Advances in

VIRUS RESEARCH

VOLUME 19

Advances in VIRUS RESEARCH

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VOLUME 19

1974

ACADEMIC PRESS New York San Francisco . London
A Subsidiary of Harcourt Brace Jovanovich Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 53-11559

ISBN 0-12-039819-2

PRINTED IN THE UNITED STATES OF AMERICA

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THE USE OF PROTOPLASTS AND SEPARATED CELLS IN PLANT VIRUS RESEARCH[1]

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I. Introduction and Scope of the Review

The prospect of using pipettable suspensions of virus-infected cells or protoplasts has elicited considerable excitement among plant virologists. The disadvantages inherent in using leaves can to some degree be overcome by the use of these newly available techniques. As is well recognized, the leaf is far from an ideal system in which to study virus replication. Much of what we must know to understand virus replication occurs within a few hours after infection. Unfortunately, in an inoculated leaf only about one ten-thousandth of the cells may be infected initially. Thus virus replication must be studied against this overwhelming background of uninfected cells. Only events where highly virus specific processes, such as the generation (or loss) of viral infectivity, or the formation of virus specific structures such as inclusion bodies, have been effectively studied using leaf tissue. Because virus spreads from cell to cell from the initial site of infection, synchrony of infection is also

[1] Abbreviations used in the review: Cowpea chlorotic mottle virus, CCMV; cucumber mosaic virus, CMV; 2,4-dichlorophenoxyacetic acid, 2,4-D; ethylenediaminetetraacetic acid, EDTA; naphthaleneacetic acid, NAA; pea enation mosaic virus, PEMV; potato virus X, PVX; tobacco mosaic virus, TMV; turnip yellow mosaic virus, TYMV.

not possible. Thus, if one wishes to study the temporal sequence of viral related events, complications arise since cells are in various stages of virus synthesis, ranging from the uninfected to cells in which virus replication has terminated. Finally, it is difficult to introduce test substances into leaves, and practically impossible to remove them subsequently as would be required in a pulse-chase experiment.

Plant virologists have devised procedures to attempt to overcome these difficulties—achieving various degrees of success. Tissues such as macerated leaf breis (Bryan *et al.*, 1964), infiltrated leaves (Zaitlin *et al.*, 1967), and bean hypocotyls (McCarthy *et al.*, 1970) facilitate the uptake of materials by virus-infected cells. An approach to synchrony of infection is provided by systems where a series of leaves are heavily inoculated but the virus replication is studied in the first systemically infected leaf (Hirai and Wildman, 1967; Nilsson-Tillgren *et al.*, 1969). Another method of speeding up viral events is that recently shown by Dawson and Schlegel (1973), in which lower leaves on a plant are infected and kept in a chamber at a temperature where the virus may replicate. The apex of the plant protrudes from the chamber and is kept at a cool temperature. In these apical leaves the viral genome is present but does not replicate to any significant degree. When the temperature of the apical leaves is later raised to a permissive level, virus replicates without a lag. Under these circumstances, TMV replication was complete by about 5 days vs 10–12 days for a "normal" system. With CCMV, maximum virus yield was achieved in 1 day, vs a "normal" 4–5 days.

We have observed, as have others, that inoculation with very high concentrations of TMV (0.5–1.0 mg/ml)—far greater than that required to saturate all the infectible sites (Siegel and Zaitlin, 1964)—on both surfaces of the leaf tends to accelerate the whole virus replication process, in contrast to earlier studies where maximum virus yield was not achieved until 21 days (Cohen *et al.*, 1957). This is somewhat surprising as it would suggest that virus particles in addition to that particle which initiates the infection center participate somehow, or aid the viral replication process.

In this review, only the uses of separated cells, protoplasts, and cultured cells of callus origin for studies in plant virology will be considered. This is a rather restricted topic at the moment as the number of published papers are few. However, in correspondence and discussions with a number of plant virologists we have ascertained that many laboratories are adopting such systems and the literature should see a dramatic growth. With a few exceptions, the studies to date have tended to define the conditions for the preparation and infection of cells and protoplasts, and to demonstrate that virus does indeed multiply therein. There has been very

little "problem solving"—attempting to answer some of the challenging questions of how plant viruses replicate and how they affect their hosts. This situation should change once the novelty of being able to infect these cells passes. We have included a "problem solving" section in the review to show what has been done to date.

Conditions for protoplast isolation and maintenance and historical aspects have been reviewed recently by Cocking (1970, 1972) and will not be considered in detail except as they refer to plant virus studies. We will also not consider the early tissue culture work using large plant callus pieces; these studies were reviewed in 1967 by Kassanis. It is generally conceded that very little of a fundamental nature has resulted from these studies as successful infections were few and minimal, and virus spread very poorly in the pieces. However, meristem tip culture techniques have enabled the production of virus-free tissue for vegetative propagation (Hollings, 1965), but these studies are beyond the scope and intent of this review. We will give consideration to the recent tissue culture studies of Murakishi and colleagues where, with modified techniques, small pieces of callus tissue have been infected, thus making them somewhat more useful than leaves for selected viral studies.

The use of insect tissue cultures for plant virus studies utilizing those viruses which replicate both in the plant and their insect vector has been reviewed by Black (1969).

A. Recent Historical Background

For plant virus studies, Zaitlin (1959) first reported virus replication in tobacco leaf cells which had been infected with TMV on the plant and then separated from one another by digestion of the middle lamella by a commercial pectinase. Virus replication was demonstrated by incorporation of labeled amino acids into the virus. The level of incorporation was low and the respiratory activity of these cells was poor when compared to the cells of the leaf (M. Zaitlin, unpublished). Several years work and many experiments were performed to improve the metabolism of these separated cells, without significant success. In 1966, Cocking demonstrated the infection of isolated tomato fruit protoplasts by TMV, but *strong* evidence for subsequent virus replication in this system was not presented until later (Cocking and Pojnar, 1969). The meaningful advance in the field came in 1968 when Takebe and his co-workers were able to solve the fundamental difficulties in the leaf cell preparation method and gave good evidence for substantial TMV replication in separated tobacco leaf mesophyll cells. These cells were infected while in the leaf and after a 24-hour incubation exhibited a 7- to 13-fold increase in virus titer. At about this time other investigators demonstrated the in-

fection of cultured tomato callus with TMV-RNA (Murakishi, 1968) and of tobacco callus with TMV (Motoyoshi and Oshima, 1968). These experiments showed that, with proper handling, small callus pieces could be made susceptible to virus infection resulting in virus titers higher than those shown by earlier workers (Kassanis, 1967).

The interest in the cell and protoplast method was significantly stimulated by the publication in 1969 of two papers from Takebe's laboratory describing their success in *infecting* protoplasts prepared from tobacco mesophyll cells. In the first paper they showed infection with TMV-RNA (Aoki and Takebe, 1969) and in the second with TMV virions (Takebe and Otsuki, 1969). They also were able to demonstrate a significant virus replication in these protoplasts. (These studies are described in Section II.) These two papers opened a new era in plant virology and almost all the work described in this review derive from them significantly.

B. Some Notes on Techniques

The methods and procedures used for the successful isolation of cells and protoplasts are adequately described in the papers cited in the relevant sections of this review and need not be repeated here. Further, one manufacturer of enzymes has prepared a booklet describing the procedure ["Enzymatic Isolation of Living Cells and Their Protoplasts from Higher Plants." Kanematsu-Gosho (U.S.A.) Inc., One World Trade Center, Suite 4811, New York, New York 10048]. However, some aspects of the procedures are worthy of note. Although the successful isolation of protoplasts and cells has been accomplished in a number of laboratories, in our own experience and in our correspondence with others, we feel that there is still some art involved in the routine production of viable cells and protoplasts. As is the case with many procedures, there are technical nuances which each investigator seems to have to uncover for himself before success is achieved. Take the selection of plant material for instance: In their 1968 report, Takebe *et al.* stated only that they used "fully expanded (tobacco) leaves 60–80 days old." Coutts *et al.* (1972b), however, suggested that "the best leaves for protoplast isolation are the ones not quite fully expanded (20 to 25 cm in length), often found in a position 4 to 6 leaves from the stem apex. Fully expanded leaves (25 to 40 cm in length) and young leaves (10 to 20 cm in length) produced unstable protoplasts." The age of the plant is very critical for the successful preparation of barley mesophyll protoplasts. Plants 7–8 days old were considered optimum, and no protoplasts could be isolated from plants more than 20 days old (Schaskolskaya *et al.*, 1973). Jensen *et*

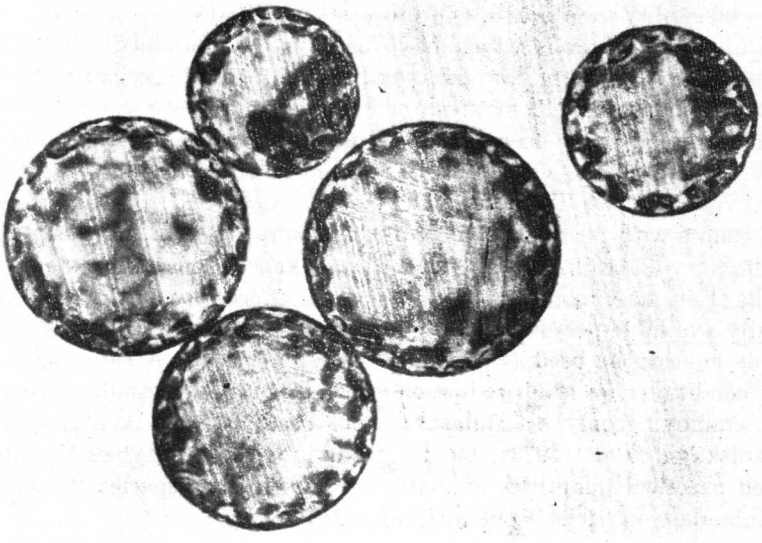

FIG. 1. Freshly isolated mesophyll protoplasts suspended in 0.7 *M* mannitol solution. × 1400. Courtesy of Dr. I. Takebe.

al. (1971) observed CO_2 fixation rates to be most consistent in cells isolated from the most recently fully expanded leaves in relatively young plants. Moreover, Shalla and Petersen (1973) found such variability in the capacity of their tobacco seedlings to yield viable protoplasts that they selected one good seedling and made rooted cuttings from it to use as a source of all the leaf material in their studies. When one does find the appropriate conditions for himself, it does seem possible to get a successful routine procedure. Motoyoshi *et al.* (1973) reported that they ran 27 consecutive successful infection experiments with CCMV and tobacco protoplasts. The success to failure ratio in other laboratories has not been revealed.

The conditions under which the plants are grown could have a bearing on the cells and protoplasts produced. For example, separated tobacco cells normally require light for the uptake of amino acids and uridine from the external medium. They will take up limited amounts of these substances in the dark if the plants are maintained in high light prior to the preparation of the cells, but virtually none if the plants are held in the dark for 48 hours prior to cell separation (Francki *et al.*, 1971). The cells seem to retain a "memory" of their previous growth conditions. Moreover, van Kammen (personal communication) observed that the

conditions of plant growth prior to protoplast preparation markedly affected his ability to infect those protoplasts with TMV.

Basically, the methods which have been used to produce cells (and subsequently protoplasts) for virus studies involve the enzymatic digestion of the middle lamella with an endopolygalacturonase—i.e., a pectinase. The methods have been largely confined to tobacco in virus studies; in fact, to date all the successful leaf protoplast infections have utilized tobacco varieties (see Table I). Cells may be separated from many species of plants with pectinase, but with the commonly used enzymes some are refractory (Zaitlin, 1959; Otsuki and Takebe, 1969a). For example, the cells from most monocots do not separate effectively with some commercially available pectinases, and alternative enzymes or cell separation methods have to be used (C.N.R.S. Symposium, 1973). A careful study giving conditions for the production of viable barley mesophyll protoplasts, employing only a cellulase without pectinase, has been given by Schaskolskaya *et al.* (1973). Gentle grinding without enzymes has also released practical quantities of viable cells from some species (Gnanam and Kulandaivelu, 1969; Rouhani *et al.*, 1973).

The pectinase and cellulase enzymes various workers have employed and the sources of these enzymes have been discussed by Cocking (1970, 1972) and Gamborg *et al.* (1973). It is apparent that the enzymes can influence the quality of the cells or protoplasts produced. For example, we have found a good deal of variation in the viability of protoplasts produced with different lots of what are apparently the same cellulase from one manufacturer. Pectinase also seems to be a variable quantity.

One critical factor essential to ensure the production of viable cells seems to be the absolute requirement for hypertonic conditions during the isolation of the cells and their subsequent handling. This essential requirement is apparently the salient difference between the early marginally successful attempts by Zaitlin (1959) to get metabolically active cells using isotonic media, and those highly rewarding studies of Takebe *et al.* (1968) using hypertonic media in which cells actively supporting virus replication were successfully isolated. To this end, when new species of plants are used as a source of cells it is important to investigate the osmotic requirements for cell production and maintenance as these appear to vary between species (Otsuki and Takebe, 1969a; Jensen *et al.*, 1971; Schaskolyskaya *et al.*, 1973). For instance, while tobacco cells showed optimum levels of photosynthetic CO_2 fixation after isolation in 0.8 *M* sorbitol and incubation at 0.6 *M*, cotton cells showed optimal photosynthesis when isolated in 0.6 *M* and incubated at 0.7 *M* sorbitol.

The addition of low molecular weight potassium dextran sulfate to the [illegible] medium seems to be important for production of viable to-

bacco cells, although it can be partially substituted by other forms of potassium (Jensen *et al.*, 1971). However, cells from a few species of plants were adversely affected when prepared with potassium dextran sulfate, although in most species good cells were observed when it was present (Otsuki and Takebe, 1969a). Exactly what K^+ and/or potassium dextran sulfate do to enhance viable cell production is unclear. Takebe *et al.* (1968) suggested that the effect of potassium dextran sulfate might be to bind basic proteins, present as contaminants in the crude pectinase, which might otherwise be damaging to the cells. One candidate for such a protein is ribonuclease which is markedly reduced in pectinase solutions by the addition of potassium dextran sulfate (Coutts, 1973b). Its effect must go beyond this, however, because of the partial substitution of its beneficial effect by inorganic forms of K^+ (Jensen *et al.*, 1971). High molecular weight potassium dextran sulfate inhibits maceration (Takebe *et al.*, 1968).

In order to get a pure population of mesophyll cells and to enhance the rate of release of the cells, Takebe *et al.* (1968) peeled the lower epidermis from the leaves prior to maceration. This procedure is not obligatory, however, as Jensen *et al.* (1971) used narrow unpeeled leaf strips that yielded cells from the cut edges. The latter procedure facilitates preparing large quantities of cells, as removing the epidermis can become quite tedious and is not practical for some plant species (Otsuki and Takebe, 1969a). With either procedure, however, it is very important to discard the first cells that are released during maceration because these are damaged and are metabolically inactive (Takebe *et al.*, 1968; Jensen *et al.*, 1971). Still another approach to protoplast isolation was used by Shilde-Rentschler (1973), who avoided the stripping of the epidermis by the use of a pectin glycosidase.

For the incubation of cells or protoplasts, to observe virus replication or other metabolic activities, most workers once again use the incubation medium and procedures of Aoki and Takebe (1969), although in recent papers from Takebe's laboratory (Otsuki *et al.*, 1972b; Otsuki and Takebe, 1973) they have modified their recipe somewhat, lowering the mannitol to 0.7 *M* and eliminating the benzyladenine. Their incubation medium also contains a mixture of salts, although it is not clear whether the essentiality of each ingredient has been tested. Jensen *et al.* (1971) found that increased potassium ion concentration (>5 m*M*), above that suggested by Takebe, is required for sustained photosynthetic CO_2 fixation.

During incubation, tobacco cells and protoplasts require light for the effective uptake of protein and nucleic acid precursors (Francki *et al.*, 1971), but virus replication in the cells goes on quite well in the dark

(Takebe *et al.*, 1968). We stress this point because if one were to measure virus replication by monitoring the incorporation of some labeled precursor molecule into virus, the results could be misleading if the uptake of the precursor itself were restricted. Furthermore, the measurement of the uptake of the precursor into the cell itself is dependent on the extent of the subsequent incorporation of that precursor into macromolecules (Jensen *et al.*, 1971). One exception to this observation seems to occur in protoplasts treated with actinomycin D, where the drug preferentially interferes with incorporation of uracil into RNA without affecting uracil uptake into the protoplasts (Sakai and Takebe, 1970). Cells do not behave this way, however; actinomycin D affects *both* uptake and incorporation to the same degree (Francki *et al.*, 1971). We have also observed (unpublished) that tobacco cells have a very restricted capacity to take up amino acids from the external medium and thus when ^{14}C-labeled amino acids are used, adding more label results in no increased uptake; small amounts of the compounds saturate the system because of their relatively low specific radioactivities. Tritiated compounds on the other hand, by virtue of their very much greater specific radioactivities, do not show this phenomenon and are therefore preferred.

Two further potential problems could occur in isotope studies. Maury and Laquerrière (1973) found that the uptake of uridine by tobacco leaf protoplasts was inhibited by the addition of other nucleosides to the incubation medium, and Watts and King (1973) found that nondividing pea protoplasts did not incorporate thymidine-^{3}H, implying no DNA synthesis in the cells.

The light intensity during incubation is also important for successful culture. Tobacco leaf cells are intolerant of prolonged periods of high light intensity. For sustained CO_2 fixation, light intensities below 500 ft-c are required (Jensen *et al.*, 1971). Light also appears to be necessary for the division of protoplasts (Takebe and Nagata, 1973).

II. Protoplasts

Protoplasts prepared from tobacco leaves have been infected with several plant viruses in a number of laboratories (Table I). Protoplasts prepared from immature tomato fruit (Cocking and Pojnar, 1969) and from a yeast, *Saccharomyces cerevisiae* (Coutts *et al.*, 1972a), have also been infected with TMV.

The replication of virus within protoplasts may be demonstrated and quantified by any one of several methods; i.e., by infectivity assays, staining with fluorescent antibodies, electron microscopy, incorporation

TABLE I

INFECTION OF LEAF PROTOPLASTS BY PLANT VIRUSES

Virus	Protoplasts prepared from	Percent of protoplasts infected	Maximum number of virions synthesized per protoplast	Reference
Tobacco mosaic virus	*N. tabacum* cv Bright yellow	3–7[a]	5.5×10^5	Aoki and Takebe, 1969
	N. tabacum cv Bright yellow	14–31; 70	10^6	Takebe and Otsuki, 1969; Takebe *et al.*, 1971b
	N. tabacum cvs Samsun and Xanthi-nc	50–80	5.8×10^6	Coutts *et al.*, 1972b
	N. tabacum cv Xanthi-nc	9–37	10^6	Hibi and Yora, 1972
	N. tabacum cv White Burley	40	9.3×10^6	Motoyoshi *et al.*, 1973
	N. tabacum cvs Samsun, Samsun NN, Xanthi, and Xanthi-nc	71–89	—	Otsuki *et al.*, 1972b
	N. tabacum cv Xanthi	80–90	—	Otsuki *et al.*, 1972a
Potato virus X	*N. tabacum* cv Burley 21	2[b]; 16[c]	—	Shalla and Petersen, 1973
	N. tabacum cv Xanthi	70	—	Otsuki *et al.*, 1974
Cowpea chlorotic mottle virus	*N. tabacum* cv White Burley	Up to 80[d]	10^7	Motoyoshi *et al.*, 1973
Cucumber mosaic virus	*N. tabacum* cv Xanthi	70–90	—	Otsuki and Takebe, 1973
Pea enation mosaic virus	*N. tabacum* cv White Burley	60–80	—	Burgess *et al.*, 1973b

[a] TMV-RNA used as inoculum.

[b] Determined by fluorescent antibody staining.

[c] Determined by thin sectioning and electron microscopy.

[d] Value cited, using virus as inoculum; with CCMV-RNA as inoculum, values up to 30% were obtained.

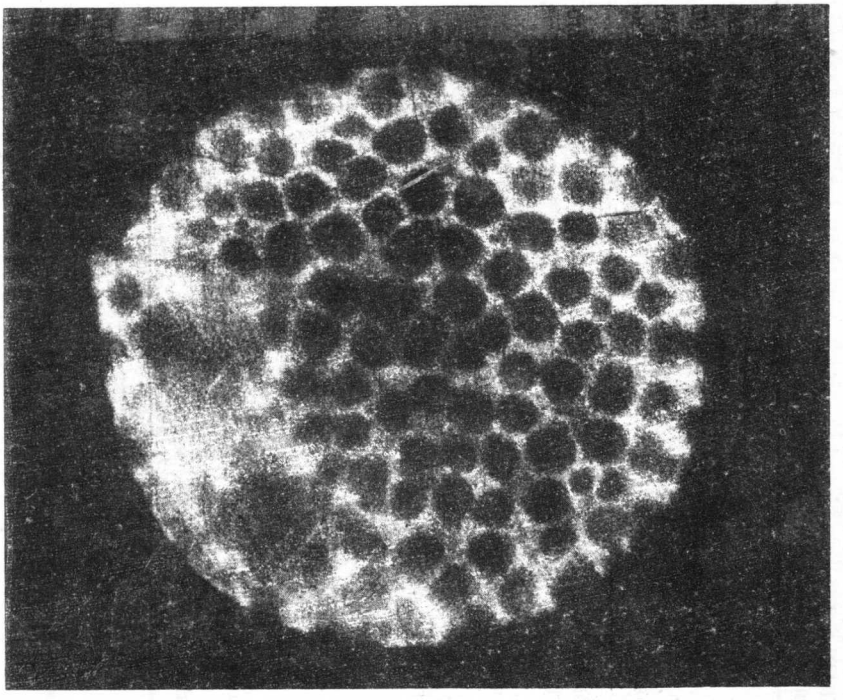

FIG. 2. Micrograph of a fluorescent antibody-stained tobacco protoplast infected with cowpea chlorotic mottle virus (CCMV)-RNA and incubated for 24 hours. Courtesy of Dr. J. B. Bancroft.

of radioactive precursors into virus, and serology. In addition, metabolic inhibitors which have characteristic effects on virus replication in leaves, show similar effects in infected protoplasts, reasserting that virus is indeed replicating there (Takebe and Otsuki, 1969; Otsuki and Takebe, 1973). It is important to note that the quantification methods enumerated above each do not have the same sensitivity and thus the same answer might not derive from each of them—particularly shortly after infection when virus concentration is low. Further, infectivity measurements are of limited sensitivity and virus inhibitors also present in the cells or the medium can give abnormally low infectivity values (Coutts *et al.*, 1972a). Moreover, all the methods do not measure the same parameter of virus replication—particularly serology, which measures protein, but not infectious units.

For assessing the proportion of protoplasts that become infected, fluorescent antibody staining (Nagaraj, 1962; Otsuki and Takebe, 1969b) seems to be the method of choice, probably because of its convenience.

It is, however, not as sensitive as examination of thin sections by electron microscopy (Hibi and Yora, 1972; Shalla and Petersen, 1973).

The time course of virus replication in protoplasts infected with TMV is shown in Fig. 3. These are data taken from several laboratories. One characteristic feature of this infection is that there is virus associated with the protoplasts at 0 time; a reduction in infectivity is frequently observed within 6–8 hours after infection, corresponding to the lag or eclipse phase of virus synthesis (Matthews, 1970). Following this reduction there is an exponential rise in infectivity which either terminates or slows considerably at about 24 hours post inoculation. In the 1969 paper of Takebe and Otsuki, where infection of protoplasts with TMV was first demonstrated, the virus growth curve showed a dramatic falloff after 24 hours (curve 1, Fig. 3), but in later studies from their laboratory, no such decline was observed (curve 2) (Takebe *et al.*, 1971b; Otsuki *et al.*, 1972b). Otsuki *et al.* attribute the loss of protoplasts in their early studies to shaking during incubation.

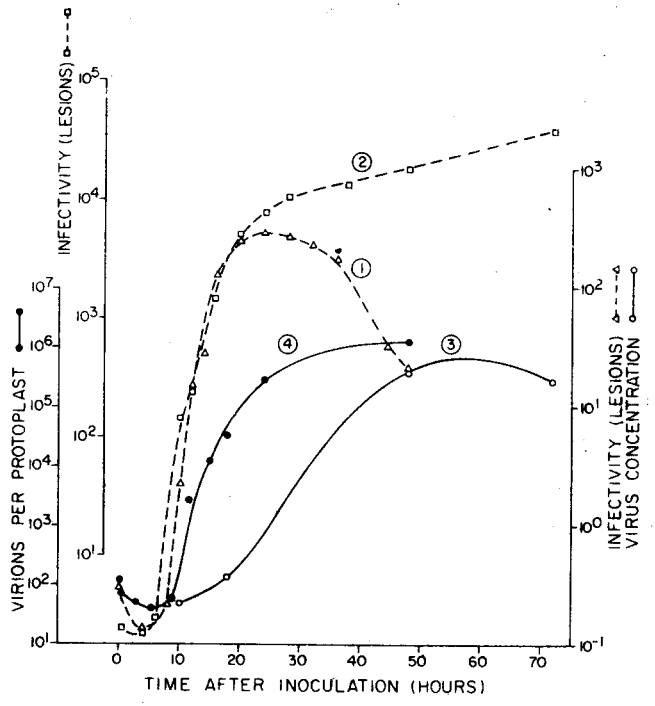

Fig. 3. Time course of the replication of tobacco mosaic virus (TMV) in tobacco protoplasts as determined in several laboratories. Data from: (1) Takebe and Otsuki, 1969; (2) Takebe *et al.*, 1971b; (3) Coutts *et al.*, 1972b; (4) Hibi and Yora, 1972.

Work from other laboratories also indicates the same general replication kinetics for TMV. In particular, the data (curve 4, Fig. 3) taken from Hibi and Yora (1972), where virus particle numbers were determined by electron microscopy of thin sections, indicate an approximate 8-hour lag period, in which virus was actually lost from the protoplasts. These workers maintain that, shortly after infection, virus is found only in "phagocytic vesicles" near the surface of the protoplast and that the loss of virus from the protoplast is entirely accounted for in the disappearance of the virus from these vesicles. They suggest that perhaps the uncoating of the TMV particles may occur in these structures, but other interpretations to explain these observations are also conceivable. Otsuki *et al.* (1972a) have also observed the loss of TMV from these vesicles.

The slower rise in virus concentration seen in curve 3 (Fig. 3), as opposed to the data from other laboratories, is postulated to reflect either a different strain of the virus, different incubation conditions, or both (Coutts, 1973a).

The kinetics of replication of CCMV in tobacco protoplasts is shown in Fig. 4. These data, taken from the work of Motoyoshi *et al.* (1973), show similar kinetics to TMV replication, but no lag was observed

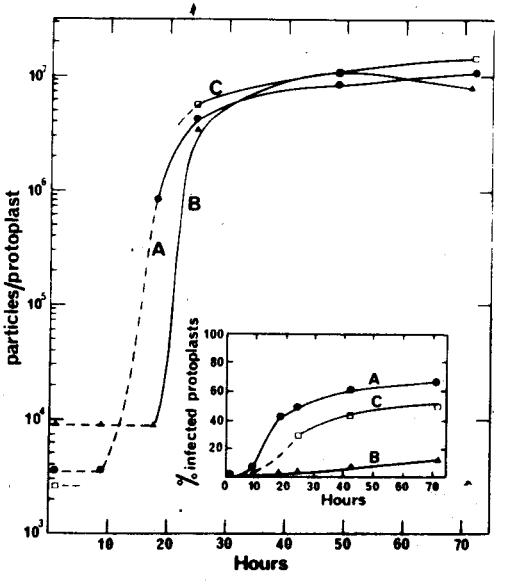

FIG. 4. Time course of the replication of cowpea chlorotic mottle virus (CCMV) in tobacco protoplasts. Data of Motoyoshi *et al.* (1973). Data from three experiments (A–C). Inset shows the percentage of protoplasts infected in each experiment.