Plant Physiol. (1984) 75, 13–20
0032-0889/84/75/0013/08$01.00/0

Phloem Unloading of Amino Acids at the Site of Attachment of *Cuscuta europaea*

Received for publication September 27, 1983 and in revised form January 6, 1984

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ABSTRACT

By washing out ^{14}C-solutes or ^{3}H-solutes in 0.5 mM $CaSO_4$ during a period of 5 to 6 hours, the release of amino acids by excised stem segments of broad bean (*Vicia faba* L. cv Witkiem) was studied. Three hours after pulse labeling with L-valine, L-asparagine, or α-aminoisobutyric acid (AIB), hollow stem segments were excised from the plant and incubated in a washout solution.

In experiments with valine and asparagine, stem segments parasitized by *Cuscuta europaea* released a higher percentage of labeled solutes into the bathing medium than control segments. This can be ascribed to enhanced phloem unloading at the site of attachment of *Cuscuta*. At low temperature (0°C) and after addition of *p*-chloromercuribenzene sulfonate to the bathing medium, parasitized segments did not release an enhanced percentage of labeled solutes, in comparison with control segments. These data suggest a metabolic control of the phenomenon of enhanced phloem unloading of amino acids. In experiments with AIB, an enhanced release of labeled solutes could not clearly be observed, but at the site of attachment of *Cuscuta* an accumulation of labeled solutes was measured. Accumulation of AIB in parenchyma cells, before the start of a washout experiment, will tend to obscure the phenomenon of enhanced phloem unloading.

The stem parasite *Cuscuta* has a deleterious influence, draining solutes from the phloem of the host. Using $^{14}CO_2$ it has been shown that *Cuscuta* can withdraw almost all photosynthate which normally moves from a photosynthesizing leaf to developing fruits of *Vicia faba* (22). In addition to a very efficient absorption by the parasite as suggested by the anatomical details (3), an enhanced unloading rate from the host phloem appears to be essential for the transfer of photosynthate from host to parasite (23, 25, 27, 28, 30). Evidence for enhanced phloem unloading of K^+ ions has been obtained (24, 25, 27). In experiments on sucrose and hexose release by excised stem segments, the stimulating influence of *Cuscuta* on sugar release appeared to be sucrose specific, supporting the view that the effect of the parasite is restricted to the sieve-tube system (30).

The process of enhanced phloem unloading is restricted to the parasitized region (27, 28) and is under metabolic control (23–25). For several years this case study of transport of solutes from host to *Cuscuta* was the only published evidence for energy-dependent phloem unloading (6–8), but most recently there have been several reports of a similar system operating in legume seeds (16, 21, 29, 31).

Since phloem exudates contain a large quantity of amino acids (13) and an intensive transfer of amino acids from host to *Cuscuta* could be demonstrated (P. Wolswinkel, unpublished data; 5), the question arose whether the transfer of amino acids from host to *Cuscuta* is comparable with the transfer of sucrose. This question has not been adequately resolved to date. In a previous report some relatively short experiments with L-alanine were discussed (23). Although quantitatively different, the effects of *Cuscuta* on the release of ^{14}C administered to the plant as amino acid (alanine), sucrose, or $^{14}CO_2$ were similar. However, ^{14}C from L-alanine can easily appear in sucrose (1), so new experiments were conducted to study efflux over a longer time and with other amino acids (AIB[1] and L-valine). Large differences were found between the results from experiments with amino acids and the results obtained in experiments with sucrose. Especially in the case of AIB, *Cuscuta* did not clearly enhance the release of labeled solutes during a washout experiment (5 to 6 h). These data have been discussed in a preliminary communication (27) and they have also been mentioned in a recent review on phloem unloading (6). Further work was needed to resolve the question. The present study presents new evidence that phloem unloading of amino acids is strongly enhanced by *Cuscuta*.

MATERIALS AND METHODS

Plant Material. Plants of broad bean (*Vicia faba* L. cv Witkiem) were grown in the soil of the laboratory garden during the summer season (experiments of Figs. 4, 5, and the experiment of Fig. 6 represented by closed symbols) and during the winter season in pots with potting compost in a glasshouse (experiments of Figs. 1, 2, 3, and the experiment of Fig. 6 represented by open symbols). Due to differences in growth conditions (*e.g.* temperature and light intensity), the morphology of the glasshouse-grown plants from the winter season was somewhat different from that of the plants grown in the garden. However, the effect of *Cuscuta* could be measured in both types of broad bean plants. Broad-bean plants grown in the garden were dug up carefully on the day of the pulse-labeling experiment and were transferred to the laboratory with their root systems in water.

Cuscuta europaea L. was grown as described earlier (22). When a seedling had attached itself to a host and had started luxuriant growth, long thin branches of about 30 cm were cut off from the extensively branching parasite and used for infecting other plants. In all experiments described in the present report, *Cuscuta* was attached to the host at only one internode. Preliminary experiments showed that, in recently infected plants, nonparasitized internodes of parasitized plants did not appreciably differ physiologically from the internodes of nonparasitized plants. Therefore, in most experiments, a parasitized stem region has been compared with nonparasitized regions of the same stem. The

[1] Abbreviations: AIB, α-aminoisobutyric acid; i, internode segment; PCMBS, *p*-chloromercuribenzene sulfonate.

coiling habit of *Cuscuta*, the development of haustoria at the contact surface between a twining *Cuscuta* stem and the host, and the anatomy of the haustorium has been reviewed by Kuijt (9). The haustorial coil represents that part of a *Cuscuta* stem which after coiling has intense interaction with the host stem.

In the figures the number of each internode used has been indicated. Leaves and internodes were numbered from base to apex. In the broad-bean plants used, leaves 1 and 2 were small leaves and leaf 3 was the first normal leaf (22). The internode number corresponds with the number of the leaf above it. In the figures, the sign u after an internode number means that an upper part of that internode was sampled and the sign l means that a lower part of that internode was sampled.

In the figures, data of some typical experiments are presented. In spite of quantitative variations between different experiments (depending, *e.g.* on the vigor of the parasite, the stage of development of host and parasite, the growing conditions, the number of windings of the haustorial coil around the parasitized stem segment of the host and the stem part used), the phenomena described appeared to be reproducible. The experiments at normal temperature (Figs. 1, 3, 4, 6) have been carried out at least five times.

Pulse-Labeling Procedure. In most experiments, ^{14}C-labeled L-valine, L-asparagine, and AIB were used. In double-label experiments (*e.g.* Fig. 3), ^{3}H-solutes and ^{14}C-solutes were administered simultaneously, by applying a mixture containing both types of labeled compounds. The radiochemicals were diluted in water without adding unlabeled amino acid. Label was administered to the plant via the petiole of a leaf (25, 28). To prepare for application of label, the leaflets of a compound leaf were removed and the rachis was severed a few millimeters below the site of attachment of the uppermost leaflets. Immediately after severing the rachis, the attached portion was placed in a narrow glass tube containing the ^{14}C-solutes or mixture of ^{14}C- and ^{3}H-solutes in approximately 0.3 ml of water. This solution was gradually taken up into the plant, usually within 15 to 30 min. The distribution pattern of labeled solutes introduced in this way indicated a rapid entry from the xylem into phloem cells (data omitted), in agreement with published data (*e.g.* 12).

L-Valine and L-asparagine were chosen to study the behavior of amino acids, since they are quantitatively very important in the phloem sap of several legume species and are readily transferred from xylem to phloem in a largely unmetabolized form (10, 11, 14, 15, 19). Moreover, an amino acid analog regularly used in studies on membrane transport, AIB, was also chosen (4, 17, 20). It is a physiologically inert amino acid (20) and it is intensively translocated from host to *Cuscuta* (P. Wolswinkel, unpublished data).

Since for our experiments amino acids were chosen which can be transferred from xylem into phloem in a largely unmetabolized form (see references in paragraph before), no attempt was made to determine the chemical identity of the ^{14}C- or ^{3}H-labeled solutes recovered. Although it can be expected that a very high percentage of the ^{14}C- and ^{3}H-labeled solutes recovered is unchanged from the form fed to the plant tissue, it cannot be excluded that some ^{14}C or ^{3}H is present in other solutes than the solute fed to the plant. Therefore, in this report, the terms 'solutes derived from valine' and 'solutes derived from asparagine' will be used several times.

Procedure for Obtaining Stem Segments and Incubation in a Washout Solution. Three h after an isotope solution had been administered to the plant, hollow stem segments of 3.0 cm (Table I), 3.5 cm (Figs. 3–5) or 4.0 cm (Figs. 1, 2, 6; Table II) were excised from the plant as described earlier (23). The stem segments were shaken 120 times per min in a horizontal direction at 25°C, in 100 ml Erlenmeyer flasks containing approximately 10 ml demineralized water, to which $CaSO_4$ had been added to a final concentration of 0.5 mM. In the case of parasitized stem segments, the haustorial coil was removed prior to incubation in the 0.5 mM $CaSO_4$ solution. After this procedure, only parts of the somewhat damaged small haustoria remained in the host stem segment. For our experiments, *C. europaea* was chosen since it is a species with relatively small haustoria (cf. 30).

After this first period of washing that permitted efflux of labeled solutes from the excised stem segments, the washing solutions were decanted and stored for radioisotope analysis. A second quantity of washing solution was added to each flask to further sample efflux. This process was repeated several more times. Initially, solution changes were frequent, but thereafter the frequency was reduced. After the efflux experiment, stem segments were extracted in 80% (v/v) ethanol at 80°C and the amounts of ^{14}C and, in some experiments, of ^{3}H in the soluble fraction were counted with a Packard TRI-CARB 2660 liquid scintillation spectrometer (22, 23, 25). The amounts of label in the series of washing solutions were added to the amounts remaining in the stem segments after the end of the washout experiment (solutes extracted in 80% ethanol), giving estimates of the amounts of ^{14}C- and ^{3}H-solutes present in the stem segments at the start of the washout experiment.

Radiochemicals. L-[U-^{14}C]Asparagine (5.5 GBq/mmol), [U-^{14}C]sucrose (370 MBq/mmol), L-[U-^{14}C]valine (10 GBq/mmol), L-[3,4(n)-^{3}H]valine (825 GBq/mmol), and 2-amino[1-^{14}C]isobutyric acid (1.8 GBq/mmol) were obtained from the Radiochemical Centre, Amersham, UK.

RESULTS AND DISCUSSION

General Properties of Amino Acid Efflux Patterns. In our experiments with excised broad bean stem segments, nonparasitized stem segments showed an efflux pattern (Figs. 1–6) similar to the data presented for several solutes by other authors, who have used the method of compartmental analysis (several papers cited in 24, 25). This pattern was also similar to that found in our experiments on the release of [^{14}C]sucrose (25, 27), K^+ (24, 25, 27), or Mg^{2+} (24, 26, 27) by broad bean stem segments.

After about 15 min, efflux from the free space (cell walls, intercellular spaces, lumina of nonliving xylem elements), and from the lumina of cells wounded during sampling has been completed (24, 25, 27, 28). The intracellular compartment, from which flux is almost complete after 2 to 3 h, is considered to be the cytoplasmic compartment (24, 25, 27, 28, and references therein). During the subsequent washing periods (the second half of an experiment of about 5 h), the efflux occurs principally from the slowest exchanging compartment, thought to be the vacuole of parenchyma cells and lumina of sieve elements (24, 25, 27, 28). The parallel between the vacuole of parenchyma cells and the lumen of sieve tubes has been discussed in previous reports (25, 28).

The efflux pattern of labeled solutes from a given stem segment was dependent on its distance from the leaf to which the labeled solutes are administered. During our pulse-labeling experiments with *Vicia faba*, stem segments obtained from a lower part of the stem released a greater percentage of the estimated total label than stem segments obtained from a higher part of the stem. In the figures, which show the percentage of labeled solutes remaining in a stem segment during the course of a washout experiment, the curves for segments obtained from the highest numbered internode are above the curves for segments obtained from more basal parts of the stem (Figs. 1, 3–6). Much evidence has been collected for the view that these differences between stem segments are related to differences in the distribution of labeled solutes over different compartments (free space, lumina of sieve elements, cytoplasmic and vacuolar compartments of parenchyma cells; P. Wolswinkel, unpublished data). At the start of the efflux experiment (3 h after label is administered to the

plant), in the case of more basal stem parts a higher percentage of labeled solutes seems to be present in the free space and the cytoplasmic compartment of parenchyma cells than in the case of stem parts at a shorter distance from the leaf to which label was administered.

Effect of the Parasite in Experiments with Valine. In the experiment from which data are presented in Figure 1, the release of ^{14}C-solutes was somewhat higher from i5^{u} (the nonparasitized upper part of a parasitized internode) than from i6 and i7, but the efflux pattern of all three segments was similar. The small differences among these three segments may be related to differences in their original position within the intact stem. No attempt was made to determine the chemical identity of the ^{14}C-solutes recovered, but it was assumed to be almost completely unchanged (see also comment in "Material and Methods").

The i5^{l} curve, representing the behavior of the parasitized stem part (Fig. 1), distinctly differed from the other three curves. Whereas about 24% was released from the nonparasitized segments i6 and i7 during the washout experiment and 27% from i5^{u}, the parasitized basal part of internode 5 (i5^{l}) released 44%. In accordance with data on the efflux of sucrose (23, 25, 30) and K^{+} (24, 27), the differences between efflux of label from parasitized and nonparasitized segments were more conspicuous during the last hours of the experiment than during the first periods. Apparently, the rate of ^{14}C-solute release decreased with time more rapidly in nonparasitized segments than in a parasitized one (Fig. 1). When, in nonparasitized stem segments, efflux of labeled solutes from the cytoplasmic compartment of parenchyma cells is complete (during the last part of our experiments), only efflux from the slowest exchanging compartment will contribute to efflux into the bathing medium. In parasitized stem segments, in addition to the normal efflux from different compartments (similar to the efflux from nonparasitized stem segments; this can be studied in detail by compartmental analysis), another phenomenon also seems to contribute strongly to the release of labeled solutes into the bathing medium. Since in pulse-labeling experiments as described in the present report a high percentage of labeled solutes will be present in phloem cells, the behavior of parasitized stem segments may be related to a change in the permeability of the plasmalemma of phloem cells.

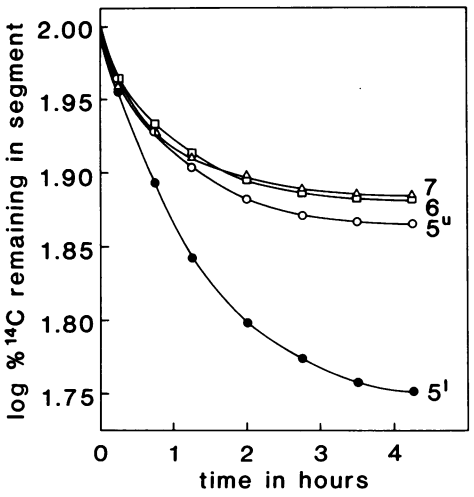

FIG. 1. Effect of *Cuscuta* on the release of ^{14}C-solutes by excised stem segments, after pulse-labeling with [^{14}C]valine via petiole of leaf 8. Data are presented on the behavior of a parasitized segment (●) and three nonparasitized segments from the same plant (○, □, △). Fresh weight of *Cuscuta* was 3 g. For the amount of ^{14}C-solutes present in an excised stem segment at the start of the washout experiment, values of 0.36 kBq (i5^{l}), 0.58 kBq (i5^{u}), 0.80 kBq (i6), and 2.27 kBq (i7) were obtained, respectively (i = internode, u = upper, l = lower).

Comparable data obtained in experiments with ^{14}C-photosynthate (23), [^{14}C]sucrose (23, 25), or unlabeled sucrose (30), have been interpreted as evidence for an enhanced phloem unloading at the site of attachment of *Cuscuta*. From data shown in Figure 1 and Table I as also from many other experiments for which data are not presented, we concluded that at the site of attachment of *Cuscuta* phloem unloading of solutes derived from valine shows a strong parallel with phloem unloading of sucrose.

In other experiments efflux of labeled solutes derived from valine was measured at 0°C (Fig. 2). With respect to the percentage of ^{14}C-solutes released during each of nine washing periods in the experiment of Figure 2, there were only very small differences between segments i4 (positioned below the parasitized stem part), i5^{l} (site of attachment of *Cuscuta*) and i5^{u} (directly above i5^{l}). In the experiment for which data are shown in Figure 2, during the last hours of the experiment efflux from i5^{l} (parasitized by *Cuscuta*) was somewhat more rapid than from i5^{u} and i4 (slope of the curves), but this cannot be used as evidence for enhanced membrane leakiness of phloem cells. The differences were only small and, in other experiments (data not presented), this pattern could not be found regularly (see also comment on Fig. 5). The release of a smaller percentage of ^{14}C-solutes from i6^{u} during the course of the experiment can be related to the shorter distance from leaf 7. As was the case in experiments on the release of sucrose (23, 25, 30) or K^{+} (24, 25), no sharp differences could be observed between parasitized and nonparasitized stem segments, when the experiments were carried out at 0°C. The fact that more ^{14}C could be measured in the parasitized stem part (i5^{l}) than in i5^{u} (legend of Fig. 2) is very typical considering the relation to the site of attachment. Normally, in nonparasitized plants, the amount of ^{14}C measured in a 4-cm stem segment decreases with distance from the leaf to which ^{14}C-solutes were administered. The accumulation of ^{14}C-solutes at the site of attachment (i5^{l}) can be attributed to enhanced phloem unloading occurring at that site, and subsequent absorption of ^{14}C-solutes from the apoplast by other cells. This phenomenon shows a parallel with the accumulation of phloem-mobile mineral elements at the site of attachment of *Cuscuta* (26, 27).

The slope of the curve which shows the behavior of i7^{l}, especially during the last 5 h of the experiment, was different from that of the other four curves (Fig. 2). Apparently, with the passage of time, release of labeled solutes derived from valine by nonparasitized stem segments was much smaller at normal temperature (25°C) than at 0°C. This can be ascribed to inhibition of the resorption of valine, at 0°C, after its efflux from intracellular compartments into the apoplast (see also data on sucrose, 30).

Although, in pulse-labeling experiments, a strong parallel could be observed between the effect of *Cuscuta* on the release of sucrose (23, 25) and the effect of *Cuscuta* on the release of valine (*e.g.* Fig. 1), important quantitative differences were measured too. In double-label experiments, more details of this difference could be studied. In all these experiments, the effect of *Cuscuta* was more pronounced in the case of solutes derived from sucrose than in the case of solutes derived from valine. In the experiment from which data are shown in Figure 3, the differences between i5^{l} (parasitized) and i5^{u} were more clear in the case of solutes derived from sucrose than in the case of solutes derived from valine. However, the accumulation of labeled solutes in i5^{l} was more clear for solutes derived from valine than for solutes derived from sucrose (legend of Fig. 3). During the course of the washout experiment, the total release of ^{3}H-solutes derived from valine from i6^{u}, i5^{u}, i5^{l}, and i4 (data not presented) was 22.4%, 26.1%, 30.6%, and 35.3%, respectively. For ^{14}C-solutes derived from sucrose these values were 6%, 14%, 27%, and 19% (for i4 no data are presented in Fig. 3).

Segment i4 released a higher percentage of ^{3}H-solutes derived

Table I. *Effect of Cuscuta on the Release of ^{14}C-Solutes by Excised Stem Segments, after Pulse-Labeling with [^{14}C]Valine*

The amounts of ^{14}C released during three successive washing periods have been expressed in a percentage of the amount present at the start of the washout experiment. In experiment A, [^{14}C]valine was administered to leaf 7 and in experiment B it was administered to leaf 8. For both experiment, data are presented on the behavior of a parasitized segment (asterisk) and two nonparasitized segments from the same plant. The length of each period (min) is presented in parentheses.

Experiment A					Experiment B				
Internode number	Total amount of ^{14}C (kBq) measured	Number of period 1 (15 min)	2[a] (120 min)	3[a] (225 min)	Internode number	Total amount of ^{14}C (kBq) measured	Number of period 1 (15 min)	2[a] (45 min)	3[a] (75 min)
i6^{u}	0.97	8.1	5.3	1.5	i8^{l}	3.67	5.6	3.0	2.0
i6^{l}*	1.45	15.6	8.2	2.5	i7^{u}*	2.96	11.2	11.0	8.7
i5^{u}	0.75	7.7	4.7	1.8	i7^{l}	1.43	8.4	5.3	3.5

[a] To restrict the dimensions of the table, data of two or more periods have been combined.

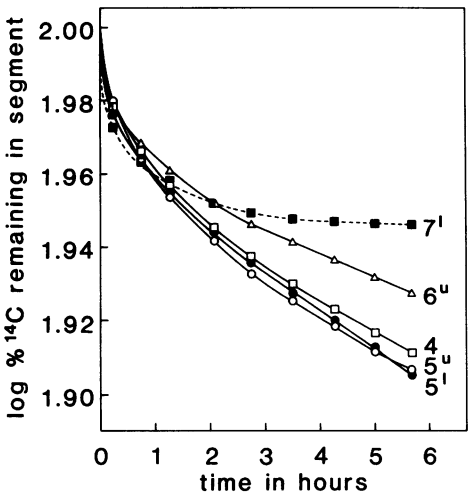

FIG. 2. Effect of *Cuscuta* and low temperature (0°C) on the release of ^{14}C-solutes by excised stem segments, after pulse labeling with [^{14}C]valine via petiole of leaf 7. Data are presented on the behavior of a parasitized segment (●) and three nonparasitized segments (○, □, △) at low temperature and also on the behavior of a nonparasitized segment at normal temperature (■---■). Fresh weight of *Cuscuta* was 10 g. For the amount of ^{14}C-solutes present in an excised stem segment at the start of the washout experiment, values of 0.89 kBq (i4), 1.75 kBq (i5^{l}), 1.32 kBq (i5^{u}), 5.32 kBq (i6^{u}), and 6.87 kBq (i7^{l}) were obtained, respectively.

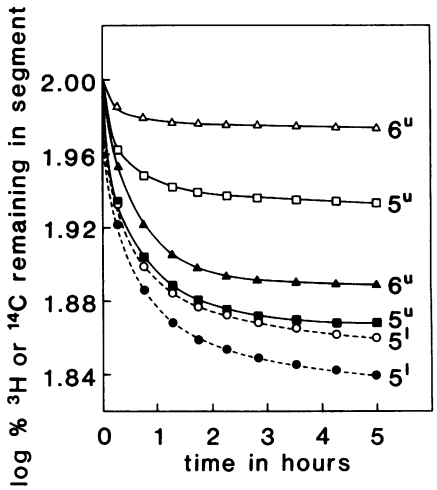

FIG. 3. Effect of *Cuscuta* on the release of ^{3}H-solutes and ^{14}C-solutes by excised stem segments, after pulse labeling with a mixture of [^{3}H] valine and [^{14}C]sucrose via petiole of leaf 7. Data are presented on the release of ^{3}H-solutes by a parasitized (●---●) and by two nonparasitized (■——■, ▲——▲) segments and on the release of ^{14}C-solutes by a parasitized (○---○) and by two nonparasitized (□——□, △——△) segments. Fresh weight of *Cuscuta* was 10 g. For the amount of ^{3}H-solutes present in an excised stem segment at the start of the washout experiment, values of 2.23 kBq (i5^{l}), 1.44 kBq (i5^{u}), and 1.50 kBq (i6^{u}) were obtained, respectively. For ^{14}C-solutes these values were 0.12 kBq (i5^{l}), 0.11 kBq (i5^{u}), and 1.23 kBq (i6^{u}), respectively.

from valine than the parasitized segment. This pattern has been observed in many experiments with amino acids. However, in experiments with labeled sucrose it has been very generally observed that the percentage of the labeled solutes present at the start of the washout experiment that is released by a parasitized segment is higher than the release by any other of the segments studied. In the case of sucrose, normally the release by a lower numbered stem segment than the site of attachment of *Cuscuta* is smaller than the release by the parasitized segment. These differences between experiments with labeled sucrose and experiments with labeled amino acids represent an important characteristic of our experimental system.

Effect of the Parasite in Experiments with Asparagine. In experiments with asparagine there was also a stimulating effect of *Cuscuta* on the release of labeled solutes (Fig. 4). The position of the curve, representing the behavior of i6, was not between those of i7 and i5 (as can be expected in nonparasitized plants), but below that of i5. However, the differences between i5 and i6 were rather small. During the last two periods of the experiment (Fig. 4) almost no differences could be measured between the release from i5 and the release from i6, the slope of the two curves becoming almost the same. The amount of ^{14}C measured in i6 was similar to the amount of ^{14}C measured in i7 (legend of Fig. 4). Since normally the amount of label measured in a stem segment decreased with distance from the leaf to which label was administered, these data point to an accumulation of label within the parasitized internode. Moreover, the relatively low amount of ^{14}C which was measured in i5 (legend of Fig. 4), is an indication for the fact that not much labeled solutes passed the site of attachment of *Cuscuta*. Apparently, solutes derived from asparagine were drained from the phloem system as a result of the infection by *Cuscuta*, a process presumably not much different from the withdrawal of ^{14}C-photosynthate (22). In the haustorial coil, detached from the parasitized segment before the start of the washout experiment, an amount of ^{14}C constituting about 50% of the amount measured in i6 (legend of Fig. 4) was present. Since the fresh weight of i6 was almost nine times as high as the fresh weight of the haustorial coil, it can be concluded from these

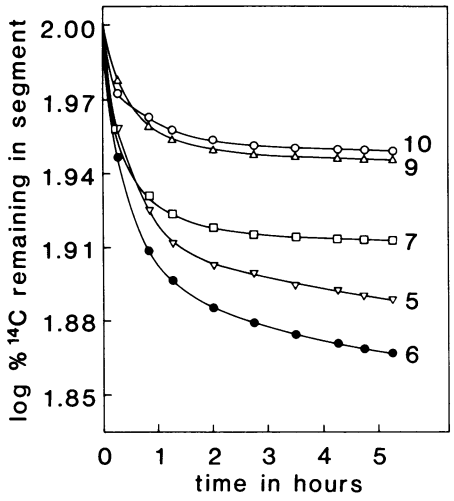

FIG. 4. Effect of *Cuscuta* on the release of ^{14}C-solutes by excised stem segments, after pulse labeling with [^{14}C]asparagine via petiole of leaf 9. Data are presented on the release from a parasitized segment (●) and from four nonparasitized segments (▽, □, △, ○). Fresh weight of *Cuscuta* was 2 g. For the amounts of ^{14}C-solutes present in an excised segment at the start of the washout experiment, values of 0.84 kBq (i5), 3.99 kBq (i6), 4.05 kBq (i7), 18.46 kBq (i9), and 3.43 kBq (i10) were obtained, respectively. In the extract of the haustorial coil of *Cuscuta* (fresh weight of 0.14 g) 1.99 kBq ^{14}C could be detected.

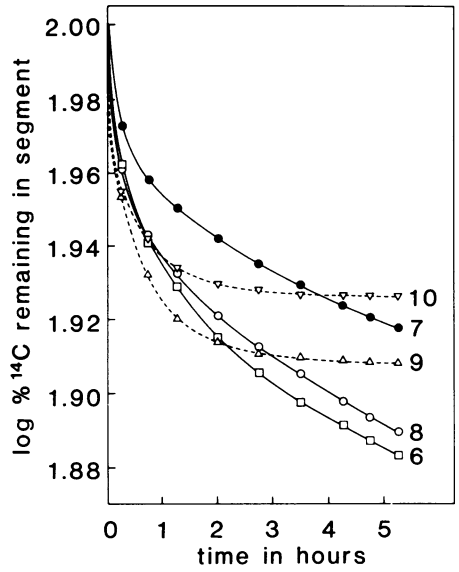

FIG. 5. Effect of *Cuscuta* and low temperature (0°C) on the release of ^{14}C-solutes by excised stem segments, after pulse labeling with [^{14}C] asparagine via petiole of leaf 10. Data are presented on the release from a parasitized segment (●——●) and from two nonparasitized segments (□——□, ○——○) at low temperature and also on the release from two nonparasitized segments at normal temperature (△- - -△, ▽- - -▽). Fresh weight of *Cuscuta* was 5 g. For the amount of ^{14}C-solutes present in an excised stem segment at the start of the washout experiment, values of 0.66 kBq (i6), 6.00 kBq (i7), 5.87 kBq (i8), 9.14 kBq (i9), and 11.87 kBq (i10) were obtained, respectively. In the extract of tissues of *Cuscuta* 27.30 kBq ^{14}C was measured.

data that during the first 3 h of the experiment, before the stem segments were excised, ^{14}C-solutes derived from asparagine became much more concentrated in a stem part of *Cuscuta* (the haustorial coil; see "Materials and Methods") than in the parasitized segment of the host. This observation supports the view that ^{14}C-solutes derived from asparagine were very intensively translocated from host phloem into tissues of the parasite.

When efflux of solutes derived from labeled asparagine was measured at 0°C, no enhanced release of ^{14}C-solutes from the parasitized segment could be measured (Fig. 5), just as was found for valine (Fig. 2). In the experiment of Figure 5, there was even an opposite trend. Segment i7 released a lower percentage of ^{14}C-solutes than i6 and i8 and during the last 2 h of the washout experiment the slope of the curve of i7 was different from those of i6 and i8. The relatively high amount of ^{14}C measured for the parasitized segment in comparison with the value measured for a stem segment obtained from a higher part of the stem, that was discussed for labeled solutes derived from asparagine (Fig. 4), was even more clear in this experiment (legend of Fig. 5). The amount of ^{14}C measured in i7 was somewhat higher than the amount measured in i8. The amount of ^{14}C measured in i6 (legend of Fig. 5) illustrated that only a very small percentage of labeled solutes could pass the site of attachment of *Cuscuta* (see also discussion of experiment of Fig. 4). In the tissues of *Cuscuta* there was almost five times the amount of ^{14}C-solutes that were found in i7, suggesting a very intensive transfer of ^{14}C-solutes from host stem to parasite.

The slope of the curves which show the behavior of ^{14}C-solutes derived from asparagine for segments i9 and i10, is different from that of the other three curves (Fig. 5), especially during the last 4 h of the experiment. Just as discussed for experiments with valine, this can be attributed to an inhibition of the resorption of solutes derived from asparagine, at 0°C, after its efflux from intracellular compartments into the apoplast.

In some experiments in which the effect of PCMBS (2, 8) on the release of ^{14}C-solutes derived from asparagine was investigated in both parasitized and nonparasitized stem segments, results were obtained that were similar to the results obtained in experiments on the effect of 0°C (data not shown). After addition of 2.5 mM PCMBS to the washout solution, no stimulating effect of *Cuscuta* on the release of ^{14}C-solutes derived from asparagine could be measured. In one experiment, the release from a parasitized segment was somewhat smaller than the release from nonparasitized segments which also had been treated with PCMBS (cf. i7 in Fig. 5). Also, especially during the last hours of a washout experiment, the release from nonparasitized segments was higher in the presence of PCMBS than in a 0.5 mM $CaSO_4$ solution. All these data show a strong parallel between the effect of 0°C and the effect of PCMBS.

Effect of the Parasite in Experiments with AIB. In washout experiments with [^{14}C]AIB it was very difficult to demonstrate a clear effect of *Cuscuta* on the release of labeled solutes from excised stem segments. Especially our early experiments with AIB led us to the conclusion, reported in a previous communication, that no clear evidence for an enhanced phloem unloading could be found (27). Data from a typical experiment are shown in Table II. However, these data seemed to be in conflict with several later observations. In many experiments with [^{14}C]AIB, AIB was translocated from host to parasite very intensively, similarly to the behavior of ^{14}C-photosynthate (22) or [^{14}C] sucrose. In some experiments with plants strongly parasitized by *Cuscuta* to the point that the growth of the stem apex had ceased, a [^{14}C]AIB solution was administered to a small part of a leaflet from which, locally, the epidermis had been removed. After a translocation period of 5 h, host and parasite were collected, lyophilized, and prepared for autoradiography. Autoradiographs showed that ^{14}C was restricted to the leaflet to which the [^{14}C] AIB solution had been administered, to the petiole, to that part of the host stem which was situated between the leaf fed with [^{14}C]AIB and the site of attachment of *Cuscuta* (site of attachment was some distance below the fed leaf), and to the tissues of *Cuscuta* (data not shown). In control plants, the same age as the parasitized plants but without *Cuscuta*, the normal distribution

Table II. *Effect of Cuscuta on the Release of ^{14}C-Solutes by Excised Stem Segments, after Pulse Labeling with [^{14}C]AIB*

The amounts of ^{14}C released during three successive washing periods have been expressed in a percentage of the amount present at the start of the washout experiment. [^{14}C]AIB was administered via petiole of leaf 7. Data are presented on the behavior of a parasitized segment (asterisk) and three nonparasitized segments of the same plant.

Internode Number	Total Amount of ^{14}C (kBq) Measured	Number of Period		
		1 (15 min)	2[a] (120 min)	3[a] (225 min)
i6[l]	4.45	4.6	2.5	2.4
i5[u]	3.40	3.8	3.0	2.2
i5[l]*	3.08	3.3	3.4	3.0
i4[u]	1.70	6.3	6.1	3.2

[a] To restrict the dimensions of the table, data of four periods have been combined.

pattern for assimilates was found. Labeled compounds moved downward to the root and up to the stem apex and developing leaves. In parallel experiments with [^{14}C]sucrose similar results were obtained. From these experiments it could be concluded that transport of AIB from host to *Cuscuta* shows a strong parallel with transport of sucrose when these solutes are applied to a small part of a leaflet from which the epidermis has been removed, in plants strongly parasitized by *Cuscuta*.

It was observed regularly that ^{14}C derived from [^{14}C]AIB was much more concentrated in the parasitized part of that internode than in other parts of the same internode. In a typical experiment, the parasitized part of an internode (1 cm) contained 637 Bq ^{14}C, whereas the adjacent 1-cm internode piece from directly above the parasitized part contained 195 Bq ^{14}C; 139 Bq ^{14}C could be detected in the internode piece 1 to 2 cm above the parasitized part.

Figure 6 shows results of two experiments in which the release of [^{14}C]AIB from a parasitized stem segment was compared with release from controls. In experiment A (open symbols), during the first hours of the experiment, the release of ^{14}C-solutes from the parasitized segment was smaller than the release from all nonparasitized segments studied (only data from two nonparasitized segments are shown in Figure 6). These results show a parallel with the first period of Table II. During the last hours of the experiment, however, a somewhat enhanced release was observed, with the slope of the curve of i4 different from those of i5 and i6. In i4 a larger amount of ^{14}C could be detected than in i5 (legend of Fig. 6), indicating an accumulation of AIB in the parasitized segment (see also comment on Fig. 5). Moreover, the small amount of ^{14}C which was measured in the basal part of internode 6 (legend of Fig. 6), is an indication for the fact that not much labeled solutes could pass the site of attachment of *Cuscuta*, presumably as a result of strong phloem unloading at the site of attachment (see also comment on Figs. 4 and 5). In experiment B (closed symbols), during the first hours of the experiment the release of ^{14}C-solutes from the parasitized segment was higher than normal. Normally, in nonparasitized plants, the release from i7 would have been smaller than from i6, but during the last hour of the experiment the release from i7 became somewhat smaller than from control segments (indicated by the slope of the curve of i7). The amount of ^{14}C measured in i7 was higher than the amount of ^{14}C measured in i8, indicating an accumulation of ^{14}C in the parasitized segment (see also experiment A).

Summarizing the data from Table II and Figure 6, it can be concluded that in parasitized segments the release of labeled AIB was somewhat different from the release by control segments. However, a clear stimulaton of AIB release could not be demonstrated.

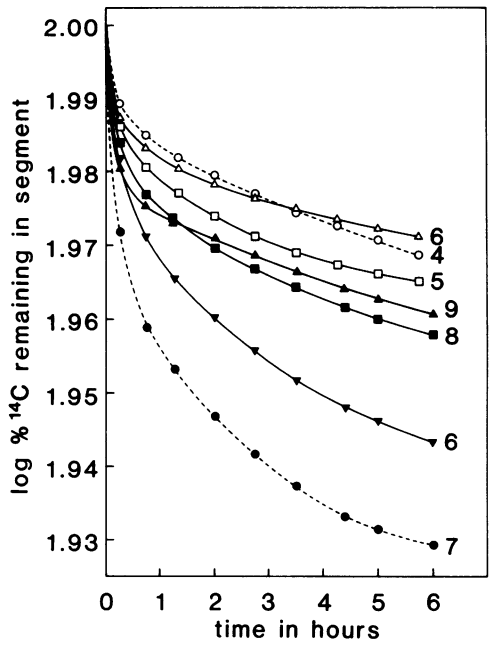

FIG. 6. Effect of *Cuscuta* on the release of ^{14}C-solutes by excised stem segments, after pulse labeling with [^{14}C]AIB. Data of two experiments are shown. In experiment A (open symbols) labeled AIB was administered via petiole of leaf 7 and *Cuscuta* was attached to the central part of internode 4 (○---○). In experiment B (closed symbols) labeled AIB was administered via petiole of leaf 8 and *Cuscuta* was attached to internode 7 (●---●). Fresh weight of *Cuscuta* was 7 g in experiment A and 16 g in experiment B. For the amount of ^{14}C-solutes present in an excised segment at the start of the washout experiment, values of 1.89 kBq (basal part of internode 4, below the parasitized segment; from this stem part no efflux data are shown), 7.27 kBq (central part of internode 4, presented as i4 in the figure), 5.49 kBq (i5), and 11.41 kBq (i6) were obtained for experiment A, respectively. For experiment B, these values were 2.65 kBq (i6), 7.70 kBq (i7), 5.04 kBq (i8), and 5.83 kBq (i9), respectively.

Differences between Valine, Asparagine, and AIB. In this report, data have been presented on three different solutes. With respect to the release of labeled solutes from excised stem segments, the stimulating effect of *Cuscuta* could be observed most clearly for valine. Here the data show a strong parallel with data on sucrose in previous reports (23, 25, 27, 30). With respect to the accumulation of labeled solutes in the parasitized segment, the effect of *Cuscuta* could be observed most clearly for AIB. Here the data show a strong parallel with data on the accumulation of Mg at the site of attachment (26, 27).

The differences in the release of labeled solutes between valine, asparagine, and AIB can presumably be explained as a result of differences in the distribution of labeled compounds over different compartments (*e.g.* lumina of sieve elements, cytoplasmic and vacuolar compartments of parenchyma cells). The strong accumulation of ^{14}C-solutes at the site of attachment of *Cuscuta* regularly observed in experiments with [^{14}C]AIB can be attributed to enhanced phloem unloading at that site, and subsequent absorption of ^{14}C-solutes from the apoplast by other cells (see also comment on Fig. 2). At the start of washout experiments, 3 h after pulse labeling, a higher percentage of labeled solutes was probably present within parenchyma cells in the case of AIB than in the case of valine and asparagine. The stimulating effect of *Cuscuta* on sugar release by cells of excised stem segments is sucrose specific, supporting the idea that the stimulating influence of *Cuscuta* on sugar release is restricted to the sieve-tube system (30). A parallel situation can be expected for amino acids.

Moreover, evidence has been presented that the efflux of solutes from parenchyma cells is smaller at the site of attachment of *Cuscuta* than in control segments (26, 27, 30).

In our view, parenchyma cells are not involved in the phenomenon of enhanced assimilate unloading into the apoplast, which occurs in a parasitized segment. Moreover, although both vacuolar compartment of parenchyma cells and the intracellular compartment of sieve elements represent the slowest exchanging compartment (25, 28), some quantitative differences have been observed. We have collected evidence that efflux from the vacuole of parenchyma cells is smaller than efflux from the intracellular compartment of sieve elements (P. Wolswinkel, unpublished data). The effect measured for a parasitized stem segment as a whole is the average of the behavior of all cells and all parts of that stem segment. It can be expected that, at the site of attachment of *Cuscuta*, there is a strongly enhanced release of labeled AIB from the sieve-tube system. However, the presence of an enhanced percentage of labeled AIB within parenchyma cells, in the parasitized segment at the start of a washout experiment, will tend to decrease the percentage of labeled AIB released into the apoplast, in comparison with the release from nonparasitized stem segments. This phenomenon may obscure the phenomenon of enhanced phloem unloading of AIB into the apoplast. When the metabolically controlled enhanced release of labeled solutes from a parasitized segment, thought to be unloading from typical phloem cells, was inhibited by low temperature or PCMBS, in some experiments a smaller release was observed in the case of a parasitized segment than in the case of control segments (segment i7 in Fig. 5, in comparison with i6 and i8, in an experiment with asparagine; see also comment on the effect of PCMBS in experiments with asparagine). These results from experiments with asparagine can also be explained by the presence of a higher percentage of label within parenchyma cells, at the start of a washout experiment.

Metabolic Control of Enhanced Amino Acid Unloading. In experiments with valine (Fig. 2) and asparagine (Fig. 5) it could be demonstrated that the enhanced release from parasitized segments is under metabolic control (see also comment on the effect of PCMBS in experiments with asparagine and discussion of low-temperature treatment of the sink region; 7). At a low temperature (0°C) and in the presence of PCMBS, a parasitized segment did not release a higher percentage of labeled solutes than inhibitor-treated nonparasitized segments. Similar data have been presented for sucrose (23, 25, 30). Observations on the metabolic control of phloem loading show also a strong parallel between sucrose and amino acids (18).

Evidence for Resorption of Amino Acids, after the Release into the Apoplast of Excised Stem Segments. During the course of a washout experiment, the release of amino acids into the solution bathing excised stem segments decreased markedly. In the case of nonparasitized segments, this could be seen very clearly during the last hours of an experiment. When inhibitors such as low temperature (Figs. 2 and 5) or PCMBS (experiments with asparagine) were added, this strong decrease was not found. Presumably, the steadily decreasing amino acid release, which is found in the absence of inhibitors, is related to a stimulation of resorption processes during the course of a washout experiment (see also data on sucrose; 30).

The possibility could be suggested that factors such as low temperature and PCMBS may affect membrane structure and increase the general permeability of cell membranes in stem segments, resulting in an enhanced efflux of amino acids, as opposed to the view of an inhibition of resorption. However, the fact that efflux of labeled amino acids and sucrose from other parts of *Vicia faba* plants (the seed coat of developing seeds, in experiments on the metabolic control of phloem unloading) could be inhibited strongly by the same inhibitors (29), presents evidence against a general larger permeability of broad bean cell membranes in the presence of these inhibitors.

The stem parasite *Cuscuta* only influences a part of the sieve tube system in stem segments (27, 28). The effect measured for a parasitized stem segment as a whole is the average of the behavior of all parts of that stem segment. At the attachment site of *Cuscuta*, the effect on amino acid unloading will be much stronger than the effect measured for the stem segment as a whole. Moreover, resorption of amino acids from the apoplast (*e.g.* by parenchyma cells) will reduce the effect of *Cuscuta* further.

Parallel between the Site of Attachment of *Cuscuta* and Seed Coats of Developing Legume Seeds. Our data on the enhanced unloading of sucrose (23, 25, 27, 30) and amino acids (this report) show a strong parallel. In experiments on the metabolic control of phloem unloading from the seed coat of *Vicia faba* (29) and *Pisum sativum* (31), a parallel could also be observed between sucrose and amino acids.

In parasitized stem segments, in addition to the normal efflux pattern from different compartments as can be studied by compartmental analysis, also metabolically controlled unloading contributes strongly to the release of labeled solutes into the bathing medium. The behavior of stem segments parasitized by *Cuscuta* shows a strong parallel with that of control seed-coat halves obtained from developing legume seeds and the behavior of control stem segments and inhibitor-treated parasitized segments shows a parallel with that of inhibitor-treated seed-coat halves (29, 31; see comment on Fig. 4 in 31). The effect of inhibiting agents (PCMBS, low temperature, KCN) on assimilate unloading from the seed coat, observed in experiments with excised seed-coat halves, was similar to the effect measured in experiments with attached seed coats (29, 31). Our experiments with seed coats, in which the results obtained with excised seed coats were similar to the results obtained with attached seed coats, confirm the usefulness of excised stem segments in the study of phloem unloading (cf. 29). In experiments on the metabolic control of ^{14}C-photosynthate unloading from the seed coat of developing legume seeds, a parallel could also be observed between attached seed-coat halves (21) and excised seed-coat halves (16). All these data lead to the conclusion that the process of phloem unloading at the site of attachment of *Cuscuta* is similar to processes occurring in the seed coat of developing seeds. The process of enhanced phloem unloading observed in stem segments parasitized by *Cuscuta* shows characteristics of a more generally occurring phenomenon.

Acknowledgments—We thank Frouke Kuyer and Yvonne van Berkel for making the figures and Lilibeth van Rhijn for typing the manuscript.

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