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Quantitative Analysis of Photosynthate Unloading in Developing Seeds of *Phaseolus vulgaris* L.[1]

I. The Use of Steady-State Labeling

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ABSTRACT

The pathway and kinetics of photosynthate unloading in developing seeds of bean (*Phaseolus vulgaris* L.) were investigated using steady-state labeling with $^{14}CO_2$. The continuous assimilation of $^{14}CO_2$ at constant specific activity produced stable tracer fluxes that facilitated straightforward analyses of photosynthate import and unloading in developing seeds. The kinetics of tracer equilibration within intact seeds were compatible with a symplastic route of photosynthate unloading in the seed coat. The import and partitioning of tracer within seeds were partially disrupted by the surgical excision of the distal halves of seeds as practiced during the preparation of "empty" seed coats for perfusion.

The mechanisms that regulate photosynthate allocation within developing legume seeds have been the focus of many recent studies (for reviews see refs. 16, 25, 30). Photosynthates accumulated by the embryo must first be unloaded to the seed coat apoplast because the seed coat and embryo of legume seeds are not symplastically linked (25). The pathway of unloading to the seed coat apoplast has not been conclusively determined. Theoretically, photosynthates could unload directly from the phloem to the apoplast (apoplastic pathway) (29, 30), or they could move symplastically from the phloem throughout the tissues of the seed coat before unloading to the apoplast (symplastic pathway) (13, 16, 17, 21). The symplastic unloading pathway has received the most support.

The study of photosynthate unloading from legume seed coats has been advanced by the development of the "empty seed coat technique," in which the embryo of a developing seed is removed through an incision in the seed coat and a perfusion solution (or agar trap) is used to collect materials released from the seed coat for subsequent analysis (20, 26, 31). The use of the empty seed coat technique is complicated by the possibility that the surgical procedure, or the introduction of a solution into the seed coat "cup," may disturb phloem import into the seed coat and/or the process of unloading to the seed coat apoplast (15). Early studies using pulse-labeling of leaves (or petioles) indicated that the total amounts of labeled photosynthates imported to perfused and intact seeds were similar (20, 26, 31). However, quantitative comparisons of pulse-label accumulation by perfused *versus* intact seeds are unreliable when made over long time periods because the tracer fluxes produced by this technique are not sustained over time.

Many problems inherent in pulse-labeling are circumvented by the use of steady-state labeling techniques. Steady-state labeling has been used for the quantitative analysis of various aspects of carbon partitioning in plants because the stable tracer fluxes produced by the continuous assimilation of $^{14}CO_2$ at constant specific activity result in relatively simple tracer kinetics at the sink (7, 9).

This study has three purposes. First, the utility of steady-state labeling methods for the study of photosynthate transport into and within developing seeds of bean (*Phaseolus vulgaris* L.) will be demonstrated. Second, the import of ^{14}C into surgically modified seeds (as required for the empty seed coat technique) will be measured to determine whether the fluxes of photosynthate within cut seeds are significantly different from those within intact seeds. Third, the properties of tracer equilibration within intact seeds will be used to examine the pathway of phloem unloading in seed coats.

MATERIALS AND METHODS

Plant Material

A determinate red kidney bean cultivar (*Phaseolus vulgaris* L., cv Redkloud, obtained from Dr. Donald Wallace, Department of Plant Breeding, Cornell University; also available from Agway, Syracuse, NY) was grown in two-gallon pots filled with a peat moss:vermiculite (1:1, v/v) mix in the greenhouse during the winter and spring of 1988. Supplemental lighting provided by 750 W mercury halide lamps (PPFD = 200 μmol photons m^{-2} s^{-1} after sundown at mid-canopy height) was used to maintain the photoperiod at 16 h. Plants were fertilized twice per week with a dilute solution of Peter's Professional Water Soluble Fertilizer (W. R. Grace & Co., Fogelsville, PA) applied to the soil, and plants were regularly sprayed with pesticides to control insects. Nodes were cleared of all vegetative lateral buds on a weekly basis, so that a uniform growth habit was achieved. Flowering began at the terminal raceme and top axil (seventh node) approxi-

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mately 33 d after planting. Seeds had ceased filling approximately 34 DAF as indicated by maximum size and the onset of cotyledon yellowing (mean final seed dry weight was 550 mg seed^{-1}). Plants were selected for experiments 15 to 23 d after flowering, at which point the seeds had reached a fresh weight of 500 to 1000 mg.

Steady-State Labeling and Carbon Fixation Measurement

Steady-state labeling of leaves with $^{14}CO_2$ was accomplished using a custom-built, steady-state labeling system similar to that of Geiger (7) (details of system construction and operation are available on request). CO_2 measurements were made using an infrared gas analyzer (LIRA model 303, Mine Safety Co., Pittsburgh, PA) coupled with an IBM PC compatible computer. Air within the labeling system was mixed by fans.

The CO_2 concentration at the inlet to the leaf chamber was maintained between 350 and 380 μL L^{-1} by computer control. However, CO_2 concentrations within the leaf chamber were influenced by the net carbon fixation rate of the leaf. Leaf chamber CO_2 concentrations ranged from 150 to 230 μL L^{-1} (mean ± SD of 28 experiments = 197 ± 32.8 μL L^{-1} CO_2), as predicted from measurements of net carbon exchange rate of enclosed leaves, given a constant flow rate and CO_2 concentration of air entering the leaf chamber. $^{14}CO_2$ of known specific activity was added to the system from a pressurized source as required to maintain a constant CO_2 concentration at the inlet to the leaf chamber.

Whole leaf carbon fixation was calculated from measurements of the CO_2 depletion rate within the labeling system by linear regression of the rate of CO_2 depletion (corrected for the internal volume of the labeling system). Carbon fixation calculations were performed by a computer at approximately 1-min intervals. Leaf areas were determined by tracing leaves on paper for subsequent measurement using a ZIDAS digitizer board (Carl Zeiss, Oberkochen, FRG). Leaf radioactivity was continuously monitored with a Geiger-Müller detector (probe model GP200, ratemeter model RLM-2; Wm. B. Johnson, Research Park, Montville, NJ) mounted approximately 1 cm from the abaxial surface of the leaf (counting efficiency was approximately 1.5%).

The RH of air entering the leaf chamber was maintained at a constant dewpoint of 13 to 18°C using a temperature-controlled condenser and water trap. However, air within the leaf chamber was probably saturated with water vapor as indicated by the condensation of water on the lower surface of the leaf chamber. The temperature of air within the leaf chamber was between 23 and 28°C for all experiments, with variation of less than 3°C during individual experiments.

Experimental Procedure

The progress of steady-state labeling was studied by harvesting separate plants after labeling for different amounts of time. To minimize the effect of plant handling on the day of an experiment, plants were readied at least 10 h in advance of experiment initiation. The evening before an experiment, the top leaf of a plant was fastened to a nylon filament support matrix within the open leaf chamber of the steady-state labeling apparatus. The terminal raceme was removed and the top axil was trimmed to a single four- or five-seeded pod. The remaining pod at the top axil was clamped in an inverted position with loosely fitting holders made from aluminum foil wrapped in Parafilm.

Experiments were conducted in a forced-draft hood, under a 1000 W metal halide lamp (M1000/C/U Metalarc, GTE Products Corporation, Manchester, NH) filtered through 2.5 cm of circulating water in a Plexiglas bath, providing a PPFD of approximately 1000 μmol photons m^{-2} s^{-1} to the top leaf. The lamp was turned on between 8:00 and 9:30 AM, and the leaf was sealed into the leaf chamber within 9 min of light initiation by surrounding the petiole with modeling clay and sealing the two halves of the leaf chamber with silicon grease. Upon leaf enclosure, steady-state labeling commenced and was maintained until the end of an experiment, when the plant was dissected for analysis.

To test the effect of seed coat surgery (as required by the empty seed coat technique) on phloem transport into seeds, seeds were surgically modified approximately 20 min after the initiation of labeling. The surgical procedure involved making a single incision that removed approximately 45% (by fresh weight) of the distal portions of two adjacent seeds, leaving at least one intact seed on either side. This was accomplished by cutting at right angles across the pod dorsal bundle (the ventral bundle provides the vascular tissue for the seeds) to a point approximately half way through the pod, cutting parallel to the pod vascular bundles until two seeds had been cut in half, and cutting back across the pod dorsal bundle. The surface of the incision was smeared liberally with silicon stopcock grease, and the entire pod was surrounded by plastic wrap to prevent drying of the incision. It was observed that, immediately following incision, some of the cut embryos tended to move slightly above the edge of the cut seed coat.

Analysis of Tissues and Extracts

Tissue samples were extracted three times with 80% (v/v) aqueous ethanol at 50°C. One leaf punch (1.1 cm in diameter) from each of the three leaflets of the labeled source leaf were pooled for extraction. Petiole and peduncle samples were cut to 2-cm lengths, and seed coats were cut in half to remove the embryo (axis + cotyledons) before extraction. An aliquot of each extract was passed through tandem Poly-Prep ion exchange columns (Bio-Rad Laboratories, Richmond, CA) consisting of an upper column of AG 1-X8 resin (formate; 200–400 mesh) and a lower column of AG 50W-X8 resin (H^+; 200–400 mesh). The neutral fraction was eluted in 80% ethanol and brought to dryness in a 70°C oven. Dried column eluates were redissolved in 3 × 0.5 mL distilled water, and 0.5 mL of 5% (w/v) $ZnSO_4$ was added, followed by the addition of 0.5 mL 0.3 N NaOH, which caused a precipitate to form. The precipitate was then sedimented by centrifugation at 500*g* for 1 min, after which the clarified supernatant was decanted into a fresh test tube for sugar analysis. The sugar recovery of this procedure was approximately 90% (± 3%), based on the recovery of sucrose standards. The ^{14}C content of aliquots of clarified supernatant was determined by liquid scintillation counting, and the total amount of glucose remaining in each sample was determined after su-

crose hydrolysis by invertase using the glucose oxidase/peroxidase, rapid assay method (1). Glucose determination following inversion quantified the total amount of sucrose + glucose. During the passage of extracts through columns, sucrose in the extracts was inverted by approximately 10% (± 5% total range) on a molar basis.

Extracted tissues, ethanol extracts, and neutral column eluates were counted using a methylcellosolve-based scintillation fluid described by Sun *et al.* (23) in a Beckman model LS-355 liquid scintillation counter with counting efficiencies determined by the external standard-channels ratio method. When ethanol extracts of leaves, petioles or peduncles were counted, 20 μL of commercial bleach (Clorox) was added to 0.2 mL of extract in a 7 mL vial at least 10 min before the addition of scintillation fluid, to decolorize the extract (23).

Embryos were dried overnight at 60°C, combusted in a Packard Sample Oxidizer (Tri-Carb B306, Downer's Grove, IL), and the ^{14}C released was trapped in Carbosorb II (Packard) for counting using Liquiscint scintillation fluid (National Diagnostics, Manville, NJ) in a Beckman LS-100C scintillation counter. The ^{14}C recovery of the sample oxidation procedure was 82 ± 1%.

Sugar Specific Activity Calculations

To determine the relative proportions of sucrose, glucose, and fructose in seed coats and leaf discs, 80% ethanol extracts were purified by a noninverting ion exchange chromatography procedure similar to that described above, consisting of an upper column of AG 2-X8 resin (formate; 100–200 mesh) and a lower column of AG 50W-X8 resin (H^+; 200–400 mesh). Neutral column eluates were collected, dried at 70°C, and rehydrated for two-dimensional TLC (similar to that described in Turgeon and Gowan [27], except that the plates were pretreated with 0.03 M boric acid in 80% [v/v] aqueous ethanol). Labeled sugars were visualized using autoradiography film (27), and were identified by reference to standards. Following identification, spots containing individual sugars were removed from the plates for liquid scintillation counting. Sucrose, glucose, and fructose comprised greater than 95% of the total ^{14}C in the neutral column eluates of leaves and seed coats, and glucose and fructose were present at approximately 4% and 5.5%, respectively, of the total sucrose + glucose + fructose in the neutral fraction. The relative abundance of sucrose, glucose, and fructose, and the approximately equal amounts of glucose and fructose in neutral column eluates allowed the calculation of carbon specific activity in the neutral fraction based on two assumptions: (a) 100% of the ^{14}C present in the neutral fraction was accounted for by sucrose, glucose, and fructose, and (b) 12 moles of carbon atoms were present per mole of sucrose + glucose assayed. (Twelve moles of carbon atoms were present per mole of glucose assayed because glucose and fructose were present in equal amounts.)

Normalization of Tracer Data

Because different $^{14}CO_2$ specific activities were used in different experiments, the tracer data were normalized so that comparisons between experiments could be made. This was done by dividing all ^{14}C and specific activity measurements within an experiment by the specific activity of $^{14}CO_2$ used in the experiment, and multiplying by the units of specific activity (GBq mol C^{-1}). Thus, the highest specific activity that could be reached was, in theory, 1.0 GBq (mol C)$^{-1}$; specific activities greater than 1.0 GBq (mol C)$^{-1}$ would result from the presence of ^{14}C in compounds other than sucrose, glucose and fructose (see discussion of specific activity calculations above).

Analysis of ^{14}C Partitioning

Tissue ^{14}C was divided into three fractions: (a) neutral fraction (column eluate primarily containing sugars), (b) nonneutral fraction (calculated as the difference between neutral ^{14}C and total ^{14}C in the extract, and consisting of organic acids and amino acids), and (c) nonextractable fraction (^{14}C remaining in tissues following ethanol extraction, and containing primarily starch and structural carbon). Transport from the leaf was calculated as the difference between net ^{14}C fixation (based on measured net CO_2 fixation assuming a constant $^{14}CO_2$ specific activity of 1.0 GBq (mol C)$^{-1}$) and net ^{14}C accumulation in the leaf.

^{14}C accumulation and seed coat sugar content of cut seeds was adjusted for reduced seed size by multiplying each cut seed measurement by the ratio of intact seed to cut seed fresh weight determined for the seeds within each pod. The typical seed size correction factor was approximately 1.6, based on an average of 62% seed fresh weight remaining after cutting.

Statistical Methods

The statistical design used for comparisons between intact and cut seeds consisted of 12 experiments divided into four time periods (2.5, 5, 8, and 10 h) with three replications per time period. Each experiment contained two cut seeds and two to four intact seeds that were pooled to produce a cut and an intact treatment for each experiment. The statistical significance of differences between cut and intact seeds between time periods and for the interaction between time periods and differences between cut *versus* intact seeds, was quantified using F test P values determined using two-way analysis of variance (22).

RESULTS AND DISCUSSION

Leaf Carbon Fixation, Accumulation, and Export

The rate of photosynthesis was fairly constant throughout the labeling period, with a slight decline (<20%) observable after 8 h in some experiments (Fig. 1). Net carbon exchange rates observed in these experiments (mean ± SD of 28 experiments = 5.14 ± 0.98 μmol CO_2 m^{-2} s^{-1}) were similar to those reported for *P. vulgaris* at the same CO_2 concentrations (28). Measurement of ^{14}C accumulation in leaves with a Geiger-Müller detector demonstrated a continuous increase in radioactivity during the labeling period (Fig. 1). A slight decrease in the slope of the radioactivity *versus* time curve (<15%) was sometimes observed after approximately 5 h of labeling. The continuous increase in leaf ^{14}C content (Fig. 1) was probably caused by the increasing specific activity of leaf carbohydrate

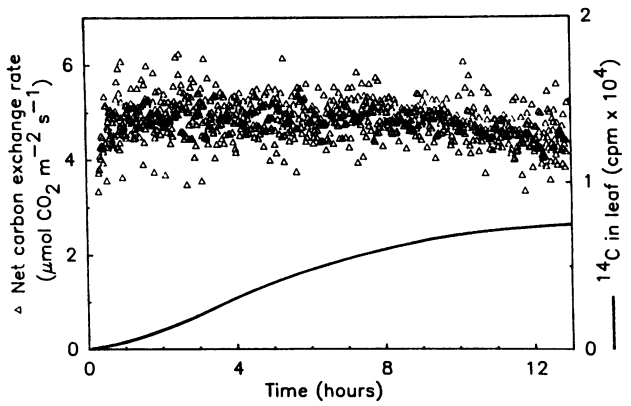

Figure 1. Net carbon exchange rate and ^{14}C accumulation by a single leaf. Measurement of net carbon exchange rate and ^{14}C accumulation (using Geiger-Müller detector) are described in "Materials and Methods." Zero time refers to the beginning of steady-state labeling. Single representative experiment.

pools (presumably to a maximum equivalent to the specific activity of the $^{14}CO_2$ used for labeling) combined with the storage of ^{14}C in the leaf as starch.

The partitioning of ^{14}C within the leaf is illustrated in Figure 2. The rates of net ^{14}C fixation, ^{14}C accumulation by the leaf, and ^{14}C transport from the leaf were relatively constant over time, as demonstrated by the high regression r^2 values calculated for these parameters over time (Fig. 2). The percentage of total fixed ^{14}C exported from the leaf was also relatively constant over time (50–60% of total ^{14}C fixed). Most of the fixed ^{14}C remaining in the leaf was present in the neutral

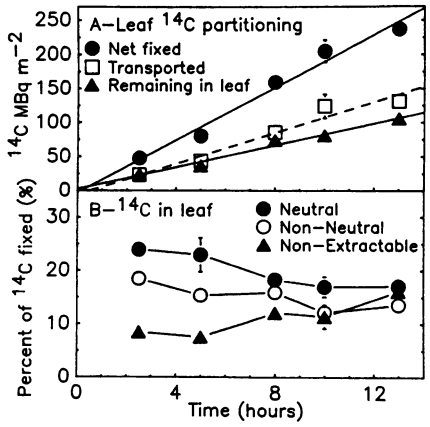

Figure 2. ^{14}C Partitioning in source leaves over time. A, Time course of net ^{14}C fixation (rate = 19.3 MBq ^{14}C m^{-2} h^{-1}; r^2 = 0.99), ^{14}C transport from the leaf (rate = 11.3 MBq ^{14}C m^{-2} h^{-1}; r^2 = 0.98), and ^{14}C accumulation in the leaf (rate = 8.0 MBq ^{14}C m^{-2} h^{-1}; r^2 = 0.99). The rates of ^{14}C fixation, transport, and accumulation were calculated from the slopes of the linear regressions shown in the figure. B, Leaf ^{14}C partitioning between the neutral fraction, nonneutral fraction, and nonextractable fraction, expressed as percentage of total ^{14}C fixed by the leaf. Points represent the mean ± SE of three experiments, except for 13-h points, which are the mean of ± SE of 16 experiments. Tracer data were normalized as described in "Materials and Methods." Zero time refers to the beginning of steady-state labeling.

fraction at all times during the labeling period, which indicates that most of the fixed ^{14}C was present in sugars (Fig. 2B). The increasing proportion of fixed ^{14}C in the nonextractable fraction (Fig. 2B) was most likely due to the accumulation of ^{14}C in starch (12).

Changes in Leaf Sugar Specific Activity Over Time

As observed previously by Fondy *et al.* (6) using *P. vulgaris*, leaf sugar content (sucrose + glucose) did not change significantly over time; the average sugar content was 4.67 ± 0.39 mmol m^{-2} (mean ± SE; 28 measurements). The leaf sugar pool appeared to be saturated with ^{14}C (no further change in specific activity) after 5 h of labeling (Fig. 3A). Thus, the amount of time required for isotopic saturation of the leaf sugar pool was longer in these experiments than the 90-min time for *P. vulgaris* presented by Geiger (7). The specific activity of the leaf sugar pool saturated at approximately 70% of the specific activity of supplied $^{14}CO_2$ (Fig. 3A), a value similar to those published for sugar beet (85%) (7) and soybean (62%) (4). Isotopic discrimination against ^{14}C is usually offered as the reason for leaf sugar specific activities lower than that of the supplied $^{14}CO_2$ (7). However, it is probable that other factors, such as the presence of a pool of slowly labeled sugar (4, 8), may also be important.

Calculation of Sugar Export Rate from the Leaf

The rate of sugar export from the leaf was calculable, given the rate of ^{14}C export, because the specific activity of leaf sugar remained at an approximately constant value of 0.65 GBq (mol C)$^{-1}$ following 5 h of labeling (see Fig. 3A). Under the assumption that 100% of the ^{14}C exported from the leaf was in sucrose, and using the leaf ^{14}C export rate of 12.6 MBq

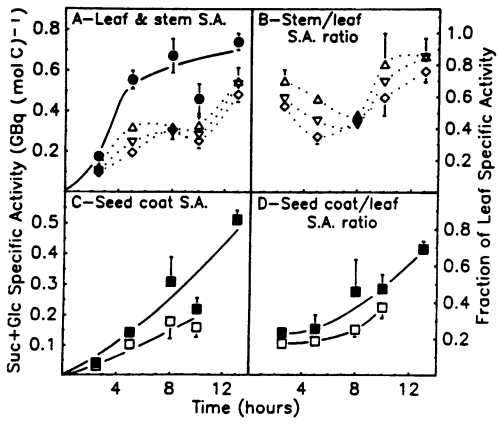

Figure 3. Tissue sugar (sucrose + glucose) specific activity *versus* time. Symbols for A and B: leaf (●), petiole segment near leaf (△), petiole segment near stem (▽), and peduncle of pod (◇); C and D: intact seed coat (■) and cut seed coat (□). A and C illustrate the sugar-specific activity of the tissues, whereas B and D display the ratio of tissue specific activity to leaf specific activity for each tissue. Points represent the mean ± SE of three experiments, except for 13-h points that are the mean of ± SE of 16 experiments. Specific activity data were normalized to a $^{14}CO_2$-specific activity of 1.0 GBq (mol C)$^{-1}$. Zero time refers to the beginning of steady-state labeling.

m^{-2} h^{-1} calculated by linear regression of the transport data in Figure 2A (omitting the 2.5-h point because a steady state had not been reached; regression $r^2 = 0.95$), the calculated rate of sucrose export was 1.6 mmol m^{-2} h^{-1}. This rate is similar to the previously reported sucrose export rate of *P. vulgaris* leaves for plants with developing pods (1.9 mmol sucrose m^{-2} h^{-1}) (5). The assumption that 100% of the ^{14}C exported from the leaf is represented by sucrose is an overestimate. However, greater than 90% of the label in the neutral fraction (sugars) of leaves was present in sucrose as determined by TLC.

Specific Activity of Sugars in the Path and Seed Coat Tissues

The specific activity of sugar in tissues along the path of photosynthate transport from the leaf to the seeds is illustrated in Figure 3. Although the difference between values was small, the specific activity of tissues nearer the leaf was consistently higher than those farther away (Fig. 3B). This may be explained by the shorter transit time for tracer movement to path tissues nearer the source, and may also be due to tracer exchange with unlabeled sugar along the path from source to sink (3). Sugar in the path tissues reached relatively high specific activities early during the steady-state labeling period, in parallel with those of the source leaf (Fig. 3, A and B). Fisher (3) estimated that approximately two-thirds of the sugar in soybean petioles was in the phloem transport stream, and thus the specific activity of sugar in path tissues would be expected to parallel the specific activity of sugar in the leaf.

At 2.5 h of labeling, the specific activity of sugar in seed coats was significantly lower than that of the path tissues (Fig. 3). In contrast to path tissues, most of the sugar in seed coats is located in tissues outside the phloem, because the concentration of sugar in the bulk symplast of seed coats is relatively high (about 100 mM) (11), and the relative volume of phloem in seed coats is extremely small (more than two orders of magnitude smaller than that of other seed coat tissues) (17). Therefore, the observation that seed coat sugar specific activity was lower than that of the path tissues at 2.5 h could be accounted for by the large pool size (13.8 μmol sucrose seed $coat^{-1}$) relative to the flux through the seed coat (2.0 μmol sucrose $seed^{-1}$ h^{-1}; see below).

After 5 h of steady-state labeling, the specific activity of seed coat sugar approached that of the path tissues (Fig. 3). Seed coat sugar specific activity could not reach that of the path tissues unless phloem-imported tracer was transported throughout the tissues of the seed coat, because a much greater proportion of tissue sugar is outside the phloem in seed coats than in path tissues (see above). Thus, the observation that seed coat sugar specific activity approached that of the path tissues after 5 h of labeling is evidence for the transport of tracer throughout the seed coat sugar pool. Tracer could have moved throughout the seed coat sugar pool via the apoplast or the symplast. Therefore, tracer movement throughout intact seed coats is suggestive of symplastic phloem unloading, but is not conclusive evidence for this pathway.

Carbon Partitioning within Intact Seeds

The time course of ^{14}C import into intact seeds is illustrated in Figure 4, A and B. The ^{14}C content of the seed coat was greater than that of the embryo during the first 5 h of labeling (Fig. 4B). This indicates that tracer accumulated in the seed coat before much transfer to the embryo occurred. Tracer "buildup" in the seed coat before transfer to the embryo was also observed in pulse-labeling experiments (21, 24). Symplastic phloem unloading would cause tracer dilution within the large seed coat sugar pool (see above) so that a significant tracer buildup in the seed coat would be required before much accumulation in the embryo could occur. On the other hand, apoplastic transport from the phloem to the embryo would enable rapid tracer accumulation in embryos without a long period of tracer buildup in seed coats. Thus, the observation of a tracer buildup in seed coats before accumulation in embryos is best explained by symplastic phloem unloading in seed coats.

^{14}C accumulation was greater in embryos than in seed coats after 5 h of labeling, and the proportion of total seed ^{14}C in the embryo increased over time (Fig. 4, A and B). The continued increase in embryo ^{14}C content is consistent with the fact that the embryo is an ultimate sink for photosynthate. In keeping with the role of the seed coat in the transfer of sugars from the phloem to the embryo, the majority of seed coat ^{14}C was present in the neutral (sugar) fraction (Fig. 4B). The increase in total seed coat ^{14}C content over time was primarily due to an increase in sugar specific activity (Fig. 3C); only a small and decreasing proportion of ^{14}C accumulated in the nonneutral (amino and organic acid) and nonextractable (starch + structural) fractions (Fig. 4B). The fact that very little ^{14}C accumulated in the nonextractable fraction (Fig.

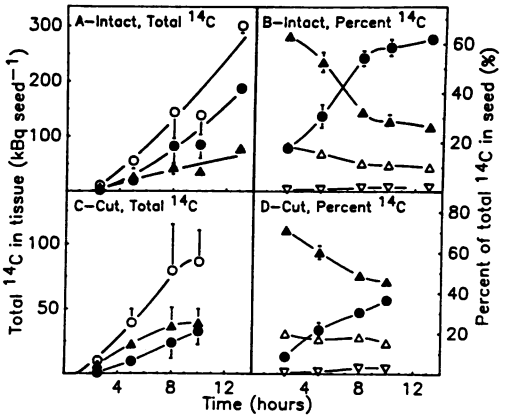

Figure 4. Partitioning of ^{14}C in intact and cut seeds over time. A and C, Total ^{14}C in seed (○), embryo (●), and seed coat (▲); B and D, percent of seed ^{14}C in embryos (●), seed coat neutral fraction (▲), seed coat nonneutral fraction (△), and seed coat nonextractable fraction (▽). Data presented on a per seed basis, as the mean of two seeds per pod for the cut seed treatment (C, D), and mean of two to four seeds per pod for the intact seed treatment (A, B). Points represent the mean ± SE of three experiments, except for 13-h points that are the mean of ± SE of 16 experiments. Tracer data were normalized as described in "Materials and Methods." Zero time refers to the beginning of steady-state labeling.

4B) indicates that the seed coats accumulated very little or no starch during the labeling period. This is consistent with the results of Fader and Koller (2) who observed that seed coat starch is not a significant source of carbon for later mobilization.

Sugar Import into Seeds

The use of steady-state-labeled tracer fluxes to calculate sugar import rates into seeds requires that all photosynthates transported to the seeds are from the labeled leaf, and that the flux from leaf to seed is constant over time. In *P. vulgaris*, the top leaf is the primary source of photosynthate to pods at the top axil (14), and pod walls are not a significant source of carbon for developing seeds (18). The labeled (top) leaf received much greater illumination than other leaves, and the removal of pods on the terminal raceme and top axil reduced the sink load of the labeled leaf. These conditions were apparently sufficient for the labeled leaf to supply the greater part of the carbon imported by the seeds because seed coat sugar specific activity attained levels similar to that of the source leaf (70% of leaf sugar specific activity at 13 h of labeling; Fig. 3D). As discussed previously, photosynthate export from the labeled leaf appeared constant (Fig. 2).

Using the conditions described above, the rate of sugar import by the embryo was calculated. First, the amount of sugar accumulated by the embryo at each point of the embryo ^{14}C-content time course (the 2.5-h point was excluded because a steady state had not been reached; Fig. 4A) was calculated by dividing embryo ^{14}C content by the specific activity of sugar in the seed coat (or the leaf; see discussion below). Linear regression was then used to determine the slope of these data, which was equivalent to the rate of sugar accumulation in the embryo. The calculated rate of embryo sugar import was 2.02 μmol sucrose seed^{-1} h^{-1} ($r^2 = 0.803$), based on the assumption that 80% of the ^{14}C taken up by the embryo consisted of sucrose (21). This rate is within the range of values previously reported for *P. vulgaris* (10, 19) and confirms that steady-state methods can reliably measure seed import rates *in vivo*.

Seed coat sugar specific activity was used for embryo sugar accumulation calculations based on the hypothesis that tracer passed through the entire seed coat sugar pool before uptake by the embryo (this would occur if phloem unloading were symplastic). When this calculation was repeated using leaf sugar specific activity as a measure of phloem specific activity (to replicate the assumptions of the apoplastic unloading pathway), the results were practically identical. The similarity of results from the two calculations was unexpected, due to the different specific activities of leaves and seed coats. However, statistical differences between the two calculations were canceled by contrasting trends in the leaf and seed coat specific activity data (leaf specific activity was constant after 5 h, whereas seed coat specific activity increased; Fig. 3).

Effect of Seed Surgery on Photosynthate Transport into Seeds

Statistical comparisons between intact and cut seeds are presented in Table I. There were statistically significant changes over time in all measured parameters except seed coat sugar content ($P < 0.05$ in the time period portion of Table I). However, time did not affect the relative differences between cut and intact seeds ($P > 0.1$ in the cutting × time interaction portion of Table I). Therefore, the data for intact and cut seeds were pooled across time periods (Table II) so that statistical comparisons between cut and intact seeds could be made (cut *versus* intact portion of Table I).

The observation that cut seed coats had greater unlabeled sugar contents than intact seed coats (Tables I and II) may be partially explained by the uneven distribution of sugar in seed coats. The basal portion of freshly cut seed coats contained more sugar than the distal portion, which was excised by the cutting treatment (the basal 62% of the seed by fresh weight contained 70% of the total seed coat sugar). Thus, the sugar content of cut seed coats was overestimated by the application of a seed fresh weight correction factor to the sugar content of the basal portion of the seed coat (see "Materials and Methods"). In the time-course experiments, the basal 55% of the seed (by fresh weight) contained an average of 73% of the intact seed coat sugar. Thus, it appears that more sugar was present in cut seed coats than could be accounted for by the application of the fresh weight correction factor alone. The higher sugar content of cut seed coats may have resulted from sugar accumulation in response to wounding, or from a blockage of seed coat sugar release to the embryo. Regardless of the explanation, the absence of statistically significant changes in sugar content over time (Table I) indicates that

Table I. *Statistical Evaluation of Differences between Intact and Cut Seeds with Respect to ^{14}C and Sugar (Sucrose + Glucose) Accumulation*

With the exception of seed coat sugar content, the data used for statistical analysis were presented in Figures 4 and 5. This table presents *F* test P values determined using two-way analysis of variance of differences between cut and intact seeds between time periods and for the interaction between time periods and differences between cut *versus* intact seeds. The smaller the P value, the greater the effect of the treatment (*i.e.* the larger the component of variability attributable to the treatment).

Component of Variability	Seed Coat Sugar Content	Seed Coat Sugar Specific Activity	^{14}C in Seed Coat Fractions				^{14}C in Embryo	Total ^{14}C in Seed
			Neutral	Nonneutral	Nonextractable	Total		
Cut *versus* intact	0.000[a]	0.091[b]	0.714	0.554	0.901	0.617	0.032[c]	0.142
Time period	0.184	0.003[a]	0.010[a]	0.004[a]	0.001[a]	0.006[a]	0.015[c]	0.010[a]
Cutting × time interaction	0.625	0.639	0.955	0.989	0.988	0.986	0.322	0.707

[a] Significant at the 99% confidence level. [b] Significant at the 90% confidence level. [c] Significant at the 95% confidence level.

Table II. *Effect of Cutting on ^{14}C and Sugar (Sucrose + Glucose) Accumulation in Seeds*

Data are averages for intact and cut seed treatments pooled across time periods using the data set analyzed statistically in Table I. Cut seed data were adjusted for the removal of a portion of the seed using a correction factor based on seed fresh weight (see "Materials and Methods"). Tracer data were normalized to a $^{14}CO_2$ specific activity of 1.0 GBq (mol C)$^{-1}$.

Seed Treatment	Seed Coat			^{14}C in Embryo	Total ^{14}C in Seed
	Sugar content	Sugar specific activity	Total ^{14}C		
	μmol	*GBq (mol C)$^{-1}$*	*kBq*	*kBq*	
Cut	18.86	0.116	40.95	16.58	53.65
Intact	13.82	0.177	36.25	47.24	87.35

the sugar must have accumulated within the first 2.5 h after cutting.

The cutting treatment appeared to cause a reduction in the specific activity of sugar in seed coats (significant at the 90% confidence level, Tables I and II; Fig. 3C), even though intact and cut seed coats had similar ^{14}C contents. The net accumulation of sugar in cut seed coats (see discussion above) may have caused the reduced sugar specific activities. However, some of the seed coat may have been damaged by cutting, such that ^{14}C-labeled sugar movement to the damaged portion of the seed coat was restricted. If this were the case, the damaged portion of the seed coat would have contained low specific activity sugar.

Another effect of seed surgery was the lowered ^{14}C content of cut embryos (Tables I and II). Surgery altered the pattern of ^{14}C accumulation within cut seeds (Fig. 4, C and D) such that the ^{14}C content of embryos never surpassed that of seed coats, as it did in intact seeds (Fig. 4, A and B). It is unlikely that the large difference between embryo and seed coat ^{14}C content was caused by the unequal surgical removal of tissue from embryos and seed coats, because the amounts of tissue removed appeared equal. Thus, the lower ^{14}C content of cut embryos relative to seed coats appeared to result from reduced photosynthate transfer from seed coat to embryo. This reduction in photosynthate transfer could have been caused by an inhibition of photosynthate unloading from seed coats, an inhibition of photosynthate uptake by embryos, or by a reduction in intimate contact between the seed coat and embryo (a possible artifact of the surgical treatment, see "Materials and Methods"). The perfused seed experiments described in the next paper of this series will distinguish between these alternatives.

CONCLUSIONS

Steady-state labeling produced stable fluxes of labeled carbon that were useful for analysis of photosynthate transport into developing seeds of *P. vulgaris*. The results of this study support the hypothesis that phloem unloading in seed coats is symplastic. Based on several criteria, including embryo ^{14}C content, seed coat sugar content and seed coat sugar specific activity, these experiments provide evidence for the disruption of photosynthate transport by the surgical excision of the distal halves of seeds, as practiced during the preparation of "empty" seed coats. The use of a quantitative method for the analysis of tracer movement within developing seeds provides an additional tool for the study of phloem unloading within developing legume seed coats.

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