Synthesis of Nucleic Acids at Infection Sites of Soybean Roots Parasitized by Heterodera glycines

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ABSTRACT

Syncytia induced in soybean roots by the soybean cyst nematode are characterized by the presence of numerous enlarged nuclei in a mass of dense cytoplasm. Observations of infected root sections made during various stages of nematode development show that syncytial nuclei are relatively quiescent in terms of deoxyribonucleic acid (DNA) synthesis. However, tritiated thymidine was incorporated in nuclei of syncytia located in both the cortical and vascular regions of infected roots. A primary site of DNA synthesis in a developing syncytium was at the leading edge of the syncytium. Nuclei of cells in this transitional zone showed high ^{3}H-methyl-thymidine uptake, signifying sites of active DNA synthesis. Phytopathology 61:395-399.

The localization of nucleic acid synthesis in syncytia induced by *Heterodera glycines* has not been reported. In the related *Meloidogyne* genus of the family Heteroderidae, studies have shown that syncytia induced by the root knot nematode cause obvious nuclear changes that can be correlated with the synthesis of DNA and RNA (14). It is well established that syncytia induced by species of *Meloidogyne* contain nuclei that undergo karyokinesis at various stages of enlargement (9). Incomplete karyokinesis without cytokinesis can give rise to the polyploid cells described by various workers (2, 7, 9, 12). In addition to direct evidence of polyploidy through chromosome counts (7), labeled precursors of DNA and RNA were used to demonstrate sites of synthesis of these nucleic acids (14). Further evidence for an increase in the levels of nucleic acids was provided through the use of specific stains for DNA and RNA (1).

In syncytia induced by *Heterodera,* no extensive karyokinesis of syncytial nuclei indicative of nucleic acid synthesis has been reported. The enlargement of nuclei and nucleoli, however, gives an indication that nucleic acid changes are taking place within the confines of the syncytial tissue during and after the incorporation of new cells through cell-wall dissolution (4, 11, 16). Mankau & Linford (10) observed that nuclei of syncytia induced by *Heterodera trifolii* show differential Feulgen staining. Anatomical studies of soybeans infected by *Heterodera glycines* revealed relatively large nuclei and dense cytoplasm within the syncytia (4). In the absence of direct evidence for an increase in chromosome number in *Heterodera*-induced syncytia, experiments were conducted to determine the sites of DNA and RNA synthesis through indirect means. This was accomplished mainly through the use of labeled precursors. Specific stains used in the study of root knot infections by others (1, 10) were used in this investigation.

The purpose of this study was to demonstrate the sites and relative amounts of DNA and RNA synthesized in syncytia and tissues of soybean roots affected by the soybean cyst nematode.

MATERIALS AND METHODS.—Susceptible, Lee, and resistant, Pickett, soybean cultivars were inoculated with larval suspensions of the soybean cyst nematode, *Heterodera glycines,* using the agar cone technique (4). This technique limits the region of infection. In one experiment, secondary root inoculations were made using the technique described for root knot infections (5).

Twenty-four hr after inoculation, the soybean roots were washed with running tap water to remove larvae that failed to enter the roots. The seedlings were transplanted to clay pots containing sand or fine (No. 4) grade vermiculite. The seedlings then were supplied with nutrient solution and allowed to grow until root samples were taken. Inoculations were made at various intervals to provide root tissues at different periods of infection. At a prescribed time after the initial inoculation, groups of soybean plants were washed with tap water to remove sand or vermiculite from the roots. Plants were then transferred to aerated nutrient solutions containing either tritium (^{3}H)-labeled thymidine or uridine with an activity of 1 to 1.25 μc/ml. The uptake period for the isotope ranged from 0.5 hr to 18 hr. The usual exposure period was 6-8 hr. Infected portions of the roots were excised and placed in vials containing either Carnoy's (1:3 acetic acid-alcohol) or FAA (Formalin-acetic acid-alcohol) fixative. After fixation, root pieces were dehydrated through a tertiary-butyl alcohol series to xylene and were embedded in 56.5 C mp paraffin. Sections were adhered to gelatin-coated slides and stored until used for radioautography or other histological procedures.

Sections for radioautography were deparaffinized in changes of xylene, rinsed in 100% ethanol, and air-dried. Slides of deparaffinized sections were coated with nuclear-track emulsion (Kodak NTB-2), and the excess emulsion was wiped from the backs and edges of the slides. After a period of 1-2 hr of air-drying in a dark

room under dust-free conditions, the slides were transferred from the drying racks to plastic slide boxes containing a small amount of a tissue paper-wrapped desiccant. The boxes were made light-tight by sealing the sides with black masking tape. The boxes were then wrapped in aluminum foil and stored at 4 C. Depending on the desired density of exposed emulsion, the slides were stored from 5-30 days. Exposed slides were brought to room temperature, developed in D-19 (Kodak) for 2 min, rinsed briefly in water, and cleared in acid fix for 10 min. After rinsing in running tap water for a minimum of 20 min, the sections were stained with gallocyanin, methyl green-pyronin, or safranin-fast green, followed by ethanol dehydration to xylene. The ethanol dehydration followed by xylene caused considerable tissue disruption. An alternative method was then used: the slides were stained, rinsed in water, air-dried, and sealed with permount and coverslips.

The following histochemical and staining techniques were used: Feulgen technique (8); modified fluorescent Feulgen (15); gallocyanin-chromalum (3, 13); methyl green-pyronin and extraction procedures for differentiating either DNA or RNA (8). In the fluorescent Feulgen technique, relative quantitation of DNA in syncytial and normal tissue nuclei was determined with an ortholux Leitz microscope equipped with a Xenon XBL 150 light source and appropriate filters. Fluorescence emitted by the specimen was recorded with a photometer.

RESULTS.—*Nuclear morphology.*—Measurement of representative root samples of 3-day-old infections showed syncytial nuclei with diameters that averaged 1.3 times larger than adjacent tissue nuclei. Syncytial nuclei of 12- and 15-day infections (Fig. 1-A, B) were approx 3 times larger in diameter than nuclei of normal tissue. Most syncytial nuclei had distorted outlines in samples taken 18 days after inoculation. Such nuclei were difficult to measure. Although irregular in outline, nuclei were large and tended to increase in size up to 21 days (Fig. 1-C). At later periods of disease development, such as 30 days, syncytial nuclei were shrunken with distorted outlines. Measurements of syncytial nuclei 12-15 days after inoculation averaged 21 μ in diam, while normal cell nuclei were about 6 μ. Measurements of syncytial nucleoli from these nuclei were often twice the size of normal-tissue nucleoli. Nucleolar size (diam) increased from 2.9 μ at 5 days after inoculation to 6.5 μ at 24 days. At periods beyond 30 days after inoculation, many syncytial nucleoli were smaller than those observed 24 days after inoculation.

Radioautography.—Radioautographs of 18 hr-infected tissues showed sites of isotope localization (Fig. 2-A). Cells with dense cytoplasm in contact with the lip region of the nematode showed very little localization of the isotope. In the same section, nuclei of cells in the vicinity of the nematode show relatively high isotope uptake, as indicated by the dense emulsion exposure over nuclei undergoing DNA synthesis. The response of roots to the early (18 hr) stages of nematode (Fig. 1-A) feeding and the normal growth pattern of the root can be compared to the cellular responses of terminal meristems (Fig. 2-B). Not only is the comparison useful for measuring relative activity of DNA synthesis, but it also provides information on the specificity of the isotopic label. The degree of isotopic label of root meristems could be regulated by the length of uptake period.

Evidence for DNA label of syncytial nuclei was

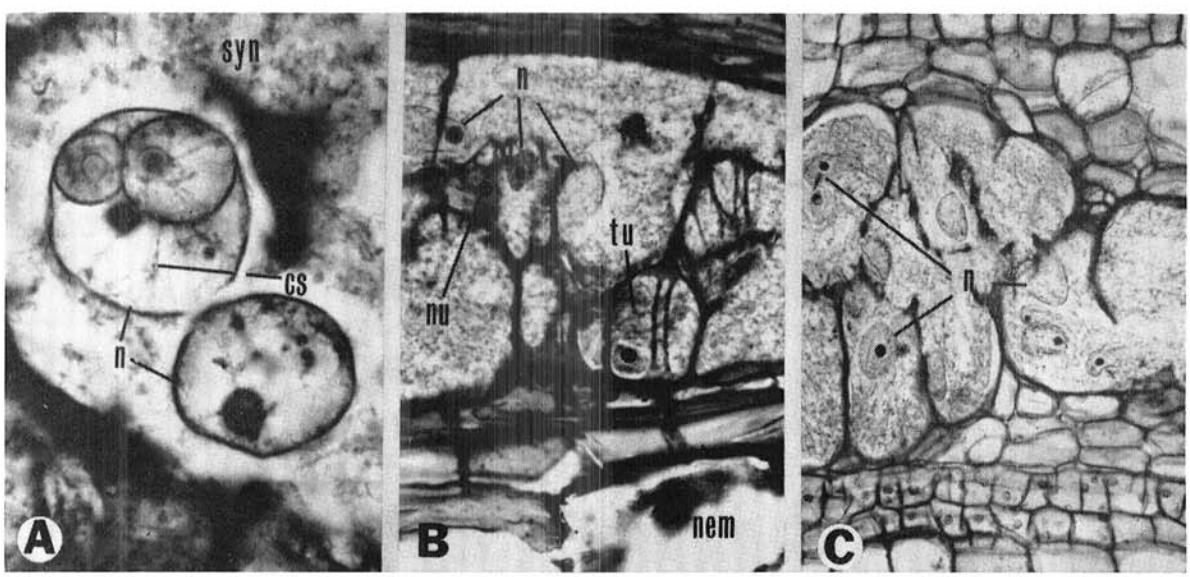

Fig. 1. Morphology of nuclei in syncytia induced in Lee soybeans by *Heterodera glycines*. **A)** Section through a syncytium, 12 days after inoculation, showing enlargement of nuclei and chromatin strands (cs). (×891) **B)** Longitudinal section through syncytium of soybean root at 15 days after inoculation. Note the irregularly shaped nuclei (n) and prominent nucleoli (nu). Tubular structures (tu) were observed at various regions of the syncytium, particularly near the nematode-feeding site. Fragments of a nematode (nem) are seen to the right of the syncytium. (×388) **C)** Tangential section into syncytium, 21 days after inoculation, showing nuclei (n) of various shapes and sizes. (×249)

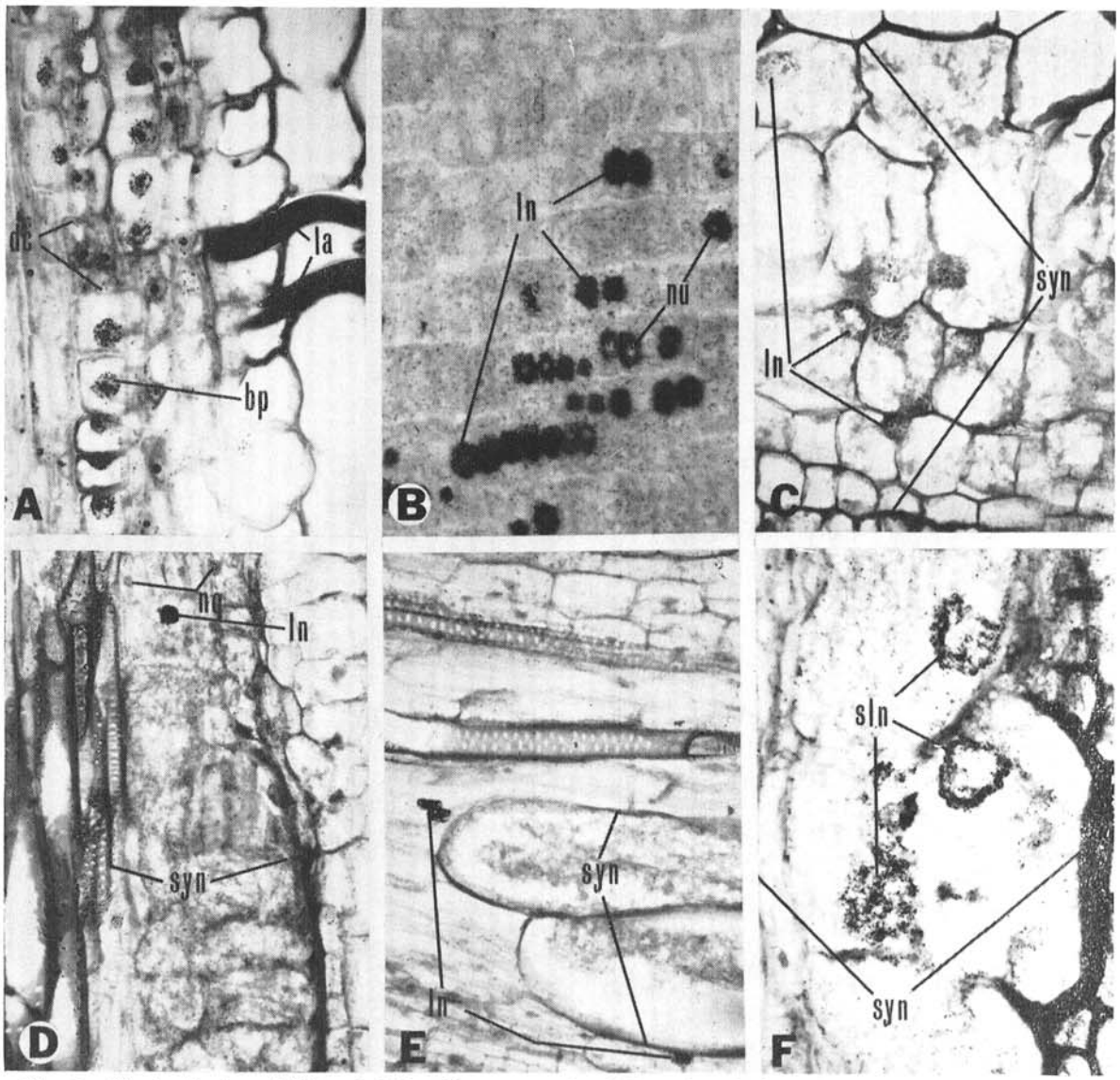

Fig. 2. The localization of ^{3}H-methyl-thymidine at normal and *Heterodera glycines*-stimulated regions of soybean roots visualized through radioautography. **A)** Second-staged larvae (la) with anterior in contact with cells of Lee soybean roots, showing dense cytoplasm (dc) and enlarged nuclei 18 hr after inoculation. Cells adjacent to region of dense cytoplasm have enlarged nuclei, and show high incorporation of ^{3}H-methyl-thymidine. Black particles (bp) over the nuclei indicate sites of emulsion exposure arising from the tritium source in the nucleus, the site of DNA synthesis. (×261) **B)** A root tip region of Pickett after 2-hr uptake of labeled thymidine, showing high incorporation of the isotope. The nucleoli (nu) appear as clear, nonlabeled regions in the center of the dense silver deposits of the emulsion caused by tritium exposure. Labeled nuclei (ln) indicated sites of DNA synthesis. (×370) **C)** A syncytium (syn) stimulated in the cortex of Lee soybean, 10 days after inoculation. The enlarged, syncytial nuclei (ln) show moderate emulsion exposure, indicating that these syncytial nuclei incorporated ^{3}H-methyl-thymidine and were synthesizing DNA at the time of sampling. (×90) **D)** A longitudinal section 12 days after inoculation through a syncytium (syn) induced in the vascular region of the soybean root. The heavy exposure of emulsion over the labeled nucleus (ln) indicates that cells along the border of syncytia are stimulated to undergo DNA synthesis. In contrast to this nucleus, there are quiescent nuclei (nq) in tissues bordering the syncytium. (×238) **E)** At 16 days after inoculation, nuclei (ln) adjacent to the nematode-induced syncytium (syn) show heavy labeling. (×238) **F)** Irregularly shaped, isotope-labeled nuclei (sln) within an extensive syncytium, 18 days after inoculation. (×370)

found in a 10-day infection of *H. glycines* in cortical tissue (Fig. 2-C). The incorporation of ^{3}H-thymidine is indicated by the silver deposits in the emulsion over enlarged syncytial nuclei.

One of the primary sites of DNA synthesis in developing syncytia was at the leading edge of the syncytium, where syncytial cytoplasm was adjacent to or in contact with normal root tissue. The incorporation rate of ^{3}H-thymidine at this interphase is striking, and probably of considerable consequence to the maintenance of the syncytium. The cell-to-cell incorporation of tissue into syncytia has been emphasized in a previous paper

(4). At 12 days after inoculation, Fig. 2-D represents the cell-to-cell change between syncytial contents and normal tissues in terms of morphology and DNA activity. Note the heavy exposure of emulsion over a nucleus which is in the transition zone of the syncytium and normal tissue. Similar sites of DNA synthesis in the form of ^{3}H-thymidine incorporation were observed for infections at various stages of nematode development (Fig. 2-E, F). The region surrounding syncytia had tissue which revealed nuclei with moderately high label 16 days after inoculation. Although many syncytial nuclei of the vascular region were quiescent in terms of DNA synthesis (absence of labeled ^{3}H-methylthymidine in nuclei), certain syncytia contained labeled nuclei (Fig. 2-F). No label was observed in the nuclei within the syncytial area in contact with some advanced stages of the female nematode (19 days), although moderately high label was found at the edge of the syncytium and at other regions of the vascular tissues. In rejuvenative tissue surrounding collapsed syncytia stimulated in the resistant cultivar, Pickett, labeled nuclei were prevalent.

Radioautographs of root sections labeled with ^{3}H-uridine showed sites of RNA synthesis in normal and infected tissue regions. Moderate RNA synthesis was detected in syncytia induced by nematodes extending from the early stages of infection until after the nematode reached maturity. A 6-day infection showed several enlarged nucleoli with good localization patterns of ^{3}H-uridine uptake (Fig. 3-A).

Gallocyanin stain showed the range of DNA present in syncytial nuclei. Nuclei of tissue bordering syncytia were often spheroid and moderately stained (Fig. 3-B), whereas many nuclei in the central region of syncytia in older infections (Fig. 3-B) were irregular in outline, enlarged, and had low-to-moderate stain, depending on the relative DNA content of each nucleus. The intensity of gallocyanin stain in syncytia of *Heterodera glycines*-infected soybeans was less than similarly stained root knot-infected soybean root sections. The presence of RNA in syncytia was demonstrated throughout the infection period. The intensity of gallocyanin stain, indicative of RNA content, corresponded to high cytoplasmic density of the syncytial contents. The RNA content and density of cytoplasm were generally high in the transitional zone of syncytia and normal tissue. Syncytial cytoplasm was easily differentiated from normal cells that were highly vacuolate.

Fluorescent feulgen.—Comparative DNA quantitation was attained, using the fluorescent Feulgen technique described by Ruch (15). Relative amounts of DNA in normal cell and syncytial nuclei were compared by recording light emitted from the fluorescent Feulgen-stained nuclei (Fig. 3-C). The ratios of the amount of light (DNA content) of syncytial nuclei to normal cell nuclei were 1.6, 1.8, 3.8, 2.8, and 3.5 for infected root samples observed at 9, 11, 13, 22, and 24 days, respectively, after inoculation.

Precautions were taken to account for background light by measuring the amount of light emitted from a region near each nucleus and subtracting this quantity from the light emitted by the nucleus. In large syncytia, measurements were made on nuclei in the central region of the syncytium to reduce the nonspecific fluorescence of lignacious tissue of the vascular system.

DISCUSSION.—Measurements of syncytial nuclei of

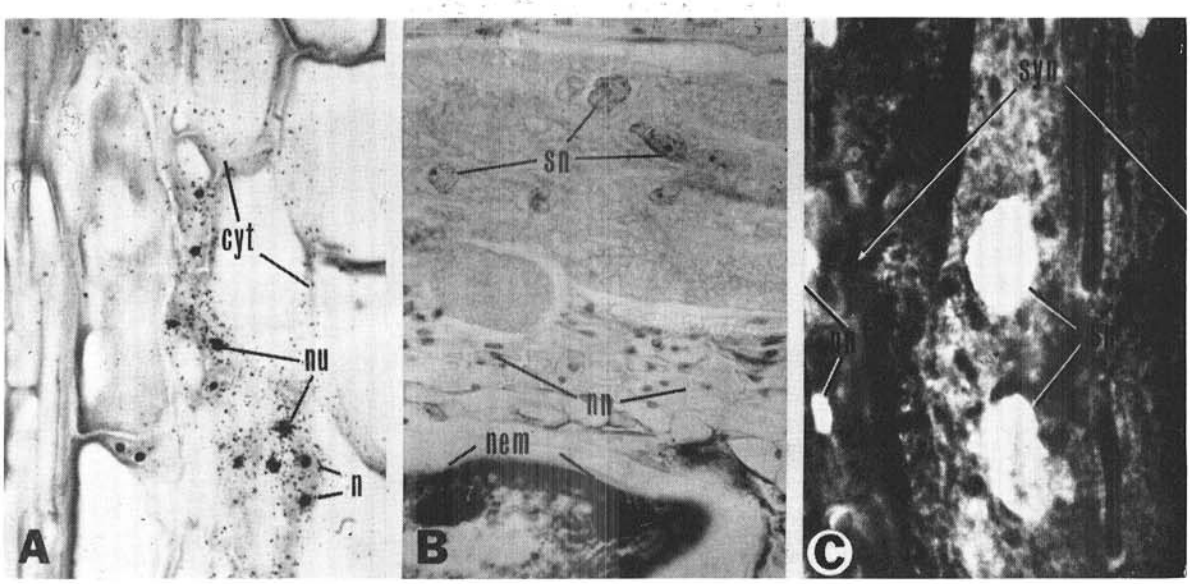

Fig. 3. Lee soybean roots inoculated with *Heterodera glycines*. **A)** A section of root 6 days after inoculation, showing a portion of a syncytium with moderate label of ^{3}H-uridine in nucleoli (nu), nuclei (n), and cytoplasm (cyt) which indicates sites of RNA synthesis. (×238) **B)** A longitudinal section of root 19 days after inoculation, stained with gallocyanin-chromalum. The nematode (nem) and root sections were stained, indicating sites of nucleic acids. Note the relative size and stain of syncytial nuclei (sn) and normal tissue nuclei (nn). **C)** Samples taken 13 days after inoculation were stained with fluorescent Feulgen technique. Fluorescence from the enlarged syncytial nuclei (sn) indicated the presence of DNA at levels higher than observed in normal tissue nuclei (nn) (×388).

soybean roots infected with *H. glycines* showed that these nuclei are considerably larger than normal tissue nuclei. Corresponding to the increased size of nuclei, the enlargement of nucleoli was noted early in disease development, and showed progressive increase in size as the syncytium developed in response to nematode feeding. Enlarged, spheroid nucleoli were observed in nuclei of various shapes and sizes. Radioautography showed ^{3}H-thymidine incorporation in syncytial nuclei at various periods of infection. It was apparent from ^{3}H-thymidine uptake and from the fluorescent Feulgen-stain reactions that DNA synthesis occurs in the nuclei of syncytia. The amount or locale of DNA synthesis in syncytia, however, showed no consistent relationship to the developmental stage of the nematode.

One could account for some nuclear enlargement if nuclei of cells adjacent to syncytia were stimulated to synthesize DNA preparatory to mitosis. Cells are usually incorporated into the syncytium through cell-wall dissolution, and stimulated nuclei may be drawn into the syncytial cytoplasm after DNA synthesis but before karyokinesis or cytokinesis. It is possible that some DNA synthesis within the syncytium is initiated in normal cell nuclei prior to or at the time of incorporation into syncytia.

Evidence for DNA synthesis as shown by radioautography was supported by photometric readings of fluorescent Feulgen-stained tissues. In the absence of mitotic figures, however, it was not possible to correlate chromosome numbers and relative emission of light to determine whether or not polyploidy had occurred.

It is apparent that, in a susceptible host, the soybean cyst nematode induces the formation of a syncytium that continues to develop during the growth of the nematode. Nuclear and cytoplasmic changes reflect the interaction between host and parasite (4, 6). But the nature of the mechanism bringing about these changes in a susceptible plant and not in a resistant plant remains unsolved. The evidence for nematode secretions mentioned in a previous paper (4) points to the need to determine changes or lack of interchange which may affect the continued development of a syncytium.

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