkelen met name wil noemen.

AB VAN KAMMEN, onder wiens supervisie het proefschrift tot stand kwam. In de wetenschap steeds met mijn problemen bij hem terecht te kunnen was ik blij met de grote mate van vrijheid, die hij mij bij het onderzoek liet. Van zijn inspirerende begeleiding bij het schrijven van het proefschrift heb ik veel geleerd. Zijn rustgevende, maar waakzame houding en zijn persoonlijke belangstelling stonden garant voor een plezierige samenwerking.

GEERTJE REZELMAN heeft een belangrijk aandeel gehad in de praktische werkzaamheden. Aan haar kritische betrokkenheid en buitengewoon experimenteel talent heb ik veel te danken.

AB HOOGEVEEN verzorgde het tekenwerk en de fotografie. Daarnaast stelde hij zijn ruime ervaring en vindingrijkheid beschikbaar voor het oplossen van alle mogelijke technische problemen. Zijn vriendelijke bereidheid en goede humeur, vaak onder voor hem moeilijke omstandigheden, heb ik zeer gewaardeerd.

ENGELIEN HEIJERMAN was in de eerste fase van het onderzoek belast met de zorg voor de planten en betrokken bij het optimaliseren van de condities voor de isolatie en infectie van de protoplasten.

De heren T. S. IE en J. GROENEWEGEN van het laboratorium voor virologie maakten de electronenmicroscopische opnamen van de protoplasten.

LIA PIEPER, JAN SCHOUTEN, ROEL DE VRIES en ANDRIES UDINK waren als student op meer of minder direkte wijze bij het onderzoek betrokken.

JEFFREY DAVIES en JOHN STANLEY corrigeerden de engelse tekst.

MARIANNE WIJERMARS verzorgde de omslag.

Waar een proefschrift ook kan worden gezien als de afsluiting van een wetenschappelijke opleiding, wil ik deze plaats ook gebruiken om mijn ouders te bedanken voor de stimulans en steun, die zij mij gedurende deze lange periode op velerlei manieren, ieder op eigen wijze, hebben gegeven.

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1. SCOPE OF THE INVESTIGATIONS

In 1968 a method was developed by TAKEBE and coworkers for the isolation of protoplasts from tobacco leaves (TAKEBE *et al.*, 1968). It was shown, that these protoplasts could be infected with virus and that the virus actively multiplied in a synchronous process (TAKEBE and OTSUKI, 1969). The availability of such protoplasts in principle should provide a means to study the molecular processes of plant virus replication. Such studies have been hampered by the low level of primary infections that can be achieved with plants or plant tissues as a result of the presence of a cell wall and by the asynchrony of the subsequent infection resulting from secondary spread of virus.

Protoplasts seemed a useful tool for the examination of problems concerning the replication of cowpea mosaic virus (CPMV). CPMV is a multicomponent virus whose RNA genome is divided between two nucleoprotein particles both of which are essential for establishing an infection (VAN KAMMEN, 1968). It is not yet known which functions are specified by the two genome parts and how they mutually interact with each other. Infection with CPMV gives rise to the appearance of characteristic cytopathic structures (DE ZOETEN *et al.*, 1974; LANGENBERG and SCHROEDER, 1975; HIBI *et al.*, 1975). These membranous structures play an essential role in CPMV replication since they contain the virus-specific double-stranded RNAs (DE ZOETEN *et al.*, 1974) and the viral replicase activity (ZABEL *et al.*, 1974 and 1976). It seems important to know how these structures are induced by the virus, how the host cell is involved, and to specify their exact functioning. Such studies might provide general information on the specific contributions of the host cell to the multiplication of plant viruses.

The aim of the work presented in this thesis was to study some aspects of CPMV multiplication in cowpea mesophyll protoplasts. Attention was focussed mainly on the detection and characterization of proteins whose synthesis is induced or is stimulated upon virus infection. Furthermore, a possible role of the host genome was investigated and attempts were made to localize functions on the viral RNA components.

A literature review on the biochemistry of plant virus multiplication in protoplasts is included in chapter 2. In addition, this chapter contains an introduction to the relevant properties of CPMV and of plant protoplasts.

The cowpea mesophyll protoplast system developed by HIBI *et al.* (1975) was adopted for the present study after several factors had been re-evaluated. A survey of the conditions that are important for the preparation of protoplasts and for their infection with CPMV is given in chapter 3. A very convenient procedure was evolved resulting in synchronous virus multiplication to high yields in the large majority of the protoplasts.

Before studying the virus-induced changes in the metabolism of infected

protoplasts, RNA and protein synthesis in healthy protoplasts were examined (chapter 4). Increasing metabolic activities were observed upon incubation of freshly prepared protoplasts and these processes could be selectively influenced by means of appropriate antibiotics. RNA and protein synthesis in the chloroplasts lasts appeared to be at a low level but their energy-generating activity was still functioning. In addition, these experiments provided practical information as to the conditions favourable for radioactive labeling.

In chapter 5, it is shown by using the antibiotic actinomycin D that CPMV replication is dependent on host genome transcription but only during the earliest stages of infection. Under conditions of complete inhibition of CPMV multiplication, synthesis of viral antigen in the form of top component still continued. Besides these coat proteins, the synthesis of other CPMV-specific proteins was observed.

In chapter 6, the detection of a number of viral-related proteins in CPMV-infected protoplasts is described. Radioactive labeling for short periods at different times during the infection cycle and subcellular fractionation of the protoplasts were found to be essential for the detection of some of them. Only two proteins appeared at the onset of progeny virus accumulation, all others were observed only in later stages of infection. Surprisingly, no viral-related proteins were observed during the actinomycin D-sensitive period, the latent phase of infection.

Our efforts in characterizing the viral-related proteins are described in chapter 7. The identification of the CPMV coat proteins and the comparison of the proteins with the translation products of B- and M-RNA synthesized in *in vitro* protein synthesizing systems are described. Although chase experiments revealed the processing of the smaller viral coat protein, no indications were obtained as to the possible existence of other precursor-product relations. Except for the viral coat proteins all viral-related proteins were produced in protoplasts infected with purified bottom component while middle component was ineffective, suggesting that the replicase function is located on B-RNA and that M-RNA may specify at least one of the coat proteins.

In chapter 8 general conclusions are drawn and a tentative view on the course of the CPMV replication process based on presently available data is presented.

A part of the results described in this thesis has already been published (ROTTIER, 1976 and 1978; ROTTIER *et al.*, 1979). Chapter 4 will be submitted for publication with A. VAN KAMMEN as co-author, chapters 6 and 7 with G. REZELMAN and A. VAN KAMMEN as co-authors.

2. INTRODUCTION

2.1. COWPEA MOSAIC VIRUS

With only a few exceptions all plant viruses have single-stranded RNA genomes. Many of these viruses are multicomponent viruses. Their genomic RNA is split into parts which are encapsidated into at least two viral particles, so far a unique feature of plant viruses (for review, see JASPARS, 1974). On the basis of genome constitution, morphology and infectivity properties some 10 different types or groups of multicomponent viruses have been recognized.

Cowpea mosaic virus (CPMV) is the type member of the comovirus group. For a detailed account of the properties of these viruses the reader is referred to the recent review of BRUENING (1977). Purified preparations of CPMV contain three types of particles, which have sedimentation values in sucrose density gradients of 115S, 95S and 58S, and which have therefore been designated bottom (B), middle (M) and top (T) component, respectively (VAN KAMMEN, 1967). Top component particles are empty capsids, the other two components each contain one RNA molecule with molecular weight 2.02×10^6 (B-RNA) and 1.37×10^6 (M-RNA), respectively (REIJNDERS *et al.*, 1974). These RNAs both have a poly(A) sequence at their 3′-end (EL MANNA and BRUENING, 1973; STEELE and FRIST, 1978) and a small protein at their 5′-terminus (STANLEY *et al.*, 1978; DAUBERT *et al.*, 1978). The icosahedral capsids of all components of CPMV are composed of two proteins of molecular weight about 44,000 and 22,000 (WU and BRUENING, 1971; GEELEN *et al.*, 1972) present in equal amounts of 60 molecules per particle.

Besides centrifugal heterogeneity, purified CPMV also exhibits electrophoretic heterogeneity. Each centrifugal component appears to resolve into two components upon electrophoresis in polyacrylamide gels (AGRAWAL, 1964; SEMANCIK, 1966), a slow migrating form present notably early in infection, which is converted into a fast form in the course of the infection process (NIBLETT and SEMANCIK, 1969). This conversion results from the specific cleavage of an oligopeptide of about 2,500 daltons from the small viral coat protein (GEELEN *et al.*, 1972).

Both virions or RNAs are necessary for CPMV infection (VAN KAMMEN, 1968) indicating that multiplication is dependent on the combined expression of the two genome pieces. This was confirmed by genetic experiments which demonstrated that the viral RNAs specify different functions (for reviews, see BRUENING, 1977, and DE JAGER, 1978) and is also in agreement with the absence of significant sequence homology between M- and B-RNA (VAN KAMMEN and REZELMAN, 1971).

The use of mutants or hybrids of comoviruses provided interesting information concerning the correlation between different characters of the viruses and the viral component by which these are specified. For instance, the regulation of the synthesis of top component seemed to be directed by middle

component (BRUENING, 1969; DE JAGER and VAN KAMMEN, 1970), conversion of slow to fast electrophoretic form by bottom component (SILER *et al.*, 1976), while symptom formation appeared to be governed by the expression of functions localized on each component (DE JAGER, 1978). This genetic work did not lead to the identification of the biochemical nature of such functions except in one case: from the combined results with CPMV (WOOD, 1972; GOPO and FRIST, 1977; THONGMEEARKOM and GOODMAN, 1978) and with the related viruses BPMV (MOORE and SCOTT, 1971) and RaMV (KASSANIS *et al.*, 1973) it may be concluded that the smaller viral coat protein is coded for by the RNA of middle component.

The RNAs of CPMV can be translated in *in vitro* protein synthesizing systems derived from wheat germ as well as from rabbit reticulocytes treated with micrococcal nuclease (PELHAM and JACKSON, 1976; PELHAM and STUIK, 1977; DAVIES *et al.*, 1977; STUIK, 1979). Proteins of large and intermediate size were found to be synthesized but no translation products the size of the viral coat proteins were produced. Moreover, no functional properties could be assigned to any of these products. Very recently, however, a polypeptide of about 30,000 daltons made in the reticulocyte system under the direction of B-RNA was observed (PELHAM, 1979) which was claimed to specifically cleave the M-RNA *in vitro* translation products (PELHAM, 1979), but the significance of this finding remains obscure since it could not be reproduced using wheat germ extracts or reticulocytes (R. GOLDBACH, pers. commun.).

CPMV-infection gives rise to the appearance of membranous, often vesiculated structures within the cytoplasm of infected cells (DE ZOETEN *et al.*, 1974; LANGENBERG and SCHROEDER, 1975; HIBI *et al.*, 1975; HUBER *et al.*, 1977). Biochemical analysis and autoradiography demonstrated that CPMV-specific replicating RNA is associated with these very structures (ASSINK *et al.*, 1973; DE ZOETEN *et al.*, 1974). Furthermore, an RNA-dependent RNA polymerase activity was detected in association with membranes after infection with CPMV (ZABEL *et al.*, 1974 and 1976). Thus, a crucial role for the virus-specific cytopathic structures seems to be evident. Attempts to identify the replicase activity by purification and polyacrylamide gel electrophoresis were, however, unsuccessful (ZABEL, 1978). Similarly, analysis of subcellular fractions enriched in cytopathic structures did not reveal the identification of virus-specific proteins clearly involved in the replication process.

In summary, CPMV is well-characterized from a physico-chemical point of view but little is known about its multiplication process *in vivo*.

2.2. THE SIGNIFICANCE OF PLANT PROTOPLASTS IN VIRUS RESEARCH

Plant protoplasts provide an experimental system which offers a good opportunity for studying fundamental processes underlying virus multiplication. In addition, they may be useful for investigating a number of specific virological problems. The ability of one virus to affect the infection by a second virus (strain) is a well-known phenomenon in plants in nature (see MATTHEWS, 1970), the

effects ranging from enhancement of multiplication of one virus by the other to protection against infection by the other. Protoplasts were shown to allow double infection with different viruses (OTSUKI and TAKEBE, 1976a; BARKER and HARRISON, 1977a, b) or strains of viruses (OTSUKI and TAKEBE, 1976b, c; BARKER and HARRISON, 1978). Thus, such interactions can be studied at the cellular level. The reduced replication of CMV in tobacco protoplasts doubly infected with TMV and CMV (OTSUKI and TAKEBE, 1976a) and the interference between strains of RRV (BARKER and HARRISON, 1978) and TMV (OTSUKI and TAKEBE, 1976c) upon successive inoculation of tobacco protoplasts suggest this to be the case.

Protoplasts may also enable the examination of questions as to susceptibility, resistance and host specificity. The multiplication of several plant viruses has been found to be sensitive to actinomycin D, *e.g.* TMV (COUTTS and WOOD, 1976), TYMV (RENAUDIN and BOVÉ, 1977), and AMV (ALBLAS and BOL, 1977). By using protoplasts this sensitivity was shown to exist only in an early stage of infection suggesting some early host-specific function to be required for the initiation of infection. Such a function might well play a role in determining whether a plant species will be a host for a virus or not.

Concerning the mechanisms underlying genetically controlled resistance, protoplasts may be less valuable. Protoplasts from some tomato lines homozygous for a gene conferring resistance to TMV infection in intact plants were tested by MOTOYOSHI and OSHIMA (1975, 1977) for their susceptibility to this virus. Immunity to infection was observed with protoplasts from only one line of tomato with one TMV strain (MOTOYOSHI and OSHIMA, 1977). It appears that resistance of plants to virus infection may rarely be due to resistance at the cellular level. This was also concluded by BEIER *et al.* (1977) on the basis of an impressive survey of more than a thousand *Vigna sinensis* lines, sixty-five of which were classified as immune to CPMV when inoculated on the primary leaves. From these only one line also expressed this immunity upon inoculation of protoplasts obtained from primary leaves.

Successful multiplication of viroids in plant protoplasts has been reported (MÜHLBACH and SÄNGER, 1977; MÜHLBACH *et al.*, 1977a, b). Protoplasts may also provide a promising system for the investigation of these infectious agents as was emphasized by experiments with inhibitors of RNA biosynthesis which suggested that enzymes preexisting in the host cell play a role in viroid replication (MÜHLBACH and SÄNGER, 1978).

The basic demands to be made upon an experimental system for the study of virus infection at the cellular level are that most of the cells can be infected simultaneously, that a synchronous virus growth curve can be established and that the appropriate biochemical techniques can be applied for the analysis of the processes occurring. Plants or plant tissue cultures (including isolated cells) are not very suitable for these purposes since the presence of a cell wall hinders the attainment of appreciable levels of initial infection while secondary spread of virus prevents the infection from being synchronous in all cells.

TABLE 2.1. Infection of leaf mesophyll protoplasts from various plant species with different plant viruses

plant virus	plant species	reference
AMV	cowpea	ALBLAS and BOL (1977)
	tobacco	MOTOYOSHI *et al.* (1975)
BBWV	broad bean	KAGI *et al.* (1975)
BMV	barley	OKUNO *et al.* (1977)
	maize	FURUSAWA and OKUNO (1978)
	oat	FURUSAWA and OKUNO (1978)
	radish	FURUSAWA and OKUNO (1978)
	wheat	FURUSAWA and OKUNO (1978)
BMV (V5)	tobacco	MOTOYOSHI *et al.* (1974)
CaMV[1]	turnip	HOWELL and HULL (1978)
CCMV	tobacco	MOTOYOSHI *et al.* (1973)
CGMMV	tobacco	SUGIMURA and USHIYAMA (1975)
CMV	cowpea	KOIKE *et al.* (1977)
	tobacco	OTSUKI and TAKEBE (1973)
CPMV	cowpea	HIBI *et al.* (1975)
	tobacco	HUBER *et al.* (1977)
CYMV	cowpea	RAO and HIRUKI (1978)
PEMV	tobacco	MOTOYOSHI and HULL (1974)
PVX	tobacco	OTSUKI *et al.* (1974)
RRSV	tobacco	BARKER and HARRISON (1977a)
TMV	cowpea	KOIKE *et al.* (1976)
	cucumber	COUTTS and WOOD (1976)
	tobacco	OTSUKI *et al.* (1972)
	tomato	MOTOYOSHI and OSHIMA (1975)
TNDV	tobacco	KUBO and TAKANAMI (1979)
TRV	tobacco	KUBO *et al.* (1975)
TRosV	turnip	MORRIS-KRSINICH *et al.* (1979)
TYMV	chinese cabbage	RENAUDIN *et al.* (1975)

1 CaMV is a double-stranded DNA virus

Protoplasts, usually isolated from leaf mesophyll tissue by enzymatic digestion of the cell wall seem to meet all the above requirements (for a recent review see TAKEBE, 1977). High levels of infection have been demonstrated using several different viruses and protoplasts isolated from a large variety of plant species (table 2.1). Since infection can be obtained by a brief contact between protoplasts and virus, usually under well-defined conditions, and since the excess virus inoculum can be removed by washing, one-step growth can be obtained. Furthermore, it has been shown that high yields of progeny virus can be produced indicative of an active synthetic process. The protoplasts being in suspension can easily be sampled by pipetting, substrates and/or inhibitors can be applied, and the medium can be changed rapidly.

Although numerous studies on plant virus infection of protoplasts have already been reported, few of them have dealt with biochemical aspects of virus replication. The large majority focussed on optimalization of conditions for

isolation and infection of the protoplasts. Obviously, this reflects the problems of obtaining reproducibly large numbers (*i.e.* 10^7 or more) of protoplasts, which are viable and readily infectable, and which can withstand incubation in a culture medium for several days without substantial losses of living protoplasts. The experience in our laboratory with two protoplast systems namely tobacco leaf (HUBER, 1979) and cowpea leaf protoplasts (HIBI *et al.*, 1975; this thesis) has indicated that it is possible to overcome these difficulties to some extent by rigorously standardizing the conditions of cultivation of the plants from which the protoplasts are isolated, but even then one has to accept that from time to time the isolations of protoplasts are not successful for apparently unaccountable reasons.

2.3. THE BIOCHEMISTRY OF VIRUS MULTIPLICATION IN LEAF CELL PROTOPLASTS

A variety of biochemical activities is induced in plant cells upon plant virus infection. On the one hand new biosynthetic processes are initiated *i.e.* the production of virus particles; on the other the normal cellular metabolism as reflected by the intracellular structure may be greatly affected: new host-specific functions may be evoked and the normal functioning of the cell may be disturbed. The pattern of all these changes appears to be virus-specific since it results from the introduction and expression of a well defined, unique piece of information, present in the genome of the virus. Rapid multiplication of virus occurs in infected protoplasts. Cytopathic changes in cellular structure similar to those found in cells of infected leaf tissue take place in these protoplasts. The fundamental processes underlying virus multiplication may therefore be studied using plant protoplasts. Certain biochemical reactions, which are often observed in leaf tissue upon virus infection (*e.g.* increases in different enzyme activities such as polyphenoloxidase, peroxidase), and which are related to the development of symptoms of disease in the whole plant, have not been found in virus-infected protoplasts. Also, a reaction like cell necrosis, which is often seen upon infection of a leaf or a whole plant, has not been observed in virus-infected protoplasts. Apparently such reactions due to plant virus infections are specific for infected tissue and protoplasts are not suitable for unravelling the biochemical mechanisms underlying these reactions.

This section reviews some results of recent studies on virus replication which have been obtained with protoplasts. Viral-related RNA and protein synthesis have been considered separately.

2.3.1. *Virus-specific RNA synthesis*

Analyses of viral RNA replication in virus-infected plants have demonstrated that plant viruses basically use the same mechanism of replication as the small animal and bacterial single-stranded RNA viruses. The viral RNA contained in the virus particles is released in the cell from its protein coat and then transcribed

into a complementary RNA strand, which in turn serves as a template for the synthesis of viral progeny RNA strands.

There are a number of specific problems in studying plant viral RNA synthesis, for which protoplasts might provide an attractive experimental system. Two examples may be given:

1. Many plant viruses have multipartite RNA genomes. Nothing is known about the coordination of the replication of the separate RNA molecules.
2. Recently it has been found with a number of plant viruses that messenger RNAs for virus coat proteins are formed during the infection. These messengers represent a specific part of the genomic viral RNA and are therefore described as subgenomic messengers. Such subgenomic messengers occur among multicomponent viruses like the bromoviruses and AMV as well as among single component viruses such as TMV and TYMV. The origin of subgenomic messengers in the virus multiplication process could be examined in protoplasts, in which virus multiplication proceeds synchronously.

So far, the replication of the RNA has been investigated in some detail only for TMV, a few bromoviruses and CMV.

2.3.1.1. Tobacco mosaic virus

Viral RNA in TMV-infected tobacco protoplasts was studied by AOKI and TAKEBE (1975). Protoplasts were allowed to incorporate ^{32}P-phosphate in the presence of actinomycin D for various times after inoculation and then RNA was extracted and analyzed by polyacrylamide gel electrophoresis. Three RNA species were detected in extracts of infected protoplasts. These RNAs were absent in the control protoplasts mock-infected with UV-inactivated virus. They were identified as newly synthesized TMV RNA and the replicative form (RF) and the replicative intermediate (RI) of TMV RNA. All three types of RNAs were already present in substantial amounts 4 h after inoculation (AOKI and TAKEBE, 1975). Their synthesis increased exponentially during the subsequent hours but at about 10 h after inoculation the rate of synthesis of TMV RNA and its RI dropped sharply, whereas the synthesis of RF ceased completely. AOKI and TAKEBE (1975) did not detect the small TMV-specific RNA, usually described as low molecular weight component RNA (LMC, mol. weight 265,000) which has been shown to be the messenger RNA for TMV coat protein (HUNTER *et al.*, 1976). The LMC nucleotide sequence is identical to that of the 3′-end part of the TMV RNA. Very recently, however, LMC has been found by the group of SIEGEL (SIEGEL *et al.*, 1978b) in TMV-infected protoplasts at a late stage of infection, when the virus synthesis was already at a relatively low rate. They were able to detect the LMC by labeling with ^{3}H-uridine for short periods. Apparently, only under such conditions was the incorporation of radioactive label into LMC sufficient to distinguish it as a separate component above the background.

Newly synthesized virus particles have been found in TMV-infected protoplasts 4 h later than the first detectable viral RNA synthesis (AOKI and TAKEBE, 1975). The rates of synthesis of viral RNA and virus nucleoprotein particles are then similar indicating that during the initial phase a significant amount of TMV

RNA molecules occurs either in a free form or only partially coated by virus proteins. But at the time that the rate of viral RNA synthesis started to decrease, production of virus particles occurred at a similar rate to RNA synthesis. These results suggest that virus coat protein synthesis starts later than viral RNA synthesis. Presumably, messenger RNA for coat protein must first be generated. There is, however, a large discrepancy between the time LMC is first detectable in protoplasts and the synthesis of the first virus particles. LMC is found only in the electrophoretic pattern at a late stage of infection (SIEGEL *et al.*, 1978b). Apparently, a more sensitive method is needed for the detection of LMC to understand its origin in the virus multiplication process and to examine the mechanism of its synthesis. It may be formed by specific cleavage of TMV RNA or by partial transcription of the complementary strand, or either followed by self-replication.

2.3.1.2 Cucumber mosaic virus and bromoviruses

The genome of CMV, the type member of the cucumovirus group, consists of three RNA molecules, referred to as RNA 1, 2 and 3 in order of decreasing molecular weights. During infection with these three RNAs a fourth RNA, RNA 4, is produced that specifies the viral coat protein. RNA 4 derives from RNA 3. RNA 1 and 2 are encapsidated in separate protein coats. RNA 3 and 4 are encapsidated together in one protein capsid. Only RNA 1, 2 and 3 are necessary for infection.

Replication of CMV RNA has been studied in tobacco protoplasts by TAKANAMI *et al.* (1977). A combination of actinomycin D treatment and UV-irradiation was used for the suppression of host RNA synthesis to achieve a low background against which the synthesis of the four viral RNAs could be examined. The newly synthesized viral RNAs became detectable 6 h after inoculation. Their rate of synthesis increased to reach a maximum at about 15 h after inoculation. RNA 3 was largely predominant throughout the infection cycle. Since RNA 3 is encapsidated together with RNA 4 in an equimolar ratio this implies that a considerable part of the synthesized RNA 3 will not be included in progeny particles. The meaning of the excess of RNA 3 production is unclear. It might indicate that the translation product of RNA 3 is needed in large quantities or that the larger amount of RNA 3 might have something to do with the origin of RNA 4 from RNA 3. Double-stranded forms of all viral RNAs were found, including double-stranded RNA of RNA 4. This might suggest that RNA 4 is synthesized by transcription of an RNA complementary to RNA 4 and not by cleavage of RNA 3. It has not however been demonstrated that the double-stranded form of RNA 4 acts as an intermediate in RNA 4 synthesis. Therefore it cannot be excluded that it is an artefact of isolation.

With some strains of CMV a satellite RNA is found. This RNA depends for its replication on CMV as a helper virus. The satellite RNA is a small RNA with a M.W. 120,000 with no sequence homology with the genomic RNAs of CMV (GOULD *et al.*, 1978). It was shown that this cucumber mosaic virus-associated RNA 5 was extensively replicated in protoplasts and the occurrence of its RF

and RI has been demonstrated (TAKANAMI *et al.*, 1977).

The genomic constitution of the bromoviruses is very similar to that of the cucumoviruses. In studies of the RNA synthesis of the bromoviruses, brome mosaic (BMV) and cowpea chlorotic mottle virus (CCMV) in tobacco protoplasts, it was found that in these cases RNA 3 was also synthesized to a much greater extent than the other three viral RNAs (BANCROFT *et al.*, 1975). Only replicative forms of the three genomic RNAs could be detected. No double-stranded RNA corresponding to RNA 4 was found. In the case of the bromoviruses the evidence therefore indicates that RNA 4 arises directly from RNA 3.

2.3.2. *Viral-related protein synthesis*

Protein synthesis in virus-infected protoplasts differs from that in uninfected cells in various respects. Firstly, there are proteins synthesized under the direction of the viral genome, the so-called virus-specific proteins. These include among others the virus coat protein(s). Secondly, there may be host-specific proteins, the synthesis of which is induced or stimulated by the virus infection and which may be involved in the virus replication process. On the other hand one might expect inhibition of the synthesis of some host-specific proteins to occur during virus infection.

Research on viral-related proteins still mainly centers around their detection. Apart from the viral coat proteins no functions can as yet be attributed to any of the other proteins found upon infection. At present, extensive studies have only been reported for TMV, CCMV and BMV.

2.3.2.1. Tobacco mosaic virus

If tobacco leaf protoplasts infected with TMV are labeled with radioactive amino acids, a very large number of labeled proteins can be detected upon electrophoretic analysis of extracts of such protoplasts on SDS-polyacrylamide gels (PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978a). The majority of the labeled proteins also occurs in uninfected protoplasts, but at least three are found only after TMV infection (SAKAI and TAKEBE, 1974; PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978a). These proteins have apparent molecular weights of about 17,500, 135,000 and 160,000. Their detection could be improved if host protein synthesis was suppressed either by adding chloramphenicol to inhibit the 70 S chloroplast protein synthesis system or by subjecting the protoplasts to UV-irradiation before labeling in order to break down messenger RNAs for host proteins.

The 17,500-MW protein could be identified as the TMV coat protein. Its synthesis started within a few hours after inoculation, at the same time that TMV RNA was first detected (SAKAI and TAKEBE, 1974; SIEGEL *et al.*, 1978a), which was prior to the detection of its messenger RNA, LMC (SIEGEL *et al.*, 1978b). The production of coat protein then increased rapidly to become almost constant after 10 to 20 h at which time its synthesis exceeded that of all other proteins. PATERSON and KNIGHT (1975) estimated that at 72 h after inoculation about 12% of the total acid-insoluble radioactivity was incorporated into viral coat protein

while SIEGEL *et al* (1978a) found as much as 70% of the radioactivity being incorporated into coat protein at 45 h after inoculation.

The 135,000- and 160,000-MW proteins are found early upon infection. Their rate of synthesis reaches a maximum at about 10 to 15 h and then decreases slowly.

If TMV RNA is translated *in vitro* in rabbit reticulocyte lysates, two proteins are produced with molecular weights of 140,000 and 165,000 (PELHAM and JACKSON, 1976). The 140,000-MW protein comigrates with the 135,000-dalton protein found in TMV-infected tobacco protoplasts and also in TMV-infected cowpea protoplasts (HUBER, 1979). Thus, this 135,000-dalton protein could well be a virus-directed protein.

Upon subcellular fractionation of the protoplasts the 135,000-MW polypeptide can be shown to be associated with the particulate fractions (SAKAI and TAKEBE, 1974; SIEGEL *et al.*, 1978a). In this fraction prepared from TMV-infected leaf tissue RNA polymerase activity can be found (ZAITLIN and HARIHARASUBRAMANIAN, 1972). This has been interpreted as indicating a role of this protein in viral RNA replication. The time-course of its appearance, which parallels the start of viral RNA synthesis (SAKAI and TAKEBE, 1974), supports this view. The identity and function of the 165,000-dalton polypeptide is, however, not clear. A protein of similar size is not normally produced upon *in vitro* translation of TMV RNA in a wheat germ system, but is produced in rabbit reticulocyte lysates. Recently, PELHAM (1978) has demonstrated that the 165,000-dalton protein is generated as a read-through product of the 135,000-MW protein. By adding tRNA, read-through was established.

Until the structures of the 165,000-dalton protein found in tobacco protoplasts and that produced in *in vitro* protein synthesizing systems have been compared more carefully, for example by comparing their fingerprints after tryptic digestion, it cannot be concluded that the 165,000-dalton protein in protoplasts is a virus-specified protein. It may well be a host-specified protein. This possibility should be seriously considered, stressed by the recent findings of HUBER (1979) who compared the proteins found after TMV-infection of tobacco and cowpea protoplasts. The larger polypeptide found in TMV-infected tobacco protoplasts did not occur in TMV-infected cowpea protoplasts. Instead a polypeptide with an apparent molecular weight 10,000 daltons less was found. In contrast, the polypeptides with apparent molecular weights of 135,000 and 17,000 (coat protein) were found in both protoplast systems. In addition, a protein of about 72,000 daltons was detected in both protoplast systems upon infection with TMV. The similarity of polypeptides synthesized in protoplasts of two different host plants after infection with TMV suggests that the synthesis of these proteins is specified by the TMV genome.

2.3.2.2. Bromoviruses

Tobacco mesophyll protoplasts were also used to study the synthesis of proteins associated with CCMV (SAKAI *et al.*, 1977) and BMV replication (SAKAI *et al.*, 1979). To reduce the level of host protein synthesis they were irradiated with

UV light before inoculation but this treatment also considerably affected the production of progeny virus (SAKAI *et al.*, 1977). Three new proteins with molecular weights of 19,000 (P_1), 34,000 (P_2) and 100,000 (P_3) were detected upon labeling with radioactive leucine and polyacrylamide gel electrophoretic analysis of proteins from protoplasts infected with CCMV. In addition to these, a fourth protein of about 107,000 daltons (P_4) appeared in protoplasts infected with BMV, strain V5. They were considered to be virus-specified since they were absent from uninfected controls, the synthesis of the CCMV-induced proteins P_1 to P_3 was insensitive to actinomycin D, and their molecular weights correspond very closely to the products of *in vitro* translation of the RNAs of CCMV (DAVIES and KAESBERG, 1974) and BMV (SHIH and KAESBERG, 1973 and 1976; DAVIES and KAESBERG, 1974).

Protein P_1 represents the viral coat protein as judged from its size and from its time-course of appearance which coincided with virus particle synthesis. The 35,000-dalton polypeptide P_2 is not a precursor of the coat protein though RNA 3 from which it is presumed to originate is known to contain the coat protein cistron. In contrast, P_2 was found to be a stable protein which was rapidly synthesized just before the onset of virus synthesis, reaching its maximum amount at the time when virus accumulation attained its maximum rate. Moreover, RNA 3 is synthesized first and most rapidly early in CCMV infection (BANCROFT *et al.*, 1975). These kinetics suggested a role as (part of the) viral replicase for P_2, in agreement with it being associated with the membranous protoplast fraction: a protein of about the same size probably related to the viral RNA polymerase has been detected in particulate fractions of BMV-infected barley leaves (HARIHARASUBRAMANIAN *et al.*, 1973). No role has as yet been established for P_3 and P_4 which are synthesized at low rates late in infection. Together the four virus-specific proteins account for over 90% of the bromovirus genome.

2.3.3. *Conclusions*

A survey of the present literature on the biochemical changes associated with plant virus infection is necessarily restricted, providing only fragmentary data on a few viruses. Some virus-specific RNAs and proteins have been detected, and their time-courses studied, but apart from the viral coat protein no functions have been clearly assigned to a specific protein or to a particular RNA of a multicomponent virus. In addition, hardly anything is known about the different effects of virus infection on host metabolism and about aspects such as uncoating and assembly. This review justifies the conclusion that plant protoplasts provide the superior system presently available to elucidate the biochemistry of plant virus infection.

3. ISOLATION OF COWPEA MESOPHYLL PROTOPLASTS AND THEIR INFECTION WITH COWPEA MOSAIC VIRUS

3.1. INTRODUCTION

Since the first isolation of tobacco mesophyll protoplasts and their infection with TMV by TAKEBE and coworkers (TAKEBE *et al.*, 1968; TAKEBE and OTSUKI, 1969) leaf mesophyll protoplasts from a wide range of plant species have been prepared and infected with a variety of plant viruses (TAKEBE, 1977; FURUSAWA and OKUNO, 1978). The production of viable protoplasts that are able to withstand different treatments and prolonged incubation often puts extremely high demands upon the growth conditions of the plant and these conditions may vary significantly for different species. The physiological state of the cells which determines their stability once isolated is not well understood.

Plant protoplasts are prepared by means of cell wall degrading enzymes. Current isolation procedures use pectinases and cellulase either as a mixture (one-step method) (KASSANIS and WHITE, 1974) or by sequential treatment (two-step method) (TAKEBE *et al.*, 1968; OTSUKI *et al.*, 1974). Media of high osmotic value are required to induce plasmolysis thereby enabling efficient enzymatic degradation of the cell wall and to prevent the protoplasts from bursting. This is normally achieved using mannitol or some other monosaccharide sugar, but inorganic salt media of proper ionic strength have also been used (MEYER, 1974; SARKAR *et al.*, 1974).

The optimal conditions for inoculation of protoplasts with plant viruses are not determined solely by the nature of the virus. They reflect the combined properties of the particular protoplast/virus combination in question. Hence the conditions favouring maximum frequency of infection must be assessed for every combination since they may vary considerably.

Cowpea mesophyll protoplasts were first isolated by HIBI *et al.* (1975). Since then they have been used to study the infection of CPMV (HIBI *et al.*, 1975; BEIER and BRUENING, 1975; ROTTIER, 1976; ROTTIER *et al.*, 1979), TMV (KOIKE *et al.*, 1976; HUBER, 1979), AMV (ALBLAS and BOL, 1977), CMV (KOIKE *et al.*, 1977), and CYMV (RAO and HIRUKI, 1978).

In this chapter a detailed account is given concerning the factors involved in the preparation of cowpea mesophyll protoplasts and their subsequent infection with CPMV. The procedures are based mainly on the original study of HIBI *et al.* (1975) which in the course of experience have been revised and modified into a more rapid and simple standard method.

3.2. Materials and methods

3.2.1. *Materials*

Cowpea seeds (*Vigna unguiculata* (L.) Walp var. 'Blackeye Early Ramshorn') were obtained from W. Atlee Burpee Co., Warminster, Pa.. Vermiculite type F2 was bought from Pull B.V., Rhenen, Netherlands. Cellulase Onozuka R-10 and Macerozyme R-10 were purchased from Kinki Yakult Manuf. Co.. Poly-L-ornithine was provided by Pilot Chemicals Inc., Boston, Mass., and 2,4-dichlorophenoxy acetic acid by Sigma Chemical Co.; gentamicin (10 mg/ml) was obtained from Schering Corporation, cephaloridin from Lilly and rimocidin sulfate from Pfizer, Inc., Groton, Conn.. Aureomycin was a product of the Nutritional Biochemicals Corporation, Cleveland, Ohio. Pectin acid transeliminase (PATE) was supplied by Hoechst A.G., Frankfurt, Germany.

3.2.2. *Cultivation of cowpea plants*

Cowpea seeds were germinated in moistened vermiculite (0.5 l H_2O/l vermiculite) for about 40 h at 25 °C in the dark. Seedlings having firm, 3 to 5-cm shoots were selected, washed under the tap and put on 6 l pots of Hoagland solution.

Plants were grown in a growth chamber (Vötsch, Miniphyt VTPH) for 14 h at 28 °C in light alternating with 10 h darkness at 22 °C and at a relative humidity between 75 and 90 %. Light was provided by 12 white fluorescent tubes (Osram L, type 40W/20) and the light intensity on the leaves was 13,500–14,500 lux during the first days of culture, increasing during growth to 15,500–17,000 lux.

3.2.3. *Isolation of protoplasts*

Fully expanded primary leaves were picked off after an 8-day culture period in the growth chamber. The lower epidermis of the leaves was abraded by brushing with carborundum powder (500 mesh) using a soft paintbrush as described by Beier and Bruening (1975) and then washed thoroughly under tap water. The midribs were removed and about 5 g of leaf tissue was infiltrated *in vacuo* for 10 min in a 100 ml Erlenmeyer flask using 25 ml of enzyme solution containing 0.45 % (w/v) Cellulase Onozuka R-10 (further purified essentially as described by Kao *et al.* (1971)), and 0.05 % (w/v) Macerozyme R-10 in 0.6 M D-mannitol, pH 5.5. The infiltrated tissue was incubated for 3.5 h at 25 °C in a shaker water bath at a frequency of 100 oscillations per min. The resulting mixture was passed through a 150-mesh stainless-steel filter and protoplasts were collected by centrifuging the filtrate for 2 min at 700 $\times g$. The supernatant was centrifuged again for 2 min at 700 $\times g$ to collect more protoplasts and the combined pellets were washed three times by resuspending in 0.6 M mannitol solution and sedimenting for 2 min at 600 $\times g$. The quality of the protoplasts was judged under the light microscope and the yield determined using a haemocytometer.

3.2.4. *Virus and virus purification*

CPMV (yellow strain) was propagated and prepared by the polyethylene glycol procedure of Van Kammen (1967) and further purified by sedimenting

through a 2.5 ml 40% (w/w) sucrose cushion in a Beckman Type 30 rotor at 30,000 rpm for 5 h at 4 °C. The pellet was dissolved in 0.01 M sodium phosphate buffer pH 7.0 and the virus was stored in liquid nitrogen until used.

3.2.5. *Inoculation of protoplasts with CPMV*

Protoplasts were suspended at a concentration of about 5×10^5 protoplasts/ml in a solution of 0.6 M mannitol, 0.01 M potassium citrate, pH 5.2, containing 5 µg CPMV/ml. The suspension was incubated for 15 min at room temperature and gently mixed once during this period.

After the inoculation the protoplasts were collected by centrifugation for 2 min at 600 $\times g$ and washed three times with sterile 0.6 M mannitol containing 10 mM $CaCl_2$.

3.2.6. *Incubation of protoplasts*

Washed protoplasts were incubated for virus multiplication at a concentration of about 5×10^5 protoplasts/ml in portions of 5–10 ml in 50-ml or 100-ml Erlenmeyer flasks for various times at 25 °C under continuous illumination of about 10,000 lux. The incubation medium was a modification of that used by AOKI and TAKEBE (1969) containing 0.6 M mannitol, 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 10 mM $CaCl_2$, 1 µM KI, 0.01 µM $CuSO_4$, 1 µg/ml 2,4-dichlorophenoxyacetic acid and 25 µg/ml gentamicin, pH 5.4. The solution was sterilized by autoclaving.

3.2.7. *Fluorescent antibody staining of infected protoplasts*

Preparation of fluorescein isothiocyanate-conjugated antibodies to CPMV and staining of infected protoplasts was carried out as described by HIBI *et al.* (1975). The preparations were inspected under a Wild fluorescence microscope and the percentage of infected protoplasts was determined.

3.2.8. *Infectivity assay of protoplast extracts*

The yield of infectious virus was determined by local lesion assay of protoplast extracts. Samples of protoplasts ($0.1–1.0 \times 10^6$) were collected by centrifugation and suspended in 0.5–2 ml of 0.1 M sodium phosphate buffer pH 7.0. The suspensions were homogenized by sonication with a Branson sonifier type B-30 (Branson Sonic Power Co.) using the microtip. The homogenates were cleared by centrifugation for 15 min at 10,000 $\times g$. After appropriate dilution with phosphate buffer the infectivity of the supernatants was assayed on the primary leaves of *Phaseolus vulgaris* (L.) var. 'Pinto' as described by DE JAGER (1976) using 6 leaves per assay.

3.2.9. *Bacterial contamination*

To estimate the degree of bacterial growth during incubation of the infected protoplasts for virus multiplication, serial dilutions of the suspension were made after the incubation and from each dilution one drop was evenly spread on an agar culture medium in a petri dish. The medium contained 0.5% bacto-peptone

and 0.5% yeast extract in 1.2% agar. The plates were incubated at 25°C for 2 days. Appropriate dishes were then selected and colonies counted.

3.3. Results

3.3.1. *Conditions for growth of cowpea plants*

The viability of cowpea mesophyll protoplasts and their susceptibility to infection with CPMV appeared to depend on the quality of the leaves from which they were isolated. The following factors were found to be of major importance.

A. Germination of cowpea seeds

Seeds were routinely germinated in vermiculite moistened with tap water, by incubation at 25°C for about 40 h. Between 80 and 95% of the seeds were then usable. Different batches of vermiculite originating from Greece as well as from Africa and Asia were used. Sometimes washing of the vermiculite with water was beneficial for the yield of viable protoplasts. Germination could equally well be achieved on or between filter paper or cotton wool. Plants from seeds germinated in perlite did not give viable protoplasts.

The degree of moistening of the vermiculite affected the yield of usable seedlings. A short measure of water gave very long, thin, and fragile shoots. Excessive watering led to short, capricious shoots frequently wound around the seed and these seeds, upon subsequent cultivation often produced plants with irregularly shaped, incompletely expanded leaves. In both cases a large proportion of the seeds did not germinate at all. Upon germination in vermiculite for more than 40 h the primary leaves started to develop. Plants grown from such seedlings usually did not produce suitable protoplasts.

B. Growth of plants on liquid nutrient medium

Cowpea plants were grown on Hoagland solution containing anorganic ions, iron (II)-chelate and trace elements (Hibi *et al.*, 1975). As far as has been tested all of the components of the nutrient solution were essential. Omission of, for instance, phosphate or sulfate ions to starve the plants for radioactive labeling of protoplasts with $^{32}PO_4$ or $^{35}SO_4$ was fatal. Starvation of phosphate was only without adverse affects to the yield of protoplasts during the last few days of plant growth, but still at the expense of their viability.

The intensity of the light for growing the plants was of crucial importance. The light intensity measured on the primary leaves must be kept within strict limits of between 13,500 and 17,000 lux depending on the age of the plants. Moreover, it appeared to be necessary to renew the TL-tubes every 4–6 months presumably because of a gradual alteration in the quality (wave-length spectrum) of the light.

Under these standard conditions the leaves were usually in optimum condition for protoplast isolation close to 10 days after sowing and this lasted for one day. This optimum could shift to an earlier or later time if the growth conditions were changed. Harvesting was done during the light period 'in the morning', but there

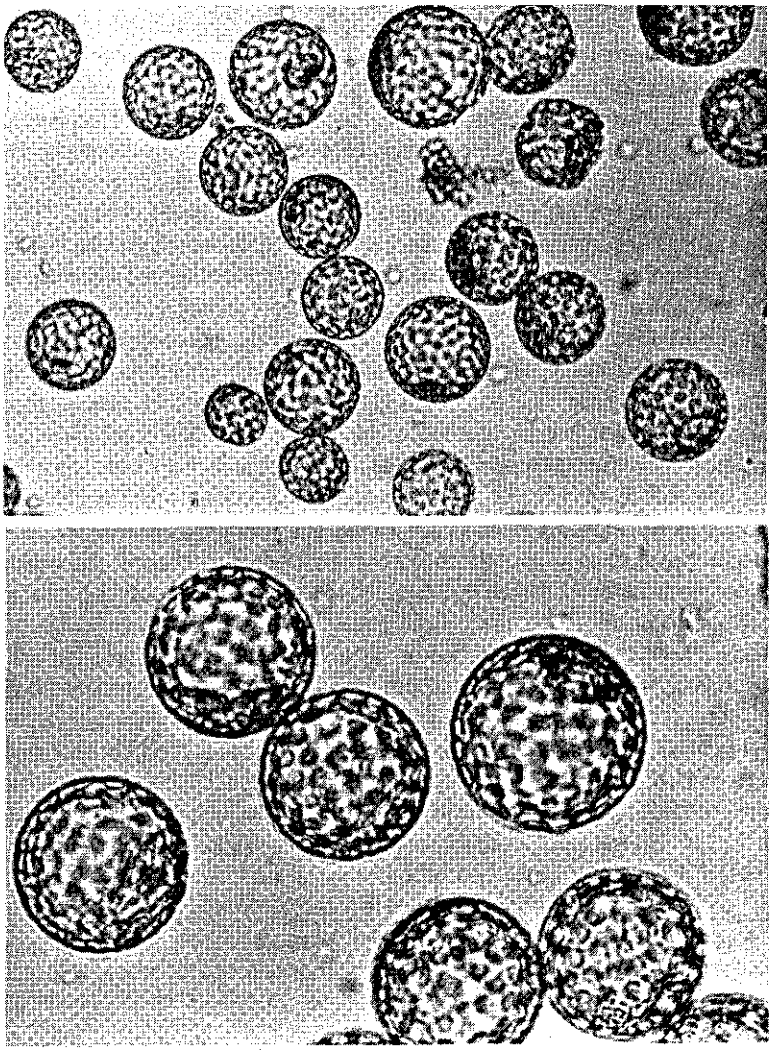

FIG. 3.1. Cowpea protoplasts freshly prepared from cowpea leaf and suspended in 0.6 M mannitol. Light microscopic view at two magnifications.

were no indications of any advantage in harvesting during any particular period of the day.

3.3.2. *Isolation of protoplasts*

To enable easy access of enzymes to the leaf tissue the lower epidermis has to be removed or damaged appropriately. Different methods were compared: peeling off the epidermis with forceps, brushing the epidermis with a paintbrush after sprinkling it with carborundum powder, or abrading the epidermis carefully with soft abrasive paper. The first two methods were the most successful

and gave equal amounts of protoplasts of similar quality. The paintbrush/carborundum method was adopted as it was the more practical.

Commercial cellulase (Onozuka R-10) usually contained too many toxic impurities for direct use in the one-step isolation of the protoplasts. Therefore, it was purified by way of salt treatment followed by column chromatography (KAO *et al.*, 1971). These enzyme preparations no longer killed the protoplasts and their specific activity had doubled. Refreshment of the medium (HIBI *et al.*, 1975) did not appear to be necessary. The debris of damaged cells was removed by washing after the enzyme treatment and rather homogeneous isolates consisting of more than 95 % of parenchyma cells were thus obtained. The yield routinely varied between 5 and 15×10^6 protoplasts/g of leaf and the percentage of living protoplasts between 80 and 95 %. Preparations with less than 80 % living protoplasts after the final washings were discarded. The microscopical appearance of a normal isolate is shown in figure 3.1.

Isolation of cowpea mesophyll protoplasts was also possible using a saline medium according to MEYER (1974) with $MgCl_2$ substituted for $MgSO_4$ as recommended by SARKAR *et al.* (1974). For obtaining reasonable quantities of protoplasts preplasmolysis of the leaves with PATE in mannitol medium appeared to be necessary but the yields were lower as compared to our standard procedure. Moreover, the susceptibility of the protoplasts to inoculation with CPMV was drastically lowered after isolation in saline medium regardless of the composition of the inoculation medium.

3.3.3. *Inoculation of protoplasts with CPMV*

Whereas HIBI *et al* (1975) reported a stimulation of infection by poly-L-ornithine in the inoculation medium such an effect could not be reproduced

TABLE 3.1. The effect of poly-L-ornithine and poly-D-lysine on infection of cowpea mesophyll protoplasts by CPMV.

Expt.	Polycation	Polycation conc. (µg/ml)	Preincubations[a]	% infected protoplasts[b]	Yield of virus (µg/10^6 living protoplasts)[c]
1	poly-L-ornithine	0.5	+	72	24
	–	–	–	71	27
2	poly-L-ornithine	0.5	+	74	n.d.
	–	–	+	80	n.d.
3	poly-L-ornithine	0.5	+	91	30
	poly-L-ornithine	1.0	+	88	n.d.
	–	–	–	89	32
	poly-D-lysine	0.5	+	77	n.d.
	poly-D-lysine	1.0	+	71	n.d.

a Preincubation of virus and protoplasts with polycation before inoculation (15 min, 25°C)
b As scored by fluorescent antibody staining about 45 h after inoculation
c As determined by local lesion assay after 41 h incubation of inoculated protoplasts (n.d. = not determined).

(table 3.1). Neither the percentage of infected protoplasts nor the yield of virus was significantly affected in several experiments. Another polycation, poly-D-lysine, caused a slight decrease in the number of infected protoplasts. Since high levels of infection could be achieved in the absence of polycations the injurious action of these agents on the protoplasts was avoided. In addition it made the preincubations of both protoplasts and virus superfluous prior to inoculation (table 3.1). Consequently, protoplasts could be suspended directly in virus solution for inoculation after the final wash.

Omitting poly-L-ornithine did not alter the concentration of virus required for maximum infection of protoplasts. In agreement with HIBI *et al.* (1975) highest frequency of infection was obtained at inoculum concentrations of 3 to 5 μg CPMV/ml. Only when virus with low specific infectivity was used were higher concentrations favourable.

As shown in table 3.2 high levels of infection could be obtained irrespective of the buffer. All buffers were tested at pH 5.2 since at more acidic pH's which favour infection of cowpea protoplasts with CPMV (HIBI *et al.*, 1975) the protoplasts become fragile. Other factors sometimes reported to be important for the infection of protoplasts with virus such as temperature of inoculation (ALBLAS and BOL, 1977; HUBER, 1979) and osmotic shock treatment immediately before or during inoculation (OKUNO and FURUSAWA, 1978; ALBLAS and BOL, 1978) had no special effect on the infection of cowpea protoplasts with CPMV. It was however favourable to inoculate the protoplasts with CPMV soon after their isolation since the efficiency of inoculation steadily decreased upon storage as was also observed by MOTOYOSHI *et al.*, 1975).

3.3.4. *Incubation of protoplasts for virus multiplication*

The concentration of protoplasts in the incubation medium did not influence the number of protoplasts that survived nor the percentage stainable with fluorescent antibodies against CPMV after a 40-h incubation but had a striking effect on yield of virus as illustrated in fig. 3.2. Up to about 4×10^5 protoplasts/ml the average amount of virus produced per infected protoplast

TABLE 3.2. Effect of different inoculation buffers on the infection percentage of cowpea mesophyll protoplasts with CPMV.

Expt.	inoculation buffer[a]	% infected protoplasts[b]
1	0.01 M citrate	81
	0.01 M acetate	88
	0.01 M phosphate	90
2	0.01 M citrate	90
	0.01 M acetate	83
	0.01 M phosphate	85
	0.005 M phosphate	90

a All buffers were potassium salt buffers at pH 5.2

b As scored by fluorescent antibody staining about 40 h after inoculation.

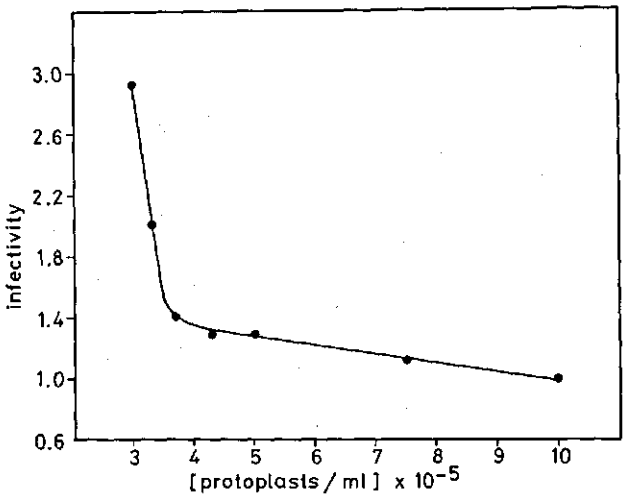

FIG. 3.2. The influence of protoplast concentration in the incubation medium on the yield of infectious virus. The relative amounts of infectivity extracted from equal numbers of protoplasts incubated at different concentrations were determined by local lesion assay and are expressed in arbitrary units.

dropped sharply. Above this concentration the decrease was much less pronounced.

Precautions such as sterilized glassware, sterile solutions or even surface-sterilization of the leaves before use do not prevent bacterial and fungal contamination, and antibiotics had to be included in the protoplast incubation medium to suppress growth of microorganisms. Incubation of the protoplasts in the absence of antibiotics resulted in a massive growth of bacteria and also fungi yielding a viscous, almost gelatinous medium after 42 h of incubation. Under these circumstances almost all protoplasts died, but essentially all of those which survived appeared to be infected, in agreement with the general finding that the more viable the protoplasts the easier they are infected with CPMV. The addition of cephaloridin (300 µg/ml), gentamicin (25 µg/ml) or aureomycin (5 µg/ml) to the incubation medium strongly inhibited bacterial growth (table 3.3), but the inhibition was not complete even when the concentrations were increased (data not shown). Gentamicin (25 µg/ml) appeared to be most effective in suppressing bacterial multiplication, its effect on protoplast viability being comparable with the other antibiotics. In addition, no difference was observed in the effectiveness with which the protoplasts supported CPMV multiplication. On the basis of these results gentamicin (25 µg/ml) was preferred to cephaloridin which was used previously by HIBI *et al.* (1975). With gentamicin fungal contamination was also suppressed and so rimocidin could be omitted. Gentamicin has been used by several groups of workers (WATTS and KING, 1973a; EVANS *et al.*, 1973; MOTOYOSHI *et al.*, 1974; KASSANIS *et al.*, 1975) for a similar purpose. It was reported to inhibit healthy protoplast metabolism (WATTS and KING, 1973b), but MOTOYOSHI *et al.* (1974) could not reproduce this effect nor did they

TABLE 3.3. Comparison of the effects of some antibiotics on bacterial growth, protoplast integrity and virus yield[a].

Antibiotics	bacterial contamination[b]	% living protoplasts	% infected protoplasts	infectivity assay[c]
Cephaloridin (300 µg/ml) + Rimocidin (10 µg/ml)	46×10^4	67	74	143
Gentamicin (25 µg/ml) + Rimocidin (10 µg/ml)	10×10^2	58	71	147
Aureomycin (5 µg/ml)	18×10^3	68	69	152
None	17×10^6	13	>90	36

[a] Infected protoplasts were incubated in the presence or in the absence of antibiotics for 42 h.
[b] Bacterial contamination assayed as described in Materials and Methods is expressed as the theoretical number of colonies per drop of undiluted protoplast suspension.
[c] Protoplasts from equal volumes of the samples were sedimented by centrifugation, homogenized and the amount of infectivity extracted was measured by local lesion assay. The results are expressed as relative values *i.e.* the number of local lesions of each assay was normalized with respect to the corresponding number on the control half leaves.

find CCMV multiplication to be affected. The inhibition of protoplast metabolism may well have been the result of some chelating activity of gentamicin since the inhibition of TMV multiplication by this antibiotic could be prevented by addition of $CaCl_2$ and other salts of divalent metals and could be mimicked by addition of EDTA (KASSANIS *et al.*, 1975).

The rate of CPMV multiplication in cowpea mesophyll protoplasts decreased at lower temperatures and became zero at about 12°C (fig. 3.3). Virus multiplication resumed upon raising the temperature to the standard temperature of 25°C (data not shown). At higher temperatures the amount of virus extractable

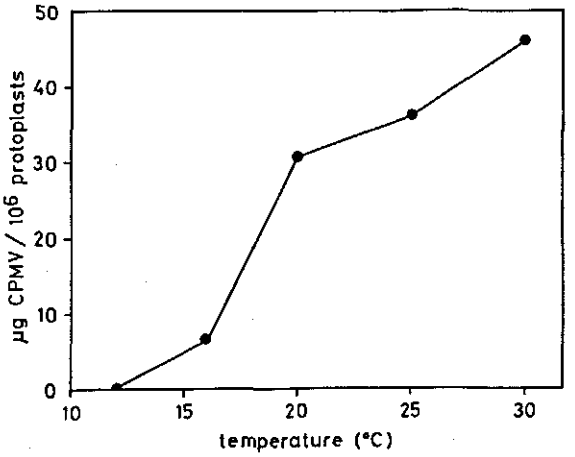

FIG. 3.3. Effect of temperature during incubation of CPMV-infected cowpea protoplasts on the yield of virus. Samples of CPMV-infected protoplasts were incubated at different temperatures and after 41 h virus yield was determined by local lesion assay.

TABLE 3.4. The effect of light during protoplast incubation on the viability of cowpea protoplasts and on CPMV multiplication[a].

Sample	Incubation	% living protoplasts	% infected protoplasts	Infectivity assay[b]
1	dark	42	30	91
2	dark	64	25	
3	light	77	76	165
4	light	79	71	

[a] Infected protoplasts were incubated as indicated for 42 h.
[b] Equal volumes of samples 1+2 and samples 3+4 were combined and assayed for infectivity as described in the footnote of table 3.3.

from the protoplasts increased, but above 30 °C protoplasts could not survive for long.

As shown in table 3.4 the percentage of living protoplasts was greatly reduced upon incubation in the dark. At the same time the number of protoplasts stainable with fluorescent antibodies to CPMV also decreased indicating a low level of CPMV multiplication in the dark. This was confirmed by local lesion assay which showed that virus yield after 42 h incubation was about 50 % lower in the dark than under light. A reduction in the percentage of stainable protoplasts to 32 % of the control was previously noted by HIBI *et al.* (1975).

3.4. DISCUSSION

The method for the preparation of cowpea mesophyll protoplasts and their subsequent infection with CPMV as has been described in this chapter provides the most convenient protoplast-virus system known today. The evaluation of several factors involved has resulted in a considerably simpler procedure than the one reported earlier by HIBI *et al.* (1975).

One major advantage of the cowpea system is the short period necessary for growing plants suitable for protoplast isolation. The growth of plants appears to be the most vulnerable stage of protoplast preparation. Even if culture conditions such as illumination, temperature, humidity and photoperiodicity were optimal, fragile protoplasts were sometimes obtained which died during isolation or upon inoculation and incubation. Not all important parameters are therefore yet understood. There are no indications that variation in the nature of the cowpea seeds, the time of the year or a too low oxygen content of the Hoagland culture solution are involved. As a consequence unsuccessful isolations had to be accepted now and then.

The leaves appeared to be suitable for protoplast isolation for at most one day under the standard conditions of growth used in the present experiments. Since the primary leaves always reached their maximal dimensions at the favourable picking-time some phase during cessation of meristematic growth, possibly

governed by a certain hormonal balance, might determine the required physiological state of the cells. No correlation could be made as to the developmental state of the secondary leaves.

A one-step procedure instead of consecutive treatment of the leaf tissue with pectinase and cellulase results in preparations of protoplasts heterogeneous with respect to cell type. Cowpea primary leaves are rich in mesophyll tissue and thus the protoplasts were mainly derived from spongy and pallisade parenchyma cells. Epidermal contamination was negligeable usually being about 1 % or less. The one-step method has been claimed to result in protoplast preparations that are more injured and exhibit a lower susceptibility to infection than after two-step isolation (TAKEBE, 1977). However, even after abrasion of the leaves instead of peeling off the lower epidermis the one-step method described here produced cowpea protoplast preparations containing few injured or dead protoplasts (normally between 5 and 15 %) or so-called subprotoplasts. The living protoplasts in these preparations could be infected with CPMV up to over 95 %. Clearly, the different types of mesophyll protoplasts had similar susceptibility to CPMV infection.

Infection of cowpea mesophyll protoplasts with CPMV was not dependent on or stimulated by the presence of polycations in the inoculation medium. Essentially with all protoplast/virus combinations studied so far, pretreatment of virus and/or protoplasts with compounds such as poly-L-ornithine and poly-D-lysine and the presence of these substances during the inoculation event is a prerequisite for successful infection or is at least greatly stimulatory. It is generally believed that these polycations serve a dual purpose *i.e.* the formation of complexes with the viral particles thus creating entities with enhanced infecting potency towards the protoplasts and the interaction with the surface of the protoplasts in a way not yet understood. Possibly they act by causing damage to the plasma membrane, by lowering its negative charge or by inducing pinocytotic activity. The fact that polycations do not stimulate CPMV infection of cowpea protoplasts might mean that the virus itself mimics the polycations in their effect on the protoplast membrane or that it enters the protoplasts by a completely different mechanism.

Based on studies with tobacco mesophyll protoplasts it was stated some years ago by OTSUKI *et al.* (1974) that 'different viruses require different inoculation conditions for infecting protoplasts at high frequency'. It is becoming evident now that these conditions are also determined by the nature of the protoplasts. For instance, polycations appeared not to be essential though stimulatory for the infection of cowpea protoplasts with TMV (KOIKE *et al.*, 1976), CMV (KOIKE *et al.*, 1977) and CYMV (RAO and HIRUKI, 1978), AMV (ALBLAS and BOL, 1977) being an exception. As far as these viruses have been studied, including CPMV (HUBER *et al.*, 1977), polycations are an essential requirement for infection of tobacco mesophyll protoplasts (TAKEBE and OTSUKI, 1969; OTSUKI *et al.* 1972; OTSUKI and TAKEBE, 1973). Presumably, cowpea protoplasts exhibit membrane properties different to those of tobacco protoplasts, and which in general seem to favour the uptake of virus particles.

The data presented in this chapter show that the cowpea protoplast/CPMV system meets all the necessary criteria to provide an excellent experimental system to examine the process of CPMV replication.

4. RNA AND PROTEIN SYNTHESIS IN COWPEA MESOPHYLL PROTOPLASTS

Uptake and incorporation of precursors and the effects of some antibiotics

4.1. Introduction

In this chapter we report on the uptake and incorporation of radioactive precursors of proteins and RNAs into healthy cowpea protoplasts. The results described show that cowpea mesophyll protoplasts have an active RNA and protein metabolism that can be studied using tracer methods and that rapidly reacts to metabolic inhibitors.

4.2. Materials and methods

4.2.1. *Materials*

Radiochemicals were purchased from the Radiochemical Centre, Amersham. Actinomycin D was a gift from Merck, Sharp and Dohme, lincomycin from Upjohn Holland (Ede, The Netherlands). Chloramphenicol, cordycepin, cycloheximide and rifampicin were obtained from the Sigma Chemical Company. Puromycin and disodium-triisopropylnaphthalene sulphonate (TPNS) were provided by Serva, and α-amanitin was kindly donated by dr. P. Zabel. Soluene-350 and Instafluor were bought from Packard Instrument Company. Hydroluma was from Lumac. The source of other materials has been mentioned in chapter 3.

4.2.2. *Isolation and incubation of cowpea mesophyll protoplasts*

Protoplasts were prepared from primary leaves of *Vigna unguiculata* (L.) Walp var. Blackeye Early Ramshorn as described (chapter 3). After isolation they were washed three times by resuspending in sterile 0.6 M mannitol solution containing 10 mM $CaCl_2$ and sedimenting for 2 min at 600 × *g*. The washed protoplasts were resuspended in the incubation medium described (chapter 3) to a concentration of about 6×10^5 protoplasts/ml and incubated at 25 °C in portions of 5 ml in 50-ml Erlenmeyer flasks under illumination of about 10,000 lux.

4.2.3. *Measurement of incorporation and uptake of radioactive precursors*

Protein was labeled by addition of ^{3}H-leucine or ^{35}S-methionine to the incubation medium, RNA was labeled using ^{3}H-uridine, ^{3}H-uracil or $^{32}PO_4$.

Incubations of protoplasts were stopped by transferring the samples into centrifuge tubes and collecting them at 4 °C by centrifugation at 600 × *g* for 3 min. The protoplasts were washed once with 5 ml cold 0.6 M mannitol containing 10 mM $CaCl_2$, 1 mM $MgSO_4$ and 1 mM of the unlabeled precursor, and were stored at –20 °C.

For determination of the uptake and incorporation of radioactivity each protoplast pellet was dissolved by incubation at 30°C for 15 min in 0.5 ml detergent solution: 0.05 M Tris-HCl, 0.1 M NaCl, 0.01 M EDTA, 2% sodium dodecyl sulfate, 1% sodium deoxycholate, 2% sodium p-aminosalicylate, 0.5% TPNS, pH 8.2. Total radioactivity taken up by the protoplasts was measured by directly counting 50-µl samples dissolved in 7 ml Hydroluma in a Packard Tricarb scintillation counter. Similar samples were spotted on Whatmann 3 MM filters to assay radioactivity incorporated. Dried filters were washed batchwise for 10 min by gently stirring in cold 10% trichloroacetic acid (TCA) containing 1 mM of the unlabeled precursor. To determine incorporation of ^{3}H-uridine or $^{32}PO_4$ into RNA, filters were washed three times with 5% TCA, three times with 1 N HCl containing 0.1 M $Na_4P_2O_7$, and twice with 80% ethanol, successively. All washings were done for 10–15 min on ice. If the amount of labeled amino acids in protein was to be measured, filters were processed through one wash in 5% TCA at 95°C, five washes in 5% TCA, two in ethanol/diethylether (1:1) and one in diethylether at room temperature. The dried filters were treated with 0.75 ml of 90% (v/v) Soluene-350 in water for 2 h at 50°C or overnight at room temperature, and counted after addition of 7 ml Instafluor.

4.2.4. *Analysis of RNA by polyacrylamide gel electrophoresis*

RNA was isolated from protoplasts by a modification of the method of GLISIN *et al.* (1974). A protoplast pellet was lysed in 3 ml 0.1 M Tris-HCl, 5 mM EDTA, 1% TPNS and 4% sarkosyl (pH 8.0) by incubation for 5 min at 50°C. In the resulting solution 3 g CsCl was dissolved and this was layered on a 1.2 ml CsCl cushion (5.7 M CsCl, 0.02M Tris-HCl, 0.1M EDTA, pH 8.2) in tubes for the SW 50.1 Beckman rotor. Centrifugation was for 12 h at 33,000 rpm and 20°C. The supernatant was pipetted off, the pellet was dissolved in 0.5 ml sterile bidest and the RNA precipitated overnight at –20°C after addition of NH_4Ac to a concentration of 0.24 M (OSTERBURG *et al.*, 1975) and two volumes of ethanol. The precipitate was collected by centrifugation at 9,800 $\times g$ for 10 min in a Janetzki centrifuge, dried and taken up in 150 µl 10% sucrose. A 50-µl aliquot was subjected to electrophoresis according to the method of LOENING and INGLE (1967). After electrophoresis the gel was scanned at 260 nm, frozen and sliced with a Mickle Gel Slicer. Sections of 1 mm were incubated in 90% Soluene-350 overnight at 50°C and counted after addition of 7 ml Instafluor.

4.3. RESULTS

4.3.1. *RNA and protein synthesis in cowpea mesophyll protoplasts*

The time-course of uptake and incorporation of ^{3}H-leucine and ^{3}H-uridine by freshly prepared protoplasts was determined by assaying total and TCA-precipitable radioactivity after various times of incubation in the presence of labeled precursor. As shown in fig. 4.1 ^{3}H-uridine initially was taken up very rapidly. The rate of uptake then decreased and the amount of free radioactivity

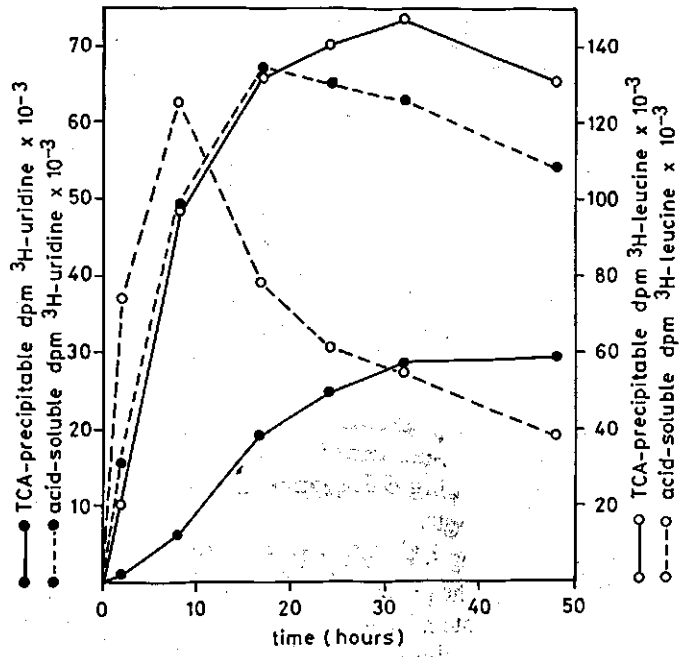

FIG. 4.1. The time-course of uptake and incorporation of ^{3}H-leucine and ^{3}H-uridine by cowpea mesophyll protoplasts.

From a batch of freshly isolated protoplasts two groups of 5 ml samples were prepared. To one group ^{3}H-leucine was added, to the other ^{3}H-uridine, 0.4 μCi/ml in each case. Protoplasts were incubated for various times and total and TCA-precipitable counts determined as described and the free radioactivity was calculated by the difference between these values.

within the protoplasts reached a maximum at about 16 h, after which time it decreased slightly. The amount of TCA-insoluble ^{3}H-uridine increased at an approximately constant rate during the first 30 h and the level subsequently maintained. The rate of incorporation of ^{3}H-leucine was also constant during the initial period but started to decrease after about 16 h. Sometimes there was even a net decline in the total amount of TCA-insoluble material. The reason for this decline is unclear. It is perhaps because some proteins were excreted into the medium but this is not easy to establish. Conversion of leucine (*e.g.* by deamination) might occur, thereby reducing its pool size. The decline may also have been caused in part by metabolic degradation of the proteins synthesized but only to a small extent, since a concomitant increase in unbound radioactivity was not observed. On the contrary, after having reached a maximum at about 8 h the amount of free ^{3}H-leucine within the protoplasts sharply decreased as did total radioactivity (fig. 4.1). This might be caused by some kind of inhibition of the uptake of ^{3}H-leucine from the medium as the protoplasts age. However, in another set of experiments in which ^{35}S-methionine was used to label protoplasts for successive 6 h periods after isolation, the opposite was found to occur, that is a significant increase in the amount of uptake of the amino acid as a function of

TABLE 4.1. Free and incorporated radioactivity in cowpea protoplasts labeled during successive 6 h periods with ^{35}S-methionine.

Labeling period (h)	Acid-soluble radioactivity (cpm × 10^{-6})	TCA-precipitable radioactivity (cpm × 10^{-6})
2– 8	49.7	4.4
9–15	55.0	7.0
16–22	67.2	9.7
26–32	68.8	12.2

Protoplast samples were labeled with ^{35}S-methionine (30 μCi/ml) for successive 6 h periods after isolation and free and bound radioactivity were determined as described.

protoplast age (table 4.1). An even more pronounced increase in the rate of protein synthesis was measured by this approach: incorporation of ^{35}S-methionine measured during 6 h periods nearly trebled during the first 30 h after isolation of the protoplasts.

From the results in fig. 4.1 it was concluded that 55% of the added ^{3}H-leucine had been taken up by the protoplasts after 17 h of incubation, 64% of this being incorporated into protein. This implies a mean specific radioactivity of the protein of about 800 dpm/μg. A similar time of labeling with ^{3}H-uridine resulted in the uptake of 21% of the label added to the protoplasts, of which 22% was found in acid-insoluble material (mean specific radioactivity of RNA about 600 dpm/μg).

Uptake and incorporation were also dependent on the amount and nature of the radioactive precursor present and on the concentration of the protoplasts. Incorporation into RNA was much more efficient using ^{3}H-uridine than ^{3}H-UTP, while the extent of protein labeling was higher with ^{35}S-methionine than with ^{3}H-leucine, which in turn was much more efficient than $^{35}SO_4$.
At lower concentrations of ^{3}H-leucine, incorporation into protein was directly proportional to the concentration of label applied. Keeping this concentration constant, incorporation of ^{3}H-leucine per protoplast increased with decreasing protoplast concentration.

RNA and protein synthesis appeared to be light-dependent. Upon incubation of the protoplasts in the dark, precursor incorporation gradually decreased.

4.3.2. *Analysis of RNA synthesis by polyacrylamide gel electrophoresis*

The electrophoretic pattern of RNAs labeled with ^{3}H-uridine during a 40 h incubation period is shown in fig. 4.2. Radioactivity was found mainly at the positions of the ribosomal RNA species of cytoplasmic ribosomes (25 S and 18 S). Only a very low level of 23 S and 16 S chloroplast ribosomal RNA synthesis was found. It is not known whether protoplasts are active in chloroplast RNA synthesis or whether ribosomal RNA synthesis in chloroplasts is affected by the gentamicin included in the protoplast incubation medium to inhibit bacterial and fungal growth. Similar results have been reported for protoplasts isolated

TABLE 4.2. Effects of inhibitors on uptake and incorporation of ^{3}H-uridine in cowpea mesophyll protoplasts.

Antibiotic	Concentration (μg/ml)	% inhibition of incorporation into RNA	% inhibition of uptake into acid-soluble pool
Actinomycin D	0.5	7	3
	5	93	33
	10	96	38
	50	99	49
α-amanitin	10	6	–3
Cordycepin	2	9	6
	5	31	10
	25	81	43
	100	96	59
Rifampicin	5	12	11
	100	57	48

Protoplast samples were incubated with or without antibiotic for 5.5 h. Then ^{3}H-uridine (0.2 μCi/ml) was applied and incubation was continued for 11.5 h. Protoplasts were then harvested and processed as described.

from tobacco leaves (SAKAI and TAKEBE, 1970; AOKI and TAKEBE, 1975) and from cucumber cotyledons (COUTTS *et al.*, 1975), whereas substantial *de novo* synthesis of these RNA species occurred in cucumber leaf protoplasts (COUTTS *et al.*, 1975) and in separated leaf cells from tobacco (JACKSON *et al.*, 1972).

4.3.3. *The effects of different inhibitors on RNA synthesis*

In table 4.2 the effects of a number of inhibitors on the amounts of ^{3}H-uridine appearing in free and incorporated form within the protoplasts are given. The results are expressed as percentages of inhibition caused by each compound in relation to untreated controls. In all cases the protoplasts were incubated with the inhibitor for 5.5 h before ^{3}H-uridine was added. Incubation was then continued for 11.5 h.

Actinomycin D at concentrations above 5 μg/ml reduced ^{3}H-uridine incorporation to less than 5 % of the controls. Uptake of ^{3}H-uridine into the acid-soluble pool was also clearly affected, but the main effect was on RNA synthesis. It has been claimed that experiments with actinomycin D must be carried out in the dark due to possible light-inactivation of the antibiotic (JACKSON *et al.*, 1972). No such inactivation was, however, detected during the present investigation: an actinomycin D solution exposed to light for several days was equally active.
Only a slight decrease in ^{3}H-uridine incorporation was caused by α-amanitin (table 4.2). This is in agreement with the limited contribution of the α-amanitin-sensitive RNA synthesis to the overall RNA synthesis (HORGEN and KEY, 1973; CHAMBON, 1975), and with our gel electrophoretic observation of predominant ribosomal RNA synthesis in cowpea protoplasts (fig. 4.2).
Cordycepin severely repressed RNA synthesis, especially at higher concentrations (table 4.2). It is a potent inhibitor since it not only inhibits rRNA and

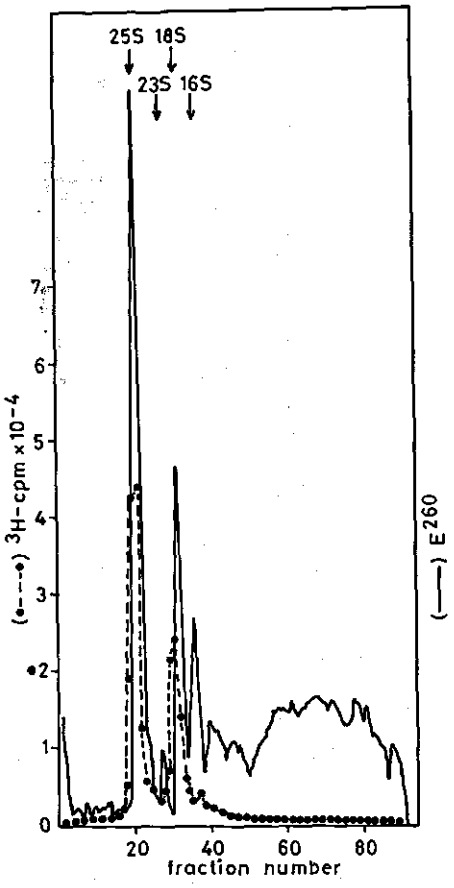

FIG. 4.2. Polyacrylamide gel electrophoresis of RNA from cowpea mesophyll protoplasts labeled with ^{3}H-uridine.

Protoplasts were labeled with ^{3}H-uridine (2 μCi/ml) for 40 h. RNA was isolated from the protoplasts and analyzed as described.

TABLE 4.3. The effect of actinomycin D and cordycepin on the time-course of uptake of ^{3}H-uridine into the acid-soluble fraction of cowpea mesophyll protoplasts.

	acid-soluble radioactivity (dpm × 10^{-3})		
Time (h)	actinomycin D	cordycepin	untreated control
2	20.2	15.4	18.2
6	61.4	51.2	53.6
10	91.4	91.2	89.2
12	93.4	96.0	125.4

Samples of protoplasts were divided into three groups: one group received actinomycin D (10 μg/ml), another group received cordycepin (25 μg/ml), while the third group was not treated. Immediately after addition of the inhibitors ^{3}H-uridine (0.2 μCi/ml) was added to each sample and incubation was started. At selected times samples of each group were harvested and acid-soluble radioactivity within the protoplasts was determined.

tRNA synthesis (FOUQUET *et al.*, 1975; DELSENY *et al.*, 1975) but also that of mRNA (SIEV *et al.*, 1969; ADESNIK *et al.*, 1972; BEACH and ROSS, 1978). Its effect on cowpea protoplasts resembled that of actinomycin D in that uptake of ^{3}H-uridine was also considerably blocked.

Although ^{3}H-uridine incorporation was diminished using rifampicin by 57 % at a concentration of 100 µg/ml this probably does not reflect impairment of chloroplast, mitochondrial or even nuclear RNA polymerase activity, since the decrease was almost completely paralleled by a reduced uptake of the radioactive precursor. A similar effect was observed by SAKAI and TAKEBE (1970) with tobacco mesophyll protoplasts isolated from mature leaves, whereas selective inhibition of chloroplast 23 S and 16 S rRNA synthesis without effect on cytoplasmic rRNA synthesis occurred in the same protoplasts isolated from young leaves (HIRAI and WILDMAN, 1977).

In order to ascertain whether the inhibitory effect of actinomycin D and cordycepin on RNA synthesis (table 4.2) was not a consequence of reduced uptake of precursor, the kinetics of accumulation of free ^{3}H-uridine within the protoplasts were studied. Label and antibiotic were applied to the protoplast incubation medium at the same time and the appearance of ^{3}H-uridine in the acid-soluble fraction was determined and compared with untreated control samples. Both in the presence of actinomycin D and of cordycepin the rate of uptake of the RNA precursor was indistinguishable from the controls during the first 10 h (table 4.3). Thereafter, however, the amount of ^{3}H-uridine in the TCA-soluble fraction of the drug-treated protoplasts no longer kept pace with that of the untreated controls, demonstrating that in both cases the antibiotics did not directly influence the uptake mechanism *per se*, but that some secondary process caused the rate of uptake of ^{3}H-uridine to decrease during the course of the treatment. In contrast, both drugs had an immediate effect on RNA synthesis (not shown). A similar decrease in uridine uptake was also observed by FRANCKI *et al.* (1971) upon actinomycin D treatment of tobacco leaf cells, but was not observed by SAKAI and TAKEBE (1970) with tobacco leaf mesophyll protoplasts.

4.3.4. *The effects of different inhibitors on protein synthesis*

In the experiments in which the effects of antibiotics on protein synthesis were measured, protoplasts were pretreated with inhibitor for 6 h and then allowed to take up and incorporate ^{3}H-leucine during 13 h of incubation. The results are summarized in table 4.4.

Actinomycin D at a concentration of 10 µg/ml which inhibits RNA synthesis by more than 90 %, reduced incorporation of ^{3}H-leucine into protein by 70 %. This inhibition of ^{3}H-leucine incorporation probably did not result from direct intervention with the protein synthesis process, but may well reflect its effect on RNA synthesis since the degree of inhibition increased with the length of the treatment.

An almost complete inhibition of ^{3}H-leucine incorporation was measured with cycloheximide at concentrations above 0.1 µg/ml, although this inhibition may have been somewhat overestimated due to a decreased uptake of label into

TABLE 4.4. The effects of inhibitors on uptake and incorporation of ^{3}H-leucine in cowpea mesophyll protoplasts.

Antibiotic	Concentration (μg/ml)	% inhibition of incorporation into protein	% inhibition of uptake into acid-soluble pool
Actinomycin D	10	71	16
Chloramphenicol	100	51	−11
	200	72	19
Cycloheximide	0.1	95	59
	1	99	89
Lincomycin	10	6	− 3
	50	8	− 5
Puromycin	200	66	17

Samples of protoplasts were incubated for 6 h in the presence of inhibitor. ^{3}H-leucine (0.2 μCi/ml) was then added to each sample and protoplasts were further incubated for 13 h. Samples were then processed for uptake and incorporation of radioactivity as described and the results related to untreated controls.

the free amino acid pool. Since cycloheximide affects cytoplasmic protein synthesis it would follow that organelle protein synthesis is at a low level in the protoplasts. This agrees well with the small effect of lincomycin, a selective inhibitor of the 70 S ribosome system (ELLIS and HARTLEY, 1971; ELLIS 1975) on ^{3}H-leucine incorporation. In contrast, chloramphenicol, once thought also to specifically interfere with this system, reduced ^{3}H-leucine incorporation by more than 50%. Obviously the antibiotic must have affected the cytoplasmic protein synthesis as has been observed in several other studies (see ELLIS, 1977a).

Puromycin, at a concentration of 200 μg/ml, reduced protein synthesis only slightly less than half the level of the control. Despite the high dosage, no complete inhibition was achieved by this non-specific inhibitor of polypeptide elongation (BOULTER, 1970; PESTKA, 1974), probably as a result of inefficient uptake of the drug into the protoplasts.

4.3.5. *The time-course of inhibition of RNA and protein synthesis by actinomycin D and cycloheximide*

In order to estimate the time required for actinomycin D and cycloheximide to reach their maximum inhibitory effect on RNA and protein synthesis, respectively, the kinetics of inhibition were studied as shown in fig. 4.3. Cowpea protoplasts were found to react rapidly to both antibiotics; their specific effects appeared to be elicited within half an hour after application. Whereas the amount of ^{3}H-leucine incorporated into protein continued to increase slowly, the amount of ^{3}H-uridine into TCA-precipitable material tended to decrease, probably reflecting turnover of RNA as has also been found by different approaches in pea protoplasts (WATTS and KING, 1973a) and tobacco protoplasts (SAKAI and TAKEBE, 1970).

No direct influence of actinomycin D on the uptake of ^{3}H-uridine into the

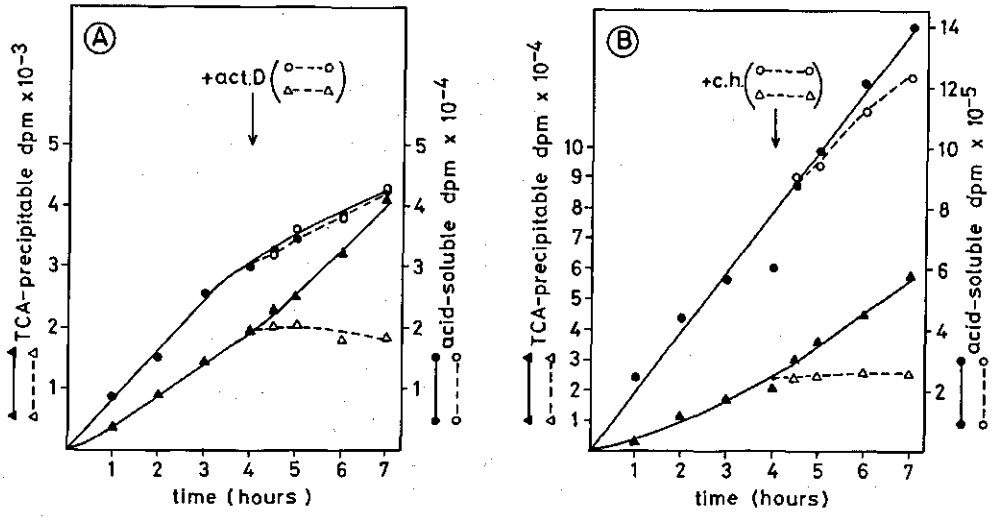

FIG. 4.3. The time-course of inhibition by actinomycin D and cycloheximide.

A. Samples of protoplasts were incubated in the presence of ^{3}H-uridine (0.4 μCi/ml). At different times samples were harvested for determination of free and bound radioactivity within the protoplasts. After 4 h of incubation the residual samples were split into two groups to one of which actinomycin D (10 μg/ml) was added. Incubation and harvesting was continued as before and radioactivity measurements were done as described.

B. Protoplast samples from the isolate were handled identically but now using ^{3}H-leucine (0.4 μCi/ml) and cycloheximide (c.h.) (1μg/ml).

acid-soluble precursor pool was observed, in accordance with the data of table 4.2. Similarly, no immediate effect of cycloheximide on the uptake of ^{3}H-leucine into the acid soluble fraction was evident.

The rate of accumulation of free radioactivity within the protoplasts appeared to decrease slowly as compared with that in untreated protoplasts during the first hours after application of cycloheximide. The drastic diminution of the amount of ^{3}H-leucine by this inhibitor must also be the result of secondary effects on the uptake process, presumably mediated by its effect on protein synthesis.

4.4. DISCUSSION

Upon continuous labeling of cowpea protoplasts with ^{3}H-leucine for long periods, the rate of accumulation of label into protein was constant during the first 15 to 20 h (fig. 4.1). Thereafter, the rate of this process slowed down. This is due in part to the remarkable, strong decrease in the amount of free ^{3}H-leucine within the protoplasts occurring at later times (fig. 4.1). It is also caused by turnover of the proteins but only to a limited extent, since the breakdown of labeled proteins would only tend to increase the amount of free label. Turnover of proteins is a well-established phenomenon in plants (BOULTER *et al.*, 1972) but has hardly been studied in protoplasts or separated cells. Using pea

protoplasts, a turnover rate of 50% in 24 h was reported by WATTS and KING (1973a), but FRANCKI *et al.* (1971) on the other hand could not demonstrate significant degradation of synthesized proteins in separated tobacco leaf cells.

The above mentioned results seem to contradict those obtained upon short-term labeling of cowpea protoplasts with ^{35}S-methionine at increasing times after isolation (table 4.1), which showed an almost threefold increase in the rate of protein synthesis during the first 30 h incubation. It could be argued that this increase is apparent because of depletion of the endogeneous amino acid pool resulting in a higher specific activity of the proteins synthesized, but one would then expect the uptake of ^{35}S-methionine into the acid-soluble fraction to be stimulated proportionally, which was certainly not the case. An early increase of the total amount of ^{3}H-leucine absorbed by tobacco mesophyll protoplasts during 2-h incubations with label, reaching a plateau after about 10 h followed by a slow decline, was reported by ROBINSON and MAYO (1975), but they did not discriminate between free and bound radioactivity. ZELCER and GALUN (1976) found that the rate of ^{14}C-leucine incorporation in these protoplasts measured during 30 min labeling-pulses decreased after a short initial rise during the first 5 hours.

No conclusive explanation can be given for the striking fall in free ^{3}H-leucine within the protoplasts upon long term labeling (fig. 4.1). Radioactivity in the medium was not at the time getting depleted since at most no more than about half of it had been taken up by the protoplasts. The ease of uptake of the amino acid was probably not affected at later times in view of the unimpaired accumulation of ^{35}S-methionine in older protoplasts (table 4.1). Finally, the possibility of leakage of ^{3}H-leucine from the protoplasts can be rejected since such an unselective process would similarly affect the amount of free ^{3}H-uridine within the protoplasts, which did not occur. One important practical conclusion that can be drawn from these experiments, is that it is unfavourable, at least with ^{3}H-leucine, to perform long labelings as the efficiency of protein labeling decreases considerably after some time.

The response of cowpea mesophyll protoplasts to metabolic inhibitors was similar to that of other cellular systems. The antibiotics exhibited their known specificity of action and appear to be useful to selectively inhibit different host functions.

Cowpea protoplasts had an apparently low level of chloroplast metabolic activity. Hardly any synthesis of chloroplast ribosomal RNA was observed (fig. 2), and labeling of the major chloroplast protein, namely the large subunit of Fraction I protein (ELLIS, 1977a) did not occur at all (see chapter 6). The results of the inhibitor studies point to the same conclusion. These observations may well reflect the presence of gentamicin in the incubation medium, added to prevent growth of microorganisms. This antibiotic was listed by PESTKA (1974) as an inhibitor of prokaryote-like protein synthesis, but was found to not seriously impair tobacco protoplast metabolism when used at a concentration of 5 µg/ml in combination with other antibiotics (MOTOYOSHI *et al.*, 1974). On the other hand chloroplast RNA and protein synthesis in cowpea protoplasts might

not be required under the conditions used. Protoplasts were isolated from fully expanded leaves which are known to usually have low chloroplast polymerase activity (ELLIS, 1977b) and Fraction I protein synthesizing activity (ELLIS, 1977a, b; BRADY and SCOTT, 1977). Despite these low activities chloroplasts appear to maintain their energy-generating function since RNA and protein synthesis ceased upon incubation of the protoplasts in the dark.

5. THE INHIBITION OF COWPEA MOSAIC VIRUS REPLICATION BY ACTINOMYCIN D

5.1. INTRODUCTION

Actinomycin D is an effective inhibitor of DNA-dependent RNA synthesis. Since replication of plus-strand RNA viruses does not involve synthesis of DNA intermediates, it has been widely used to study whether the multiplication of these viruses is somehow dependent on a functionally active host genome. The multiplication of a number of animal viruses was found to be sensitive to actinomycin D. This was demonstrated for picornaviruses like poliovirus (COOPER, 1966; GRADO *et al.*, 1965; SCHAFFER and GORDON, 1966), mengovirus (PLAGEMAN and SWIM, 1966) and echovirus (KORANT and HALPEREN, 1975) as well as for togaviruses such as Japanese encephalitis virus (ZEBOVITZ *et al.*, 1972). General conclusions cannot be drawn, however, since a number of viruses from these classes appeared either to be resistant to the drug (CALIGUIRI and TAMM, 1970; MACNAUGHTON *et al.*, 1976; STOLLAR *et al.*, 1966; KÄÄRIÄINEN and GOMATOS, 1969) or were sensitive depending on the time of addition, dose of the drug or other experimental conditions (COOPER, 1966; KOCH *et al.*, 1967; SCHAFFER and GORDON, 1966).

Contradictory results have also been obtained with plant cells infected with plant RNA viruses. Whereas in leaf tissue the multiplication of tobacco mosaic virus and cowpea chlorotic mottle virus was found to be strongly inhibited by actinomycin D (SMITH and SCHLEGEL, 1965; SEMAL, 1967; LOCKHART and SEMANCIK, 1969; DAWSON and SCHLEGEL, 1976; DAWSON, 1978), no effect on virus production could be observed using tobacco mesophyll protoplasts (TAKEBE and OTSUKI, 1969; AOKI and TAKEBE, 1969; BANCROFT *et al.*, 1975). The multiplication of cucumber mosaic virus in tobacco protoplasts was also not affected by actinomycin D (OTSUKI and TAKEBE, 1973). On the other hand the production of potato virus X, alfalfa mosaic virus and turnip yellow mosaic virus in different protoplast systems was found to be sensitive to the drug provided that it was applied during the first hours after inoculation (OTSUKI *et al.*, 1974; ALBLAS and BOL, 1977; RENAUDIN and BOVÉ, 1977). The same pattern of early sensitivity was shown for CPMV and BPMV using hypocotyl tissue, but this effect appeared to be host-dependent in the case of BPMV (LOCKHART and SEMANCIK, 1968 and 1969).

We have investigated the effect of actinomycin D on the multiplication of CPMV in cowpea protoplasts under conditions where the host-dependent RNA synthesis was inhibited by more than 90%. We have found that viral RNA multiplication, but not viral protein synthesis, is prevented by actinomycin D if the antibiotic is added at the time of inoculation of the protoplasts. The application of actinomycin D enabled us to identify six virus-specific proteins in CPMV-infected protoplasts.

5.2. Materials and methods

5.2.1. *Materials*

Disodium-triisopropylnaphthalene sulfonate (TPNS), acrylamide and methylene bisacrylamide were obtained from Serva. Phenylmethylsulphonylfluoride (PMSF) was bought from Merck, tetraethylmethylenediamine (TEMED) from Koch-Light and Coomassie Brilliant Blue R250 from Sigma Chemical Co.. The source of all other chemicals has been mentioned in the previous chapters.

5.2.2. *Isolation, inoculation and incubation of cowpea protoplasts*

The procedures for the isolation of the protoplasts, their inoculation with CPMV and the subsequent incubation of the protoplasts for virus multiplication have been described in chapter 3.

5.2.3. *Measurement of incorporation of radioactive precursors*

Incorporation of RNA and protein precursors by protoplasts was determined by means of TCA precipitation as described in chapter 4.

5.2.4. *Sucrose density gradient analysis*

To determine the amount of virus produced, homogenates of infected protoplasts were analyzed on sucrose density gradients. Protoplasts ($3–5 \times 10^6$), labeled with either ^{32}P or ^{35}S-methionine, were homogenized for 5 min in 0.4 ml 0.01 M sodium phosphate buffer in a 2 ml minipotter homogenizer. The homogenate was centrifuged for 10 min at 9,800 $\times g$ in a Janetzki TH12 centrifuge and the supernatant was layered onto a 12 ml isokinetic sucrose density gradient, containing 0.01 M sodium phosphate and 0.05 M KCl, pH 7.0. Isokinetic gradients were prepared in tubes of the SW41 rotor by running in sucrose solutions from a mixing vessel containing 12.1 ml of 15.9 % (w/v) sucrose to which was added 12.0 ml of 39.9 % (w/v) sucrose. Centrifugation was for 3.5 h at 40,000 rpm and at 4°C. Fractions were collected manually from the bottom of the tube, while monitoring the absorbance pattern at 254 nm. Radioactivity in each fraction was determined by Cerenkov counting (^{32}P) or by liquid scintillation counting after the addition of 0.5 ml of water and 7 ml of Hydroluma.

5.2.5. *Polyacrylamide slabgel electrophoresis of proteins*

Portions of 3×10^6 protoplasts were collected by centrifugation and homogenized in 0.5 ml buffer containing 0.05 M Tris-HCl, 0.01 M KCl, 0.001 M EDTA, 0.005 M $MgCl_2$, 0.06 M β-mercaptoethanol, 0.001 M phenylmethylsulphonylfluoride, 0.4 mM methionine and 10 % (w/v) sucrose. The homogenates were centrifuged for 30 min at 30,000 $\times g$ in a Sorvall SS34 rotor. A 1/2 volume of a three times concentrated solution of Laemmli sample buffer (Laemmli, 1970) was added to the supernatants. After heating for 3 min at 100°C samples of at most 100 µl were analyzed by electrophoresis on polyacrylamide gradient slabgels, essentially according to Laemmli (1970), using a 7–15 % linear gradient of

acrylamide in the separating gel and a 4 % acrylamide spacer gel. Electrophoresis was performed for 6 h at 200 V (constant voltage). Gels were stained with Coomassie Brilliant Blue R250, destained as described by KEDINGER *et al.* (1974) and dried for autoradiography.

5.3. RESULTS

5.3.1. *Effect of actinomycin D on the incorporation of ^{32}P and ^{3}H-uridine*

In table 5.1 the incorporation of ^{32}P and ^{3}H-uridine into portions of uninfected and CPMV-infected protoplasts from the same batch is compared. The amount of incorporation of ^{32}P or ^{3}H-uridine in infected and uninfected protoplasts was usually of the same order, but was significantly higher in infected samples in the case of very active CPMV multiplication.

In the presence of actinomycin D at a concentration of 10 µg/ml the incorporation into the infected and the healthy protoplasts decreased to below 10 % of the untreated controls, demonstrating that RNA synthesis was inhibited effectively. Lower concentrations of the drug resulted in lower levels of inhibition and were not used in the present experiments. No significant differences in the degree of inhibition occurred between ^{32}P and ^{3}H-uridine, indicating that DNA synthesis in the protoplasts is negligeable.

5.3.2. *Effect of actinomycin D on CPMV multiplication*

CPMV multiplication in protoplasts was measured by analyzing homogenates on sucrose density gradients. This technique enabled the separate detection of middle (M) component (95S) and bottom (B) component (115S) without interference of 80S ribosomes, since the latter became completely dissociated upon homogenization in the absence of magnesium ions. The virus components appear in a region of the gradient into which no material sediments in the corresponding gradient run with a homogenate of uninfected protoplasts (fig. 5.1).

TABLE 5.1. Effect of actinomycin D on RNA synthesis in healthy and CPMV-infected protoplasts[a].

Protoplast sample	Expt. 1 (^{32}P)[b]		Expt. 2 (^{32}P)[c]		Expt. 2 (^{3}H-uridine)	
	cpm	%	cpm	%	cpm	%
Healthy	25000	100	7450	100	34750	100
Healthy + act. D	2350	9	500	7	2900	8
Infected	33200	133	6800	91	34150	98
Infected + act. D	2100	8	400	5	2800	8

[a] Inhibition of incorporation of ^{32}P and ^{3}H-uridine into healthy and CPMV-infected protoplasts by actinomycin D (10 µg/ml).

[b] In experiment 1 samples (6 ml) containing 8.3×10^5 protoplasts/ml were used; actinomycin D was added 2.5 h after inoculation, ^{32}P (184 µCi) was given 0.5 h later.

[c] In experiment 2 samples (7 ml) containing 7.1×10^5 protoplasts/ml were used; actinomycin D and label (5 µCi ^{32}P + 5 µCi ^{3}H-uridine) were added 2 h and 7.5 h after inoculation, respectively.

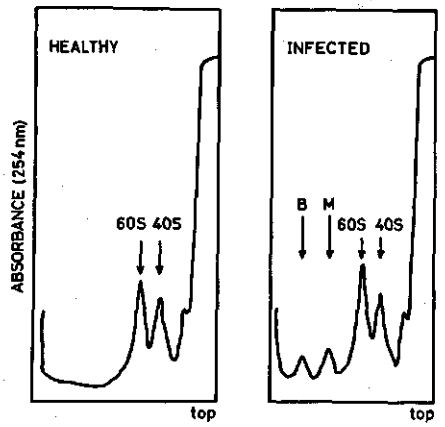

FIG. 5.1. Sucrose density gradient centrifugation of healthy and CPMV-infected protoplast homogenates. Samples (5 ml) of protoplasts (7.0×10^5/ml) were incubated for 44 h and processed as described. Positions of ribosomal subunits and viral nucleoprotein particles are indicated.

If healthy and infected protoplasts, to which actinomycin D was added at various times after inoculation, were labeled with ^{32}P and analyzed on sucrose gradients, a time-dependent effect of the drug on the synthesis of viral nucleoprotein particles was observed (fig. 5.2). Virtually complete inhibition of production of viral nucleoprotein components occurred when the antibiotic was present immediately from the time of inoculation. When actinomycin D was added 8 hours after inoculation (or later), no significant effect on virus yield could be detected. The degree of inhibition increased the shorter the interval between inoculation and addition of the drug. The synthesis of both viral nucleoprotein particles was affected equally; selective inhibition of one of the components was not observed.

5.3.3. *Effect of actinomycin D on synthesis of virus antigen*

Addition of actinomycin D 8 hours after inoculation (or later during infection) had no effect on the percentage of protoplasts stainable with fluorescent antibodies against CPMV (table 5.2), confirming our data on virus yield. Upon earlier administration of actinomycin D there was a gradual decrease in the percentage of stainable protoplasts. Surprisingly, however, a considerable number of protoplasts, usually between 20 and 50 %, could still be made fluorescent when actinomycin D was present throughout the procedure, indicating that accumulation of virus antigen had occurred although no infectious virus was produced. This percentage of stainable protoplasts could not be further reduced by addition of the drug prior to inoculation. The intensity of fluorescence in protoplasts in which virus synthesis was fully or partly inhibited by actinomycin D sometimes seemed somewhat fainter as compared to the untreated control, but in general no clear difference was visible.

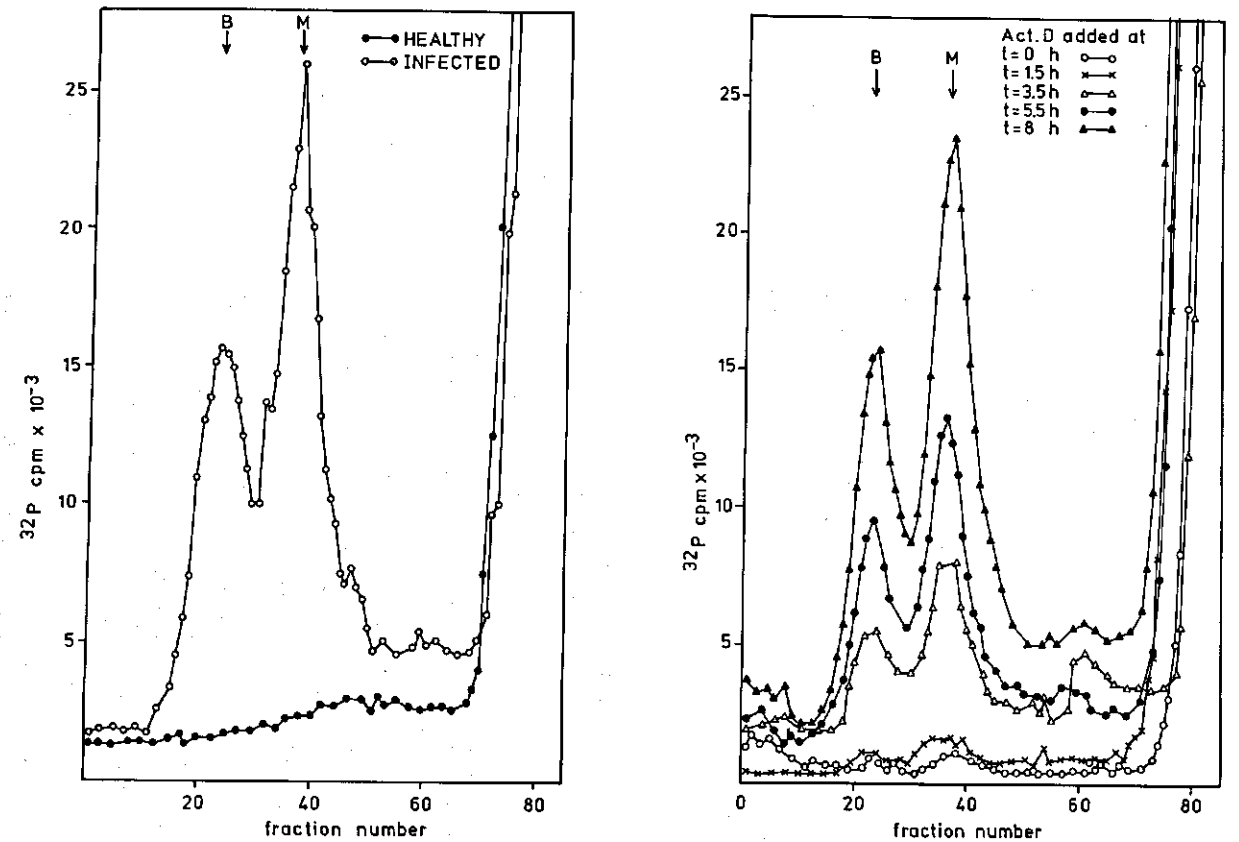

FIG. 5.2. The effect of actinomycin D on CPMV multiplication; analysis by sucrose density gradient centrifugation. Protoplast samples of 6 ml containing 6.8 × 10^5 protoplasts/ml received 120 μCi of ^{32}P 1.5 after inoculation. Actinomycin D (10 μg/ml) was added at various times after inoculation as indicated (right panel). Samples incubated without actinomycin D are shown in the left panel. Incubation was for 42 h.

TABLE 5.2. Effect of actinomycin D on synthesis of virus antigens.

Time of addition (h)[a]	% Fluorescent protoplasts[b]	
	Expt. 1	Expt. 2
0	48	33
0.5	54	–
1.5	61	41
3.5	66	52
5.5	66	69
8.0	75	89
no act. D	82	84

[a] Effect of actinomycin D on the percentage of fluorescent protoplasts when added at various times after inoculation with CPMV.
[b] Protoplasts were stained with fluorescent antibodies against CPMV after incubation for 42 h.

5.3.4. *Synthesis of CPMV top component in actinomycin D-treated protoplasts*

In order to distinguish between viral RNA and protein synthesis, CPMV-infected and healthy protoplasts, treated and not treated with actinomycin D, were labeled with ^{32}P and ^{3}H-leucine.

Upon incubation in the absence of actinomycin D the CPMV-infected protoplasts showed the production of M and B components (fig. 5.3). Whereas incorporation of ^{32}P into 40-60S material was similar in healthy and infected protoplasts, the amount of ^{3}H-radioactivity found in this region of the gradient was strikingly higher in infected protoplasts. In the presence of actinomycin D there was considerable incorporation of ^{3}H-leucine into material sedimenting at 40-60S, while ^{32}P radioactivity in these fractions was relatively low. No viral nucleoprotein particles were produced. The incorporation of ^{3}H-leucine into 40-60S material was about twice as high in the CPMV-infected sample as compared to the control. This might indicate that virus top component, which has a sedimentation coefficient of 58S, is synthesized in the CPMV-infected protoplasts after treatment with actinomycin D.

This supposition was confirmed by analysis of the polypeptide composition of the 60S material on polyacrylamide slabgels. Samples of healthy and infected protoplasts were labeled with ^{35}S-methionine in the presence or absence of actinomycin D. Homogenates of the protoplasts were run on sucrose density gradients and the 60S region of each gradient collected and subjected to gel electrophoresis. Figure 5.4 shows the presence of both viral coat proteins in the 60S region from infected, actinomycin D-treated protoplasts, demonstrating the presence of CPMV top component.

5.3.5. *Synthesis of CPMV-specific proteins in actinomycin D-treated protoplasts*

As virus coat proteins were synthesized in the absence of virus RNA replication, the possible synthesis of other virus-specific proteins was also investigated. Healthy and CPMV-infected protoplasts were labeled with ^{35}S-

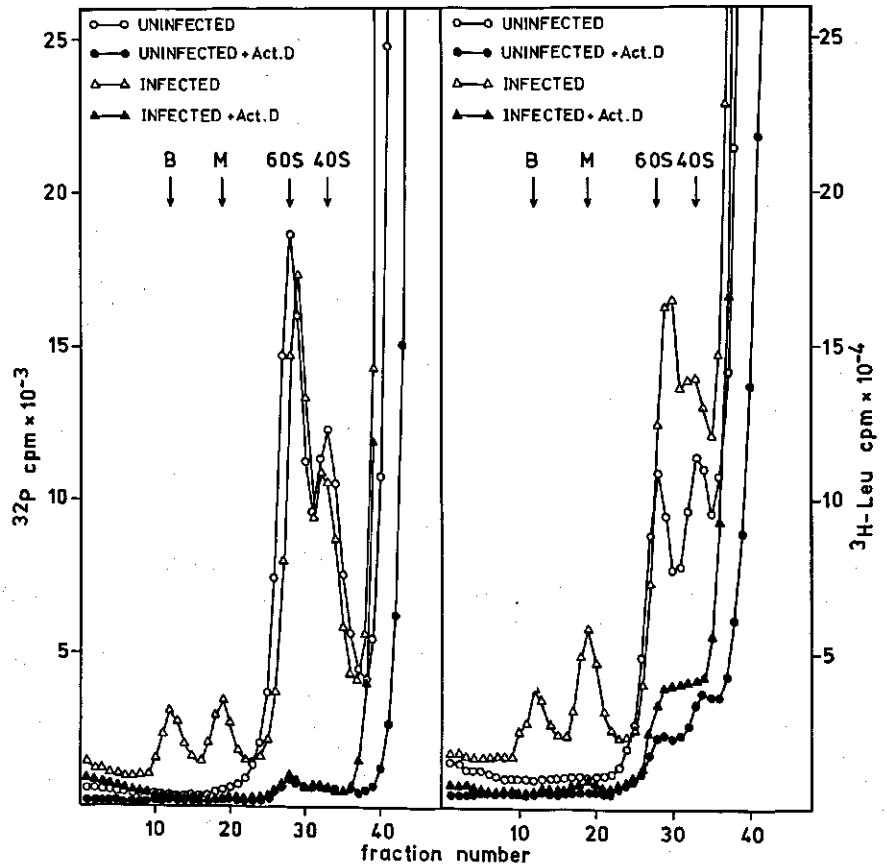

FIG. 5.3. Sucrose density gradient analysis of homogenates of healthy and infected protoplasts labeled at the same time with ^{32}P and ^{3}H-leucine in the presence or absence of actinomycin D. Protoplasts were incubated in 7 ml-portions at a concentration of 6.0×10^5/ml. Samples + actinomycin D were treated with actinomycin D (10 µg/ml) from the time of inoculation. All samples were labeled at 7.5 h after inoculation with a combination of ^{32}P (25 µCi) and ^{3}H-leucine (250 µCi). At t = 44 h protoplasts were harvested and processed as described.

methionine in the presence or absence of actinomycin D. Supernatant fractions (30,000 $\times g$) were prepared and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

The autoradiogram of the electrophoretic pattern of the labeled proteins is shown in figure 5.5. Several differences between the pattern of the infected protoplasts as compared to that of the healthy ones can be observed. Two proteins, whose molecular weights were estimated to be 170,000 and 21,000 daltons, are clearly distinguishable from host components. In addition, four proteins appear at places in the gel where there are also weaker protein bands in the samples of uninfected protoplasts. Their molecular weights were estimated to be 110,000, 84,000, 37,000 and 22,000. The 37,000 and 22,000 dalton proteins

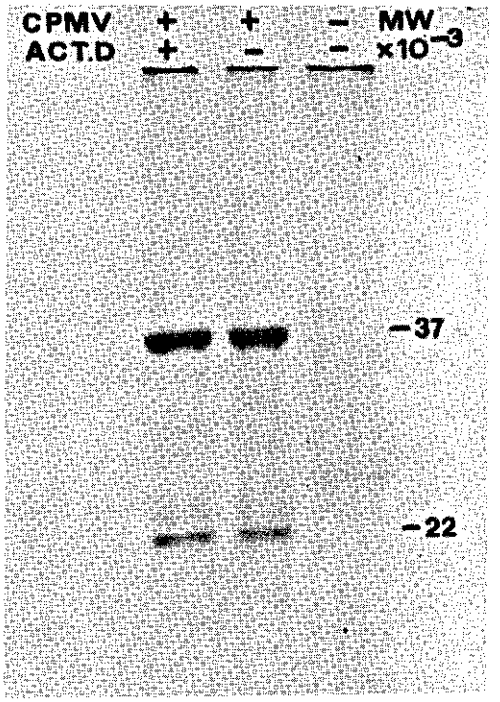

FIG. 5.4. Analysis of the polypeptide composition of the 60S fraction from cowpea protoplasts in which virus multiplication was inhibited by actinomycin D.

Actinomycin D (10 μg/ml) was added to 7.5 ml of CPMV-infected protoplasts immediately after inoculation; 2.5 h later 75 μCi ^{35}S-methionine was added and the protoplasts were incubated for 44 h. The protoplasts were then homogenized and analyzed by sucrose density gradient centrifugation. The 60S fraction of the gradient was collected and centrifuged for 16 h at 50,000 rpm in a Spinco Ti75 rotor. The pellet was dissolved in Laemmli sample buffer and, after heating, analyzed by electrophoresis on a 12.5 % polyacrylamide slabgel and by autoradiography (left). Samples of infected (middle) and healthy protoplasts (right), incubated without actinomycin D, were similarly processed and run on the same gel. Approximately equal amounts of radioactivity were applied to each slot. The positions of CPMV coat proteins, used as unlabeled markers, are indicated.

were found to have electrophoretic mobilities identical to the large and small viral coat protein (not shown). These two proteins, together with the 170,000 molecular weight polypeptide, always appeared as the most prominent virus-specific protein bands under the labeling conditions used.

Upon inhibition of CPMV multiplication with actinomycin D the synthesis of all virus-specific proteins was still very evident. Moreover, the relative intensities of their synthesis did not seem to be altered. It should be kept in mind, however, that on early administration of the drug total incorporation of ^{35}S-methionine was reduced to about half the amount of the untreated controls. Since in Fig. 5.5 equal amounts of radioactivity were applied to each slot, it can be deduced that under these conditions of labeling and inhibition of CPMV multiplication, virus-specific as well as host-specific protein synthesis were equally decreased to about half the amount of the untreated controls.

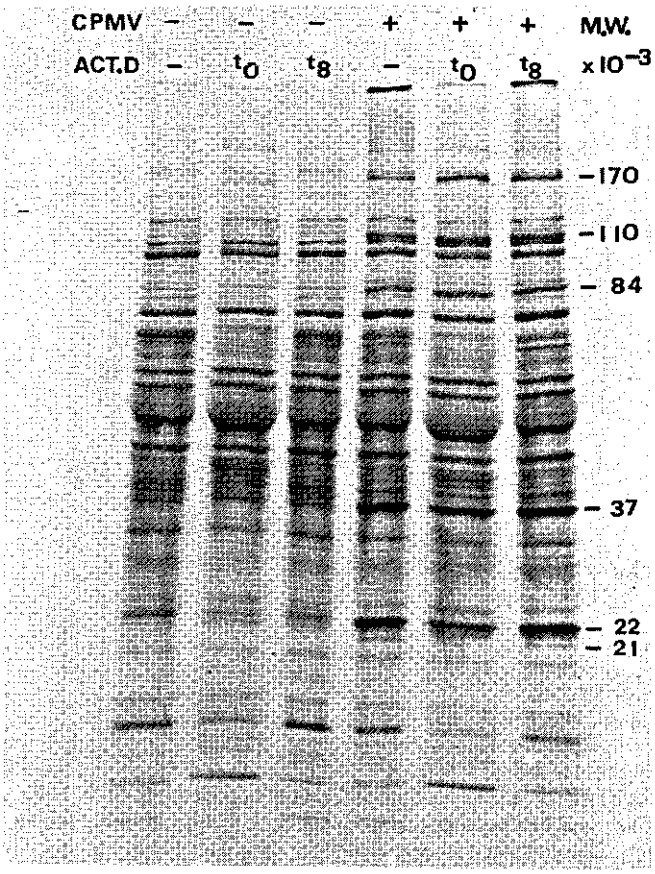

FIG. 5.5. Autoradiogram of ^{35}S-labeled proteins from the soluble fractions of protoplasts analyzed by polyacrylamide gel electrophoresis. Samples of healthy and CPMV-infected protoplasts (5 ml, 7.0 × 10^5/ml) were labeled with 80 µCi ^{35}S-methionine and incubated for 44 h.

Some samples were treated with actinomycin D (10 µg/ml) from the time of inoculation (t_0); to others the drug was added 8 h thereafter (t_8). Soluble protein fractions were prepared and equal amounts of TCA-precipitable counts were analyzed in 7–15% polyacrylamide slabgels as described.

5.4. DISCUSSION

From the pattern of actinomycin D inhibition it can be concluded that CPMV multiplication is in some way dependent on host DNA-specified RNA synthesis during the earliest stage of infection. Actinomycin D at a concentration of 10 µg/ml inhibited RNA synthesis in infected as well as uninfected protoplasts by more than 90% and prevented the production of CPMV nucleoprotein particles if the drug was added at the time of inoculation or immediately thereafter. The inhibitory effect of actinomycin D rapidly decreased when added at progressively later times after inoculation, and at 8 hours after inoculation, a stage at

which newly synthesized virus is not yet detectable, replication had become completely insensitive to the drug. These findings were confirmed by local lesion assays of extracts of infected protoplasts which were similarly treated (data not shown). They are in agreement with the results of LOCKHART and SEMANCIK (1968 and 1969) who reported that CPMV replication in cowpea hypocotyl tissue was inhibited by actinomycin D provided that the antibiotic was administered shortly after inoculation, but not in late phases of infection.

Inhibition of CPMV multiplication by actinomycin D is probably not due to interference with the process of viral RNA replication *per se*, since addition of the drug during the period of active RNA replication did not affect virus synthesis. Moreover, it has been shown that *in vitro* the enzymatic activity of partially purified CPMV replicase is negligibly sensitive to actinomycin D (ZABEL *et al.*, 1974).

DNA-dependent RNA synthesis as a prerequisite for RNA replication suggests the involvement of some essential virus-induced, but host-specified component. Two potential explanations may be proposed. One possibility is that a host-derived factor is required for a functional virus replication complex. Some such situation has been shown for the bacteriophage Qβ replicase, consisting of four different polypeptides (KAMEN, 1970; KONDO *et al.*, 1970) three of which are host-specified. The other possibility concerns the virus-specific cytopathic structures, the formation of which is induced by CPMV infection (DE ZOETEN *et al.*, 1974). These structures are likely to play an essential role in viral replication in view of the finding that CPMV-specific double-stranded RNA mainly appears in association with them. Involvement of host-directed protein synthesis is therefore imperative, since the necessary genetic information needed for this vast synthesizing activity is not likely to originate from the virus.

The expression of a specific part of the host DNA as one of the first steps in the multiplication of CPMV might well determine which cells are susceptible to the virus and which are not. As such, this host-specified component might play a decisive role in the determination of the host range of this virus.

Six virus-specific proteins were detected in CPMV-infected protoplasts. They were estimated to be 170, 110, 84, 37, 22 and 21 kilodaltons. From coelectrophoresis with solubilized proteins of purified CPMV the 37,000 and 22,000 dalton proteins probably represent the viral structural proteins. The continued synthesis of all six virus-specific proteins under conditions of inhibition of CPMV multiplication by early treatment with actinomycin D indicates that they are coded for by the viral genome. This means that the total coding capacity of the viral RNAs is greatly exceeded. *De novo* synthesis of virus-specific proteins under conditions of complete inhibition of multiplication was also demonstrated by the production of radioactively labeled top component. A similar situation in which viral RNA and nucleoprotein synthesis was inhibited, while the synthesis of empty capsids continued or even increased upon treatment of infected leaves with thiouracil has been reported for TYMV (FRANCKI and MATTHEWS, 1962; RALPH *et al.*, 1965). In these experiments, however, the same treatment had little effect on the synthesis of cellular nucleic acids (RALPH *et al.*, 1965).

Addition of actinomycin D resulted in a significant reduction of the incorporation of ^{35}S-methionine, whereas qualitatively the patterns of the proteins synthesized remained nearly unchanged. If this decrease in incorporating activity was only accounted for by depletion of host messenger RNA by the antibiotic this would indicate that mRNA in these cells is very stable having an average half life in the order of days. However, great care must be taken in interpreting such results since in animal cells it has been shown that actinomycin D can hamper the initiation of ribosomes on mRNA (SINGER and PENMAN, 1972). Moreover, we have found that actinomycin D treatment reduces the amount of acid-soluble radioactive amino acids within cowpea protoplasts, possibly by interference with the uptake of label (chapter 4).

As already stated, several plant RNA viruses have been shown to exhibit early sensitivity to actinomycin D. In cases in which this sensitivity could not be demonstrated, this may have escaped detection as a result of too late a time of addition or too low a dose of the drug. It is tempting to speculate, therefore, that inhibition of multiplication by actinomycin D is a common feature of all plant RNA viruses and, consequently, that the involvement of an early nuclear function is a general phenomenon.

6. PROTEIN SYNTHESIS IN COWPEA MOSAIC VIRUS INFECTED COWPEA PROTOPLASTS

I Detection of viral-related proteins

6.1. Introduction

Plant protoplasts appear to be suitable for the identification of virus-encoded proteins produced in the course of the infection process. Protoplasts are attractive for such a study as they can be infected synchronously to a high percentage. In addition, the infection of plant protoplasts with virus may lend itself to trace host proteins the synthesis of which is induced or stimulated by virus infection and which may play a role in the virus multiplication process.

Though there are now numerous reports on the infection of isolated leaf cell protoplasts of several plant species by a number of plant viruses, only a few papers have been published on the biochemical aspects of plant virus infection and multiplication in protoplasts. Protein synthesis in protoplasts after virus infection has only been studied with tobacco mosaic virus (TMV), cowpea chlorotic mottle virus (CCMV), and brome mosaic virus (BMV), all in tobacco mesophyll protoplasts. Infection with TMV revealed two apparently virus-specific proteins with molecular weights of 165,000 and 135,000 besides the 17,500-dalton virus coat protein (Sakai and Takebe, 1974; Paterson and Knight, 1975; Siegel *et al.*, 1978a). Sakai *et al.* (1977) reported the synthesis in CCMV-infected protoplasts of two proteins with molecular weights of 35,000 and 100,000 in addition to the 19,000-MW capsid protein. Proteins of similar size plus a 107,000-dalton species were observed upon infection with the related virus BMV (Sakai *et al.*, 1979).

In the previous chapter we have presented evidence that in cowpea mesophyll protoplasts infected with cowpea mosaic virus (CPMV) seven virus-specific proteins with molecular weights of 170,000, 110,000, 87,000, 84,000, 37,000, 22,000 and 21,000 daltons are synthesized. In the present chapter these results have been extended and detailed. By fractionation of the protoplasts in several fractions and by applying short labeling periods at different intervals during the infection cycle more than ten viral-related proteins were demonstrated.

6.2. Materials and methods

6.2.1. *Materials*

Digitonin was obtained from Sigma Chemical Co.. Proteins used as markers for polyacrylamide gel electrophoresis were phosphorylase A (M.W. 92,500), catalase (M.W. 57,000) and lactate dehydrogenase (M.W. 35,000) from Boehringer Mannheim GmbH; β-galactosidase (M.W. 116,000), transferrin (M.W. 80,000), γ-globulins (M.W. 54,000 and 23,500) and ovalbumin (M.W. 46,000)

from Sigma Chemical Co.; bovine serum albumin (M.W. 68,000) from Schwarz/Mann; and myosin (M.W. 200,000) which was a gift from Dr. H. Pelham, Cambridge, England.

6.2.2. *Isolation and inoculation of protoplasts*

Protoplasts were isolated from leaves of *Vigna unguiculata* (L.) Walp. var. 'Blackeye Early Ramshorn' and inoculated with CPMV as described in chapter 3. Protoplasts mock-infected with UV-inactivated CPMV were used as controls. Complete inactivation of virus was achieved by exposing purified CPMV in an open petri dish for 30 min to a TUV 15W Philips lamp at a distance of 30 cm.

6.2.3. *Incubation and labeling of protoplasts*

Inoculated protoplasts were washed three times with sterile 0.6 M mannitol containing 10 mM $CaCl_2$, suspended in the medium described in chapter 3 at a concentration of $5-7 \times 10^5$ protoplasts/ml and divided into samples of $3-5 \times 10^6$ protoplasts. They were incubated at 25°C under constant illumination of about 10,000 lux. At selected intervals samples of protoplasts were labeled with 30 μCi/ml ^{35}S-L-methionine for 6 hours. At the end of the incubation period the protoplasts were collected by centrifugation and stored frozen at –80°C. The percentage of infected protoplasts was determined by fluorescent antibody staining of samples of unlabeled protoplasts incubated for 44 hours. After such an incubation period more than 70% of the protoplasts were still living and the percentage of infected protoplasts was about 85%.

6.2.4. *Subcellular fractionation of protoplasts*

Protoplasts were fractionated and prepared for electrophoresis by the procedure depicted in fig. 6.1. The frozen protoplast samples were thawed and homogenized for 5 min in a minipotter with 0.5 ml of homogenization buffer (HB) composed of 0.05 M Tris-CHl (pH 7.4), 0.01 M KCl, 0.001 M EDTA, 0.005 M $MgCl_2$, 0.06 M β-mercaptoethanol, 0.001 M phenylmethylsulfonylfluoride, 0.4 mM L-methionine and 10% (w/w) sucrose. Unbroken cells were removed by centrifugation for 30 sec at 500 × *g*. The homogenate was centrifuged at 1,000 × *g* (3,000 rpm) for 15 min at 4°C in a Sorvall SS 34 rotor. The 1,000 × *g* pellet was washed once by resuspension in 0.2 ml HB and centrifugation. The washed pellet was extracted with 0.4 ml HB lacking $MgCl_2$, to which 0.2% (w/v) digitonin had been added (detergent buffer). The suspension was centrifuged for 15 min at 1,000 × *g* to give the 1,000 × *g* pellet and 1,000 × *g* extract (fig. 6.1).

To the 1,000 × *g* supernatant was added 1/3 volume of glycerol and the solution was then centrifuged at 15,500 rpm for 30 min at 4°C in a Sorvall SS 34 rotor to give the 30,000 × *g* supernatant (fig. 6.1) and a pellet. The pellet was washed with 0.1 ml HB and subsequently extracted twice with 0.04 ml of detergent buffer. Both extracts were combined to give the 30,000 × *g* extract, whereas the residue constituted the 30,000 × *g* pellet (fig. 6.1).

Prior to electrophoresis the 1,000 × *g* and 30,000 × *g* pellet residues were

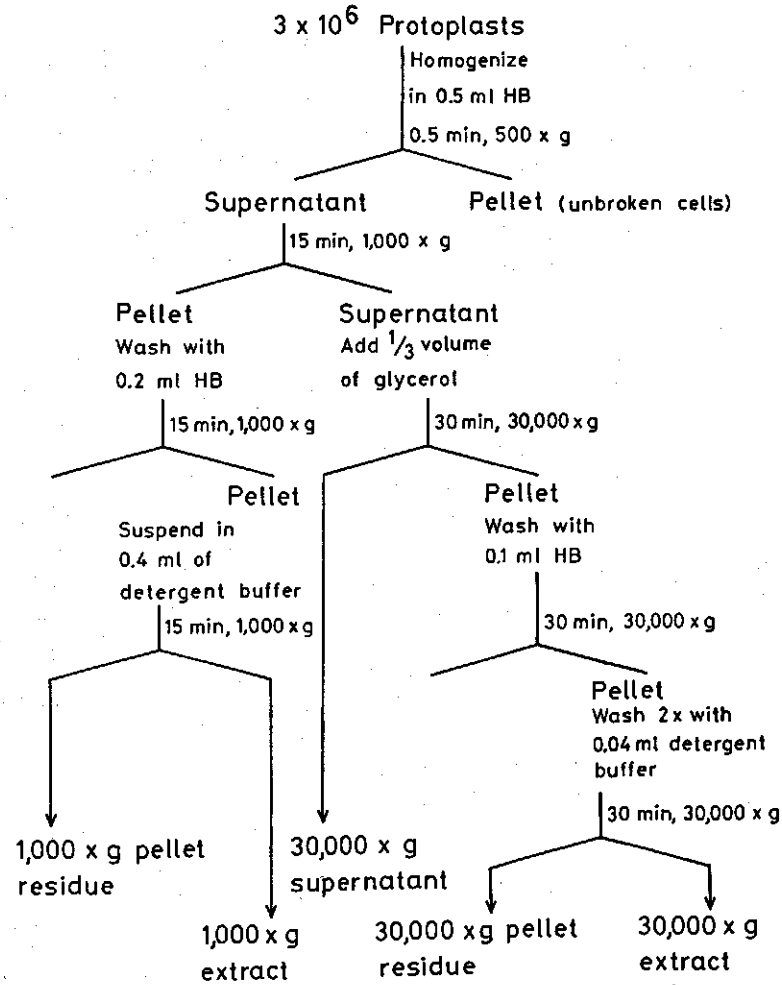

FIG. 6.1. Scheme for the subcellular fractionation of cowpea mesophyll protoplasts.

suspended in 0.6 ml and 0.1 ml of Laemmli sample buffer (LSB), respectively, (LAEMMLI, 1970) and heated at 100°C for 3 min to obtain a clear solution. To the 30,000 $\times g$ supernatant and the 1,000 $\times g$ and 30,000 $\times g$ extracts was added $\frac{1}{2}$ volume of three times concentrated LSB and the solutions were then heated at 100°C for 3 min. The amount of trichloroacetic acid (TCA) precipitable radioactivity in each fraction was determined as described previously (chapter 4).

6.2.5. *SDS-polyacrylamide slabgel electrophoresis*

Proteins were analyzed in slabgels by the discontinuous SDS-gel system as described by LAEMMLI (1970) and modified by MARSDEN *et al.* (1976) using the Pharmacia Gelelectrophoresis Apparatus GE4. The dimensions of the separating gel were 0.25 × 8 × 12 cm on top of which a 1 cm stacking gel was layered. In all experiments a 7–15% linear acrylamide gradient was used in the separating

gel and the stacking gel contained 4 % acrylamide. Samples not larger than 75 µl were loaded into the wells. Electrophoresis was at 75 V until the bromophenol blue dye had entered the separating gel. The voltage was then increased to 200 V and electrophoresis was continued for about 6 h. Gels were stained with Coomassie Brilliant Blue R250, destained as described by KEDINGER *et al.* (1974) and dried for autoradiography. The molecular weights of CPMV-related proteins were determined by comparing their electrophoretic mobilities in polyacrylamide gels with the marker proteins enumerated above (see Materials).

6.3. RESULTS

6.3.1. *Radioactivity incorporation into subcellular fractions of protoplasts*

In order to examine if a specific subcellular fraction is particularly involved in the multiplication of CPMV the amount of ^{35}S-methionine which was incorporated into various fractions of CPMV-infected and uninfected protoplasts was compared.

Healthy and infected protoplasts were labeled with ^{35}S-methionine from 19–25 h after inoculation when the rate of virus multiplication is at its maximum (HIBI *et al.*, 1975). The protoplasts were fractionated according to the scheme in fig. 6.1 and the total amount of TCA precipitable radioactivity in each fraction was determined. The results given in table 6.1 show that more than half of the incorporated ^{35}S-methionine is found in the 30,000 × *g* supernatant. More than 30 % is in the 1,000 × *g* fraction the major part of which remained bound to the pellet residue after extraction with detergent buffer. No significant differences occurred in the distribution of incorporated radioactivity between corresponding fractions of healthy and CPMV-infected protoplasts. No such differences were found upon labeling for a shorter period or at a different stage of infection, but the relative incorporation into the 30,000 × *g* supernatant fraction tended to

TABLE 6.1. Distribution of radioactivity incorporated into different subcellular fractions of CPMV-infected and uninfected protoplasts.[a]

Fraction	Healthy		Infected	
	cpm × 10^{-6}	%	cpm × 10^{-6}	%
1,000 × *g* pellet residue	10.3	22	18.1	29
1,000 × *g* extract	4.7	10	4.4	7
30,000 × *g* pellet residue	2.8	6	3.1	5
30,000 × *g* extract	1.6	3	1.0	2
30,000 × *g* supernatant	27.0	58	35.0	57
	46.4	100	61.6	100

[a] Samples of 3 × 10^6 protoplasts in 5 ml incubation medium were labeled with ^{35}S-methionine from 19–25 h after inoculation and processed as described.

increase during the course of the incubation, especially in the infected sample, probably as a result of virus accumulation. Total radioactivity incorporation was always found to be higher in CPMV-infected as compared to uninfected protoplasts but this divergence only became apparent around 15 h after inoculation when rapid virus multiplication resulted in accumulation of virus particles.

The rate of protein synthesis in all fractions gradually increased during incubation both in infected and uninfected protoplasts. Radioactivity incorporation measured during various 6 h periods gradually increased and reached a level about three times the starting value 30 h after inoculation (see chapter 4). Although the uptake of labeled amino acid into the acid-soluble fraction of the protoplasts also slightly increased during incubation this cannot explain the higher incorporation.

6.3.2. *Polyacrylamide gel electrophoresis of viral-related proteins*

Samples of subcellular fractions of uninfected and CPMV-infected protoplasts labeled with ^{35}S-methionine during different 6 h periods in the infection cycle were analyzed on 7–15 % polyacrylamide gradient slabgels in the presence of SDS.

A. 30,000 × *g* supernatant. Two viral-related proteins were observed in CPMV-infected protoplasts labeled between 9 and 15 h after inoculation (fig. 6.2). Their molecular weights were estimated to be 170,000 and 30,000. They represented the first detectable differences in the electrophoretic patterns of healthy and infected protoplasts since no such differences were observed during the first 9 hours of infection. When labeling from 16–22 h after inoculation the synthesis of seven more viral-related proteins became apparent. They were estimated to be 130, 110, 87, 84, 37, 24 and 23 kilodaltons[1]. Compared with the 9–15 h interval the intensities of the 170,000 and 30,000 dalton protein bands had increased during this labeling period. A further increase in the rate of synthesis of these and the other proteins was apparent between 26 and 32 h after inoculation during which phase exactly the same set of viral-related proteins was found. A few more proteins in the samples of CPMV-infected protoplasts were sometimes detected which were not present in uninfected protoplasts such as the protein bands appearing in the gel just below the 170,000 dalton protein band. Their significance and the reason for the variability of their synthesis is still unclear.

Only the 170,000 and 130,000 dalton proteins in the pattern from infected protoplasts were clearly distinguishable from host proteins. The other seven polypeptides appeared to have electrophoretic mobilities similar to those of proteins also present in uninfected protoplasts. These proteins may either be newly synthesized or they may be cellular proteins, whose synthesis is stimulated upon infection.

[1] The 24 and 23 kilodalton proteins correspond with the two polypeptides previously estimated to be 22 and 21 kilodaltons, respectively (chapter 5; ROTTIER *et al.*, 1979).

The overall pattern of cellular protein synthesis was not greatly affected by CPMV infection since essentially all normal host proteins were present in infected protoplasts in comparable quantities. Only a few minor changes were observed, the disappearance of a host protein with a molecular weight of about 145,000 being the most pronounced. These changes became apparent only at later stages of infection.

B. 30,000 × *g* and 1,000 × *g* pellet residues. The autoradiogram of the electrophoretic analyses of 30,000 × *g* pellets after extraction with detergent buffer (*fig.* 6.3) showed a diffuse dark background but some obvious differences between samples of healthy and infected protoplasts emerged. Again a protein with

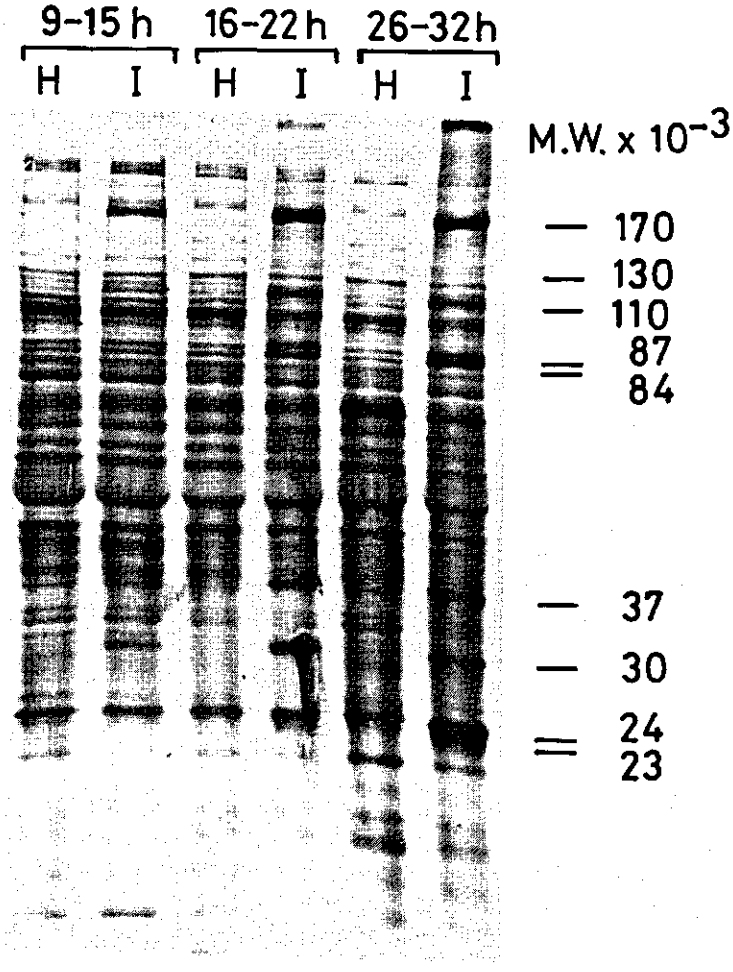

FIG. 6.2. Autoradiogram of SDS-polyacrylamide slabgel electrophoretic analysis of 30,000 × *g* supernatant fractions of CPMV-infected (I) and uninfected (H) protoplasts which had been labeled with ^{35}S-methionine during various 6 h periods after inoculation.

an apparent molecular weight of 170,000 appeared as the first detectable difference when protoplasts were labeled from 9–15 h after inoculation, but the 30,000 dalton protein was absent. Soon after, upon labeling between 16 and 22 h post inoculation, more viral-related protein bands became visible: polypeptides with molecular weights of 110,000, 37,000 and 23,000 also found in the supernatant fraction; the 130,000, 87,000, 84,000, 30,000 and 24,000 dalton polypeptides that were present in the soluble fraction did not appear here. On the other hand one extra protein band was observed, which was always somewhat broad and diffuse and present in the molecular weight region of 68,000. All these proteins were synthesized at higher rates later in infection during the 26–32 h labeling period (fig. 6.3).

The 1.000 × *g* pellet residues revealed the same set of viral-related proteins with the exception of the 110,000 dalton polypeptide, the latter probably due to the even higher background of radioactivity than was the case in the 30,000 × *g* pellet residues (data not shown).

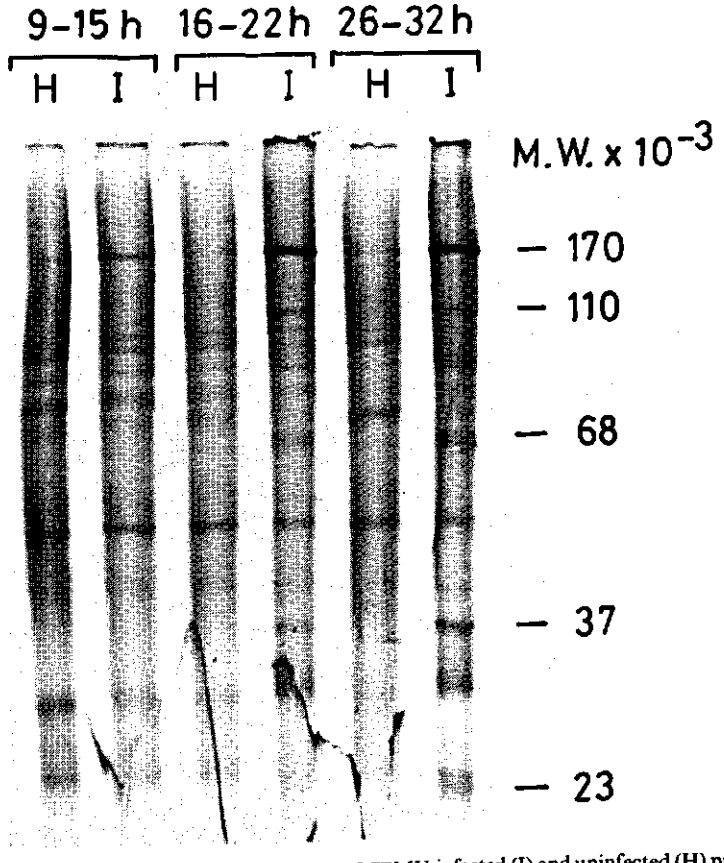

FIG. 6.3. Analysis of 30,000 × *g* pellet residues of CPMV-infected (I) and uninfected (H) protoplasts labeled with ^{35}S-methionine during various 6 h periods after inoculation.

C. 30,000 × *g* and 1,000 × *g* extracts. As is clear from figures 6.4 and 6.5 treatment of the true pellets with buffer containing digitonin without magnesium ions resulted in extracts having low backgrounds of radioactivity upon polyacrylamide gel electrophoresis. Six viral-related polypeptides were detected in both extracts with molecular weights of 170,000, 112,000, 110,000, 37,000, 24,000 and 23,000; of these only the 112,000- and 110,000-dalton polypeptides were distinct from host proteins. In these, as in other fractions, the 170,000-MW polypeptide was the first viral-related protein that was detectable, the others becoming increasingly apparent about 16 h after infection.

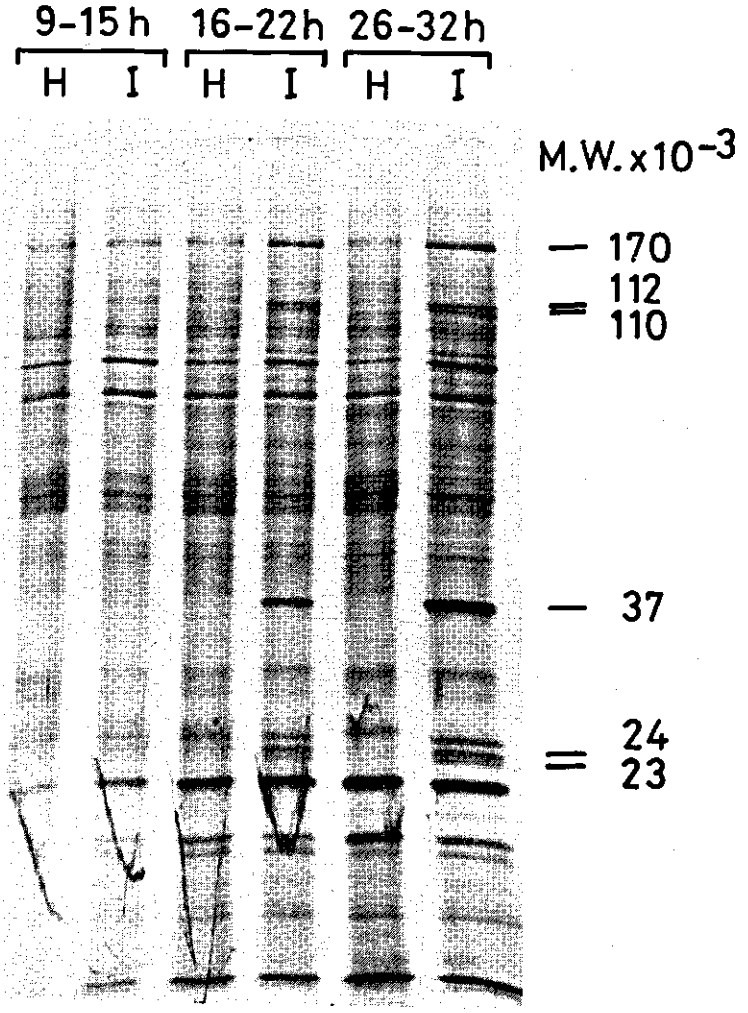

FIG. 6.4. Analysis of extracts of 30.000 × *g* pellets from CPMV-infected (I) and uninfected (H) protoplasts labeled with ^{35}S-methionine during various 6 h periods after inoculation.

6.3.3. *Comparison of viral-related proteins from different fractions*

The viral-related proteins found in the various subcellular fractions were compared by simultaneous electrophoresis on the same slabgel. It was found that the proteins to which the same molecular weights had been assigned migrated with equal mobilities in gels of different acrylamide composition (data not shown). From this it was concluded that they were indeed identical resulting in the overall picture of viral-related proteins shown in table 6.2. Eleven such proteins were reproducibly detectable. At least those which were found in the supernatant fraction could also be detected upon labeling with ^{3}H-leucine.

If a viral-related protein has no equivalent in the healthy protoplast sample it

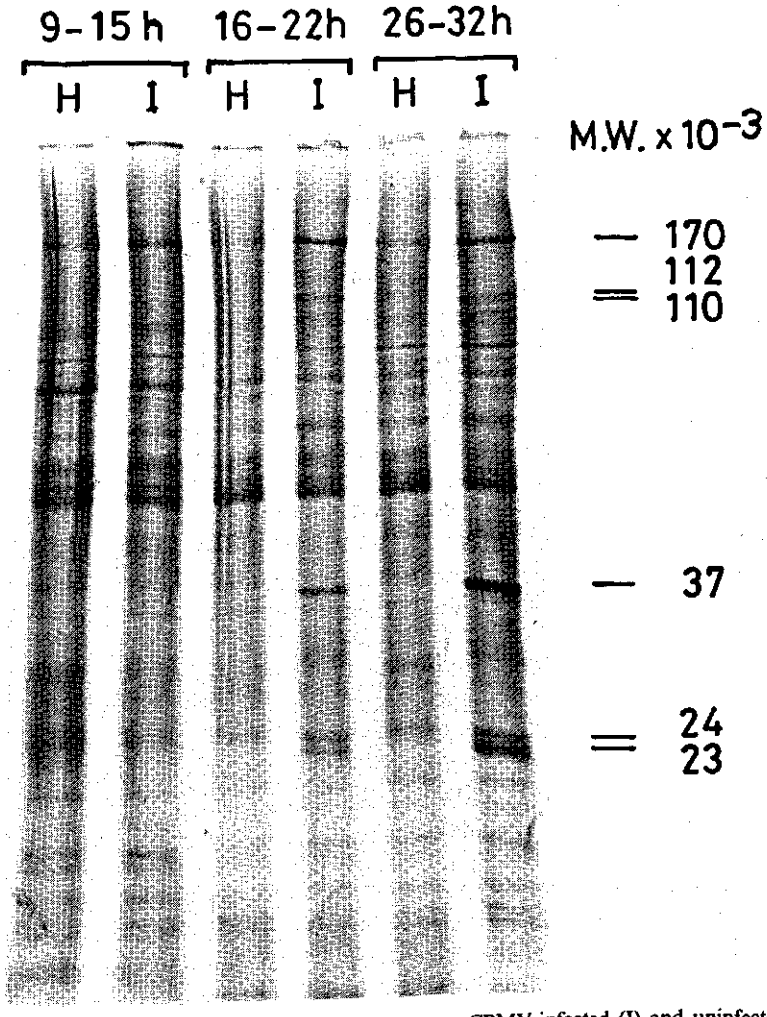

FIG. 6.5. Analysis of extracts of 1,000 $\times g$ pellets from CPMV-infected (I) and uninfected (H) protoplasts labeled with ^{35}S-methionine during various 6 h periods after inoculation.

TABLE 6.2. CPMV-related proteins in fractions of CPMV-infected cowpea protoplasts.[a]

MW × 10^{-3}	30,000 × *g* supernatant	30,000 × *g*		1,000 × *g*	
		pellet residue	extract	pellet residue	extract
170	+ (n)	+	+	+	+
130	+ (n)	−	−	−	−
112	−	−	+ (n)	−	+ (n)
110	+	+ (n)	+ (n)	−	+ (n)
87	+	−	−	−	−
84	+	−	−	−	−
68	−	+ (n)	−	+ (n)	−
37	+	+ (n)	+	+ (n)	+
30	+	−	−	−	−
24	+	−	+	−	+
23	+	+ (n)	+	+ (n)	+

[a] The presence of a particular protein in a particular fraction is indicated by + and −, respectively, while n denotes that there is no protein with the same electrophoretic mobility in the corresponding preparation of uninfected protoplasts.

may be considered to be induced by virus infection. The results obtained with different fractions of CPMV-infected and uninfected protoplasts were not always consistent. The 170,000-dalton polypeptide, for instance, is clearly a novel protein, since no host protein of the same size is detectable when comparing the 30,000 × *g* supernatant fractions. In the pellet fractions, however, this polypeptide was accompanied by a host protein of similar electrophoretic mobility. The reverse holds true for the 110,000-MW protein among others. As judged from the pellet fractions, this protein seems to be virus-induced but the presence of a protein of apparently similar size in the soluble fractions of uninfected protoplasts suggests differently. This feature has been included in table 6.2 from which it can be inferred, mainly due to the fractionation, that at least seven of the viral-related proteins are probably synthesized *de novo*.

6.4. DISCUSSION

The synthesis of eleven viral-related proteins during infection of cowpea mesophyll protoplasts with CPMV has been demonstrated. Their detection was strongly enhanced by or in some cases (*e.g.* 112,000-dalton polypeptide) even dependent on two conditions: (i) radioactive labeling of the protoplasts for only a few hours at an appropriate point during the infection cycle; and (ii) preparation of subcellular fractions with subsequent treatment of the particulate fractions with a weak detergent in the absence of magnesium ions. Protoplasts appeared to be effectively fractionated as judged both by visual inspection of the fractions (*e.g.* an almost colourless supernatant fraction) and of the polyacrylamide gel patterns. This contrasts sharply with the problems encountered when

using intact leaf tissue. With unfractionated protoplast preparations the analysis of the autoradiograms was greatly hampered by the presence of a general high background-blackening which, according to the developed procedure, remained confined exclusively to the pellet residues upon fractionation. A similar continuous background of radioactivity, which could not be removed by extraction of lipids with acetone, has been reported by ELLIS (1977a) after electrophoresis of the thylakoid fraction of isolated pea chloroplasts incubated with ^{35}S-methionine.

As previously mentioned, studies of protein synthesis in plant virus-infected protoplasts revealed only three or four viral-related polypeptides (SAKAI and TAKEBE, 1974; PATERSON and KNIGHT, 1975; SAKAI *et al.*, 1977; SIEGEL *et al.*, 1978a; SAKAI *et al.*, 1979). Our observations suggest that the possible detection of other proteins may well have been impeded by experimental conditions since in all cases analysis was done using unfractionated solubilized samples of protoplasts which were usually labeled for relatively long periods.

All CPMV-related proteins were most clearly observed at the stage of infection when new virus is actively being synthesized. Only two proteins, namely the 170,000- and 30,000-dalton proteins, were demonstrable earlier in the infection process, from about 10 h after inoculation, *i.e.* before the onset of rapid virus accumulation. The rate of radioactivity incorporation into these two proteins increased more than the general rise in incorporation that occurred during protoplast incubation. Thereafter, between about 16 and 32 h post-inoculation, the intensities of their labeling seemed to roughly parallel or slightly exceed this increase in overall radioactivity incorporation. These observations suggest that their rates of synthesis rapidly increase between about 10 and 15 h after inoculation but then decrease or become constant. The same considerations seem to apply to the other viral-related proteins with the difference that their synthesis probably starts later. This might suggest that the preceding synthesis of the 170,000- and/or 30,000-dalton protein is a prerequisite for the other viral-related proteins to appear.

No viral-related polypeptides could be observed during the latent phase of the infection cycle. This is surprising since some virus-induced protein synthesis might well be expected to precede the formation of the first progeny particles. The pronounced sensitivity of CPMV multiplication to actinomycin D during the very first hours after inoculation (see chapter 5) strengthens this view. Also unexpected was the absence of detectable viral coat proteins immediately after this latent phase when the first infectivity becomes extractable, *i.e.* between 9 and 15 h after inoculation (HIBI *et al.*, 1975). Presumably, the levels of such proteins do not exceed the lower detection limit of the present methods of analysis.

CPMV infection gives rise to the formation of virus-specific cytopathic structures (DE ZOETEN *et al.*, 1974; HIBI *et al.*, 1975) which are presumed to have an essential function in viral RNA replication (DE ZOETEN *et al.*, 1974). The synthesis of these structures, however, was not reflected by a greatly altered pattern of protein synthesis in the membrane fractions or a stimulated synthesis of host proteins involved in membrane development. Two virus-induced polypeptides

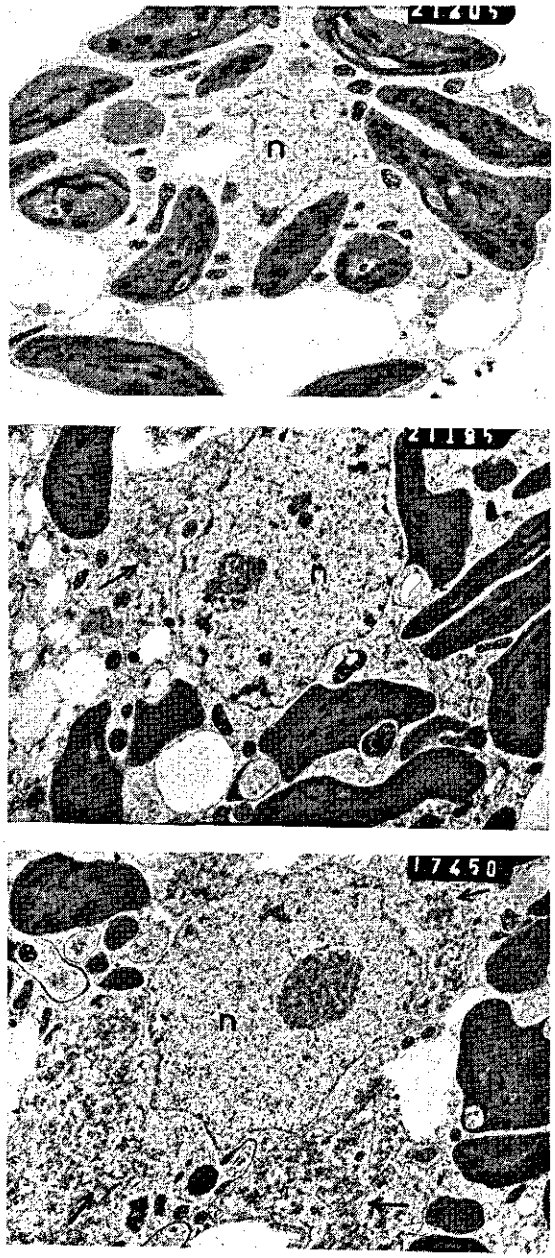

FIG. 6.6. Electron micrographs of cowpea protoplasts infected with CPMV. Samples of protoplasts were fixed at 8 h, 16 h and 24 h after inoculation as described by HIBI *et al.* (1975). Arrows indicate thy cytopathological structures that start to clearly appear at 16 h after inoculation and predominate the cytoplasm at 24 h after inoculation (n = nucleus).

were specifically found in the particulate fractions: a 112,000-MW polypeptide, only detectable in the extracts of these fractions, while a 68,000-dalton protein was present in the pellet residues. Their time-course of appearance paralleled that of the cytopathic structures no sign of which was visible 8 h after inoculation and only traces 16 h after inoculation, but which were clearly developing 24 h after inoculation (fig. 6.6). The synthesis of more membrane-associated proteins may well have been obscured by the high background of the pellet residues, especially that of the 1,000 $\times g$ pellet. Application of chloramphenicol to the protoplasts to inhibit mitochondrial and chloroplast protein synthesis as has been used by some authors (SAKAI and TAKEBE, 1974; PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978a) did not reduce this background of radioactivity.

The synthesis of protoplast proteins did not seem to be drastically affected by CPMV infection. Essentially all host proteins that are produced in healthy protoplasts are equally well synthesized during infection. This contrasts with the observations on animal picornaviruses, among others, where a general inhibition of host protein synthesis is usually found (CARRASCO, 1977), but is in agreement with results obtained using tobacco mesophyll protoplasts infected with TMV (PATERSON and KNIGHT, 1975; SIEGEL *et al.*, 1978a). Incorporation of radioactive amino acids was always found to be higher in CPMV-infected as compared to uninfected cowpea protoplasts. Together with the observed unaffected synthesis of the normal host proteins this supports the hypothesis put forward by SIEGEL *et al.* (1978a) that virus-specific protein synthesis is not at the expense of host protein synthesis, but in addition to it.

Clearly the sum of the molecular weights of the observed CPMV-related proteins largely exceeds the total coding capacity of the viral RNAs. In the subsequent chapter we report on our efforts to identify some of the virus-related proteins and there the implications for the mechanism of the synthesis of CPMV-specific proteins will be discussed.

7. PROTEIN SYNTHESIS IN COWPEA MOSAIC VIRUS INFECTED COWPEA PROTOPLASTS

II Further characterization of viral-related proteins

7.1. INTRODUCTION

In the preceding chapter it was demonstrated that in cowpea mesophyll protoplasts infected with CPMV eleven proteins are synthesized which either do not occur in healthy cells or are synthesized in much lower amounts. In this chapter experiments are reported which aim at the identification of the origin of the proteins: are they coded by the viral genome or the host genome and, if viral-coded, are they translated from M-RNA or B-RNA of CPMV?

As the sum of the molecular weights of the 11 proteins characteristic for CPMV-infected protoplasts by far exceeds the coding capacity of the CPMV RNAs, some of the proteins may be specified by the host. The possibility of the induction of synthesis of host proteins after CPMV infection is also supported by the finding that the multiplication of CPMV RNA is prohibited by actinomycin D (chapter 5) indicating the need for the expression of some host function(s) for virus replication. On the other hand, CPMV RNAs may be translated into large precursor polypeptides from which different virus-specific proteins arise by processing of the precursor possibly via several intermediate polypeptides. This possibility is supported by the results of *in vitro* translation experiments with CPMV RNAs. In cell-free protein synthesizing systems derived from wheat germ (DAVIES *et al.*, 1977) and rabbit reticulocytes (PELHAM and JACKSON, 1976; PELHAM and STUIK, 1977) M-RNA of CPMV produces two polypeptides with molecular weights of 105,000 and 95,000. Translation of B-RNA produces 170,000- and 30,000-MW polypeptides. In neither *in vitro* system are the capsid proteins with molecular weights of 37,000 and 24,000 synthesized. These results at least suggest the possibility that the virus coat proteins arise by processing from a precursor protein although other explanations are possible as well; the synthesis of the coat proteins may, for example, be mediated by subgenomic messenger RNAs generated only *in vivo* during virus multiplication.

In this chapter an attempt is made to characterize the viral-related proteins found in CPMV-infected protoplasts by comparing their mobility with those of the virus coat proteins, and with the products of *in vitro* translation of the CPMV RNAs by electrophoresis in SDS-polyacrylamide gels, and further by immunoprecipitation with CPMV antiserum. Inoculation of cowpea protoplasts with separate bottom and middle component of CPMV was used to trace the role of the component RNAs in the synthesis of the various proteins. Finally, some attempts to demonstrate processing of precursor proteins or the occurrence of subgenomic viral messenger RNAs are reported.

7.2. Materials and methods

7.2.1. *Materials*

The amino acid analog, DL-p-fluorophenylalanine, the protease inhibitors, L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK) and N-α-p-tosyl-L-lysine-chloromethyl ketone hydrochloride (TLCK), and the sugar analog, 2-deoxy-D-glucose (grade III) were purchased from Sigma Chemical Co.. Oligo(dT)-cellulose type 7 was obtained from P-L Biochemicals and protein A-Sepharose CL-4B was from Pharmacia. Products of *in vitro* translation of CPMV RNAs were kindly donated by Drs. J. W. Davies, R. Goldbach and E.Stuik. The sources of other materials have been mentioned in the preceding chapters.

7.2.2. *Preparation, inoculation and culture of protoplasts*

Protoplasts were prepared from *Vigna unguiculata* (L.) Walp. var. 'Blackeye Early Ramshorn', inoculated with the yellow Nigerian strain of CPMV and incubated for virus multiplication as described in the previous chapters. For labeling with $^{32}PO_4$ the incubation medium was replaced by a phosphate-deficient medium one hour before the addition of ^{32}P-labeled phosphate.

7.2.3. *Subcellular fractionation of protoplasts and polyacrylamide gel electrophoresis of protein fractions*

Unless otherwise stated the same techniques were used as before (chapter 6).

7.2.4. *Isolation of CPMV components from protoplast homogenates by sucrose density gradient centrifugation*

Protoplasts were homogenized, the homogenates were clarified by low-speed centrifugation and run on isokinetic sucrose density gradients as described in chapter 5. After centrifugation the gradients were fractionated and the zones containing the viral components, as shown by the radioactivity profile and by comparison with the 254-nm extinction profile of purified CPMV run on a parallel gradient, were collected.

7.2.5. *Immunoprecipitation*

Aliquots of 15 or 20 µl of 30,000 × *g* supernatant fractions of ^{35}S-methionine-labeled protoplasts were diluted to 50 µl with water and an equal volume of buffer containing 0.02 M sodium phosphate, 1.8% NaCl, 2% Triton X-100, 1% sodium deoxycholate, 0.2% SDS (pH 7.2) was added, followed by the addition of 1 µl of anti-CPMV serum. The titre of the antiserum was 1/512 as determined by agar immunodiffusion test with purified CPMV at a concentration of 1 mg/ml. The mixture was incubated overnight at 4 °C. Then 20 µl of a 10% (v/v) suspension of protein A-Sepharose in immunoprecipitation buffer (0.01 M sodium phosphate, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.2) was added and the sample was further incubated for 30 min at room temperature. The suspension was centrifuged for 3 min at 9,800 × *g* in a Janetzki

centrifuge. The supernatant was removed and the pellet was washed three times by suspending in 0.5 ml immunoprecipitation buffer and centrifugation. The final pellet was suspended in 75 µl Laemmli sample buffer (LAEMMLI, 1970), heated for 3 min at 100 °C and the supernatant obtained after clarification by low-speed centrifugation was analyzed by polyacrylamide gel electrophoresis.

7.2.6. *Purification of CPMV components*

Middle and bottom components of CPMV were prepared by sucrose density gradient centrifugation of purified virus in a Ti-15 zonal rotor (Beckman) using 15–30% (w/w) linear sucrose gradients essentially as described by DE JAGER (1978).

7.2.7. *Isolation, oligo(dT)-cellulose chromatography and polyacrylamide gel electrophoresis of RNA*

RNA from $^{32}PO_4$-labeled protoplasts was isolated by cesium chloride centrifugation essentially as described by GLISIN *et al.* (1974). To a frozen protoplast pellet, 3 ml of dissociation buffer and 3 g of solid CsCl was added. The sample was briefly mixed on a Vortex to dissolve the protoplasts and the salt and heated for 5 min at 65 °C. The composition of the dissociation medium was 0.05 M Tris-HCl, 0.1 M NaCl, 0.01 M EDTA, 2% sodium lauryl sarcosinate, 1% sodium deoxycholate, 2% sodium p-aminosalicylate, 0.5% disodium triisopropylnaphtalene sulphonate, pH 8.2. The solution was then layered onto 1.2 ml 5.7 M CsCl, 0.02 M Tris-HCl, 0.1 M EDTA (pH 8.2) in a polyallomer tube and centrifuged for 20 h at 35,000 rpm and 18 °C in a Beckman SW 50.1 rotor. The RNA pellet was dissolved in 0.4 ml 0.01 M Tris-HCl, 0.01 M EDTA (pH 8.0), precipitated with ethanol, dried and dissolved in 0.1 ml 0.01 M Tris-HCl (pH 8.0). Samples for direct electrophoretic analysis were diluted 1:1 with 0.01 M EDTA, 0.1% bromophenol blue in 100% formamide and heated for 3 min at 60°C. Fractionation of RNA into poly(A)$^-$ and poly(A)$^+$ fractions by oligo(dT)-cellulose chromatography was carried out according to PEMBERTON *et al.* (1975). RNA was analyzed by electrophoresis in 2.7% polyacrylamide slabgels (15 × 11 × 0.2 cm) at 160 V as described by PEACOCK and DINGMAN (1968). Gels were dried on Whatman 3 MM paper and exposed to Kodak Royal X-Omat film for autoradiography.

7.3 RESULTS

7.3.1. *Identification of CPMV coat proteins*

When the solubilized proteins of purified CPMV were compared by SDS-polyacrylamide gel electrophoresis with the viral-related proteins of CPMV-infected protoplasts, the large capsid protein comigrated with the 37,000-dalton polypeptide. The small capsid protein was found to vary considerably in size depending on the particular virus isolate; it sometimes migrated with the 24,000- or 23,000-MW polypeptide but was often apparently smaller (data not shown).

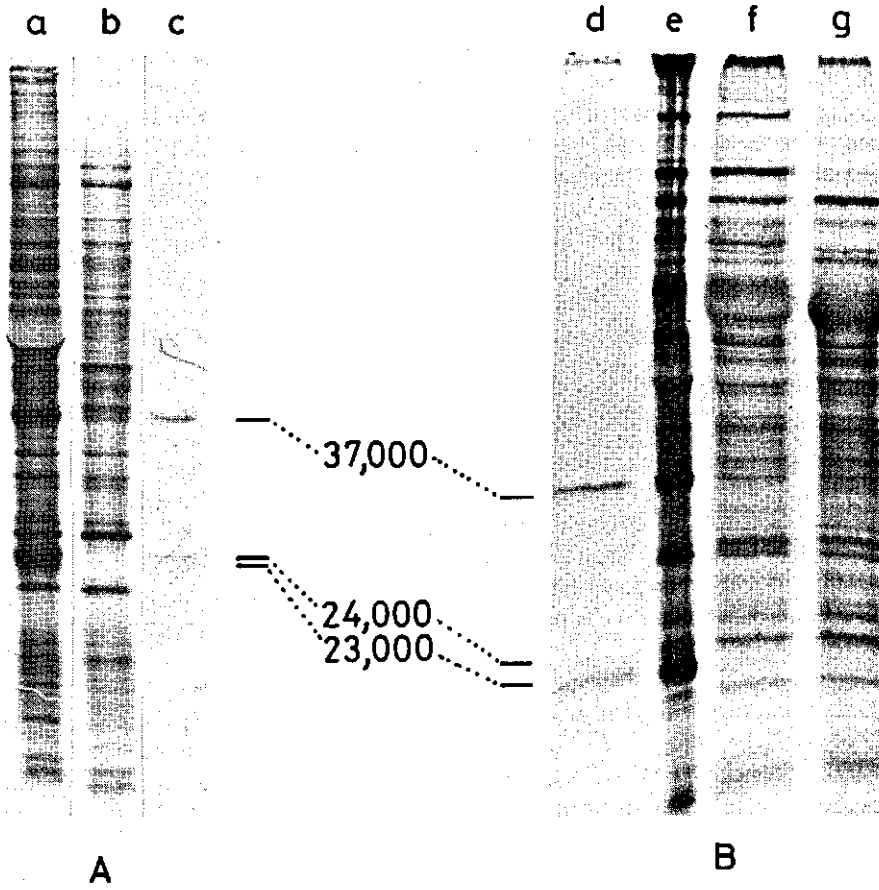

FIG. 7.1. Identification of CPMV coat proteins.

A. CPMV-infected protoplasts were labeled with ^{35}S-methionine from 21 to 26 h after inoculation. They were then harvested, homogenized and the homogenate was run on a sucrose density gradient. Samples were taken for electrophoretic analysis of the labeled proteins:

- track a: sample from the homogenate before centrifugation
- track b: sample from the top layer of the gradient after centrifugation
- track c: sample from a fraction of the gradient containing CPMV middle component.

B. Electrophoretic analysis of samples of a 30,000 $\times g$ supernatant fraction of CPMV-infected protoplasts labeled with ^{35}S-methionine from 25 to 32 h after inoculation, before and after immunoprecipitation with anti-CPMV serum:

- track d: sample from the immunoprecipitate
- track e: sample from the 30,000 $\times g$ supernatant fraction before addition of the antiserum
- track f: sample from the 30,000 $\times g$ supernatant fraction after immunoprecipitation
- track g: sample from a comparable 30,000 $\times g$ supernatant fraction from uninfected protoplasts after immunoprecipitation.

If a 30,000 $\times g$ supernatant fraction of CPMV-infected protoplasts was layered on a sucrose density gradient and centrifuged, the 37,000-, 24,000- and 23,000-dalton proteins (fig. 7.1A) were recovered from the regions of the gradient containing the centrifugal components of CPMV as shown by centrifugation of purified virus on a parallel gradient. Of the other viral-related proteins, the 170,000- and 30,000-MW proteins in one experiment were shown to remain at the top of the gradient; the others were not resolved.

The same three polypeptides disappeared from the electrophoretic pattern after immunoprecipitation of capsid protein with anti-CPMV serum (fig. 7.1B). The immunoprecipitate showed only the 37,000-, 24,000- and 23,000-dalton polypeptides upon electrophoresis in SDS-gels and none of the other viral-related proteins of the 30,000 $\times g$ supernatant.

Similar results were obtained when extracts of the particulate fractions of CPMV-infected protoplasts prepared by treatment with a buffer containing digitonin (chapter 6) were analyzed. Obviously, the polypeptides with molecular weights 37,000, 24,000 and 23,000 were present in CPMV particles, since no proteins of similar size were detectable after removal of virions by ultracentrifugation or immunoprecipitation. No other viral-related protein seems to have serological relationships with the coat proteins, as they all remain soluble under conditions in which the coat proteins are immunoprecipitated with anti-CPMV serum.

7.3.2. *Comparison of viral-related proteins with in vitro translation products of CPMV RNAs*

In fig. 7.2 the CPMV-related proteins detectable in 30,000 $\times g$ supernatant fractions of infected protoplasts are compared by SDS-polyacrylamide gel electrophoresis with the polypeptides synthesized under the direction of CPMV RNAs in two *in vitro* protein synthesizing systems. The 170,000- and 30,000-dalton proteins induced upon infection *in vivo* migrate with the same mobility as the polypeptides translated from B-RNA in a nuclease-treated lysate of rabbit reticulocytes. The products obtained with B-RNA in a wheat germ extract did not migrate to the same positions. M-RNA in both *in vitro* systems is translated into two polypeptides of molecular weights of about 105,000 and 95,000. These do not correspond to any of the viral-related proteins produced in CPMV-infected protoplasts. Although fig. 7.2 suggests that the mobility of the larger M-RNA product from the rabbit reticulocyte lysate is identical to the *in vivo* 110,000 dalton protein, further examination by electrophoresis in other gels proved this not to be the case. No mature coat proteins were detectable among the polypeptides synthesized in any of the *in vitro* translation systems. Treatment of the *in vitro* products with anti-CPMV serum confirmed this finding: no specific polypeptides were precipitated. It would appear that the proteins with molecular weights 170,000, 105,000, 95,000 and 30,000 which are synthesized *in vitro* do not bear antigenic determinants of the coat proteins.

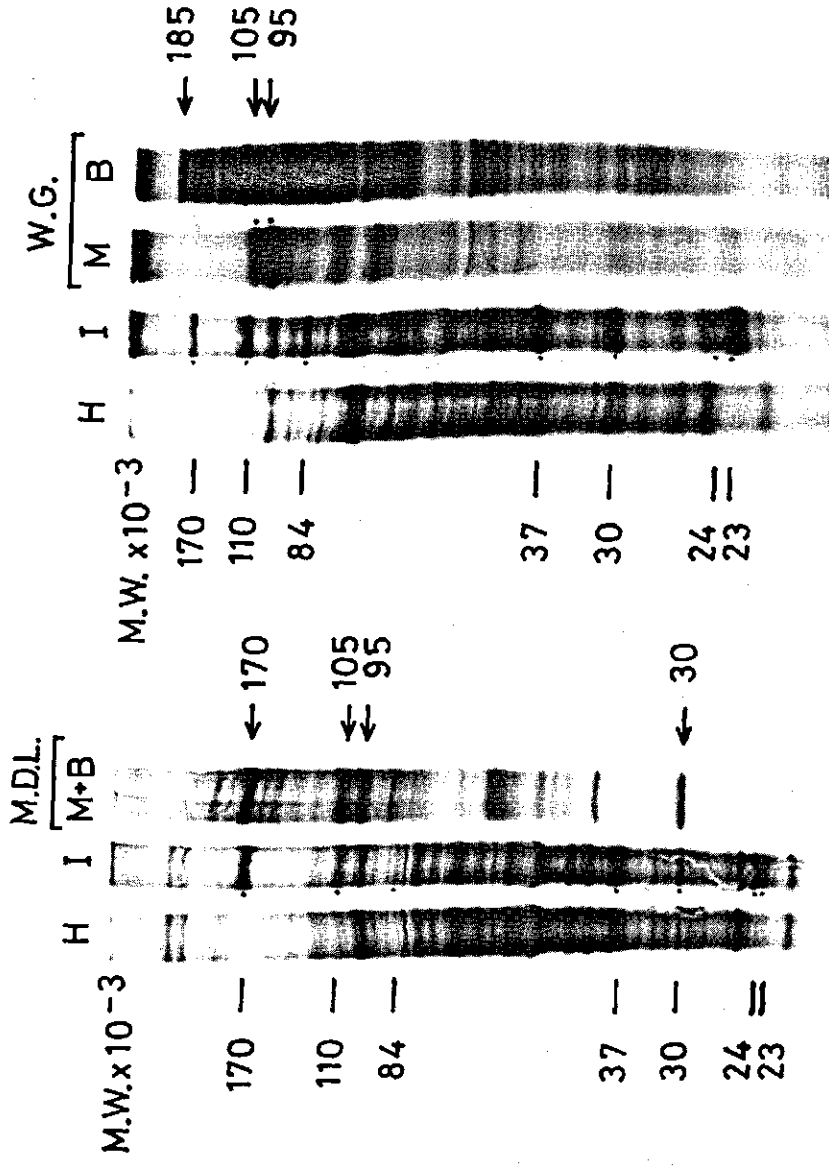

FIG. 7.2. Comparison of viral-related proteins from CPMV-infected protoplasts with *in vitro* translation products of CPMV RNAs.

Proteins present in 30,000 × *g* supernatant fractions from CPMV-infected (I) and uninfected (H) protoplasts labeled with ^{35}S-methionine during a 25–32 h interval after inoculation were compared by polyacrylamide gel electrophoresis with the *in vitro* translation products obtained with a mixture of CPMV M- and B-RNA in the messenger-dependent lysate (M.D.L.) of rabbit reticulicytes (left) and with the separate RNAs in the wheat germ (W.G.) system (right).

7.3.3. *Protein synthesis in cowpea protoplasts inoculated with separate components of CPMV*

Bottom and middle components of CPMV were purified by zonal sucrose density gradient centrifugation and the purity of the components was checked by local lesion assay on Pinto half leaves (table 7.1). After two cycles of centrifugation, the middle component had only very little residual infectivity. Similar preparations of bottom component were also only slightly infectious. Infectivity was fully restored by mixing the separate middle and bottom components of the virus. The residual infectivity of both component preparations could not be significantly reduced by further recentrifugation in sucrose density gradients while equilibrium density gradient centrifugation in cesium chloride resulted in unacceptable loss of biological activity of the preparations. Problems in obtaining bottom component free of infectivity have also been reported by others (VAN KAMMEN, 1968; DE JAGER, 1978).

When cowpea protoplasts were inoculated with separate CPMV components, almost no infectivity was produced as determined by local lesion assay of protoplast homogenates. Control levels of infectivity were obtained upon inoculation of protoplasts with the mixture of the two components.

The low yield of virus produced with the separate component preparations was also evident from immunoprecipitation experiments with CPMV antiserum (table 7.1): the radioactivity precipitable from the homogenates of ^{35}S-methionine-labeled protoplasts inoculated with middle or bottom component hardly exceeded the background.

Protein synthesis in protoplasts inoculated with separate CPMV nucleoprotein components was studied as before, except that after radioactive labeling and homogenization protoplasts were fractionated by centrifugation only into a

TABLE 7.1. Assay of purity of CPMV nucleoprotein components.

Inoculum	Local lesion assay[a]		immunoprecipitable radioactivity (cpm)[b]
	directly	protoplast homogenate	
M	0.1	0.2	1512 (2.7)
B	3.8	1.3	2044 (3.6)
M + B	100	100	56700 (100)
buffer	–	–	1400 (2.5)

[a] Preparations of M- and B-component of CPMV were assayed for infectivity directly on 6 Pinto half leaves either separately or in combination. Alternatively, samples of protoplasts were inoculated with the separate components (7 µg/ml) or with a mixture of the two (both 7 µg/ml) and infectivity generated during a 24 h incubation was determined by local lesion assay of protoplast homogenates. The results are expressed as percentages of local lesions relative to the inoculum containing both components.

[b] Similar samples of protoplasts were incubated and labeled with ^{35}S-methionine (30 µCi/ml) from 15 to 25 h after inoculation. Radioactivity immunoprecipitable with anti-CPMV serum from 15 µl aliquots of 30,000 × *g* supernatant fractions prepared from the protoplasts was measured. Figures in parentheses represent the percentages of ^{35}S-methionine in the immunoprecipitates calculated with reference to the sample from protoplasts inoculated with the mixture of both components.

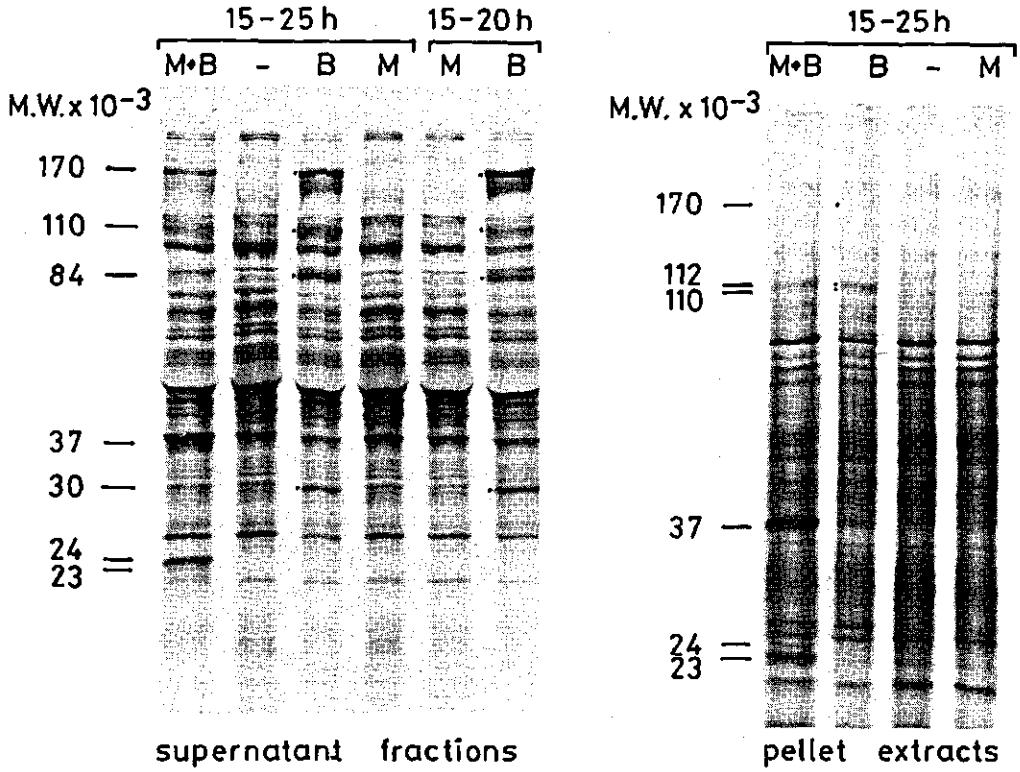

FIG. 7.3. Analysis of protein synthesis in protoplasts inoculated with separate nucleoproteins of CPMV.

Samples of protoplasts inoculated with M-component (7 μg/ml), B-component (7 μg/ml), a mixture of M- and B-component (7 + 7 μg/ml) or buffer only were incubated and labeled with ^{35}S-methionine (30 μCi/ml) either from 15–20 h or from 15–25 h after inoculation. Homogenates of the protoplasts were divided by centrifugation into a 30,000 $\times g$ pellet and a 30,000 $\times g$ supernatant fraction. The left part of the figure shows an autoradiogram of the electrophoretic analysis of supernatant fractions. The autoradiogram on the right shows the electrophoretic patterns of extracts from 30,000 $\times g$ pellet fractions of protoplasts prepared by extraction of the pellets with detergent buffer.

30,000 $\times g$ pellet and a 30,000 $\times g$ supernatant. Gel electrophoretic analyses of fractions thus obtained are shown in fig. 7.3. No viral-related proteins were detected in protoplasts upon inoculation with middle component alone, the electrophoretic patterns being identical with those of uninfected controls. In contrast, inoculation with bottom component gave rise to the synthesis of all viral-related proteins that were produced upon infection using a mixture of both components, except for the proteins with molecular weights of 37,000, 24,000 and 23,000 previously identified as the viral coat proteins. As far as can be judged from the autoradiograms, the proteins of 170, 112, 110, 84 and 30 kilodalton appeared to be synthesized in quantities comparable to control protoplasts infected with both components. The 130, 87 and 68 kilodalton polypeptides were not resolved in these experiments.

7.3.4. *The stability of the CPMV-related proteins*

In order to examine the possible occurrence of precursor-product relations between the different viral-related proteins, pulse-chase type experiments were performed. To obtain sufficient incorporation of radioactivity into the relevant proteins, labeling periods of 4–6 h were required since the uptake of amino acids by cowpea protoplasts appeared to be too slow to allow shorter pulses. For the same reason, incubation for several hours in the presence of unlabeled methionine was necessary to get a chase effect. The autoradiogram of fig. 7.4 illustrates the results of such an experiment in which protoplasts were labeled for 6 h with ^{35}S-methionine which was then chased by further incubation of the protoplasts for 19 h in a medium without radioactivity but containing 10 times as much unlabeled methionine. Protoplasts harvested directly after the labeling period and after the chase were fractionated and equal amounts of TCA-precipitable radioactivity were analyzed by polyacrylamide slabgel electrophoresis. Almost all viral-related proteins were very stable: no appreciable turnover occurring during the long chase

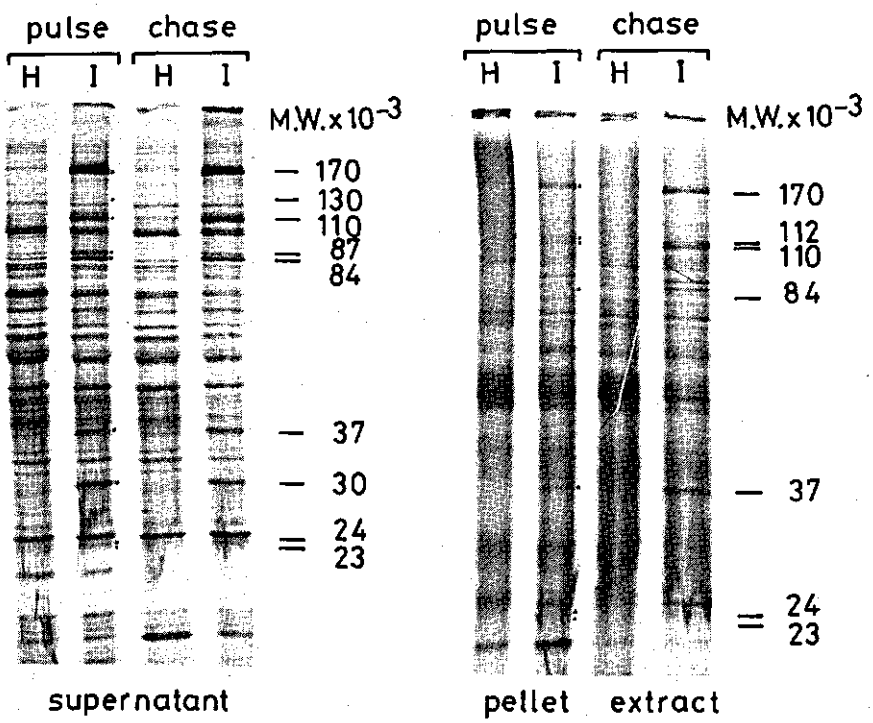

FIG. 7.4. Pulse-chase experiments with CPMV-infected protoplasts.

Protoplasts were incubated in the presence of ^{35}S-methionine (30 μCi/ml) from 19–25 h after inoculation with CPMV. The incubation medium was then removed, protoplasts were washed once with 0.6 M mannitol containing 10 mM $CaCl_2$ and 1 mM $MgSO_4$, and further incubated for 19 h in incubation medium containing unlabeled methionine (0.5 mM). Samples of healthy (H) and CPMV-infected (I) protoplasts harvested immediately after the pulse and after the chase period were fractionated and analyzed by polyacrylamide gel electrophoresis.

period was observed. This was also the case with the 68,000-dalton protein present in the pellet residue fractions (not shown). An exception was the polypeptide with a molecular weight of about 87,000 daltons detectable in the soluble fraction, the intensity of which was much weaker after the chase. Since the corresponding protein in the sample of uninfected protoplasts showed the same behaviour, this 87,000-dalton protein is probably host-coded. Another exception was the polypeptide migrating with an apparent molecular weight of 24,000 known to represent the small viral coat protein, but the decrease in the intensity of this band was not always discernable due to the presence of host proteins migrating with similar electrophoretic mobilities. Since previous experiments had indicated that this protein does not occur free in the cytoplasm of the protoplasts, but is present in viral particles, its fate was studied by analysis of these particles. Again, ^{35}S-methionine labeling and chase experiments were carried out as before and the CPMV components were isolated from sucrose density gradients run with homogenates of infected protoplasts. They were subsequently disrupted in SDS and analyzed by electrophoresis in a 10% polyacrylamide slabgel. When virion polypeptides were studied after protoplasts had been labeled for 5 hours, the small coat protein appeared to be present mainly as a polypeptide with an estimated molecular weight of 23,600 as shown for middle component in fig. 7.5. This was

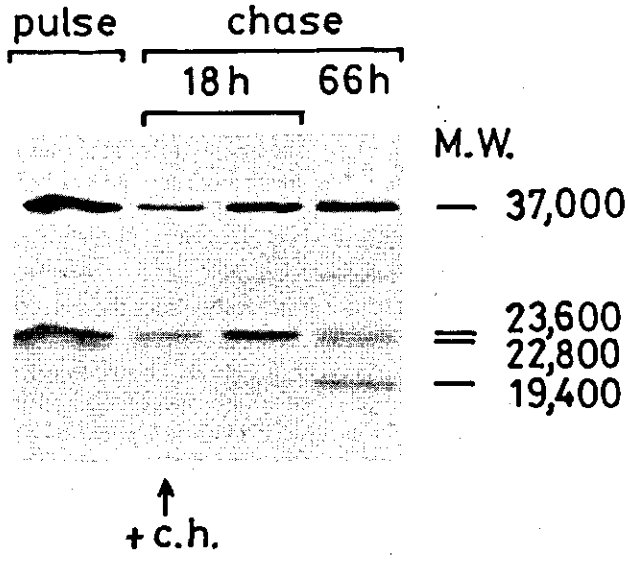

FIG. 7.5. Conversion of the smaller CPMV coat protein.

CPMV-infected protoplasts were labeled with ^{35}S-methionine from 21–26 h after inoculation. The radioactive medium was then removed, protoplasts were washed once with 0.6 M mannitol containing 10 mM $CaCl_2$ and 1 mM $MgSO_4$, and further incubated in incubation medium containing unlabeled methionine (10 mM). To one sample cycloheximide (c.h.) (1 µg/ml) was added. Samples of the protoplasts were taken immediately after the labeling period and after an 18 h or 66 h chase period. Protoplasts were homogenized and run on sucrose density gradients as described. The proteins present in the fractions containing the viral components were analyzed by polyacrylamide gel electrophoresis in a 10% gel as is shown for CPMV M component.

observed irrespective of the time after inoculation at which the labeling was done. Usually a faint band at a position of about 22,800 dalton was also visible, especially after longer exposure of the protoplasts to the radioactivity. When after the labeling, the protoplast incubation was continued in the presence of unlabeled methionine, the intensity of the 23,600-dalton polypeptide decreased, and the 22,800-dalton band often became more pronounced. If chasing was continued long enough, a new protein species of about 19,400 dalton clearly appeared. This was found to occur similarly with all three centrifugal CPMV components. The processing of the 23,600-MW protein into the smaller species was inhibited when after the labeling period protein synthesis was shut off by means of cycloheximide (fig. 7.5). A protein band of variable intensity was often visible in the gel in the region between the large and small coat proteins. Its presence did not depend on the method of preparation of the viral components since it was also observed when virus was isolated by immunoprecipitation. The nature of this protein which migrated distinct from the 30,000-MW viral-related polypeptide detectable in the supernatant fraction of CPMV-infected protoplasts is unclear, but it might be a degradation product of the large viral coat protein.

In CPMV preparations isolated from virus-infected cowpea leaves, the same set of coat protein species could be detected. In addition, two more proteins were stained in gels run with virus preparations that had been isolated late in infection, especially when plants were grown at higher temperatures, *e.g.* 30°C. The molecular weights of these proteins were estimated to be 18,000 and 16,500.

The above experiments indicate that the 170, 130, 112, 110, 84, 37 and 30 kilodalton viral-related proteins are stable products, that the 87,000 dalton protein is an unstable host-specific species and that the small viral coat protein undergoes a number of distinct cleavages occurring sequentially after assembly into the viral particles.

7.3.5. *Attemps to demonstrate precursor polypeptides*

The above type of experiment did not inherently allow the detection of short-living precursor polypeptides. To further investigate the possible occurrence of such protein species the effects of several compounds, known in other systems to interfere with proteolytic cleavage, were studied. They were selected and applied at concentrations so as to significantly inhibit CPMV multiplication at any time of the infection cycle without affecting the integrity of the protoplasts. They were added 24 h after inoculation, *i.e.* one hour before the 7 h labeling period of the protoplasts with ^{35}S-methionine was started. The polyacrylamide gel electrophoretic analysis of the proteins synthesized during this period is shown in fig. 7.6 for the 30,000 $\times$ *g* supernatant fractions, and similar conclusions could be drawn from the analyses of the others.

Treatment of protoplasts with the amino acid analog p-fluorophenylalanine (1 mM) markedly changed the pattern of proteins synthesized when compared with control samples, but the resulting patterns of CPMV-infected and uninfected protoplasts were indistinguishable: neither the known viral-related proteins nor any possible virus-specific precursor proteins appeared. The almost complete

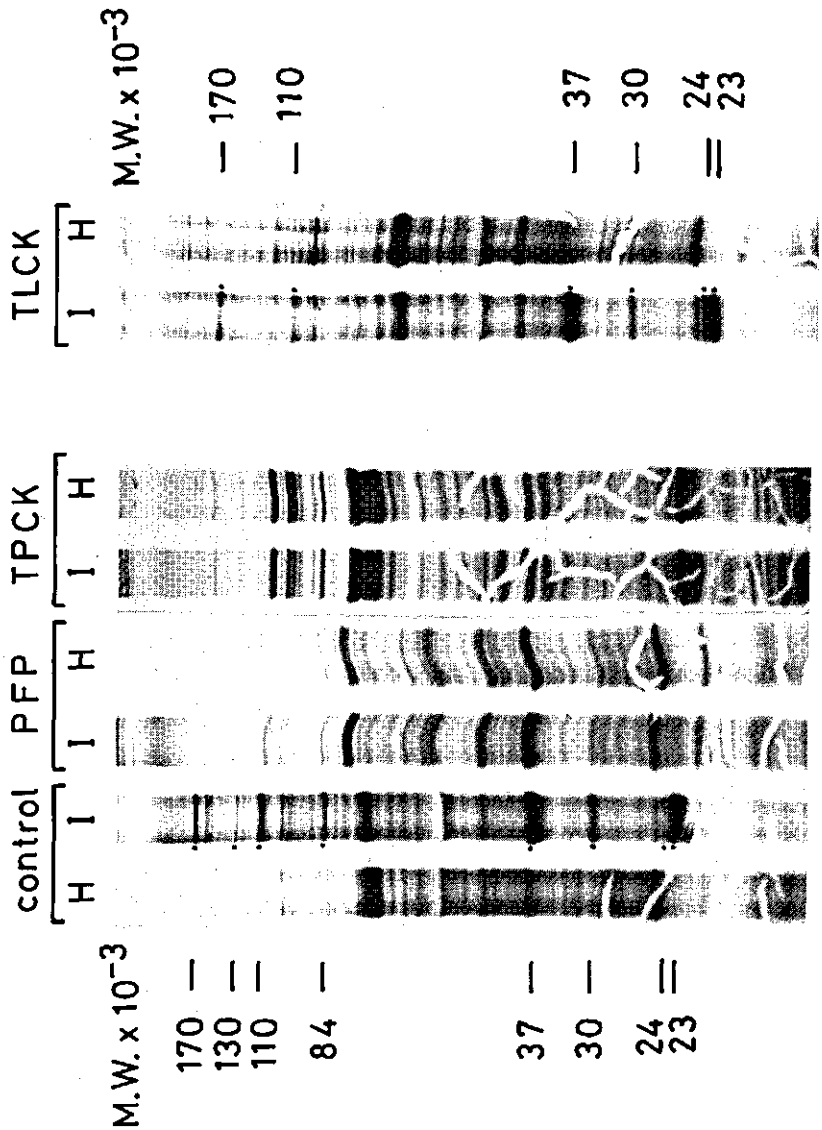

FIG. 7.6. The effect of p-fluorophenylalanine, TLCK and TPCK on the synthesis of viral-related proteins in CPMV-infected protoplasts.

Samples of healthy (H) and CPMV-infected (I) protoplasts were prepared and incubated in parallel. After a 24 h incubation period p-fluorophenylalanine (PFP), TLCK or TPCK was applied to different samples to a concentration of 1 mM (PFP and TLCK) or 50μM (TPCK). One hour later ^{35}S-methionine (20 μCi/ml) was added and incubation continued for 7 h. Protoplasts were then harvested and processed for polyacrylamide gel electrophoretic analysis of the labeled proteins.

reduction in virus yield caused by the analog might well be linked up with its effect on protein synthesis, since the incorporation of ^{35}S-methionine was lowered by about 80%. Similar results were obtained with the protease inhibitor TPCK (50μM). In contrast, TLCK (1 mM) hardly affected protein synthesis in the protoplasts. In the presence of this protease inhibitor, almost all viral polypeptides normally appeared except for the species with molecular weights of 130,000, which was absent, and 84,000, which was considerably inhibited. These specific effects which were not accompanied by the simultaneous accumulation of any possible precursor(s) for these proteins presumably play a role in the approximately 75% decrease in CPMV production caused by this drug. TLCK expressed its protease-directed property as shown by the observation that the processing of the small viral coat protein was affected by the drug: whereas most of this protein was present at

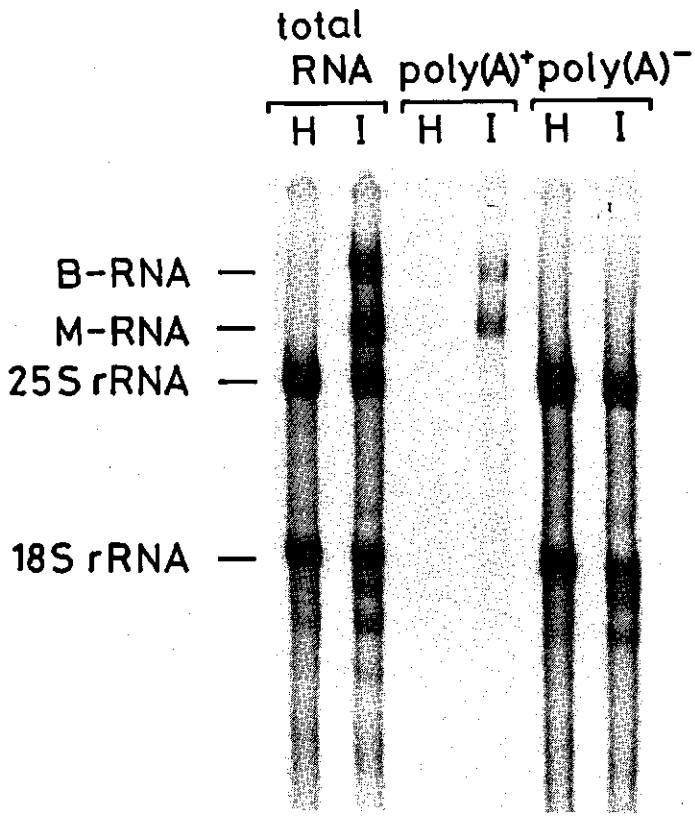

FIG. 7.7. Analysis of virus-specific RNA synthesis in CPMV-infected protoplasts.

Samples of healthy (H) and CPMV-infected (I) protoplasts were incubated in the presence of $^{32}PO_4$ (80 μCi/ml) from 22–25 h after inoculation. One hour before addition of the label the protoplasts were transferred to an incubation medium lacking phosphate. Protoplasts were harvested, RNA was isolated and fractionated into a poly(A)-containing and a poly(A)-deficient fraction by oligo(dT)-cellulose chromatography. Samples were taken before and after fractionation and analyzed by polyacrylamide slabgel electrophoresis.

23,000-dalton size in the infected control sample, the quantity of its 24,000-dalton precursor was clearly increased by TLCK treatment. No significant effect on protein synthesis was observed upon incubation of CPMV-infected protoplasts in the presence of 2-deoxy-D-glucose, an inhibitor of animal virus glycoprotein processing (data not shown). All viral-related proteins were synthesized including the 130 and 84 kilodalton polypeptides though the yield of CPMV was reduced by about 80%.

7.3.6. *Polyacrylamide gel electrophoretic analysis of CPMV-specific RNA synthesis*

In order to investigate the possible occurrence of subgenomic viral messengers such as have been found with some animal and plant RNA viruses, protoplasts were labeled with $^{32}PO_4$ at different time intervals in the infection cycle. RNA was isolated and analyzed by electrophoresis in 2.7% polyacrylamide slabgels. An example is given in fig. 7.7. Upon labeling from 22–25 h after inoculation, the two full-length CPMV RNAs clearly appeared. No other differences were found between the electrophoretic patterns of healthy and CPMV-infected samples. Fractionation of the RNA by oligo(dT)-cellulose chromatography into a poly(A)-containing and a poly(A)-deficient fraction led to the same conclusion.

7.4. Discussion

For some of the 11 viral-related proteins which are found in cowpea protoplasts after infection with CPMV it has been possible to determine whether they are viral-coded or host-coded proteins; for others circumstantial evidence was obtained.

The 37,000-, 24,000- and 23,000-dalton polypeptides were identified as viral coat proteins by immunoprecipitation with anti-CPMV serum and by comparing their mobilities on polyacrylamide gels with those of the capsid proteins of purified CPMV. The viral coat proteins occurred in the homogenates of CPMV-infected protoplasts only in the capsids of virus particles. If the homogenate was subjected to sucrose density gradient centrifugation, the coat proteins were completely removed from the homogenate and sedimented at the rate of CPMV particles. No other stable forms of the coat proteins were detected in CPMV-infected protoplasts. Besides the coat proteins, none of the other proteins were precipitated by anti-CPMV serum.

The 170,000- and 30,000-MW polypeptides appeared to be virus-specific proteins encoded by CPMV B-RNA. They migrated with the same mobility as the polypeptides which are synthesized in a messenger-dependent rabbit reticulocyte lysate under the direction of B-RNA. They are also synthesized in cowpea protoplasts inoculated with purified bottom component. We have previously shown that the 170,000- and 30,000-dalton polypeptides appear in CPMV-infected protoplasts early in the infection period, before any of the other viral-related proteins was detectable (chapter 6). This suggests that the 170,000- and 30,000-dalton

proteins provide early functions in the virus infection and multiplication process. An early function of B-RNA products is also suggested by the observation that inoculation of protoplasts with purified bottom component results in the production of a number of viral-related proteins, whereas inoculation with middle component does not reveal any newly induced proteins. Apparently bottom component specifies the viral (part of the) replicase and can function more or less independently, while middle component to function requires the presence of bottom component.

Inoculation of cowpea protoplasts with separate bottom component produced; in addition to the 170,000- and 30,000-dalton polypeptides, the 112, 110 and 84 kilodalton species but not the viral coat proteins. This negative evidence suggests that the viral coat proteins are encoded on M-RNA. The presence of the sequences coding for (one of) the capsid proteins in M-RNA was also implicated by genetic work with CPMV (WOOD, 1972; GOPO and FRIST, 1977; THONGMEEARKOM and GOODMAN, 1978) and other members of the comovirus group (MOORE and SCOTT, 1971; KASSANIS *et al.*, 1973).

The finding that more proteins were induced in protoplasts after inoculation with CPMV as well as with separate bottom component than can theoretically be specified by the infecting genome, stimulated the search for precursor-product relations. Processing has recently been shown *in vitro* by PELHAM (1979) who found that the products translated from CPMV M-RNA in a nuclease-treated rabbit reticulocyte lysate were specifically cleaved by B-RNA *in vitro* translation products. Accumulation of precursor polyproteins was not observed when CPMV-infected cowpea protoplasts were incubated in the presence of amino acid analogs or protease inhibitors that were selected for their ability to block CPMV multiplication. Similarly, no precursor-product relations could be demonstrated by the type of pulse-chase experiments performed except for the different forms of the small viral coat protein. This CPMV protein, once assembled into viral particles, appeared to be subject to several successive proteolytic cleavages, a process which might relate to the occurrence of electrophoretic heterogeneity of the CPMV particles (SEMANCIK, 1966; NIBLETT and SEMANCIK, 1969; GEELEN *et al.*, 1972 and 1973). With one exception all other viral-related proteins were found to be stable end-products. Only the 87,000-MW polypeptide appeared to be turned over, and since the corresponding protein present in uninfected protoplasts was similarly affected this polypeptide probably is host-coded. The nature of these experiments does not allow firm conclusions as to the possible involment of proteins with molecular weight of 130,000, 112,000, 110,000, 84,000 and 68,000 in precursor-product relations, since fast processing events for instance may have escaped detection. Of these proteins the 110,000- and 84,000-dalton species have previously been shown to be virus-coded on the basis of their appearance under conditions of actinomycin D inhibition of CPMV multiplication (chapter 5). The genetic origin of the 130-, 112- and 68-kilodalton polypeptides thus remains obscure.

Another mechanism for the expression of the viral genome that has been observed with many plant viruses involves the generation of virus-specific sub-

genomic messengers during the infection process. CPMV-infected protoplasts were therefore labeled with $^{32}PO_4$ and RNA was analyzed by polyacrylamide gel electrophoresis. Besides the genome-size RNAs, no other virus-specific RNAs were detected. This result is in agreement with those of STUIK (1979), who also could not find evidence for the existence of messengers smaller than genome size by sucrose density gradient fractionation of RNA from CPMV-infected cowpea leaves and subsequent *in vitro* translation of the RNAs from these fractions. This seems to be unique since subgenomic messengers specifying the viral coat proteins have been demonstrated for almost all plant RNA viruses studied,including monocomponent viruses such as TMV (SIEGEL *et al.*, 1973 and 1976; HUNTER *et al.*, 1976) and TYMV (KLEIN *et al.*, 1976; PLEY *et al.*, 1976; RICARD *et al.*, 1977) as well as multicomponent viruses like the three-component group (see VAN VLOTEN-DOTING and JASPARS, 1977).

8. CONCLUSIONS

The study reported in this thesis clearly demonstrates the value of plant protoplasts for the investigation of the multiplication of plant viruses. Infection of cowpea mesophyll protoplasts with CPMV was found to induce or stimulate the synthesis of 11 proteins apparently involved in viral multiplication. They were estimated to be 170, 130, 112, 110, 87, 84, 68, 37, 30, 24 and 23 kilodaltons. By fractionation of protoplast homogenates it was shown that some of the proteins were present in all subcellular fractions (*e.g.* 170 and 37 kilodaltons) but that others were confined to some particular fraction(s) (*e.g.* 130 and 30 kilodaltons only in the 30,000 $\times g$ supernatant fraction; 112 and 68 kilodaltons only in particulate fractions). Except for the viral coat proteins (37, 24 and 23 kilodaltons) all viral-related proteins that were found after normal CPMV infection also seemed to be synthesized when protoplasts were infected with bottom component alone. Protoplasts inoculated with middle component did not show any change in protein synthesis and no virus-specific polypeptides were observed. It should be pointed out that it is hard to imagine such results being obtained using whole leaves.

We have found some evidence about the distribution of functions among bottom and middle component for CPMV multiplication. The appearance of the nonstructural viral-related proteins after inoculation with separate bottom component suggests that this component is able to independently replicate RNA. Middle component seems to be required to produce progeny virus which lends support to the view that the CPMV capsid proteins are encoded by M-RNA, and that replication of this RNA depends on the expression of B-RNA. The presumptive translation products of B-RNA, namely the 170,000-and 30,000-MW proteins, are the first viral-related proteins detectable in CPMV-infected protoplasts. Altogether these observations suggest that bottom component is involved in specifying early viral functions while middle component becomes essential only late in infection.

Our results on the multiplication of CPMV in protoplasts confirm earlier findings that CPMV multiplication is dependent on host DNA transcription. By using actinomycin D it was demonstrated that host RNA synthesis is a prerequisite for the infection process to start. The early inhibitory effect of this drug was shown to act on viral RNA replication and not on viral protein synthesis.

By comparison with the products of CPMV RNA *in vitro* translation, the 170 and 30 kilodalton proteins synthesized in CPMV-infected protoplasts were assigned to B-RNA. Therefore, almost the complete coding capacity of this RNA is accounted for. Assuming both viral capsid proteins to be specified by M-RNA, about half of the information in this RNA remains obscure. Comparison of protein synthesis profiles from CPMV-infected protoplasts with those from M-RNA directed *in vitro* translation was of no help here: neither the 105 and 95 kilodalton polypeptides synthesized in different cell-free systems nor their *in*

vitro cleavage products (PELHAM, 1979) were recognized among the proteins derived from protoplasts.

No firm conclusions can be drawn as to the mechanism by which the CPMV RNAs are translated. Neither precursor polypeptides nor virus-specific subgenomic messengers were identified in CPMV-infected protoplasts but the evidence is still too incomplete to postulate expression of the RNAs by means of multiple internal initiations. The occurrence of such a mechanism in eukaryotic cells has only been demonstrated for the animal Kunjin virus (WESTAWAY, 1977) while two or three initiation sites operative in protein synthesizing systems *in vitro* were observed on TRV RNA 2 (FRITSCH *et al.*, 1977), CarMV RNA (SALOMON *et al.*, 1978), CPMV M-RNA (PELHAM, 1979) and poliovirus RNA (CELMA and EHRENFELD, 1975; JENSE *et al.*, 1978). The significance of these findings for the *in vivo* situations is still questionable. To obtain further insight into these processes a homologous *in vitro* system from cowpea protoplasts *e.g.* by mild detergent treatment should be prepared. Such a system which could be prepared from healthy as well as from CPMV-infected protoplasts in different stages of infection would probably provide a more practical and reliable tool.

Infection with CPMV induces the formation of characteristic membranous structures in the cytoplasm of infected cells (ASSINK *et al.*, 1973; DE ZOETEN *et al.*, 1974; HIBI *et al.*, 1975; HUBER *et al.*, 1977). Viral RNA replication has been shown to occur in these structures (ASSINK, 1974; DE ZOETEN *et al.*, 1974; ZABEL *et al.*, 1974 and 1976). The only viral-related proteins possibly involved in their formation are the 112,000- and 68,000-MW proteins as these were specifically confined to the pellet fraction of infected protoplasts. It may be assumed, however, that the cytopathic structures are composed of many more constituents. In view of their probable relevance to CPMV multiplication further examination of the membranous fractions of infected protoplasts is desirable for instance by further purification using discontinuous sucrose density gradients according to DE ZOETEN *et al.* (1974).

Besides the two coat proteins CPMV virions contain a third structural protein. Results have demonstrated the presence of a protein covalently bound to the 5′-termini of both M and B RNA components of CPMV (STANLEY *et al.*, 1978; DAUBERT *et al.*, 1978). Such proteins were not detected in CPMV-infected protoplasts. The significance of the virion RNA-bound proteins of CPMV is unknown. They are not involved in some early step in virus multiplication since removal of the protein from the RNA does not affect its infectivity nor does it alter its *in vitro* translation behaviour (STANLEY *et al.* 1978). By their extremely lipophylic nature the proteins are likely to be associated with membranes within the cell and as such they might function to attach the RNA. It is therefore tempting to speculate that the role of the cytopathic structures is not limited to viral RNA replication alone, but that they provide the matrix on which the coordinate synthesis and assembly of all virion components occurs. In this view the RNA plus-strands synthesized in the membrane-bound replication structures become anchored to the membranes at the start of or during their synthesis. This arrangement might enable the efficient translation of the coat protein

cistrons. By their very low solubility in water these coat proteins during or at the end of their synthesis are likely to dissolve in the membranes and assembly of CPMV particles then occurs by the successive association of coat protein molecules. During its growth the capsid shell becomes increasingly water-soluble and gradually emerges from the membrane. The RNA is then incorporated into the empty shell, the particle is closed and released into the cytoplasm. It seems likely that the burial of the RNA is a late event in assembly in view of the occurrence of empty particles: top component.

Although this picture of the CPMV production process is admittedly tentative there are several observations that are in agreement with it. For instance, no free coat protein molecules were observed in the soluble fraction of CPMV-infected protoplasts, which is not surprising in view of their low solubility in water, and no soluble precursors of these proteins were detected with anti-CPMV serum. When the RNA of free polysomes from CPMV-infected cowpea leaves labeled with ^{3}H-uridine or $^{32}PO_4$ was analyzed by polyacrylamide gel electrophoresis no full-length CPMV RNAs appeared to be present. Moreover, these polysomes, though active in *in vitro* translation, neither produced CPMV coat proteins nor were other known CPMV-specific proteins clearly synthesized (unpublished observations). Association of the 5′-terminal protein of the CPMV RNAs with membranes might prevent it from being cleaved off from the RNA. An enzymatic activity removing the corresponding protein from poliovirus RNA has been found not only in extracts of animal cells but also in wheat germ (AMBROS *et al.*, 1978). Similar cytological abnormalities as found in CPMV-infected cells have also been observed with plant nepoviruses (ROBERTS and HARRISON, 1970; HARRISON *et al.*, 1974) and with animal picornaviruses (DALES *et al.*, 1965; AMAKO and DALES, 1967; LENK and PENMAN, 1979). These are the only viruses so far found to bear a virion RNA-bound protein.

SUMMARY

In contrast to the situation concerning bacterial and, to a lesser extent, animal RNA viruses, little is known about the biochemical processes occurring in plant cells due to plant RNA virus infection. Such processes are difficult to study using intact plants or leaves. Great effort has therefore been spent in developing *in vitro* cultures of plant protoplasts, but the use of these protoplasts has been seriously hampered by various technical problems.

It is clear that plant RNA virus infections give rise to a number of specific reactions in infected cells, particularly at the level of RNA and protein synthesis. The multiplication of these viruses implies a manifold copying of their structural components, namely RNA and protein. These activities clearly require the involvement of other RNA and protein species in view of, for instance, the drastic cytopathic effects that are often caused by infection. Thus, detailed knowledge of virus-induced RNA and protein synthesis is a prerequisite for an understanding of the infection process.

In this thesis investigations are described concerning the proteins involved in the multiplication of cowpea mosaic virus (CPMV) in cowpea protoplasts.

The relevant properties of CPMV are summarized in chapter 2. Research on CPMV is interesting not only in the field of plant virology as the virus is also of general virological significance. Structurally, for example, CPMV shares a number of striking features with the animal picornaviruses like poliovirus and foot-and-mouth disease virus. From a molecular biological point of view the multiplication of CPMV presents several intriguing specific questions. CPMV is a multicomponent virus: its genome consists of two different single-stranded RNA molecules occurring in separate nucleoprotein particles, both of which are essential for infection. It would be of interest to learn which functions are specified by the two genome pieces, whether the separate viral components can give rise to some virus-specific biochemical expression within the cell and how they complement each other during normal infection. Characteristic cytopathic structures are induced in plant cells upon infection with CPMV in the form of vesicular membranes embedded in amorphous electron-dense material. It is now well established that replication of the viral RNA is localized in these structures. Information about their development and functioning is thus required.

Chapter 2 also contains an introduction to the essential features of plant protoplasts and an enumeration of the diverse possibilities they can offer for the investigation of specific virological problems. Finally, the present state of knowledge about the biochemistry of multiplication of plant viruses, as obtained mainly through the use of protoplasts, is reviewed.

The isolation of viable protoplasts from the leaf mesophyll tissue of cowpea requires the stringent control of the growth conditions of the plants (chapter 3). Various factors appeared to influence the quality of the protoplasts and their

infectability with CPMV. The combination of cowpea mesophyll protoplasts and CPMV appeared to offer a very attractive system. Based on the original study of HIBI *et al.* (1975) a procedure was developed for the preparation and infection of the protoplasts, the most rapid and simple one so far described (chapter 3). The major reasons for this are:

- a short growth period (about 10 days under the conditions used) is required to obtain suitable leaf material
- for an efficient enzymatic digestion of the leaves it is sufficient to damage the lower epidermis by means of carborundum powder and paint-brush instead of stripping off the epidermis with forceps
- enzymatic one-step isolation of protoplasts can be achieved by simultaneous digestion with pectinase and cellulase
- inoculation of cowpea protoplasts with CPMV does not require the use of polycations, making the preincubations of protoplasts and virus superfluous.

Using this standard procedure, synchronous infection in virtually all protoplasts could be routinely established.

Radioactive precursors of RNA and protein added to the cowpea protoplast incubation medium were taken up and incorporated into macromolecules to high specific activities (chapter 4). This light-dependent metabolic activity increased during the first 30 h after isolation of the protoplasts. It could be influenced by several metabolic inhibitors. RNA synthesis, for instance, was strongly inhibited by actinomycin D and cordycepin, whereas α-amanitin and rifampicin had only a slight effect. Protein synthesis in the protoplasts could be blocked completely by means of cycloheximide, and was also sensitive to the action of antibiotics such as chloramphenicol, puromycin and, to a lesser degree, lincomycin. These inhibitor studies, as well as the results of analyses of RNAs and proteins synthesized in the protoplasts, indicated that the metabolic activity of the chloroplasts is low and mainly limited to light-dependent energy generation.

The multiplication of CPMV in cowpea mesophyll protoplasts relies upon host-coded DNA-dependent RNA synthesis during the earliest stage of infection. As described in chapter 5, propagation of the virus was completely prevented when protoplast DNA transcription and thereby almost all RNA synthesis was inhibited by means of actinomycin D, provided that the drug was applied at the time of inoculation or immediately thereafter. The degree of inhibition of CPMV replication by actinomycin D rapidly decreased when the antibiotic was added later after inoculation; the production of viral nucleoprotein particles became progressively more resistant to the drug, showing complete resistance at about 8 h after inoculation, a time at which infection was still in its latent phase.

Surprisingly, synthesis of CPMV antigen was still demonstrable by immunofluorescent antibody staining techniques in protoplasts in which virus replication had been completely blocked by actinomycin D. Under these circumstances *de novo* production of viral top component (empty capsids) was observed. In addition to the CPMV capsid proteins, these experiments revealed another 3

viral-related polypeptides in infected protoplasts (molecular weights 170,000, 110,000 and 84,000). Since their synthesis also clearly continued during inhibition of host RNA synthesis by actinomycin D it was concluded that they were coded for by the viral genome.

A more detailed analysis of the proteins involved in CPMV multiplication is described in chapter 6. At least 11 proteins not present in mock-infected protoplasts or present in much lower amounts were detected in infected protoplasts. Their molecular weights were estimated to be 170,000, 130,000, 112,000, 110,000, 87,000, 84,000, 68,000, 37,000, 30,000, 24,000 and 23,000. Host-specific protein synthesis appeared to be hardly affected by CPMV infection. Virus-specific proteins therefore had to be distinguished from a large variety of protoplast proteins. This appeared to be greatly facilitated by radioactive labeling of the protoplasts for short periods during a suitable phase of the infection cycle and by subcellular fractionation of the protoplasts. The treatment of the particulate fractions with the mild detergent digitonin yielded extracts in which proteins could be detected by polyacrylamide gel electrophoretic analysis that otherwise remained hidden by a general high background of radioactivity upon direct analysis of the untreated particulate fractions.

All of the proteins apparently concerned in CPMV infection were most clearly observed late in infection. None of them was detectable during the actinomycin D-sensitive latent period. The 170 and 30 kilodalton proteins were the first detectable, namely from about 10 h after inoculation. All others became apparent about 6 h later.

The induction of the characteristic cytopathic structures in CPMV-infected cells suggests the induction and/or stimulation of synthesis of a large number of proteins by the virus. Upon fractionation these proteins should appear in the particulate fractions of the protoplasts. However, only 2 virus-induced polypeptides (112 and 68 kilodalton) were demonstrated exclusively in these fractions. Other components of the cytopathic structures presumably remained in the extracted pellet residues.

In chapter 8 further characterization of the viral-related proteins found in CPMV-infected protoplasts is described. The 37, 24 and 23 kilodalton proteins were identified as viral capsid proteins by comparing their electrophoretic mobility in polyacrylamide gels with those of proteins from purified CPMV and by immunoprecipitation with anti-CPMV serum. They occurred in the soluble fraction of protoplasts only in viral particles, not as free proteins. The 170 and 30 kilodalton polypeptides also appeared to be virus-specific and to be coded for by CPMV bottom component RNA: they comigrated in polyacrylamide slab-gels with the main products of *in vitro* translation of this RNA in a messenger-dependent lysate of rabbit reticulocytes. These polypeptides were also induced in protoplasts after inoculation with purified CPMV bottom component. Under these conditions some other viral-related proteins were also synthesized, but not the viral capsid proteins. In contrast, inoculation with purified CPMV middle component did not detectably affect protoplast protein synthesis. These experiments strongly suggest that CPMV B-RNA specifies one or more early functions

one of which being the replicase function, while M-RNA specifies late functions, particularly the viral capsid proteins.
Since the total size of the viral-related proteins by far exceeded the theoretical coding potential of the CPMV genome, the occurrence of precursor-product relations was investigated by means of pulse-chase experiments and by attempting to accumulate possible precursors by blocking their proteolytic processing. The results of the latter method were negative. Upon chasing the ^{35}S-methionine label with unlabeled amino acid the 87,000 dalton polypeptide appeared to be unstable, as was a protein with the same electrophoretic mobility synthesized in uninfected protoplasts. This protein thus probably represents a host-specific protein the synthesis of which is stimulated by CPMV infection. Furthermore, these experiments revealed that several discrete oligopeptides were cleaved from the smaller CPMV coat protein after assembly into a virion. Although the involvement of precursor-product relations could not be definitely excluded on the basis of the methods used, another possible mechanism of viral gene expression was also investigated, namely the occurrence of subgenomic viral messengers. Upon analysis of ^{32}P-labeled RNA from CPMV-infected protoplasts by polyacrylamide gel electrophoresis, 2 RNAs of genome length were the only detectable virus-specific polynucleotides. These results suggest a mechanism for the translation of the CPMV genome using internal initiation sites. Further research is, however, required to unequivocally elucidate the strategy of the viral gene expression.

SAMENVATTING

In tegenstelling tot de situatie bij bacteriële en, in mindere mate, dierlijke RNA-virussen is onze kennis over de biochemische processen, die zich afspelen in plantecellen als gevolg van infektie door plante-RNA-virussen nog erg beperkt. Een belangrijke reden hiervoor is, dat zulke processen moeilijk zijn te bestuderen met behulp van intakte planten of bladeren. Weliswaar is daarom hard gewerkt aan de ontwikkeling van *in vitro* cultures van protoplasten, maar het gebruik ervan is tot nu toe beperkt gebleven als gevolg van allerlei technische problemen.

Het staat vast, dat plante-RNA-virusinfekties aanleiding geven tot een aantal specifieke reakties in de geïnfekteerde cellen, in het bijzonder op het nivo van RNA- en eiwitsynthese. Immers, de vermenigvuldiging van deze virussen impliceert een veelvoudige kopiëring van hun strukturele komponenten RNA en eiwit. Bovendien vereist deze aktiviteit ongetwijfeld de betrokkenheid van andere RNA- en eiwitspecies gezien bijvoorbeeld de ingrijpende cytopathologische effekten, die de infektie vaak tot gevolg heeft. Een goed inzicht in het infektieproces vooronderstelt derhalve een gedetailleerde kennis van de virusgeïnduceerde RNA- en eiwitsynthese.

In dit proefschrift wordt een onderzoek beschreven naar de eiwitten, welke een rol spelen bij de vermenigvuldiging van cowpea mozaïekvirus (CPMV) in cowpea protoplasten.

De belangrijkste eigenschappen van CPMV zijn samengevat in hoofdstuk 2. Studie aan CPMV is niet alleen van plantevirologisch belang, ook uit een algemeen virologisch oogpunt is het virus interessant. Zo heeft het virus struktureel een aantal opvallende kenmerken gemeen met de dierlijke picornavirussen als poliovirus en mond-en-klauwzeervirus. Ook molekulair-biologisch biedt de vermenigvuldiging van CPMV enkele intrigerende specifieke vragen. CPMV is een multikomponentvirus: het genoom bestaat uit twee verschillende enkelstrengs RNA-moleculen, die in afzonderlijke nucleoproteïnedeeltjes voorkomen en die beiden essentieel zijn voor infektie. Het is interessant te weten, welke funkties gespecificeerd worden door de twee genoomdelen, of de afzonderlijke komponenten in de cel nog tot enige virus-specifieke biochemische expressie in staat zijn, en hoe zij elkaar tijdens een normale infektie aanvullen. Tijdens een infektie met CPMV ontstaan in de cel karakteristieke cytopathologische strukturen, woekeringen van membranen in het cytoplasma. Het staat vast, dat de replicatie van het virale RNA is gelokaliseerd in deze strukturen. Informatie over hun ontstaan en funktioneren is dus vereist.

Hoofdstuk 2 bevat verder een inleiding in de essentiële kenmerken van protoplasten en een uiteenzetting over de diverse mogelijkheden, die zij bieden voor de bestudering van specifieke virologische problemen. Tenslotte wordt middels een litteratuur-review een overzicht gegeven van de huidige kennis over de biochemie van vermenigvuldiging van plantevirussen zoals die voornamelijk dank zij het

gebruik van protoplasten is verworven.

Het isoleren van levensvatbare protoplasten uit het bladmesophyll van cowpea stelt zeer hoge eisen aan de kweekomstandigheden van de planten (hoofdstuk 3). Allerlei faktoren bleken van invloed te zijn op de kwaliteit van de protoplasten en op hun infekteerbaarheid met CPMV. De kombinatie van cowpea bladmesophyll protoplasten en CPMV bleek een bijzonder aantrekkelijk systeem op te leveren. Gebaseerd op de oorspronkelijke studie van HIBI *et al.* (1975) werd een procedure ontwikkeld voor de bereiding en infektie van de protoplasten, welke de snelste en eenvoudigste is die tot nu toe werd beschreven (hoofdstuk 3). De belangrijkste redenen hiervoor zijn:

- de planten leveren al na een korte groeiperiode (ca. 10 dagen onder de gebruikte omstandigheden) geschikt bladmateriaal
- voor een efficiënte enzymatische afbraak van het blad is het voldoende de onderepidermis te beschadigen mbv. carborundumpoeder en penceel ipv. het te moeten afstrippen van deze epidermis met een pincet
- enzymatische éénstaps-isolatie van protoplasten is mogelijk door gelijktijdige behandeling met pectinase en cellulase
- voor inoculatie van cowpea protoplasten met CPMV zijn geen polykationen vereist, hetgeen preïncubaties van protoplasten en virus overbodig maakt.

Met deze standaardprocedure kon routinematig een synchrone infektie in bijna alle protoplasten tot stand worden gebracht.

Radioaktieve bouwstenen van RNA en eiwit, toegevoegd aan het incubatiemedium van cowpea protoplasten, worden door de protoplasten opgenomen en tot hoge specifieke radioaktiviteit ingebouwd in de betreffende makromolekulen (hoofdstuk 4). Deze licht-afhankelijke metabolische aktiviteit bleef gedurende de eerste 30 uren na isolatie van de protoplasten toenemen. Zij kon worden beïnvloed door allerlei metabole remmers. Zo kon de synthese van RNA sterk worden geremd door actinomycine D en cordycepine, waar α-amanitine en rifampicine slechts een gering effekt hadden. De eiwitsynthese in de protoplasten kon dmv. cycloheximide volledig worden onderdrukt, maar was eveneens gevoelig voor antibiotica als chloramphenicol, puromycine en, in mindere mate, lincomycine. Deze studies, alsook de analyses van door de protoplasten gesynthetiseerde RNAs en eiwitten, toonden aan, dat de metabole aktiviteit van de chloroplasten gering is en zich voornamelijk beperkt tot het vastleggen van licht-energie.

De vermenigvuldiging van CPMV in cowpea protoplasten is afhankelijk van door de gastheer gekodeerde DNA-afhankelijke RNA synthese tijdens het vroegste stadium van infektie. Zoals beschreven in hoofdstuk 5 werd de vermeerdering van het virus volledig verhinderd, wanneer men met actinomycine D de protoplast-DNA transcriptie en daarmee vrijwel alle RNA synthese blokkeerde vanaf of onmiddellijk na de inoculatie. De mate van remming van de CPMV replicatie door actinomycine D nam snel af, wanneer het antibioticum later na inoculatie werd toegediend en de produktie van virale nucleoproteïnedeeltjes was resistent tegen de drug geworden rond 8 uur na de inoculatie, een tijdstip overigens waarop de infektie nog in de latente fase is.

Verrassend genoeg bleek dmv. immunofluorescentie-experimenten toch synthese van CPMV-antigeen aantoonbaar in protoplasten, waarin de virusreplicatie volledig was geremd door actinomycine D. Inderdaad werd onder deze omstandigheden de *de novo* synthese van virale topkomponent (lege capsiden) waargenomen. Afgezien van de CPMV-manteleiwitten werden bij deze proeven nog 3 virus-specifieke eiwitten (molekuulgewichten 170.000, 110.000 en 84.000) aangetroffen in geïnfekteerde protoplasten. Daar ook hun synthese tijdens inhibitie van de gastheer RNA-synthese door actinomycine D nog duidelijk voortging werd gekonkludeerd, dat zij worden gekodeerd door het virale genoom.

Een meer gedetailleerde analyse van de bij de vermenigvuldiging van CPMV betrokken eiwitten is beschreven in hoofdstuk 6. Tenminste 11 eiwitten werden opgespoord, welke in ongeïnfekteerde protoplasten niet of in veel mindere mate voorkomen. Hun molekuulgewichten werden bepaald op 170.000, 130.000, 112.000, 110.000, 87.000, 84.000, 68.000, 37.000, 30.000, 24.000 en 23.000. De gastheer-specifieke eiwitsynthese bleek door CPMV-infektie nauwelijks te worden beïnvloed. Dit had tot gevolg, dat de virus-specifieke eiwitsynthese moest worden onderscheiden van een grote varieteit aan protoplast-eiwitten. Dat niettemin zoveel met de infektie samenhangende eiwitten konden worden gedetekteerd was vooral te danken aan de uitvoering van korte radioaktieve labelingen tijdens een geschikte periode van de infektiecyclus en aan de verdeling van de protoplasten in een aantal subcellulaire frakties. In het bijzonder de bereiding van extracten van de deeltjesfrakties door behandeling met het milde detergens digitonine leverde preparaten op, welke bij polyacrylamide gel electroforetische analyse leidden tot de detektie van eiwitten, die bij direkte analyse van onbehandelde pelletfrakties schuil gaan achter een sterke algemene achtergrond aan radioaktiviteit.

Alle voor CPMV-infektie specifieke eiwitten werden met name laat in infektie waargenomen. Geen van hen was detekteerbaar tijdens de actinomycine D-gevoelige, latente periode. Het vroegst aantoonbaar was de synthese van het 170 en 30 kilodalton eiwit, nl. vanaf ca. 10 uur na inoculatie. Vanaf ca. 16 uur na inoculatie werden ook de andere eiwitten aangetroffen.

De inductie van de karakteristieke cytopathologische strukturen in CPMV-geïnfekteerde cellen suggereert de inductie en/of stimultatie van een groot aantal eiwitten. Bij fraktionering zouden deze eiwitten in de deeltjesfrakties van de protoplasten moeten verschijnen. Niettemin werden slechts 2 virus-geïnduceerde eiwitten (112 en 68 kilodalton) exclusief in deze frakties aangetoond. Wellicht blijven andere komponenten van de cytopathologische strukturen achter in de geëxtraheerde pellet-residuën.

In hoofdstuk 8 wordt de verdere karakterisering van de voor CPMV-infektie specifieke eiwitten beschreven. De 37, 24 en 23 kilodalton eiwitten werden geïdentificeerd als zijnde de virale manteleiwitten door vergelijking van hun electroforetische mobiliteit met die van de eiwitten uit gezuiverd CPMV en door immunoprecipitatie met anti-CPMV serum. Zij komen in de oplosbare protoplastenfraktie slechts voor in de virale deeltjes en niet als vrije eiwitten. Ook de 170 en 30 kilodalton eiwitten bleken virus-specifiek en gekodeerd door CPMV

bodemkomponent RNA: zij comigreerden bij polyacylamide gel electroforese met de hoofdprodukten van de *in vitro* translatie van dit RNA in een messenger-afhankelijk lysaat van konijne-reticulocyten. Deze eiwitten werden ook geïnduceerd in protoplasten na inoculatie met alleen CPMV bodemkomponent. Ook een aantal andere voor CPMV-infektie specifieke eiwitten werden onder deze omstandigheden gesynthetiseerd met als opvallende uitzonderingen de virale manteleiwitten. Inoculatie met gezuiverde CPMV middenkomponent daarentegen had geen detekteerbaar effekt op de protoplasten-eiwitsynthese. Deze proeven suggereren, dat CPMV B-RNA één of meer vroege funkties specificeert, waaronder tenminste de replicase-funktie, terwijl M-RNA voor late funkties kodeert, in het bijzonder de virale capside-eiwitten.
Daar de grootte van de 11 eiwitten gezamenlijk de theoretische koderende kapaciteit van het CPMV-genoom verre overtreft werd het bestaan van precursor-produkt relaties onderzocht, enerzijds dmv. pulse-chase experimenten, anderzijds door te pogen eventuele precursors op te hopen door hun proteolytische processing te blokkeren. Het resultaat van de laatste methode was negatief. Bij een chase van het ^{35}S-methionine label met ongelabeld aminozuur bleek het 87.000 dalton eiwit instabiel te zijn evenals een eiwit met dezelfde electroforetische mobiliteit in gezonde protoplasten. Dit eiwit representeert dus waarschijnlijk een door CPMV gestimuleerd gastheer-specifiek polypeptide. Verder bleek bij deze experimenten, dat van het kleine CPMV-manteleiwit na assemblage in een virion achtereenvolgens een aantal diskrete oligopeptidefragmenten werd afgesplitst. Hoewel de aard van de gebruikte methoden het bestaan van precursor-produkt relaties niet kon uitsluiten werd ook het optreden van een ander mechanisme voor de virale genexpressie onderzocht, nl. het voorkomen van subgenomische virale messengers. Bij analyse van ^{32}P-gelabeld RNA uit door CPMV geïnfekteerde protoplasten dmv. polyacrylamide gel electroforese waren 2 RNAs van genoom-lengte de enige virus-specifieke polynucleotiden, die konden worden gedetekteerd. Deze resultaten suggeren een mechanisme voor de translatie van het CPMV-genoom middels interne initiatie op meerder startplaatsen. Voor de definitieve opheldering van de strategie van de virale genexpressie is echter meer onderzoek noodzakelijk.

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ABBREVIATIONS

AMV	alfalfa mosaic virus
BBWV	broad bean wilt virus
BMV	brome mosaic virus
BPMV	bean pod mottle virus
B-RNA	CPMV bottom component RNA
CaMV	cauliflower mosaic virus
CarMV	carnation mottle virus
CCMV	cowpea chlorotic mottle virus
CGMMV	cucumber green mottle mosaic virus
Ci	curie
CMV	cucumber mosaic virus
cpm	counts per minute
CPMV	cowpea mosaic virus
CYMV	clover yellow mosaic virus
DNA	deoxyribonucleic acid
dpm	desintegrations per minute
E_{260}	optical density at 260 nm
EDTA	ethylenediaminetetraacetate
g	acceleration of gravity
LMC	low molecular weight component RNA
LSB	Laemmli sample buffer (LAEMMLI, 1970)
M	molar
M-RNA	CPMV middle component RNA
mRNA	messenger RNA
MW	molecular weight
nm	nanometer
oligo(dT)	oligodeoxythymidylic acid
PEMV	pea enation mosaic virus
poly(A)	polyriboadenylic acid
RaMV	radish mosaic virus
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
RRV	raspberry ringspot virus
S	Svedberg unit
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TLCK	N-α-p-tosyl-L-chloromethyl ketone hydrochloride
TMV	tobacco mosaic virus
TNDV	tobacco necrotic dwarf virus
TPCK	L-1-tosylamide-2-phenylethyl-chloromethyl ketone

TPNS	disodium-triisopropylnaphtalene sulphonate
Tris	tris(hydroxyl)aminomethane
tRNA	transfer RNA
TRosV	turnip rosette virus
TRV	tobacco rattle virus
TYMV	turnip yellow mosaic virus
UV	ultraviolet
V	volt

CURRICULUM VITAE

Peter J. M. Rottier werd op 24 januari 1947 geboren te Hontenisse (Z). In 1966 behaalde hij het gymnasium-β diploma aan het Jansenius Lyceum te Hulst. In datzelfde jaar ging hij chemie studeren aan de Katholieke Universiteit te Nijmegen. In 1970 behaalde hij het kandidaatsexamen S2 en in 1974 het doctoraalexamen (hoofdvak biochemie met als nevenrichting klinische chemie; bijvakken fysiologie en farmacochemie).

Vanaf 1974 tot 1978 was hij in dienst van de Stichting Scheikundig Onderzoek Nederland (S.O.N.) werkzaam bij de vakgroep Moleculaire Biologie van de Landbouwhogeschool te Wageningen, alwaar het in dit proefschrift beschreven onderzoek werd verricht.

Sinds 1979 is hij als wetenschappelijk ambtenaar verbonden aan het Instituut voor Virologie van de Faculteit der Diergeneeskunde van de Rijksuniversiteit te Utrecht, waar hij is betrokken bij het onderzoek aan de vermenigvuldiging van coronavirussen.

Peter,
ik heb Ruben en Wieger laten
plassen en ben nog even
naar Troost.

Hans