Microautoradiographic Studies on Host-Parasite Interactions

II. The Exchange of ^3H -Lysine Between *Uromyces phaseoli* and *Phaseolus vulgaris*

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Abstract. To study the function of bean rust haustoria either the host, *Phaseolus vulgaris*, or the parasite, *Uromyces phaseoli*, were labeled with ^3H -lysine. 24 h and 48 h after inoculation samples of the infected leaves were taken and processed for electronmicroscopic autoradiography. When the host was labeled, silver grain densities over the structures of host and parasite were compared. Very low grain densities were found over developing haustoria of the bean rust and higher grain densities over mature and old haustoria. Compared with mature haustoria, reduced grain densities were observed over intercellular hyphae. It is assumed that a flow of labeled metabolites from the host cell to the haustorium and then to the intercellular hypha took place. When sporelings originating from labeled uredospores infected the leaf, no detectable amount of ^3H -lysine or its metabolites were found to be transferred from the intercellular hyphae or haustoria to cells of the leaf parenchyma.

Key words: Bean rust — *Uromyces phaseoli* — *Phaseolus vulgaris* — Autoradiography — Electronmicroscopy — Haustorium.

When a fungal parasite such as bean rust infects a leaf, various substances accumulate in the infected area some days later (Shaw and Samborski, 1956). Hyphae, haustoria and spores of the fungus appear to take up and accumulate metabolites from the host cells. Recent autoradiographic studies suggest that a continuous flow of nutrients from the uninfected tissue via the infected tissue into fungal structures occurs (Lewis, 1973). However, we do not know what happens during the early stages of infection and which role rust haustoria play during that time (Bushnell, 1972). Furthermore, little information is available on the

kinetics of metabolite uptake during development and aging of haustoria and on the possible transfer of similar metabolites from fungus to host tissue at the early stage of infection (see Sivak and Shaw, 1970; Mendgen and Heitefuss, 1975). Such information is needed for a description of haustorial function.

In this study, ^3H -lysine was selected as model metabolite since Jäger and Reisener (1969) have shown that ^{14}C -lysine, when applied to a wheat leaf, was readily transported to the parasite and incorporated into proteins without major metabolism. These authors reported that over 84% of the detectable lysine in labeled uredospores was present as ^{14}C -lysine incorporated into proteins. Furthermore, both free and bound ^3H -lysine are readily and irreversibly fixed to the tissue during glutaraldehyde fixation (Peters and Ashley, 1967). Trace amounts of unfixed ^3H -lysine are washed out during processing and therefore unspecific labeling of cell structures is avoided (Williams, 1969). Therefore, in the present study it was assumed that lysine would be a suitable metabolite for an electronmicroscopic autoradiographic study of metabolite exchange between host and parasite without major problems of unspecific diffusion during processing. Some of the first results have already been published (Mendgen, 1977).

Materials and Methods

1. Labeling of Host Tissue

Bean plants (*Phaseolus vulgaris*, cv. Favorit) were grown in compost soil at 21°C under approximately 4000 lx fluorescent light for 16 h/day. 12 days following planting a primary leaf was removed and trimmed to a diameter of 3 cm. The petiole was then immersed in a vial containing 0.5 ml water with 350 μCi ^3H -lysine (250 mCi/nmol, L-[4,5(n)- ^3H] lysine monohydrochloride, TRA 232, Amersham-Buchler). The leaf took up the water with labeled lysine within 6 h. Then, the vial was refilled with tap water. 18 h after application of the label, the leaf was inoculated with bean rust (*Uromyces phaseoli* (Pers.) Wint. var. *typica* Arth.) uredospores (Mendgen, 1973). 24 h and 48 h after inoculation samples of $1/2 \times 2$ mm leaf tissue were fixed for 4 h with 3.5% glutaraldehyde in 0.08 M cacodylate buffer pH 7.2 with 0.5% sucrose. After washing in buffer and postfixation in

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buffered 2% osmic acid, the tissue was dehydrated with ethanol. Samples of the ethanol used for dehydration were checked in a scintillation counter for radioactivity. The tissue was embedded in resin (Spurr, 1969) when all unbound ^3H -lysine was washed out.

2. Labeling of Fungal Spores

Bean plants were raised as described above. Labeled uredospores were produced by immersing the petiole of a trimmed primary leaf ($\varnothing$ 3 cm) into 1 ml water containing 500 μCi ^3H -lysine. This leaf had been inoculated with bean rust 4 days prior to trimming and label application (Mendgen, 1973). When the ^3H -lysine had been taken up by the leaf, water with 50 mg/l benzimidazole was added. Spores, developing within the next 10 days were harvested.

Primary leaves of healthy, ten day old bean plants were inoculated with the labeled uredospores. 24 h and 48 h after inoculation samples of the leaf were excised, processed and embedded in epoxy-resin as described above.

3. Microautoradiography

Golden coloured sections were cut with a Reichert Ultracut and mounted on copper grids with a "pioloform" film (Wacker Chemie, München). The sections were stained with uranyl-acetate and lead citrate and then coated with a thin carbon film. Following coating they were covered with the Ilford L 4 photoemulsion, applied as a gelled monogranular layer after Caro and van Tubergen (1962). The grids were exposed for 1–4 months at 4°C over silica gel and developed after gold latensification in photorex-ascorbic acid developer prepared according to Salpeter and Szabo (1972). The sections were examined in a Zeiss EM 10 A electronmicroscope operating at 80 and 100 KV.

For light-microscopy, 2 μm thick sections were cut with glass knives, mounted on microscope slides, covered with the Ilford L 4 photoemulsion and developed in Agfa-Rodinal after 2–4 weeks exposure time. Photographs were taken with a Leitz microscope and interference-contrast T optics.

4. Statistical Evaluation of Grain Distribution

For the determination of ^3H -lysine uptake by bean rust, grain densities over host and fungal structures were measured and compared. Since grain density varies considerably as a consequence of variation in section thickness (Williams, 1969), exposure time and development of autoradiographs, sections for statistical analysis were treated identically. Furthermore, a "standard" was used for every autoradiograph and consisted of the grain density over the host cytoplasm including nucleus and mitochondria, but excluding plastids and vacuoles. The grain density over this "standard-area" was determined by measuring the area of the cytoplasm with a compensating polar planimeter and the silver grains were counted using the "probability circle" method (Salpeter and McHenry, 1973). Silver grains over fungal walls in contact with the host (junctional components) were not counted. The "standard" was compared with grain densities over the haustorium or over an intercellular hypha near the infected host cell in the same autoradiograph. 10–20 autoradiographs per variant were analysed and subjected to a paired t -test. The number of grains counted and grain density per variant is shown in Table 1.

Results

Uredospores of *Uromyces phaseoli* germinated and formed appressoria over host stomata 10–14 h after inoculation. From the enlarged appressorium an infection peg differentiated and grew through the guard cells into the substomatal chamber to form a substomatal vesicle; the latter gathered its cytoplasm and then elongated to form the infection hypha (Mendgen, 1978). At 24 h after inoculation, the first haustorial mother cell had already formed at the tip of the infection hypha in contact with a host cell; in most instances this haustorial mother cell was vacuolated. Within the host cell a haustorial neck and haustorial body with two nuclei had differentiated (Mendgen, 1978). Such haustoria were about 8 h old ("mature haustoria"). In some cases, stomatal penetration had occurred later and fungal development was retarded. In these instances, the haustorial mother cell had no large vacuoles, was binucleate and the haustorial body was just developing within the host cell ("developing haustoria"). At 48 h after inoculation, infection hyphae had branched and up to four haustorial mother cells with haustoria were differentiated (Mendgen, 1978). The first formed haustorium was recognized by its large diameter, many lobes and a prominent, vacuolated haustorial mother cell. These haustoria were ca. 30 h old and are referred to in this paper as "old haustoria".

Uptake of ^3H -lysine by Uromyces phaseoli

For these studies the labeled metabolite was supplied to the leaves approximately one day before inoculation with bean rust. This treatment resulted in an even distribution of labeled material within the leaf before formation of haustoria.

A developing haustorium is shown in Fig. 1. In this micrograph one nucleus of the dikaryotic haustorial mother cell is seen partially migrated into the haustorial neck. In contrast to the high silver grain density over the host cytoplasm, the fungal cytoplasm, which includes the haustorial mother cell and the haustorium, is only covered by a few silver grains. In contrast to the grain densities over developing haustoria, mature haustoria (Fig. 2) and old haustoria, including pertinent intercellular hyphae (Fig. 3), exhibited much higher grain densities.

The grain density over fungal structures was determined and compared to the grain density over the

Fig. 1. An infection hypha (*ih*), a haustorial mother cell (*hmc*), a haustorial neck (*arrow*) and a developing haustorium (*h*) in host tissue labeled with ^3H -lysine. One of the two nuclei (*fn*) in the haustorial mother cell is seen in the neck region. A high grain density over the host cytoplasm, and a very low grain density over fungal structures is evident ($\times 9600$)

Fig. 2. Mature, ca. 8 h old haustorium (*h*) with nucleus (*fn*) in a host cell. As compared with the haustorium shown in Fig. 1, it is covered by more silver grains. ($\times 12500$)

Fig. 3. An irregularly lobed, ca. 30 h old haustorium (*h*) inside a host cell. The corresponding intercellular hypha (*ih*) and haustorial mother cell (*hmc*) are above in the intercellular space. Grain density over fungal structures is lower than the grain density over the host cell. Fungal nucleus (*fn*). ($\times 7700$)

host cytoplasm (Table 1). Since the developing haustoria in the autoradiographs examined were relatively different in their morphological development, a broad variation in grain density was found. Nevertheless, these haustoria exhibited a lower grain density than mature haustoria. Mature haustoria had similar grain densities as 30 h old haustoria. However, since the older haustoria were much larger in size, the data indicate that a constant uptake of ^3H -lysine, paralleling the increase of haustorial body size, occurred. Intercellular hyphae, in comparison to 30 h old haustoria, were clearly covered by fewer grains per area unit.

^3H -lysine Transfer from Labeled Fungal Structures to the Host Cell

These experiments were performed similarly to an earlier study with ^3H -orotic acid (Mendgen and

Table 1. Grain density (%) over fungal structures in relation to the grain density over host cytoplasm, nucleus and mitochondria

Fungus	Grain density %	Grains counted	t-test
Developing haustoria	26 ± 5	350	$t = 3,11$ $P < 5\%$
Mature haustoria	36 ± 4	733	
ca. 30 h old haustoria	36 ± 3	1255	$t = 3,75$ $P < 5\%$
ca. 30 h old intercellular hypha	27 ± 2	598

Fig. 4. An intercellular hypha (*ih*) which originated from a labeled uredospore, is seen between leaf parenchyma cells 24 h after inoculation. All fungal structures including their nuclei (*fn*), are heavily labeled, however, no label over host cells is present. ($\times 7000$)

Fig. 5. Light microscopic autoradiograph of an intercellular hypha (*ih*) and two haustoria (arrows) at 48 h after inoculation. No label is seen in the cells or intercellular spaces of the leaf mesophyll. ($\times 1200$)

Heitefuss, 1975). However, in the present experiment, the uredospores were labeled with ^3H -lysine before they were allowed to germinate on an unlabeled leaf.

At 24 h after inoculation, highly-labeled infection hyphae in the intercellular space were seen in intimate contact with mesophyll cells. At this time, no silver grains over host structures were observed, indicating that movement of major amounts of labeled material into host cells had not occurred (Fig. 4). Similar results were found when evenly labeled, mature haustoria within host cells were observed (Fig. 6). At 48 h after infection, radioactivity in the fungal structures was too low for electron microscopic autoradiography and could only be demonstrated by light microscopic autoradiography. Using such methods, at 48 h following infection, silver grains were only found over hyphae and haustoria (Fig. 5).

Discussion

A study of nutrient transfer between host and parasite during development of the first haustoria can be performed by electronmicroscopic autoradiography.

After labeling the host plant with ^3H -lysine, it was assumed that a constant pool of ^3H -lysine was available to the haustorium in the infected cell. This assumption can reasonably be accepted, since Sempio et al. (1975) did not observe major fluctuations of free or bound lysine concentrations in infected leaf tissue during the early stages of pathogenesis of the bean rust fungus. The ^3H -lysine pool in host cells corresponds approximately to the standard area used in the autoradiographs as the basis for comparison of grain densities. As standard area, the host cytoplasm including nucleus and mitochondria were chosen. The plastids had to be

Fig. 6. An evenly labeled haustorium (*h*) that originated from a labeled sporeling at 24 h after inoculation. There is no label over host structures. ($\times 12500$)

excluded because they were overexposed in our autoradiographs (Salpeter, pers. comm.). Also, it appeared not useful to include the vacuoles because of the very low grain density over that area.

The quantitative evaluation of grain densities over fungal structures indicated that developing haustoria have lower grain densities than older haustoria. This suggests that a mature haustorium is required for optimal nutrient uptake. Since grain densities over mature and old haustoria were identical, it appears that a continuous flow of lysine occurred from host to parasite during haustorial development (i.e. increase in size).

The lower grain density over intercellular hyphae in comparison with adjacent old haustoria, and the relatively high grain density over the host cell structures suggests that a transfer of ^3H -lysine, or its metabolites, had occurred from the host cell into the haustorium and through the haustorial mother cell to the intercellular hypha. This observation supports the assumption that most label in the fungus, in host tissues, originated from

metabolites taken up by the haustorium. Unfortunately, the data give no indication as to whether diffusion of label from host cells into intercellular hyphae is possible by direct contact and if so, how much. However, uptake originating from such contact probably plays only a minor role since bean rust mycelium growing with dead haustoria in a labeled resistant bean, exhibited only very low grain densities (Mendgen, 1979). Although our experiments can only account for ^3H -lysine in the bean rust host-parasite system (see also Onoe et al., 1973), comparable results have been obtained in nutritional studies with other haustorial parasites such as *Bremia lactucae* (Andrews, 1975) and *Erysiphe graminis* (Martin and Ellingboe, 1978). It seems that in biotrophic parasitism a functional relationship, e.g. a developed haustorium, is needed for a normal nutrient transfer, especially of amino acids.

In comparison with the results presented here up to 48 h after infection, intense accumulation of different metabolites has been shown in fungal structures (main-

ly bean rust) several days after infection (Staples and Ledbetter, 1958; Fuchs and Tschen, 1969; Heitefuss, 1970; Favali and Marte, 1973; Manocha, 1975). At this stage of pathogenesis an increase of enzyme activities of many metabolic pathways can be measured (Heitefuss and Williams, 1976). Leakage of various metabolites begins at the fourth day after bean rust infection (Hoppe and Heitefuss, 1974). Thus, at this time after infection, the fungus may be able to take up nutrients easier and store them at a high rate.

In this study, transfer of ^3H -lysine and its derivatives from parasite to host was not detected. This corresponds to earlier work with ^3H -orotic acid (Mendgen and Heitefuss, 1975) and to results observed after infection of *Solanum tuberosum* with *Phytophthora infestans* (Sivak and Shaw, 1970). Such studies, however, cannot exclude the possibility that secretion or transfer of minor amounts of label from the parasite occurs, as it is difficult in electronmicroscopic autoradiography to differentiate a few silver grains over a large structure from background grains (Mendgen and Heitefuss, 1975). Furthermore, water soluble compounds which are not immobilized by standard fixatives cannot be demonstrated by electronmicroscopic autoradiography.

This paper demonstrates the "normal" function of a bean rust haustorium, with ^3H -lysine used as a model transfer substance. The results were the basis for experiments with rust haustoria in resistant cultivars (Mendgen, 1977, 1979).

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