# Infection of Isolated Plant Protoplasts with Potato Virus X

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ABSTRACT

Tobacco leaf protoplasts were inoculated in vitro with potato virus X (PVX) and monitored for newly formed infection products by fluorescent antibody staining, electron microscopy, and infectivity measurements. Viral antigen, PVX particles, and laminate inclusion body components (beaded sheets) in the cytoplasm were observed at 38, 56, and 62 hr, respectively, after inoculation. Intracytoplasmic particles were identified as PVX by ferritin-antibody labeling. Microscopic indications of infection were associated with a sixfold increase of infectious material.

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*Additional key words:* virus multiplication.

Potato virus X (PVX) in tobacco leaf cells provides a model system for study of intracellular aspects of virus replication. Infected leaves contain a soluble viral antigen (free-protein) which is antigenically similar to depolymerized structural protein of PVX but antigenically distinct from whole virus particles (11, 14). Thus, the use of free-protein-specific antibodies should make it possible to localize newly formed viral antigen in infected cells and identify sites of structural protein synthesis. Previous attempts to do so have failed, probably because inoculation by conventional mechanical means did not give synchronous infection among the leaf cells. Recent introduction of the use of plant protoplasts for study of tobacco mosaic virus (TMV) multiplication (1, 2) prompted us to explore the feasibility of using tobacco leaf protoplasts as hosts of PVX. This report presents evidence for successful infection of isolated protoplasts with PVX and describes preliminary observations on the internal location and successive formation of some virus-specific products during the course of infection.

MATERIALS AND METHODS.—*Virus and host plant.*—A mottle strain of PVX (13) was purified and stored in distilled water at 4 C for use as inoculum. Protoplasts were obtained from fully expanded leaves of *Nicotiana tabacum* L. 'Burley 21'. Since different tobacco seedlings varied greatly in their capacity to yield viable protoplasts, rooted cuttings were made from a seedling which consistently provided good protoplast preparations. This vegetatively propagated clone of tobacco was the source of protoplasts for all experiments described here.

*Protoplasting and inoculation.*—Protoplasts were prepared as described by Aoki & Takebe (1), in a maceration medium containing 1% potassium dextran sulfate (mol wt 560). Conversion of free cells to protoplasts was complete 2-6 hr after exposure to cellulase (Yokult Biochemicals, Tokyo). For inoculation, 0.25 ml of protoplasts packed by low-speed centrifugation were dispersed in 3 ml of a solution containing 0.9 M mannitol, 13 mM $CaCl_2$, 7 mM $ZnSO_4$, 1.3 mM $MgSO_4$, and 10 $\mu$g/ml poly-L-ornithine (mol wt 150,000, Pilot Chemicals, Inc., Watertown, Mass.), and then 1 ml of PVX at 0.1 mg/ml in distilled water was added. After standing for 30 min, the inoculated protoplasts were washed twice with 0.8 M mannitol and suspended in standard incubation medium (1) to which Loridine (300 mg/ml) and Rimocidin (1 mg/ml) were added to suppress microbial contamination. Incubation was at room temperature under cool-white fluorescent light, with occasional stirring. Most protoplasts remained alive (as indicated by cytoplasmic streaming) for about 4 days after inoculation.

*Monitoring infection.*—Protoplasts were monitored for newly generated PVX at various intervals after inoculation by electron microscopy, fluorescent antibody staining, and infectivity measurements.

For assays of infectivity, 10-ml portions were removed from the incubation medium and submitted to mild centrifugation (ca. 100 *g*). The sedimented protoplasts were dispersed in 4 ml of 0.05 M borate buffer, pH 8.2, and frozen until shortly before being used to inoculate assay plants. After thawing, the protoplasts were further disrupted by mild sonication for 3 min (Disontigrator System Forty, Ultrasonic Industries, Long Island, N.Y.) and grinding in a ground-glass homogenizer. The resulting homogenates were used to inoculate corundum-dusted leaves of *Chenopodium amaranticolor* Coste & Reyn. (four- to eight-leaf stage), which react to PVX infection by forming localized chlorotic lesions 8-10 days after inoculation.

Protoplasts were conveniently prepared for staining with fluroescent antibodies as follows. Two drops of protoplast suspension (in incubation medium) were placed on an ovalbumin-coated glass slide and immediately overlaid with two drops of buffered 3% glutaraldehyde. After drying overnight on a warming table (48 C), the slides were washed thoroughly with phosphate-buffered saline (PBS), stained for 2 hr with fluorescent antibody, and again washed with several changes of PBS. The stained protoplasts were then mounted in glycerol-PBS (1:1, v/v) under a coverslip and examined with a Zeiss GFL research microscope equipped with a mercury-vapor lamp and filters for observing fluorescence in the range of 400-650 nm. Labeled antibody was prepared

by conjugating gamma-globulins from a PVX-immunized goat with fluorescein isothiocyanate by the method of Marshall et al. (7). The antiserum was cross-reactive with both PVX and its depolymerized structural protein. Used in controls was fluorescein-labeled gamma-globulins from a nonimmunized goat. The specificity of the fluorescent antibody preparations was confirmed by staining protoplasts isolated from tobacco plants systemically infected with PVX.

For electron microscopy, protoplasts were fixed in standard BEEM capsules (Polysciences) with 3% glutaraldehyde which was buffered to pH 6.8 and contained 0.7 M mannitol. They were packed by mild centrifugation for subsequent manipulation. After washing with neutral buffer, they were postfixed with buffered 2% $OsO_4$ (pH 7.0), washed with distilled water, rapidly dehydrated by transferring them directly into 95% acetone followed by two changes of 100% acetone, and embedded in a low-viscosity epoxy resin. Thin sections were mounted on copper grids, stained with uranium and lead, and examined with an RCA EMU-3H electron microscope.

*Ferritin-antibody labeling.*—Ferritin-labeled antibodies were used to identify, as PVX, filamentous particles observed with the electron microscope in the cytoplasm of inoculated protoplasts. Ferritin-antibody conjugates were prepared as described previously (10, 12) using purified rabbit antibodies specific against either PVX or (for controls) TMV. Protoplasts were fixed for 1 hr with 3% glutaraldehyde containing 0.7 M mannitol, washed thoroughly with neutral phosphate buffer, and suspended in a small volume of ferritin-antibody conjugate dispersed in 0.05 M sodium phosphate buffer, pH 7.0. At this point, the mixture was subjected to mild sonication for 3 min (see above) to help the ferritin-antibody conjugates penetrate the protoplasts. After standing for an additional hr at room temperature they were washed twice with buffer, treated with $OsO_4$, dehydrated, embedded in epoxy resin, and sectioned for electron microscopy.

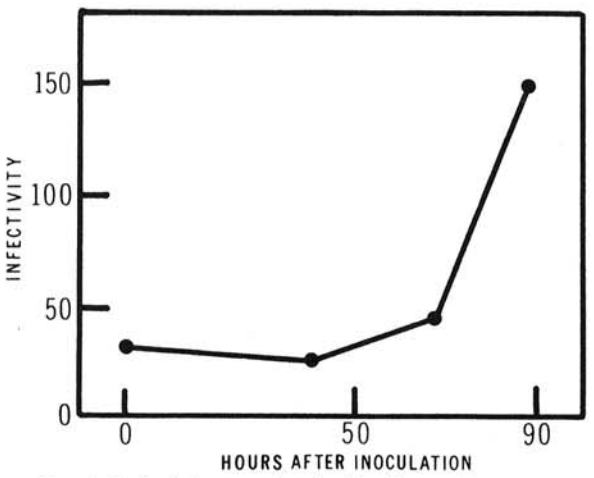


**Fig. 1.** Infectivity associated with disrupted protoplasts sampled at different intervals after inoculation with PVX. Infectivity refers to the total number of local lesions produced on six leaves of *Chenopodium amaranticolor* per sample.

RESULTS.—The following data clearly indicate that some of the protoplasts from healthy tobacco leaves became infected with PVX and supported virus multiplication. After inoculation and incubation for several hr, protoplast preparations contained a high multiplicity of infectious units. Also, heavy concentrations of viral antigen, PVX particles, and virus-specific inclusion-body components accumulated inside individual protoplasts in response to infection.

Infectivity associated with disrupted protoplasts at various times following inoculation is shown in Fig. 1. Samples taken at 0 and 41 hr postinoculation (PI), respectively, induced 31 and 25 lesions on six leaves of *C. amaranticolor*, probably the result of residual inoculum in the protoplast preparations. At 64 hr there was a slight increase in lesion number (43/6 leaves) and by 88 hr there was about a six-fold increase (156/6 leaves). The lesion numbers at 64 hr may not represent a significant increase in infectivity, but those at 88 hr definitely show a marked increase, indicating that new infectious units of PVX had been formed.

When protoplasts were stained with fluorescent antibodies, viral antigen was demonstrated in a small proportion of them beginning 38 hr PI (Fig. 2, 3, 4). When stained with fluorescent antibody, 1-2% of the protoplasts sampled at 38, 46, 63, and 90 hr exhibited a network of yellow-green fluorescence surrounding the chloroplasts (Fig. 4, Table 1). Only traces of nonspecific fluorescence were observed in inoculated protoplasts which were sampled before 38 hr and stained with fluorescent antibody or in any of the inoculated protoplasts which were stained with FITC-labeled normal goat-globulin (Fig. 3). Also, no positive fluorescent antibody reaction was seen in noninoculated protoplasts which had been incubated for 62-90 hr. These results indicate that a substantial amount of PVX structural protein was formed by 38 hr and was distributed uniformly through the cytoplasm of protoplasts which were successfully infected. Furthermore, the proportion of infected protoplasts did not change significantly over the 52 hr of incubation following the first appearance of viral antigen.

Table 2 summarizes electron-microscope observations on inoculated protoplasts. From about 1 hr to 52 hr PI, no ultrastructural differences were observed between inoculated and noninoculated protoplasts. They were bounded by a single unit membrane (the plasmalemma) and lacked any visible cell wall (Fig. 5). Chloroplasts were often distorted and contained paracrystalline bodies believed to result from the hypertonic conditions required for integrity of the protoplasts (8). Otherwise, subcellular organization and morphology appeared the same as in intact leaf cells and as previously described for uninfected protoplasts of Xanthi tobacco (8).

At 56 hr, and at all sampling times thereafter, filamentous particles similar to PVX were observed in

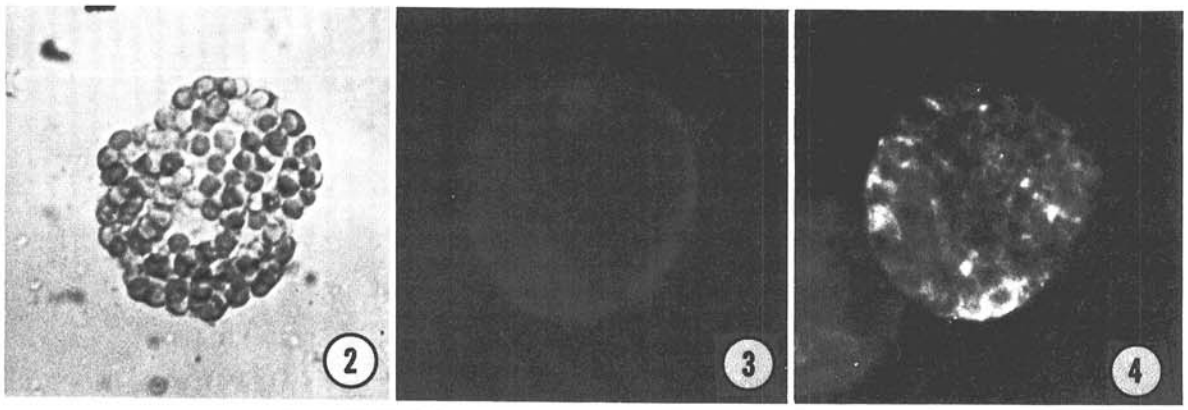

Fig. 2-4. Protoplasts prepared for fluorescent-antibody staining. 2) Brightfield view of a protoplast similar to those shown in Fig. 3-4. Chloroplasts are evident. Protoplast is distorted because of postfixation drying (× 1,500) 3) Fluorescence microscopy showing the characteristic appearance of an inoculated protoplast (62 hr postinoculation) stained with FITC-labeled normal serum. No virus-specific staining is evident (× 1,500). 4) Fluorescence microscopy of an infected protoplast stained with labeled antibody specific for PVX-antigen. Immune fluorescence appears as a light network surrounding the chloroplasts. Part of an unstained protoplast is shown at lower left (× 1,500).

the cytoplasm of about 16% of the protoplasts (Fig. 6, 7, 8, 9, Table 2). In agreement with fluroescent antibody observations, these particles were distributed uniformly throughout the cytoplasm of all protoplasts which contained them. They were not observed in noninoculated protoplasts which had been incubated for 62 hr. Tagging experiments with ferritin-labeled antibodies confirmed the viral nature of these particles. They were heavily tagged when protoplasts (at 62 hr PI) were treated with PVX-specific antibody conjugates (Fig. 8) but were not tagged with TMV antibody conjugates. The morphology of the particles, their antigenicity, and appearance only in inoculated protoplasts after prolonged incubation indicated that the particles were progeny of PVX.

Another definite indication that the protoplasts became infected with PVX was the occurrence, in some, of cytopathic changes characteristic of PVX-infected cells. After 62 hr PI, some of the infected protoplasts contained, in addition to virus particles, bundles of laminated inclusion body components (Fig. 9, Table 2). These consisted of beaded sheets with virus particles interspersed between collateral layers identical to those found in cells of plants systemically infected with PVX (4, 12, 15). No inclusion body components were found in noninoculated protoplasts, even after prolonged incubation.

DISCUSSION.—In pioneering the use of protoplasts as a host for TMV, Takebe & co-workers (1, 8, 16) used three principal criteria to prove virus multiplication: (i) generation of new infectious material; (ii) formation of fluorescent antibody-

TABLE 1. Incidence of fluorescent antibody staining in protoplasts at different times after inoculation with potato virus X

| Hours after inoculation | FITC[a] conjugate | | | |
|---|---|---|---|---|
| | Normal serum | | PVX-antiserum | |
| | No. observed[b] | % Positive[c] | No. observed | % Positive |
| PVX-inoculated | | | | |
| 0 | 160 | 0 | 400 | 0 |
| 15 | 600 | 0 | 590 | 0.1 |
| 22 | 570 | 0 | 530 | 0.2 |
| 38 | 460 | 0 | 290 | 1.4 |
| 46 | 440 | 0 | 210 | 1.4 |
| 63 | 620 | 0.1 | 410 | 1.9 |
| 90 | 340 | 0 | 490 | 1.6 |
| Noninoculated | | | | |
| 63 | | | 140 | 0 |
| 90 | | | 110 | 0 |

[a] For staining protoplasts, conjugate of fluorescein isothiocyanate and IgG from an nonimmunized (normal serum) or PVX-immunized (antiserum) goat.
[b] Total number of protoplasts observed.
[c] Percent protoplasts exhibiting FITC-fluroescence in the cytoplasm.

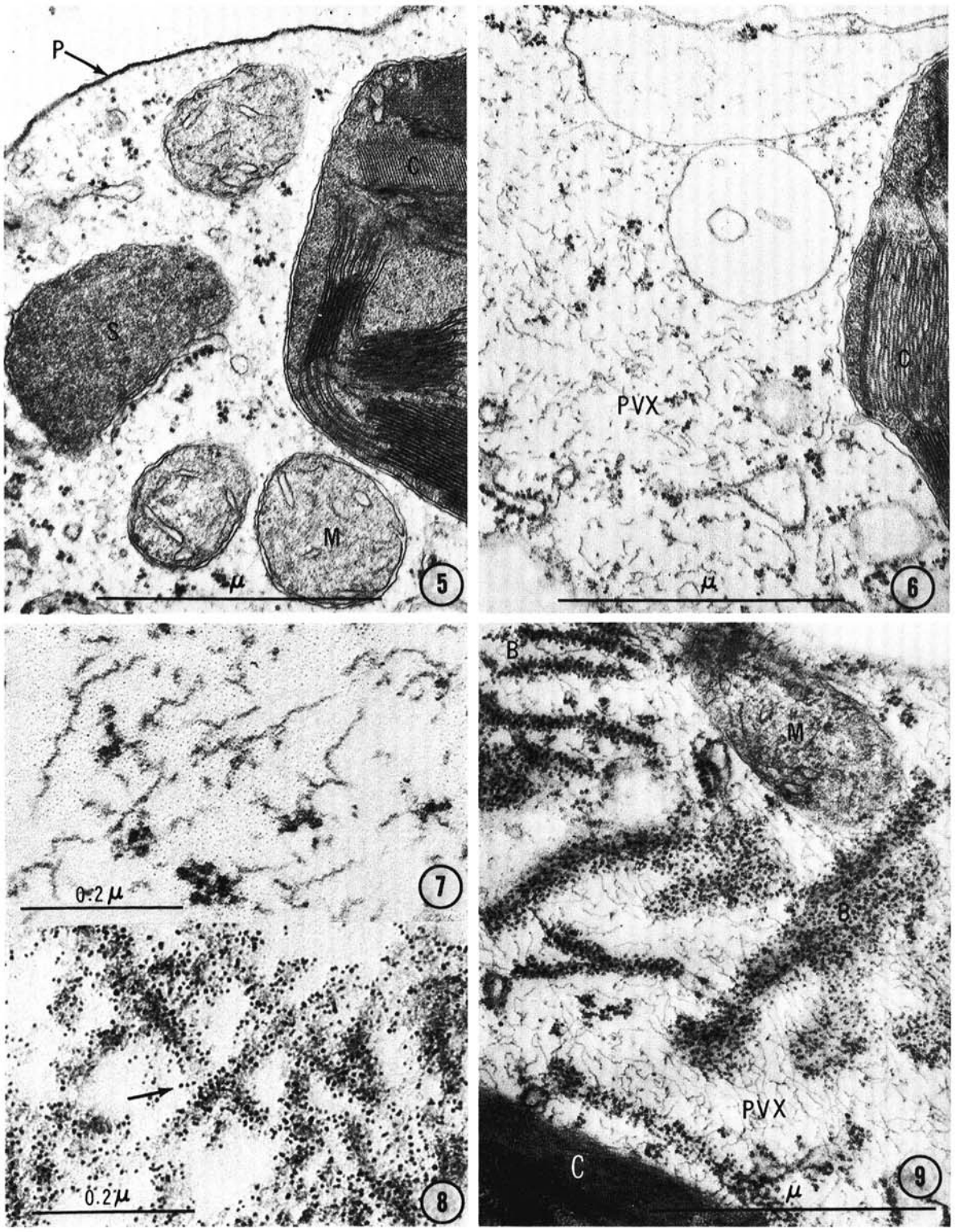


Fig. 5-9. Electron micrographs of sectioned protoplasts. 5) Noninoculated protoplast showing normal cytoplasmic structure. (C) crystalline material in a chloroplast; (M) mitochondrion; (P) plasmalemma; (S) spherosome (× 47,000). 6) Portion of a protoplast sampled 62 hr after inoculation. Filamentous particles of PVX are scattered throughout the cytoplasm (× 47,000). 7) Enlarged view of cytoplasmic PVX particles similar to those shown in Fig. 6 (× 182,500). 8) Intracytoplasmic particles of PVX tagged with virus-specific ferritin-antibody conjugate, 62 hr post-inoculation (PI). Ferritin granules appear as dark dots (arrow) (× 182,500). 9) Infected protoplast at 88 hr PI containing beaded-sheet inclusion-body components (B) in the cytoplasm. Virus particles (PVX) are scattered around and between the beaded sheets (× 47,000).

specific deposits in inoculated protoplasts; and (iii) internal accumulation of particles with a morphology similar to that of the infecting virus. By these criteria we have demonstrated that tobacco leaf protoplasts are also capable of being infected with PVX and supporting its multiplication. Infection was further confirmed by the specific identification of the cytoplasmic particles as PVX by ferritin-antibody tagging and by the presence in inoculated protoplasts of characteristic inclusion body components.

Protoplasts were less efficient hosts for PVX than for TMV since a lower proportion were infected and longer incubation times were required for generating infection products. Takebe & Otsuki (16) observed that as much as 31% of those inoculated with TMV stained with fluorescent antibody, and from observations with the electron microscope, Otsuki et al. (8) estimated that a "great majority" of the protoplasts were infected. In our system, only about 2% stained with fluorescent antibody and about 16% were found to contain PVX particles with the electron microscope. Also in the TMV system, new virus particles were found in sections of inoculated protoplasts by 6 hr (8) and infectivity reached a maximum at about 24 hr PI (16). By comparison, PVX particles and new infectivity in PVX-inoculated samples were not detected before 52 and 88 hr, respectively.

The apparent delay in production of infectious material after recognizable PVX particles appeared in the cytoplasm was probably due to the low sensitivity of *C. amaranticolor* as a local-lesion host for this virus. This also accounts for the relatively small number of lesions which formed. In one experiment, no increase in infectivity could be detected even though many PVX particles were observed in the cytoplasm of some protoplasts. The greater sensitivity of electron microscopy for detecting virus particles also explains the discrepancy between the proportion of protoplasts containing PVX particles and that which stained positively with fluorescent antibody.

Although the efficiency of infection with PVX was relatively low, we believe the system is reasonably synchronous. Since the proportion of protoplasts which stained with fluorescent antibody did not increase over the 52-hr incubation period following the first appearance of antigen (38 hr PI), there probably was little or no secondary infection after the initial period of inoculation. Also, the appearance of all infected protoplasts in the electron microscope was identical in any given sample, as would be expected if they were all at the same stage of infection. Therefore, despite the low percentage and long time course of infection, this is a valid and useful system for studying the sequence of events in virus replication as a function of time after inoculation.

Viral antigens appeared in the cytoplasm of PVX-inoculated protoplasts about 14 hr before the appearance of whole virus particles. After 38 hr, the cytoplasm of some protoplasts stained heavily with fluorescent antibody, whereas PVX particles could not be found in thin-sectioned protoplasts until 56 hr PI.

TABLE 2. Virus-specific cytopathic changes observed with the electron microscope in sections of PVX-inoculated protoplasts

| Hours after inoculation | PVX[a] | LIC[b] |
|---|---|---|
| <1 | –[c] | – |
| 15 | – | – |
| 38 | – | – |
| 52 | – | – |
| 56 | + | – |
| 62 | +[d] | + |
| 64 | + | + |
| 88 | + | + |

[a] Profuse filamentous particles in the cytoplasm.
[b] Laminate inclusion components (beaded sheets).
[c] Ultrastructure of protoplasts appears normal.
[d] Filamentous particles specifically tagged with ferritin antibody.

Since electron microscopy was the more sensitive method for detecting infection (i.e., at 62 hr PI, 16% of the protoplasts contained PVX particles, compared with 2% which stained with fluorescent antibody), it is unlikely that the time differential in the appearance of antigen and whole PVX was due to sampling error. The earlier-formed antigen probably comprised a cytoplasmic pool of unassembled structural protein destined, at least in part, for incorporation into whole PVX particles. It is widely accepted that formation of a large pool of structural proteins precedes intracellular assembly and maturation of some bacteriophages (6) and mammalian viruses (3). It is not known whether similar events occur during replication of any plant virus. There is cytological evidence for the accumulation of viral antigens, other than the virus particles themselves, in cells infected with TMV (5, 10) and clover yellow mosaic virus (9). However, those studies were with asynchronously infected leaf cells, so no definite temporal relation between antigen formation and viral assembly could be determined. The data with PVX-infected protoplasts are less ambiguous and provide more convincing evidence for the formation of a precursor pool of viral protein in plants.

Multilayered bundles of proteinaceous sheets (4, 12, 15) are characteristic features of PVX-infected cells. The sheets are often studded with ribosomelike particles (beads) and are prominent components of amorphous inclusion bodies. The similarity of beaded sheets, when viewed in cross section, to polysomes prompted Kozar & Sheludko (4) to speculate that they might be sites of active viral protein synthesis. More recently, we reported that the beads were distinctly smaller than cytoplasmic ribosomes and that the beaded sheets were neither composed of, nor associated with, unassembled structural proteins of PVX (12). These structures were observed in PVX-infected protoplasts in the present study but only very late in the course of infection (62 hr PI). Their appearance long after that of viral antigen and subsequent to the formation of PVX particles

themselves, is further evidence against their involvement in structural protein synthesis.

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