THE REPLICATION OF TOBACCO NECROSIS VIRUS IN COWPEA LEAF PROTOPLASTS

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A THESIS SUBMITTED IN PART FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE UNIVERSITY OF LONDON

DEPARTMENT OF PURE AND APPLIED BIOLOGY,
IMPERIAL COLLEGE OF SCIENCE AND TECHNOLOGY,
LONDON, UNITED KINGDOM. 1982

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Abstract Tobacco necrosis virus (TNV) is an icosahedral plant virus with a single stranded RNA genome; it has been extensively studied both in whole plants and in vitro investigations of RNA transcription and translation. TNV is of considerable interest because some strains serve as "helpers" for a very small, serologically unrelated virus, satellite tobacco necrosis virus (STNV). For the successful production of STNV particles TNV must be present in the infected cells. A protoplast system has been developed with a view to investigating this particular relationship and its potential application to elucidating the control processes of viral RNA replication.

Cowpea primary leaf protoplasts have been selected because they can be isolated rapidly, and reproducibly utilised in controlled experiments. Short plant growth periods allow for rigorous physiological control of culture procedures. The single-step protoplast isolation, with a direct virus infection, osmotic "step-up", and temperature reduction is optimal for TNV infection. This rapid procedure, and the degree of control afforded at each stage, is essential in producing material for biochemical analysis.

The course of virus replication in protoplasts has been investigated by infectivity assay of nucleoprotein and viral RNA; UV absorption by protoplast extracts on sucrose gradients; and virus specified proteins incorporating radiochemicals, as detected by polyacrylamide gel electrophoresis and fluorography. The rate of virus synthesis has been demonstrated by these criteria to peak at 18 hours post-inoculation. At this stage, RNA dependent RNA polymerase activity has been extracted from the protoplasts and assayed in vitro; the activity extracted from infected protoplasts is clearly increased over that extracted from "mock-infected" or healthy protoplasts.

Fluorography revealed three protein species stimulated by TNV infection. One protein of 30,000 M_r corresponds to the capsid protein, while two other proteins also stimulated correspond to 23,000 M_r and 100,000 M_r. The proteins were not characterised further.

The advantages of the protoplast system, and the possibilities of extending the system to analyse the "satellite/helper"relationship are considered.

SYMBOLS AND ABBREVIATIONS

AMV	= Alfalfa mosaic virus
ATP	= Adenosine-5'-triphosphate
BMV	= Brome mosaic virus
BSA	= Bovine serum albumin
Car MV	= Carnation mottle virus
CMV	= Cucumber mosaic virus
CPMV	= Cowpea mosaic virus
CTP	= Cytidine-5'-triphosphate
ds(RNA)	= Double stranded (RNA)
EDTA	= Ethylene diamine tetra-acetic acid
GTP	= Guanosine-5'-triphosphate
I	= Infected
IB	= Incubation buffer
MI	= Mock inoculated
N.P.	= Nucleoprotein
PBS	= Phosphate buffered saline
PEG	= Polyethylene glycol
PGB	= Protoplast grinding buffer
PI	= Post inoculation
PLO	= Poly-L-ornithine
POPOP	= 1,4-di-(2-(5-phenyloxazolyl))-benzene
PPO	= 2,5-diphenyl oxazole
rf(s)	= Replicative form(s)
SDS	= Sodium dodecyl sulphate
ss(RNA)	= Single stranded (RNA)
STNV	= Satellite tobacco necrosis virus
or SV	= Satellite virus
TCA	= Trichloro acetic (acid)
TEMED	= NNN'N'-tetramethylethylenediamine
TNE	= Tris/NaCl/EDTA buffer
TNV	= Tobacco necrosis virus
$TNV_{D(or\ A)}$	= Tobacco necrosis virus strain D (or A)
TYMV	= Turnip yellow mosaic virus
VIM	= Virus incubation medium

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1 INTRODUCTION

Studies of plant virus multiplication have lagged far behind similar studies with bacteriophage and animal viruses, partly because for many years a synchronous infection system for isolated cells has not been available. Consequently we know relatively little of the methods by which RNA plant viruses redirect host cells to synthesize virus specific products. Nevertheless, in common with animal virus and bacteriophage systems it is clear that the molecular processes of virus proliferation: translation, nucleic acid synthesis and virus assembly are precisely related to the viral genome structure.

Accordingly, much work has gone into examining primary RNA sequences that are able to serve as messengers, and more recently into considering how their secondary structures participate in the replication events (Kaesberg et al., 1981). Fundamentally the plant virus genome must be compatible in primary and secondary structure with the synthetic machinery of the eukaryotic host in which it replicates. In general this means that plant viral RNAs will conform to the restrictions usually shown by eukaryotic ribosomes in only translating 5' cistrons directly and not initiating protein synthesis at internal sites (Kozac, 1978).

With respect to RNA replication, viral RNAs and their protein products must be compatible with (and more or less dependent upon) host-coded RNA-dependent RNA polymerases (Fraenkel-Conrat,1979) to account for the rapid and specific synthesis of viral RNA following the infection of a host cell with a plus-strand RNA virus. Viral-coded proteins (Paterson & Knight, 1975; Sakai et al., 1977) have been postulated to confer transcriptional control on a host replicase core (Romaine & Zaitlin, 1978), possibly in conjunction with membrane binding (Stussi-Garaud et al., 1977) but demonstrations of this mechanism are still lacking for plant viruses.

Translational Strategy There are three basic methods by which plant viral RNAs overcome the restrictions of eukaryotic ribosomes and code for viral proteins in the host cell. 1) The viral genome may be divided into subgenomic RNAs at some or all stages of the replication cycle. Subgenomic RNAs may then be translated from a previously closed initiation site at the 5' end. 2) The viral RNA may allow read-through of one or more stop codons to give more than one protein from a single region of RNA. 3) The whole of the viral RNA may be translated as a

monocistronic messenger into a single polyprotein. Functional viral proteins are then obtained by proteolysis of the polyprotein.

Plant virus translation strategies usually involve a combination of the above mechanisms and the programming of protein synthesis *in vitro* using plant viral RNAs has revealed the extent of translational strategic diversity (Davies, 1979). Turnip yellow mosaic virus (TYMV) employs all three mechanisms (Morch & Benicourt, 1980) for example, whereas brome mosaic virus (BMV) and cowpea mosaic virus (CPMV) each employ a single mechanism: subgenomic RNAs (Shih & Kaesberg, 1973) and proteolysis of a polyprotein (Davies *et al.*, 1977) respectively. Most other viruses appear to adopt similar or intermediate strategies; however two possible exceptions have been reported to the usual restrictions on internal initiation.

The *in vitro* translation of internal cistrons has been reported for both carnation mottle virus (CarMV) (Salomon *et al.*, 1978) and tobacco necrosis virus (TNV) (Salvato & Fraenkel-Conrat, 1977) each in a wheat-germ cell-free system. Both viruses have been subject to tests for subgenomic RNAs, and their messenger function is associated only with the respective genomic RNAs. Translation products include the respective coat proteins for CarMV and two proteins not yet detected in infected tissues. TNV specifies a range of protein products including the capsid protein and these will be considered in detail later.

Viral RNAs have been used as models to probe mechanisms of plant gene expression and the regulation of plant messenger translation (Hall, 1979). The 80 S cytoplasmic ribosomes of wheat germ extracts can translate polycistronic messenger RNA from bacteriophage Qβ (Davies & Kaesberg, 1973) and some plant messenger RNAs may be polycistronic. Although the divergence of virus strategies, and the multipartite character of some viruses (Jaspars, 1974), has been related to the problems of plant cells in handling large polycistronic messengers (Reijnders, 1978), some viruses may have evolved or retained a polycistronic genome. Such a strategy would have implications in any hypothesis concerning virus evolution, and the analysis of virus products *in vivo* may clarify the nature of the proteins produced by *in vitro* translation (Davies, 1979).

RNA-Dependent RNA Polymerases The rapid synthesis of viral RNA following infection of a host cell by a plus-strand RNA virus requires the presence of an active RNA-dependent RNA polymerase. For many years

it was generally assumed that, since no role was postulated for an RNA replicase in healthy cells, RNA viruses must contain the genetic information necessary for RNA replication. However, since the demonstration of RNA replicase activity in healthy Chinese cabbage (Astier-Manfacier & Cornuet) in 1971, similar activity has been extracted from all normally metabolising plant tissues tested (Astier-Manfacier & Cornuet, 1981). Studies of poliovirus (Flanegan & Baltimore, 1979) and the bacteriophage Qβ (Kamen, 1975) have shown that host proteins may be involved in RNA replication in conjunction with virus products. The involvement of a hybrid enzyme complex in virus replication is consistent with the small size and limited coding capacity of many plant viruses.

The enzymes stimulated in plant cells as a result of virus infection do not apparently replicate cellular RNAs and must be able to select RNA templates. A distinction might thus be expected between healthy and virus-stimulated replicase activities based on their specificity for template RNAs of viral origin. Viral proteins stimulated maximally in the early stages of infection (Hariharasubramanian et al., 1973; Paterson & Knight, 1975; Sakai et al., 1977) have been postulated to play a role in determining template specificity by modifying the host replicase core (Romaine & Zaitlin, 1978). However, the only virus-coded protein so far detected in association with a replicase complex is the capsid protein of alfalfa mosaic virus (AMV) (Clerx & Bol, 1978). In the case of CPMV, replicase function has been associated with a single RNA species and by analogy with poliovirus (Etchison & Ehrenfeld, 1980), may turn out to be a membrane-bound (M_r= 58,000) polypeptide. Generally there is no clear distinction between RNA polymerase preparations from healthy and infected tissues (Ikegami & Fraenkel-Conrat, 1978a,b; Ikegami & Fraenkel-Conrat, 1979; Duda, 1979).

Viral replicase activity is usually associated with membranes and possesses an endogenous viral template (Fraenkel-Conrat, 1976; Weening & Bol, 1975; Zaitlin et al., 1973; Van Vloten-Doting & Jaspars, 1977; White & Dawson, 1978); for TNV, membrane binding has been shown to have no effect on RNA elongation (Stussi-Garaud et al., 1977) but is probably crucial for specific initiation. Most replicase preparations show little template specificity and low specific activity (about 1 % of that described for Qβ) which probably reflects the loss of cellular localisation and membrane binding during the purification procedure.

In contrast an enzyme has been described (Hardy et al., 1979) which

displays a high level of activity and template specificity when extracted using mild isolation techniques from infected tissue. This enzyme was prepared from barley leaves infected with BMV, and showed more than fifty times the activity detected in corresponding extracts from healthy tissue. A similar procedure was used to investigate replicase activity in BMV-infected and uninfected protoplasts (Okuno & Furusawa, 1979). Four proteins were shown to be specific to BMV infection in various protoplast systems, three of which were located in the membrane-bound polymerase fraction. The protoplast system appears to overcome many of the problems encountered in studying early events in virus replication (Takebe, 1975), including RNA polymerase activity.

Tobacco Necrosis Virus (TNV) TNV is the type member of the loosely related Tobanecro virus group. Many members of the group have little or no serological relationship, and some TNV isolates have such widely differing nucleotide compositions that they may be distinct viruses rather than strains. In general, strains of TNV occur in the roots of otherwise seemingly normal plants. The virus is transmitted between roots by zoospores of the fungus Olpidium Brassicae, the mechanism of transmission has been discussed by Temmink et al. (1970) and by Kassanis and Macfarlane (1964, 1965, 1968). Strains of TNV have been found responsible for a necrotic disease of cucumber (Dias & Doane, 1968), and associated with the "rusty-root" disease of carrots (Barr & Kemp, 1975; Kemp & Barr, 1978). Almost all strains elicit a hypersensitive response when inoculated onto the leaves of host plants.

The properties of TNV were examined closely in the early 1960s with regard to thermal inactivation, serological relationships, and behaviour in plants (Kassanis, 1960; Kassanis & Nixon, 1961; Babos & Kassanis, 1963 a, b & c; Kassanis & Welkie, 1963). In particular, TNV was shown to support the replication of a satellite virus (satellite tobacco necrosis virus ; STNV) which modifies the host response to infection (Kassanis, 1962), by reducing lesion size as STNV concentration increases.

When virus interactions were reviewed by Kassanis in 1963, STNV represented the only known example of the satellite phenomenon, in which one virus is obligately dependent upon another for its replication. Since then, satellites have been found associated with many plant viruses, including the associated satellite of cucumber mosaic virus, CMV-CARNA 5 (Kaper & Waterworth, 1977). In this case the effects on symptom expres-

sion make CARNA 5 economically important. The defective interfering particles of predominantly animal viruses often show similar properties, but they are derived from (and therefor serologically related to) their respective helper viruses (Huang & Baltimore, 1974). Satellite viruses are distinct in origin and have no obvious evolutionary relationship to their respective helper viruses.

There are several serologically distinct strains of STNV which are activated only by specific serotypes of TNV (Kassanis & Phillips, 1970; Uyemoto et al., 1968), and the ability to activate different satellite strains (Rees et al., 1970) has been related to the host range of the helper strain rather than serological relationships between helpers. This specificity is probably analagous to the compatability of RNA components among divided genome viruses (Lane, 1979) and takes similar advantage of vector transmission. Competition or interference occurs between coinfected strains of the satellite (Kassanis & White, 1972) as evidenced by the change in relative yields of progeny virus. The presence of a compatible satellite also reduces the yield of TNV in the infected tissue (Kassanis, 1962), although the propogation host is important in regulating relative virus yields (Salvato & Fraenkel-Conrat, 1977) and the two viruses can differ markedly in their optimum requirements (Kassanis, 1968). The Urbana, Yarwood and AC 36 strains of TNV have been variously used as STNV activators (Roy et al., 1969; Uyemoto & Grogan, 1969; Fraenkel-Conrat, 1976; Lesnaw & Reichmann, 1970), and TNV_D has been shown to neither contain nor support a satellite virus strain (Babos & Kassanis, 1963b; Kassanis & Phillips, 1970).

TNV has isometric particles of 26 to 27 nm diameter with a molecular weight of about 7×10^6. Each particle contains a single-stranded (ss) RNA molecule of 1.3×10^6 to $1.6 \times 10^6 M_r$ (Bishop et al., 1967; Lesnaw & Reichmann, 1969; Uyemoto & Grogan, 1969). The capsid sub-units have been reported as $M_r = 33,500$, probably grouped as 32 morphological units arranged in a T = 3 lattice (Lesnaw & Reichmann, 1969). Alternatively, the sub-units have been determined as $M_r = 22,600$ (Uyemoto & Grogan, 1969) and postulated to form a T = 4 lattice or a T = 3 lattice with an internal protein core. The forces responsible for stabilising the virion have not been characterised in any great detail. Changes in sedimentation coefficient have been described for TNV_D (McCarthy et al., 1980) that accompany alterations in the pH between 5 and 7. These changes are reversible and suggest that virion stability and diameter are dependent on salt lin-

kages between protein and RNA at pH 7, whereas at pH 5 other types of bond may predominate. This is consistent with observations of thermal inactivation (Babos & Kassanis, 1963a) and a pH dependent response to sodium dodecyl sulphate (SDS) (Ronald & Tremaine, 1976) and has consequences for virus recovery and identification on sucrose gradients. The lack of systemic hosts for almost all strains of TNV restricts virus propagation to hypersensitive hosts where conditions are optimal for any particular TNV strain (McCarthy et al., 1976; Wieringa-Brants, 1978; McCarthy et al., 1980). Virus yield can be optimised by maintaining a suitable temperature and humidity regime during propagation.

Satellite Tobacco Necrosis Virus (STNV) STNV is better characterised than TNV and has an icosahedral shell (Sjoberg, 1977) built of 60 protein sub-units. The sub-unit has been estimated as M_r= 22,800 by amino acid analysis (Rees et al., 1970) and as M_r= 22,900 and M_r= 22,700 by neutron (Chauvin et al., 1977) and x-ray (Sjoberg, 1977) scattering respectively. This gives a particle molecular weight of about 1.85×10^6 and a diameter of 18 nm incorporating ssRNA of M_r= 2.8 to 4.0×10^5 (Reichmann, 1964; Chauvin et al., 1977).

The complete nucleotide sequence of STNV RNA has been derived from the direct sequencing of 5' (Leung et al., 1979) and 3' (Merregaert et al. 1979) terminal regions, and the sequencing (Ysebaert et al., 1980) of a DNA copy comprising some 80 % of the genome (van Emmelo et al., 1980). In total the genome contains 1,239 residues and much of the RNA is probably of non-coding function.

The antigenicity of STNV is a feature of the satellite strain and is not influenced by the activator TNV strain (Kassanis, 1966). It thus appears that STNV RNA codes for its own coat protein. Additional features of the RNA must account for replication, control, packaging and maintaining the stability and overall shape of the molecule. Thermal denaturation kinetics suggest suggest that a high proportion of STNV nucleotides are involved in base pairing (Mossop & Francki, 1979). Although the satellite has never been isolated from plant tissue without TNV being present (Kassanis, 1968), STNV RNA is extremely stable in vivo and has been shown to survive for up to 10 days in the host cell without degradation, before being "rescued" by TNV and subsequently replicating. This persistence of STNV RNA in the host cell would be favoured by a high degree of secondary and tertiary structure as indicated by base pairing,

and must be of great strategic importance to the virus at the cellular level (Mossop & Francki, 1979).

The major differences between TNV strains and STNV strains facilitates efficient procedures for their separation and purification. These include sucrose density gradient centrifugation (Salvato & Fraenkel-Conrat 1977) and differential precipitation by ammonium sulphate from crude extracts (Fraenkel-Conrat, 1976). Alternative propagation hosts may be required in order to regulate the yield of the respective viruses (Salvato & Fraenkel-Conrat, 1977; Kassanis, 1968).

In Vitro Translation Although both TNV and STNV lack the 5'-terminal cap structure (Wimmer et al., 1968; Lesnaw & Reichmann, 1970a) usually associated with eukaryotic messenger function (Kozac, 1978), both RNAs function as messengers in cell-free translation systems. STNV RNA has been translated in prokaryotic (Clark et al., 1965; Klein et al., 1972; Rice & Fraenkel-Conrat, 1973) and eukaryotic (Klein et al., 1972; Leung et al., 1976; Salvato & Fraenkel-Conrat, 1977) in vitro translation systems with greater or lesser fidelity. Analysis of the amino-terminal tripeptides of the STNV coat protein gives identical results whether synthesized in vivo (Klein et al., 1972) or in vitro in a variety of systems (Lundquist et al., 1972; Klein & Clark, 1973) indicating a unique initiation site. No other significant translation products have been reported for STNV RNA (Salvato & Fraenkel-Conrat, 1977). The addition of a 7-methyl guanosine cap to the 5'-terminus does not enhance STNV RNA activity as a messenger in a wheat germ translation system (Smith & Clark, 1979), and the RNA presumably contains compensating features for ribosome binding and translation initiation. The RNA may also contain features which reduce the efficiency of translation when intact RNA is used as a messenger.

Models of STNV secondary structure (Browning et al., 1980; Ysebaert et al., 1980) suggest that the coat protein initiation site can be brought into close proximity to the 5'-terminus by the formation of a terminal hairpin loop. A segment of RNA around this initiation site is protected by wheat germ ribosome binding against ribonuclease digestion (Leung et al., 1976; Browning et al., 1980). In addition, the ribosome-protected region could bind to a complementary region about 1,000 nucleotides downstream (Ysebaert et al., 1980). If by such a long range interaction these regions were stably base paired, then a cryptic ribosome binding site

would be produced. The formation of such a structure would account for the similar messenger efficiency of STNV RNA in the wheat germ system, both with and without a 5' cap (Smith & Clark, 1979).

The model also includes a tRNA clover-leaf-like structure at the 3' terminus, similar to many plant viral RNAs. It is not yet known if the RNA will accept the methionine residue indicated by the anticodon of the clover-leaf sequence; the structure certainly assists in protecting the RNA from ribonuclease digestion, a role postulated for the terminal poly (A) segments common to many eukaryotic mRNAs (Huez et al., 1977) and lacking in TNV and STNV RNAs.

Translation of TNV RNA in an in vitro wheat germ system (Salvato & Fraenkel-Conrat, 1977) yields coat protein as evidenced by size, tryptic digest pattern and antigenicity. Additionally, a series of proteins are produced in quantities too small to represent structural proteins. This messenger function is retained if TNV RNA is heated and fractionated under conditions designed to dissociate RNA complexes of turnip yellow mosaic virus (TYMV) (Klein et al., 1976; Pleij et al., 1976). Messenger function in TNV appears to be associated with the genomic RNA. The non-structural proteins presumably serve a catalytic or regulatory function, they include polypeptides of 63,000 M_r and 43,000 M_r and one of 35,000 M_r which has been attributed to the endogenous wheat germ residue. Proteins of 26,000 M_r and less were shown to share coat protein antigenicity and were assumed to represent degradation products. The TNV genome could carry the information to specify three polypeptides including 63,000 and 43,000 M_r proteins together with the 30,000 M_r coat protein and additional degradation products.

Co-translation of TNV and STNV in vitro showed that STNV has a translational advantage over TNV. Much less STNV RNA is required than TNV RNA for maximal translation, and STNV RNA is preferentially translated in the presence of excess TNV RNA; STNV coat protein and coat protein degradation products (which share coat antigenicity) prevail greatly over TNV-specific products in vitro.

In Vivo Replication TNV and STNV replication has also been studied extensively in plant leaves as a means of resolving the translational strategy (Condit & Fraenkel-Conrat, 1979) and in order to investigate the competitive satellite relationship (Jones & Reichmann, 1973). Three double-stranded (ds) RNAs have been isolated from TNV-infected tobacco leaves

which by RNA-RNA hybridisation and fingerprinting techniques appear to be of viral origin (Condit & Fraenkel-Conrat, 1979). The smaller RNAs represent subsets of the genomic RNA derived from the 3' terminus, and the molecular wights of the ds forms are reported as 2.6×10^6, 1.05×10^6 and 0.94×10^6 M_r. The dsRNAs fit the criteria of replicative forms (rfs) for viral mRNA synthesis, and their size and location would lead to messengers capable of specifying polypeptides of 77,000, 6000 and 43,000 M_r at maximum. In an *in vitro* translation system the smallest dsRNA has been shown to specify a product similar to the coat protein in electrophoretic mobility. The method by which the dsRNAs may be synthesized *in vivo* is not known, but their interpretation as rfs for mRNA synthesis would classify TNV as a virus requiring processing and/or cleavage prior to translation of the 3' portion of the RNA.

Double stranded forms of TNV RNA and two smaller components have also been isolated from the replication products made *in vitro* by the membrane-bound replicase fraction from TNV-infected tobacco (Fraenkel-Conrat, 1976; Stussi-Garaud *et al.*, 1977). This replicase fraction has high polymerase activity, and contains a 60,000 M_r polypeptide which resembles the larger *in vitro* product in the wheat germ translation system, and possibly corresponds to the maximum coding capacity ($\leq$77,000 M_r) for TNV mRNAs. A similar product has also been identified as a result of TNV infection in tobacco leaves (Jones & Reichmann, 1973).

Six virus-induced or virus-stimulated proteins were detected in tobacco leaves infected with TNV, including coat protein, and proteins of 64,000, 41,000, 23,000, 15,000 and 12,000 M_r. Co-infection with STNV resulted in the additional synthesis of STNV coat protein and a suppression of TNV coat protein synthesis. The degree of suppression by STNV was shown to depend on the strain of TNV used as the helper virus with more efficient helper strains being most sensitive to interferance. Due to the asynchronous nature of leaf infection, the proteins could not be divided into products of early or late message translation. Similarly, no distinction could be made between host- and viral-coded protein species in the products detected as a result of TNV infection, except in the case of viral coat proteins.

Clearly, a synchronous infection system would be an advantage in studying the replication events and translation strategy of TNV. The relationship, and apparent competition, between TNV and STNV may also prove advantageous as a tool in studying virus replication in general.

Isolated Protoplasts Studying the early events in virus replication is difficult in intact plant tissues because infection efficiency is low and replication proceeds asynchronously (Jones & Reichmann, 1973; Takebe, 1975). Cultured cells have been used to study TMV replication (Murakishi et al., 1971; Jackson et al., 1972) and more recently soybean mosaic virus (Fang-Sheng Wu & Murakishi, 1978), but since the initial observations by Cocking (1966) and exploitation by Takebe (1968) isolated protoplasts have made the major contribution to molecular studies of infection. Techniques involving protoplasts have revolutionised plant virus studies, enabling for the first time simultaneous inoculation of large numbers of cells, which then support synchronized, one-step viral replication. A high proportion of cells can be infected and the material can be controlled precisely and handled quantitatively, yielding high virus titres or virus-related products. This technique has been used to investigate the synthesis of virus-related products during infection (Zaitlin et al., 1981), genome organisation (Franssen et al., 1981), and the mechanisms of resistance (Boulton et al., 1981), necrosis (Kopp et al., 1981), and cross protection (Barker & Harrison, 1981). The applications of protoplast technology to plant virology have been extensively reviewed (Zaitlin & Beachy, 1974; Takebe, 1975, 1977; Wood et al., 1979) and the scope continues to increase, however there are still limitations to the use of protoplasts in virus research.

Initially, isolated protoplasts may prove difficult to handle, and reproducible results difficult to achieve. In addition, results obtained using a protoplast system cannot be taken as representing the situation in the intact plant. Major physiological and structural differences between the two systems must be recognised and a number of "short-term" stress responses induced by protoplast isolation taken into account.

Isolation Response Many of the treatments used in protoplast isolation lead to marked changes in cellular metabolism. Osmotic stress has been shown to reduce amino acid (Ruesink, 1978) and nucleic acid (Premecz et al., 1978) incorporation by protoplasts, and induce changes in ribonuclease and protease levels (Lazar, et al., 1973). Both RNA and protein precursors are lost from protoplasts into the culture medium (Rubin & Zaitlin, 1976) and cellulase treatment has been shown to cause the plasmamembrane to become leaky (Taylor & Hall, 1976). However, different cellulase preparations used in protoplast isolation vary considerably in their effects on subsequent amino acid incorporation (Ruesink, 1978).

Some metabolic responses to protoplast isolation might be regarded as "short-term". Protoplasts derived from cell suspension cultures have been shown (Kulikowski & Mascarenhas, 1978) to adopt a pattern of RNA synthesis high in poly(A)-containing sequences and deficient in rRNA processing. However, within 24 hours these protoplasts resume a pattern of RNA synthesis similar to that in whole cells. Transport studies on similarly derived protoplasts (Mettler & Leonard, 1979a, b) show them to have similar transport properties to cells in suspension culture, despite cellulase treatment.

Isolation of protoplasts from leaf tissue results in cellular changes not easily related to physiological conditions, such as the altered protein profile immediately protoplasts are incubated in culture medium (Fleck et al., 1979). A general stimulation of carrier synthesis has been postulated (Robinson & Mayo, 1975) during the first hours of protoplast culture and the polysome pattern differs markedly from that in the intact leaf (Ruzicska et al., 1979). The isolation of the cell from its normal environment has been suggested as the most important factor in these alterations in metabolism. Burgess (1978) has reviewed some of the work on isolated protoplasts which illustrates the "short-term" responses. However, while some metabolic changes clearly result from the stress of isolation (Burgess et al., 1978), others might be regarded as "long-term" responses to the isolated state, and represent an adaptation to the new environment.

For plant virus studies, most interest is generally directed at the initial minutes or hours following protoplast isolation, since at this stage the protoplasts are most amenable to virus infection. This is also the period when isolation responses play a major role in the biochemistry of the cell. Accordingly, attempts must be made to minimise their effect on the analysis of virus infection. If protoplasts are derived from cell cultures or suspensions (Jarvis et al., 1981) then much of the short-term stress of isolation is avoided. However, the cellular metabolism of the cultured state makes the model less representative of the intact plant, and must reflect accordingly in the response to virus infection.

Allowing the protoplasts recovery time, to regain their normal physiology, after isolation from leaves (Burgess, 1978) would necessitate a second enzyme treatment to remove the regenerating cell wall before infection. Alternatively, a rapid isolation and infection procedure permits a recovery period between protoplast infection and subsequent ex-

perimental procedures (e.g. addition of labelled precursors) without requiring prolonged enzyme treatment. This would enable many stress-induced responses to be avoided and the cell to resume normal biochemical behaviour prior to the onset of virus induced phenomena.

Comparative Systems Also with a view to achieving representative and reproducible protoplast material, stresses and variations in plant growth conditions must be avoided if responses to virus infection are to be compared. For protoplasts to survive the isolation procedure, and for efficient virus infection the initial physiological condition of the plants is important. Plant material must be reproducible with respect to age and any physiological variables encountered during the growth period. Particular attention must be given to photoperiod, temperature and water stress immediately prior to protoplast isolation. Such parameters are difficult to regulate over the sixty to seventy days required to grow tobacco plants suitable for protoplast isolation (Takebe et al., 1968; Huber, 1979).

A system has been described (Hibi et al., 1975) which employs cowpea seedlings, with a much shorter growth period than tobacco, as a source of protoplast material. The primary leaves of cowpea plants are suitable for protoplast isolation within nine to eleven days of sowing under controlled light and temperature conditions. Plants can be maintained in nutrient solutions and fully expanded primary leaves yield protoplasts by a one-step digestion procedure.

Although the tolerance limits for the plant growth period are considerably reduced - protoplasts from older or younger leaves have a low survival rate and give a lower percentage of viral infection - this is compensated by the degree of environmental control which can be achieved over the shorter growth period. A single-step enzyme treatment is more convenient than a sequential procedure (Takebe et al., 1968), and appears to give increased protoplast yields from several plants (Evans et al., 1972; Hibi et al., 1975).

Cowpea protoplasts used for viral infection consist mainly of palisade, and some spongy mesophyll protoplasts. Most epidermal and a proportion of spongy mesophyll protoplasts are removed by centrifugal washing procedures. Virus infection techniques differ markedly between cowpea and tobacco protoplasts even when using the same virus, and this has been taken to reflect differences in the plasmalemma (Alblas & Bol, 1977; Hibi

et al., 1975). The differences in protoplast response and the mechanisms of virus infection are not well understood. However, since the cowpea mesophyll system draws substantially on experience gained with tobacco protoplasts, then comparisons can be usefully made in terms of infection techniques and parameters.

Protoplast Infection The infection of isolated leaf protoplasts with either virus or viral RNA is promoted by the prior incubation of virus or RNA in a buffered polycation solution (Takebe, 1975). The polycation (frequently poly-L-ornithine ; PLO) is thought to aggregate the virus and buffer ions at pH values which depend on the isoelectric point of the virus. A dual polycation role would also involve the formation of surface lesions on protoplasts as well as making the cells attractive to virus aggregates (Burgess, 1973a, b). A differential response has been observed between tobacco and cowpea mesophyll protoplasts inoculated with CPMV or AMV in the presence of PLO.

PLO is obligatory for the infection of tobacco mesophyll protoplasts with CPMV, whereas cowpea protoplasts have been inoculated with CPMV in the absence of polycation. However, in low concentration PLO does have a stimulatory effect on cowpea protoplast infection (Hibi et al., 1975) which is limited by high toxicity as the concentration is increased. The infection of cowpea and tobacco protoplasts with AMV in a buffered inoculum with PLO shows a differential pH dependence (Alblas & Bol, 1977) and comparative infections with strains of TMV have revealed a differential response to poly-D-lysine (Huber et al., 1981). Although the use of buffered cations has become a routine infection procedure, the uncertainty surrounding the mechanism (Burgess et al., 1973b), and the toxicity of the polycations (Cassels & Barlass, 1978) has prompted some workers to investigate alternative procedures.

The initiation of infection with polyethylene glycol (PEG) has been described as such an alternative (Cassels & Cocker, 1980), and may provide other methods of analysing the infection mechanism as may artificial membrane and liposome studies (Cassels, 1978). However, the initiation of infection with PEG may depend on similar phenomena to those already recognised as factors in buffered polycation systems. Certainly PEG serves to aggregate virions and induces osmotic changes at the cell surface; an osmotic "step-up" is now widely used (Okuno & Furusawa, 1978; Alblas & Bol, 1978) to enhance infection in conjunction with polycation treat-

ment. The contributions of localised plasmolysis, endocytosis, surface lesions and simple electrical phenomena are largely conjectural. Measurements of protoplast charge (Grout & Coutts, 1974), and the use of non-physiological temperatures with ionic and osmotic variations (Okuno & Furusawa, 1978) indicate a combination of several of the proposed mechanisms (Kassanis et al., 1977). Although the high rates of infection reported for many experimental systems suggest that techniques can be combined and adapted, there are also apparent contradictions. Cowpea protoplasts, for example, show enhanced replication of AMV (Alblas & Bol, 1977) in response to an osmotic "step-up" and a decrease in the amount of TMV synthesised (Huber et al., 1981). On this basis a judicious combination of infection techniques must always be optimised for each virus-host combination.

Divalent cations, usually Ca^{2+}, are an important feature of most protoplast infection systems. In plant tissue Ca^{2+} ions have been shown to retain membrane structure and ionic integrity (Kassanis & White, 1974; Tu, 1978), and in protoplast systems assist in stabilising the plasmalemma. After virus infection using PLO, Ca^{2+} or Mg^{2+} ions have been shown to assist in disaggregating the infection complexes, and may play an important role in virus disassembly (Durham et al., 1977) and subsequent cellular regulation (Durham, 1978). Virus multiplication in protoplasts has been shown to stop if the divalent-ion concentration in the culture medium is reduced (Kassanis et al., 1975). Thus a washing solution of $Ca\ Cl_2$ in osmotically stabilised medium is often used directly after protoplast inoculation (Hibi et al., 1975) or high Ca^{2+} concentrations are incorporated into the protoplast culture medium (Takebe, 1979; Alblas & Bol, 1977) to support virus replication.

TNV Replication in Protoplasts Cowpea protoplasts have been infected with TMV and TNV using a buffered polycation system (Wieringa-Brants et al 1978). The protoplasts were maintained for two days in a simple medium ($CaCl_2$ in a mannitol osmoticum) with added antibiotics, and virus replication was assessed by local lesion assay of protoplast extracts. These initial observations suggest that the infection conditions might be optimised further and TNV replication studied by more rigorous methods.

Such a system should avoid the problems of asynchronous infection and tissue necrosis which accompany whole plant infections, and thus be more compatible with biochemical analysis.

2 MATERIALS AND METHODS

2.1 Viruses and Virus Purification Tobacco necrosis virus strain D (TNV_D) (Babos & Kassanis, 1963) was originally supplied in French bean leaves (Phaseolus vulgaris var. The Prince) by Dr. S.R.Chant and Dr.D. McCarthy and was propagated through single lesion isolates in primary leaves of cowpea (Vigna Sinensis Endl. cv. Blackeye No.5). Highly purified virus preparations were obtained from heavily necrosed primary leaves of cowpea or French bean (var. The Prince or Pinto).

Purification techniques involved differential centrifugation (Kassanis & Phillips, 1970) or polyethylene glycol precipitation of virus (Gooding & Hebert,1967) but most consistent results were obtained using a centrifugation method based on the procedure of Salvato and Fraenkel-Conrat (1979).

Five days after inoculation with TNV_D, necrosed primary leaves of Pinto beans were harvested and frozen at -20°C. All subsequent procedures were carried out at 0 to 4°C. The frozen tissue was homogenised in a Kenwood homogeniser containing 0.1 M sodium phosphate buffer, pH 7.5 with 1% 2-mercaptoethanol to give a 1:1 ratio (w/v) of infected tissue to buffer.

The resulting homogenate was strained through 3 layers of muslin into a measuring cylinder and ethanol was added at 25ml/100ml original extract. This mixture was stirred for 30 minutes and clarified by centrifugation at 10,000xg for 20 minutes.

The supernatant was filtered through 3 layers of muslin to remove coagulated material and recentrifuged at 30,000xg for 30 minutes. This clarified supernatant was ultracentrifuged for 120 minutes at 105,000xg to pellet the virus.

The virus pellet was resuspended overnight in 20ml distilled H_2O / 100ml original extract and subsequently clarified by centrifuging at 30,000xg for 30 minutes. Virus was re-pelleted from the clarified supernatant by a further ultracentrifugation at 105,000xg for 120 minutes.

The resulting pellet was then resuspended in 0.9ml distilled H_2O/ 100ml original extract and clarified at 30,000xg for 20 minutes. The virus content of the supernatant was estimated spectrophotometrically using a Beckman model 35 spectrophotometer and employing the extinction coefficient : $E^{1\,cm}_{260}$ 0.1% = 5.5 (Babos & Kassanis, 1963). Prior to

storage in aliquots at -20°C, a sample of the virus suspension was taken to determine the end-point titre on cowpea primary leaves.

Virus was purified from cowpea leaves by a similar method using necrosed primary leaves 3 to 4 days after inoculation.

Tobacco necrosis virus strain A (TNV_A) was originally provided by Mr. R.F.White and was similarly propagated in cowpea primary leaves. Highly purified virus preparations were obtained from primary leaves of Pinto beans 6 days after inoculation, using the method described for TNV_D purification.

An additional sucrose gradient centrifugation was employed to separate TNV_A and SV. The virus content of the highly purified preparation was estimated as for TNV_D, and 0.5 to 1 ml of the solution was layered onto 23 ml, 10 to 40 % (w/v) sucrose gradients and centrifuged at 25,000 rpm for 210 minutes in a Beckman SW 30 rotor.

The gradients were sampled by sucrose displacement and monitored using a Uvicord gradient analyser measuring absorbance at 260 nm. The collected fractions were dialysed overnight against distilled water and and the dialysed extract was analysed for virus content as before using the extinction coefficient $E^{1\,cm}_{260}$ o.1 % = 6.5 (Babos & Kassanis, 1963) for samples containing SV. Prior to storage at -20°C, samples were inoculated onto cowpea primary leaves to verify the TNV_A and SV designation of the fractions.

All clarification steps were carried out in an MSE Hi-spin 21 centrifuge using a 6 x 30 ml rotor at 11,000 rpm and 19,000 rpm (to give 10,000xg and 30,000xg respectively). Ultracentrifugation was variously carried out in a Beckman L8 using a 45 Ti rotor or a Spinco ultracentrifuge using a type 50 rotor, depending on the volume to be centrifuged.

2.2 Plants and Plant Cultivation Cowpea (Vigna sinensis Endl. cv. Blackeye No.5) seeds were obtained from Ferry-Morse Seed Company, Mountain View, California, U.S.A., and germinated in seed trays containing "Levington's All Peat Potting Compost". High humidity was maintained during germination and the seedlings were subsequently grown under glasshouse conditions at 25 ± 3°C with supplementary lighting from Phillips 400 W mercury vapour lamps, providing a 16 hour photoperiod of light intensity between 10 and 20,000 lux. Plants were selected for inoculation 8 to 10 days after germination, when the primary leaves were fully ex-

panded but prior to emergence of the first trifoliate leaf.

French bean (Phaseolus vulgaris L. cv. Pinto) seeds were obtained from The Botanic Supply Unit, Royal Holloway College, Surrey, U.K., and germinated under similar conditions to cowpea seeds. Plants were selected for inoculation 10 to 12 days after germination, when the primary leaves were fully expanded but prior to emergence of the first trifoliate leaf. After inoculation the plants were sprayed regularly with water to reduce desiccation of the tissue and high humidity was maintained.

Cowpea plants used as a source of protoplasts were grown under more rigorously defined conditions. Seeds were germinated in pots of moistened vermiculite under a 16 hour photoperiod providing 10,000 lux illumination from 24 warm white fluorescent tubes and 9 x 150 W incandescent tungsten bulbs. The temperature was maintained at 25°C throughout and relative humidity at approximately 50 %.

Four days after seeding, germinated plantlets were transferred to bottles of culture solution which was formulated according to Heller (1953), and stored as 3 stock solutions (see appendix 1). Seedlings in culture were then maintained under similar conditions for 4 to 8 days until the primary leaves were fully expanded and suitable for protoplast isolation. Primary leaves were selected just prior to the emergence of the first trifoliate leaf (Hibi et al., 1975).

2.3 Surface Sterilisation Detached primary leaves of cowpea were surface sterilised by a procedure similar to that of Coutts and Wood (1975). Using a "Pathfinder" laminar air-flow cabinet the leaves were washed for 5 seconds in 70 % ethanol. They were then immersed for 15 minutes in a 5 % solution of "Domestos" bleach (Lever Brothers, Ltd; a sodium hypochlorite solution with 10 % available chlorine).

The washing procedure was carried out using large casserole dishes for ease of handling and sterilisation. Leaves were submerged in the washing solutions by weighting with sterile Petri dishes. After sterilisation, the leaves were rinsed in sterile distilled water and maintained in the third wash until protoplast isolation.

2.4 Protoplast Isolation Sterilised leaves were allowed to dry in the laminar air-flow and the lower epidermis was detached using fine jewellers forceps (5 INOX). The peeled leaves were floated (peeled surface

down) in 7.5 cm diameter sterile plastic Petri dishes containing 25 ml of 0.5 M mannitol solution. After 10 to 30 minutes the mannitol was decanted and replaced with 25 ml of enzyme solution containing 0.1 to 1.0 % (w/v) Cellulase Onozuka R-10, 0.05 to 0.5 % (w/v) Macerozyme R-10 (Kinki Yakult Chemicals, Japan) and 0 to 0.1 % (w/v) bovine serum albumin (Sigma) (Beier & Bruening, 1976) in 0.5 M mannitol at pH 5,6, freshly prepared and filter sterilised before use.

The tissue was incubated in Petri dishes sealed with "Parafilm" (Gallenkamp) in darkness for 2 to 4 hours. Incubation was carried out in a shaking water bath at 25 to 35°C and 0 or 25 excursions/minute. For optimum isolation conditions see results section.

After incubation the protoplast suspension was filtered through 77 um nylon bolting cloth (Henry Simon, Ltd.) into sterile MSE, HR 50 ml tubes and centrifuged at 200xg for 3 minutes in a BTL bench centrifuge. The suspension was transferred using sterile plastic Pasteur pipettes (Alpha Laboratories) with enlarged tips to reduce friction damage to the protoplasts.

The sedimented protoplasts were washed by decanting the enzyme solution and resuspending in 0.5 M mannitol. The resuspended protoplasts were washed in 2 further changes of 0.5 M mannitol by centrifugation, and resuspended in a measured volume of solution. Protoplast yield was determined by counting samples from the measured volume in a modified Fuchs-Rosenthal haemocytometer.

2.5 Determination of Protoplast Viability Protoplast viability was determined using the exclusion dyes phenosafranine (Widholm, 1972), and Evan's Blue (Gaff & Okong'O-ogola, 1971) or the vital stain fluorescein diacetate (Widholm, 1972).

Phenosafranine was dissolved in 0.5 M mannitol containing 0.1 mM $CaCl_2$ to give a 0.1 % (w/v) solution. Samples of protoplast suspension were mixed with equal volumes of the stain and examined after 10 minutes using a haemocytometer. Protoplasts which remained green were regarded as intact and viable, damaged protoplasts stained red.

Evan's Blue was similarly dissolved in mannitol and $CaCl_2$ solution and mixed with equal volumes of protoplast suspension. After 10 minutes viable protoplasts which excluded the blue stain appeared yellow/green.

The metabolic stain fluorescein diacetate was dissolved to give

2.5 mg in 0.5 ml of acetone. This was added to 10 ml of 0.5 M mannitol containing 0.1 mM $CaCl_2$ and mixed with protoplast samples in equal volumes. The mixture was allowed to stand in darkness for 10 minutes and examined under a fluorescence microscope fitted with exciter filter KP500 and barrier filters LP520 and LP540 using a Fuchs-Rosenthal haemocytometer. Live protoplasts fluoresced bright yellow/green.

Protoplast viability was assessed in each case using the mean percentage of viable protoplasts in 10 microscope fields.

2.6 Culture Media and Incubation Washed protoplasts were resuspended in one of 3 liquid media for incubation under controlled conditions for upto 64 hours. Takebe's simple salts medium was prepared essentially as described by Aoki & Takebe (1969) and contained 1.0 mM KNO_3, 0.1 mM $MgSO_4$ 0.1 mM $CaCl_2$, 1.0 µM KI, 0.01 µM $CuSO_4$, and 0.2 mM KH_2PO_4 in 0.5 M mannitol at pH 5.4. This medium was designated virus incubation medium (or VIM).

Root culture medium after White (1943) and modified by Street & McGregor (1952) and Sheat et al. (1959) (Flow Laboratories) was prepared without sucrose or supplements in 0.5 M mannitol for protoplast culture.

A simplified medium (Alblas & Bol,1977) was also used which contained 0.5 M mannitol and 10 mM $CaCl_2$. All media were sterilised before use by autoclaving at 121°C for 10 to 15 minutes.

Protoplasts were incubated in 10 ml batches in 100 ml Erlenmeyer flasks for sequential sampling experiments. For discrete samples or "pulse labelling" experiments protoplasts were incubated in 1 to 5 ml aliquots in 10 ml scintillation vials. All culture vessels were washed in chromic acid prior to sealing with aluminium foil and heat sterilising at 120°C for 15 minutes.

Unless otherwise stated, protoplasts were incubated under controlled conditions of 3,000 lux continuous light supplied by "warm white" fluorescent tubes and 150 W incandescent tungsten bulbs at 22°C. Incubation density was determined using a haemocytometer and by making suitable dilutions of the protoplast suspension.

2.7 Antibiotics Both Nystatin (E.R.Squibb & Sons Ltd.) and chloramphenicol (Sigma) were added to culture media before and/or during protoplast incubation to control microbial contamination (Watts & King, 1973).

Nystatin stock solution containing 75 units of Nystatin in 1 ml of VIM was stored at -20°C and appropriate volumes added to the incubation medium before or after protoplast suspension.

Chloramphenicol stock solution was prepared on the day of use. By dissolving initially in a small volume of ethanol 0.05 g of chloramphenicol was added to 25 ml of VIM. The solution was filter sterilised and added in appropriate volumes to the protoplast incubation medium.

2.8 Uptake and Incorporation of Protein and RNA Precursors The uptake of L-(1-^{14}C)Leucine at 59 mCi/mmol and (5-^{3}H)Uridine at 30 Curies/mmol (Radiochemical Centre, Amersham) into isolated protoplasts was determined essentially as described by Rubin & Zaitlin (1976). Both total incorporation into the protoplasts and incorporation into acid-insoluble products were determined.

Unless otherwise stated, 15 µCi of (^{14}C)-leucine and/or 150 µCi of (^{3}H)-uridine were added to 10 ml of incubated protoplasts in Erlenmeyer flasks. Labelled precursors were added immediately after protoplast isolation or after preincubation in unlabelled medium. In pulse-chase experiments, excess of unlabelled precursor was added after being dissolved in a small volume of incubation medium following the labelled incubation period.

In experiments to determine incorporation into acid-insoluble products, 200 µl aliquots were withdrawn at intervals from the incubated samples. Nucleic acid and protein synthesis was arrested by adding 5 ml of ice cold 0.5 M mannitol followed by an equal volume of 20 % trichloroacetic acid. The samples were maintained at 4°C overnight or until the complete time-course was available for analysis.

Stored samples were filtered onto Whatman GF/C glass fibre filters and washed 3 times with 5 ml aliquots of cold 10 % trichloroacetic acid (TCA), followed by 2 washes with 80 % (v/v) ethanol and finally with 5 ml of diethyl ether. After drying, the radioactivity on the filters was measured in 8 ml of a toluene-based scintillation cocktail containing 2 volumes of toluene to 1 volume of absolute ethanol with 0.4 % (w/v) 2, 5-diphenyl oxazole (PPO) and 0.01 % 1,4-di-(2-(5-phenyloxazolyl))-benzene (POPOP) (both Sigma). The activity was determined using a"Tracerlab"coruspectromatic -200 with corrections made for (^{14}C) "cross-over" counts registered in the (^{3}H)-counting channel.

In experiments to compare total uptake of precursors with incorporation into acid-insoluble products a comparative sampling procedure was followed. Aliquots of 500 µl were withdrawn from incubated protoplast samples and resuspended in 2 ml of 0.5 M mannitol. The protoplasts were centrifuged at 200xg for 3 minutes and the supernatant decanted.

After a further wash in 0.5 M mannitol the protoplast pellets were homogenised in a grinding buffer containing 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 50 mM tris/HCl at pH 7.6, using 1,500 ul of buffer to 500 µl of washed protoplasts. The homogenised samples were centrifuged at 6,000xg for 15 minutes and the supernatants retained for analysis. All procedures were carried out at 0 to 4°C.

Total incorporation into the protoplast samples was determined by mixing 70 µl of the supernatant with 300 µl of Soluene 100 and 10 µl of glacial acetic acid to reduce the quenching effects of Soluene 100 (Paus, 1970). The solubilised samples were dissolved in 8 ml aliquots of the toluene scintillant described above and counted similarly in 10 ml vials with Whatman GF/C filters inserted. Duplicate samples were taken.

Incorporation into TCA acid-insoluble fractions was determined from 500 µl of the homogenate supernatant added to 500 µl of ice-cold 20 % TCA and collected on Whatman GF/C filters. The filters were washed using 3 aliquots of TCA and 2 of 80 % ethanol as previously described and counted as above. Incorporation was corrected for the relative protoplast concentration in each sample counted.

2.9 Infection of Protoplasts with TNV Initial infection procedures were similar to those described by Wieringa-Brants et al. (1979), these were optimised by independently varying the different infection parameters and assaying the virus infectivity recovered. Individual infection conditions are described in the results section.

A known number of washed protoplasts were sedimented from 0.5 M mannitol by centrifugation at 200xg for 3 minutes and resuspended in 10 ml aliquots of inoculation medium in 100 ml Erlenmeyer flasks. The inoculation medium contained 1 to 100 mM phosphate buffer (or citrate buffer) at pH 5.2 to 6.4; 0.5 to 16 ug/ml poly-L-ornithine (Sigma, M_r = 122,000) and 1 to 20 µg/ml TNV_D in 0.5 to 0.7 M mannitol. The mannitol and buffer solutions were sterilised by autoclaving and dispensed aseptically immediately before use. The buffer solutions were formulated in mannitol to reduce osmotic changes in the inoculation medium. Poly-L-ornithine was

freshly dissolved in mannitol solution and the inoculation medium was pre-incubated for a time at 25°C before protoplast addition. In "mock-infections" TNV was omitted from the mixture.

Freshly sedimented protoplasts were incubated in the inoculation medium for 5 to 30 minutes at 0 or 25°C in a reciprocating water bath at 25 excursions per minute.

After inoculation the protoplasts were sedimented by centrifugation and washed 3 times in 0.5 M mannitol containing 0.1 mM $CaCl_2$. Washed protoplasts were resuspended in VIM and after adjusting the incubation density, decanted into culture vessels and incubated as described above.

2.10 Infectivity Assay of Protoplast Extracts Infected protoplast suspensions were harvested in 1 ml aliquots and assayed for infectious virus as both protoplast pellets and incubated samples including incubation medium.

Aliquots for assay as whole suspensions were frozen directly at -20°C and stored until a complete series was available for assay. The samples were then thawed and dialysed overnight against distilled water at 4°C. Protoplast disruption was ensured by ultrasonication for 1 minute and the samples were diluted in distilled water in order to moderate counts in local lesion assay (dilution ± 1 in 10). Samples were inoculated onto primary leaves of 10 to 12-day old cowpea plants (Coutts, 1978) grown as described above. Each sample was inoculated onto at least 10 leaves using sterile cotton-tipped applicators (Johnson & Johnson) and 500 carborundum (The Carborundum Company Ltd.) as an abrasive. Lesions were counted 3 to 5 days post-inoculation.

Aliquots for assay as protoplast pellets were centrifuged directly after harvesting (200xg for 3 minutes) and the supernatant was decanted. The protoplast pellets were frozen at -20°C for storage and assayed as a complete series. Pellets were thawed and diluted in distilled water as above before ultrasonication. Inoculation procedures were carried out as above using the diluted, sonicated pellets.

2.11 Infectivity Assay of Protoplast RNA Extracts RNA was extracted from sedimented protoplasts essentially as described by Coutts et al. (1975). The protoplasts from 1 ml aliquots of culture medium were collected by centrifugation and resuspended in 500 µl of TNE buffer cont-

taining 0.1 M tris, 0.1 M NaCl, and 0.01 M Na_2EDTA at pH 8.5. An equal volume of water saturated phenol was added containing 15 % (v/v) m-cresol with 0.1 % para-hydroxy-quinoline. Also added to the mixture were 42 µl of 20 % sodium dodecyl sulphate solution (SDS) and 2 µl of 4 % bentonite prepared according to Fraenkel-Conrat (1956), and the complete mixture was shaken at 4°C for 10 minutes using a "Griffin" flask shaker. The aqueous phase was removed after centrifugation at 300xg for 10 minutes at 4°C and re-extracted with 500 µl of the phenol mixture as before. The re-extracted aqueous phase was recovered and excess phenol was removed by shaking with 4 changes of water-saturated ether. Remaining ether was finally removed in a continuous air stream. All buffers and glassware were autoclaved before use and maintained at 4°C during the experimental procedures.

The extracted aqueous phase was diluted with distilled water at 4°C and inoculated immediately onto cowpea primary leaves as described above.

2.12 Sucrose Gradient Analysis of Protoplast Extracts Protoplast pellets sedimented from 5 ml aliquots of incubation medium were resuspended in 500 µl aliquots of protoplast grinding buffer (PGB) comprising 10 mM KCl, 2.0 mM EDTA, 5 mM 2-mercaptoethanol and 50 mM tris at pH 7.6. The resuspended pellets were stored at -20°C until a series of samples were available for analysis.

After thawing, the samples were macerated using a Teflon, Potters-Elversheim bulb arrangement and the homogenate was centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was shaken vigorously with an equal volume of chloroform and the emulsion was separated by centrifuging at 1,000xg for 10 minutes. The clear, aqueous phase was retained and 200 to 500 µl samples were layered onto 4.5 ml 10 to 40 % (w/v) sucrose gradients in distilled water.

The gradients were centrifuged at 30,000 rpm and 4°C for 210 minutes in a Beckman SW 40 rotor and analysed using a Varioperpex peristaltic pump and LKB Uvicord gradient analyser. Collected fractions were dialysed overnight against distilled water at 4°C and inoculated onto cowpea primary leaves as described above.

The areas of the peaks on the absorbance traces were compared to peaks produced by known quantities of virus added to healthy protoplasts which were similarly extracted and analysed. Virus peaks were located

by comparing traces from gradients loaded with purified virus from a variety of sources. The comparative peak areas were used to quantify virus yield from infected protoplasts at various times post-inoculation.

2.13 SDS-Polyacrylamide Gel Electrophoresis of Protoplast Extracts At various times post-inoculation, infected and "mock-infected" protoplasts incubated in 2 ml aliquots of VIM were labelled either continuously or in 2 hour pulses with (U-^{14}C) protein hydrolysate (57 mCi/milliatom carbon; Radiochemical Centre Amersham) to give 12.5 µCi/ml. After labelling duplicate 1 ml samples were centrifuged at 200xg for 3 minutes and the protoplast pellets stored in liquid nitrogen.

When required, the pellets were macerated in 20 µl of PGB using a glass rod to thoroughly grind the tissue. The resulting homogenate was centrifuged at 10,000xg for 3 minutes and the supernatant was boiled for 5 minutes in an equal volume of loading buffer (Laemmli, 1970; see appendix 2). The homogenate pellet was solubilised by macerating in 20 µl of PGB with 1 % Triton X-100 (BDH Chemicals Ltd.) added and after incubating overnight at 4°C the samples were centrifuged and the supernatant boiled with loading buffer as described above.

From the disrupted pellet and supernatant fractions 2 µl aliquots were withdrawn and spotted onto 1 cm squares of Whatman 3MM paper. The squares were boiled for 15 minutes in a solution of 20 % TCA, 8% NaH_2PO_4 and 8 % $Na_4P_2O_7$ (all w/v) and washed in 80 % (v/v) ethanol. The radioactivity in the squares was assessed in 8 ml aliquots of toluene scintillant as described above, using a Packard Tri-Carb scintillation spectrometer.

The scintillation data was used to load similar levels of radioactivity in each lane when the samples were loaded onto discontinuous, 15 % polyacrylamide slab gels (Laemmli, 1970; see appendix 2). The gels were also loaded with 2 µl of (^{14}C) methylated protein mixture (Radiochemical Centre Amersham; see appendix 3) in 1 or 2 lanes to serve as protein molecular weight markers, and electrophoresed at 100 V for 4 to 6 hours.

After fixing the gels overnight in 7 % acetic acid (v/v), radioactive proteins were detected by fluorography using pre-flashed film (Bonner & Laskey, 1974; see appendix 4). Kodak X-omat film was exposed for 14 days to the PPO-impregnated gels at -70°C. After developing, the

film was sliced into strips along the gel lanes and these were scanned individually on a Gilford recording spectrophotometer fitted with a gel-scanning attachment.

2.14 RNA Replicase Assay of Protoplast Extracts At various times post-inoculation healthy, infected and "mock-infected" protoplasts incubated in 5 ml aliquots of VIM were sedimented by centrifugation at 200xg for 3 minutes and washed in 2 changes of 0.5 M mannitol with 0.1 mM $CaCl_2$. After the final wash the pellets were resuspended in 1 ml of PGB with 10 % (w/v) glycerol added and homogenised thoroughly using a Teflon, Potters-Elversheim apparatus. The resulting homogenate was centrifuged at 1,000xg for 10 minutes and the supernatant was further pelleted at 20,000xg for 15 minutes. All steps were carried out at 0 to 4°C and all buffers and glassware were autoclaved before use at 121°C for 15 minutes.

Each 15,000xg pellet was resuspended in 400 µl of incubation buffer (IB) containing 10 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 7.5 mM 2-mercaptoethanol, 1 mM EDTA, and 5 % (v/v) glycerol in 50 mM tris pH 8.4. A glass rod was used to ensure complete disruption of the pellet before the suspension was assayed for RNA replicase activity.

The enzyme suspension was added in 90 µl aliquots to a reaction mixture containing 0.05 µ mol each of adenosine-5'-triphosphate (ATP), cytidine-5'-triphosphate (CTP) and guanosine-5'-triphosphate (GTP), 1.5 µg of actinomycin D (all Sigma Chemicals) and 2.5 uCi (5,6-^{3}H) uridine-5'-triphosphate ammonium salt ((^{3}H)UTP) specific activity 10 Ci/mmol (Radiochemical Centre Amersham).

ATP, CTP and GTP were added as their respective ammonium salts dissolved in IB and stored at -20°C until ready for use. Actinomycin D was dissolved in IB by extensive shaking on a Griffin flask-shaker and stored at -20°C. (^{3}H)UTP was stored at -20°C in ethanol/water (1:1 v/v); immediately before use an aliquot was evaporated to dryness using a fine air stream and the label was resuspended in IB for addition to the reaction mixture.

Reaction mixtures were incubated for 60 minutes in darkness at 30°C in a water bath and the reaction was stopped by adding 500 µl of ice-cold termination solution containing 10 % (w/v) TCA, 4 % (w/v) $Na_4P_2O_7$ and 4 % NaH_2PO_4. Termination solution was added to zero-time control reactions immediately after addition of the enzyme to the mixture.

After termination, reaction mixtures were maintained at 4°C and collected on Whatman GF/C glass fibre filters with 10 µl of 10 % (w/v) bovine serum albumin (BSA) added to the mixtures to serve as a "carrier" in precipitation.

The reaction precipitates were washed by drawing a series of wash solutions through the filters. Initially 30 ml of 5 % (w/v) TCA with 2 % (w/v) $Na_4P_2O_7$ and 2 % (w/v) NaH_2PO_4 was used, followed by 30 ml of 100 mM $Na_4P_2O_7$ in 1N HCl; and 10 ml of 80 % (v/v) ethanol. The filters were dried after passing through 6 ml of diethyl ether and were assessed for radioactivity using the scintillant and Packard Tri-carb described earlier.

2.15 Composite-Gel Electrophoresis of Protoplast RNA Extracts At various times post-inoculation, 5 ml aliquots of infected and "mock-infected" protoplasts were supplemented with 250 µCi of carrier-free (^{32}P)-orthophosphoric acid and 50 µg of actinomycin D. After incubation the protoplasts were washed with 0.5 M mannitol by centrifugation, pelleted and stored at -70°C. (^{32}P)-orthophosphoric acid was supplied in dilute HCl solution at pH 2 to 3 by Amersham International Limited.

The frozen pellets were thawed and extracted as described for the RNA infectivity assay except that after removing the ether by evaporation the RNA was precipitated in 2 volumes of cold ethanol. The RNA precipitate was collected by low speed centrifugation and the ethanol was removed in a nitrogen stream. The precipitate was resuspended in distilled water and 2 µl aliquots were withdrawn from each sample and dried onto Whatman 3MM squares. These were assessed for radioactivity by scintillation counting in 8 ml aliquots of the scintillant previously described.

The RNA samples were adjusted to contain equal amounts of radioactivity and heated at 60°C for 15 minutes in 5 M urea, 10 % (w/v) sucrose, 4 % bromophenol blue (BDH Chemicals) and 2 x normal strength TAE buffer mixed 1:1 (v/v) with the RNA samples.

These samples were subject to electrophoresis at 25 mA for 2 to 2.5 hours at 4°C on slab gels containing 2.4 % acrylamide, 0.12 % methylene-bisacrylamide and 0.45 % agarose in TNE buffer pre-electrophoresed for 10 to 15 minutes before loading. Electrophoresis was carried out in TNE buffer with 0.2 % (w/v) SDS added. After electrophoresis the SDS was removed by equilibrating in changes of TNE buffer before staining with toluidine blue (0.1 % (w/v) in water) for 3 minutes and destaining in water.

Radioactive bands were detected by autoradiography for 2 to 5 days at -70°C using Kodak X-omat film and an intensifying screen (Kodak regular).

2.16 Cylindrical-Gel Analysis of TNV Virus samples purified from both cowpea and French bean were analysed by electrophoresis in 7.5 x 0.5 cm diameter cylindrical gels cast in glass tubing. Virus protein was resolved on 10 % acrylamide using 0.1 M sodium phosphate electrode buffer at pH 7.2 containing 0.1 % (w/v) SDS.

TNV was denatured in 10 mM sodium phosphate buffer at pH 7.2 containing 1 % (v/v) 2-mercaptoethanol, 1 % (w/v) SDS, 0.5 M sucrose and 0.12 % (w/v) bromophenol blue by heating in a water bath at 80°C for 2 minutes. 25 µl aliquots of denatured TNV were loaded individually or mixed 1:1 with similarly denatured mixed marker solution containing cytochrome C (M_r= 12,384), ovalbumin (M_r= 47,000), human gamma globulin heavy (M_r= 55,000) and light (M_r= 23,500) chains and bovine serum albumin (M_r= 67,000).

Electrophoresis was carried out at 4 mA per gel for 1 hour and subsequently increased to 8 mA per gel until the bromophenol blue left the gel tubes. After electrophoresis the gels were extruded and stained in a mixture of methanol : 7 % acetic acid (1:1 v/v) containing 0.25 % Coomassie blue (BDH Chemicals) and stored in 7 % (v/v) acetic acid. The gels were scanned at 280 nm using the Gilford spectrophotometer described previously.

2.17 Production of ^{3}H-Labelled TNV To obtain ^{3}H-labelled virus preparations, French bean leaves cultivated as described previously were cut off 2 to 3 days after inoculation. Each was immersed in and allowed to imbibe 25 µCi of (^{3}H) amino acid mixture at 1 mCi/ml (Radiochemical Centre Amersham) in 500 ul of water. The leaves were maintained in small sample tubes and allowed to imbibe several changes of water before harvesting. High humidity was achieved by regular spraying and the leaves were kept in an enclosed cabinet with a 16 hour photoperiod at 5,000 to 10,000 lux and a temperature of 25°C. After 3 days the leaves were frozen at -20°C and TNV was purified by the differential centrifugation method described.

2.18 Preparation of Antiserum to TNV Two methods were used to raise rabbit antiserum to TNV_D using pooled samples of highly purified virus

in distilled water at 0.44 mg/ml.

In procedure "A", 700 µl of virus suspension was mixed with 700 µl of Freund's complete adjuvant and injected into a rabbit intramuscularly in each hind leg on 3 occasions at 2 week intervals. The rabbit was bled 10 ml from the ear before each injection and bled completely by cardiac puncture 3 weeks after the last injection. The blood was allowed to clot at room temperature for 4 hours then stand at 4°C overnight before it was centrifuged at 1,000xg for 15 minutes and the supernatant serum stored at -20°C.

Procedure "B" required 2.25 ml of virus suspension injected into a rabbit intraveinously after a 10 ml bleed. A further 2.25 ml was injected intraveinously after 3 weeks, and a 2.25 ml intramuscular injection after 7 weeks. The rabbit was bled 10 ml before each injection and also 1 week before the final injection. A final complete bleed was taken 4 weeks after the final injection and the blood was treated as in the previous method.

Antiserum to plant products was adsorbed using acetone-extracted powderprepared from frozen French bean leaves directly after harvesting, and extracted with 200 % (v/w) ethanol (80 % v/v) at 80°C for 10 minutes. The powder was washed in 3 changes of phosphate buffered saline (PBS), comprising 1.45 M NaCl in 4.5 mM sodium phosphate buffer.

Washed powder was added at 1 % (w/v) to the rabbit serum and stirred at room temperature for 1 hour. The mixture was centrifuged at 1,000xg for 20 minutes and the supernatant was again mixed with 1 % (w/v) washed powder. After stirring for 1 hour and centrifuging, the serum was stored in aliquots at -20°C.

Antisera were assessed by Ouchterlony tests using 0.5 % (w/v) Oxoid Ionagar No.2 in 10 mM sodium phosphate buffer at pH 7 containing 0.02 % (w/v) sodium azide layered onto clean microscope slides. Double diffusion gel-precipitation tests, using wells cut in the agar with Pasteur pipettes were carried out with serial dilutions of virus and antiserum. The slides were kept for 2 to 4 days in Petri dishes at room temperature with high humidity to develop precipitin bands. After equilibrating in distilled water the slides were stained with Coomassie blue as described for cylindrical gels.

3. RESULTS: PROTOPLAST ISOLATION AND INCUBATION

3.1 Introduction The techniques of Hibi et al.(1975) provide a convenient starting point for cowpea protoplast isolation, and open up the possibility of investigating TNV replication in vivo, in isolated protoplasts. Accordingly, the protoplast isolation procedures were optimised for maximum yield under the conditions available, and the protoplasts investigated with respect to viability and metabolic activity.

The effects of isolation procedures on cultured protoplasts have been investigated (Ruesink, 1978) and shown to cause changes in protoplast metabolism. Variations in precursor incorporation into protein (Rubin & Zaitlin, 1976) or RNA (Premecz et al., 1978) would strictly limit the application of labelling techniques to studying virus replication (Paterson & Knight, 1975; Siegel et al., 1978; Rottier et al., 1979). Thus, the factors influencing precursor incorporation required investigation before studying virus replication. Protoplast incubation medium (Zelcer & Galun, 1976), incubation density (Rubin & Zaitlin, 1976) and the addition of antibiotics (Watts & King, 1973; Wieringa-Brants, 1978) all influence precursor uptake, and rates may change with the duration of incubation (Robinson & Mayo, 1975). In addition, plant cultivation conditions can affect protoplast quality (Zaitlin & Beachy, 1974; Huber, 1979) and required standardisation in order to give reproducible, comparative results in the present study.

Gross responses to isolation treatment were initially considered in terms of protoplast yield and viability. More sophisticated changes were monitored in precursor uptake and incorporation rates at different times after isolation.

3.2 Plant Cultivation Initially, protoplasts were isolated from primary leaves of cowpea plants cultivated in either greenhouse or a variety of growth cabinet conditions. Reproducible leaf material was obtained using the conditions described in detail under Materials and Methods, and the isolation procedures were optimised for such material (Fig. 1.).

Seasonal variations in the environment accelerated or retarded plant development, and the sampling criteria for maximum protoplast yield were accordingly based on primary leaf expansion and the emergence of the trifoliate leaf rather than a strict comparison of plant growth periods. Protoplast yield was low if a leaf was not fully expanded, whereas plants

Fig. 1. Cultivation of Plants for Protoplast Isolation

Cowpea plants used as a source of protoplasts were grown under a 16 hour photoperiod providing 10,000 lux illumination from 24 warm white fluorescent tubes and 9 x 150 W incandescent tungsten bulbs. The temperature was maintained at 25°C throughout and relative humidity at approximately 50 %.

Seeds were germinated in pots of moistened vermiculite, and transferred to bottles of liquid culture solution after 4 days growth. After 4 to 8 days in liquid culture, fully expanded primary leaves were selected for protoplast isolation, just prior to the emergence of the first trifoliate leaf.

harvested after trifoliate development produced fragile, unstable protoplasts. All harvested leaves were sterilised by the methods of Coutts & Wood (1975) before protoplast isolation techniques were investigated.

3.3 Enzyme Treatments Pre-plasmolysis and enzyme treatments were carried out as described in Materials and Methods using 0.5 M mannitol after preliminary observations of protoplast stability. Variations in enzyme concentration, incubation temperature and duration were investigated by comparing the yield of viable protoplasts per gram of leaf material. The use of a shaking water bath accelerated protoplast release at the minimum oscillation rate (i.e. 25 excursions/minute), whereas more vigorous shaking caused protoplast damage and reduced yield and viability. The data in table 1 were obtained using a $3\frac{1}{2}$ hour incubation at 30°C and 25 excursions/minute.

Table 1. **Protoplast Isolation Using Different Enzyme Concentrations**

Protoplast isolation enzymes in 0.5 M mannitol adjusted as necessary to pH 5.6 :		Protoplast yield per gram of leaf tissue:
Cellulase Onozuka R-10	Macerozyme R-10	
(% w/v)	(% w/v)	(x 10^{-6})
0.6	0.075	3.37
0.5	0.075	4.95
0.5	0.100	6.87
0.5	0.125	5.77
0.4	0.075	4.44
0.4	0.100	6.13

An incubation temperature of 25°C required an overnight digestion to give comparable protoplast release and a temperature of 35°C reduced the viability of the protoplasts released after $3\frac{1}{2}$ hours incubation. Using the optimum enzyme concentrations from the data above (i.e. 0.5 % w/v Cellulase Onozuka R-10 and 0.1 % w/v Macerozyme R-10) a reduced incubation time at 30°C resulted in a large yield of cells retaining a cellulose wall. Incubation for 4 hours or more reduced the viability of the protoplasts released.

Bovine serum albumin (BSA) was added to the enzyme solution (Beier & Bruening, 1976) prior to pH determination and the effect on protoplast viability was recorded (Table 2.).

Table 2. Effect of Bovine Serum Albumin on Protoplast Yield

Protoplast isolation solution containing 0.5 M mannitol at pH 5.6 with:			Viable protoplast yield per gram of leaf tissue:
Cellulase Onozuka R-10	Macerozyme R-10	Bovine serum albumin (BSA)	
(% w/v)	(% w/v)	(% w/v)	(x 10^{-6})
0.5	0.1	-	6.87
0.5	0.1	0.1	9.48

A routine isolation procedure was adopted which involved incubation for $3\frac{1}{2}$ hours at 30°C in a shaking water bath at 25 excursions/minute. The isolation medium contained 0.5 % Cellulase Onozuka R-10, 0.1 % Macerozyme R-10 and 0.1 % BSA in 0.5 M mannitol at pH 5.6. This treatment routinely gave protoplast yields of around 10^7 protoplasts per gram of leaf material, assessed as described in Materials and Methods.

3.4 Viability Determination Protoplast viability was determined after washing in osmotically stabilised 0.1 mM $CaCl_2$ using the techniques described under Materials and Methods. The vital stain fluorescein diacetate gave the most rigorous determination of protoplast viability, but proved time consuming as a routine technique. For routine analysis the exclusion stains phenosafranine and Evan's Blue gave comparative results. With experience, protoplast viability could be determined in unstained material from observations of protoplast integrity; similar values were obtained when the mean percentage of viable protoplasts was assessed from 10 microscope fields using all the above methods (Fig 2).

3.5 Incubation Density (a) A preliminary experiment was carried out to determine the effect of different incubation densities on protoplast viability. Freshly isolated protoplasts were washed and resuspended in 0.5 M mannitol with 0.1 mM $CaCl_2$ at various densities. The cultures were incubated at 23°C in 10 ml aliquots in continuous light (3,000 lux). After 48 hours incubation in the absence of any antibiotics the cultures were assessed for protoplast viability.

The mean protoplast viability as assessed for a series of cultures is shown in table 3. From this data a concentration of 1.5×10^5 protoplasts per ml was selected for experiments to determine antibiotic levels and precursor incorporation.

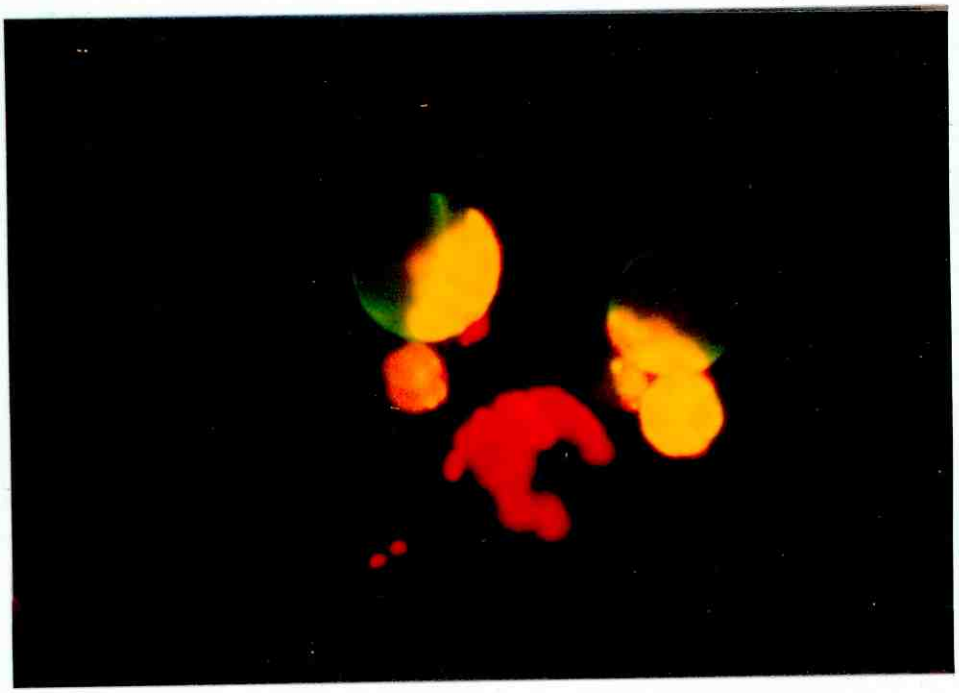


Fig. 2. Protoplast Viability Determination Using Fluorescein Diacetate

Cowpea protoplasts were viewed under high power using a fluorescence microscope fitted with exciter filter KP500 and barrier filters LP520 and LP540. The protoplasts were incubated for 10 minutes in darkness with 0.0125 % fluorescein diacetate in osmotically stabilised 0.1 mM $CaCl_2$ before examination using a Fuchs-Rosenthal haemocytometer.

Live protoplasts fluoresce yellow/green. Red fluoresence is due to autofluorescence of the chlorophyll and is observed in unstained material.

Table 3. Effect of Incubation Density on Protoplast Viability

Protoplast incubation density in 0.5 M mannitol with 0.1 mM $CaCl_2$:	percentage viability after 48 hours of protoplast culture :
($\times 10^{-5}$/ml)	(%)
3.72	32.0
3.10	35.0
2.48	36.6
1.86	41.6
1.24	39.0

3.6 **Antibiotic Treatments** Antibiotics were added to control microbial contamination and selected on the basis of minimal effects on virus nucleoprotein (McCarthy et al., 1972), and their application in similar experimental systems (Robinson & Mayo, 1975; Siegel et al., 1978; Wieringa-Brants, 1978). Dosage levels of 200 µg/ml chloramphenicol and 75 units/ml Nystatin were investigated with repeated treatments during incubation (Wieringa-Brants, 1978). Antibiotics were not added during the short enzyme treatment. Treatments were assessed on the basis of their effect on protoplast viability and the protoplasts were incubated in osmotically stabilised 0.1 mM $CaCl_2$ at 23°C in 10 ml aliquots under continuous light.

Table 4. Effect of Antibiotics on Protoplast Viability

Treatment of incubated protoplasts with antibiotics after 0 and 24 hours of incubation using 1.5×10^5 protoplasts/ml			percentage viability after 48 hours of protoplast culture :
Treatment	0 hours	24 hours	(%)
A	N/C	N/C	38
B	N/C	N	67
C	N/C	C	42
D	N/C	-	90

(N = 75 units/ml Nystatin; C = 200 µg/ml chloramphenicol)

For routine experiments a single treatment was adopted at the start of protoplast incubation, using 200 µg of chloramphenicol and 75 units of Nystatin per ml of culture medium. In cultures treated in this way microbial contamination was observed only after 48 hours of incubation and restricted to a small percentage of culture vials.

3.7 Pulse-Chase Labelling Using the optimised isolation and provisional incubation procedures, protoplast metabolism was investigated by the addition of radio-labelled protein and RNA precursors to the incubation medium. Protoplasts were incubated in 10 ml aliquots of osmotically stabilised 0.1 mM $CaCl_2$ in the light (3,000 lux) at 23°C. Labelled precursors were added at the start of the incubation period, and samples of protoplasts were withdrawn at set time intervals for cold TCA acid-insoluble radioactivity determination, as described under Materials and Methods.

After a lag period of about 4 hours, (^{3}H)-uridine (Fig. 3.) and (^{14}C)-leucine (Fig. 4.) were incorporated in almost linear fashion into TCA acid-insoluble products. The decline in (^{3}H)-uridine incorporation after 15 hours of incubation probably reflects the dilution effect of unlabelled precursor molecules released from broken protoplasts (Rubin & Zaitlin, 1976), and the rapid nucleic acid turnover rate in freshly isolated protoplasts (Watts & King, 1973). Excess unlabelled uridine added to the medium after 4½ hours incubation resulted initially in reduced incorporation, and subsequently, in the loss of labelled products from the TCA precipitable material. This may similarly reflect a rapid rate of nucleic acid turnover (Fig. 3.).

The incorporation of (^{14}C)-leucine did not show a marked decline after 15 hours of incubation and continued to increase slowly after the addition of unlabelled leucine to the medium. Such continued incorporation may reflect a longer period required for the dilution of the intracellular amino acid pool, or a turnover rate for protoplast proteins which is slower than that for nucleic acids. Further experiments were carried out to determine the role of the precursor pool in incorporation, by assessing uptake of labelled material as well as incorporation into RNA or protein.

The reduced initial uptake observed as a lag period in both protein and RNA synthesis may reflect the time taken to recover from high osmotic pressure and protoplast isolation procedures (Robinson & Mayo, 1975). Since these procedures are largely unavoidable in any isolation technique, alternative incubation media were investigated as a means of stabilising protoplast metabolism soon after isolation (Coutts et al., 1975). The osmotic "step-up" was also investigated, however incubation for 10 minutes in 0.7 M mannitol before resuspension in 0.5 M mannitol with 1 mM $CaCl_2$ did not influence the incorporation pattern of labelled precursors.

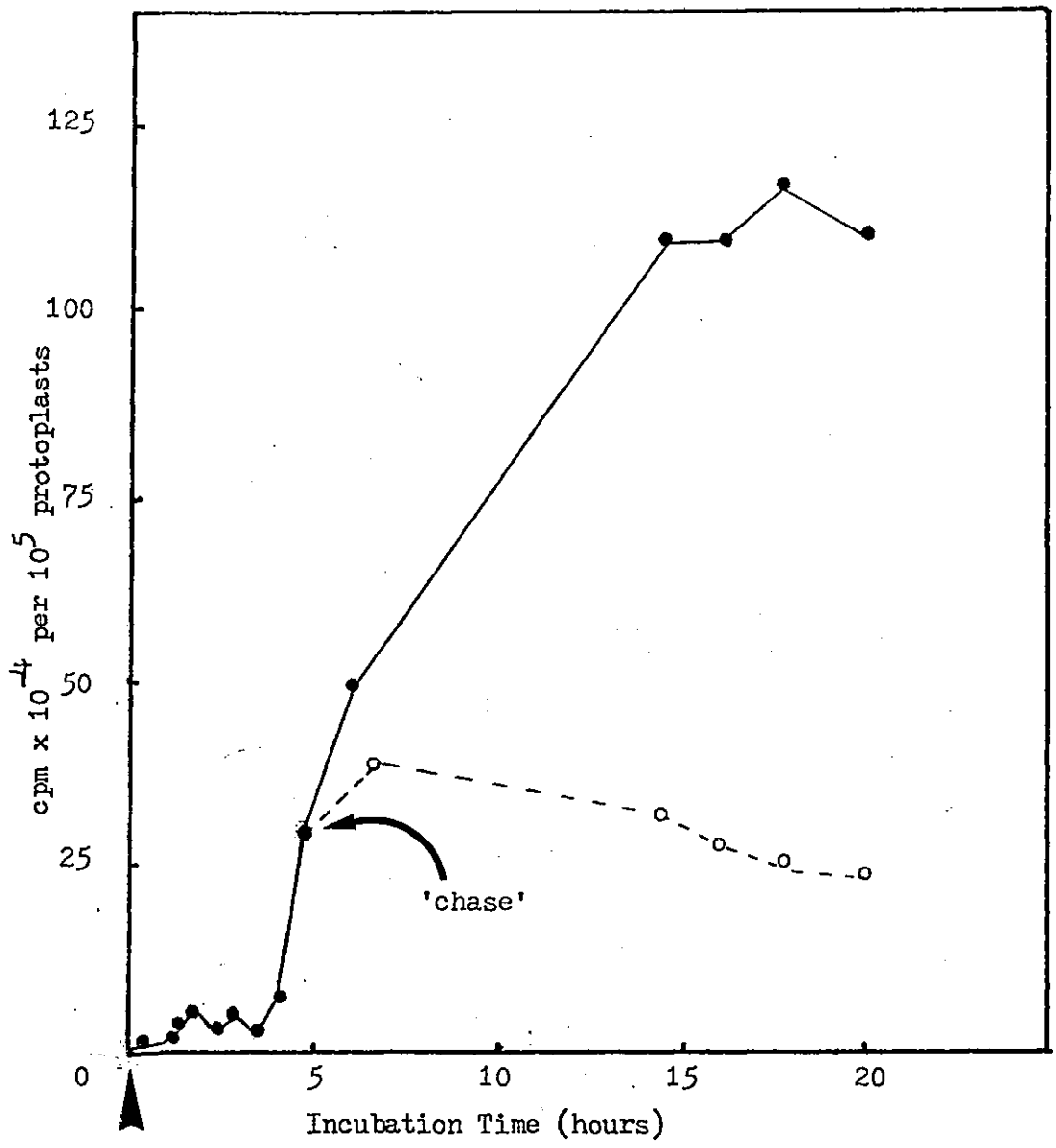


Fig.3 Effect of adding an excess of unlabelled uridine on the incorporation of (^{3}H)-uridine into isolated protoplasts incubated in 10 ml of 0.5 M mannitol with 0.1 mM Ca Cl_2 (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C. Samples were labelled with 150 µCi of (^{3}H)-uridine at the start of the incubation. After 4½ hours, unlabelled uridine (700 fold dilution of radioactivity) was added to the chase portion of the experiment.

Aliquots (200 µl each) were withdrawn at various time intervals and radioactivity incorporated into cold TCA acid-insoluble material was determined. ●—● continuous (^{3}H)-uridine; o--o chase portion of experiment.

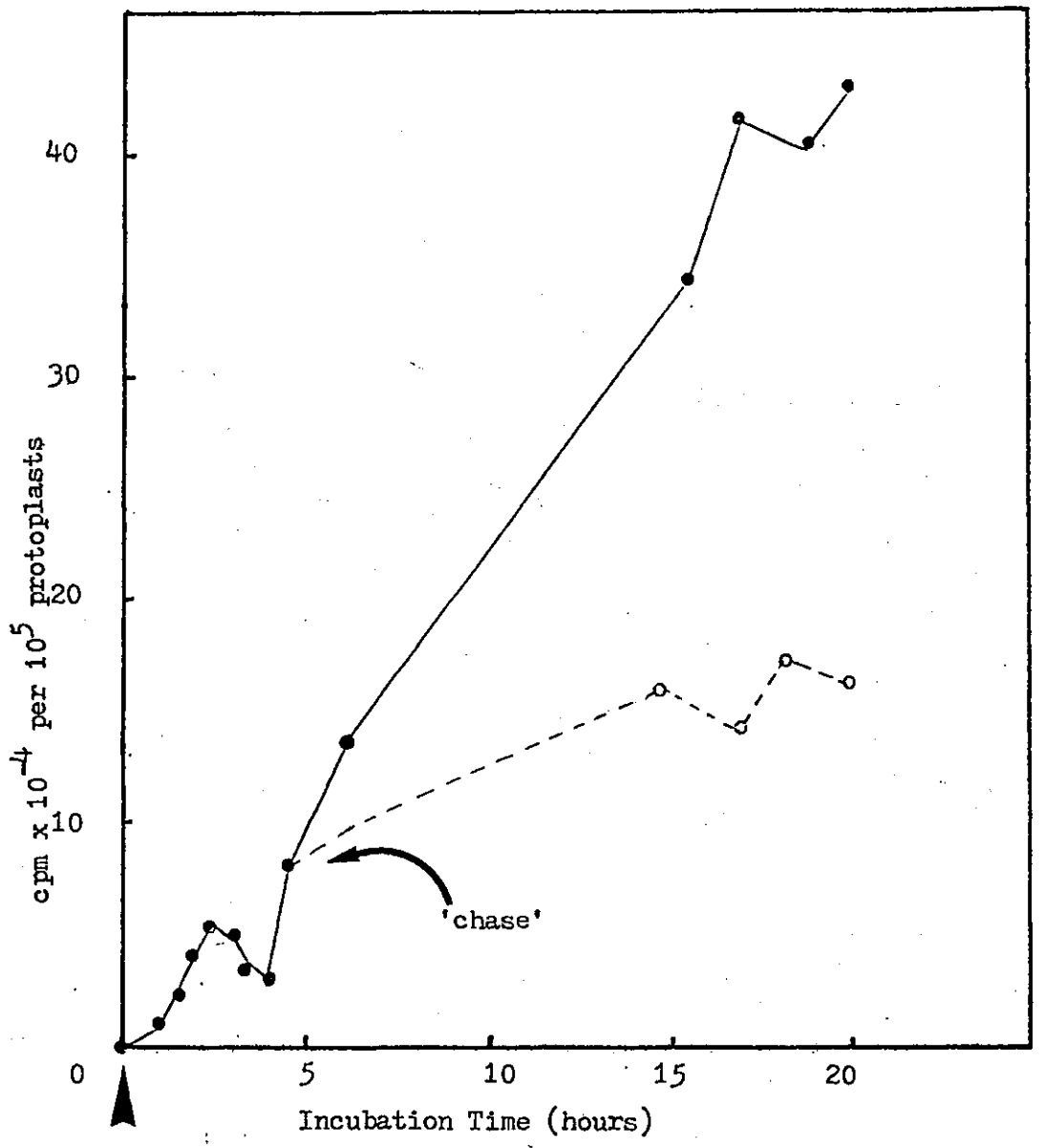


Fig.4 Effect of adding an excess of unlabelled leucine on the incorporation of (^{14}C)-leucine into isolated protoplasts incubated in 10 ml of 0.5 M mannitol with 0.1 mM Ca Cl_2 (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C. Samples were labelled with 15 µCi of (^{14}C)-leucine at the start of the incubation. After 4½ hours, unlabelled uridine (700 fold dilution of radioactivity) was added to the chase portion of the experiment.

Aliquots (200 µl each) were withdrawn at various time intervals and radioactivity incorporated into cold TCA acid-insoluble material was determined. ●—● continuous (^{14}C)-leucine; o--o chase portion of experiment.

3.8 Uptake and Incorporation in Different Media A preliminary series of experiments was carried out to investigate the incorporation of protein precursors into protoplasts incubated in different media.

Protoplasts were isolated as described previously and washed in 0.1 mM $CaCl_2$ in 0.5 M mannitol. Sedimented protoplasts were resuspended to give 2×10^5 protoplasts /ml in each of 3 different incubation media containing (^{14}C)-leucine. Duplicate cultures were sampled after 22 hours of incubation as described under Materials and Methods, and the mean incorporation into TCA acid-insoluble products per 10^5 protoplasts was recorded in table 5. Modified White's medium (Beier & Bruening, 1975), a simple salts medium (Aoki & Takebe, 1969) and 0.1 mM $CaCl_2$ (Alblas & Bol, 1977) were osmotically stabilised in 0.5 M mannitol for protoplast culture.

Table 5. **Incorporation of (^{14}C)-leucine by Protoplasts Incubated in Different Culture Media.**

Protoplast incubation medium, osmotically stabilised in 0.5 M mannitol with added antibiotics:	Mean incorporation into TCA acid-insoluble products : (cpm $\times 10^{-4}$ per 10^5 protoplasts)
Modified White's medium	57.7
Simple salts medium (VIM)	75.8
0.1 mM $CaCl_2$ medium	42.5

Precipitable incorporation and total uptake were investigated for RNA and protein precursors in VIM and $CaCl_2$ media. The initial rate of uptake was higher for both precursors using the VIM rather than $CaCl_2$ medium (Figs. 5 and 6), and a similar pattern was followed by TCA acid-insoluble incorporation, (Figs. 7 and 8).

This rapid uptake and incorporation by protoplasts incubated in the simple salts medium directly after isolation, is more suitable for sequential pulse labelling, provided a similar rate of incorporation is maintained during culture. The increased incorporation rate may be due to metabolic stability being achieved more rapidly, and the rate of uptake may reflect depletion of the cellular pool as a controlling factor (Francki et al., 1971). Thus, factors responsible for the initial rates of uptake may be a consequence of the protoplast isolation procedure acting directly on the membrane and transport system (Robinson & Mayo, 1975), or may reflect the altered protein profile in the isolated cell and the consequent depletion of the cellular precursor pools.

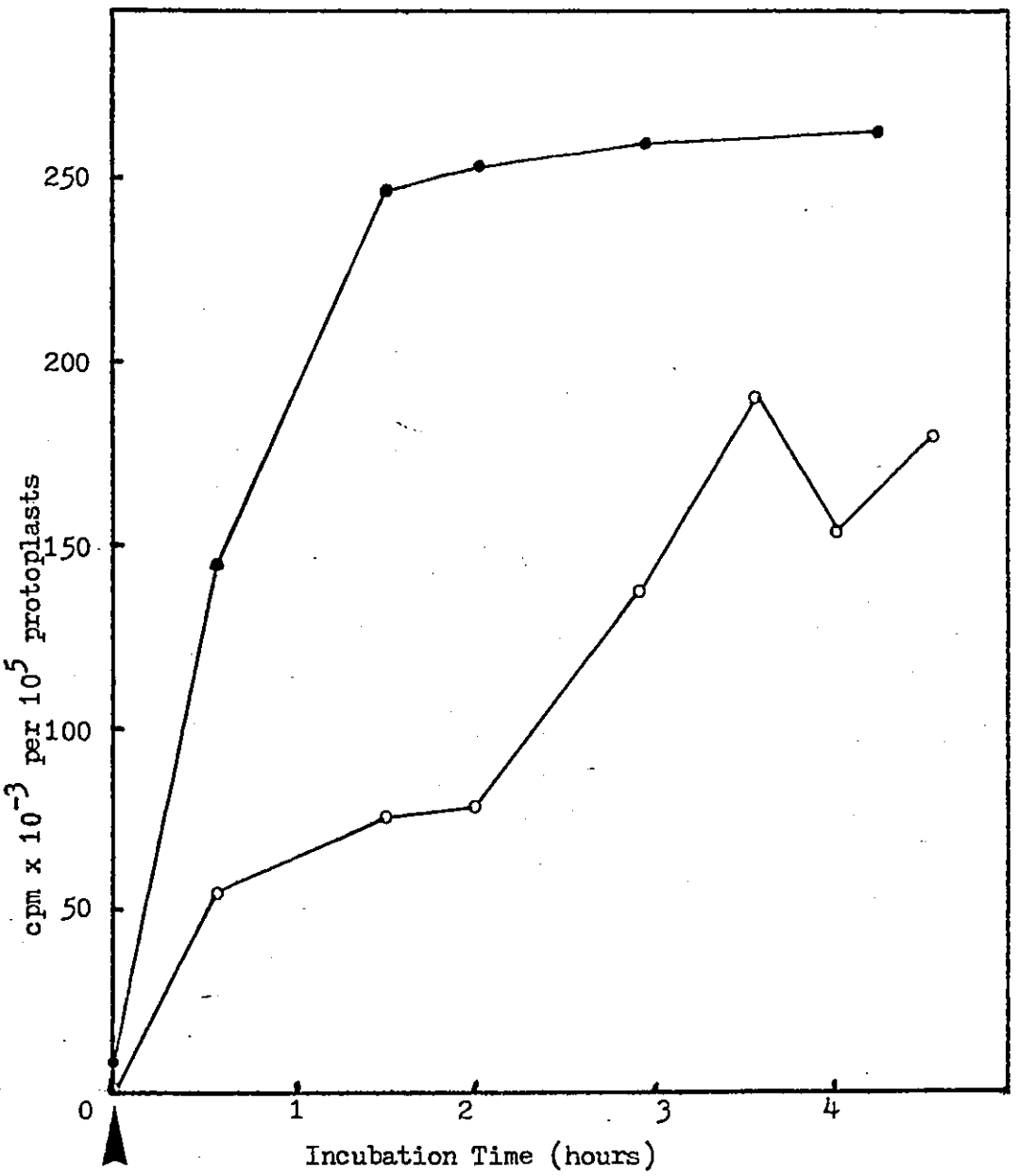


Fig.5 Uptake of (^{14}C)-leucine by isolated protoplasts incubated in different media. Protoplasts were incubated (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C in 10 ml aliquots of Takebe's simple salts incubation medium or 0.5 M mannitol with 0.1 mM Ca Cl_2 . Samples were labelled with 15 µCi of (^{14}C)-leucine and aliquots of 500 µl were withdrawn at intervals and washed in 0.5 M mannitol. These protoplasts were homogenised and extracts were solubilised and assessed for radioactivity by scintillation counting. ●—● uptake by protoplasts incubated in Takebe's simple salts medium; o—o uptake by protoplasts incubated in 0.5 M mannitol with 0.1 mM Ca Cl_2.

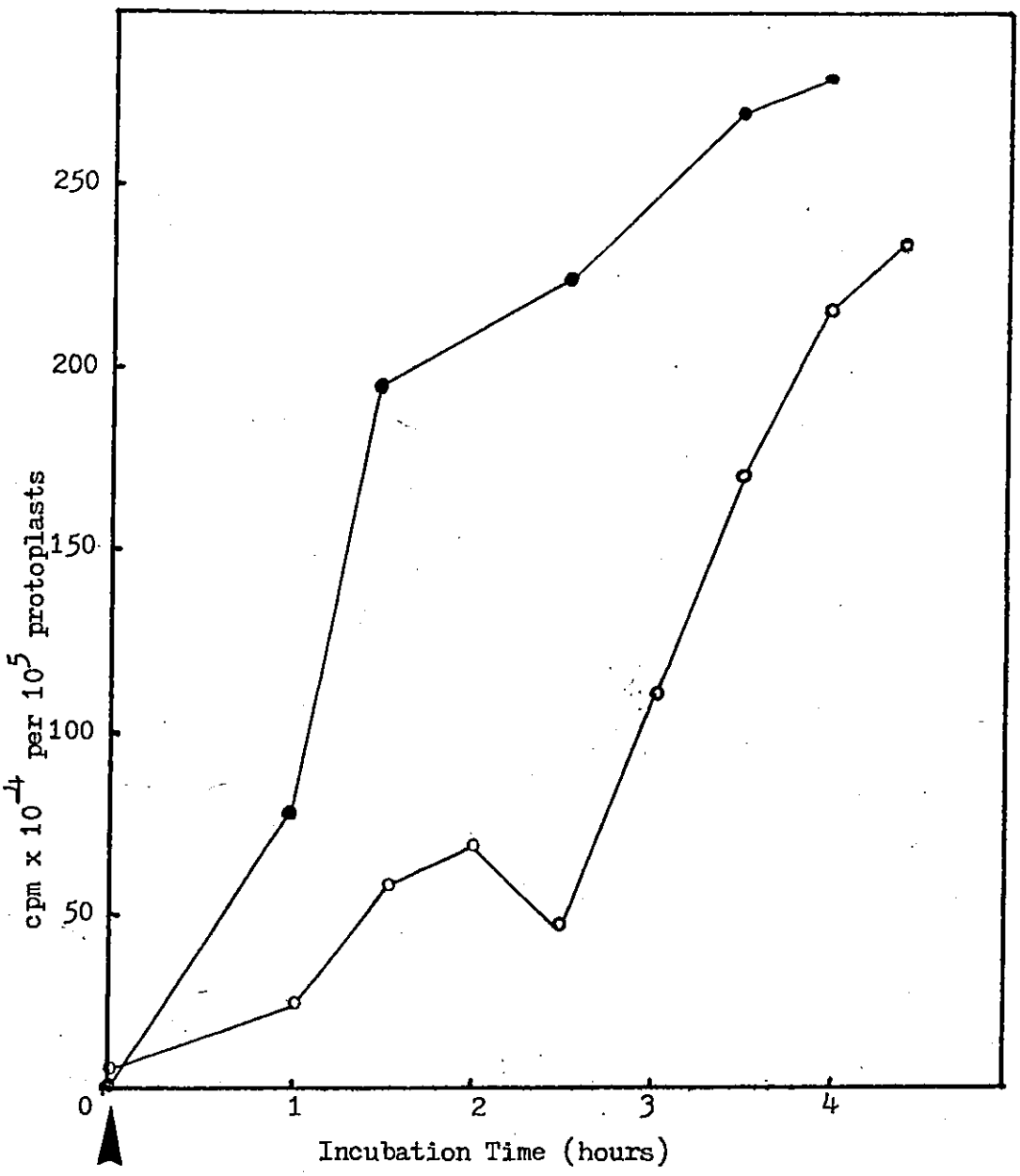


Fig.6 Uptake of (^{3}H)-uridine by isolated protoplasts incubated in different media. Protoplasts were incubated (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C in 10 ml aliquots of Takebe's simple salts incubation medium or 0.5 M mannitol with 0.1 mM Ca Cl_2. Samples were labelled with 150 µCi of (^{3}H)-uridine and aliquots of 500 µl were withdrawn at intervals and washed in 0.5 M mannitol. These protoplasts were homogenised and extracts were solubilised and assessed for radioactivity by scintillation counting. ●—● uptake by protoplasts incubated in Takebe's simple salts medium; o—o uptake by protoplasts incubated in 0.5 M mannitol with 0.1 mM Ca Cl_2.

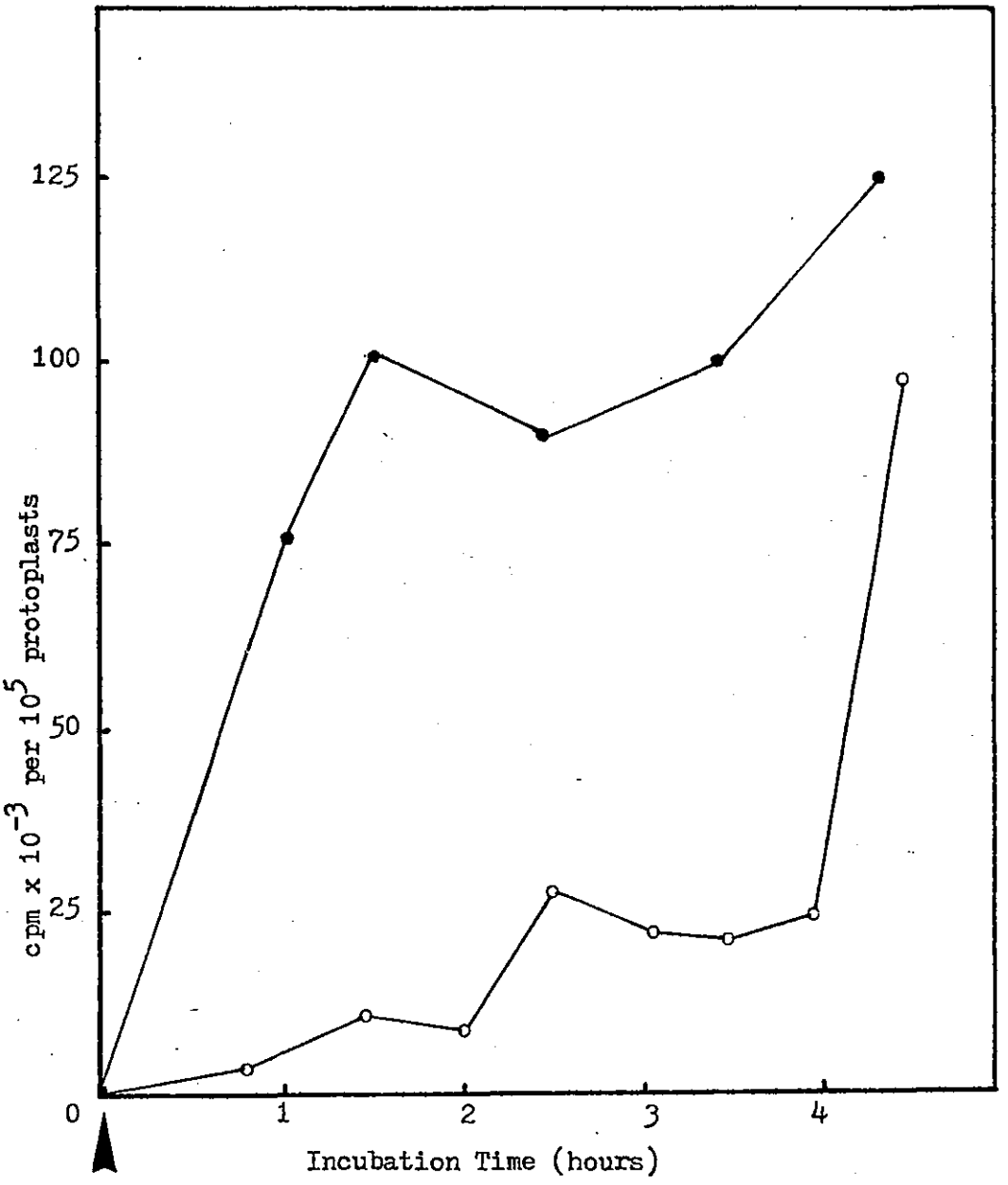


Fig.7 Incorporation of (^{14}C)-leucine into TCA acid-insoluble products by isolated protoplasts incubated in different media. Protoplasts were incubated (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C in 10ml aliquots of Takebe's simple salts incubation medium or 0.5 M mannitol with 0.1 mM Ca Cl_2. Samples were labelled with 15 µCi of (^{14}C)-leucine and aliquots of 200 µl were withdrawn at intervals and radioactivity incorporated into TCA acid-insoluble material determined. ●—● incorporation by protoplasts incubated in Takebe's simple salts medium; o—o incorporation by protoplasts incubated in 0.5 M mannitol with 0.1 mM Ca Cl_2.

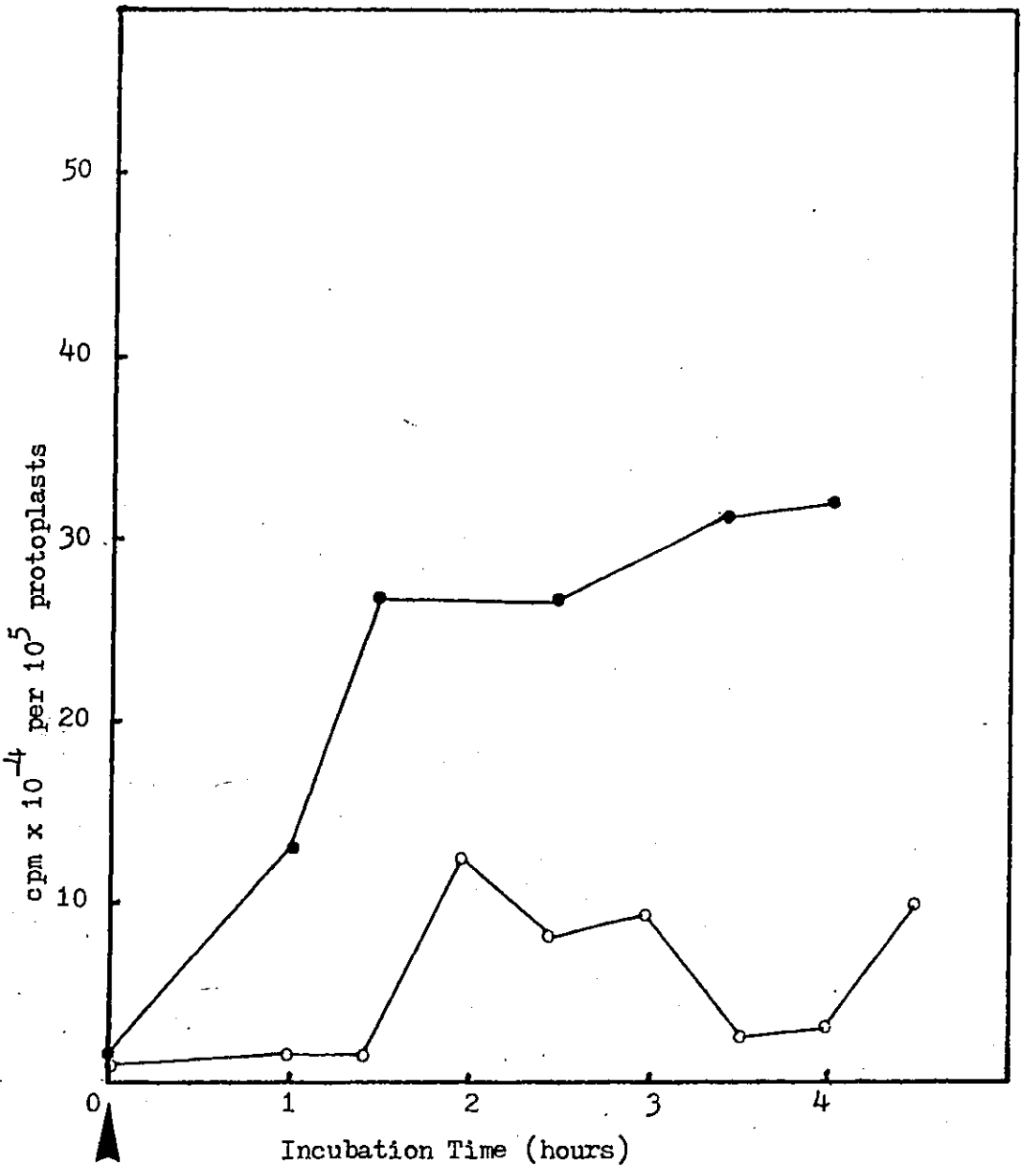


Fig.8 Incorporation of (^{3}H)-uridine into TCA acid-insoluble products by isolated protoplasts incubated in different media. Protoplasts were incubated (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C in 10 ml aliquots of Takebe's simple salts incubation medium or 0.5 M mannitol with 0.1 mM Ca Cl_2. Samples were labelled with 150 µCi of (^{3}H)-uridine and aliquots of 200 µl were withdrawn at intervals and radioactivity incorporated into TCA acid-insoluble products was determined. ●—● incorporation by protoplasts incubated in Takebe's simple salts medium; o—o incorporation by protoplasts incubated in 0.5 M mannitol with 0.1 mM Ca Cl_2.

3.9 Incorporation Rates During Different Labelling Periods Comparable rates of incorporation during the incubation period determine whether pulse labelling is a quantitative method for analysing protein synthesis. Accordingly, uptake and incorporation were investigated for protein and RNA precursors after a period of protoplast incubation. Protoplasts were incubated in simple salts medium in orded to maximise the incorporation rate (as in Figs. 6 and 8), and the radiolabelled precursors were added at the start of incubation or after 18 hours of incubation under similar conditions. Samples were taken at set time intervals and the incorporation of radioactivity into TCA acid-insoluble material was determined (Figs. 9 and 10).

Protoplasts labelled at the start of the incubation period showed a decline in the incorporation rate after 18 hours of culture. This decline was more marked for (^{3}H)-uridine, and similar to that observed for protoplasts incubated in osmotically stabilised $CaCl_2$ medium.

However, for both protein and RNA precursors the initial rates of incorporation were similar whether the protoplasts were labelled at the outset or after 18 hours of incubation. This similarity was taken as a basis for pulse labelling experiments during the time course of virus replication.

3.10 Incubation Density (b) As well as culture medium and incubation period, the effect of cell concentration was investigated as a factor in precursor incorporation by protoplasts. Although incubation density was initially optimised for viability at 48 hours, prior to antibiotic treatment, cell concentration may have major effects on precursor dilution in the culture medium (Rubin & Zaitlin, 1976). Thus precursor incorporation was determined for protoplasts incubated in simple salts medium for 18 hours at a range of concentrations. Cultures were labelled with (^{3}H)-uridine at the start of the incubation period, and duplicate samples were analysed after 18 hours of incubation. Incorporation into TCA acid-insoluble products was determined per ml of culture medium and per 10^5 protoplasts. The results for a range of protoplast concentrations are expressed in Fig.11.

At increasing protoplast concentrations the incorporation of (^{3}H)-uridine into protoplast RNA is observed to remain stable or decrease per ml of culture. The incorporation per protoplast decreases sharply as the density is increased.

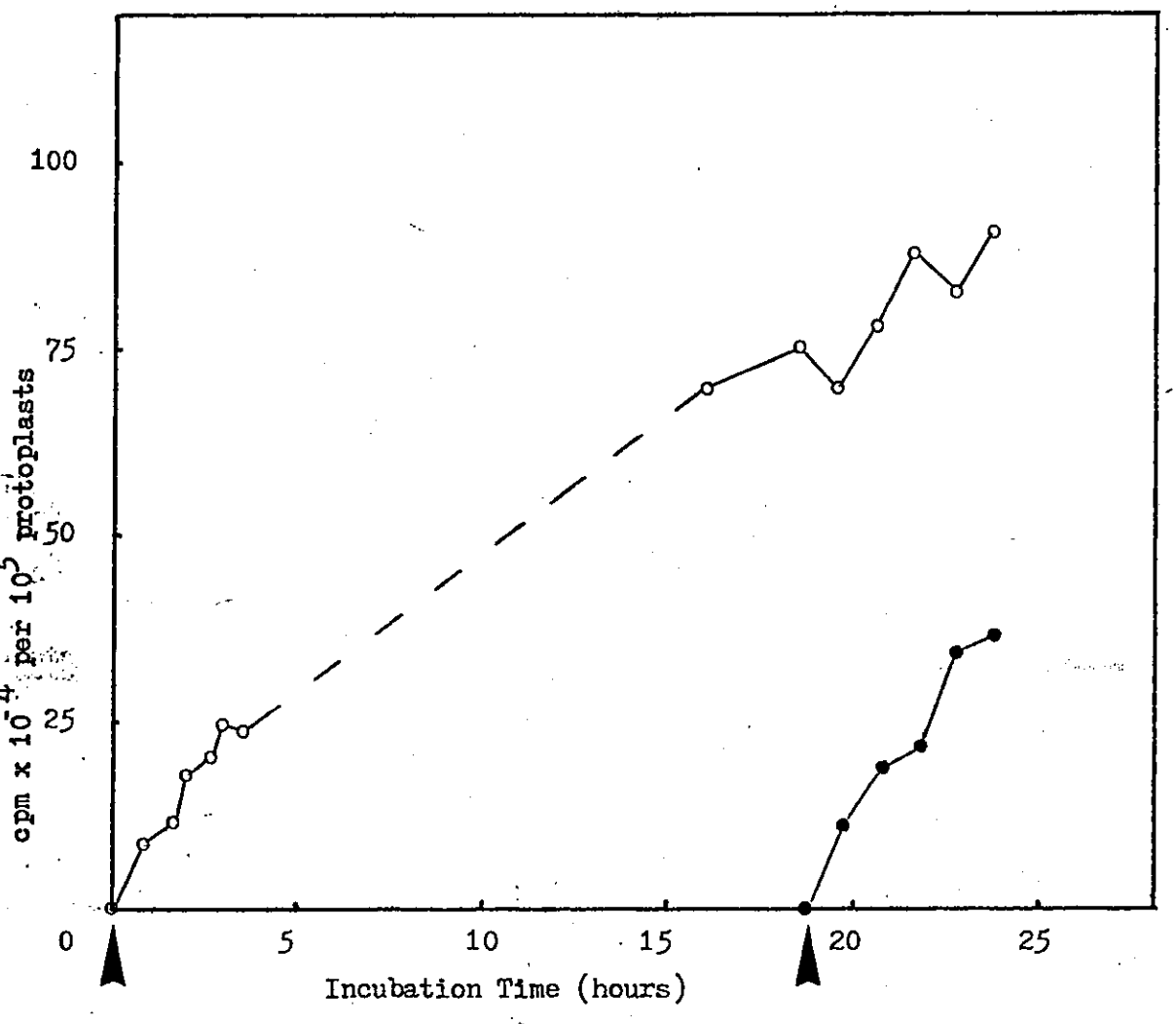


Fig. 9 Incorporation of (^{14}C)-leucine into isolated protoplasts incubated in 10 ml of Takebe's simple salts incubation medium (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C. Samples were incubated in the presence of 15 µCi of (^{14}C)-leucine added at time 0 or after 18 hours of incubation. Aliquots (200 µl each) were withdrawn at intervals and radioactivity incorporated into cold TCA acid-insoluble material determined.
o—o labelled at time 0; •—• labelled after 18 hours incubation.

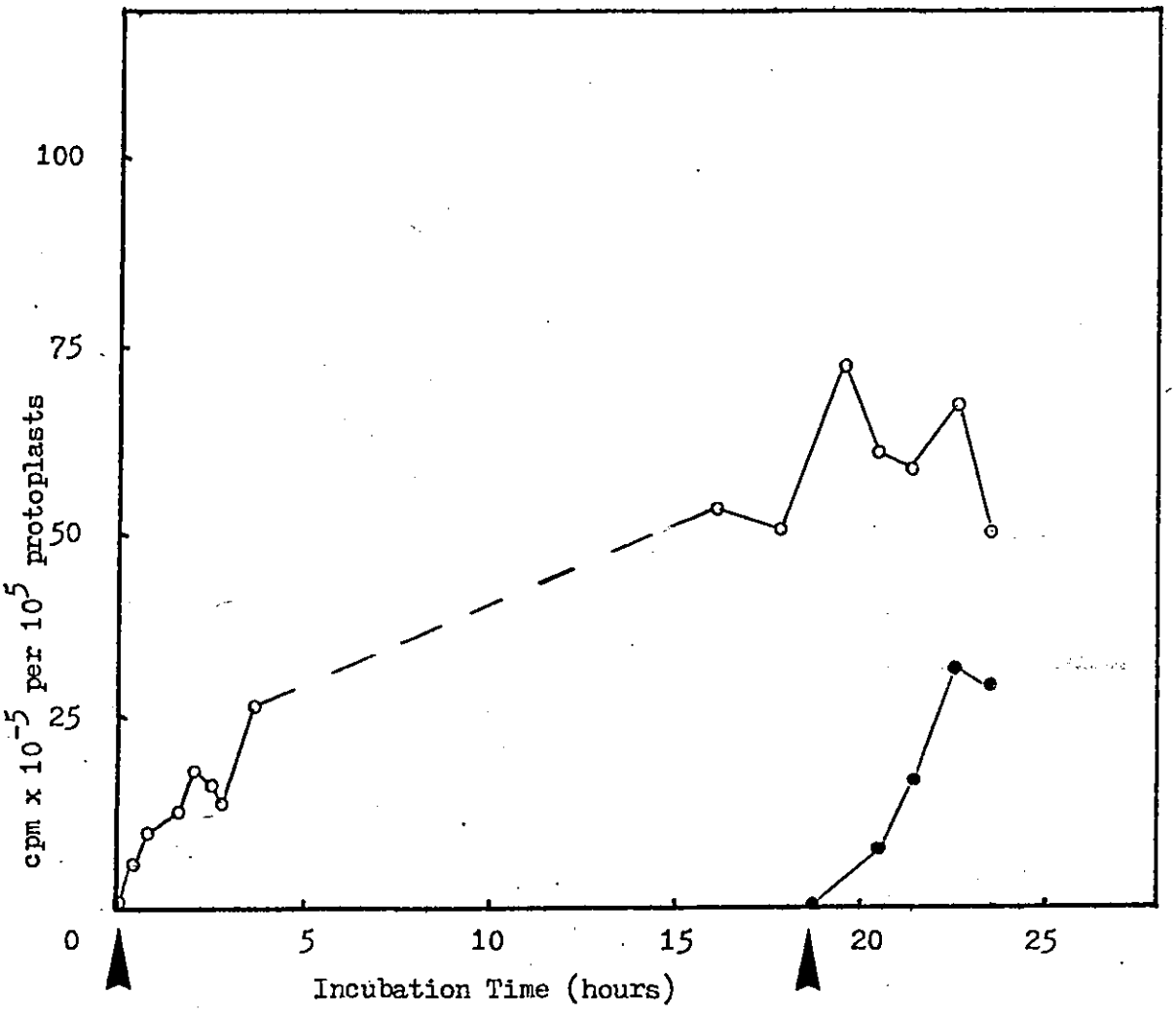


Fig. 10 Incorporation of (^{3}H)-uridine into isolated protoplasts incubated in 10 ml of Takebe's simple salts incubation medium (1.5 x 10^5/ml) in the light (3,000 lux) at 23°C. Samples were incubated in the presence of 150 µCi of (^{3}H)-uridine added at time 0 or after 18 hours of incubation. Aliquots (200 µl each) were withdrawn at intervals and radioactivity incorporated into cold TCA acid-insoluble material determined.
o—o labelled at time 0; ●—● labelled after 18 hours incubation.

High rates of precursor incorporation are thus favoured by dilute protoplast cultures, although at low densities protoplast viability may become limiting. The phenomenon may be related to the dilution of labelled precursors in the incubation medium in a similar way to the decline in rate of uptake with incubation time, observed for (^{3}H)-uridine (Fig.9).

3.11 Actinomycin D Because of its potential application to studying virus RNA replication, the effect of actinomycin D on precursor incorporation was investigated. After 5 hours of incubation, actinomycin D and labelled precursors were added to protoplast suspensions which were subsequently incubated in darkness for 12 hours (since actinomycin D is photosensitive). Control cultures were incubated with radiolabelled precursors but without the antibiotic, and incubated both in darkness and under normal lighting conditions as described in Materials and Methods. The incorporation for protoplasts incubated with and without 10 µg /ml actinomycin D is shown in table 6.

Table 6 Effect of Actinomycin D on Precursor Incorporation

Actinomycin D concentration	TCA acid-insoluble incorporation : (^{14}C)-leucine		(^{3}H)-uridine	
(ug/ml)	(cpm)	(% inhibition)	(cpm)	(% inhibition)
0	6,720	-	36,515	-
10	6,352	8.5	33,391	5.4

Over the 12 hour period tested and after 5 hours incubation under normal conditions, precursors were incorporated into protoplasts at a similar rate in darkness or in light. The addition of actinomycin D resulted in only slight inhibition of either (^{14}C)-leucine or (^{3}H)-uridine incorporation into TCA acid-insoluble products, although the dosage level was similar to that employed to suppress DNA-dependent RNA synthesis.

In long-term incubations protoplast viability was reduced by darkness or by high light intensities (8,000 to 10,000 lux). Cultures were consequently maintained under the regime described in Materials and Methods for most experimental purposes.

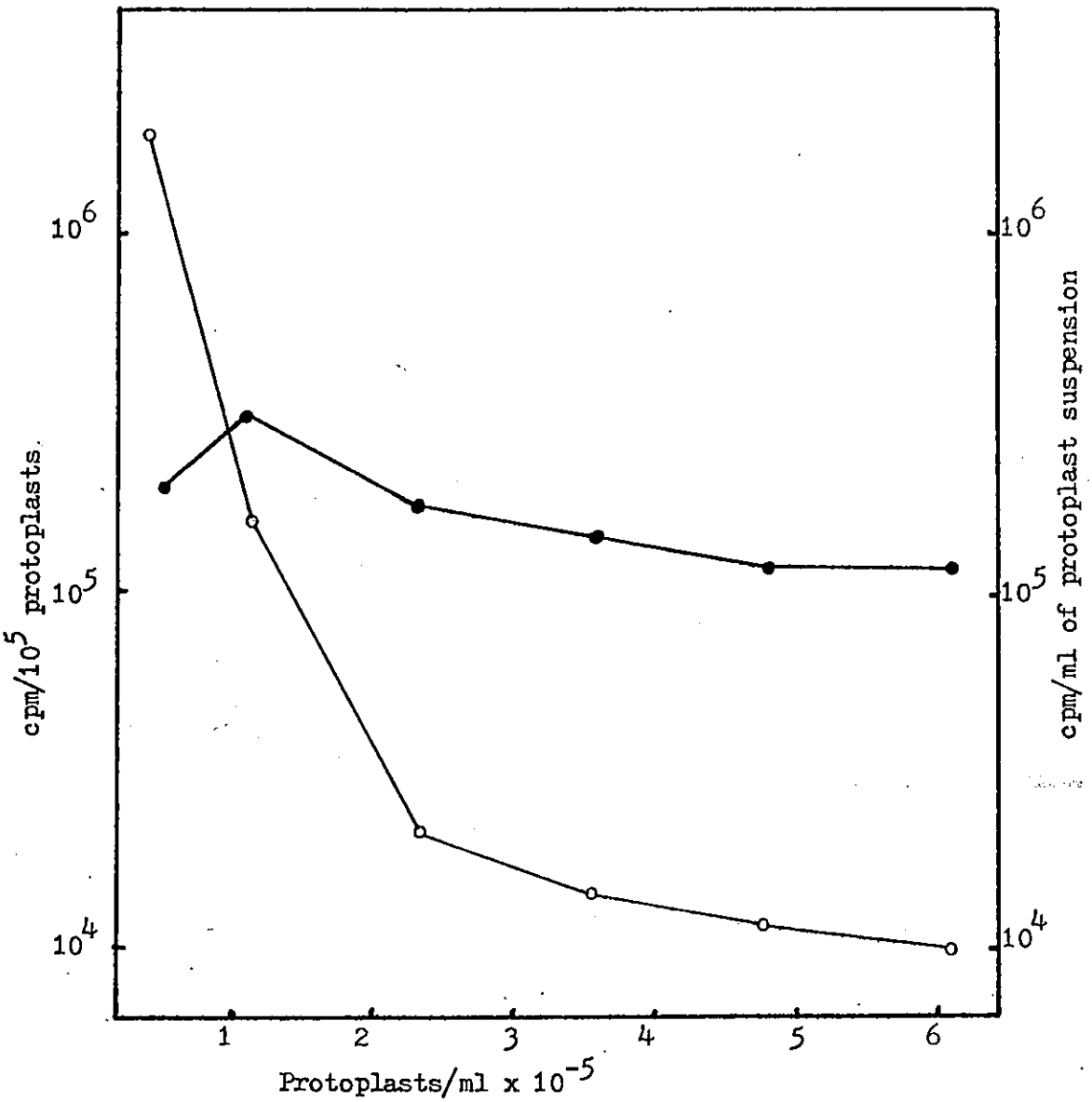


Fig. 11 Incorporation of (^{3}H)-uridine into TCA acid-insoluble products at increasing protoplast concentrations in Takebe's simple salts incubation medium. Protoplasts were incubated at various concentrations in 10 ml samples in the light (3,000 lux) at 23°C. Samples were labelled with 150 µCi of (^{3}H)-uridine at the start of the incubation and duplicate aliquots (200 µl each) were removed after 18 hours incubation. The incorporation of radioactivity into TCA acid-insoluble material was determined by scintillation counting. ●—● incorporation/ml; o—o incorporation/10^5 protoplasts.

4. RESULTS: INFECTION OF PROTOPLASTS BY TNV

4.1 Introduction Wieringa-Brants et al. (1978) have shown that cowpea protoplasts can be inoculated with the A strain of TNV. The method provides a useful demonstration of infection, but for biochemical investigations of virus replication, procedures which ensure a more efficient and reproducible infection would be necessary. The D strain of TNV, which does not support satellite replication, would also be a more convenient strain with which to initiate replication studies. Investigation of the A strain and STNV replication could then follow once the infection conditions were established.

Accordingly, TNV strains A and D were purified from various sources using several procedures, and the purified virus was investigated for infectivity and sedimentation characteristics. The buffered polycation system employed by Wieringa-Brants et al. (1978) was then used to establish infection in protoplasts isolated and incubated as described in the previous section.

The initial inoculation procedure was as follows :
10 ml of inoculation medium containing 20 µg of poly-L-ornithine (PLO), 160 µg of TNV_D and 0.01 M phosphate buffer pH 5.6 in 0.7 M mannitol was pre-incubated for 10 minutes at 20°C in a 100 ml Erlenmeyer flask.

6×10^5 protoplasts were sedimented from 0.5 M mannitol washing solution and resuspended for 15 minutes in the inoculation medium at 20°C.

The non-adsorbed virus was removed by 3 cycles of centrifugation and resuspension in osmotically stabilised $CaCl_2$, and the protoplasts were incubated in culture medium at 23°C and 3,000 lux constant illumination.

Virus replication was monitored by sampling the protoplast suspension culture at various times post-inoculation (PI), and virus content was assessed by local lesion assay on cowpea primary leaves. Individual infection parameters such as pH, virus concentration, osmotic "step-up" and polycation concentration were investigated, using the protoplast - infectivity assay to determine the optimum procedure.

4.2 Virus Purification Highly purified virus preparations were obtained from heavily necrosed primary leaves of cowpea or French bean (var. The Prince or Pinto). Purification was by differential centrifugation as described by Kassanis & Phillips (1970) or polyethylene glycol pre-

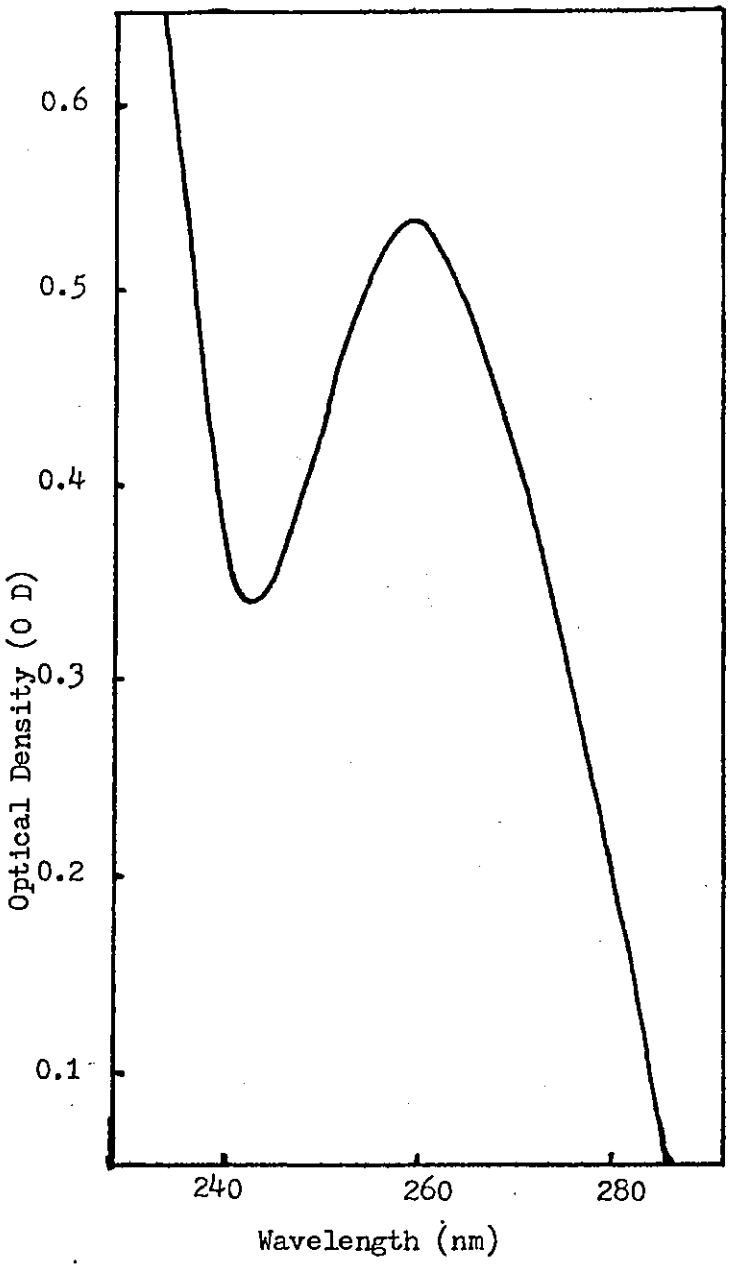


Fig.12 Ultraviolet-absorption spectrum of a TNV_D preparation purified by differential centrifugation. Using the extinction coefficient $E^{1\,cm}_{260}$ 0.1% = 5.5 (Babos & Kassanis, 1963) the purified preparation which gave the above trace at 1/10 dilution represents 1mg/ml of virus.

cipitation of the virus (Gooding & Hebert, 1967) but most consistent results were obtained with the centrifugation method described under Materials and Methods and based on the procedure of Salvato & Fraenkel-Conrat (1979).

Virus content in the highly purified preparation was assayed spectrophotometrically as illustrated in Fig. 12. Using the differential centrifugation method of purification, yields in excess of 10 mg/ml were obtained routinely from French bean or cowpea leaf material. For polyethylene glycol purified preparations, a maximum yield of 0.5 mg/ml was obtained and for ammonium sulphate precipitation 0.15 mg/ml. The respective dilution end points on cowpea primary leaves were 10^{-7} for differential centrifugation, 10^{-4} for polyethylene glycol and 10^{-3} for ammonium sulphate.

The yield of virus was similar for equivalent weights of French bean and cowpea leaves. French beans Pinto and The Prince were compared as propagation hosts for TNV, and although virus yields were similar, Pinto was selected for initial purification on account of improved germination.

4.3 Sucrose Gradient Analysis Samples of both TNV strains were sedimented on 10 to 40 % sucrose gradients and UV absorbance at 260 nm was recorded (Fig. 13) as described under Materials and Methods. When dialysed overnight against distilled water and inoculated onto cowpea primary leaves, fractions from the single TNV_D peak gave characteristic local lesions after 5 days. After similar treatment, the rapidly sedimenting fraction of TNV_A produced a similar local lesion response. However, the slower sedimenting component did not elicit a lesion response on test plants and was assumed to be satellite virus (STNV).

Sucrose gradient fractionation of some virus samples purified from Pinto bean leaves resulted in a more complex absorbance pattern (Fig. 14). Gradient fractions (500 µl) were collected from the absorbing region and analysed individually at 260 and 280 nm; the absorbance ratios for individual fractions are shown in Fig. 14. In addition, 500 µl aliquots were dialysed against distilled water and inoculated onto test plants. Further samples were loaded onto Ouchterlony slides and precipitated against antiserum to TNV in double diffusion tests as described under Materials and Methods.

Local lesions were elicited by all fractions taken from the absorb-

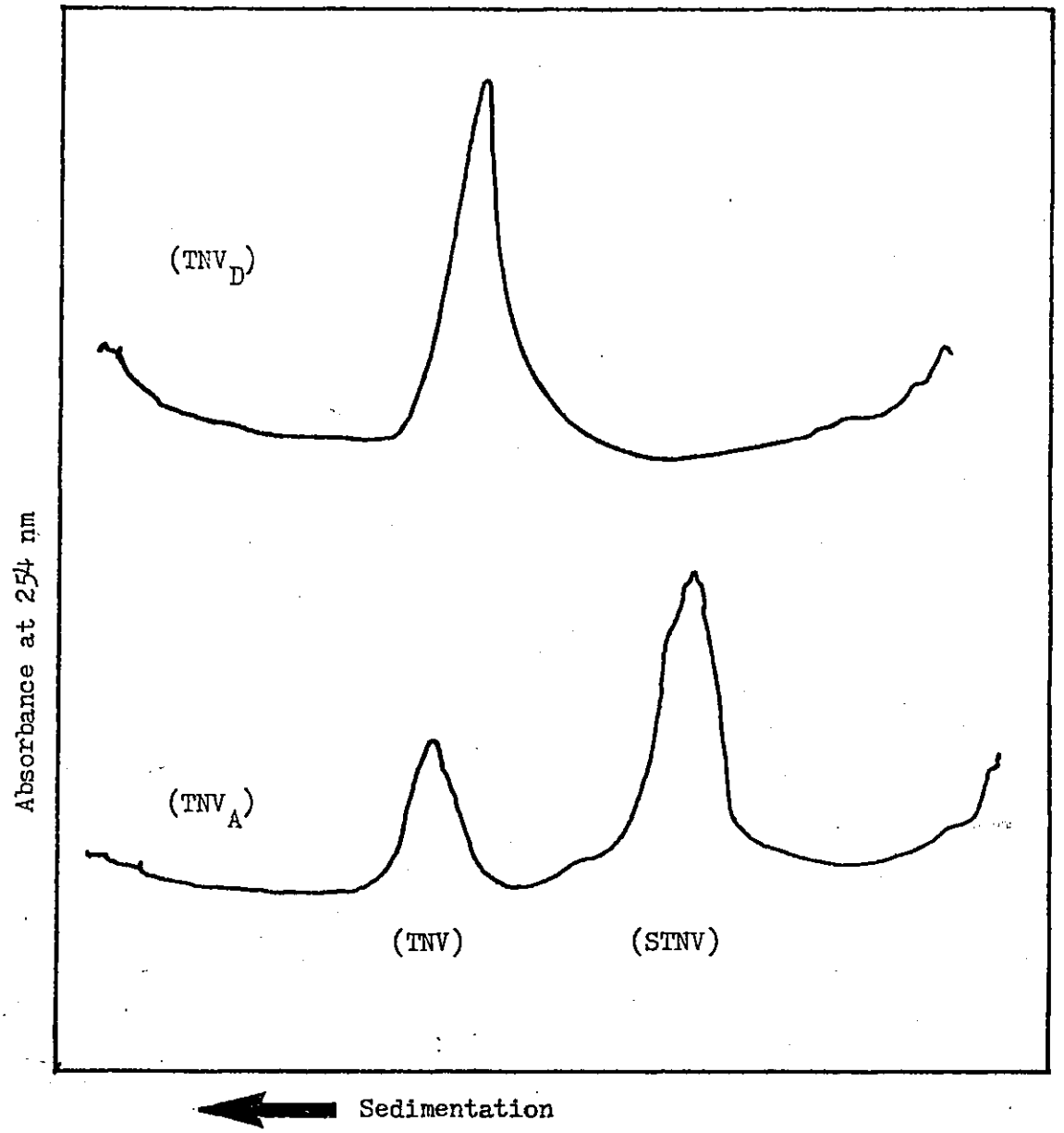
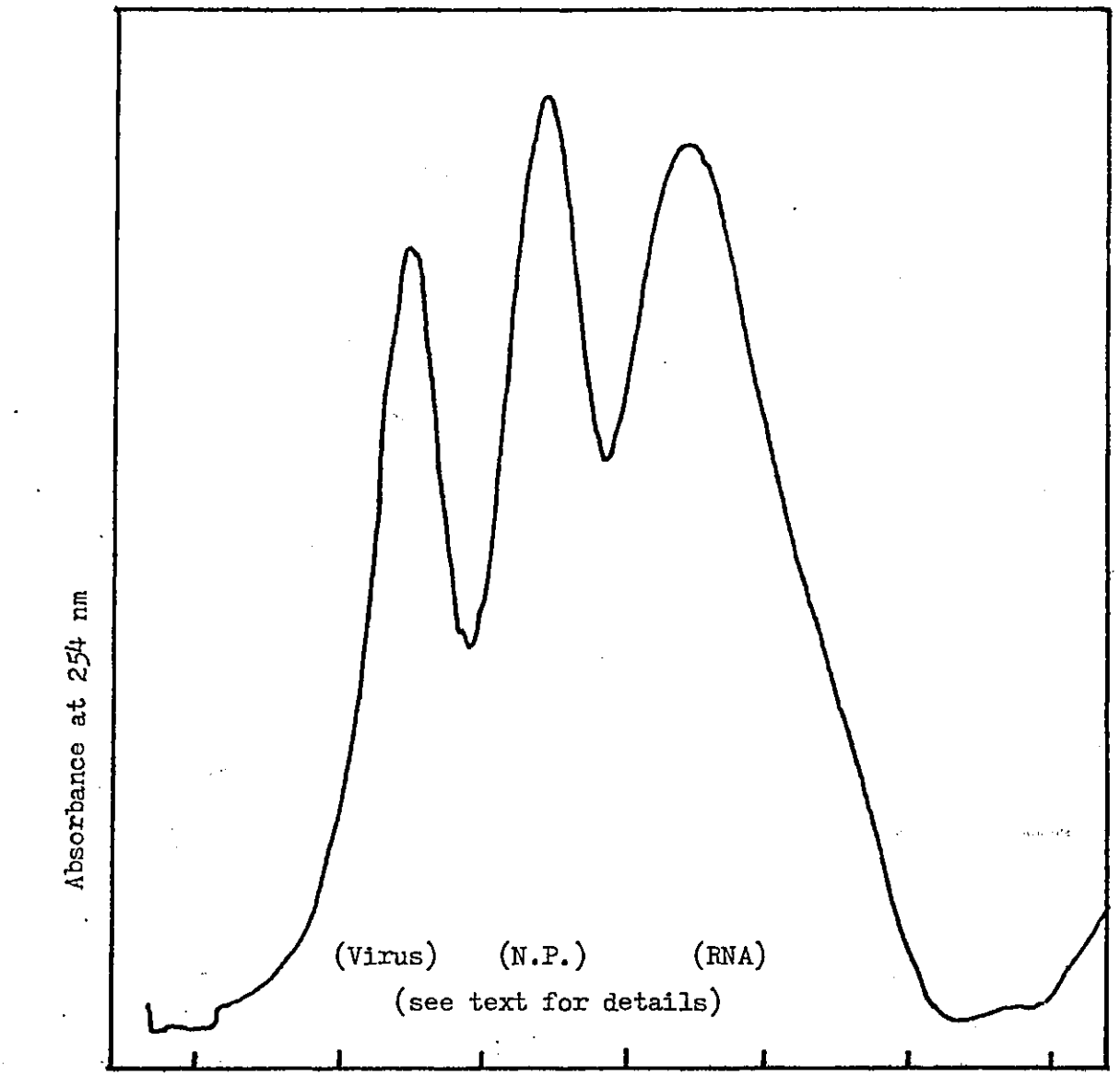


Fig.13 Sedimentation-absorbance profile of 2 strains of TNV purified from cowpea leaves in phosphate buffer pH 7.5 and stored at -20°C in distilled water. Virus (50 µg in 500µl) was layered onto 4.5 ml 10 to 40 % (w/v) sucrose gradients in distilled water and centrifuged at 30,000 rpm and 4°C for 210 minutes. The gradients were analysed on a Uvicord gradient analyser at 254 nm and fractions corresponding to virus peaks were collected using an LKB fraction collecter.



$A_{260} : A_{280}$	1.650	1.657	1.753	1.675	1.813	1.571

500 µl Gradient Fractions Absorbance Ratios

Fig.14 Sedimentation-absorbance profile showing the dissociation of TNV_D purified from Pinto bean leaves in phosphate buffer pH 7.5 and stored at -20°C in distilled water. Virus (15 µg in 500µl) was layered onto 4.5 ml 10 to 40 % (w/v) sucrose gradients in distilled water and centrifuged at 30,000 rpm and 4°C for 210 minutes. The gradients were sampled using a Uvicord gradient analyser and collected fractions were analysed individually in Beckman model 35 spectrophotometer to give $A_{260} : A_{280}$ absorbance ratios for each 500 µl fraction.

ing region, although maximum infectivity was associated with the more rapidly sedimenting region. The rapidly sedimenting fractions also gave precipitin bands against TNV antiserum.

The absorbance ratios of the gradient fractions are consistent with the typical absorbance spectra of the virus ($A_{260}:A_{280}$= 1.75) a nucleoprotein ($A_{260}:A_{280}$= 1.67) and a nucleic acid ($A_{260}:A_{280}$= 1.81). On this basis, the gradient pattern probably represents dissociation of the virus particles and may be related to pH or salt concentration during the purification procedure. Similar sedimentation patterns have been investigated by McCarthy et al., (1980).

Dissociation of the virions was not observed in virus preparations from cowpea leaves, and cowpea was subsequently used as the major propagation host.

4.4 Protoplast Infection Protoplasts were initially inoculated with TNV_D, using the procedure described by Wieringa-Brants et al. (1978). The inoculated protoplasts were washed and incubated as described under Materials and Methods using 1 ml aliquots of protoplast suspension in 10 ml scintillation vials. Discrete aliquots incubated in this way were convenient for pulse labelling studies and easily sampled for infectivity determinations.

At set times after inoculation, vials were withdrawn and stored at -20°C until the experiment was terminated. The samples were thawed and dialysed overnight against distilled water before inoculating onto test plants. When samples were diluted before inoculation to moderate the lesion counts, an appropriate multiplication factor was applied to the infectivity results.

A time course of virus replication using the initial protoplast inoculation procedure is shown in Fig. 15. High levels of virus infectivity were observed directly after inoculation, which may reflect an excess of virus particles remaining adsorbed to the protoplasts after the $CaCl_2$ washing procedure. The continuing presence of infectious virus throughout the time course may also indicate a degree of asynchrony in the inoculation procedure which would reflect on a biochemical analysis of replication. Nevertheless, virus replication progressed rapidly between 24 and 48 hours post-inoculation.

Because of the high virus concentration in the inoculum, and the

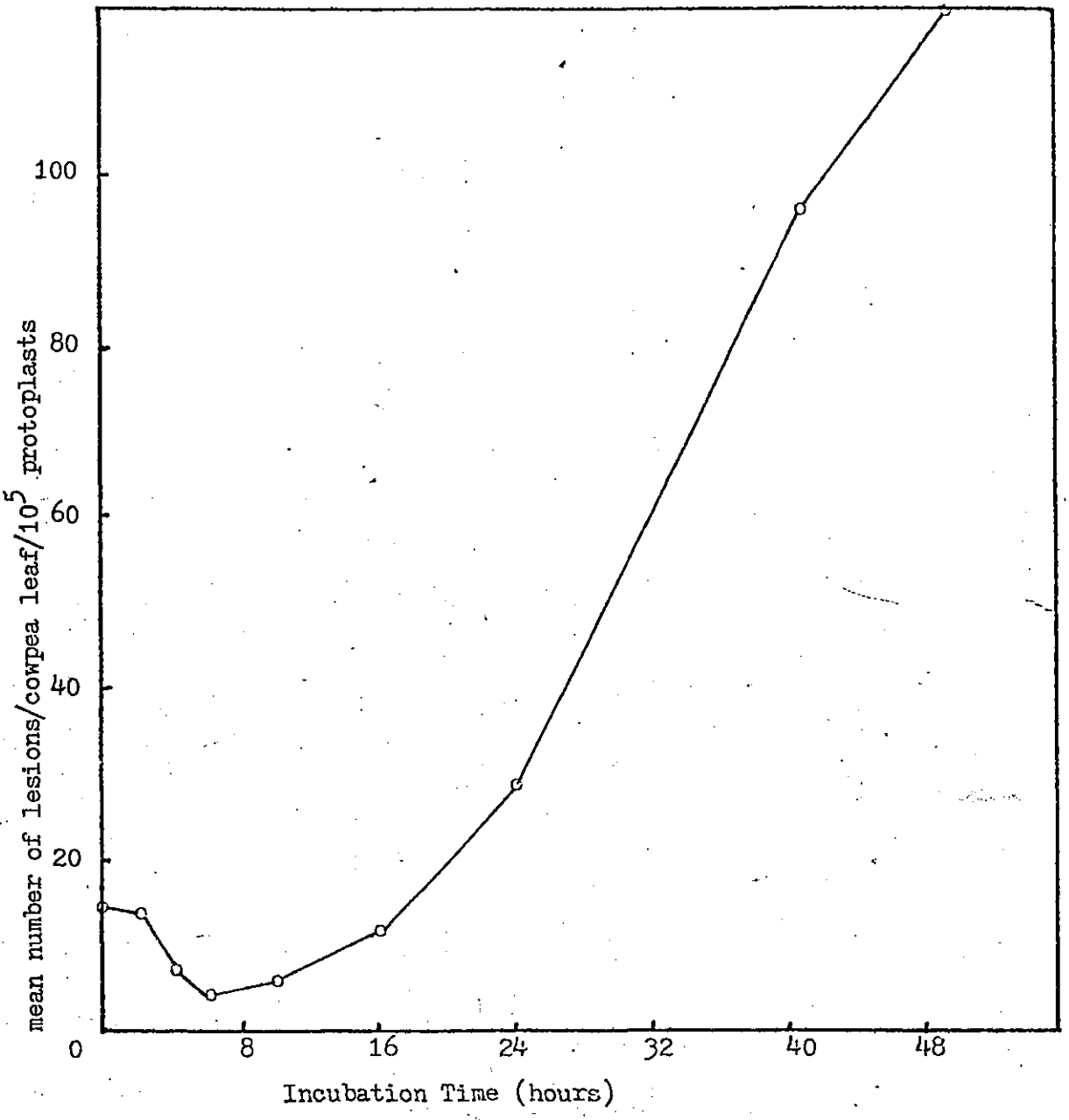


Fig.15 Time course of TNV replication in protoplasts inoculated as described by Wieringa-Brants et al. (1978). Incubated protoplast aliquots were withdrawn at various times PI and inoculated after dialysis onto test plants. Lesion counts were multiplied by appropriate dilution factors, and expressed as mean number of lesions/cowpea leaf/10^5 protoplasts.

lengthy purification procedure required to obtain sufficiently concentrated virus, optimisation experiments were initiated using a range of TNV concentrations. For convenience the sampling procedure was reduced to 3 salient points on the time course. In order to assess the excess virus in the inoculation medium, protoplasts were sampled prior to incubation. Aliquots were then withdrawn at 24 and 48 hours PI to determine the rate of virus replication.

4.5 Influence of Virus and Poly-L-Ornithine Concentration Protoplasts were inoculated with TNV using the method described previously except that a range of TNV concentrations were applied. At 0, 24 and 48 hours PI samples were withdrawn for inoculation onto test plants. Table 7 shows a typical series of results assayed on cowpea primary leaves. Each treatment was inoculated onto 10 cowpea leaves and the results are expressed as mean number of lesions per cowpea leaf per 10^5 protoplasts.

Table 7. **Effect of Virus Concentration on Protoplast Infection**

Concentration of TNV in standard inoculum:	Mean number of lesions per cowpea leaf per 10^5 protoplasts at various times PI		
(µg/ml)	(0 hours PI)	(24 hours PI)	(48 hours PI)
2	1	3	87
5	0	36	168
10	3	75	198
20	14	52	211

Because there is evidence that poly-L-ornithine in the inoculum (PLO) interacts with negatively charged virus particles, the optimal concentrations of virus and PLO were regarded as interdependent. Nevertheless, virus concentration may be critical in infection. Although repeated experiments established a virus optimum for synchronous infection of 4 to 5 µg/ml, (using 2 µg/ml of PLO) a range of PLO concentrations was investigated using 5 µg/ml of TNV.

Using a similar inoculation procedure, a series of PLO concentrations were added to the pre-incubation mixture with 5 µg/ml of TNV. After infection the protoplasts were incubated and analysed as above and the results are expressed in similar terms in Fig. 16 for a representative series of overlapping experiments which were assayed together.

The optimum concentration of PLO for protoplasts inoculated with 5

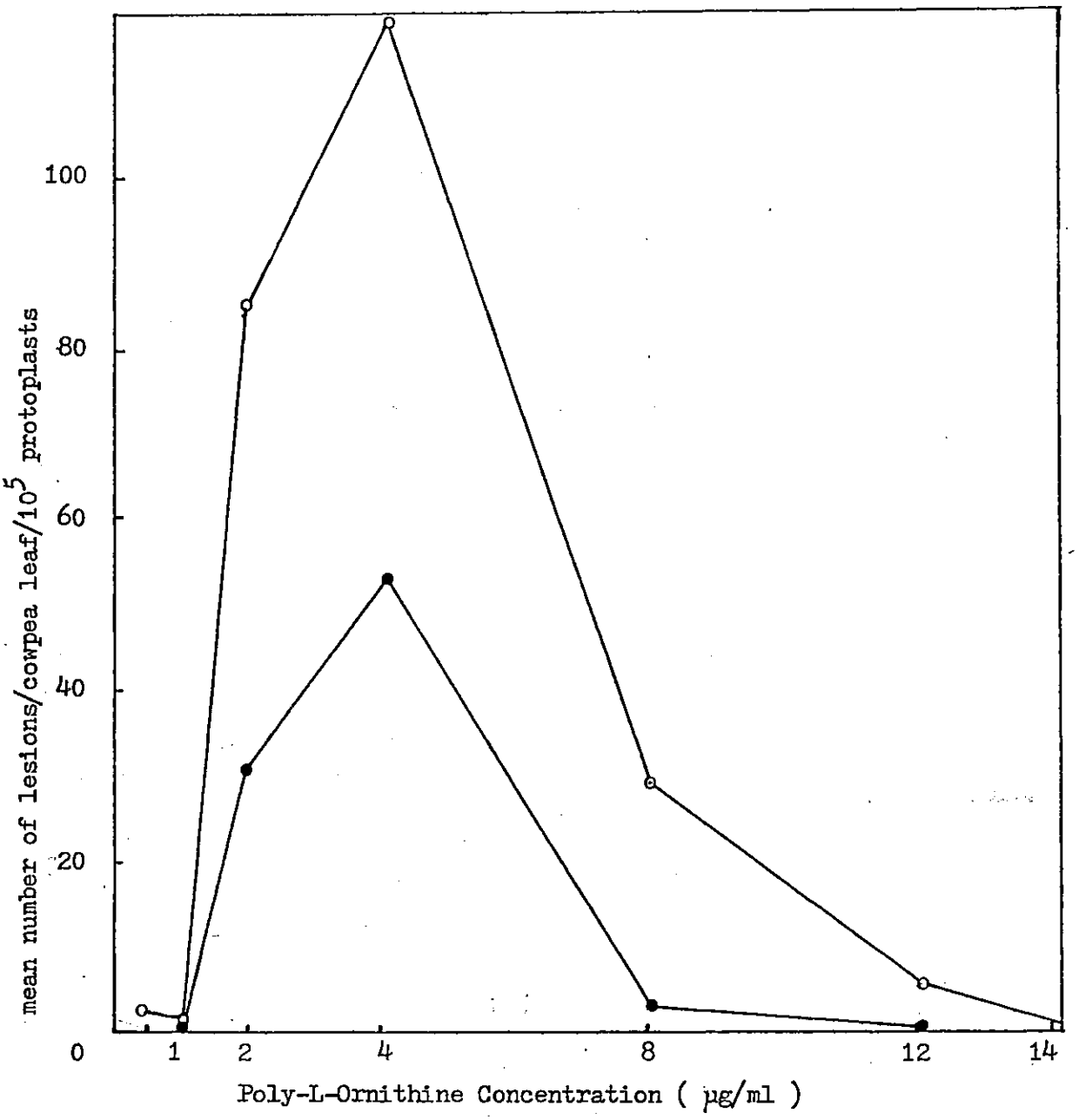


Fig.16 Effect of PLO concentration on protoplast infection using standard inoculation procedure and 5 µg/ml TNV. Protoplasts were assayed by inoculation onto cowpea primary leaves after (●—●) 24 hours and (o—o) 48 hours incubation post-inoculation. At time 0 all treatments gave less than 2 lesions per leaf per 10^5 protoplasts.

µg/ml of TNV was observed to be around 4 µg/ml throughout a series of experiments. This optimum level is close to the 1:1 ratio reported for many protoplast/virus interactions (Otsuki & Takebe, 1976; Maule et al., 1980). High levels of PLO (above 8 µg/ml) were accompanied by a significant loss in protoplast viability but for protoplasts inoculated in the absence of PLO no virus replication was observed.

Since buffer ions in the inoculation medium might influence not only the formation of infection complexes but also protoplast stability, a series of pH variations and buffer concentrations was investigated. Preliminary results suggested that protoplast viability was reduced when inoculated in citrate buffers, and consequently inoculations were carried out using phosphate buffers over a broad pH range.

4.6 Influence of pH and Buffer Concentration Using the optimised PLO and virus concentrations, a series of inoculations was performed using 10 mM phosphate buffer at a range of pH values. The protoplast infectivity results are shown in Fig. 17, at 24 and 48 hours PI; infectious virus was not detected immediately after inoculation.

Phosphate buffer at pH 6.0 consistently resulted in the maximum yield of infectious virus, and was tested at a range of concentrations using a similar inoculation procedure. The influence of buffer concentration on infectivity is shown in table 8.

Table 8. Influence of Buffer Concentration on Protoplast Inoculation

Concentration of phosphate buffer at pH 6.0 in the inoculation medium :	Mean number of lesions per cowpea leaf per 10^5 protoplasts at various times PI		
	(0 hours PI)	(24 hours PI)	(48 hours PI)
2 mM	0	4.5	235
5 mM	0	3.0	92
10 mM	1	4.5	15
15 mM	0	9.0	16
20 mM	0	3.0	14
30 mM	0	1.5	9

All subsequent experiments employed an inoculation mixture containing 2 mM phosphate buffer pH 6.0, 5 µg/ml TNV and 4 µg/ml PLO in 0.7 M mannitol. The inoculation temperature and incubation times were then adjusted to give maximum virus yields.

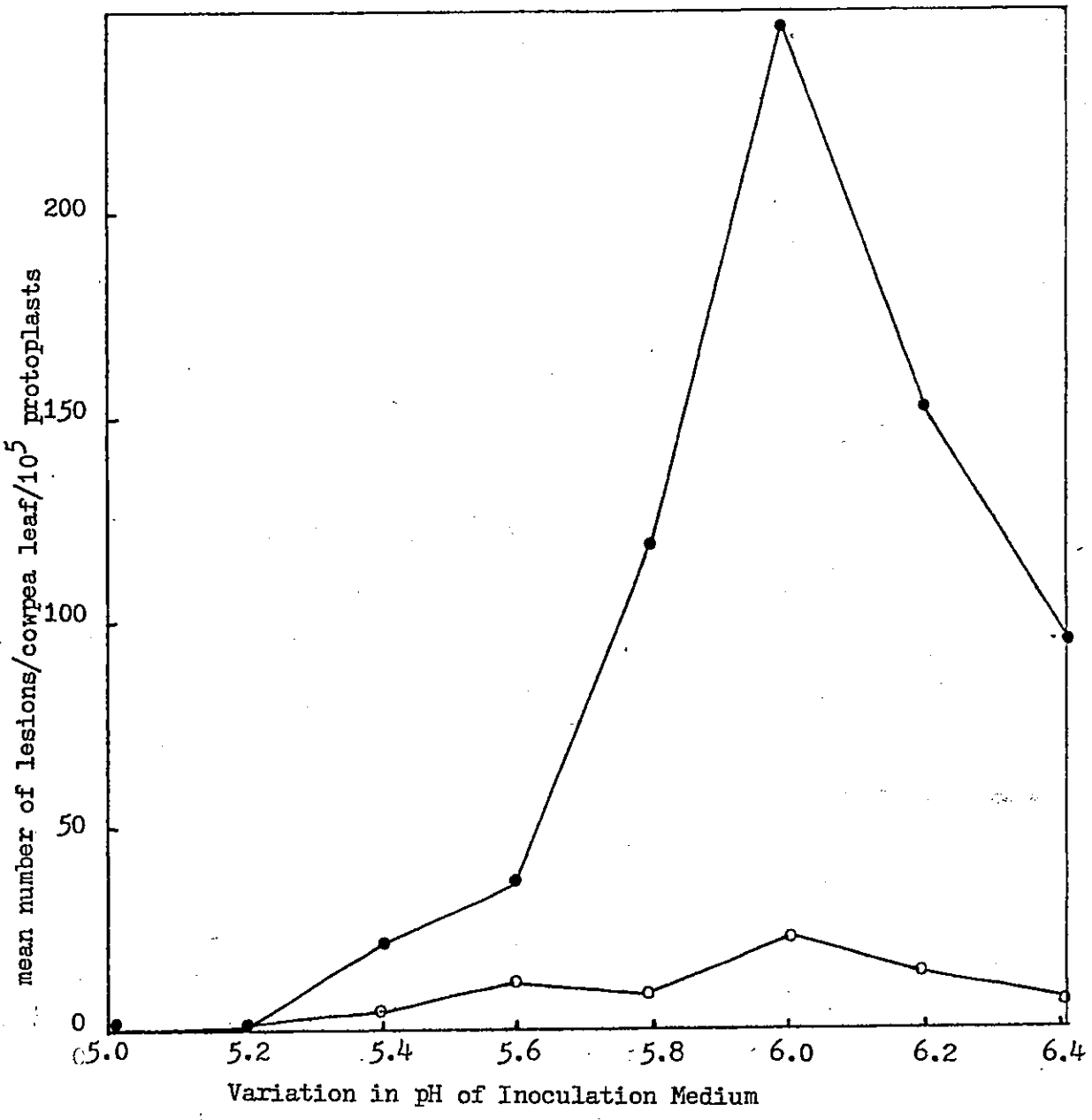


Fig.17 Using the optimised PLO and virus concentrations, protoplasts were inoculated using 10 mM phosphate buffer at the above pH values. Protoplast infectivity was assayed by local lesion counts after 24 (o—o) and 48 (●—●) hours of incubation. The counts are expressed as mean number of lesions per cowpea leaf per 10^5 protoplasts. No infectivity was detected immediately after inoculation.

4.7 Influence of Incubation and Pre-incubation Times Using a 15 minute inoculation period and the modified inoculation mixture described above, experiments were carried out to investigate the effects of pre-incubation on protoplast infection. Freshly sedimented protoplasts were resuspended in inoculation medium, pre-incubated for 0 to 37.5 minutes at 20°C in Erlenmeyer flasks. The cultures were maintained at 20°C for 15 minutes before washing and incubating as previously. After 48 hours the cultures were dialysed and assayed on cowpea leaves; the results are shown in Fig. 18, for a representative series of pre-incubation times.

An optimum pre-incubation time of 30 minutes was observed for media subsequently employed in a 15 minute inoculation. Accordingly, a 30 minute pre-incubation time was used to investigate a series of inoculation times. The range of inoculation times and the corresponding infectivity data at 24 and 48 hours PI are shown in Fig. 19. No infectious virus was recovered from the protoplasts directly after inoculation.

Although infectivity increased in protoplasts incubated for up to 25 minutes in the inoculation mixture, an inoculation time of 10 minutes was selected for use in subsequent experiments. The extended incubation was thought to increase the possibility of unspecific virus attachment to the protoplasts, this might lead to a loss of synchrony in the infection. The short incubation also leads to an infectivity peak; this may reflect a rapid association and Ca^{2+}-assisted disassembly of the virus, contiguous with the protoplast membrane. Such a process would lead to a more synchronous infection of the protoplasts, and reduce the chances of subsequent infections by associated virus particles.

Accordingly, the 30 minute pre-incubation was investigated with a 15 minute inoculation period to determine the effects of temperature variations on the protoplast infection mechanism.

4.8 Influence of Inoculation Temperature. Protoplast inoculations were carried out using the optimised conditions and employing a temperature shift to 0°C for the pre-incubation and/or the inoculation period. Using the 30 minute pre-incubation period, no difference was observed between preparations pre-incubated at 20°C and those pre-incubated at 0°C.

Observations of cultures, and microscopic examination of inoculated protoplasts showed an aggregation of the protoplasts which was proportional to the PLO concentration in the inoculum. At high concentrations

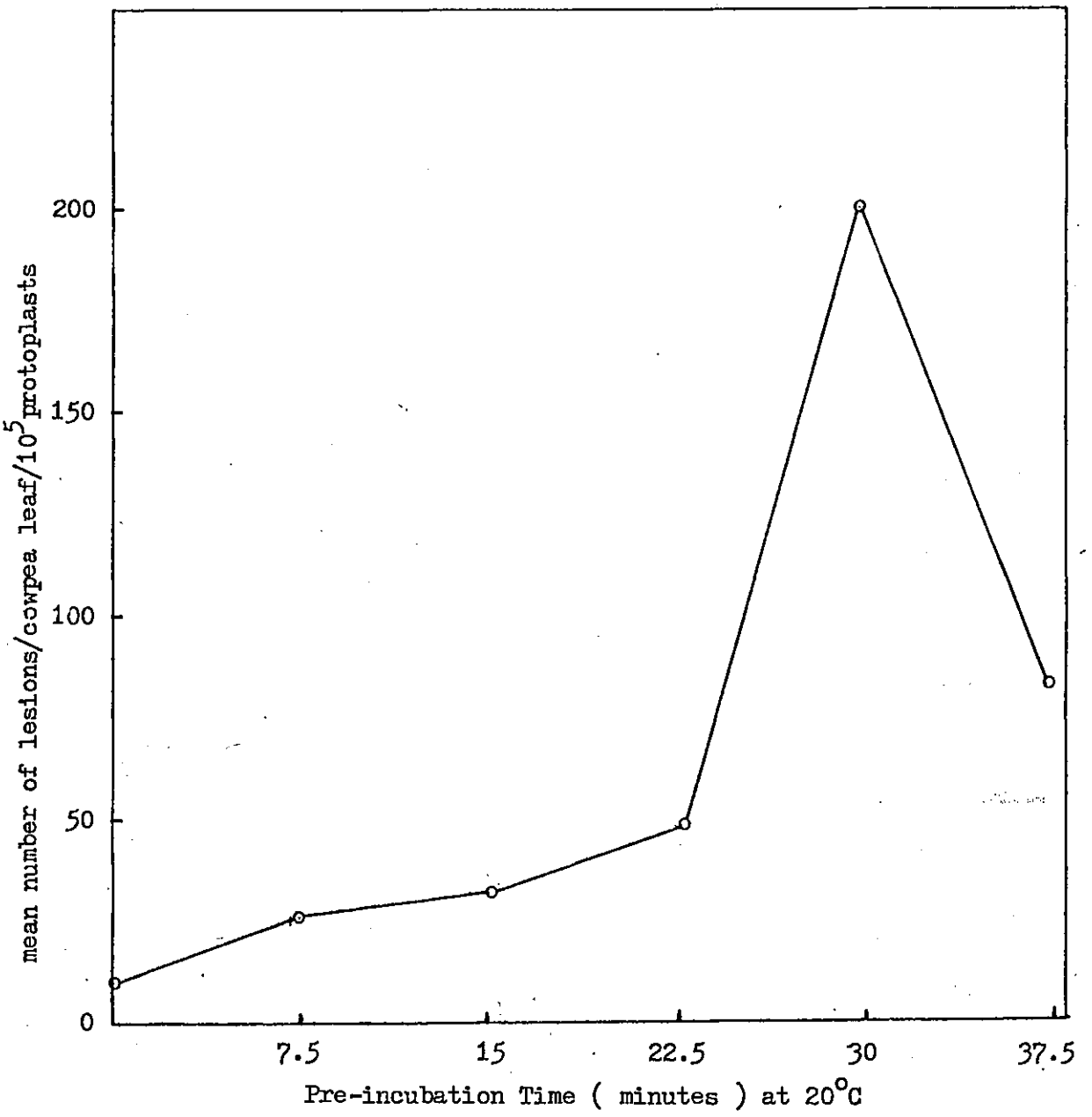


Fig.18 Effect of pre-incubation time on protoplast infection. Protoplasts were infected using the modified inoculation medium and a 15 minute inoculation time at 20°C. Prior to infection, the inoculation medium was pre-incubated for various periods as shown above. Protoplast cultures from each treatment were assayed for infectivity after 48 hours of incubation. The infectivity results are expressed as mean number of lesions per cowpea leaf per 10^5 protoplasts.

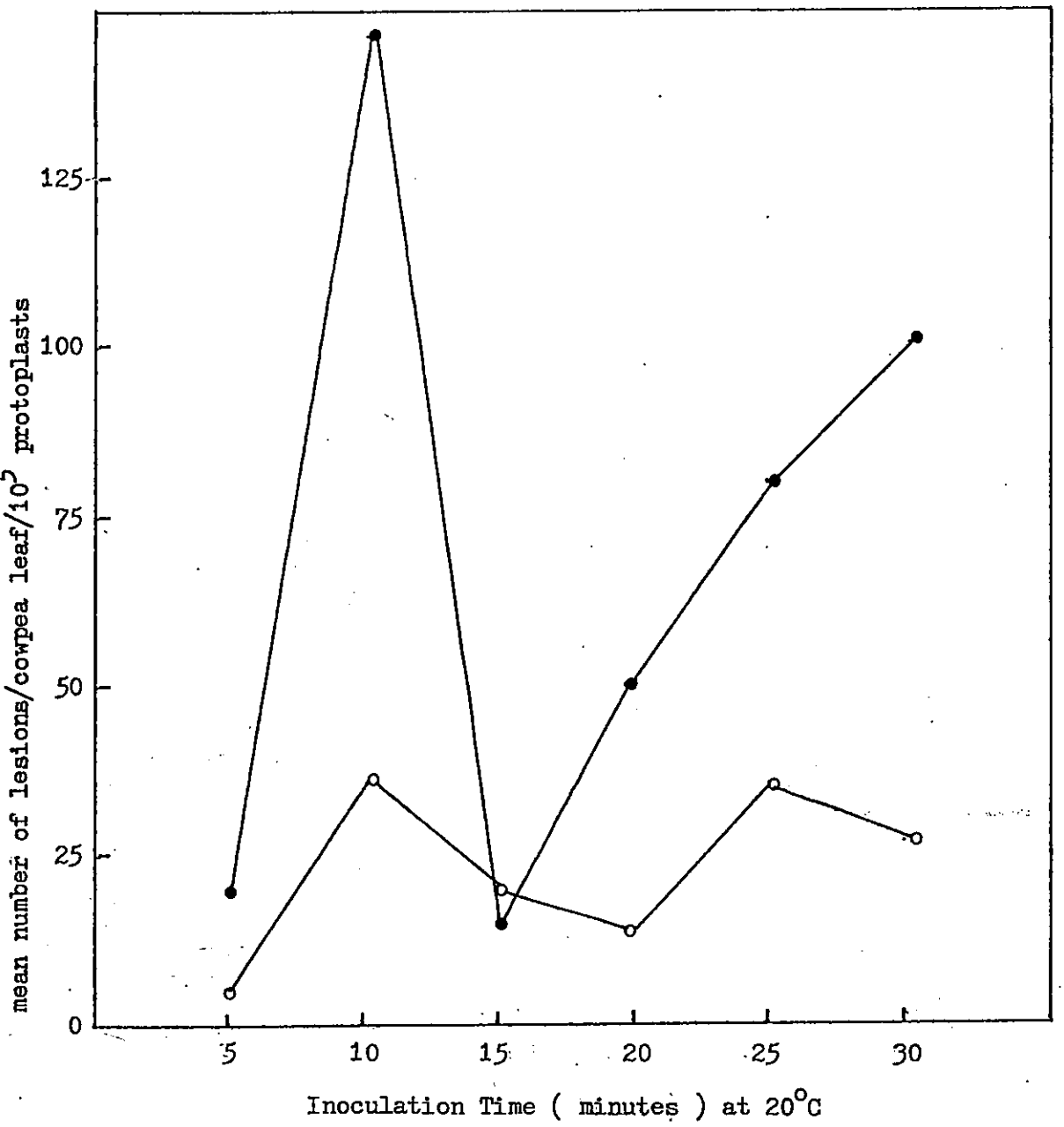


Fig.19 Effect of inoculation time on protoplast infection. Protoplasts were inoculated using the modified inoculation medium pre-incubated at 20°C for 30 minutes. A range of inoculation times was investigated and protoplasts were incubated for 24 (o—o) and 48 (•—•) hours, prior to dialysis and infectivity assay on cowpea primary leaves. The infectivity results are expressed as mean lesion number per cowpea leaf per 10^5 protoplasts.

(more than 8 µg/ml) protoplasts were damaged by the polycation and lost viability. At low PLO concentrations (less than 2 µg/ml) protoplast aggregation was not observed, and little or no infectious virus was recovered after incubation. In general, a degree of protoplast aggregation was always observed in cultures which subsequently yielded infectious virus, although the aggregates were often dissociated by washing in low molarity $CaCl_2$ solution in 0.5 M mannitol. (10 mM $CaCl_2$ resulted in a similar protoplast aggregation and 0.1 mM $CaCl_2$ was more suitable as a protoplast washing solution).

Cultures which were inoculated at 0°C showed an increase in protoplast aggregation over those inoculated at 20°C using a similar level of PLO. Reproducibly high yields of infectious virus resulted from the 0°C temperature shift although the increase over cultures inoculated at 20°C was not always statistically significant. Nevertheless, on the basis of the infectious virus recovered,and the observations of protoplast aggregation using non-toxic levels of PLO, the temperature shift was employed in subsequent experiments.

4.9 Optimal Inoculation Conditions The influence of the osmotic "step-up" was similarly difficult to assess statistically. However, protoplast stability during incubation was greater if an osmotic change was employed in the inoculation procedure. Although protoplast loss during inoculation increased as a consequence of the step-up, many of the broken cells were removed in the washing procedure. Thus a more robust population of cells was incubated and this selection was reflected in protoplast stability, at 24 and 48 hours PI. On this basis the osmotic step-up was retained in the inoculation procedure which in subsequent experiments was as follows:

10 ml of inoculation medium containing 40 µg of PLO and 50 µg of TNV,with 2 mM phosphate buffer pH 6.0 in 0.7 M mannitol was pre-incubated for 30 minutes at 20°C in a 100 ml Erlenmeyer flask.

Protoplasts were sedimented from 0.5 M mannitol solution and resuspended in the inoculation medium for 10 minutes at 0°C.

The non-adsorbed virus was removed by washing in 0.1 mM $CaCl_2$ solution in 0.5 M mannitol as described previously.

10 ml of inoculation was used to infect between 0.6 and 1 x 10^6 protoplasts without any significant difference in the infectivity recovered.

5. RESULTS: VIRUS REPLICATION IN PROTOPLASTS

5.1 Introduction Throughout the optimisation experiments, the recovery of infectious virus was assessed by local lesion assay as a measure of virus replication. This procedure was effectively used to compare treatments and thus develop a more efficient infection method. However, in order to study virus synthesis in detail, more rigorous parameters of replication were required.

Thus, the time course of virus replication was investigated by assessing the yield of infectious RNA (Coutts & Wood, 1976) as well as the yield of infectious whole virus. In addition, TNV synthesis was followed by analytical sucrose gradient centrifugation of extracts from protoplasts at various times after inoculation (Gonda & Symons, 1979). The virus yield was quantified by comparison with similar extracts of healthy protoplasts, supplemented with highly purified virus.

RNA-dependent RNA polymerase activity was also investigated during the course of viral replication. Following a method similar to that used by Okuno and Furusawa (1979), extractable polymerase activity was assayed at various times after inoculation or "mock-inoculation".

Protein synthesis during replication was studied using radiolabelled precursors and techniques similar to those applied in TMV replication studies (Paterson & Knight, 1975; Siegel et al., 1978; Huber, 1979). The protoplasts were fractionated prior to analysis in order to improve the detection capacity of the analysis techniques. Protein synthesis was investigated using polyacrylamide-SDS slabgel electrophoresis and labelled products were visualised by fluorography. Densitometer traces were also obtained by scanning the fluorogram lanes to give comparative data.

The molecular weights of the protein products were estimated by using labelled marker proteins and co-electrophoresing labelled capsid protein.

5.2 Infectivity Time Course During subsequent time course experiments, protoplasts were harvested from the incubation medium by a 200 xg centrifugation step prior to analysis. This was necessary to achieve the concentration required for biochemical analysis of the protoplast products. Since comparative data were required from both infectivity and biochemical assays of virus replication, a similar sampling procedure

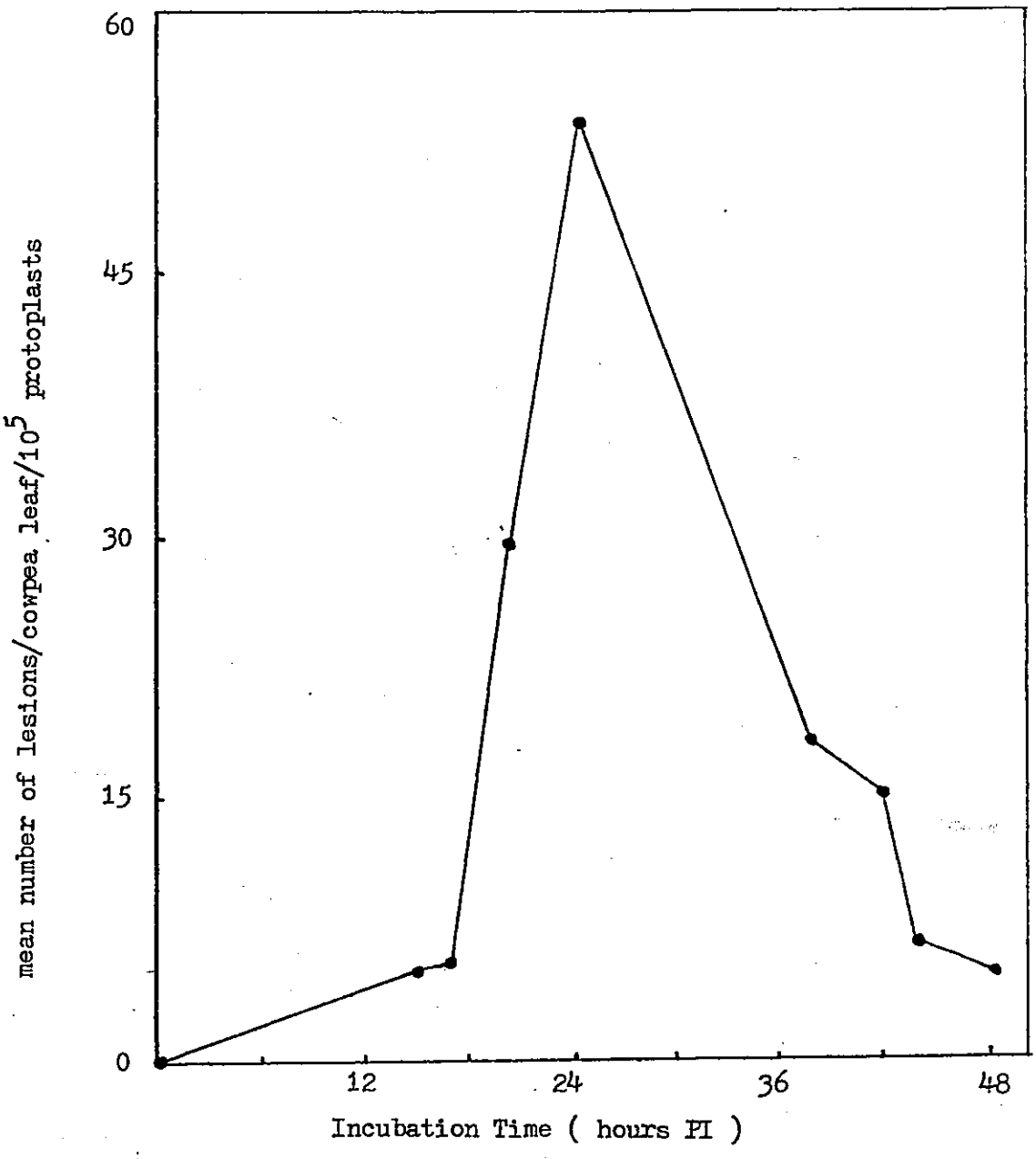


Fig. 20 Nucleoprotein- infectivity time course of virus replication using protoplast pellet extracts. Protoplasts were harvested by centrifugation after various periods of incubation. The pellets were sonicated and diluted in distilled water before inoculation onto test plants. The infectivity data are expressed as lesions per cowpea leaf per 10^5 protoplasts.

was adopted for both techniques, involving a preliminary centrifugation step. Accordingly, for local lesion assays of the replication time course protoplasts were sedimented from the inoculation medium, resuspended in distilled water at 4°C, and sonicated before inoculation onto cowpea primary leaves. The infectivity data from such a time course are expressed in Fig.20.

By comparison with Fig. 15, a sharp decline in the recovery of infectious virus was detected after 24 hours of incubation. In other, similar experiments infectious virus was observed to increase for a period upto 30 hours PI in a similar pattern to Fig. 15, before falling sharply until the end of the incubation period, similar to Fig. 20.

This rapid decrease in virus content was observed in all experiments sampled by the centrifugation procedure, and may reflect the increasingly fragile state of the protoplasts after prolonged (i.e. beyond 24 to 30 hours) incubation. In this case, protoplast material which is retained in the dialysis procedure would be lost by centrifugation, and discarded with the inoculation medium.

However, the initial time course studies indicated that virus replication precedes rapidly between 14 and 30 hours PI. Certainly RNA-dependent RNA polymerase activity would be expected early in the replication cycle, perhaps decreasing in activity while virus replication continued (Okuno & Furusawa, 1979), and virus specific protein synthesis might follow a similar pattern. Thus, over the period of maximum interest both dialysis and centrifugation procedures give similar results.

Accordingly, sedimented protoplast samples were also investigated for infectious RNA content. Over a similar incubation period, protoplasts were harvested and phenol extracted as described under Materials and Methods. The resultant aqueous phase was diluted in distilled water at 4°C and inoculated immediately onto test plants. The results from such a time course are shown in Fig. 21.

The infectivity recovered from phenol extracted protoplasts was in general about half of that present in nucleoprotein extractions. This difference probably reflected the efficiency of the extraction procedure and the instability of the RNA. The rapid increase in infectious RNA preceded the nucleopreotein increase in all experiments although there was variation in the onset of detectable replication between experiments. The infectivity peak was further studied by sucrose gradient analysis.

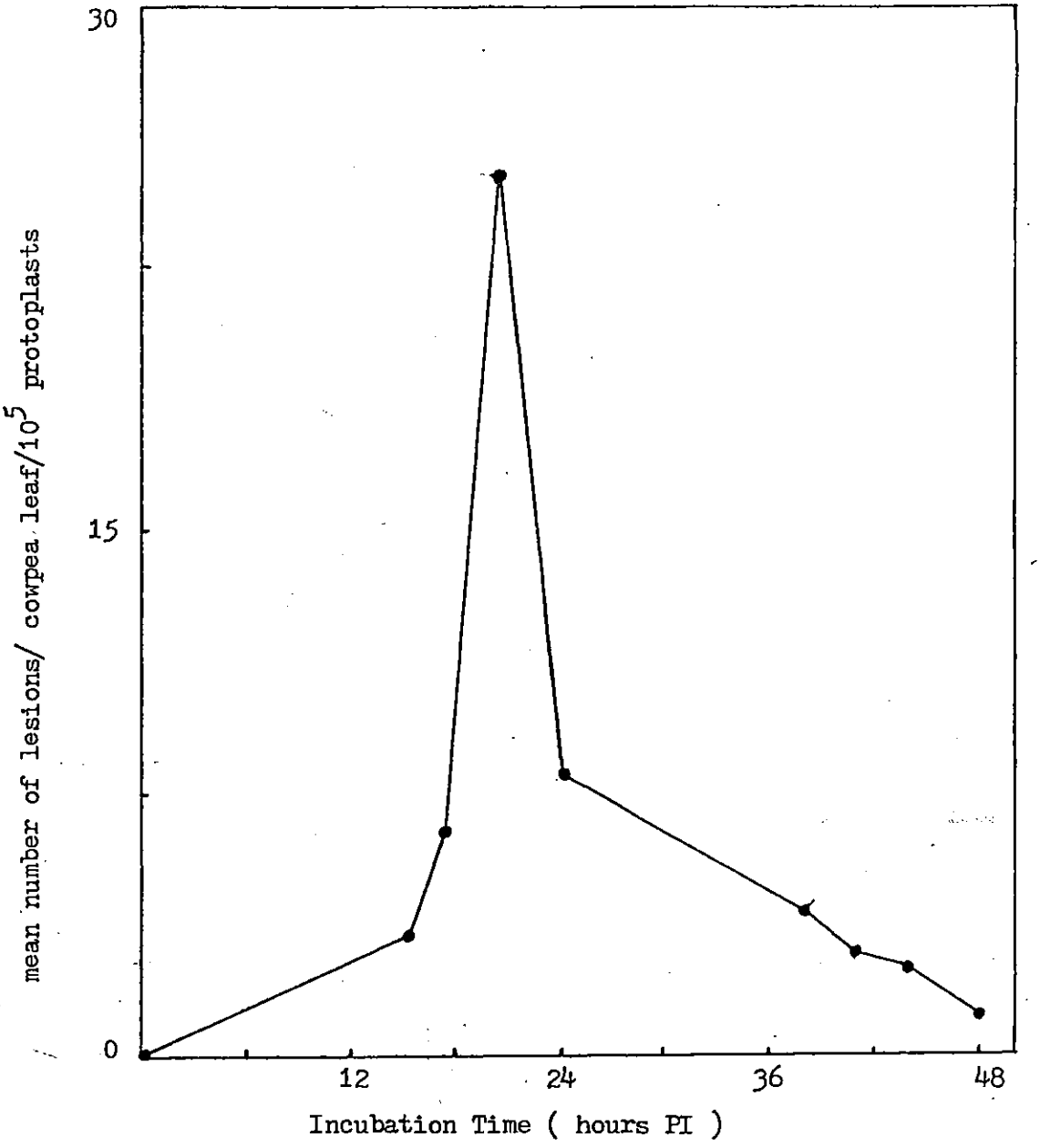


Fig. 21. RNA-infectivity time course of virus replication using protoplast pellet extracts. Protoplasts were harvested by centrifugation after various periods of incubation. The pellets were phenol extracted as described under Materials and Methods and the resultant aqueous phase diluted in distilled water. These diluted samples were inoculated onto test plants for local lesion assay. The results are expressed as mean number of lesions per cowpea leaf per 10^5 protoplasts.

5.3 Sucrose Gradient Analysis Inoculated protoplasts were pelleted after various incubation periods and extracted in PGB as described under Materials and Methods, for analytical sucrose density gradient centrifugation. When healthy protoplasts were mixed with a known concentration of highly purified virus, and analysed by this method, characteristic peaks were observed on absorbance-sedimentation profiles at 254 nm (Fig. 22.), sedimenting close to the bottom of the gradient.

Viral protein antigenicity as evidenced by Ouchterlony tests, and infectivity as shown by local lesion assay, were observed to co-sediment with the UV absorbing material. The absorbance-sedimentation peak area, as measured by comparing the weight of cut-out traces, was also proportional to the amount of virus added to the protoplasts. Thus, the corresponding peak areas from infected protoplast extracts were used as a quantitative estimate of virus yield (Fig. 23).

When virus content as estimated by UV absorbance-peak areas was plotted on the time course of protoplast incubation, a pattern was observed similar to the infectivity data from a parallel nucleoprotein assay (Fig. 24). The virus peak was also similarly located between 24 and 30 hours PI in other experiments and followed the course of protoplast infectivity in each experiment.

From these data, it appears that virus replication occurred synchronously in the protoplasts and although the peak was variable in its timing the onset of rapid viral synthesis occurred between 15 and 20 hours PI, in all cases. Accordingly, a period between 15 and 25 hours after inoculation was selected for analysing protoplasts with respect to RNA-dependent RNA polymerase activity. Viral replicase activity might be expected throughout this period to account for the observed synthesis of viral RNA.

5.4 RNA Replicase Assay During the period of rapid virus synthesis, between 15 and 25 hours PI, protoplast pellet extracts were investigated for RNA replicase activity by a method similar to that described by Okuno and Furusawa (1979).

Virus infected, "mock-inoculated" and healthy protoplasts were collected by centrifugation and washed before being ground and clarified by centrifugation prior to extraction. The membrane-bound enzyme fraction was obtained as a 20,000xg pellet as described under Materials and Meth-

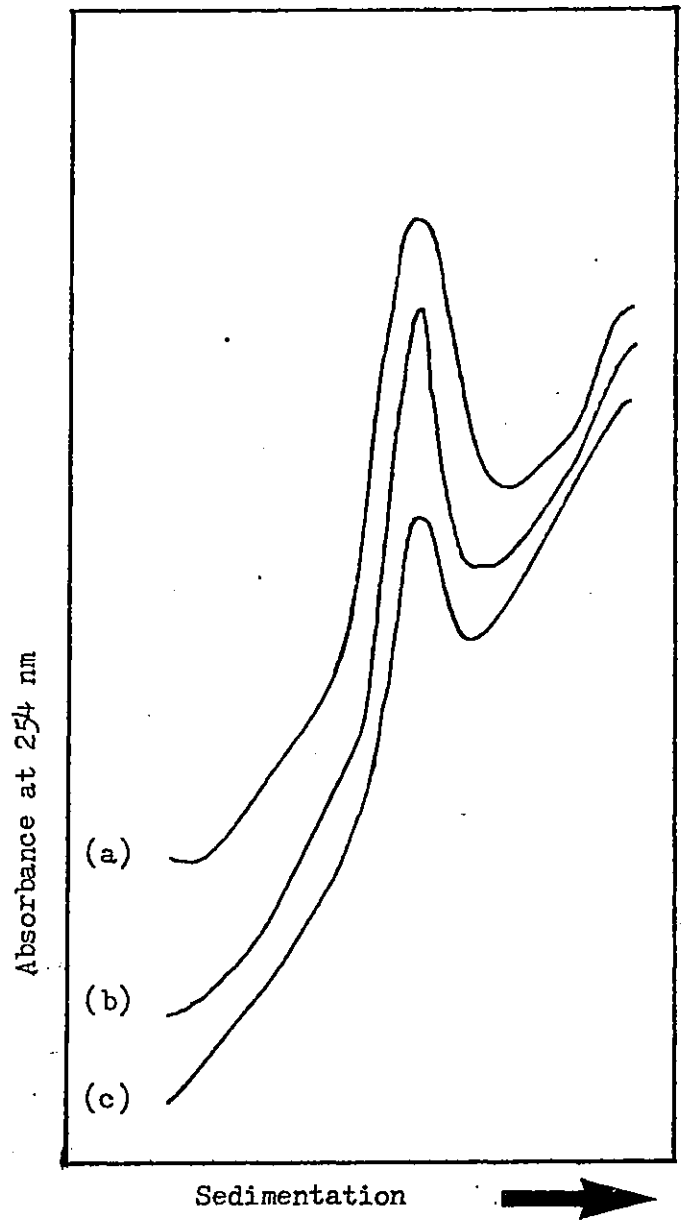


Fig. 22 Calibration of sucrose gradient analysis for protoplast extracts. Standards of (a) 30 µg, (b) 20 µg, and (c) 10 µg of purified virus were added to suspensions of healthy protoplasts immediately prior to extraction in grinding buffer (PGB). The extracts (for details see text) were layered onto 10 to 40 % (w/v) 4.5 ml sucrose gradients in distilled water and centrifuged at 30,000 rpm and 4°C for 210 minutes. Gradients were sampled using a Uvicord gradient analyser and the relative peak areas were used to calculate virus content in infected protoplast samples.

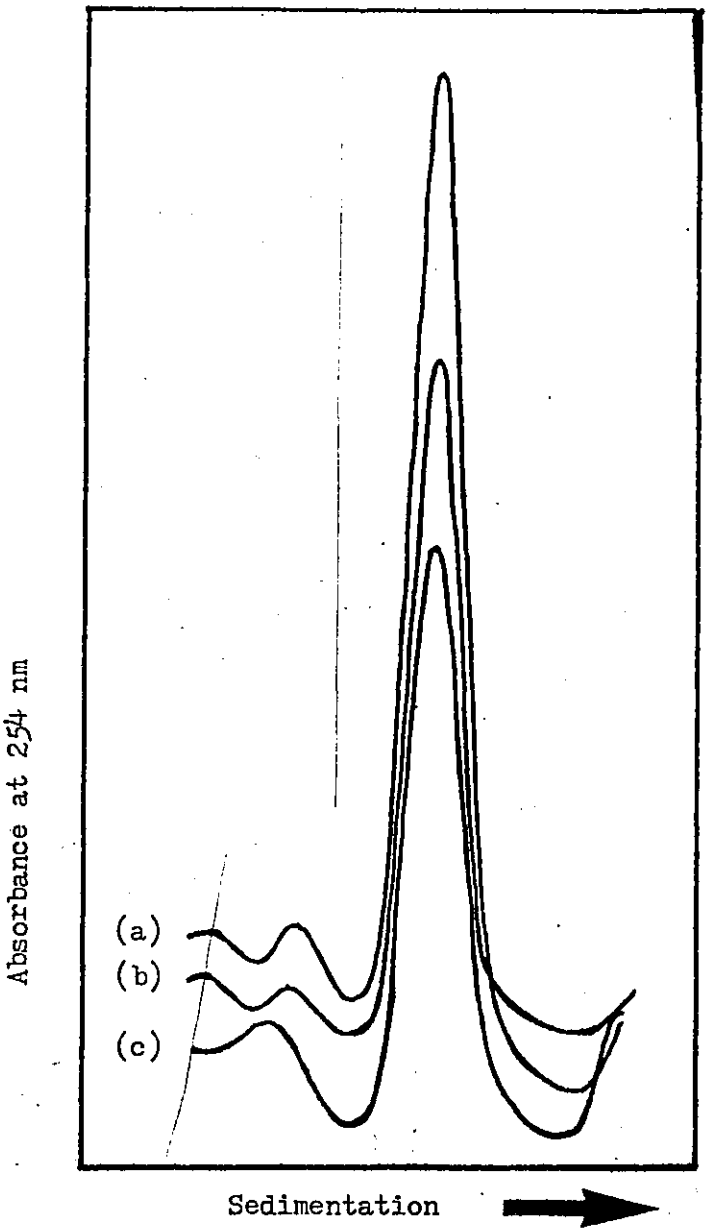


Fig. 23 Sedimentation-absorbance profile of protoplast extracts at various times after inoculation with TNV_D. Protoplast pellets were sedimented from 5 ml aliquots of incubation medium and resuspended in 500 µl aliquots of PGB. The pellets were ground and 200 µl extracts (for details see text) were layered onto 4.5 ml 10 to 40 % (w/v) sucrose gradients and centrifuged at 30,000 rpm and 4°C for 210 minutes.

The absorbance peak areas were measured to quantify the virus yield by comparison with virus standards. Samples were taken at (c) 15, (b) 17 and (a) 19 hours hours post-inoculation.

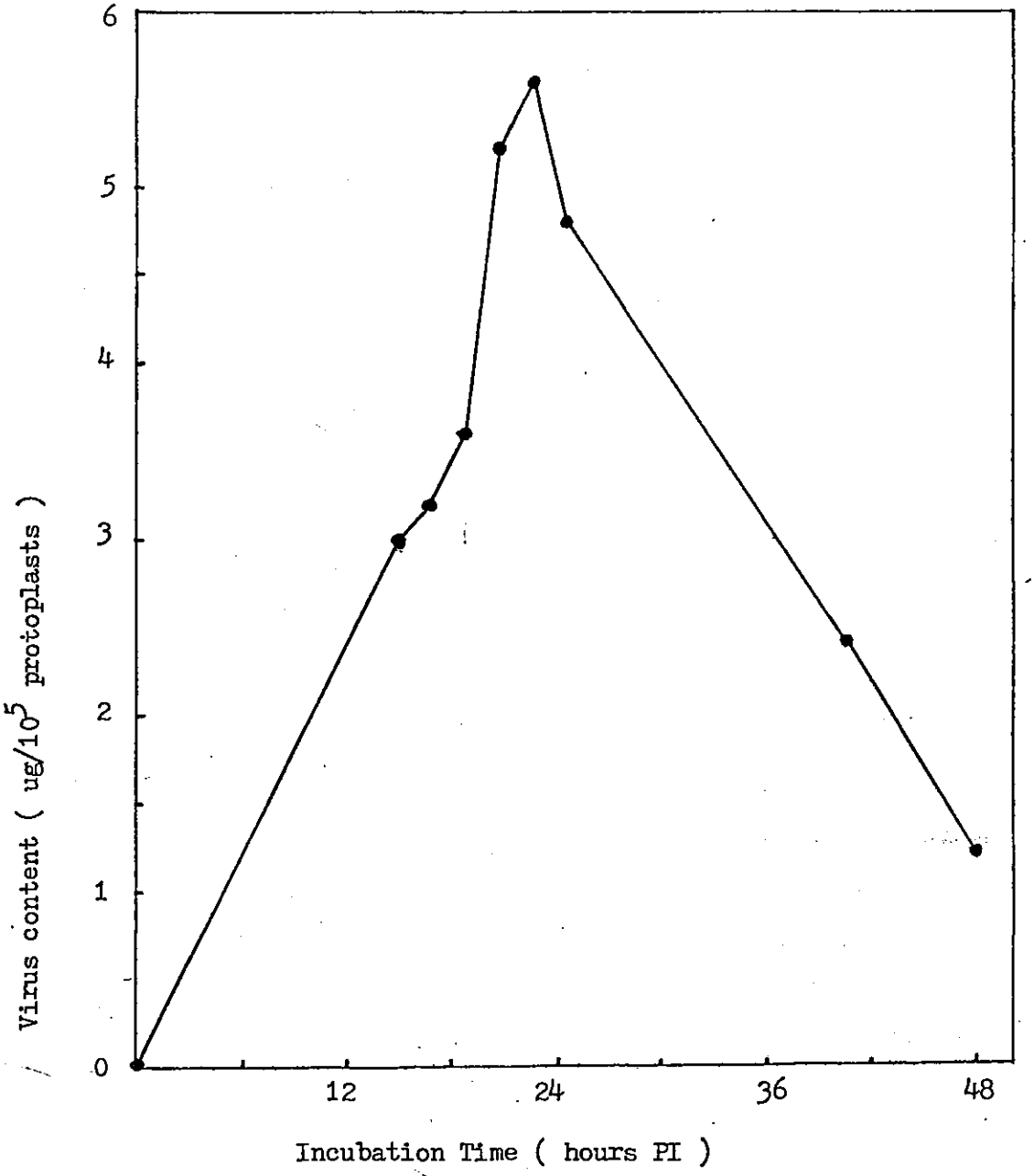


Fig. 24. Time course of virus replication as assessed by UV absorbance of protoplast pellet extracts on sucrose gradients. Protoplasts were harvested at various times post inoculation and analysed by analytical sucrose density gradient centrifugation (see Materials and Methods). Virus yields were calculated from the sedimentation-absorbance profile peak areas, by comparison with calibration standards. The virus content is plotted on a similar time course to a parallel infectivity assay of nucleoprotein (Fig. 20.) and RNA (Fig. 21.) extracts from infected protoplasts.

ods and was taken up in the reaction buffer IB. No replicase activity was detected in the soluble fraction or associated with the 1,000xg pellet.

Membrane preparations from healthy,"mock-inoculated" and infected protoplasts were assayed for RNA replicase activity by measuring the incorporation of (^{3}H) UTP into TCA acid-insoluble products in a standard reaction mixture. Extracts were incubated for 60 minutes at 30°C in the presence of actinomycin D to inhibit DNA-dependent RNA synthesis, while duplicate reaction mixtures were incubated with TCA and tetra-sodium pyrophosphate solution to serve as control experiments.

The incorporation of (^{3}H) UTP into TCA acid-insoluble products by protoplast membrane preparations taken at 18 hours PI is shown in table 9, fractions were prepared from healthy, infected and "mock-inoculated" protoplast cultures.

Table 9. **Polymerase Assay of Protoplast Pellet Extracts**

Membrane fractions after 18 hours incubation of protoplast cultures :	(^{3}H) UTP incorporated into TCA acid-insoluble products (cpm x 10^{-2})	
	control*	after 1 hour at 30°C
Healthy	2.49	1.96
"	2.44	2.34
Mock-inoculated	2.58	4.26
"	2.46	3.30
Infected	2.97	6.97
"	2.16	7.67

*control assay was incubated with termination solution, for details see text.

When healthy protoplast membrane fractions were assayed for RNA-dependent RNA polymerase activity, no incorporation of (^{3}H) UTP was observed above the control background level. Fractions extracted from mock-inoculated protoplasts resulted in a 1½ to 2 fold increase in the level of incorporation, whereas infected protoplast fractions showed a 3 to 4 fold increase in incorporation.

Polymerase activity was assayed at 2 hour intervals throughout the

period of rapid virus replication. However, the increased level of replicase activity in infected protoplasts was only detected at 18 hours PI. Since replicase activity must extend over a long period to account for the observed virus synthesis, both the duration and level of incorporation detected probably reflect a far from optimal assay system for this enzyme. Nevertheless, the incorporation detected may represent the peak of enzyme activity during virus synthesis, and protein synthesis during the early replication period might thus include components of the replicase.

Accordingly, protein synthesis was investigated during the period of virus replication using pulse labelling techniques with radio-labelled protein hydrolysate or amino acid mixtures.

5.5 Protein Synthesis Analysis Healthy, infected and "mock-inoculated" protoplasts were pulsed for 2 hour periods at various intervals after the appropriate inoculation and/or incubation procedure. Protoplast proteins were analysed by polyacrylamide gel electrophoresis and fluorography after the labelling period, as described under Materials and Methods.

When protoplasts were homogenised directly in disruption buffer and electrophoresed on 15 % acrylamide SDS slab gels, fluorography revealed the presence of a polypeptide of approximately 30,000 M_r in infected samples, which was not present in healthy samples (Fig. 25).

The 30,000 M_r product was taken to represent the viral coat protein, which was observed as a 30,000 M_r polypeptide on both polyacrylamide slab and cylindrical gels (Fig. 26). However, fluorogram definition was poor and many possible bands from the protoplast preparation were obscured by diffuse darkening of the film.

Resolution was improved if the protoplast preparations were partitioned against chloroform (similar to the sucrose gradient analysis) before loading onto gels, and an extraction procedure similar to the polymerase preparation was adopted in order to maximise the detection capacity for replicase components. In general the high lipid content of the protoplasts disturbed the polypeptide pattern and resulted in a diffuse darkening of the fluorograms. Efficient resolution was aided by PGB extraction and by increased SDS in the disruption buffer, however polypeptide resolution in the 10,000xg pellet was not improved by SDS or Triton X-100 and the pellet fraction was not investigated further. The 10,000xg supernatant was analysed throughout the time course.

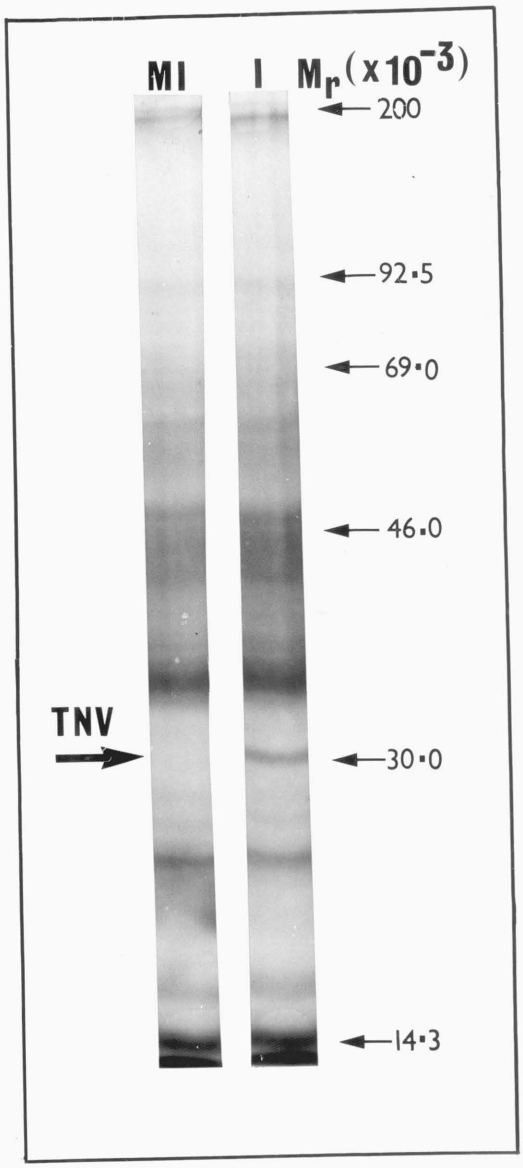


Fig. 25. A fluorogram of (^{14}C)leucine-labelled protein synthesis during 48 hours incubation of "mock-inoculated" (MI) and infected (I) protoplasts. After incubation protoplasts were disrupted and electrophoresed on 15 % polyacrylamide SDS slab gels, The molecular weights of co-electrophoresed ^{14}C-labelled marker proteins are indicated in the right-hand margin. The expected position of authentic TNV coat protein is indicated in the left-hand margin.

The protoplasts were not fractionated extensively before electrophoresis.

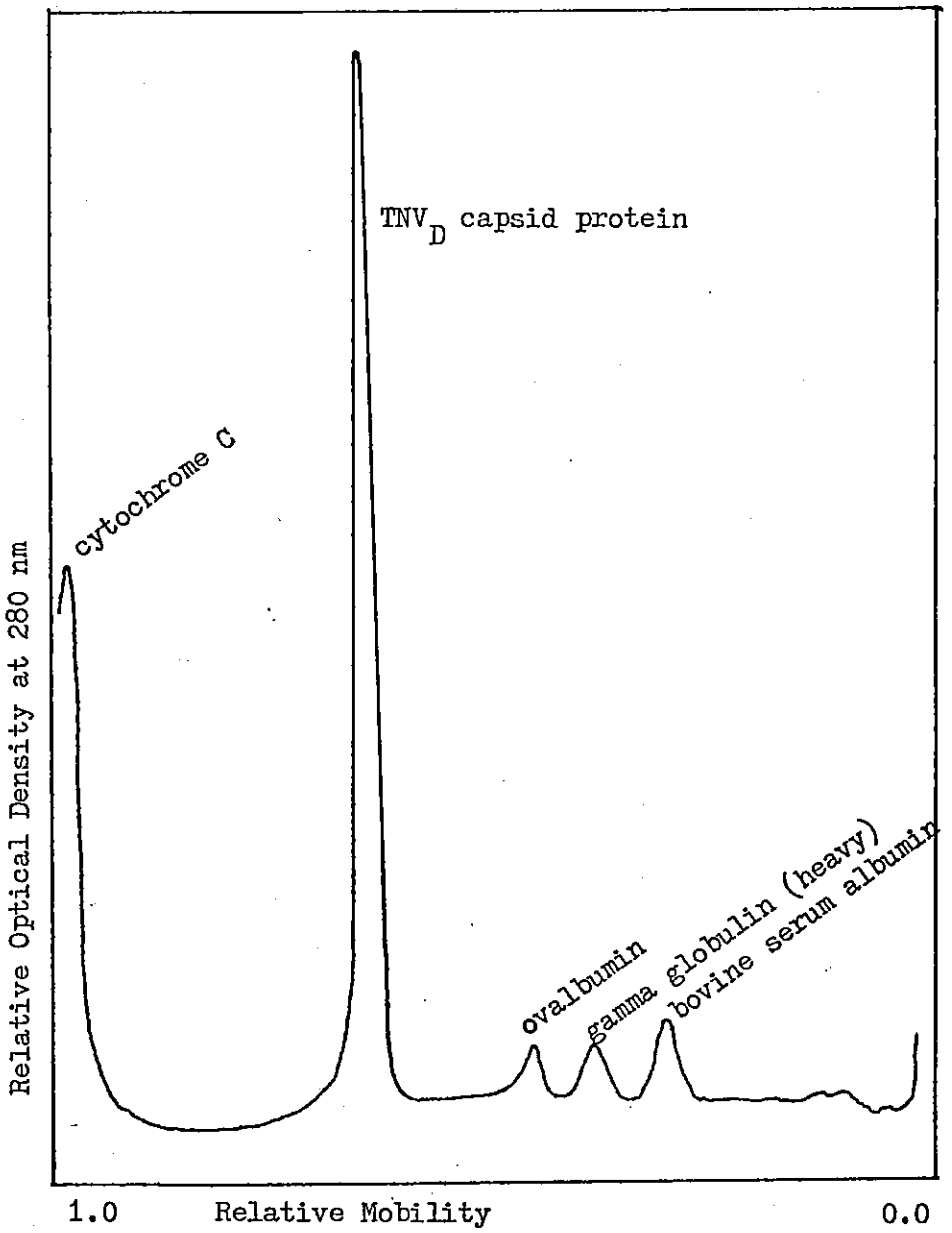


Fig. 26 Cylindrical-gel analysis of TNV coat protein. Denatured TNV was resolved on 10 % acrylamide with 0.1 % SDS, and co-electrophoresed with marker proteins. After electrophoresis as described under Materials and Methods, gels were stained with bromophenol blue and scanned at 280 nm using a Gilford spectrophotometer.

Relative mobilities were used to estimate the coat protein molecular weight.

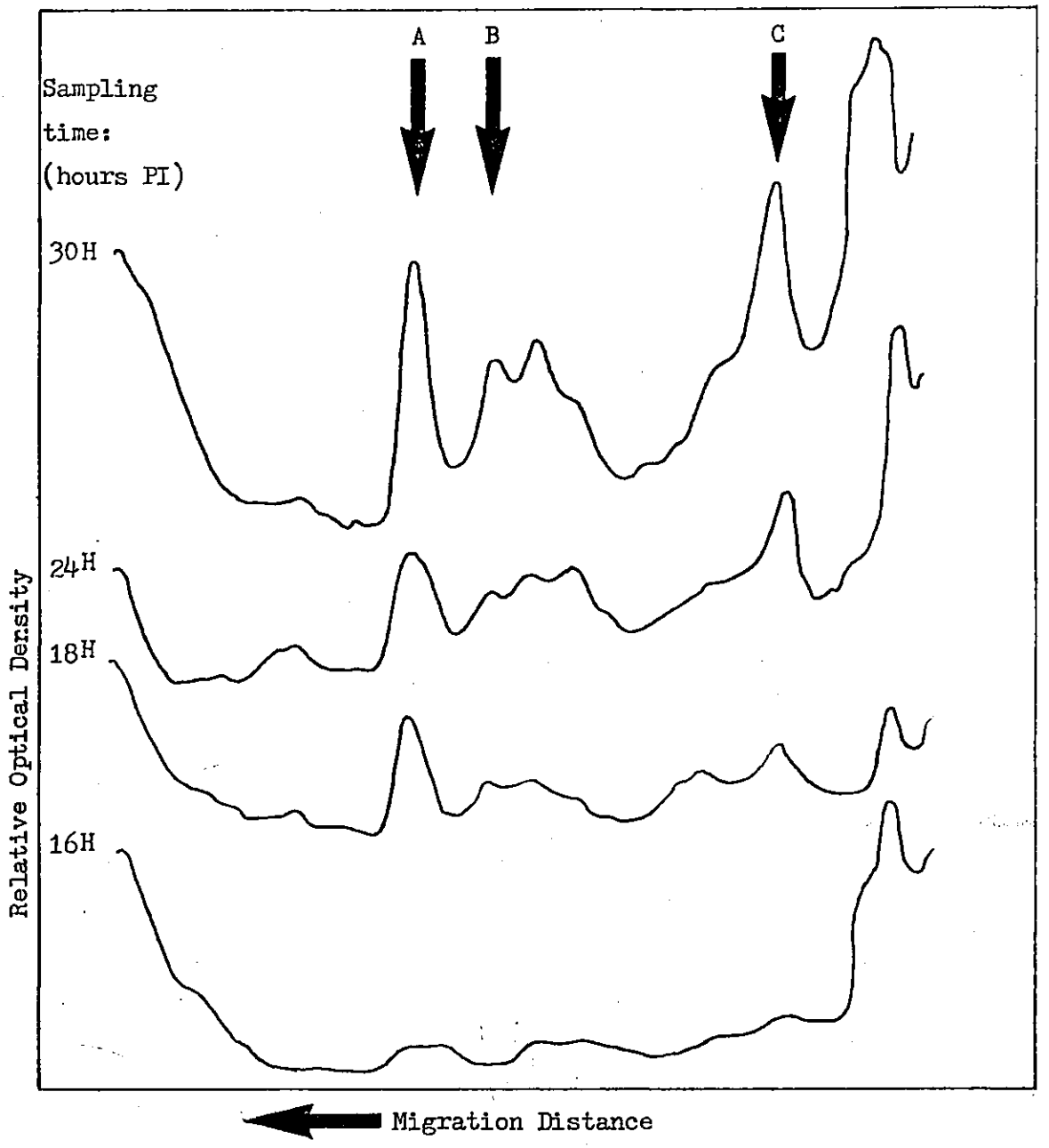


Fig.27 15 % polyacrylamide-SDS gel loaded with 2 hour (^{3}H)-amino acid-pulsed protein extracts. Infected protoplasts were pulsed for 2 hour periods at various times after inoculation. The protoplast pellets were fractionated (see text) and loaded onto 15 % polyacrylamide-SDS slab gels. After electrophoresis, the gels were fluorographed and the lanes were scanned using a densitometer. Protoplast samples were taken at 16, 18, 24 and 30 hours after inoculation.

Arrows represent polypeptides of A, 23,000 M_r; B, 30,000 M_r; and C, 100,000 M_r.

Although the extraction procedure reduced the labelling intensity of the 30,000 M_r polypeptide, electrophoresis of fractionated protoplast pellets revealed a number of discrete bands which were not identified in whole protoplast preparations. The scarcity of these bands was probably due to the low specific activity in the radio-labelled amino acid mixture and inefficiencies in the fluorographic techniques, nevertheless three polypeptides were revealed in infected protoplasts which were not detected in healthy or mock-inoculated protoplasts.

Fig. 27 shows densitometer scans of protein fractions synthesised in 2 hour labelling periods during the course of rapid viral replication. Fig. 28 illustrates the profile of synthesis at 40 hours after inoculation in both infected and mock-inoculated protoplasts. The proteins detected only in infected protoplasts are indicated and their molecular weights shown as 23,000 M_r; 30,000 M_r; and 100,000 M_r. These molecular weights were estimated from relative mobility plots and co-electrophoresis of marker proteins as described under Materials and Methods.

Synthesis of all three proteins increased during the period of virus replication, whereas at 40 hours PI over a longer labelling period coat protein synthesis was much reduced together with the 23,000 M_r protein. The 100,000 M_r product was a major component of protein synthesis at 40 hours PI although at this stage the overall level of protein synthesis was much reduced.

5.6 **Analysis of RNA Synthesis** RNA synthesis was investigated by a similar pulse labelling technique using (^{32}P)-orthophosphoric acid as described under Materials and Methods. Protoplasts were pulsed for 2 hours at various times after incubation with (^{32}P)-orthophosphoric acid and 10 µg/ml actinomycin D. After harvesting protoplast pellets were phenol extracted and the aqueous phases were electrophoresed on agarose/acrylamide composite gels for autoradiography.

Autoradiography of the protoplast pellet extracts revealed only ribosomal RNA species synthesised in either healthy or infected protoplasts. The incubation conditions were modified for (^{32}P) containment purposes, and the pH of the incubation medium was markedly reduced by the addition of radiolabelled orthophosphoric acid. Only a single actinomycin D concentration was used, and the technique was not investigated further in view of the experimental difficulties and limitations.

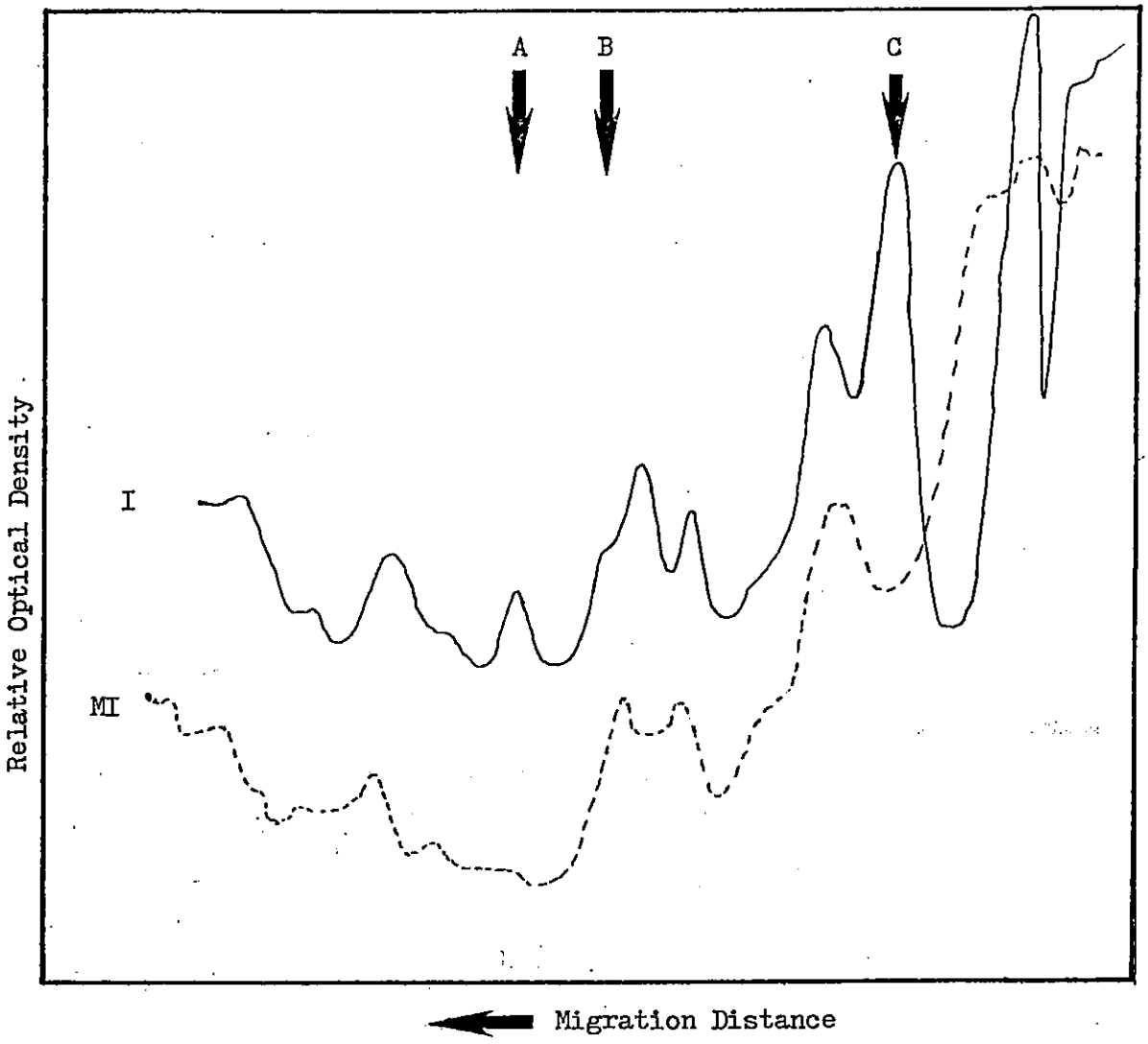


Fig. 28 15 % polyacrylamide-SDS gel loaded with 24 hour (^{3}H)-amino acid-pulsed protein extracts. Infected (I) and mock inoculated (MI) protoplasts were pulsed for 24 hours at 40 hours after inoculation. The protoplast pellets were fractionated (see text) and loaded onto 15 % polyacrylamide-SDS slab gels. After electrophoresis, the gels were fluorographed and the lanes were scanned using a densitometer.

Arrows represent polypeptides of A, 23,000 M_r; B, 30,000 M_r; and C, 100,000 M_r.

6 DISCUSSION

Cowpea primary leaf protoplasts were selected to investigate the replication of tobacco necrosis virus because they avoid the biochemical limitations of necrosis and asynchronous infection encountered in whole plant studies. In addition, cowpea seedlings can be cultivated more rapidly and reproducibly than tobacco plants to give material suitable for biochemical analysis.

The investigation of protoplast isolation and incubation was based on many reports (Hibi *et al.*, 1975; Beier & Bruening, 1976; Alblas & Bol, 1977; Koike *et al.*, 1977) that cowpea seedlings can be used as a convenient and efficient source of leaf mesophyll protoplasts to study virus replication. Consistent with these accounts, protoplasts suitable for virus infection were found to be dependent on strictly defined plant growth conditions and a standard isolation method.

Using a rapid isolation technique to minimise enzyme exposure, an optimum procedure was developed to isolate protoplasts from plant material 8 to 12 days old. Protoplast viability was improved by adding bovine serum albumin as described by Beier & Bruening (1976), and using this procedure over 10^7 protoplasts were routinely obtained per gram of leaf material from plants cultivated under the defined regime. This represents an efficient yield of experimental material, comparable to those described by Hibi *et al.* (1975) using a two-step enzyme treatment or Beier & Bruening (1976) using an abrasive treatment in isolation prior to enzyme incubation. In order to avoid abrasive contamination of the cultures, the technique of epidermis detachment using fine forceps was retained throughout, with a single-step enzyme treatment.

Many physiological studies have been carried out on tobacco (Rubin & Zaitlin, 1976) cucumber (Coutts *et al.*, 1975) and other (Ruesink, 1978) protoplast systems, but few comparative data are available for cowpea protoplasts. In view of the requirement for biochemical analysis after protoplast infection, preliminary investigations were carried out using different labelling techniques and a variety of culture media and conditions. These results indicate the similarity between cowpea and other protoplast systems in culture metabolism and responses.

The osmotically stabilised $CaCl_2$ medium described by Alblas & Bol (1977) for cowpea protoplast incubation resulted in protoplast aggreg-

ation when used at 10 mM $CaCl_2$. This caused difficulties in determining protoplast yield and viability. When used at 0.1 mM, osmotically stabilised $CaCl_2$ served as an efficient washing solution after inoculation. However, uptake and incorporation of protein and nucleic acid precursors immediately after isolation were more rapid in Takebe's simple salts medium than in modified $CaCl_2$ solution. Incorporation by protoplasts incubated in Takebe's simple salts medium during 22 hours of culture was also found to be greater than with $CaCl_2$ or White's medium over a similar period. Consequently, Takebe's simple salts medium was considered more suitable for labelling studies.

In a similar result to that described by Coutts et al. (1975) for cucumber protoplasts, the incorporation of (^{3}H) uridine into cowpea protoplasts was observed to reach a plateau at 15 to 20 hours after the addition of labelled uridine to the culture medium. The plateau may be a reflection of a diluted intracellular (^{3}H) uridine pool due to the metabolic turnover rate, and comparative evidence from pulse labelling and (^{14}C) leucine uptake would support this. Measurements of total uptake by isolated protoplasts indicated the presence of large intracellular pools of both (^{3}H) uridine and (^{14}C) leucine, not incorporated into TCA acid-insoluble products.

Pulse labelling showed that incorporated (^{3}H) uridine was lost from TCA acid-insoluble products when a cold uridine "chase" was applied to labelled protoplasts. However, (^{14}C) leucine-labelled protoplasts continued to incorporate radiolabel, albeit at a reduced rate, after a similar cold leucine "chase". The continued incorporation was presumably from the large intracellular pool of labelled leucine. Since uridine metabolism is more diverse and turnover is rapid, the intracellular pool was more quickly diluted with cold uridine and labelled metabolites were lost from the cell during the "chase" period. After 15 to 20 hours incubation of protoplasts with (^{14}C) leucine, incorporation was reduced but did not plateau or achieve a net loss of label as observed for protoplasts incubated with (^{3}H) uridine.

The different reductions in incorporation are unlikely to reflect changing rates of precursor uptake, since label added after 18 hours of protoplast culture was incorporated at a similar initial rate to label added immediately after protoplast isolation, using both (^{3}H) uridine and (^{14}C) leucine. On this basis, the analysis of virus replication would

be best carried out using discrete labelling periods at various times throughout the incubation period .

The effects of metabolic turnover and precursor dilution are probably substantial components of the variation in observed incorporation rate with changes in protoplast incubation density, a phenomenon also described for tobacco protoplasts (Rubin & Zaitlin, 1976). Cowpea protoplasts incubated at low densities incorporated more radiolabel per protoplast than those incubated under similar conditions at higher densities.

Protoplasts incubated at high densities would release more metabolites into the medium both as a result of biochemical turnover, and from associated protoplast fragments. These metabolites would dilute the concentration of added precursor and thus reduce the observed rate of radiolabelled precursor incorporation. However, for experimental purposes the low protoplast density required for optimum incorporation is balanced by the loss of protoplast viability at low incubation densities, and the number of protoplasts required to give adequate yields for both biochemical and infectivity analysis. Accordingly, for routine experiments protoplasts were incubated at 1.5 to 2.5 x 10^5 per ml, within the range of incubation densities reported for cowpea protoplasts by Hibi et al. (1975) and Beier & Bruening (1975), and with high specific incorporation.

Actinomycin D was investigated in view of its application to viral RNA replication studies. Accordingly, the dosage level of 10 ug/ml was similar to that employed to suppress DNA-dependent RNA synthesis and represents a relatively low concentration (Francki et al., 1971). When actinomycin D was added to protoplast cultures after 5 hours of incubation, only slight inhibition of (^{3}H) uridine and (^{14}C) leucine incorporation was observed after a further 12 hours of culture.

Since the site of uridine incorporation was not investigated it is difficult to account for such a high level of incorporation, although a variety of alternate pathways exist, in addition to RNA synthesis. The level of leucine incorporation may reflect a long messenger half-life, and suggests that in viral replication studies, protein synthesis might be effectively studied during periods of inhibited RNA synthesis.

During the period of actinomycin D treatment, incorporation was similar in both light and dark-incubated protoplasts. However, prolonged incubation in darkness reduced protoplast viability severely. These

results are probably more consistent with reports by Francki et al. (1971) using separated leaf cells, than with protoplast studies carried out by Sakai & Takebe (1970) although both were isolated from tobacco leaves.

Once protoplast isolation and incubation techniques were established together with different labelling procedures, protoplasts were inoculated with TNV. The D strain was selected for initial studies because it does not support the replication of a satellite strain. Optimised infection conditions were different from those reported by Wieringa-Brants et al. (1978) using TNV_A, however TNV_A supports the replication of STNV with a higher isoelectric point than TNV. Thus the optimum pH value and polycation levels may reflect the presence of an extra component in the inoculum as well as possible differences between virus strains.

Working from the conditions described by Wieringa-Brants et al.(1978) the interdependence of virus and polycation levels was investigated, and virus concentration in the inoculum was reduced to give an experimental optimum of almost 1:1. At this ratio, the level of virus used in the inoculum was less than $\frac{1}{3}$ of that used in the initial infection experiments, and residual infectivity was reduced to almost zero immediately after inoculation. Low residual virus levels would reduce the chances of subsequent, asynchronous protoplast infection, and the steep increase in infectious virus recovered after 18 hours of incubation suggested a high degree of synchrony in virus replication.

Ion concentration and pH variations in the inoculum were investigated using phosphate buffer because initial observations suggested that protoplast viability was reduced by citrate buffer. Reduced protoplast viability was also observed at low pH values and at high phosphate buffer concentrations. Thus the optimisation results for these conditions appear to represent an equilibrium, similar to that achieved with PLO, between protoplast damage leading to reduced viability and virus infection leading to replication.

These experiments provide little evidence regarding the mechanism of virus infection, although observations of protoplast aggregation during the most efficient infection procedures probably reflect the electrical charge phenomena involved. One of the results of inoculating protoplasts at $0^{o}C$ was to increase this aggregation response to a given level of polycation. Thus protoplast viability was increased by using less toxic con-

centrations of PLO in experiments carried out at low temperatures. In addition, alterations in membrane fluidity could be important in virus attachment, and virus stability might also be increased at 0°C as suggested for AMV (Alblas & Bol, 1977).

Using a 15 minute infection period, pre-incubation of virus and PLO assisted protoplast inoculation although infectivity was reduced after 30 minutes pre-incubation. By contrast, after an initial infectivity peak using a 10 minute inoculation, a steady increase in infectivity was observed as the inoculation period was increased from 15 to 30 minutes.

In view of the models for Ca^{2+}-assisted virus dissociation (Durham et al., 1977), a $CaCl_2$ washing solution was employed after each inoculation procedure. Thus, the initial infection peak may represent rapid virus attachment and subsequent disassembly aided by Ca^{2+}; whereas with increasing inoculation periods, membrane damage is more likely to accompany protoplast infection, with the possibility of non-specific virus attachment and a loss of infection synchrony.

Investigations using electron microscopy or sensitive serological techniques such as ELISA which can detect infecting virus levels might indicate a differential attachment mechanism between the long and short inoculation procedures. However, since efficient virus replication was achieved using the 10 minute inoculation, this method was adopted for routine use irrespective of the mechanism.

The infection efficiency would also be more accurately assessed by using fluorescent antibody staining (Sarkar, 1977). Unfortunately, this technique did not give satisfactory results despite experiments with a variety of antisera, conjugated antibodies and fixation techniques. Accordingly the optimum inoculation procedure was assessed only on the basis of infectious virus recovery and local lesion assay. Nevertheless reproducibly infected protoplasts were routinely obtained and analysed by more rigorous techniques, in order to study virus replication.

In view of the inconsistencies such as differential responses to osmotic changes (Huber et al., 1981) between many host/virus combinations, these results provide little information on the mechanism of virus entry. However, a more efficient protoplast infection technique provides a useful system for studying this virus in detail, and could potentially be adapted to study related TNV and SV strains.

Since local lesion assays can only be used as a measure of infectious virus material recovered, an analytical sucrose gradient technique (Gonda & Symons, 1979) provided a useful alternative assay based on physical properties. This technique was also selected because of the differential behaviour of TNV and SV on sucrose gradients. Thus the relative yields of TNV and SV might be assessed in the products of mixed protoplast infections when local lesion assay data would be inadequate.

Using the sedimentation-absorbance profile on sucrose gradients as a measure of virus yield, a time course of synthesis was observed which increased sharply at 18 hours post-inoculation. This resembled the profile of infectivity data taken from nucleoprotein extracts of similar protoplast pellets.

The profile of infectivity data from protoplast RNA extracts showed an increase in infectivity detected at 16 hours post inoculation, earlier than that observed for nucleoprotein extracts. An early synthesis of infectious RNA might be expected prior to capsid assembly. For TMV, the delay in coat protein synthesis (Aoki & Takebe, 1975) leads to the presence of free RNA molecules throughout much of the replication period. However,for a spherical virus such as CMV in which synthesis occurs coordinately (Gonda & Symons, 1979) any initial excess of RNA is rapidly encapsidated. The infectivity due to TNV RNA was rapidly overtaken by infectious nucleoprotein, although this result must reflect the efficiency of recovery as well as the rate of virus assembly.

In order to investigate the mechanism of TNV replication further, RNA-dependent RNA polymerase activity was assayed during the period of rapid virus synthesis. On the basis of the infectivity and absorbance data, replicase activity was assayed at intervals between 15 and 24 hours post inoculation in healthy, infected and mock-inoculated protoplasts. Although a series of protoplast fractions were analysed for polymerase activity, significant incorporation of (^{3}H) UTP into TCA acid insoluble products was only detected for a membrane fraction which sedimented at 20,000xg.

Experiments carried out by Okuno & Furusawa (1979) showed that RNA replicase activity is stimulated in barley protoplasts from 7 to 8 hours after BMV infection. At 30 hours after infection, the level of activity is increased 30-fold and decreases thereafter although virus synthesis continues.

Using a similar experimental protocol, polymerase activity was detected in cowpea protoplast extracts 18 hours after TNV infection but not at 16 or 20 hours. The activity represented a 2 fold increase over that found in mock-inoculated protoplasts, and a 3 fold increase over the level in healthy protoplast extracts. It seems unlikely that this period of synthesis would account for the observed levels of virus replication.

Both the specific activity of the polymerase and the observed duration of its activity probably reflect the limitations of the assay procedure, in which case the activity detected at 18 hours PI may represent the peak of synthesis. In order to investigate this further, a systematic study of the assay conditions with particular reference to Mg^{2+}, pH and KCl concentration might lead to greater sensitivity. Once these conditions were optimised, the polymerase activity of different protoplast fractions and the time course of synthesis might be considered in more detail. Replicase preparations from different sources (Duda, 1979; Zabel et al., 1979; Ikegami & Fraenkel-Conrat, 1979) reveal considerable variation in their optimum assay requirements, and a matrix of concentrations for each component would be required to optimise the system.

During the period of rapid virus replication, protein synthesis was investigated using 2 hour labelling pulses with (^{3}H) amino acids. Protein products were analysed by polyacrylamide gel electrophoresis and fluorography to compare synthesis at different times during virus replication. Synthesis of 3 major proteins was observed to increase in infected protoplast fractions during the period from 16 to 30 hours post inoculation, whereas corresponding polypeptides were not observed in healthy fractions. One of these products was a 30,000 M_r polypeptide band, which resembled the viral capsid protein in electrophoretic migration. Synthesis of this product increased from 16 to 30 hours post inoculation but was much reduced at 40 hours PI.

Two other products of 23,000 M_r and 100,000 M_r were also observed to increase during the period of rapid virus replication, and synthesis of the 100,000 M_r product was observed as a major component at 40 hours PI, although overall synthesis was much reduced at this time. Polypeptides corresponding to both 23,500 and 92,500 M_r have been isolated from RNA polymerase preparations stimulated by CPMV infection of cowpea leaves (Huber, 1979), and purified by the methods of Zabel et al. (1974,1976, 1978, 1979). These polypeptides have also been detected in cowpea leaves

infected with C-TMV or mock inoculated (Huber, 1979).

Since only the 30,000 M_r product corresponds to the various estimates of TNV in vitro-coded polypeptides and the potential coding capacity of the viral genome, the additional polypeptides may represent host-coded products. As such they may correspond to the replicase components described by Huber (1979) and detected in this case as a result of stimulation by TNV infection. The failure to detect synthesis of these products in mock inoculated protoplasts would reflect the degree of stimulation by virus infection, and the threshold of synthesis required for detection by this technique.

In order to investigate the presence of other virus-specific polypeptides in infected protoplasts, and to improve the resolution of host-coded products in mock inoculated material, more sensitive techniques could be employed. In particular, further fractionation of the protoplasts might reveal products at present obscured by polypeptides of a similar size or character. While pulse labelling with (^{35}S) methionine or (^{3}H) amino acids of higher specific activity and the use of polyacrylamide gradient gels might improve resolution at the level of fluorography. An alternative approach might be to inhibit host protein synthesis using UV irradiation or antibiotics, but this would be less favoured because of the possible effects on the pattern of virus synthesis. However, with some refinements to the analytical techniques TNV might be studied in cowpea protoplasts in a similar manner to CMV (Gonda & Symons, 1980), CPMV (Goldbach et al., 1980) and CPsMV (Beier et al., 1981).

The difficulties associated with labelling protoplasts directly using (^{32}P)-orthophosphoric acid were both technical and physiological. The protoplast incubation conditions were modified with regard to light and temperature control because of the (^{32}P) containment requirements. In addition, (^{32}P)-orthophosphoric acid caused a major shift in the pH of the protoplast incubation medium. This method of RNA analysis involved extensive periods of incubation and handling of (^{32}P) before the RNA was extracted and the concentration of (^{32}P) reduced.

In view of these difficulties and the use of complementary (c) DNA transcripts to sequence STNV RNA (Ysebaert et al., 1980; Van Emmelo et al., 1980), cDNA probes might provide a more efficient means of detecting RNA species synthesised as a result of TNV infection. Genomic RNA species (Gonda & Symons, 1979) for CMV and subgenomic messengers (Goelet &

Karn, 1982) for TMV have been investigated using cDNA or RNA probes. "Randomly" primed cDNA TNV probes could be similarly used to identify possible subgenomic TNV RNAs, for subsequent translation *in vitro*.

Of more specific interest in this system are the methods by which the satellite/helper relationship might be investigated. A mechanism of interaction has been postulated by Ysebaert *et al.* (1980) which requires that a TNV coded product should bind to STNV RNA. The TNV protein would bind to the base paired region of STNV RNA thus freeing the initiation region for ribosome binding. This mechanism could be studied using a protoplast system capable of supporting TNV and STNV replication.

Translation-initiation complexes with 80s and 40s ribosome subunits have been investigated using $STNV_A$ (^{125}I) RNA, labelled by Commerford's (1971) procedure (Browning *et al.*, 1980). The RNA regions protected by ribosome binding from specific ribonuclease degradation were characterised by comparison with the known 5'-terminal nucleotide sequence of STNV RNA (Leung *et al.*, 1979). This suggests that a protection analysis of $STNV_A$ (^{125}I) RNA in protoplasts superinfected with TNV_A might reveal regions of STNV RNA protected from ribonucleases by association with a TNV_A-coded product *in vivo*. Such regions could then be compared with the known sequence of STNV RNA, particularly over the long-range base-paired region. Ysebaert *et al* (1980) consider that the protein involved in this interaction may well be a virus-coded replicase component.

Thus the demonstrations of reproducible and synchronous infection and replication, polymerase activity and protein synthesis using TNV_D provide a convenient starting point to analyse the satellite/helper interaction in cowpea protoplasts. Based on these results, an optimum inoculation and superinfection procedure would have to be developed for both satellite and helper strains. Protoplasts could then be inoculated with labelled $STNV_A$(^{125}I)RNA without helper virus. Superinfection with TNV_A, and treatment with actinomycin D would then initiate the synthesis of virus-coded proteins (Goldbach, 1980) while replication of the respective RNAs would be inhibited. Thus an undiluted population of $STNV_A$ (^{125}I)RNA molecules would be present *in vivo* in association with the products of virus-coded protein synthesis. Protoplasts could then be disrupted and ribonuclease treated. Phenol extraction and fractionation of the products could then be carried out as for ribosome protected fragments, finally resolved by two-dimensional electrophoresis and sequence analysis.

In this approach, the potential contribution of protoplast studies to a biochemical problem is particularly evident. The viral RNA would be present in its in vivo configuration, and any association with viral-coded proteins due to membrane binding would be retained. The TNV_A translation products would also be processed if required within the system, and the protection study could be carried out without further characterisation of the proteins involved.

Although relatively little can be deduced from the data presented, this study represents a system with considerable potential for investigating the satellite relationship with TNV, and its implications for plant virus replication in general.

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APPENDIX 1

Heller's salts (Heller, 1953) were prepared by mixing 3 stock solutions stored at room temperature and formulated as follows (g/l):

Solution 1; KCl, 7.5; $CaCl_2$, 0.75; $NaNO_3$, 6.0; $MgSO_4.7H_2O$, 2.5; $NaH_2PO_4.2H_2O$, 1.25.

Solution 2; $FeCl_3.6H_2O$, 1.0.

Solution 3; $MnSO_4.4H_2O$, 0.1; $ZnSO_4.7H_2O$, 1.0; H_3BO_3, 1.0; KI, 0.01; $CuSO_4.5H_2O$, 0.03; $NiCl_2.6H_2O$, 0.03.

To make 1 l of Heller's salts solution 100 ml of solution 1, 1.0 ml of solution 2 and 1.0 ml of solution 3 were mixed and made up to 1 litre with distilled water.

APPENDIX 2

SDS-polyacrylamide slab gels for subsequent fluorography were formulated as follows using solutions stored at 4°C. Ammonium persulphate solution was prepared on the day of use and 20 % SDS was brought to room temperature before addition to the mixture.

For 30 ml 15 % acrylamide running gel;

30 % (w/v) acrylamide solution .....15 ml
1.0 % (w/v) methylaminebisacrylamide solution.....2.6 ml
1 M tris/HCl pH 8.7 at 25°C .....11.2 ml
20 % (w/v) SDS solution .....150 µl
distilled water .....1.0 ml
NNN'N'-tetramethylethylenediamine (TEMED) .....10 µl
10 % (w/v) ammonium persulphate .....100 µl

For 8.725 ml 5 % acrylamide stacking gel;

30 % (w/v) acrylamide solution .....1.67 ml
1.0 % (w/v) methylaminebisacrylamide solution ....1.3 ml
1 M tris/HCl pH 6.8 at 25°C .....1.25 ml
20 % (w/v) SDS solution .....50 µl
distilled water .....4.4 ml
TEMED .....5 µl
10 % (w/v) ammonium persulphate solution .....50 µl

Electrode buffer was stored as 5 x concentrate and formulated as follows:

glycine, 144 g; tris base, 30 g; 20 % (w/v) SDS solution, 25 ml made up to 1 litre with distilled water. The solution was diluted 5 fold for use, and stored at room temperature.

Samples were denatured in loading buffer formulated as follows:

1 M tris/HCl pH 6.8 at 25°C .....4.0 ml
20 % (w/v) SDS solution .....5.0 ml
0.2 % (w/v) bromophenol blue in ethanol ....300 µl
2-mercaptoethanol .....500 µl
sucrose .....17 g
distilled water to make final volume up to..50 ml

The buffer was stored in aliquots at -20°C.

APPENDIX 3

(^{14}C) methylated protein mixture as supplied by The Radiochemical Centre Amersham contained the following molecular weight markers:

Marker protein	Molecular weight
Methylated myosin	200,000
Methylated phosphorylase-b	100,000 & 92,500
Methylated bovine serum albumin	69,000
Methylated ovalbumin	46,000
Methylated carbonic anhydrase	30,000
Methylated lysozyme	14,300

Radioactive concentration: 0.833 µCi each protein/ml (total 5 µCi/ml)

APPENDIX 4

Fixed polyacrylamide gels were prepared for fluorography by washing for 30 minutes in each of 2 changes of dimethyl sulfoxide (DMSO Fisons Chemicals). Gels were washed in developing dishes covered with aluminium foil shaking gently over a water bath at 30°C. DMSO from the first wash was discarded after use; DMSO from the second wash was used for the first wash in a subsequent experiment. In each case, enough DMSO was used to cover the gel.

After washing the gels were immersed in 2,5-diphenyl oxazole (PPO, Sigma Chemicals) dissolved in DMSO (360 ml of DMSO added to 100 g of PPO was used for up to 5 gels). The gel was shaken in a water bath, as in

the washing procedure for 3 hours.

After 3 hours in DMSO/PPO the gel was washed in running water for 1 hour and in distilled water for 30 minutes before placing on a sheet of Whatman No.1 filter paper cut to overlap the gel on all sides. The filter paper and gel were covered by a sheet of "Cling-film" and placed paper side down on a "Raven" gel-dryer. The apparatus was made air-tight using plastic bags and "cling-film" and connected to an aspirator. Once a seal was established the apparatus was left under Osram reflector lamps for upto 18 hours or until the gel was dry.

When dry, the gel was taped (gel side up) to a glass plate. A sheet of Kodak X-omat film was cut to the same size as the plate and exposed to a yellow safe light for 20 seconds. The film was placed over the gel and secured by a second glass plate and masking tape. The plates were wrapped in light-proof bags and placed at -70^{o}C for upto 14 days.

After a suitable period of exposure the film was removed and developed automatically using Kodak Royal X-omat developer and fixer.

ACKNOWLEDGEMENTS

I gratefully acknowledge the support of the Agricultural Research Council throughout this project, and I am also grateful to The Royal Society and Imperial College for financial support which allowed me to attend the Fifth International Virology Congress in Strasbourg.

I would like to thank the virology group at Imperial College, particularly Dr. R.H.A. Coutts, Dr. E.E. Wagih, Dr. R. Sanders, Ms. R. Collins, Mr. G.O. Adejare, and Mr. C. Knight. I also thank all the staff and students of the plant pathology and genetics laboratories, for their support and encouragement throughout my time spent at Imperial College.

Thanks are also due to Dr. T.M.A. Wilson at the University of Liverpool, for his consideration during the writing of this thesis.