The Tracing of Ingested TMV ^{125}I in the Aphid Myzus persica

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ABSTRACT

The advisability of using a fixative known to fix a virus in situ in plants for studying plant virus localization in insects was demonstrated. Green peach aphids (*Myzus persicae*) were fed ^{125}I-labeled and nonlabeled tobacco mosaic virus (TMV), ^{125}I-labeled rabbit gamma globulin, and Na ^{125}I through parafilm membranes. Virus was detected in the first ventriculus after fixation at pH 3.8 with osmic acid, a fixative which preserves TMV inclusion bodies in plant cells. No virus was detected in the first ventriculus after fixation with neutral phosphate buffered glutaraldehyde followed by osmic acid, a fixative which does not preserve TMV inclusions in plant cells. After the pH 3.8 osmic acid fixation, large amounts of TMV were found in the first ventriculus, lesser amounts in the rest of the digestive tract, and none in the rest of the aphid.

^{125}I was incorporated only in the trachea and cuticle in aphids fed Na ^{125}I. No label was found in the trachea and cuticle in aphids fed ^{125}I-labeled virus or gamma globulin. No ^{125}I was released from the labeled proteins. Phytopathology 61:15-21.

Additional key words: Radioautography, thin sections, dissection versus "whole" body fixation.

The mechanisms of plant virus transmission by aphids and the reasons for vector specificity are largely unknown, despite extensive research. Microradioautography can be used at a light microscope level for a rapid survey of virus localization over a large area, and at the electron microscope level for an unambiguous identification and detection of very small amounts of virus. Application of this technique to the study of the localization of plant viruses that are transmitted by aphids in comparison with the localization of those not transmitted might provide clues to the mechanisms.

The location of viruses in insects has been studied by assay of dissected organs (2), electron microscopy (9), serology (19), and radioautography (5). In one case, impermeability of the gut wall seemed to prevent transmission (20), but in another case, a virus was found in the hemolymph of a nonvector insect (15). It has been shown repeatedly that aphids will imbibe viruses that they do not transmit (4, 5, 7, 13, 21).

If the results of microradioautography are to be reliably interpreted, procedures for fixation and embedding must be available which preserve the localization of the virus. Furthermore, the label must remain attached to the virus. No good criterion is available for quality of fixation of a plant virus in an aphid, but a criterion is available for fixation of a virus that forms inclusions in plant cells. Such inclusions, visible in the phase microscope, should be preserved through fixation and embedding. Though there are many differences in the cells of plants and aphids, procedures that do not preserve the viruses in plants probably would not preserve the same viruses in aphids.

For our study and radioautographic approach, we chose the aphid *Myzus persicae* because of the great number (11) of circulative viruses this aphid transmits (6). Tobacco mosaic virus was chosen because it already had been shown to survive the environment of the gut (7, 13), its size, and ready availability in quantity. Tobacco mosaic virus has also been labeled with high specific activity (10). We reasoned that to use a small spherical virus as a model (e.g., brome mosaic virus) would increase the difficulties unnecessarily, since it is similar in size with ribosomes and thus would require radioautographic procedures in all steps instead of just a few. It occurred to us that TMV ^{125}I, therefore, would be a convenient vehicle to test the feasibility of following a labeled virus in its passage through the aphid *M. persicae*.

MATERIALS AND METHODS.—*Labeling procedures.*—Rabbit gamma globulin was purified and labeled with an iodine monochloride method (1). The rabbit globulin had a final concn of 60 μg protein/ml, and had an activity of 7 μc/ml.

One mg of TMV was labeled when needed with 1 mc ^{125}I by the IC1 method (1). Unreacted ^{125}I was removed by placing the reaction mixture on a density-gradient column and centrifuging for 2.25 hr in a Spinco SW-25.1 rotor at 23,000 rpm. Columns were prepared by layering 4-, 7-, 7-, and 7-ml solutions of 75, 150, 225, and 300 mg sucrose/ml in 0.001 M phosphate, pH 7.0, and allowing equilibration overnight at 4 C. The virus band was dialyzed against several changes of 0.001 M neutral phosphate. The dialysate was free of unbound ^{125}I after the completion of the dialysis. The final virus concn was 600 μg/ml. One μliter of this concn gave a count of 20,000 cpm.

The density-gradient purification was chosen in order to minimize breakage of the TMV particles. The presence of small labeled pieces could conceivably present an erroneous picture of the intracellular penetration of aphid gut tissue. Labeled material was counted with a well-type NaI scintillation crystal (Tracer Lab Inc.).

In the labeling with ^{125}I with the IC1 method (1), a slight modification was made because our total volumes were small. Instead of aspirating the reactants in the reaction vessel, as described in the original procedure,

we squirted the required amounts into the vessel while the contents were rapidly stirred in an ice bath with a magnetic stirrer. The total volume of the reactants was routinely kept at 2 ml or less.

Aphid feeding.—Aphids (*Myzus persicae* Sulz.) were fed in feeding cages via parafilm membranes (11). Final concn of the sucrose in the mixtures fed was from 10-20%. Young and wingless adult aphids imbibed the following for 2-4 days at 21 C: 0.4-0.6 mg TMV/ml, 0.3 mg TMV/ml labeled with ^{125}I (14 μc/ml), Na ^{125}I in 0.025 M neutral phosphate (14 μc/ml), rabbit gamma globulin 45 μg/ml, labeled with ^{125}I (5 μc/ml). The rabbit globulin was fed because some radioautographs showed a presence of silver grains above background in tissues of aphids fed on TMV ^{125}I (Fig. 1, 5). The possibility of incorporation in aphid tissue of labeled digests of protein had to be investigated. Since we already had on hand labeled rabbit gamma globulin, this was fed to test the above hypothesis.

Aphids were given access to the various solutions for 2-4 days to be certain that all aphids processed were ones that had imbibed the solutions offered. No aphids survived 2 days in control cages without a sugar solution. Some died even in the cages where they had access to the sucrose solutions. Only turgid insects were chosen for fixation.

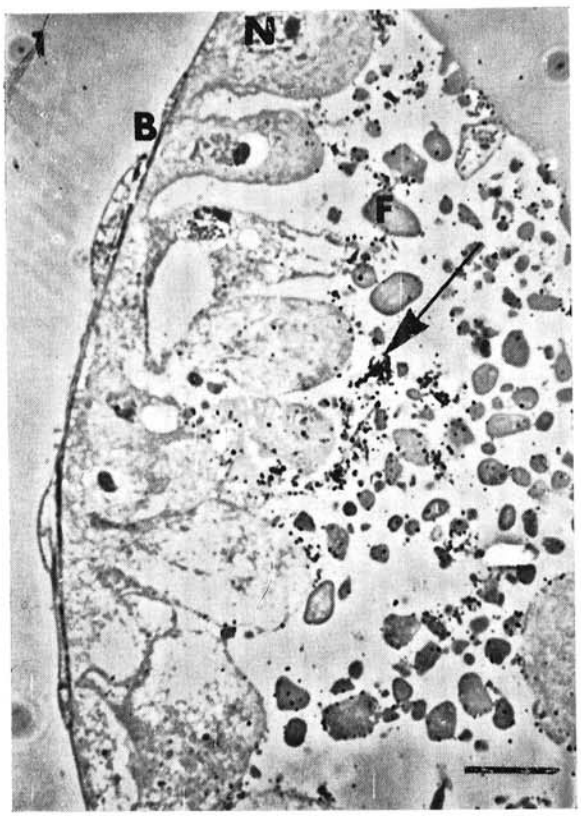

Fig. 1. A radioautograph of a dissected pH 3.8 fixed first ventriculus wall. Radioautographic exposure, 7 days. The aphid had been fed on tobacco mosaic virus labeled with ^{125}I. The lumen can often be seen descending nearly to the basal membrane (B). See also Fig. 7-B. N = nucleus in a large cell lining the ventriculus; F = food particles in lumen; arrow indicates label. Scale = 15 μ.

Fixation and embedding of aphids.—Aphids were fixed in four different fixatives. (i) Aphids were dissected in insect Ringer (11g NaCl, 1.4 g KCl, 1.1 g $CaCl_2$/1,000 ml) following an elegant dissecting method (7). The dissected first ventriculus, second ventriculus, proctodaeum, and salivary glands were fixed in 0.1% osmic acid in insect Ringer, pH 3.8, for 1.5-3 hr at 24 C. The pH of the fixation fluid was lowered to 3.8 with 2% acetic acid. (ii) Aphids were placed whole in the same fixative as in (i) after removal of the legs and antennae to aid in fixative penetration. (iii) Dissected ventriculi were fixed for 3 hr in cold 3% glutaraldehyde in 0.05 M neutral phosphate, rinsed 3 times for 15 min each in cold buffer, and postfixed in 0.1% osmic acid in the same buffer for 1 hr. (iv) Aphids were also fixed in neutral glutaraldehyde [fixatives as (iii)], but not dissected. Aphids were vacuum-infiltrated after removal of legs and antennae. The difference thus between fixations (i, iii) and (ii, iv) is the removal of the various parts and their separate fixation versus "whole" aphid fixation where the intestinal tract, etc., remained intact. Complete green peach aphids could not be fixed and embedded without removal of the legs and antennae to allow the fixative and embedding medium to penetrate. Complete aphids, vacuum-infiltrated with cold 1% osmic acid, could be seen walking around unfixed 24 hr later at the bottom of the fixation vessel. After the appropriate fixation, dehydration commenced with a graded acetone series, followed by embedment in Epon 812. The tissues were brought to Epon from 100% acetone.

Light and electron microscope radioautography.—One-micron sections were cut on glass knives on a Servall MT1 microtome, and ultrathin sections (silver to gold) on a Servall MT2 with a diamond knife. The micron sections were floated on a drop of distilled water on clean slides and allowed to dry at room temp. Thin sections were supported by a carbon-backed Parlodion membrane on nickel or copper grids. Staining of thin sections took place according to standard procedures with uranyl acetate and lead citrate (14). Sections used in radioautography were stained after development of the radioautograph.

A Zeiss Standard Universal microscope with planachromatic phase lenses was used for light microscopy, and for electron microscopy, an RCA 3 G electron microscope at 100 kv.

A 1:1 dilution of Ilford L4 nuclear emulsion was used for both 1 μ and thin sections. The emulsion contained 0.04% dioctyl sodium sulfosuccinate (12). This greatly reduced the number of films bursting, and aided in spreading the silver halide crystals in the emulsion. A monolayer of silver halide crystals was applied with the loop method (3). Grids with thin sections and slides bearing 1-μ sections were stored over silica gel under Freon gas in airtight containers at −20 C until development. Development for the 1-μ sections took place in full strength Kodak D19 or Dektol for 2 min. Fixation for 5 min in Kodak rapid fix was followed by a 5-min

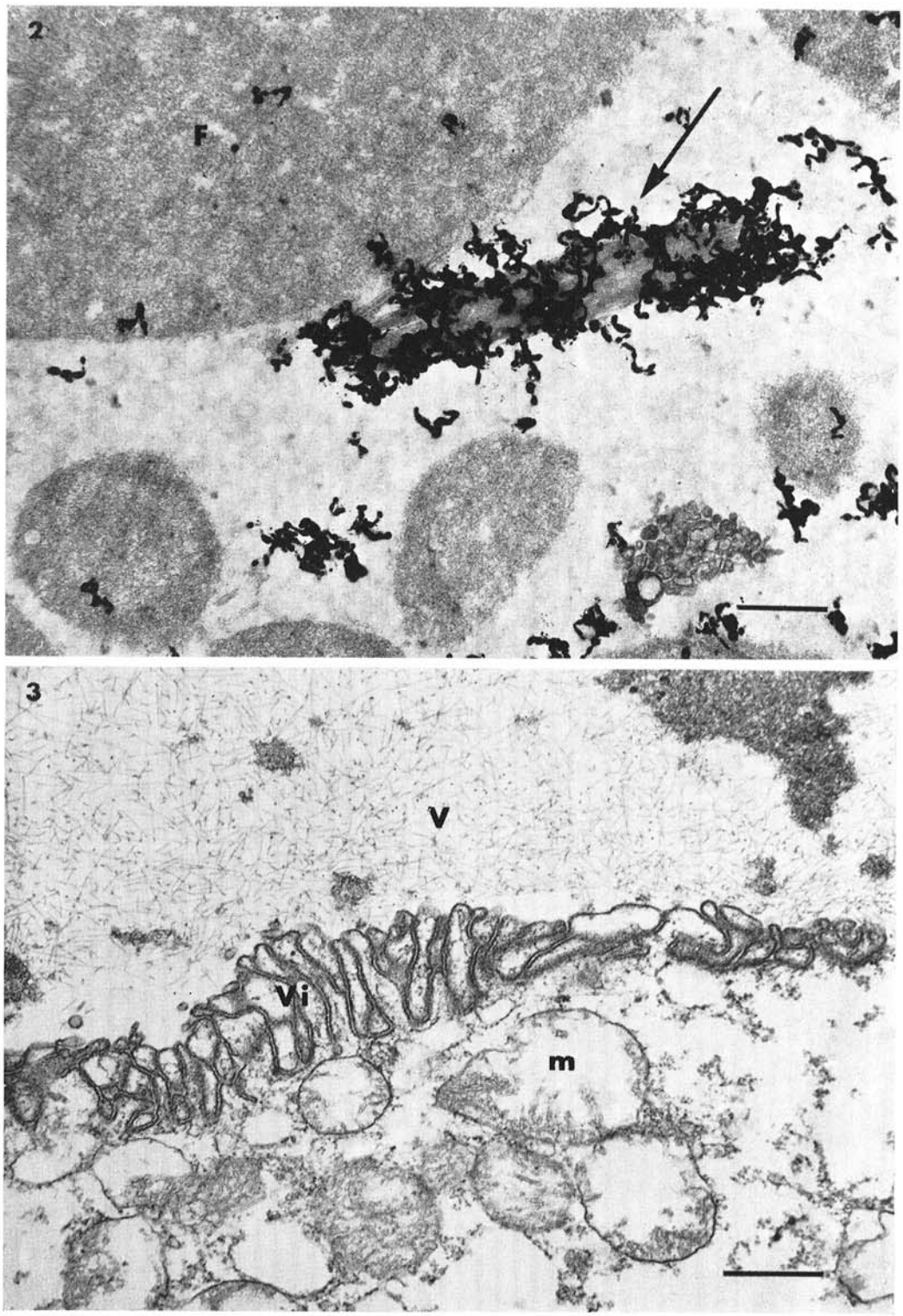

Fig. 2-3. 2) Electron microscope radioautograph of ^{125}I-labeled tobacco mosaic virus (TMV) paracrystal (arrow) in the lumen of a dissected pH 3.8 fixed first ventriculus. Radioautographic exposure, 3 months. F = food bolus. Scale = 0.5 μ. **3)** Unlabeled TMV (V) present as individual rods in the lumen of a dissected pH 3.8 fixed first ventriculus. Vi = villi of a ventriculus cell; m = mitochondrion. Scale = 0.3 μ.

wash in running water and 1-min rinse in distilled water.

RESULTS.—*Dissected aphid organs, pH 3.8 fixation.* —The first ventriculus contained large amounts of TMV (Fig. 2, 3). With the electron microscope, the virus could be seen in paracrystalline form or as individual virus rods in the lumen (Fig. 2, 3). The association with the villi in the first ventriculus was sometimes very close, but no virus particles were at any time seen inside the first ventriculus cells. In the second ventriculus, little virus could be detected in a longitudinal section after long exposures (Fig. 4), and a cross section of 1 μ showed none. No virus could be detected in dissected salivary glands or the proctodaeum or body cavities after feeding with unlabeled or labeled virus. Aphids of varying ages from nymphs to wingless adults were used. In none has TMV been seen past the gut wall and in the hemolymph. The influence of age had to be considered because Simons (18) has shown that differences exist between nymphs and adults in the same species of aphids.

A few silver grains were detected above the cells of the first ventriculus indicating intracellular label (Fig. 1, 5). This label could be attached to short virus rods or onto subunit protein produced by degradation of virus after the density-gradient centrifugation used to separate 300 micrometer rods for feeding the aphids. Since so little intracellular label was found, it is doubtful that full length virus rods were taken up by the cells. Little label appeared intracellularly or in the lumen in the first ventriculus after uptake of labeled gamma globulin by the aphids. None appeared in other parts of the digestive tract or in the salivary glands with either fixation method (pH 3.8 and pH 7.0). The lack of label cannot be explained, unless labeled antibody was not fixed and leached out during fixation and embedding. The first ventriculus at least should have contained labeled globulin after 2- to 4-day continuous access, but no label could be found even in whole body sections. The specific activity of the solution was high enough to be detected by radioautography of sections. Whatever the reason for the lack of label, apparently no globulin was digested and utilized by the aphids; otherwise it would have appeared as labeled tissue or incorporated in the cuticle.

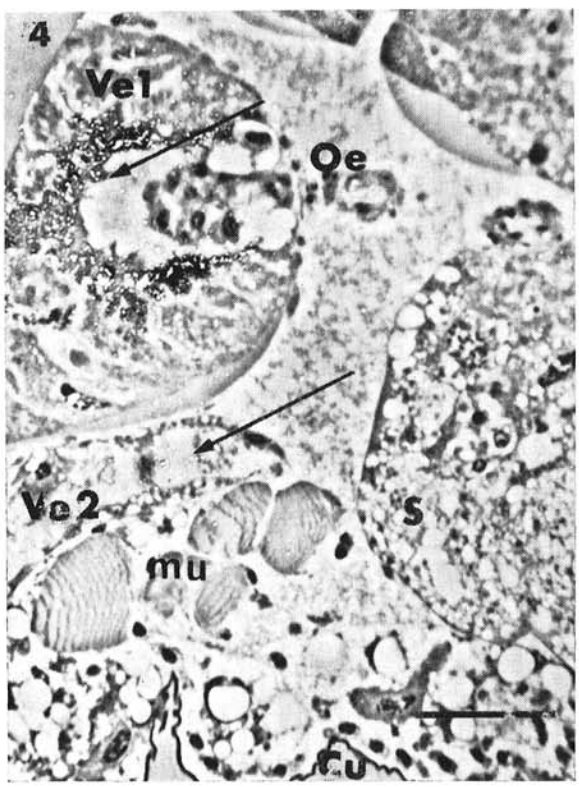

Fig. 4. Light microscope radioautograph of whole body, pH 3.8 fixed aphid fed on ^{125}I-labeled tobacco mosaic virus. Radioautographic exposure was 16 days. Heavy label (upper arrow light spots) is present in the lumen of the first ventriculus (Vel). The aphid has been sectioned frontally. The esophagus (Oe) entry into the first ventriculus can be seen. Light label (lower arrow) only is present in the second ventriculus (Ve 2). S = salivary gland frontally of the ventriculi; mu = striated muscle; Cu = cuticle. Scale = 25 μ. With 1 μ phase microscope radioautography, the silver grains are often over focused when the tissue is in focus and then appear as bright spots (17).

"Whole" body fixation at pH 3.8.—The results were the same as by fixing dissected organs at pH 3.8 (Fig. 4). It was, however, far more difficult to orient oneself in whole body aphid sections at the electron microscope level; and it proved far more time consuming than scanning sections of dissected organs.

Fixation of dissected ventriculi in pH 7.0 glutaraldehyde, followed by postfixation in osmic acid.—Virus was not found in the dissected first ventriculi or second ventriculi after fixation at pH 7.0. This was not surprising, since previous experience (16) had already shown that glutaraldehyde fixation did not fix TMV in situ in plants. Unfixed virus had ample time to exit via the cut ends during fixation and subsequent washing and postfixation of the dissected organs. Aphids used for this experiment were fed on unlabeled TMV only.

Whole body fixation at pH 7.0.—That virus had been present in aphids before dissection and fixation at pH 7.0 was shown by the fact that virus was still present in the first ventriculus after whole body fixation of aphids fed on TMV solutions. The virus, even though unfixed, apparently could not exit in the absence of cut ends, and remained in the first ventriculus (Fig. 6). No virus could be detected in the second ventriculus or proctodaeum of whole body fixed aphids. Some virus undoubtedly was present, but since the aphids in these experiments were not fed on radioactive virus, the very small amounts of virus that pass beyond the first ventriculus could easily escape detection.

Uptake of inorganic ^{125}I.—Generally, the cuticle of insects absorbs iodine (8); however, ratios of ^{131}I intermediates varied greatly within the class Insecta. Aphids fed on inorganic ^{125}I showed heavy incorporation of ^{125}I only in the cuticle (Fig. 7) and trachea. A separate paper on the iodine utilization of the green peach aphid is is preparation.

Aphids fed on ^{125}I-labeled TMV and gamma globulin showed no incorporation of ^{125}I in the cuticle,

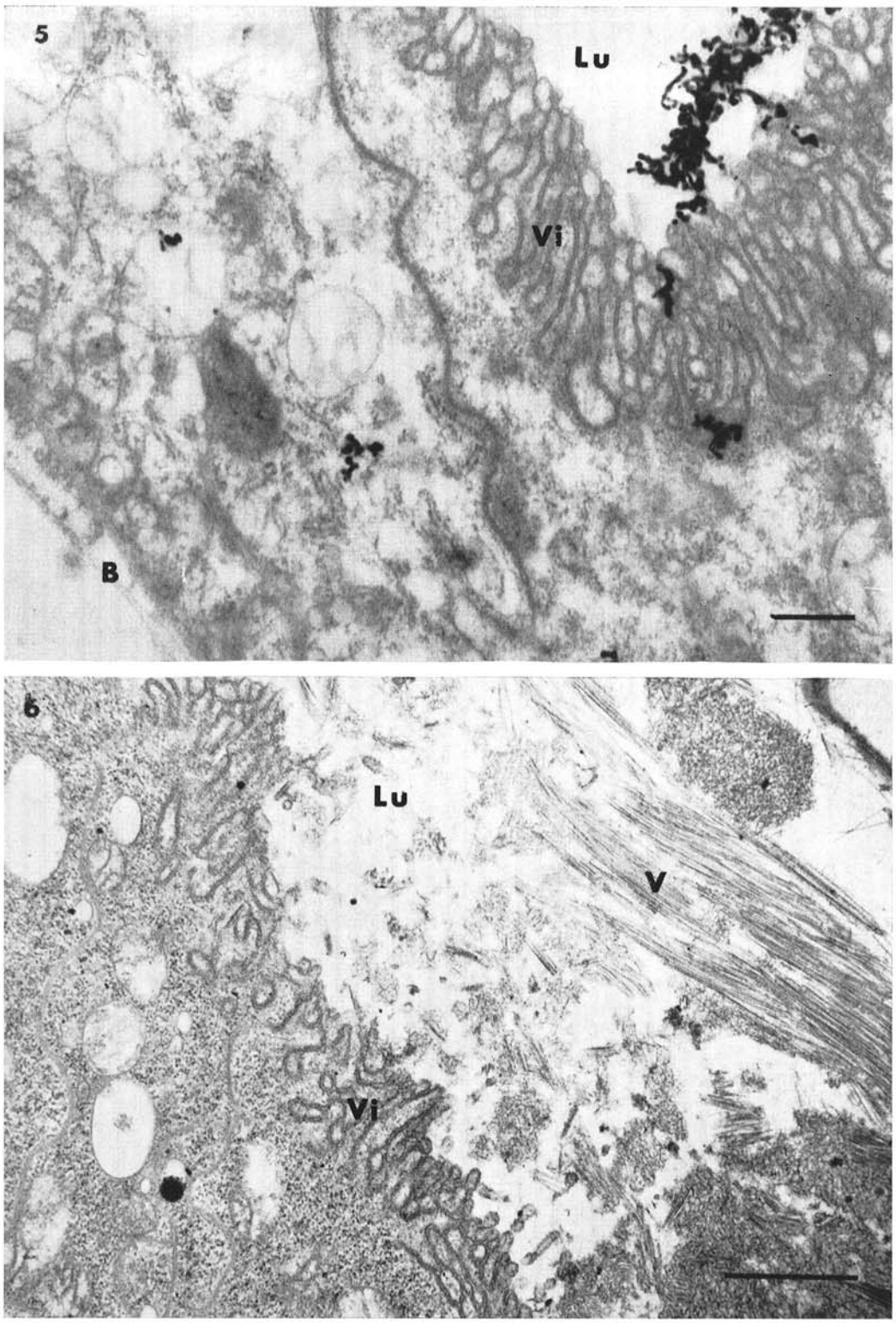

Fig. 5-6. 5) Electron microscope radioautograph of the region near the basal membrane (B) between two cells of a dissected aphid first ventriculus. Aphid fed on tobacco mosaic virus (TMV) ^{125}I. Intracellular label was higher than generally encountered background, indicating possible breakdown products of TMV. Vi = villi; Lu = lumen. Radioautographic exposure 100 days. Scale = 0.5 μ. **6)** Whole body neutral pH fixed aphid. Large amounts of TMV (v) present in the lumen (Lu) of the first ventriculus. Aphids had been fed on 300 μg TMV/ml for 2 days. Vi = villi. Scale = 0.8 μ.

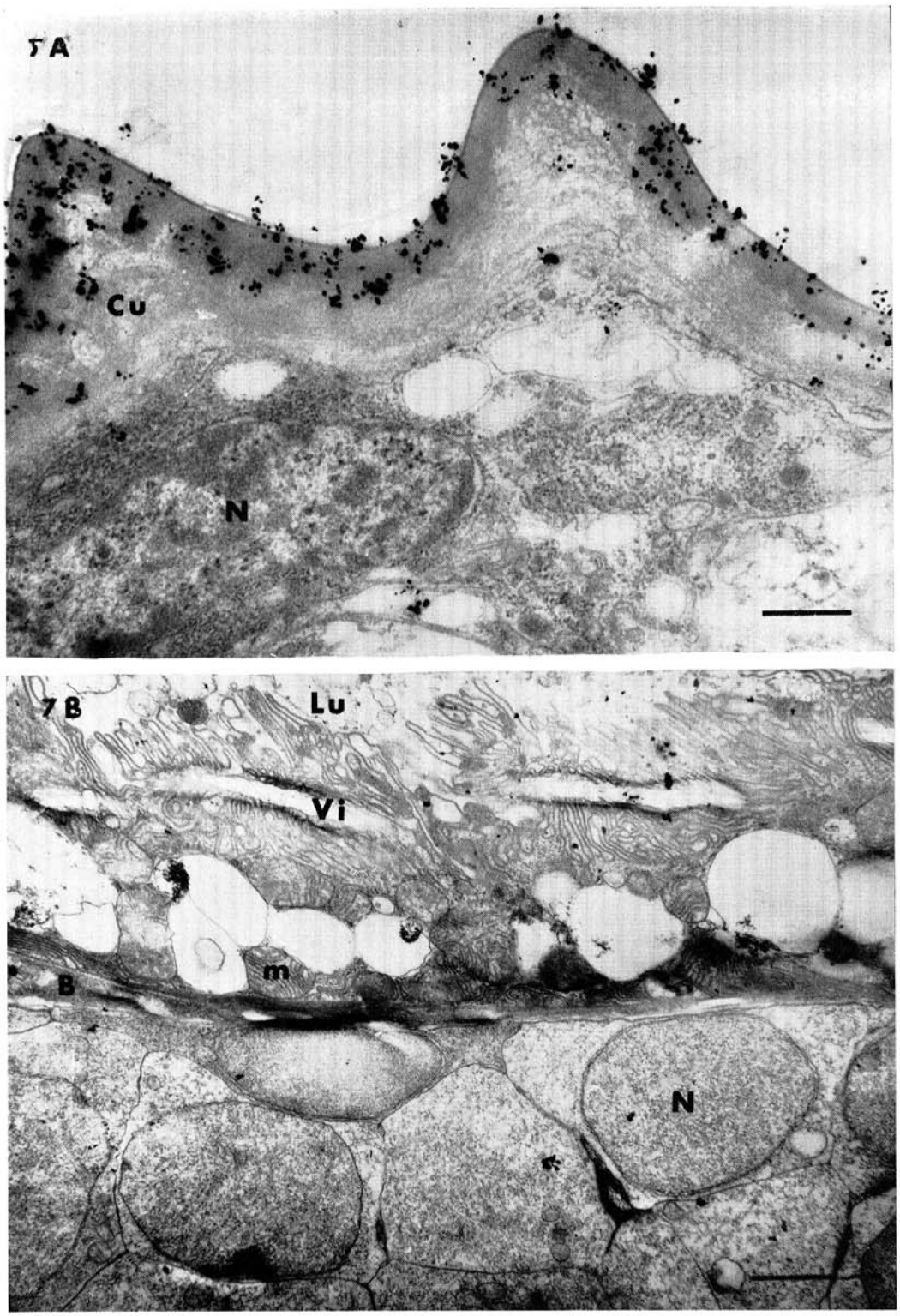

Fig. 7. A) Electron microscope radioautograph of an aphid cuticle in the thorax region showing incorporation of inorganic ^{125}I. Aphid fed on Na ^{125}I in 20% sucrose for 2 days. Radioautographic exposure, 27 days. N = nucleus; Cu = cuticle. **B)** Radioautograph of a whole body fixed and embedded aphid in the thorax region showing the close relationship often found between the first ventriculus and a salivary gland. This aphid was fed on the same solution of inorganic ^{125}I as (A) above. No incorporation of ^{125}I in tissues is evident. Radioautographic exposure, 27 days. B = basal membrane; Lu = lumen of ventriculus; Vi = villi; m = mitochondrion; N = large nucleus of salivary gland cell. Scale = 15 μ.

trachea, or anywhere else. Therefore, no ^{125}I in detectable quantities was dislodged from the labeled proteins, or it would have been encountered in sections of whole body fixed and embedded aphids.

DISCUSSION.—The above experiments were undertaken to find a general procedure that could be used to follow the passage through an insect vector of a purifiable virus. Several problems with the fixations, embedding, and scanning of sections of whole aphids in the electron microscope have been overcome by isolation of organs of interest and fixation near the isoelectric point of TMV. Aphid tissue was fixed more poorly at pH 3.8 than at neutrality, but this is a secondary consideration in a study of the localization of virus. Neutral glutaraldehyde fixation was completely unsatisfactory for fixing virus in dissected organs, just as it is unsatisfactory for fixing virus in inclusion bodies in plant cells (16). TMV found in the ventriculus of aphids fixed whole probably was not fixed and insoluble, but merely retained because it could not escape from the intact organ. It is highly desirable to use a fixative that will fix virus in dissected organs, because it is much easier to identify tissue and cells at the electron microscope level if the starting organ is known.

There is no assurance that all plant viruses will be fixed by acid fixatives. However, most rod-shaped viruses have an acid isoelectric point. Among the aphid-transmitted polyhedral viruses, both potato leaf roll virus and turnip yellow mosaic virus seem to be less soluble at a low pH than at neutrality.

A rod-shaped virus such as TMV can usually be detected in thin sections by electron microscopy. Polyhedral viruses are less easily detected, especially when they are present in low concn. The present results as well as published reports (5, 7, 13) show that aphids can take up a virus that they do not transmit, and that this virus is readily identified in parts of the digestive system. But such virus may be irrelevant to transmission. Virus that will be transmitted may be present in small amounts and in backgrounds less suitable for detection than the gut. Labeled virus particles properly fixed can be located with great precision. It is for detection of small amounts of virus, particularly polyhedral viruses, against densely staining backgrounds that microradioautography should be particularly useful.

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