

A Study of Tobacco Etch Virus-Induced Inclusions Using Indirect Immunoferritin Procedures

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The technical assistance of G. A. Secor, Montana State University and F. B. Diez, University of Wisconsin is gratefully acknowledged.

This research was completed while the first author was on leave in the Department of Plant Pathology, University of Wisconsin, Madison, and was supported in part by National Science Foundation Grant GB-26415-A2 to the first author, by Hatch project 1494 directed by G. A. DeZoeten of the University of Wisconsin, and by NSF Grant GB-32093 to the third author and E. Hiebert.

Montana Agricultural Experiment Station Journal Series Paper No. 464.

Accepted for publication 29 October 1973.

ABSTRACT

Intranuclear and cytoplasmic inclusions within cells infected by tobacco etch virus (TEV) were tested for immunochemical cross-reactivity with rabbit antibody prepared against TEV, TEV-dissociated capsid protein (TEV D-protein), and TEV cylindrical inclusion protein. Indirect immunoferritin experiments were conducted with fractured plant cells using ferritin-tagged sheep anti-rabbit immunoglobulin G (R-IgG) antibody subsequent to rabbit antibody. Examination of treated cellular regions with the electron microscope revealed that nuclear crystalline

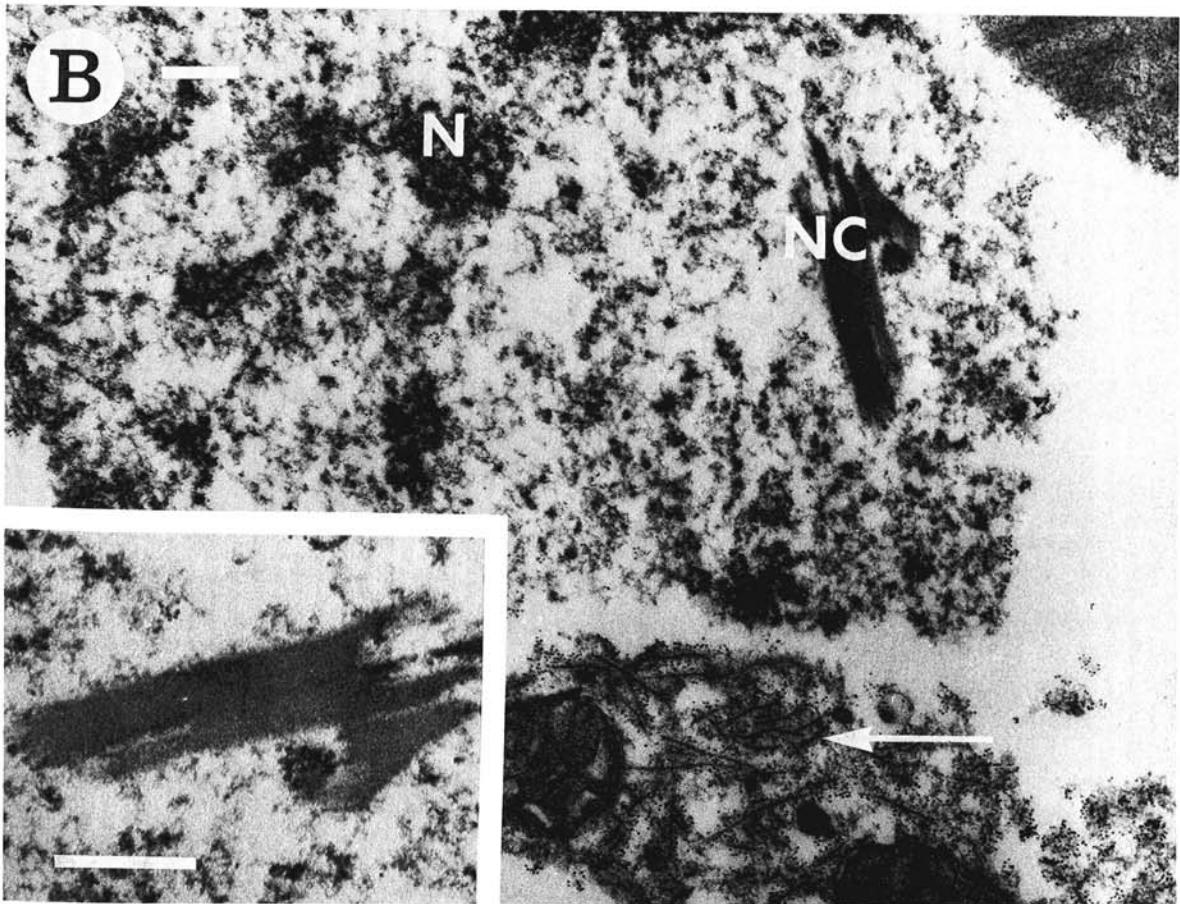
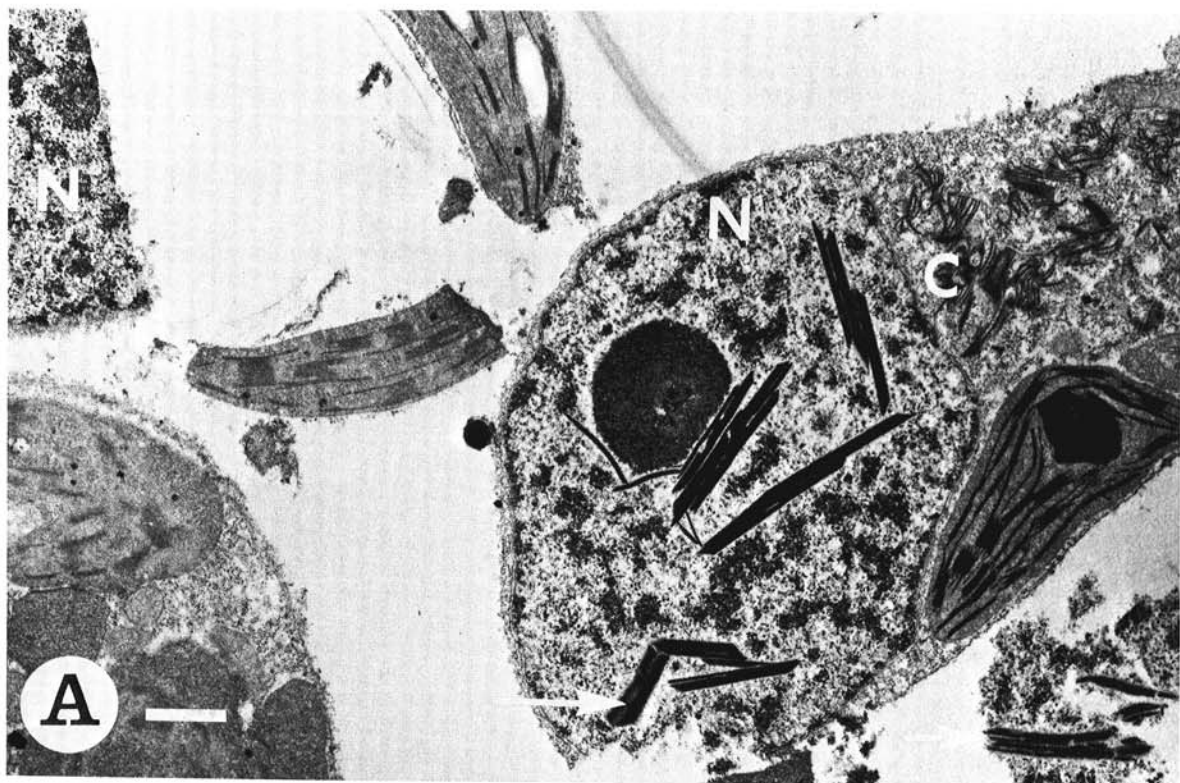
inclusions were not ferritin-tagged following exposure to any of the antibody preparations tested. However, TEV particles and TEV-induced cylindrical inclusions were intensely labelled after incubation with first their homologous antibody, and then ferritin tagged sheep anti-R-IgG antibody. These results suggest that TEV-induced nuclear protein crystals possess an immune specificity distinct from both TEV capsid protein and cylindrical inclusion protein.

Phytopathology 64:418-425

Intracellular inclusions induced by tobacco etch virus (TEV) have been the subject of research for many years, and the results of the previous studies have been summarized in numerous review articles (2, 11). Two basic types of abnormal structures (exclusive of virus particles) develop within TEV-infected cells: cylindrical

inclusions consist of pinwheel and/or laminated aggregates restricted to cytoplasmic regions (4) and nuclear inclusions (7) composed of crystallized protein (14). Cytoplasmic inclusions have been shown to be immunochemically distinct from viral capsid protein (6, 17). However, no direct evidence has been reported

Fig. 1-(A, B). **A)** Low-magnification view of fractured cells, chloroplasts, and nuclei after glutaraldehyde fixation, freezing, and pulverization. Tobacco etch virus (TEV)-induced nuclear crystals (denoted with arrows) are evident in two fractured nuclei, and TEV cylindrical inclusions are also visible. Scale bar represents 4 μ . N = nucleus, C = cylindrical inclusions. **B)** Fractured nuclear and cytoplasmic regions of an infected cell illustrating ferritin-tagged virus particles (arrows) after exposure to anti-TEV antibody. The TEV-induced nuclear crystal is not labelled as is more clearly evident in the enlarged inset. Scale represents 500 nm. N = nucleus, NC = TEV-induced nuclear crystalline inclusion.



regarding the nature of nuclear crystalline protein, despite suggestions that it may be TEV capsid protein (11).

Four distinct possibilities seemed plausible to us regarding the nature of TEV-induced nuclear crystalline protein. The inclusion (i) may indeed be crystallized TEV capsid protein, (ii) capsid protein which possesses a different molecular conformation and hence antigenic specificity from that of the protein in assembled TEV particles, (iii) crystalline protein of cylindrical inclusions, or (iv) protein(s) distinct from each of the aforementioned. To test these possibilities, indirect immunoferritin procedures were employed in experiments which ultimately suggested that TEV-induced nuclear inclusions are composed of protein(s) distinct from capsid and cytoplasmic inclusion proteins.

MATERIALS AND METHODS.—*Virus-host combinations.*—Tobacco etch virus (TEV), the same strain of which was used in prior studies (17), was maintained in *Nicotiana tabacum* L. 'White Burley.' Potato virus X (PVX) was also increased in tobacco (18) for use in comparative and control experiments.

Viral and dissociated capsid protein antibody.—TEV was purified according to the method previously described for PVX (19) except that 5% polyethylene glycol solutions were used for virus precipitation rather than 7% solutions. TEV-dissociated capsid protein (D-protein) was prepared by the pyrrolidine degradation procedure described for other rod-shaped plant viruses (15, 16) and used for the immunization of rabbits (16). Resultant antisera were assayed for reactivity in double immunodiffusion tests (16) against TEV D-protein prepared by either 2.5% pyrrolidine or 1% sodium dodecyl sulfate solutions. Serum titers (reciprocal of the highest reactive dilution) were identical in tests against TEV D-protein prepared by either method and in one rabbit reached a maximum of 256. Antiserum against nondegraded TEV was elicited in rabbits using the glutaraldehyde stabilization procedure previously reported for PVX (18). Early serum collections (3-4 wk after initial administration of immunogen) displayed either no, or only a trace of, reactivity against TEV D-protein whereas an anti-TEV titer of 1024 was recorded in tube precipitin tests with pooled early collections. PVX and PVX D-protein antibody were previously prepared (18).

Specifically purified anti-viral and anti-D-protein antibody were isolated from immune precipitates as before (13). Homologous titers in double diffusion with anti-TEV D-protein and anti-PVX D-protein purified antibody were 32 with only a trace of reactivity against their respective nondissociated virus. Purified antibody against intact TEV and PVX reacted with homologous virus at a 1/1024 dilution but not at all with their respective D-protein.

Anti-inclusion serum.—Antiserum against TEV-induced cylindrical inclusions was previously prepared in rabbits (6) and stored in the freezer after lyophilization. For use in the present study, the antiserum was dissolved and brought to one-half its starting volume in 0.1 M phosphate buffer pH 7.0 containing 0.85% NaCl (PBS). An equal volume of a partially purified TEV preparation in PBS was added to the antiserum to remove TEV

reactive antibody, and after 1 h incubation, TEV particles and bound antibody were removed from the serum by centrifugation at 100,000 g for 2 h in a Beckman SW 39 rotor. Centrifuged antiserum was carefully removed with a syringe avoiding the film at the meniscus.

Preparation and purification of sheep anti-rabbit IgG antibody.—Antibodies against highly purified rabbit immunoglobulin G (R-IgG) were elicited in a 9-mo-old sheep. Fifty mg of R-IgG (Miles Laboratories, Kankakee, Illinois) were emulsified in Freund's incomplete adjuvant and administered intramuscularly at 7-day intervals for 4 wk. Bleedings were made from the jugular vein after 5 wk of immunization with 300 ml of blood collected per drawing. Antiserum pooled from several collections displayed a titer of 64 in immunodiffusion tests against R-IgG at 1 mg/ml.

For the preparation of specific ("purified") sheep antibody against R-IgG, an insoluble R-IgG immunoabsorbent was made with glutaraldehyde in the manner developed by Avrameas and Terynck (1). This consisted of dissolving 1 gm of lyophilized R-IgG (Miles Laboratories) in 20 ml of 0.1 M potassium phosphate buffer pH 7.0 followed by the dropwise addition of 4.0 ml of a 2.5% (w/v) glutaraldehyde-phosphate buffer solution. After incubation at room temp for 1 h, the heavy gel was thoroughly ground in a mortar with a pestle in PBS. The gel particles were sedimented at 10,000 g for 10 min and the pellets rinsed and resedimented four additional times. Gels were ground in a mortar between each centrifugation. Final pellets were dispersed in a small volume of PBS and the immunoabsorbent was then mixed with 100 ml of sheep anti-R-IgG serum. The immunoabsorbent-antiserum mixture was vigorously stirred at room temp for 30 min after which the absorbent was removed by centrifugation. No anti-R-IgG reactivity was detected in the supernatant. The immunoabsorbent was rinsed three times in PBS and finally in 0.85% NaCl (saline) alone with sedimentation between each rinse. The final pellet was washed for 10 min with stirring in 10 ml of 0.1 M glycine-saline buffer pH 2.5 each of four times to elute sheep antibody. After each wash and low-speed centrifugation, eluted antibody was precipitated from the supernatant by adding an equal volume of saturated NaCl. Most of the antibody was recovered from the first two washes. Precipitated antibody was pelleted by low-speed centrifugation, rinsed once in 0.1 M glycine pH 2.5 containing half saturated NaCl, repelleted, and then dissolved in a small volume of PBS. Antibody was dialyzed for 24 h against several changes of PBS and then adjusted to a concn of 3 mg/ml. Antibody concns were determined spectrophotometrically at 278 nm as before (18). The activity of antibody preparations was confirmed in double immunodiffusion tests against R-IgG.

Following the four elution washes, the immunoabsorbent was rinsed in PBS and sedimented three times. It was then suitable for reuse or storage at 4 C.

Preparation of tissues for antibody treatment.—The peripheral continuity of plant cells must be perturbed if antibody molecules are to diffuse into cytoplasmic regions (9). Previously, this has been accomplished by either cutting thick sections of glutaraldehyde-fixed tissue with a freezing microtome (12) or shaving small tissue squares in a frozen droplet of water with a razor blade

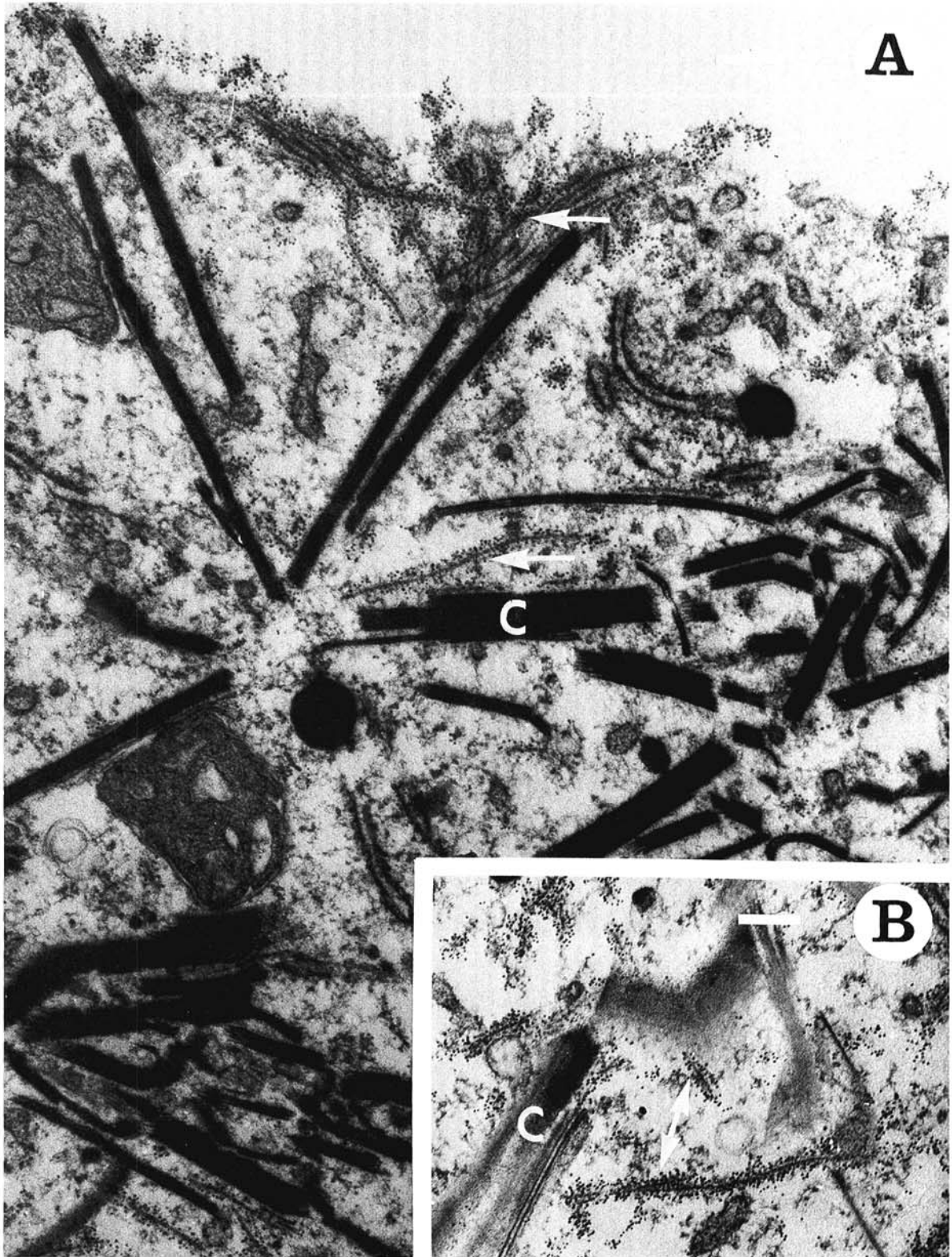


Fig. 2-(A, B). **A)** A cytoplasmic region within a tobacco etch virus (TEV)-infected cell which contains numerous laminated aggregates. Immunoferritin procedures with anti-TEV antibody resulted in tagging of TEV particles (arrows), but not of the cylindrical inclusions. Scale represents 200 nm. **C** = cylindrical inclusions. **B)** Tobacco etch virus (TEV) particles (arrows) which are heavily tagged with ferritin following exposure to anti-TEV antibody. Portions of cylindrical inclusions present are not labelled. Scale represents 200 nm. **C** = cylindrical inclusions.

(17). In the present study, both methods were inadequate for exposing a sufficient number of fractured nuclei into which ferritin-tagged antibody would diffuse. Hence, another method was devised to fracture cells, nuclei, and cytoplasmic regions in sufficient quantity to permit tagged antibody studies (Fig. 1-A). Tissue strips 2-4 mm wide were cut with a razor blade from TEV infected and noninfected leaves, and fixed for 2-3 h in a 3% glutaraldehyde solution buffered to pH 7.0 with 0.1 M phosphate. The strips were rinsed 1-2 h in several changes of phosphate buffer and then placed in a mortar immersed in and containing liquid nitrogen. Frozen tissue was thoroughly pulverized with a pestle; and then the powder, in the presence of a small volume of nitrogen, was poured into a clinical centrifuge tube. When the nitrogen had nearly evaporated, the tube was filled with phosphate buffer. The larger tissue pieces were allowed to settle (ca. 5 min), and then the buffer which contained smaller fragments was decanted into a second centrifuge tube. Cell fragments were sedimented at 100 g for 10 min, and the pelleted material was used for subsequent antibody labelling experiments.

Indirect immunoferritin procedures.—Purified sheep anti-R-IgG antibody at 3 mg/ml was conjugated with ferritin according to Singer and Schick (21). With modifications to be detailed later, the procedures used to ferritin tag the various antigens are as follows. Aldehyde-fixed, pulverized leaf tissue was pelleted at 100 g in a clinical swinging bucket rotor with only enough tissue used to form a firm pellet. The supernatant was discarded and the pellet dispersed in 1.0 ml of 0.1 M phosphate buffer pH 7.0. One ml rabbit antibody (at varying experimental dilutions) was added to the slurry and gently agitated with a magnetic stirrer for 1 h. The pulverized tissue was separated from unreacted antibody

by sedimentation at 100 g and then rinsed and resedimented three or four times over the course of 2 h. Following the final rinse, the pellet was dispersed in 2.0 ml of phosphate buffer to which was then added 0.5 ml of ferritin-tagged sheep anti-R-IgG antibody. Exposure of cell fragments to tagged antibody was continued for 1 h with gentle stirring, after which the tissue was sedimented and rinsed four times during the ensuing 2 h. The final pellet was covered with a 1% solution of osmium tetroxide and postfixed in the cold overnight. The specimen was then dehydrated with acetone, stained with uranyl acetate in acetone, and embedded in Spurr's low viscosity medium (22). Ultrathin sectioning was done with a Reichert OMU-2 ultramicrotome and postsection staining was with lead citrate.

All antiserum and purified antibody preparations were dialyzed overnight in the cold against 0.1 M phosphate buffer pH 7.0 before use.

RESULTS.—*Control of nonspecific antibody binding.*—Preliminary treatments of fractured cells from TEV-infected or noninfected tobacco with rabbit antibody and then ferritin-tagged sheep antibody often resulted in relatively high levels of nonspecifically bound ferritin. Similar results were observed after primary treatment with normal (nonimmune) rabbit serum. It is recognized that any nonspecific binding of rabbit antibody will result in significant levels of background since numerous tagged sheep antibodies may be complexed with each rabbit antibody. Hence, other workers (20) have suggested that rabbit antibody should be used at as high a dilution as possible, and that the antibody oligomers be eliminated from preparations before use. Anti-TEV and all other antibody preparations were centrifuged at 100,000 g for 2 h and then tested in increasing dilutions against pulverized tissue from

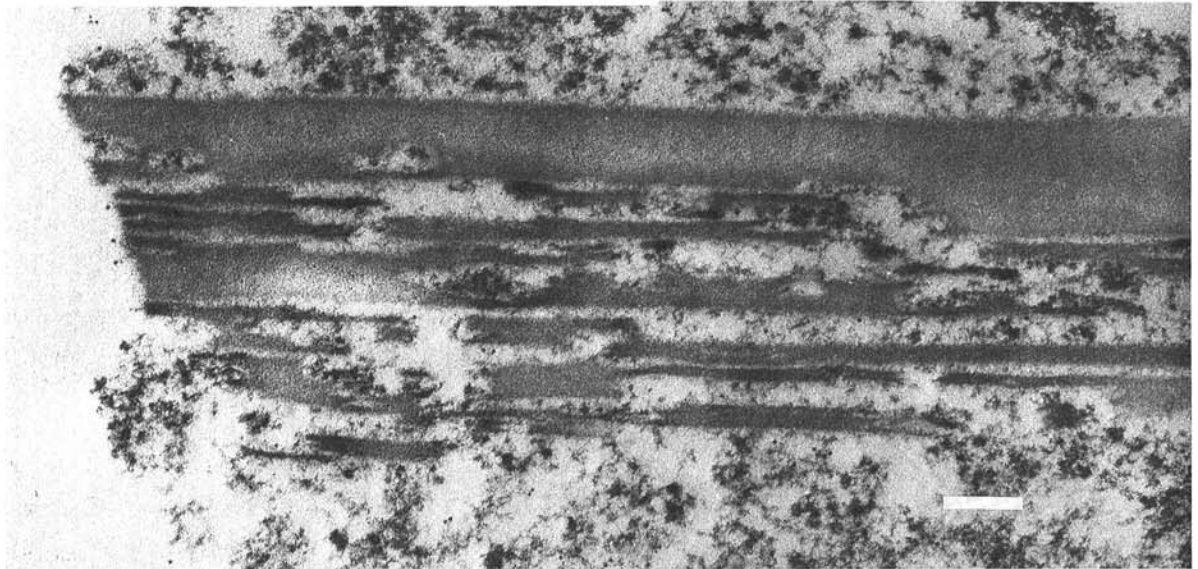


Fig. 3. Tobacco etch virus (TEV)-induced nuclear crystals following exposure to anti-TEV-dissociated capsid protein (D-Protein) antibody and then ferritin tagged sheep anti-rabbit immunoglobulin G (R-IgG) antibody. No labelling of nuclear inclusions occurred. Scale represents 200 nm.

infected and noninfected plants. The results suggested that higher antibody dilutions did reduce background ferritin significantly; but frequently, the dilution required

to eliminate background also significantly decreased the level or consistency of specific tagging.

Other studies (10) have revealed that glutaraldehyde-

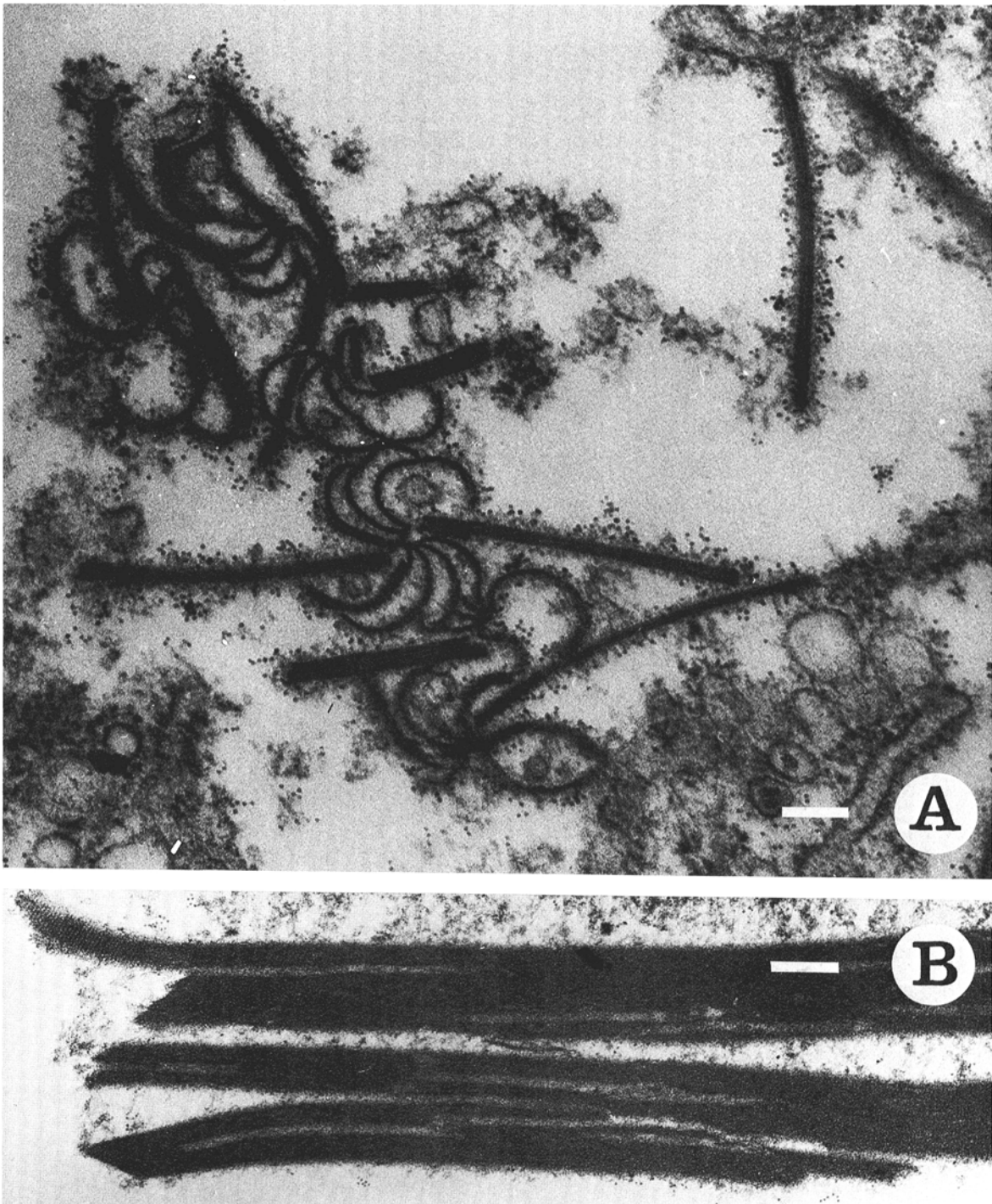


Fig. 4-(A, B). A) Ferritin-tagged tobacco etch virus (TEV) cylindrical inclusions and laminated aggregates after treatment with homologous antibody. Scale represents 150 nm. B) TEV nuclear inclusions treated as in A). No ferritin label is in evidence. Scale represents 300 nm.

fixed tissue is "sticky" and may even bind large amounts of free amino acids. Consequently, we attempted to saturate nonspecific protein (antibody) binding sites prior to the exposure of tissue to antibody. One percent solutions of the following amino acids and proteins were tested: L-leucine, L-alanine, bovine serum albumin (BSA), egg albumin, and normal sheep immunoglobulin. The amino acids were found to be essentially ineffective for reducing ferritin background, whereas each of the proteins was very effective. Of these proteins, however, the sheep normal immunoglobulin fraction seemed to be the most efficient when used in the following schedule. Pulverized leaf tissue preparations were sedimented and the pellet(s) dispersed with gentle stirring in 2 ml of sheep immunoglobulin buffered to pH 7.0 with 0.1 M phosphate. After 30 to 60 min, 0.1 - 0.2 ml of undiluted anti-TEV or other rabbit antibody was added directly to the mixture and incubated with stirring for 1 - 2 h. Unreacted antibody was subsequently removed by phosphate buffer rinses and sedimentation, and the final pellet dispersed in 2 ml of phosphate buffer. A 0.5-ml aliquot of ferritin-tagged sheep antibody was introduced, incubated 1 - 2 h with gentle stirring, and unbound ferritin removed by rinsing. Relatively consistent results were obtained with this procedure with respect to both low levels of background and pronounced specific ferritin antibody tagging. Therefore, it was used in all further studies.

TEV and TEV D-protein antibody labelling.—Indirect immunoferritin tagging experiments with anti-TEV antibody resulted in the extensive labelling of TEV particles within cytoplasmic regions (Fig. 1-B, 2). Also heavily tagged were virions in close proximity to the cylindrical inclusions induced by TEV infection (Fig. 2). In agreement with prior reports (17), no ferritin labelling was detected on cylindrical inclusions following their exposure to anti-TEV antibody (Fig. 2). TEV-induced protein crystals situated within cell nuclei including the fully exposed crystals of fractured nuclei (Fig. 1-B) were not at all labelled in these experiments, nor were they ferritin tagged after contact with TEV D-protein antibody (Fig. 3). Indeed, anti-TEV D-protein antibody produced no definitive patterns of label within infected cell fragments except for an occasional retention of ferritin on the surface of a few virus particles.

TEV cylindrical inclusion antibody.—Pronounced tagging with ferritin occurred on both the curved plates and laminate components of TEV-induced cylindrical inclusions following their exposure to homologous anti-inclusion antibody (Fig. 4-A). No ferritin attachment to TEV particles was observed with TEV-absorbed anti-inclusion serum. Also, ferritin tagging of TEV induced nuclear crystals was not observed following their coinubation with anti-inclusion serum (Fig. 4-B).

Experiments were also conducted with pulverized tissue from infected leaves which contained PVX-induced cytoplasmic inclusions. These inclusions have been suggested (13) to bear a morphological resemblance to those induced by potato virus Y group viruses (5), and it was of interest, therefore, to test for the possibility of immunochemical cross-reactivity between the two types of inclusions. Exposure of PVX-induced laminated inclusion components (13) to TEV inclusion antibody, however, failed to provide evidence of specific ferritin

tagging. Reciprocal tests could not be performed since anti-PVX inclusion serum was not yet available.

Control experiments.—A variety of experiments were conducted to assure that the label observed in indirect immunoferritin studies was indeed specific. Nonimmune rabbit serum, anti-PVX antibody, and anti-PVX D-protein antibody all failed to produce patterns of label within TEV-infected cells upon subsequent treatment with ferritin-tagged sheep anti-R-IgG antibody. Conversely, anti-TEV antibody did not result in the label of components with PVX-infected cells.

DISCUSSION.—The immunocytochemical evidence presented in this investigation suggests that TEV-induced protein crystals within nuclei of infected cells possess an antigenic specificity wholly distinct from that of either assembled or dissociated capsid protein. From this, it seems probable that nuclear inclusion protein is also chemically distinct and, hence, not TEV capsid protein. There is, however, a remote possibility that capsid protein might possess an extraordinarily unique molecular conformation when crystallized which would endow it with an antigenic specificity distinct from the assembled or dissociated forms. We suggest that the second argument is less than plausible, however, for the following reasons. It is well recognized that proteins may display a variety of molecular forms, each of which may possess a different antigenic specificity (3). Nevertheless, the molecular forms are normally immunochemically cross-reactive except in certain cases where one form represents severely denatured protein. In the case of TEV capsid protein, assembled structure unit protein is but weakly cross-reactive with dissociated protein, and since subunit dissociation with SDS (a potent protein denaturant) results in the assumption of a unique antigenic specificity, it is probable that denaturation does accompany the dissociation of TEV protein subunits. Thus, two extreme forms of molecular configuration; i.e., "native" and denatured, are accounted for by homologous antibody preparations; and if a form intermediate between the two extremes was in evidence, some cross-reactivity with either or both antibody populations, would be anticipated. A molecular conformation not intermediate between, but totally distinct from the two extremes and simultaneously possessing a complement of unrelated antigenic determinants, is unlikely. Similar logic should pertain to studies with anti-TEV inclusion serum. The protein of cylindrical inclusions after SDS dissociation is strongly cross-reactive with antibody produced against nondissociated inclusions (6). This, then, would rule out the possibility that nuclear inclusions are crystallized cylindrical inclusion protein. An independent study (8) simultaneous with this one has been successful in the purification of TEV-induced nuclear inclusions. These authors confirm the absence of immunochemical cross-reactivity among the three types of TEV-induced proteins observed in the present investigation.

The indirect immunoferritin procedure when nonspecific ferritin binding is controlled, has several advantages over direct methods for the intracellular localization of antigen. Probably the primary procedural benefit relates to the preparation of purified (specific) antibody and its subsequent conjugation with ferritin. Sheep anti-R-IgG antibody may be prepared (or

purchased) in large amounts and specific antibody purified with relative ease. Following ferritin conjugation, a single tagged antibody preparation may be used in conjunction with small volumes of nonfractionated antiserum against the antigen(s) under study. This feature obviates the need for purifying and conjugating antibody for each antigen, and is particularly useful for those antigens or antibodies which are difficult to isolate in large quantities. A second, though less regular, feature of the indirect immunoferritin technique is that the intensity of specific tagging may conceptually be greater than in direct procedures. Each bound rabbit antibody molecule possesses several antigenic determinants each of which is capable of complexing a ferritin-tagged anti-rabbit antibody. Hence, an antigenic determinant present on the surface of a virus particle, for example, may be associated with at least three or four ferritin molecules rather than just one.

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